ABSTRACT

Title of Document:	THE AGARASE SYSTEM OF *SACCHAROPHAGUS DEGRADANS* STRAIN 2-40: ANALYSIS OF THE AGARASE SYSTEM AND PROTEIN LOCALIZATION
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Saccharophagus degradans (formerly "*Microbulbifer degradans*") strain 2-40 is a Gram-negative marine bacterium isolated from the Chesapeake Bay. Analysis of 16s rDNA sequence indicated that *S. degradans* is related to a group of marine proteobacteria adept at degrading complex polysaccharides (CPs). *S. degradans* can depolymerize at least ten CPs including agarose. Agarose, an algal galactan, is degraded by few organisms. The agarase system of *S. degradans* was shown to be composed of five enzymes AgaA, AgaB, AgaC, AgaD and AgaE. These proteins contain glycoside hydrolase domains GH16, GH50 and GH86. *S. degradans* is the only organism known to collectively encode agarases with at least one of these domains. Unusual for agarases, AgaB and AgaE also contain multiple type-six carbohydrate binding modules. Furthermore, AgaE contains four thrombospondin type-three repeats whose function in prokaryotic proteins were unknown. The predicted agarases were characterized using a variety of methods including genomics, biochemical assays, proteomics and a newly described mutagenic technique. Agar degradation by *S. degradans* includes two depolymerases, AgaB and

AgaC, a β-agarase II (AgaE) and a possible α-neoagarobiose hydrolase (AgaA). AgaB was found to be freely secreted while AgaC and AgaE were surface associated. AgaC is a predicted lipoprotein while AgaE did not have domains characteristic of surface localization. The Tsp-3 repeats, which are similar to repeats found on other cell surface enzymes, are the proposed cell surface anchoring sequences of AgaE.

THE AGARASE SYSTEM OF *SACCHAROPHAGUS DEGRADANS* STRAIN 2-40: ANALYSIS OF THE AGARASE SYSTEM AND PROTEIN LOCALIZATION.

By

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Dedication

This work is dedicated to my wife Kristin. Her love and constant patience is my guide.

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Chapter 1: Introduction

Marine strain 2-40

Marine strain 2-40 was isolated from the surface of decaying salt marsh grass, *Spartina alterniflora* found in the lower Chesapeake Bay, U.S.A (7). It is a gram-negative, pleomorphic rod shaped organism and motile by a single polar flagellum. Strain 2-40 requires 1-10% sea salts for growth with optimal growth occurring at 2.3%. Growth occurs optimally at 28°C and pH 7.5 with a range of 4-35°C and pH 4.5-10, respectively (94). The organism is oxidase and catalase positive. Colonies are white to cream colored and turn black upon the production of eumelanin (83) during late stages of growth. Strain 2-40 can degrade at least 10 complex polysaccharides (CPs) including agar, alginate, β-glucan, chitin, cellulose, laminarin, pectin, pullulan, starch, and xylan (50, 70, 94, 126). These CPs can be derived from numerous sources including algae, land plants, crustaceans, bacteria, and fungi. Strain 2-40 is the only described marine organism with this array of degradative capabilities.

The phylogenetic position of strain 2-40 was difficult to establish because 16S rDNA sequence of closely related organisms was not available (56, 94). Based on available evidence, it was tentatively classified as "*Microbulbifer degradans"* strain 2-40. Chapter two will discuss an updated analysis of the inferred phylogenetic position of strain 2-40

and defend the placement of this organism in a new genus related to other CP degrading marine organisms.

Complex Polysaccharide Degradation

CPs are composed of repeating sugar units and function primarily as structural or energy storage substances. Some exist in long chains, arrayed in resilient and nearly insoluble states, for example, crystalline cellulose (21). Other CPs, such as agar, are soluble at high temperatures, but under physiological conditions are found in a relatively insoluble state. CPs are typically found intermeshed with other polysaccharides adding to the difficulty of their degradation (9). For example, the cell walls of vascular plants are primarily composed of cellulose microfibrils embedded within a matrix of hemicellulose and lignin (21). CPs do not occur in homogenous solutions and represent recalcitrant forms of carbon that only specialized organisms can access.

CP degradation in terrestrial environments is mediated by several known microorganisms (11, 12, 16, 17, 19, 22, 30, 57, 124, 142); however, the microbes that mediate the mineralization of these substances to CO_2 in marine environments are largely unknown. In pelagic zones, where non-living matter is a major food source, dynamic microbial communities have been isolated from solid aggregates (58, 60, 85) composed of CPs and other organic and inorganic substances. This matter is referred to as “marine snow”. The microbial communities found attached to it contain various bacteria belonging to multiple genera (60, 84), many of which produce hydrolytic enzymes i.e. chitinases to access the

available carbon of marine snow. Much less is known about the microorganisms that degrade CPs in estuarine environments such as the Chesapeake Bay. Estuaries are partially enclosed regions where fresh water and ocean water mix creating areas where high influxes of nutrients and varying salinity are present. Unlike pelagic zones, estuaries are areas of high primary production much of which occurs in the salt marshes present in these areas (122). Salt marshes similar to where strain 2-40 was isolated are typically flooded by tidal waters and are colonized by the marsh grass *Spartina alterniflora*. They contain plentiful sources of CPs derived from vascular plants, algae and crustaceans. These areas are supported by detritus-based ecosystems where CPs derived from plant matter are decomposed by communities of fungi and bacteria (100).

Relatively little is known about the microbial communities present in the *S. alterniflora* decay system. It was thought that the degradation of *Spartina spp.* initially occurs by fungi (24). These organisms reduce up to 60% of the organic mass present by producing copious amounts of exoenzymes, including lignases, and cellulases (106). As the partially decomposed detritus settles into the marsh sediments bacteria become the predominant decomposers (24). Recently though, bacterial and fungal communities have been shown to cohabitate suggesting that the colonization scheme presented above may not always occur as simply (28).

Much of the study of salt marsh microbial ecology has focused on ecologically dominant fungal and bacterial constituents with little data on how the actual degradation of CPs occurs. Furthermore, this research has been based on organisms involved in

lignocellulose degradation while other plentiful sources of CPs have not been addressed, including CPs derived from resident algae and arthropods. Although it is yet to be established whether strain 2-40 constitutes an ecologically relevant organism in salt marshes from the Chesapeake Bay it can be used as a model organisms for how multiple CPs are degraded in these areas.

Strategies of Complex Polysaccharide Degradation

Organisms known to degrade CPs do so by two general means. One strategy, utilized by *Sphignomonas* sp. imports high molecular weight alginate by means of an invagination of its outer membrane within which a specific transporter is present (98). The alginate is imported and degraded intracellularly (97, 99). More commonly bacteria and fungi use secreted enzymes to mediate the initial degradation of CPs to soluble saccharides which are then imported into the cell. These enzymes must remain active in the extracellular environment where changes in pH, salinity and proteolytic substances may be present. The location of these enzymes in relation to the cell and substrate plays a critical role in the degradation of the substrate and subsequent carbon acquisition by the organism.

Secreted CP degrading enzymes can be presented by two different means, either attached to the cell surface or freely secreted into the extracellular milieu. Freely secreted enzymes allow for the degradation of distant substrates. A disadvantage of this strategy is that if excessive diffusion of the enzymes occurs then the products of their activity may be lost.

This challenge would be compounded in marine environments where mixing due to tidal currents could greatly disperse the desired products. An alternative strategy is the attachment of enzymes to the surface of the bacterium. Although these enzymes cannot access distant substrates the resulting soluble sugars would be in close proximity to the cell.

Preliminary data suggested that strain 2-40 attaches certain agarases, chitinases and alginate lyases to its cell surface by an unknown mechanism (139). The remainder of this report will focus on addressing the apparent method of cell surface attachment of agarases utilized by strain 2-40.

Cell Surface Enzyme Attachment in Bacteria

Bacteria are known to utilize several mechanisms to attach proteins to their cell surface (88). One example is the direct anchoring of the proteins to the outer-membrane by means of anchoring domain present in the protein, for example, EngE produced by *Clostridium cellulovorans* (131). This protein is an endoglucanase, which utilizes an S-layer homology domain (SLH) to anchor to the peptidoglycan layer resulting in the exocellular display of the protein. These domains are highly similar to the S-layer protein RsaA from the Gram-negative bacterium *Caulobacter crescentus* (26), and can be found on various surface associated proteins from gram-positive bacteria (89, 113). In fact,

SLH domains have only been identified as anchoring domains in proteins produced by Gram-positive bacteria.

Another mechanism to anchor proteins to a bacterial cell surface is through the use of transmembrane domains. These domains are typically multi-pass domains characteristic of outer membrane porins and receptors (88). These domains have not been identified in hydrolytic proteins, but are common in integral outer membrane proteins, such as LamB from *E. coli* and OmpA proteins from various gram-negative bacteria (88).

Alternatively, the cell surface attachment of secreted proteins may be dependent on a specific post-translational modification. For example, PulA, a pullulanase from *Klebsiella pneumoniae*, utilizes a lipid anchor to attach to the outer membrane of this organism (116). Characterization of this protein sequence has shown that there is a 19 amino acid residue signal sequence that is cleaved upon export to the periplasm at a site termed the lipobox. This exposes an amino terminal cysteine. The sulphydryl group of the cysteine is modified by the attachment of a diacylglycerol and the amino group of cysteine is acylated with a fatty acid (62). The modified protein is then transiently embedded in the outer membrane (36). The mature enzyme is released into the medium without the removal of the covalently attached lipid moiety (34, 35, 37).

Another strategy of bacterial cell surface protein display is the cellulosome. Cellulosomes are high molecular weight cell surface protuberances capable of hydrolyzing complex polysaccharides including cellulose, pectin, and xylan (43). These structures are

produced by various cellulose degrading *Clostridia* and fungi (3, 4, 11-22, 38, 39, 41-43, 51, 53, 77, 107, 125, 141). Cellulosomes array carbohydrases upon cell surfaces via a well characterized protein-protein interaction. The characteristics of cellulosomes are well established. Each cellulosome can range from 1-3 mDa and contains multiple non-catalytic organizing scaffold proteins termed scaffoldins (21). Scaffoldins are high molecular weight proteins that provide platforms to which degradative proteins and a cellulose binding domain are both anchored to the cell wall and exposed to the environment (13, 17, 19). Scaffoldin proteins contain cohesion domains (cohesins) that bind to repetitive docking sequences within hydrolytic enzymes termed dockerin domains. All hydrolytic enzymes that bind to scaffoldins share species-specific dockerin repeat sequences. Dockerin sequences are well characterized and easily identified by their conserved repetitive signature sequences (13, 17, 19). The scaffoldin itself is bound to an SLH-containing anchoring protein that attaches the entire complex to the cell surface (104). The cellulosomes are exposed to the extracellular milieu and are thought to allow the organism to simultaneous bind and degrade CPs while keeping released soluble sugars in close proximity to the cell (17). The organisms that utilize cellulosomes appear to be exclusively from terrestrial habitats.

The attachment of enzymes to the cell surface of strain 2-40 was proposed to be similar to that of cellulosomes (139). Growth with agarose as a sole carbon source coincided with the apparent production of cell surface protuberances (139). Some of these protuberances appeared to contain a 98kDa agarase. The protuberances also appeared to cross-react with a scaffoldin specific anti-serum suggesting that strain 2-40 also produced

structures similar to cellulosomes (139). It was unknown, however, if the enzymes of strain 2-40 contained dockerin-like signature sequences. The agarase system was chosen for further study because little was known about the protein sequences and functional domains found within these proteins.

Agars and Agarose

Agars belong to a heterogeneous family of algal polysaccharides containing the repeating units of 1,3-D-galactose and 4-linked 3,6,-anhydro-α-L-galactose, termed agarose (47). Agarose is an uncharged polysaccharide with well known gelling characteristics. Agarose polymers can be cleaved into two distinct disaccharide units, neoagarobiose and agarobiose. Neoagarobiose contains D-galactose at the reducing end while agarobiose contains the L-galactose derivative at its reducing end (33) (Figure 1.1).

Agars belong to a larger family of algal galactans termed agarocolloids. Those with strong gelling capabilities are termed agars and those with weakly gelling capabilities are termed agaroids (33). Agarocolloids are found in nature as methyl ether, pyruvate acetal and sulphate ester substituted derivatives dependent on the originating species (33). Porphyran, for example, is a highly sulphated weakly gelling agaroid derived from *Porphyran* and *Bagia* species (33, 102). The discrepancy in gelling is due to the amount and nature of various chemical substitutions that may take place along the agarose polymer, for example, 3,6, anhydro-L-galactose can be replaced with L-galactose, methyl

ethers, or sulfate hemi esters. Pyruvic acid ketals may be added to either monosaccharide, xylose or glucose may also be attached to the C-6 sugar of the 3 linked saccharide (33). Other sugars may also be present, in a species specific manner such as 4-O-methyl-α-L-galactopyranose from *Gracilaria tikvahiae* (33).

Agars and some agaroids are major cell wall constituents of various red algae belonging to the *Rhodophyceae* family, such as some members of *Gracilaria* and *Porphyra* genus (33, 102). Most red algae utilize galactans as a major constituent of their cell walls of which agar can constitute 70% (33). Other galcatans present in the cell walls of these algae are largely unknown, but appear to consist of various forms of agars and a similar polysaccharide termed carrengeenan (β-(1,4)-D-galactose-α-(1,3)-D-galactose) (32).

Algal cell walls are complex substrates consisting of a matrix of agars and other galactans along with embedded xylans and cellulose microfibrils. Removal of the gelatinous portion leads to the collapse of the cellulose microfibrils (33). The complete degradation of algal cell walls requires a consortium of enzymes with differing catalytic capabilities. This complexity may be the first line of defense for rhodophytes presenting a sufficiently complex substance that requires the function of multiple enzymes for its degradation.

Agarolytic Organisms

Agar-degrading organisms were first isolated from the Norwegian coast by Gran in 1902. Since that time period at least 30 organisms have been reported that are capable of pitting agar plates. The vast majority of these organisms are aquatic isolates belonging to *Cytophaga* (45, 46, 136), *Microbulbifer (110-112)*, *Pseudomonas* (61, 80), *Pseudoalteromonas* (23, 74, 119, 137), *Microscilla* (148), *Vibrio* (9, 10, 127-129), *Alterococus* (121), *Alteromonas* (115), *Thalassomonas* (109) and *Zobellia* (5, 6, 76). Agarase activity has also been observed in terrestrial organisms, such as *Paenibacillus* (67), *Streptomyces* (25), as well as an unidentified hospital contaminant (130). Currently there is only one report of an agarase purified from a Eukaryote, the mussel *Littorina mandshurica* (134, 135). Because agarolytic bacteria have been isolated from various Far Eastern Mussels (74, 75), it is likely that the agarase activity of *L. mandshurica* is due to resident bacteria.

Agarase activity was thought to be solely associated with saprophytic organisms. Recently it has been found that agarolytic members of algal epiphytic communities can cause disease. Agarolytic bacteria belonging to *Vibrio* (127), *Pseudomonas* and *Pseudoalteromonas* (75) genera have been isolated from the surface of certain agar-producing red algae (agarophytes). *Pseudomonas* sp. SK38 is the causative agent of green spot rot in *Porphyra dentata* (80, 117) while *Pseudoalteromonas gracilis* strain B9

has been implicated in a major die-offs of cultivated agarophytes in South Africa (119). Further study revealed that *P. gracilis* produces a β-agarase that localizes to the cell walls of *Gracilaria gracilis* where it causes bleaching and weakening of the cell wall structure (119). Agarases can now also be considered pathogenicity factors that affect the cellular structure of red algae and pose threats to algal culture stocks. Cell wall degrading enzymes are also used as pathogenicity factor by terrestrial plant pathogens such as *Erwinia chrysanthemi* (81).

Agarases

Agarases can be classified into two major categories, β and α. β-agarases cleave agarose at the β 1,4 linkages into series of neoagarobiose with D-galactose at the reducing end while α agarases yield sugars of the agarobiose series with 3,6, anhydro-L-galactose at the reducing (63, 115, 143).

The more common β-agarase system has been well characterized in *Pseudoalteromonas atlantica*. This system utilizes three classes of agarases to depolymerize agarose to soluble monosaccharides (Figure 1.1) (23). The first is a β-agarase I that cleaves agarose into neoagarotetraose as a major product. Larger neoagaro-oligosaccharides may also be present. Neoagarobiose can also be detected when reactions contain excessive concentrations of the enzyme. This enzyme, however, cannot cleave neoagarotetraose. The second enzyme is a β-agarase II. It cleaves agarose to an early accumulation of

neoagarohexaose and neoagarotetraose and can also cleave these products into neoagarobiose (101, 103). This enzyme, thus, has both endo- and exo-lytic activity. A third enzyme, α-neoagarobiose hydrolase, acts upon the 1,3 linkage of neoagarobiose to yield D-galactose and 3,6 anhydro-L-galactose (23). This enzyme cannot degrade sugars larger than neoagarobiose. Galactose enters the glycolytic pathway while the biochemical fate, if any, of 3,6 anhydro-L-galactose is unknown (23). β-agarase enzymes have been purified from numerous organisms (Table 1.1).

Figure 1.1

The degradation of agarose by β-agarase systems:

Agarose is a algal polysaccharide composed of repeating units of 1,3-D-galactose and 4-linked 3,6,-anhydro-α-L-galactose. Neoagarobiose and agarobiose are noted. β-agarase systems utilize three enzymes to degrade agarose. The major depolymerase is the β-agarase I enzyme that produces predominately neoagarotetraose. This sugar can be degraded by a β-agarase II enzyme to yield neoagarobiose. An α-neoagarobiose hydrolase cleaves neoagarobiose to D-galactose and 3,6 anhydro-L-galactose (Figure adapted from Belas, (23)).

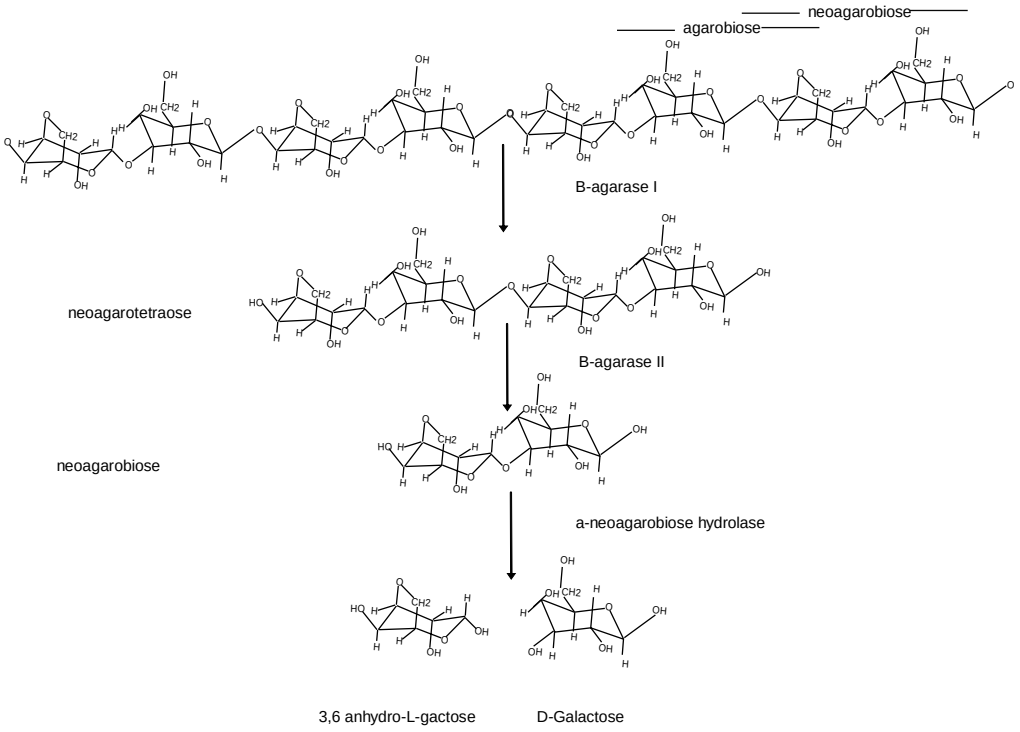

neoagarobiose
agarobiose
B-agarase I
neoagarotetraose
B-agarase II
neoagarobiose
a-neoagarobiose hydrolase
3,6 anhydro-L-gactose
D-Galactose

Table 1.1

Characterized agarases

Organism	Activity	Name	MW	Cellular Location	Reference
Pseudomonas SK38	β-I	*pagA*	37		(80)
Pseudomonas isolate	β-I β-I	I IIb	105 63	Supernatant Supernatant	(93)
P. atlantica	β-I β-II		55 32	Supernatant Membrane	(23, 101-103)
Microbulbifer JAMB-A94	β-I	*agaA*	48		(112)
Microbulbifer JAMB-A7	β-I	*agaA7*	49		(110)
Agarivorans JAMB-A11	β-II	*agaA11*	105		(108)
Alteromonas agarlyticus GJ1B	αI		180	Supernatant	(63)
Vibrio AP-2	β-II		20	Supernatant	(9)
Vibrio sp. PO-303	βI βII β?	a b c	88 115 57	Supernatant	(10)
P. atlantica str T6c	β-I	*agrA*	55	Supernatant	(23)
Cytophaga sp.	β			Supernatant	(45, 46)
Pseudomonas str, w7	β-I	*pjaA*	59		(61)
S. coelicolor	β-I	*dagA*	35	supernatant	(25)
Vibrio JT0107	β-II	*agaA* (0107) *agaB*	105		(127-129)
Pseudoalteromonas gracilis str. B9	β-I	*agaA*	30	Supernatant	(119)
Cytophaga flevensis	β-I		26	Cell Bound & Supernatant	(96, 136)
Pseudoalteromonas antartica	β-I		33	Supernatant	(137)

There are only two described α-agarase systems (63, 109). The most well known is that of *Alteromonas agarlyticus* strain GJ1B(63). This organism appears to utilize a depolymerizing α-agarase that yields predominately agarotetraose as the major product. This sugar is then partially degraded by a presumed β-galactosidase acting upon the reducing end to yield agarotriose (63). Considering the dramatic agar degrading characteristics reported for this organism, additional enzymes could be present. Most notably absent is an α-agarase II that would degrade agarotriose.

Modular Structure of Agarases

Agarases belong to a family of proteins termed O-glycoside hydrolases that hydrolyze bonds between carbohydrate units. These proteins are typified by a modular structure that contain distinct catalytic, and occasionally, carbohydrate binding modules. Currently the most widely accepted method for the classification of these modules is based upon primary amino acid sequence, as opposed to catalytic or binding properties. Using this method O-glycoside hydrolase catalytic (GH) domains have been categorized into 99 families while carbohydrate binding modules (CBM) now belong to 43 families (65, 66). Distinct domains are typically separated by repetitive linker sequences as has been observed in numerous proteins from strain 2-40 (69) and *Cellvibrio japonicus* (114).

Modules of the same family, either GH or CBM, are thought to share structural and mechanistic features as well as evolutionary lineages (65, 66). GH domains and CBM that share protein folds are grouped together into clans. Interestingly, though, conserved sequences or protein folds are not indicative of binding or catalytic characteristics. There are also reports of proteins containing multiple GH domains from different families each with distinct catalytic properties (65, 66). Until recently, as will be discussed in Chapter 3, GH domains within agarases had not been established. Furthermore, CBM domains capable of binding agarose have not been described.

Uses of Agar and Agarases

The cultivation of agar-producing red algae (agarophytes) is an agricultural industry estimated to be worth billions of dollars ($US) worldwide. The cultivation of agarophytes is encouraged by the Food and Agricultural Organization of the United Nation as a profitable and sustainable industry. Current statistics show that 55,000 dry tons of red algae are collected annually yielding 7,500 tons of agar. The value of this industry is currently estimates at 132 million dollars world-wide with Chile, Spain and Japan as the major producers. Other agarophytes, such as *Porphyra sp.* (*nori*, Japanese), are sources of food and not necessarily used for the extraction of agar. The annual production of *Porphyra sp.* is estimated at 90,000 dry tons with a value of 1.5 billion dollars(http://www.fao.org/documents/showcdr.asp?urlfile=/DOCREP/004/Y3550E/Y35

50E 04.htm). The study of agarolytic organism may become increasingly important as these potentially valuable crops are further cultivated.

Agar and agarose have been used in many applications, including as gelling agents for bacteriological media and electrophoretic gels. Agar is also used extensively as texturizing, emulsifying, and thickening agents in a variety of foods allowing for a plant source of a gelatin-like substance. Agar can also be used as an ointment base for creams and salves (1). Reports exist of the beneficial characteristics of neoagaro-oligosaccharides such as moisturizing characteristics and their ability to stimulate macrophage populations (147).

An emerging use of agarases is in the production of protoplasts from agarophytes (10). Protoplasts can be used for plant breeding and genetic manipulation (29). Enzymatic mixtures containing agarase, xylanase, cellulase, mananase, and proteolytic enzyme activity are typically needed to efficiently produce high quality protoplast preparations (10). A challenge is the complex algal cell wall that is modified in a species specific manner. Because of this, a single cocktail of enzymes does not perform well on all sources. If strain 2-40 is shown to degrade certain red algae species in culture then cellular extracts and supernatant preparations from these cultures may form the basis of new enzyme cocktails. Strain 2-40 may represent an organism that would enable harvesting of proteins from a single source.

The 2-40 Agarase System

The agarase system of strain 2-40 has been preliminarily characterized on the basis of enzyme activity. It appears to produce at least three proteins that depolymerize agarose in zymograms (140). Previous studies detected β-agarase I, β-agarase II, and α-neoagarobiose hydrolase activities in cellular fractions by reducing sugar assays (126). Total agarase activity appeared to be cell-associated through logarithmic growth then was primarily detected in the supernatant fraction as the cultures entered stationary phase (126). The majority of the cell-associated activity was present in crude membrane preparations while little was detected in periplasmic or cytoplasmic fractions (126, 140).

Agarase activity was detected in culture supernatants from cultures grown with agar, agarose, neoagarohexaose, neoagarotetraose and neoagarobiose as sole carbon sources (126). Agarase activity was also detected to a lesser degree in culture supernatants after growth in the following substrates: pullulan, xylan, chitin, starch, alginic acid, β-glucan, glucose, and, carboxy methyl cellulose (126). Interestingly, agarase activity was detected in cultures entering stationary phase growth with glucose as a sole carbon source (OD_{600}=0.7) and was nearly 3x more as the cultures aged (OD_{600}=1.2) (126, 140). This suggested that glucose only partially represses agarase activity perhaps by repressing certain, but not all, of the agarases encoded by strain 2-40.

A β-agarase I was partially purified from strain 2-40 (139, 140). This enzyme was 98kDa and released neoagarotetraose as the major product from the degradation of agarose. A β-agarase II of 42kDa agarase was co-purified and released neoagaotetraose and small amounts of neoagarobiose when reactions contained excessive amounts of enzyme (139, 140). A neoagarobiose hydrolase has not been purified from strain 2-40 but its activity has been detected by reducing sugar assays (126).

Cell Surface Agarase Activity in strain 2-40

Growth in the presence of agarose appears to coincide with a dramatic cell morphological change. While cells grown with glucose were typified by smooth surfaces, those grown with agarose had surfaces covered by protuberances (139). This was also observed when the cells were grown with chitin and alginate as a sole carbon sources (139). Antibodies, specific to the 98kDa β-agarase I, were raised and shown by immuno-localization to bind to discreet areas of the cell surface. These areas were thought to correspond to the cell surface protuberances and suggested that strain 2-40 anchored hydrolytic enzymes to its surface (139, 140). However, the mechanisms of attachment and the nature of the protuberances remained unknown.

There are multiple reports alluding to the presence of membrane bound agarases from other organisms such as *Cytophaga* sp. (44, 46), *Cytophaga flevensis* (136), *Zobellia galactanivorans* (6, 76) and *Pseudoalteromonas atlantica* (23) . This best data is from *P.*

atlantica (59, 101, 103) which by characterization of various cellular fractions indicate that the β-agarase I was a freely secreted protein (101, 103) while the β-agarase II was only found in soluble fractions of cellular lysates suggesting a membrane localization for this protein (59). The nature of this apparent cell surface attachment is currently unknown. Protuberances have not been described for any of these organisms.

Statement of Purpose

The degradation of complex polysaccharide is a critical process in the carbon cycle. In terrestrial habitats it is carried out by communities of microorganisms some of which array enzymes on their cell surface. Much less is known about the organisms that perform this critical function in marine habitats. The marine isolate, *S. degradans,* appears to be unusually adept at degrading complex polysaccharides and can be used as a model to study the strategies by which marine bacteria degrade these substances. This work was performed to identify the constituents of the agarase system and characterize their activity and cellular placement. The model of agar degradation by *S. degradans* may be conserved in other enzymes systems produced by this and perhaps related organisms.

Chapter 2: *Saccharophagus degradans* gen. nov., sp. nov., a Versatile, Marine Degrader of Complex Polysaccharides

Introduction

The carbon cycle in marine habitats has not yet been elucidated as many of the microorganisms that degrade complex polysaccharides (CPs), especially those derived from higher plants and algae, have not been identified. These include highly specialized microorganisms that recycle CP, a critical step in the marine food web. Within the past decade a number of such bacteria have been discovered.

In 1997 a gram-negative bacterium that degrades cellulose, xylan and chitin was isolated by Gonzalez et al. (55) from a salt marsh in Georgia USA and named *Microbulbifer hydrolyticus* IRE-31^{T}. In 2002 Distel et al. (40) reported taxonomic criteria for a shipworm symbiont that degrades cellulose and found them to be sufficiently different from the *Microbulbifer* taxon to be accorded a new genus, *Teredinibacter*. Within the past two years six strains related to *Microbulbifer* have been deposited in the GenBank database.

In 1986 Andrykovitch (8) isolated bacteria involved in the degradation of a salt marsh grass, *Spartina alterniflora* found in the lower Chesapeake Bay, U.S.A. One of these was

designated strain 2-40 ^{T}. Based upon phenotypic characteristics, it was placed with the alteromonads, where it resides today in order Alteromonadales of the Gammaproteobacteria, in family Alteromonadaceae. Later, based on sequence analysis of 16S rDNA, distinctive cellular morphology, and CP degradative capabilities, 2-40 ^{T} was considered to be closely related to the genus *Microbulbifer.* A more meaningful classification of strain 2-40 ^{T} had been hampered by the limited availability of 16S rDNA sequences from closely related organisms. Now, on the basis of more than 20 available sequences a new genus is proposed, *Saccharophagus*, to accommodate the most versatile marine carbohydrate degrader yet identified.

Strain 2-40 ^{T} can degrade at least 10 CPs, including agar, alginate, chitin, cellulose, fucoidan, laminarin, pectin, pullulan, starch, and xylan (50, 70). These CPs are derived from numerous sources including algae, land plants, crustaceans, bacteria and fungi. Because of its involvement in ocean and estuarine carbon cycles, the US Department of Energy has recently (February 2005) completed sequencing the genome (http://genome.jgi-psf.org/draft_microbes/micde/micde.home.html). Genomic analysis has predicted that there are >130 open reading frames, encoding enzymes involved in the depolymerization of CPs. Additionally, it is likely that at least another 100-200 genes are involved in signaling, regulation and further metabolism of CPs.

Strain 2-40 ^{T} clusters with a marine CP degrading genera. Its 16S rDNA sequences is most closely related to those of *Microbulbifer hydrolyticus* (90.5%) and *Teredinibacter turnerae* (91.5%); it shares the ability to degrade complex polysaccharides with both *M.*

hydrolyticus and *T. turnerae* and it has similar cellular morphology to that of *M. hydrolyticus*, most notably the copious production of membrane blebs and vesicles when grown with a complex polysaccharide as the sole carbon source (56). It is proposed that these organisms form a cluster of Gram-negative marine gamma-proteobacteria with the ability to degrade CPs. The question remained whether strain 2-40 ^{T} is sufficiently different from members of these genera to warrant its placement in a new genus.

Materials and Methods

Phylogenetic analysis: The16S rDNA sequence of strain 2-40 ^{T} was obtained from GenBank (AF055269). The 16S rDNA tree was generated using ClustalW 1.82 (31) for sequence alignment and the neighbour-joining program in the PHYLIP package (52). Before analysis, a filter was applied to exclude positions with less than 50% conservation within the sequences being aligned. Only positions 110 to 1,265 (*E. coli* numbering) were considered. All additional sequences were obtained from the GenBank database. It should also be noted that a metagenomic study of Sargasso Sea prokaryotes revealed putative proteins attributed to isolate Microbulbifer SAR-1 (http://www.ncbi.nlm.nih.gov/Web/Newsltr/Spring04/sargasso.html). However, 16S rDNA sequences from the Sargasso Sea shared <90% similarity with any isolate included in this analysis. To date (October 2005) other related sequences have not been reported in the GenBank database.

Substrate Utilization: Gelatin utilization and nitrate reduction were determined by API 20NE test strips supplemented with sea salts medium (2.5% sea salts, 0.05% yeast extract). Assays for the depolymerization of cellulose, pullulan and xylan utilized azurine-crosslinked polysaccharides (Megazyme; http://www.megazyme.com/) incorporated into 1/3 strength Marine Agar (Difco 2216) were performed by M. Howard. Degradation of these insoluble substrates by endo-hydrolases produced soluble dye-labeled fragments that were easily observed as blue halos around active colonies. *M. hydrolyticus* was used as a positive control for xylan and cellulose utilization. To assay for growth on alginate or pectin, strains were grown in minimal sea salts liquid media incorporating the polysaccharide of interest (0.2% final conc.). The disappearance of the polysaccharides and a reduction of broth viscosities indicated the degradation of alginate and pectin. Negative controls included media without polysaccharide. Strain 2-40 ^{T} was used as a positive control.

Results

Since the initial report of strain 2-40 ^{T} (56), 18 additional 16S rDNA sequences of related organisms have become available in GenBank. These permitted clarification of the phylogenetic position of strain 2-40 ^{T} when considered along with available phenotypic analyses. The inferred position of strain 2-40 ^{T} falls outside of two branches of the tree (Figure 2.1). The first is a *Microbulbifer* cluster that contains seven members including *Pelagiobacter variabilis* (72). This cluster is supported by a high bootstrap score. Six members were isolated from aquatic marine habitats, while strain *M. arenaceous* was

isolated within coastal sandstone (133). The type species of the genus *Microbulbifer* is *M. hydrolyticus* IRE-31^{T}.

The second branch is more diffuse and contains organisms from diverse marine niches, predominantly endosymbionts. Only one of these, *T. turnerae*, has been given a validly published name (40). *T. turnerae*, LP1, LP2, LP3, and AF102866 are endosymbionts of shipworms from the bivalve family *Teredinidae* while "*Candidatus* Endobugula glebosa" and "*Candidatus* Endobugula sertula" were isolated from the pallial sinuses of the bivalve *Bugula neritina* (64, 92). Strain NKB4 was isolated from deep sea sediments (90). The 16S rDNA sequences of strains SCB11 and BD2-13 were derived from gene clones from marine environmental samples (91). Strain characteristics for these organisms have not been reported. Strain 2-40 ^{T} shares a maximum of 91% 16S rDNA similarity with the *Microbulbifer* genus and 93% similarity with the endosymbiont cluster (Table 2.1). It shares 91.9% and 91.4% to BD2-13 and SCB11 respectively. The generated phylogenetic tree does contain nodes with weak bootstrap scores. This is due to the low available sampling size of 16s rDNA sequences. These data suggest that strain 2-40 ^{T} is related to the other members included in the 16S tree; however, there is no strong support that strain 2-40 belongs within the two clades represented in this tree and thus may represent a new genus and species. This is in agreement with previously published data (40, 56, 133, 146).

The suggestion that strain 2-40 ^{T} is distinct from the other *Microbulbifer/Teredinibacter* isolates is also supported by comparison of phenotypic characteristics (Table 2.2). Most notably the %G+C content of strain 2-40 ^{T} is significantly lower than that estimated for the other organisms, 46.7% as reported by Gonzalez and Weiner (56) and 45.8% as determined by draft genomic sequence compared to 57.7% for *M. hydrolyticus* and 49-51% for several *T. turnerae* strains (40). Furthermore the major fatty acid of strain 2-40 ^{T} is Iso-$C_{16:0}$ (37%) (56), while Iso-$C_{15:0}$ is the primary fatty acid of *M. hydrolyticus* (55), and *M. salipaludis* (146). Strain 2-40^{T} reduces nitrate to nitrite (this study) and synthesizes eumelanin via tyrosinase activity (82) both traits reported for only one other strain, *M. arenaceous* (133), which does not degrade agar and shares only 90.6% 16S rDNA sequence similarity with strain 2-40^{T}.

Figure 2.1

Phylogenetic position of strain 2-40^T based on its 16S rRNA gene sequence: The tree was generated using CLUSTAL W 1.82 (31) for sequence alignment and the neighbor-joining program in the PHYLIP package (52). *Burkholderia cepacia* served as the outgroup (not shown). Numbers at nodes indicate percentage bootstrap values above 50 (100 replicates). Bar, Jukes–Cantor evolutionary distance of 0·05.

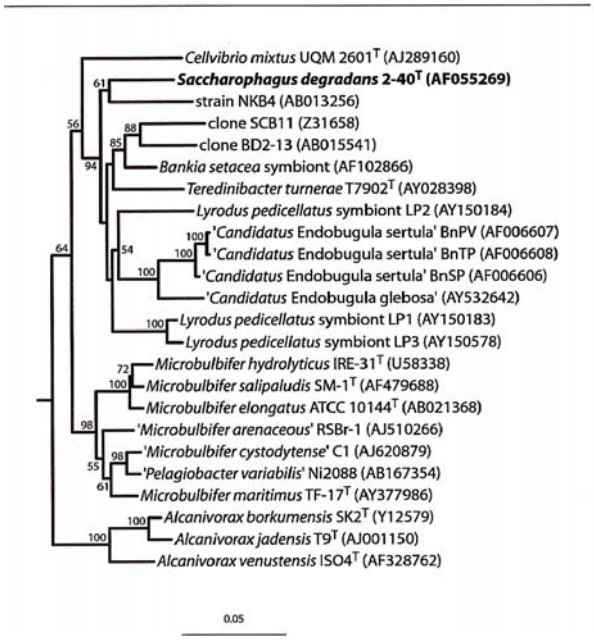

Cellvibrio mixtus UQM 2601^T (AJ289160)
***Saccharophagus degradans* 2-40^T (AF055269)**
strain NKB4 (AB013256)
clone SCB11 (Z31658)
clone BD2-13 (AB015541)
Bankia setacea symbiont (AF102866)
Teredinibacter turnerae T7902^T (AY028398)
Lyrodus pedicellatus symbiont LP2 (AY150184)
'*Candidatus* Endobugula sertula' BnPV (AF006607)
'*Candidatus* Endobugula sertula' BnTP (AF006608)
'*Candidatus* Endobugula sertula' BnSP (AF006606)
'*Candidatus* Endobugula glebosa' (AY532642)
Lyrodus pedicellatus symbiont LP1 (AY150183)
Lyrodus pedicellatus symbiont LP3 (AY150578)
Microbulbifer hydrolyticus IRE-31^T (U58338)
Microbulbifer salipaludis SM-1^T (AF479688)
Microbulbifer elongatus ATCC 10144^T (AB021368)
'*Microbulbifer arenaceous*' RSBr-1 (AJ510266)
'*Microbulbifer cystodytense*' C1 (AJ620879)
'*Pelagiobacter variabilis*' Ni2088 (AB167354)
Microbulbifer maritimus TF-17^T (AY377986)
Alcanivorax borkumensis SK2^T (Y12579)
Alcanivorax jadensis T9^T (AJ001150)
Alcanivorax venustensis ISO4^T (AF328762)
0.05

Table 2.1

Pair-wise alignment of related 16srDNA sequences

Organism	**GenBank Accession number**	**% Similarity to 2-40^T**
Cellvibrio mixtus UQM 2601^T	AJ289160	91.00
Strain NKB4	AB013256	92.60
Gene clone SCB11	Z31658	91.36
Gene clone BD2-13	AB015541	91.89
Bankia setacea symbiont	AF102866	93.08
Teredinibacter turnerae T7902 ^{T}	AY028398	91.52
Lyrodus pedicellatus symbiont LP2	AY028398	91.15
"*Candidatus* Endobugula sertula BnPV"	AF006607	89.62
"*Candidatus* Endobugula sertula BnTP"	AF006608	89.57
"*Candidatus* Endobugula sertula BnSP"	AF006606	90.32
"*Candidatus* Endobugula glebosa	AY532642	91.40
Lyrodus pedicellatus symbiont LP1	AY150183	92.04

Lyrodus pedicellatus symbiont LP3	AY150578	93.10
Microbulbifer hydrolyticus IRE-31 ^{T}	U58338	90.54
Microbulbifer salipaludis SM-1 ^{T}	AF479688	91.09
Microbulbifer elongatus ATCC10144 ^{T}	AB021368	90.75
Microbulbifer arenaceous RSBr-1	AJ510266	90.57
Microbulbifer cystodytense C1	AJ620879	90.23
Pelagiobacter variabilis Ni2088	AB167354	90.90
Microbulbifer maritimus TF-17 ^{T}	AY377986	90.22
Alcanivorax borkumensis sk2T	Y12579	87.68
Alcanivorax jadensis T9 ^{T}	AJ001150	87.36
Alcanivorax venustensis ISO4 ^{T}	AF328762	87.84

Table 2.2

Distinguishing characteristics of strain 2-40 as compared to *Microbulbifer spp*. and *T. turnerae*

	Strain 2-40^T	*M. hydrolyticus*	*M. salipaludis*	*M. elongatus*	*M. maritimus*	*M. arenaceous*	*T. turnerae*
Trait							
%G+C	45.8	57.7	59	nd	59.9	nd	49-51
Major FA	Iso-C $_{16:0}$	Iso-C $_{15:0}$	Iso-C $_{15:0}$	Iso-C $_{15:0}$	Iso-C $_{15:0}$	nd	nd
Eumelanin	+	-	-	-	-	+	-
NO_3 reduction†	+*	-*	nd	-*	nd	+	nd
Gelatin†	+*	+*	nd	+*	nd	nd	nd
Substrate§							
Agar	+	-	+	+	-	-	-
Alginate	+	+**	+**	+	nd	nd	nd
Cellulose	+	+	-**	+	nd	nd	+
Chitin	+	+	-	+	-	+	nd
Fucoidan	+	nd	nd	nd	nd	nd	nd
Laminarin	+	nd	nd	nd	nd	nd	nd
Pectin	+	+**	-**	+**	nd	nd	+
Pullulan	+	+**	+**	+**	nd	nd	nd
Starch	+	+	+	+	nd	+	nd
Xylan	+	+	+	-**	nd	nd	+
Reference	(56)	(55)	(146)	(144)	(145)	(133)	(40)

* as determined in this study; ** as determined by M. Howard§ Substrates tested for depolymerization and utilization; +, substrate depolymerized; -, substrate not depolymerized under conditions tested; †, API 20NE test strips; nd, not described;

Discussion

Strain 2-40 ^{T} was known to degrade at least 10 CP, many more than had been reported for *T. turnerae* and strains of *Microbulbifer* (Table 2.2). However, because this required additional confirmation, the differences in CP degradative capabilities within the *Microbulbifer/Teredinibacter/ Saccharophagus* group of bacteria were investigated to establish the possibility other conserved genes. *M. hydrolyticus*, *M. elongatus*, and *M. salipaludis* were tested for their abilities to degrade alginate, cellulose, pectin, pullulan and xylan as compared to strain 2-40 ^{T}. The results are summarized in Table 2.2.

Strain 2-40 ^{T} depolymerized all the tested substrates shown. Each of the other related strains was negative for at least one of the substrates even considering the fact that not every strain was available. The versatility of strain 2-40 ^{T} CP degradation is unique among the other strains of the *Microbulbifer/ Teredinibacter/ Saccharophagus* group and even among any other known bacteria. The phylogenetic and phenotypic analyses presented here, together with other published recommendations (40, 123) support the classification of strain 2-40 ^{T} as gen. nov. *Saccharophagus* sp. nov. *S. degradans* 2-40^{T}.

Description of *Saccharophagus* gen. nov.

Saccharophagus (Sac′a.ro.pha.gus. Gr.n. *saccharon* sugar; Gr.masc. n. phagos, glutton; N.L. masc. n. *Saccharophagus*).

Gram-negative, motile, heterotrophic, pleomorphic, rod shaped, aerobic, catalase positive, oxidase positive. Numerous cell surface blebs and vesicles are produced. Degrades numerous complex polysaccharides. Requires sea salts for growth. The type species is *S. degradans*.

Description of *Saccharophagus degradans* sp. nov.

(de.gra′dans L. part. adj. *degradans*, bring back into the former order).

Cells are pleomorphic rods, averaging 1.5 to 3.0 μm long x 0.5μm wide during the log growth in glucose. In media containing complex polysaccharides as sole carbon sources, cells can be pleomorphic and produce surface protuberances and vesicles. Cells form coils and filaments when grown at high salinity. Colonies are cream color then turn black upon eumelanin production. Colonies rapidly pit agar plates. Capable of utilizing the following complex carbohydrates as a sole carbon source agar, alginate, chitin, cellulose, fucoidan, laminarin, pectin, pullulan, starch, and xylan. Hydrolyzes tyrosine. Temperature range for growth is 4-37 degrees with an optimum of 30 degrees. Optimum pH for growth is 7.5 with a range of 4.5-10. Requires sea salts for growth with range of 1-10% and an optimum of 3.5%. Secretes proteases. The %G+C content is 45.8 as determined by genomic sequencing. Isolated from the surface of degrading salt marsh cord grass, *Spartina alterniflora* in the lower Chesapeake Bay, Mathews County VA and deposited in the American Type Culture Collection (ATCC43961 ^{T}) and in the German Collection of Microorganisms and Cell Cultures collection (17024^{T}). The type strain (and only strain to date) is 2-40$^{T.}$

Chapter 3: Characterization of the Agarase System of *S. degradans*

Prior to this work little was known about the structure of agarases. It was unknown whether these proteins shared conserved amino acid sequence; in fact, previous attempts to classify agarases based on primary amino acid sequence were unsuccessful (127, 148). The modular structure of agarases as a whole was thus unknown including conserved glycoside hydrolase (GH) domains and carbohydrate binding modules (CBM). The agarase system of *S. degradans* was chosen for study because it represented a relatively unknown class of glycoside hydrolase enzymes that likely contained unusual GH and possibly CBM domains.

Preliminary data suggested that *S. degradans* degrades agarose as a sole carbon source with multiple secreted β-agarases (140). Activities suggestive of β-agarase I, β-agarase II, and α-neoagarobiose hydrolase enzymes were detected (126) in culture supernatants and whole cell lysates. The agarase system from *S. degradans* appeared unusually efficient; growth occurred more rapidly than with chitin or cellulose (unpublished observations; N. Ekborg, L. Taylor, M. Howard). Furthermore, *S. degradans* degrades agar at a faster rate than *P. atlantica* (R. Weiner, unpublished results). *S. degradans* appeared to utilize at least three proteins to depolymerize agarose; however, the amino acid sequences of these proteins were unknown. Acquiring these protein sequences would allow for the further understanding of how *S. degradans* degrades agarose.

Interestingly, immunolocalization studies suggested that *S. degradans* attached a 98kDa β-agarase to its cell surface (139). This protein appeared to localize to cell surface protuberances that appeared similar to the cellulosomes encoded for by the cellulolytic *Clostridia* (139). Furthermore, *S. degradans* cross-reacted with an anti-sera specific for the cellulosomes signature protein CipA from *C. thermocellum* (139). This suggested that the agarases from *S. degradans* may contain dockerin repeats similar to those found in cellulases from *C. thermocellum* that allow for cell surface localization.

It was thought that the agarases of *S. degradans* contained domains that allowed these proteins to efficiently degrade agarose and, perhaps for some, to be attached to the cell surface. Because a feasible genetic system was not available (e.g. transformation, mutagenesis protocols were not established) for *S. degradans* several alternative approaches were utilized to elucidate the protein sequences of these enzymes. These included many techniques not previously performed on *S. degradans* including genomic library construction and screening, expression of target proteins in *E. coli*, genomic analysis, proteomic analysis, and a newly developed mutagenic technique capable of creating site specific null mutations in *S. degradans*. This is the first report of an efficient mutagenic technique for use in S. *degradans*. The data generated in this chapter have led to a model of agarose degradation by several proteins containing unusual GH domains and CBM.

Materials and Methods

Bacterial strains and plasmids: Strains and plasmids are described in Table 3.1. *S. degradans* strain 2-40^{T} was grown in minimal medium containing: 2.3% Instant Ocean (Aquarium Systems, Mentor, Ohio), 0.5% ammonium chloride and 50 mM TrisCl, pH 7.6. Carbon sources were added to a final concentration of 0.2%. 1.5% agar was added to solid media. Cultures of *S. degradans* were incubated at 30°C. *Escherichia coli* EC300^{TM}, DH5α-E, and Tuner strains were grown at 37°C in Luria-Bertani (LB) broth or agar supplemented with the appropriate antibiotics. Antibiotics were added to media at the indicated concentrations (in μg/ml): ampicillin (200), kanamycin (50), and chloramphenicol (30) where indicated.

Molecular biology protocols: DNA manipulations were performed using standard protocols (118). Restriction enzymes and T4 DNA ligase were obtained from New England Biolabs (Ipswich, MA). The pETBlue-2 expression vector was purchased from Novagen (Madison, Wis.). All other reagents and substrates were obtained from Sigma-Aldrich (St. Louis, Mo.) unless otherwise noted. Polymerase chain reactions (PCR) were performed using a Hybaid PCR Sprint Thermal Cycler and employed either Taq (Invitrogen, Carlsbad, CA) or ProofPro (Continental Lab Products, San Diego, CA) polymerases using the manufacturer's recommended conditions. The nucleotide sequence of plasmid DNA or gel-purified PCR products was obtained at the UMBI sequencing facility.

GenBank Accession Numbers: The sequences for *agaA*, *agaB*, *agaC agaD*, *agaE* and *galA* are reported under the GenBank accession numbers ZP_00067773, Bankit637526, ZP_00068378, ZP_00064995, Bankit628083, and ZP_00067772.

Chromosomal DNA Isolation: A 50ml culture of *S. degradans* was harvested at OD_{600} = 0.5 to 0.8 by centrifugation (8000xg, 10 min). The pellet was resuspended with 4.75ml of TE buffer pH8.0, transferred to a Corex™ tube and placed on ice. To this suspension, 62.5µl of 20% SDS and 12.5µl of a 20% proteinase K solution was added. This was incubated at 37°C for 1 hour. To this solution 600µL of 5M NaCl was added and the solution slowly mixed, 375µL of 10% CTAB in 0.7M NaCl prewarmed at 65°C was added and the solution incubated at 65°C for 20min. Six ml of a chloroform isoamyl alcohol solution (24:1) was added and mixed by pipeting then centrifuged at 8,000xg for 15 min. The aqueous layer was transferred to a fresh tube and extracted with the same solution until a minimal amount of debris was present at the solvent interface. The aqueous solution was transferred to a fresh tube and 0.65 volumes of isopropyl alcohol were added to precipitate the DNA. The DNA was collected by spooling onto a glass capillary tube, rinsed with 1ml of 100% ethanol, and air-dried for 15 minutes. The spooled DNA was placed into 1ml of TE buffer along with 1µL of an RNase solution (100mg/ml) and incubated for 1 hour at 37°C. The DNA was precipitated with 0.3M sodium acetate (final concentration) and two volumes of 100% ethanol. After incubating at -20°C for 30 minutes the precipitated DNA was spooled, washed with 0.5ml of ethanol, and air-dried until there was no scent of ethanol. The DNA was eluted in 1ml of TE buffer overnight at 4°C.

Construction and initial screen of *S. degradans* genomic library: The genomic library was constructed using a CopyControl™ Fosmid Library Production Kit (Epicentre Technologies, Madison, WI) by following the manufacture's recommended protocol. Briefly, randomly sheared DNA were generated by multiple passes through a 10µl glass syringe (Hamilton; Reno, NV). Fragments of approximately 40kb were isolated by gel extraction using a provided proprietary gel-digesting gelase™ enzyme. The extracted DNA was treated with an end-repair enzyme mix and ligated with Fast-Link™ DNA ligase into the linear fosmid, pCC1FOS™. The fosmid was packaged into lambda phage using MaxPlax™ Lambda packaging extracts and used to transfect *E. coli* EPI300™.

Sequencing of pNE10 insert: pNE10 was isolated and digested with Sau3A and fragments of 5-10Kb were isolated by gel purification. These fragments were ligated into BamHI- digested pUC19 and transformed into *E. coli* DH5α-E. Random pUC19 derivatives were isolated and a partial DNA sequence of the insert obtained by using the M13REV (CAGGAAACAGCTATGACC) and the M13(-21) (TGTAAAACGACGGCCAGT) primers. After similarity searches, the nucleotide sequence of the ORFs of interest were completed by primer walking using synthetic oligonucleotides (Table 3.2).

Bioinformatic tools: Protein modules and domains were identified in deduced products using the Simple Modular Architecture Tool (SMART), pFAM database (www.smart.embl-heidelberg.de), and the CAZYsite. Similarity searches were performed using the BLAST algorithm at the National Center for Biotechnology Information (NCBI)

server (www.ncbi.nih.nlm.gov). Type II secretion signals were identified using the SignalP version 1.1 program (www.cbs.dtu.dk/services/SignalP). Multiple-sequence alignments were performed using the ClustalW (www.searchlauncher.bcm.tmc.edu). Molecular masses of polypeptide products were estimated using the Peptide Mass Tool at the ExPASy server of the Swiss Institute of Bioinformatics (www.us.expasy.org).

Zymograms: Concentrated culture supernatants of *S. degradans* were prepared from 50ml cultures grown at 30°C for 16h in minimal medium supplemented with 0.2% agarose as the sole carbon source. All subsequent steps were performed at 4°C. Cultures were harvested at 10,000 x *g* for 20 min and the supernatants collected. Supernatants were filtered through a 0.22-μm pore filter and the cell-free filtrates were concentrated approximately 100-fold using a centrifugal concentrator with a 10-kDa cutoff filter (Millipore, Billerica MA).

The concentrated culture supernatants were fractionated by SDS-PAGE in an 8% polyacrylamide gel supplemented with 0.1% agarose. Gels were washed twice in 20ml Pipes-Triton buffer (20mM PIPES and 2.5% Triton X-100) for 20 minutes at room temperature and incubated in PIPES-Triton buffer overnight at 4°C. Gels were washed twice with 20 ml PIPES Buffer and incubated at 42°C for 2 hours. Zymograms were stained with Gram's iodine solution to identify agarase activity.

Protein expression and purification: Genes of interest were amplified by PCR using tailed primers (Table 3.2). Each fragment was digested with the designed restriction enzyme, ligated into the pETBlue-2 and transformed into *E. coli* Tuner or *E. coli* DH5α-E

cells. A 50ml culture of each transformant carrying a clone of interest was grown at 37°C to an OD_{600} of 0.5 and induced with 1 mM (final concentration) isopropyl-ß-D-thiogalactopyranoside (IPTG). After growth for an additional 4 h at 37°C, cells were harvested and frozen at -20°C. His fusions were purified from lysed cells by using Ni-NTA resin (Qiagen, Valencia CA) according to the manufacturer's recommendations.

Assay of agarase activity and identification of reaction products: Reactions with purified agarase and agarose were performed in 50 µl reactions containing 40ul of purified agarase and 5 µl of 1% agarose. Neoagarohexaose, neoagarotetraose, neoagarobiose and D-galactose (5 µg) were used as standards. The reactions were incubated at 42°C for two hours. The reaction mixture was applied to a Whatman silica gel 60A plate with a 250 µm layer. The plates were developed with 2:1:1 n-butanol:acetic acid:water solution and stained with 2:1 ethanolic sulfuric acid: naphthresorcinol solution as previously described (47). Degradation products were visualized by baking at 80°C for 10 minutes.

Mass spectrometry: Culture supernatants were fractionated on 8% SDS-PAGE gels and stained with Sypro Ruby Red (Molecular Probes, Eugene OR). Gel slices of interest were excised and chopped into 1-2 mm cubes. The samples were submitted to the Stanford University Mass Spectrometry Laboratory (SUMS) for LC-MS/MS analysis following trypsin digestion. Mascot analysis of the protein fragments was used to identify the protein fragments.

Immunoblots: Proteins were fractionated by SDS-PAGE as described above and electroblotted onto supported nitrocellulose (0.45µm pore; Osmonics Trevose, PA). Membranes were blocked with 1% alkali-soluble casein (Novagen) and incubated with mouse anti-His (Novagen). The membranes were washed twice and incubated with goat anti-mouse conjugated horseradish peroxidase (HRP). The immunoreactive proteins were visualized using an ECL detection kit (Amersham Pharmacia Biotech).

Generation of Site Directed Null Mutations: The procedure used to generate the *agaE* mutants was similar to that previously described (95, 105). Briefly, the 1kb upstream and downstream flanks of the agarase of interest were amplified by PCR with primers containing 5' tails complementary to the ends of an amplified kanamycin resistance cassette. The three products were mixed and a splicing PCR was employed to generate a 3kb product. This product was mixed with an actively growing culture of *S. degradans* grown in minimal medium with glucose and allowed to incubate with shaking at 28°C for 2 hours. The entire culture was plated onto solid medium with glucose and kanamycin. After 3-4 days growth small colonies were observed and streaked for purification onto fresh media. Single colonies from these plates were screened by PCR for the presence of the kanamycin resistance cassette confirming the presence and location of the insertion.

Table 3.1

Strains and plasmids used

Strain or Plasmid	Description or use	Source
E. coli EPI300™	F- *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80d*lacZ*ΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139 Δ(*ara*, *leu*)7697 *gal*U *gal*K λ- *rps*L *nup*G *trf*A *ton*A *dhfr*	Epicentre
E. coli Tuner™ (DE3)(pLacI)	F– *ompT gal* [*dcm*] [*lon*] *hsdSB* (r_B^- m_B^-; an *E. coli* B strain) with DE3, a λ prophage carrying the T7 RNA polymerase gene, *lacZY* deletion; Cm^R	Novagen
E. coli DH5α-E™	$F^–$ 80d*lacZ*ΔM15 Δ(*lacZYA-argF*) U169 *rec*A1 *end*A1 *hsd*R17($r_k^–$, m_k^+) *gal*– *pho*A *sup*E44 $λ^–$ *thi*–1 *gyr*A96 *rel*A1	Invitrogen
pCC1-Fos™	copy-control fosmid cloning vector; Chl^R	Epicentre
pET-Blue2™	IPTG inducible expression vectore; creates hexa-His carboxy terminal fusions	Novagen

Table 3.2

Oligonucleotide primers used

Primer	Sequence	Use
agaF1	TATTTAAAGAGCCGTAT	Primer walking
agaR1	ATTAATCATTGCCACTGCTT	Primer walking
agaF2	GTTGGAATTCTGGGTTTGC	Primer walking
agaR2	CTCTGCCCATCCACAAAAT	Primer walking
agaF3	GCACTATATAGTCGACGTTTT	Primer walking
agaR4	AGAGTAAGGAGCTATCCATG	Primer walking
agaR3	AGTTGGCAGAGTAATGCGC	Primer walking
agaF2-1	ATGCCGATGTTGTAAGTTA	Primer walking
agaR8	AGACTTGCGCCTTACACAC	Primer walking
agaF7	TTCACTGGCCGTCGTTTTAC	Primer walking
agaF6	GATCGCAATCACCCTTCCAT	Primer walking
agaR7	TGGTAGCATAGAGTTTTTGG	Primer walking
agaF5	GCGTTTAACTCACCAAGATTG	Primer walking
agaR6	TTAGGGATTGGGCAGACT	Primer walking
agaF4	GGATGGTGCAACAGCGTATT	Primer walking
agaR5	GACTACCAACCGAAAGATGC	Primer walking

agaB-f	AACTGCAGATCCATGAAAACCACAAATGC	cloning agaB
agaB-r	CCATCGATCTTATCTAGGTTCCACTGCCA	cloning agaB
agaB968R	CCATCGATACCTGTGGCAGAGAAGTTG	truncation analysis of agaB
agaB853R	CCATCGATGTAGAATCGCACCCAGTCAAT	truncation analysis of agaB
agaB736F	CTTGGCGCGCCGGCGCGACGAACAAGGTA	truncation analysis of agaB
agaB1371R	CCATCGAATGTACTGGGTGGATTGGTG	truncation analysis of agaB
agaE-f	CTTGGCGCGCCGAGTCGCTTTTATCAT	cloning agaE
agaE-r	CCATCGATTCTATTTGGCTCAGAAGT	cloning agaE
agaB767R	CCATCGATGCGCCAAGGCTGATGCTGT	cloning agaE
agaE-CF	CTCTCATCAACCGTGGCGAGGTCGGCGCAAACTGTCA	Null mutation of AgaE
agaE-CR	ACACATTGCGATAGTCACGC	Null mutation of AgaE
agaE-NF	CCGCTGCGCTGTGAGTATC	Null mutation of AgaE
agaA-CF	CTCTCATCAACCGTGGGCTTATTTACGCAGTGTTAGG	Null mutation of agaA
agaA-CR	CTCTTTCGCGTTAGCATCTAA	Null mutation of agaA
agaA-NF	CAGAGCCTTCTTTACCTGTG	Null mutation of agaA
agaA-NR	CGATGATGGTTGAGATGTGTTTATGTCTGATGGCTAAACGA	Null mutation of agaA

Results

Development of an *S. degradans* Genomic Library

A genetic approach was undertaken to identify the agarases encoded by *S. degradans*. *S. degradans* was initially assessed for the use of common genetic tools, such as electroporation, conjugation, and transposon mutagenesis. These tools, unfortunately, did not function in *S. degradans*. Consequently, genomic libraries of *S. degradans* were expressed in *E. coli* and screened for agarolytic activity as indicated by an agar pitting phenotype. Initially, the libraries were constructed in the cosmid pWEB as well as pACYC184 and pBR322. Pitting colonies were not identified in these libraries after extensive screening (3-5x genome coverage). It was surmised that the agarases encoded by *S. degradans* might be toxic to the *E. coli* host and thus not detected.

Because the previous vectors were multi-copy plasmids it was thought that reducing the copy number might reduce the expression of any potentially toxic gene products. This was accomplished using a fosmid genomic library constructed in a beta-trial version of pCC1FOS^{TM} (gifted from Epicentre Technologies). This vector could be maintained as a single or multicopy plasmid in *E. coli* EPI300 by the absence or presence of a proprietary inducing agent. Pitting colonies, indicative of the hydrolysis of agar, were observed in the pCC1-240-NE genomic library at a frequency of 1.7×10^{-3} when cells were grown under single copy conditions for the fosmid. This was the expected frequency for a

single gene in this library. This library was subsequently used to identify multiple CP degrading *E. coli* clones. *E. coli* EPI300 transfectants capable of degrading chitin, pectate, starch, cellulose, β-glucan and xylan were identified in multiple screens of this library (70). A total of nine transformants that pitted agar were identified in multiple library screens. Agarase positive clones were not identified during multi-copy conditions. In fact, growth of multiple randomly selected clones (17/20) was inhibited under multi-copy conditions. *E. coli* EPI300 (pNE10) pitted agar plates the quickest and was chosen for further analysis.

Identification of *agaA* and *agaB*

Phenotypic instability of *E. coli* pNE10 was observed as spontaneous agarase negative colonies appeared at a high frequency (See Appendix I). This precluded the use of transposon mutagenesis to identify the agarase encoded by pNE10. The agarase encoding nucleotide sequence was, therefore, obtained by random sequencing of pNE10. Three ORFs that were likely involved in the degradation of agar were identified (Table 3.3). The first, *agaA*, encoded a deduced product of 776 amino acids (aa) with a predicted molecular weight (MW) of 87kDa. AgaA was 44% identical and 62% similar to a β-agarase from *Vibrio* sp. strain JT0107 (SR6651). The second ORF, *agaB* was predicted to encode a 593 aa product with a MW of 64kDa. AgaB was 54% identical/69% similar to the β-agarase from *Microbulbifer* sp. JAMB-A7 (BAC990221). *agaA* appeared to be divergently expressed from *agaB* from a shared promoter region. The third ORF, *galA*,

was 304 bp downstream of *agaA*. It encoded an 859 aa deduced product with a predicted MW of 97kDa that exhibited 30% identity and 45% similarity to the β-galactosidase from *Clostridium perfringens* (BAB80972.1). PCR primers were constructed and used to screen the remaining eight agarolytic *E. coli* ECI300 clones for the presence of *agaA* and *agaB*. Each of the clones yielded products consistent with the presence of *agaA* and *agaB* indicating that similar fragments were present in each clone. However, the agarase activity observed for *E. coli* EC300 (pNE10) did not account for the entire activity observed in supernatants of *S. degradans*. The supernatant contained an additional approximately 100kDa protein, suggesting, the presence of other unidentified agarases encoded by *S. degradans* (Figure 3.1).

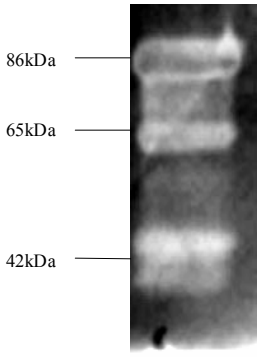
Genomic Sequence of *S. degradans*

Concurrently with the identification of *agaA*, *agaB,* and *galA* by conventional library screens, a genomic sequencing project was initiated with the US Department of Energy. Nucleotide sequence was derived by random shotgun sequencing of the pCC1-240-NE genomic library described in the previous section. This library was used because it represented the most complete assemblage of *S. degradans* genomic DNA as indicated by the various complex polysaccharide degrading clones, including agarases, detected. Multiple unfinished versions of this data were made available throughout the project. Fragments of *agaA* and *agaB* were identified; however, they were located at the ends of two different contigs.

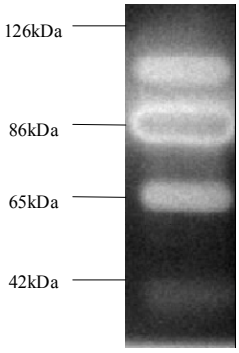
Figure 3.1

Zymogram analysis to detect agarase active proteins in cell lysates of *E. coli* (pNE10)

A cell lysate of *E. coli* (pNE10) was fractionated on an agarose zymogram and compared to a concentrated culture supernatant of *S. degradans*. The proteins were refolded in gel, incubated and stained with an iodine solution to detect agarase depolymerizing proteins. Prestained molecular weight standards (left) were used and marked on the gel before iodine staining.



E. coli pNE10



S. degradans

The sequences derived from pNE10 were used to link these two contigs. The completed genomic sequence of *S. degradans* became available in February 2005. It currently exists as one contiguous strand of DNA assembled in a linear unclosed state. The completed genome is 5.05 Mb constructed from sequence with 9x coverage. Currently there are 3982 open reading frames. The %G+C content is 45.8%.

The data generated with the pCC1-240-NE genomic library has allowed for the prediction of enzymes from various complex polysaccharide (CP) degradation pathways including chitin (68-71), cellulose, β-glucan, xylan (LE Taylor; unpublished data) and alginic acid (2), as well as multiple predicted CP degrading enzymes. The annotation data obtained from Computational Biology Program at Oak Ridge National Labs led to the prediction of 130 proteins involved in the degradation of CPs. These ORFs appeared scattered through out the genome in small loosely defined gene clusters.

Identification of candidate agarases in the *S. degradans* genomic sequence

Previous studies indicated that *S. degradans* secretes at least three agarases; however, only two candidate agarases, AgaA and AgaB, had been identified in the preceding genomic library screen. Surprisingly, the annotated genomic data did not predict the presence of any putative agarases. Using the agarase protein sequences available in the

GenBank database, three additional predicted agarases were identified by BLAST similarity searches. AgaC was predicted as a 86 kDa protein sharing 38% identity and 55% similarity to a β-agarase from *Pseudoalteromonas atlantica* (AAA25696). AgaD had a predicted MW of 89 kDa and shared 43% identity and 60% similarity to the *Vibrio sp*. JT0107 β-agarase (S46651). AgaE was the largest agarase encoded by *S. degradans* of 146 kDa and shared 60% identity and 72% similarity 42% to a β-agarase from *Microbulbifer* sp. JAMB-A7 (Table 3.3). This data suggested that *S. degradans* may produce five agarases, indicating redundant activity for at least some enzymes.

Genomic arrangement of agarase genes encoded by *S. degradans*

Upon the availability of the completed genomic sequence of *S. degradans* it was found that the five putative agarases were located in two regions of the genome. The first region (nt1470946-nt1484318) (Figure 3.2) contained the aforementioned *agaB*, *agaA* and *galA*. Additionally, a tandem repeat downstream of *agaB* was identified (Dr. Ilya Borovich, personal communication). Other proteins predicted to function in the degradation of agar were not identified adjacent to these genes. The second region (Figure 3.3) was larger (nt3304997-nt3338165) and contained several genes conserved in other agarolytic organisms. This area of 33.2kb was defined simply by the presence of *agaD*, *agaC*, *agaE,* and other predicted proteins conserved in agarolytic organisms. This included an apparent operon containing several dehydrogenases and a conserved hypothetical protein. A nearly identical dehydrogenase operon is encoded by *Microscilla* PRE1 (148) while a similar conserved hypothetical protein is encoded by *Microscilla* PRE1 (148), *Pirellula* strain 1 (54) and multiple unidentified soil strains (138), all of which are agarolytic.

Table 3.3

Predicted agarases identified in *S. degradans*

ORF	GenBank accession number	Molecular Weight (KDa)	Closest match	GenBank accession number	% Similarity	% Identity
agaA	ZP_00315251	87	β-agarase *Vibrio* sp. str. JT0107	S46651	62	44
agaB	AAT67062	64	agarase *Pseudomonas* sp. ND137	BAB88713	69	54
agaC	ZP_00031652	86	β-agarase *Pseudoalteromonas atlantica*	AAA25696	55	38
agaD	ZP_00031360	89	β-agarase *Vibrio* sp. JT0107	S46651	60	43
agaE	ZP_00031657	146	β-agarase *Microbulbifer* JAMB-A94	BAD86832	72	60

Molecular weights were calculated using ExPASy proteomics server molecular weight calculator; Closet match, % similarity and % identity were calculated by using the BLAST algorithm.

Figure 3.2

Gene arrangement of *agaA* and *agaB* region

Sequences derived from the genomic annotation of *S. degradans* were assembled. Annotation of each predicted protein is noted above the predicted open reading frames (arrow). Inverted and direct repeats are also noted (arrow heads). The position of the genes is noted by nucleotide (nt) number.

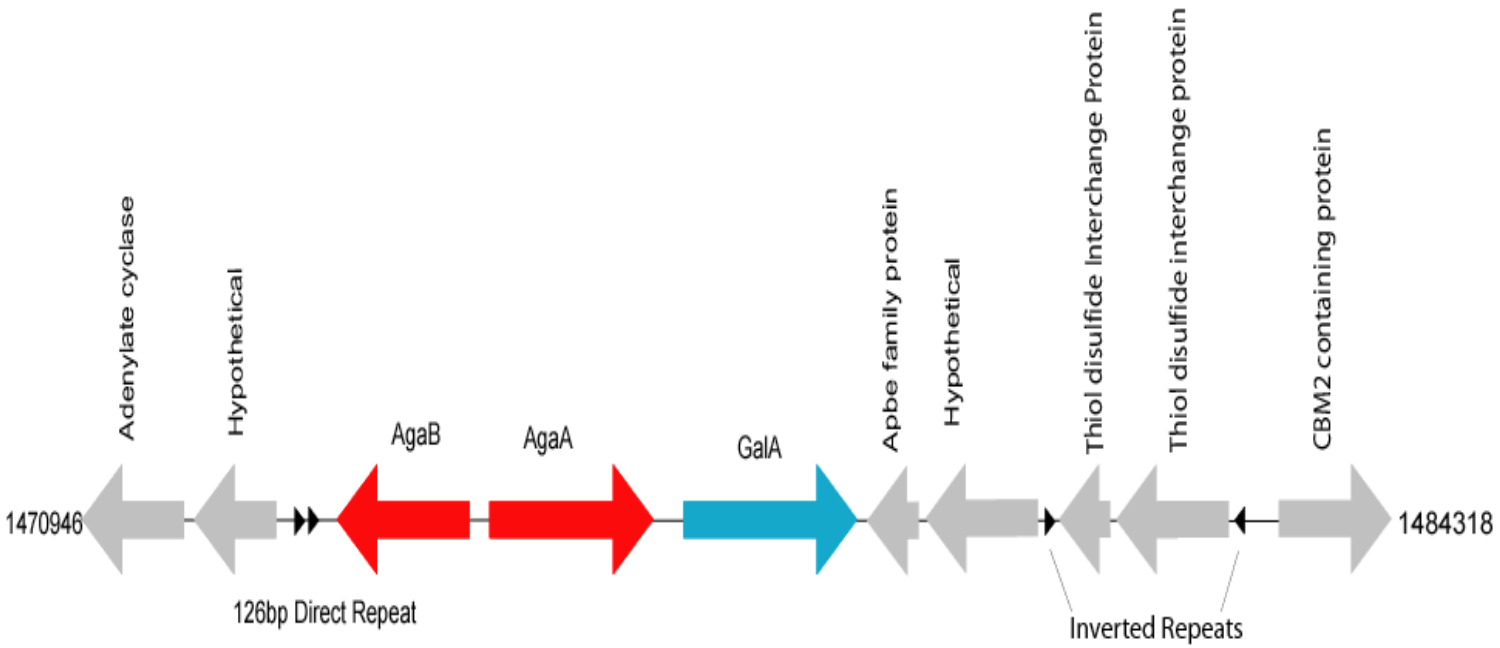
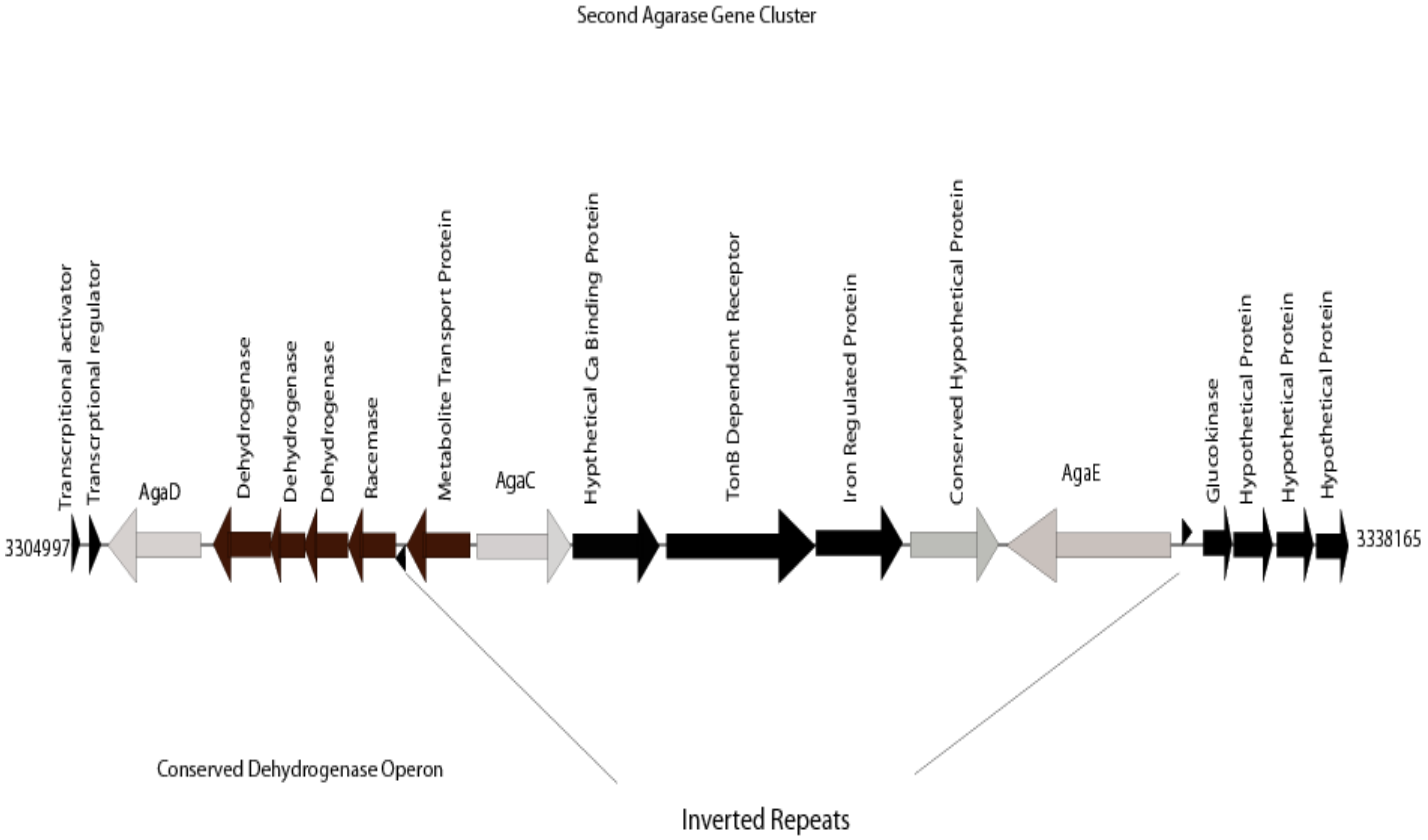

Adenylate cyclase
Hypothetical
AgaB
AgaA
GalA
Apbe family protein
Hypothetical
Thiol disulfide Interchange Protein
Thiol disulfide interchange protein
CBM2 containing protein
1470946
1484318
126bp Direct Repeat
Inverted Repeats

Figure 3.3

Gene arrangement of the *agaD*, *agaC* and *agaE* region

Sequences derived from the genomic annotation of *S. degradans* were assembled. Annotation of each predicted protein is noted above the predicted open reading frames (arrow). Inverted and direct repeats are also noted. The position of the genes is noted by nucleotide (nt) number


Second Agarase Gene Cluster
Transcrpitional activator
Transcrptional regulator
AgaD
Dehydrogenase
Dehydrogenase
Dehydrogenase
Racemase
Metabolite Transport Protein
AgaC
Hypthetical Ca Binding Protein
TonB Dependent Receptor
Iron Regulated Protein
Conserved Hypothetical Protein
AgaE
Glucokinase
Hypothetical Protein
Hypothetical Protein
Hypothetical Protein
3304997
3338165
Conserved Dehydrogenase Operon
Inverted Repeats

The function of these predicted proteins in the degradation of agar is unknown. The agarase system of *S. degradans* appears to be loosely organized lacking operons or defined gene clusters. Although it does appear that other uncharacterized proteins may be involved in the degradation of agarose.

Identification of domains present in the agarases encoded by *S. degradans*

The five predicted agarase identified in *S. degradans* allowed for the first time the analysis of several agarases from the same organism. It was thought that these enzymes would share conserved sequences that could be used to define their glycoside hydrolase (GH) domains. Whole protein alignments, however, demonstrated that the agarases from S. *degradans* shared little primary amino acid sequence similarity.

In order to simplify these alignments small sequence blocks of each predicted agarase were aligned. The blocks consisted of overlapping 50-200 amino acid regions. These analyses revealed that the agarases from *S. degradans* contained three distinct regions of shared sequence similarity. Region 1 was defined by the amino terminal region of AgaB that was not similar to the other agarases encoded by *S. degradans*. Region 2 was defined by the alignment of AgaA and AgaD. Region 3 was defined by the alignment of AgaC and AgaE. To verify these results, each agarase in the GenBank database was aligned to Region 1, Region 2 or Region 3 and categorized based on sequence similarity. It was

found that each agarase tested contained one of these three regions that existed as contiguous domains in which size and sequence were conserved. It was assumed that these regions contained the catalytic GH domains of these agarases. Later, these domains were defined as GH16 (Region 1; AgaB), GH50 (Region 3; AgaA, AgaD) and GH86 (Region 2; AgaC, AgaE) (65, 66).

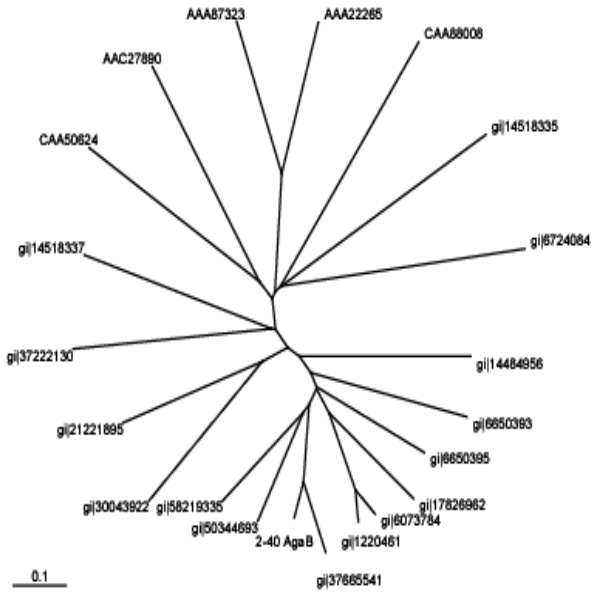
Additional domains were also identified in the agarases encoded by *S. degradans*. Unusual for agarases two type 6 carbohydrate binding modules (CBM6) were identified within the carboxy terminus region of AgaB, and three CBM6 were located within the amino terminus of AgaE. The CBM6 of AgaE were separated by repetitive linker sequences consisting of $(Glu\text{-}Pro)_{17}$ and $(Pro\text{-}X)_4$.

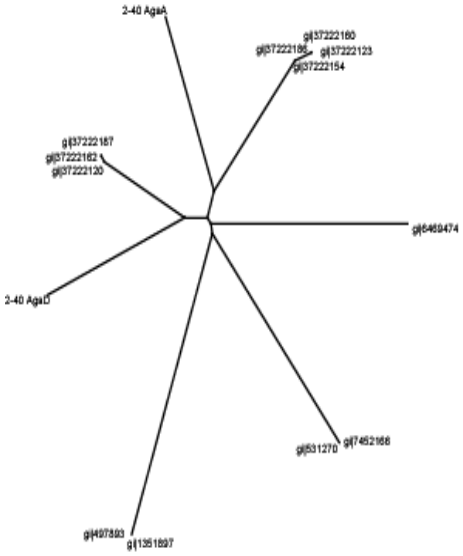
Small sequence motifs were also detected in the agarases from *S. degradans*. AgaE contained four aspartic acid rich repeats termed thrombospondin type 3 (Tsp-3) repeats between aa 511 and aa 643 (B. Henrisatt personal communication). The function of these repeats is unknown. Secretion signals were predicted for each candidate agarase by SignalP 1.1 suggesting either a periplasmic or freely secreted localization for these proteins. Additionally, a lipoprotein acylation site was identified within AgaC by the DOLOP algorithm. This suggested that AgaC could be attached to the outer-membrane of *S. degradans*.

Figure 3.4

GH16, GH50 and GH86 are found in all known and predicted agarases

Agarase sequences were retrieved from the GenBank database with the gi numbers noted. Multiple sequence alignments are graphically displayed as a radial tree to shown that each known or predicted agarase can be categorized in one of three GH families: GH16, GH50 or GH86. Agarases from *S. degradans* are labeled as AgaA, AgaB, AgaC, AgaD, and AgaE. Sequence alignments and tree construction were performed with ClustalX. The radial tree was visualized with TreeView1.1 **GH16**: (top tree, clockwise) 37222130, uncultured bacterium; 14518337 *Microscilla* PRE1; 6724084, α-agarase, *Alteromonas agarilytica;* 37665541, β-agarase, *Microbulbifer* JAMB-A7; 50344693, β-agarase, *Microbulbifer* JAMB-A94; 58219335, β-agarase Pseudomonas sp. ND137; 6650393, β-agarase, *Z. galactanivorans;* 6073784, β-agarase, *Aeromonas* sp; 1220461, *Pseudoalteromonas atlantica;* 17826962, β-agarase *Pseudomonas* sp. ND137; 6650395, β-agarase, *Zobellia galactanivorans;* 14484972, *Microscilla* PRE1; 30043922, agarase, *Pseudomonas* sp. CY24; 21221895, *Streptomyces coelicolor*; **GH50** (bottom left, clockwise**)**: 7452166, β-agarase, *Vibrio* sp. JT0107; 13518967, β-agarase *Vibrio* sp 0107;6469474, *S. coelicolor;* 3722162 and 37222120 and 37222187, uncultured bacterium;37222186 and 37222123 and 37222160 and 37222154, uncultured bacterium; **GH86**: (bottom right, clockwise) 14518314 & 14484949, Microscilla PRE1; 32443741, *Pirellula* strain 1; 14518320, *Microscilla* PRE1; 57864209, β-agarase, *Microbulbifer* JAMB-A94; 94831, β-agarase *P. atlantica*;.


AAA87323
AAA22265
CAA88008
AAC27890
gi|14518335
CAA50624
gi|6724084
gi|14518337
gi|37222130
gi|14484956
gi|6650393
gi|21221895
gi|6650395
gi|17826962
gi|30043922
gi|58219335
gi|6073784
gi|50344693
2-40 AgaB
gi|1220461
0.1
gi|37665541


2-40 AgaA
gi|37222160
gi|37222186
gi|37222123
gi|37222154
gi|37222187
gi|37222162
gi|37222120
gi|6469474
2-40 AgaD
gi|531270
gi|7452166
gi|497893
gi|1351897
0.1

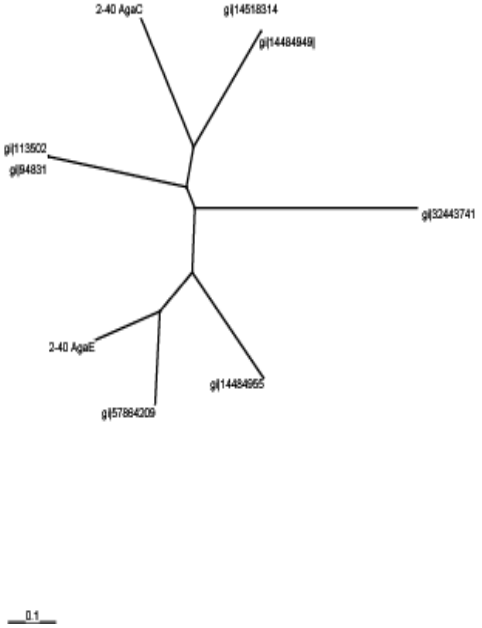
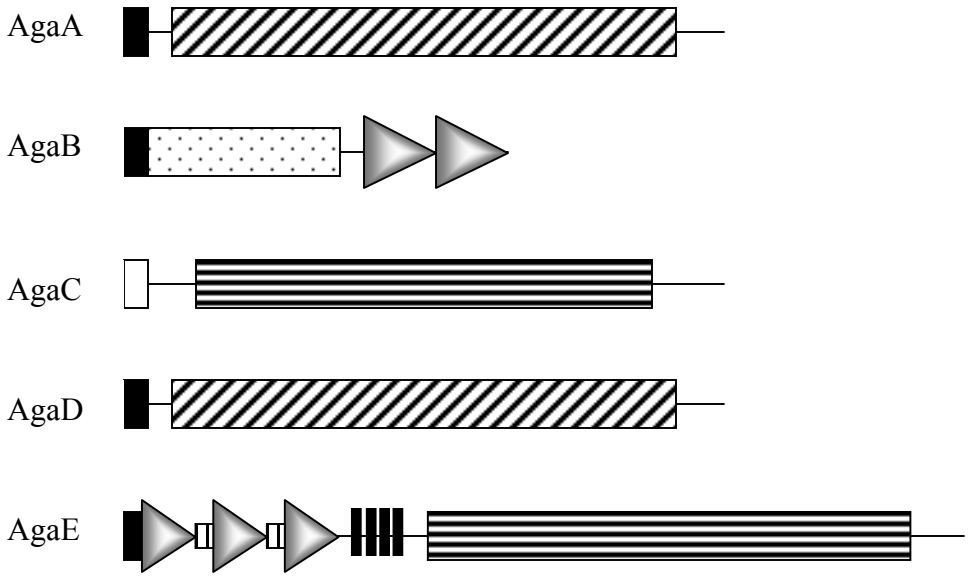

2-40 AgaC
gi|14518314
gi|14484949|
gi|113502
gi|94831
gi|32443741
2-40 AgaE
gi|14484955
gi|57864209
0.1

Figure 3.5

Domains and sequence motifs identified in the predicted agarases encoded by *S. degradans*

AgaA, AgaB, AgaC, AgaD, AgaE (top to bottom) all contained predicted Type II secretion signals (filled box) at their amino-terminus. AgaC containing an additional putative lipoprotein acylation site (open box). CBM6 domains (triangles) were identified in AgaB and AgaE by SMART domain analysis. GH domains were established by multiple sequence alignments. GH50 domains were present in AgaA and AgaD. GH86 domains were found in AgaC and AgaE and a GH16 domain was present in AgaB. Repetitive linker sequences (striped boxes) and four thrombospondin type three repeats (arrows) were present in AgaE. The sequences of the four thrombospondin type three repeats are shown below. The highlighted residues are conserved.



Tsp-1 DGDNDGVPDTSDNC
Tsp-2 DTDEDGINDKIDQC
Tsp-3 DGVLNGADQCGNTP
Tsp-4 DADNDGVANSEDTC

Biochemical Activity of the Predicted Agarases

The previous analysis indicated AgaA, AgaB, AgaC, AgaD, and AgaE contained GH domains suggestive of agarase activity. In order to determine whether these proteins were active against agarose, attempts were made to express each agarase encoded by *S. degradans* in *E. coli* as carboxy-terminal His-tagged derivatives. The purified proteins could then be tested for agarase activity by zymography and *in vitro*.

AgaB-His was purified from *E. coli* Tuner pAgaB. The purifications revealed two products, an expected 65kDa product and an 85kDa derivative (Figure 3.6) in Western blots. The *agaB-his* construct carried by pAgaB was delimited by the translational start and stop signals of the vector and should have only produced a 65 kDa product. Sequencing confirmed that the plasmid carried the correct insert. Surprisingly, agarase activity was associated with the larger 85kDa product in zymograms (Figure 3.7). The anomalous molecular weight estimation suggested that AgaB-His may be modified in an unknown fashion. Extensive analyses of this apparent modification were performed (See Appendix II). It is currently thought that the anomalous molecular weight derivative may be due to a non-denatured region of AgaB-His.

The products of AgaB-His activity upon agarose were identified by thin layer chromatography (TLC) (Figure 3.8). Typical of β-agarase I activity, neoagarotetraose and neoagarohexaose were released from agarose. Neoagarobiose could not be detected

after extended incubations. This activity defines AgaB as a β-agarase I enzyme a major depolymerase of this system.

AgaE-His was similarly prepared and analyzed. Multiple products were detected in immunoblots including the expected 146kDa protein and apparent degradation products (Figure 3.6). AgaE-His derivatives with masses of 100 and 86 kDa exhibited agarase activity, but activity was not detected for the full length protein (Figure 3.7). It was unclear whether the absence of activity for the full length protein represented a precursor state of the enzyme or a failure to renature the full length polypeptide. Similar patterns of agarase activity were detected in lysates of *E. coli* (pAgaE). Alternative translational initiation sites that could produce the observed derivatives were not present within *agaE*. The degradation of AgaE-His was unaffected by a commercially available protease inhibitor cocktail that lacked chelating agents (Sigma P8849). The resulting agarase active derivatives of 85kDa and 100kDa could suggest that the processing had occurred between the CBM6 perhaps at the sites of the repetitive linker sequences. Cleavage at these sites would yield products consistent with these observed molecular weights.

The products released by AgaE-His activity against agarose were determined by TLC. Only neoagarobiose was detected after overnight incubation with agarose (Figure 3.8). This activity is consistent with that of a β-agarase II capable of producing neoagarobiose from neoagaro-oligosaccharides in this case polymeric agarose.

The previous data suggested that the all of the CBM6 of AgaE were not necessary for the degradation of agarose in zymograms as spontaneously arising truncated derivatives of AgaE not containing the CBM6 domains were agarase active. To determine whether they were necessary for the degradation of agarose by AgaB multiple truncated derivatives of AgaB-His were constructed (Figure 3.9). Colonies expressing the derivative of AgaB-His were patched onto plates containing IPTG to induce expresson and compared to colonies expressing full length AgaB-His. Colonies containing the predicted full length GH domain pitted agar plates to the same extent as the full length AgaB-His. This indicated that the GH domain of AgaB was functional independently of the CBM6 and further supported the analysis presented previously that AgaB contained a functional GH16 domain.

The activities of AgaA, AgaC and AgaD could not be established as their expression appeared toxic to *E. coli* Tuner cells. Each was cloned in non-expressing *E. coli* DH5α-E but *E.coli* Tuner transformants could not be obtained under any condition. Similar results were obtained when only the GH50 domain of AgaA was cloned and tested in this expression system. Because of these results alternative strategies to determine their role in the degradation of agarose were used.

Figure 3.6

Purification of AgaB-His and AgaE-His from *E. coli* Tuner

AgaB-His and AgaE-His were expressed in *E. coli* Tuner and purified on a Ni-NTA column under denaturing conditions. The samples were fractionated on an 8% SDS-PAGE gel, electrotransfered to nitrocellulose membranes and probed with anti-His antibody. AgaB-His purification (left) yielded two products of 65kDa along and 85kDa. AgaE-His purification (right) yielded the expected 146kDa product along with multiple apparent degradation products of various smaller sizes. Molecular weight standards are noted in kDa.

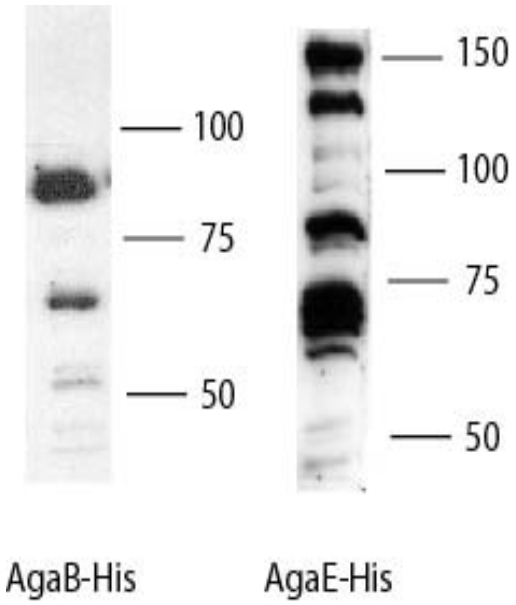

100
75
50
150
100
75
50
AgaB-His
AgaE-His

Figure 3.7

AgaB-His and AgaE-His degrade agarose in zymograms

Purified AgaB-His and AgaE-His were fractionated on an agarose zymogram, refolded *in gel*, incubated then stained with an iodine solution to detect depolymerized agarose. These samples were compared to the pattern of activity observed in samples of a concentrated *S. degradans* supernatant (left).The supernatant (left) contained three predominant active proteins of approximately 100kDa, 85kDa and 60kDa. The activity of AgaB-His (middle) was associated with the unexpected 85kDa protein. Multiple active proteins were observed with AgaE-His (right). The full length 146kDa protein did not retain activity but three apparent degradation products of 100kDa, 85kDa and 42kDa were agarase positive.

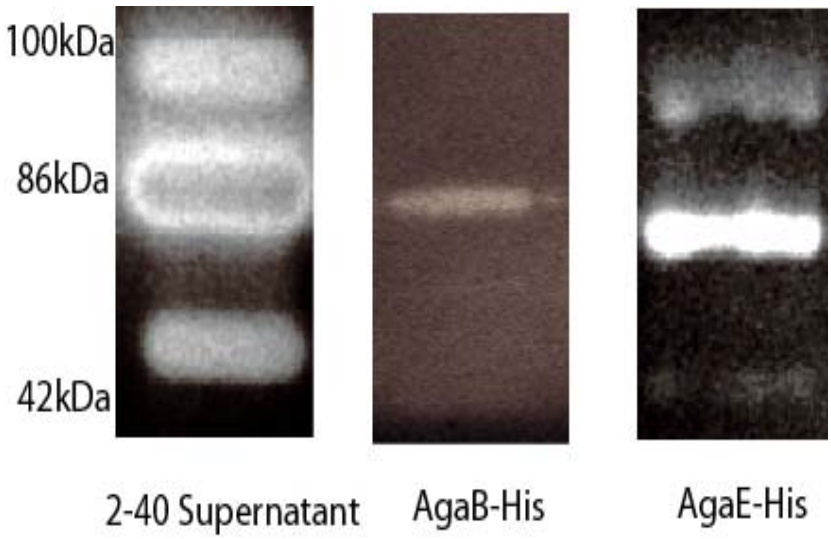

100kDa
86kDa
42kDa
2-40 Supernatant
AgaB-His
AgaE-His

Figure 3.8

Reaction products released from agarose by the activity of AgaB-His or AgaE-His

Reactions composed of agarose and purified agarase were incubated for two hours with AgaB-His or 1-2 days with AgaE-His. The sugars released by the degradation of agarose by AgaB-His and AgaE-His were resolved by TLC and compared to sugar standards. Samples: D-Galactose, lane 1; neoagarobiose, neoagarotetraose, and neoagarohexaose, lane 2, top to bottom; whole cell lysate of *S. degradans* lane 3; AgaB-His reaction, lane 4; AgaE-His reaction from a separate TLC plate, lane 5.

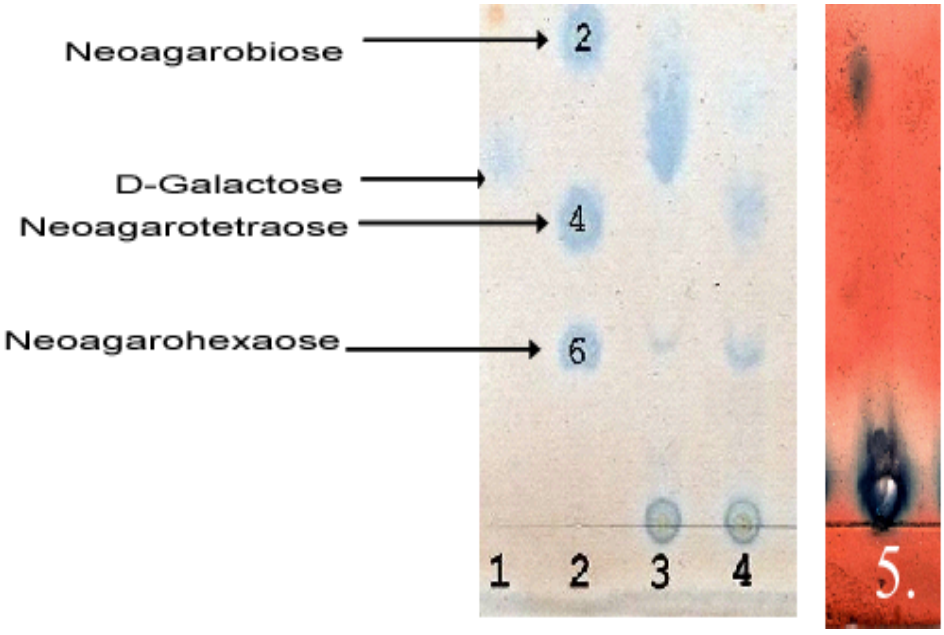
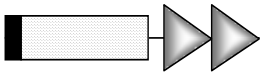

Neoagarobiose
D-Galactose
Neoagarotetraose
Neoagarohexaose
2
4
6
1
2
3
4
5.

Figure 3.9

Analysis of the agarase activity of truncated derivatives of AgaB-His

Derivatives of AgaB-His were constructed and expressed in *E. coli* Tuner. The colonies were patched onto solid media containing IPTG to induce expression. Agarase expression was observed as pitting (+) or non-pitting colonies (-). The modules present in AgaB are shown. Solid lines below indicate the regions included in the truncated derivatives. Legend: Secretion signal, black box; GH16 domain, spotted rectangle; CBM6 domains, triangles; regions included in derivatives are noted by the line below AgaB.



Agarase Activity

1 ———————— +

2 ———————— +

3 ——— -

4 ———————— -

Detection AgaB, AgaC, and AgaE by mass spectrometry

Because previous attempts to express AgaA, AgaC and AgaD did not succeed in *E. coli* alternative approaches to characterize the agarases produced by *S. degradans* were used. This included a concerted effort by multiple members of the Hutcheson and Weiner laboratory to identify the proteins secreted by *S. degradans* during growth with a complex polysaccharide as a sole carbon source. Cell free supernatants were analyzed for the constituents by HPLC coupled tandem mass spectrometry. These initial trials detected AgaB, AgaC and AgaE in supernatants from cultures grown with agarose as a sole carbon source (L. Taylor, unpublished results). Interestingly, AgaE was also detected in supernatants from cultures grown with glucose as the sole carbon source. Unfortunately, these samples were excessively complex and contained proteins that were not predicted to be secreted (e.g. DNA polymerase). This analysis, furthermore, did not allow for the assignment of specific agarase activity to the detected proteins. For these reasons a targeted survey of the agarase active proteins in culture supernatants was performed.

A selective survey of agarase active proteins was performed to decrease the complexity of the samples analyzed. Identical samples were fractionated on an SDS-PAGE-agarose gel and on a separate agarose zymogram gel. The 85kDa and 100kDa bands from the SDS-PAGE-agarose gel that corresponded to active bands in the zymogram were excised.

A 150kDa band was also excised and included in the analysis. These samples were analyzed by HPLC Tandem MS/MS following trypsin digestion (Table 3.4). The molecular weights of the detected fragments were used to search the non-redundant GenBank database which contained the predicted protein sequences from S. *degradans*. Using this technique multiple proteins were detected. The probability (P) of a random match was calculated by (-10*Log(P)) for each detected fragment. Individual ions score >52 indicate identity or extensive homology ($p<0.05$). Only proteins with ions scores greater than 50 were reported. Keratin, a common contaminant in dust, was detected, but not reported in the table of results. Each of the detected proteins had a minimum of two protein fragments identified. Three proteins were identified in the 85kDa band, AgaC, CbmX and PecX. This data suggests that AgaC is responsible for the enzyme activity observed at 85kDa as the Cbm protein did not contain a predicted GH domain and PecX contained a well conserved pectate lysase domain. The 100kDa band revealed five proteins: a xylanase, CbmX, and 3 distinct TonB dependent receptors. A predicted agarase was not detected at this molecular weight. The xylanase detected did not encode for a GH domain typical of agarase enzymes. The agarase responsible for this activity remains unknown. The 150kDa band revealed AgaE and a TonB dependent receptor. Although agarase activity was not present at this molecular weight it indicates that AgaE is produced by *S. degradans*.

Table 3.4

Proteins identified by mass spectrometry in cultures supernatants of *S. degradans*

Gel Slice	Protein Identified	Predicted MW	Queries Matched	Ions Score	Predicted Function
85kDa	2478	73	3	196	CBM4_9
	2752	75	2	88	Pectate Lyase
	AgaC	86	2	65	Secreted Agarase
100	1168	58	5	234	TBDR
	2478	73	5	193	CBM4_9
	1167	101	3	137	TBDR
	2607	98	2	89	Xylanase
	862	100	1	54	TBDR
150	AgaE	147	32	772	β-AgaraseII
	2477	131	2	64	TBDR

A concentrated culture supernatant was fractionated on an SDS-PAGE gel and specific gel fragments were excised and analyzed by Tandem MS/MS. Queries matched refer to the number of protein fragments that were detected per protein. Ions score is -10*Log(P), where P is the probability that the observed match is a random event. Individual ions score >52 indicate identity or extensive homology ($p<0.05$). Scores below 50 were not reported. Only protein fragments with high similarity to proteins encoded by *S. degradans* are included e.g. common contaminants such as keratin were not reported in this table.

Construction of null mutations in *S. degradans*

The lack of efficient genetic tools to create mutations in *S. degradans* hindered the progress of identifying other agarolytic proteins in *S. degradans*. It was unknown if AgaA or AgaD were expressed by *S. degradans* or if they were necessary for the degradation of agar. Recently, a method for the use of double stranded linear DNA to generate site specific null mutations was reported for use in naturally competent *Acinetobacter spp* (95). These organisms use so-called Com proteins to assemble a type four pilus structures that bind and imports linear DNA. Four genes predicted to act as type four pilus biogenesis proteins were identified in *S. degradans* (nt 1079246-1083698) (S. Hutcheson, personal communication). Three of these predicted proteins (ZP_00315067) were similar in sequence to several Com proteins identified in *Bacillus spp*. suggesting that *S. degradans* was naturally competent.

To test whether *S. degradans* was naturally competent a protocol similar to that used with *Acinetobacter spp* was attempted using *agaE* (Figure 3.10). First, a linear double stranded DNA fragment was constructed by PCR. The flanking sequences of *agaE* were amplified from genomic DNA by PCR using primers with 5' tails complementary to the ends of a kanamycin resistance cassette. The upstream and downstream products were spliced by PCR to the cassette creating a linear double stranded product. The desired spliced

products of 3kb along with multiple larger and smaller products were typically present (Figure3.11) after splicing reactions.

Next, a transformation protocol for *S. degradans* was optimized with respect to culture conditions. An overnight culture of *S. degradans* was used to inoculate a fresh culture (500µl) and allowed to grow for 4.5 hours. To this culture 100µl of the splicing PCR reaction was added. The culture was incubated for two additional hours then plated onto selective media containing kanamycin and glucose. A control transformation not containing DNA was included.

The resulting transformants were screened for the presence and location of the kanamycin resistance cassette by PCR. Primers specific for the kanamycin resistance cassette and the flanking sequences were used to determine whether the transformation and homologous recombinational event had occurred (Figure 3.11). This revealed that the kanamycin cassette specifically integrated into the genome of *S. degradans* within the *agaE* gene. Typical transformation frequencies were only slightly greater than that of spontaneous mutants (Table 3.5). This suggested that further optimization of the transformation protocol may be necessary for larger scale application of this method. This construct was confirmed through the use of an anti-AgaE antisera to be discussed in the following chapter, which did not cross-react with *agaE* deletion mutants. Using this method a null mutant of *agaE* was acquired and demonstrated for the first time the natural competency of *S. degradans*. This method was repeated and a null mutation of *agaA* was also acquired.

The *agaA* and *agaE* null mutants of *S. degradans* shared similar phenotypes. Both were unable to grow on solid media only containing agar as a carbon source while they did grow on solid media containing glucose and agar. The colonies slightly pitted agar plates, but much less than that observed for wild type *S. degradans*. Furthermore, when analyzed by zymography, cellular lysates of the *agaE* and *agaA* deletion mutants were similar to those of wild type *S. degradans*. This suggested that neither AgaA nor AgaE were required for the depolymerization of agarose, but likely required for the metabolism of agar. Interestingly, null mutations of *agaB, agaC* and *agaD* were not acquired although similar protocols were attempted for each for unknown reasons. Although it is possible that these mutations could lead to lethal phenotypes it is more likely that the protocol described above requires further optimization to increase the frequency of recombination.

Figure 3.10

Generation of double stranded linear DNA to transform *S. degradans*

The DNA fragment was generated by splicing PCR. First a 1kb flanking region was amplified from the 5' and 3' regions of *agaE* using the primer pairs A, X' and B, Y'. These regions were designed to have tails complementary to the 5' and 3' region of a kanamycin resistance cassette noted in black (top). These products were mixed with a kanamycin resistance cassette (Kan^R) containing regions complementary to X' and Y' denoted as X and Y (middle). A splicing PCR was performed using primers A and B generating a linear fragment containing the Kan^R cassette in place of AgaE (bottom).

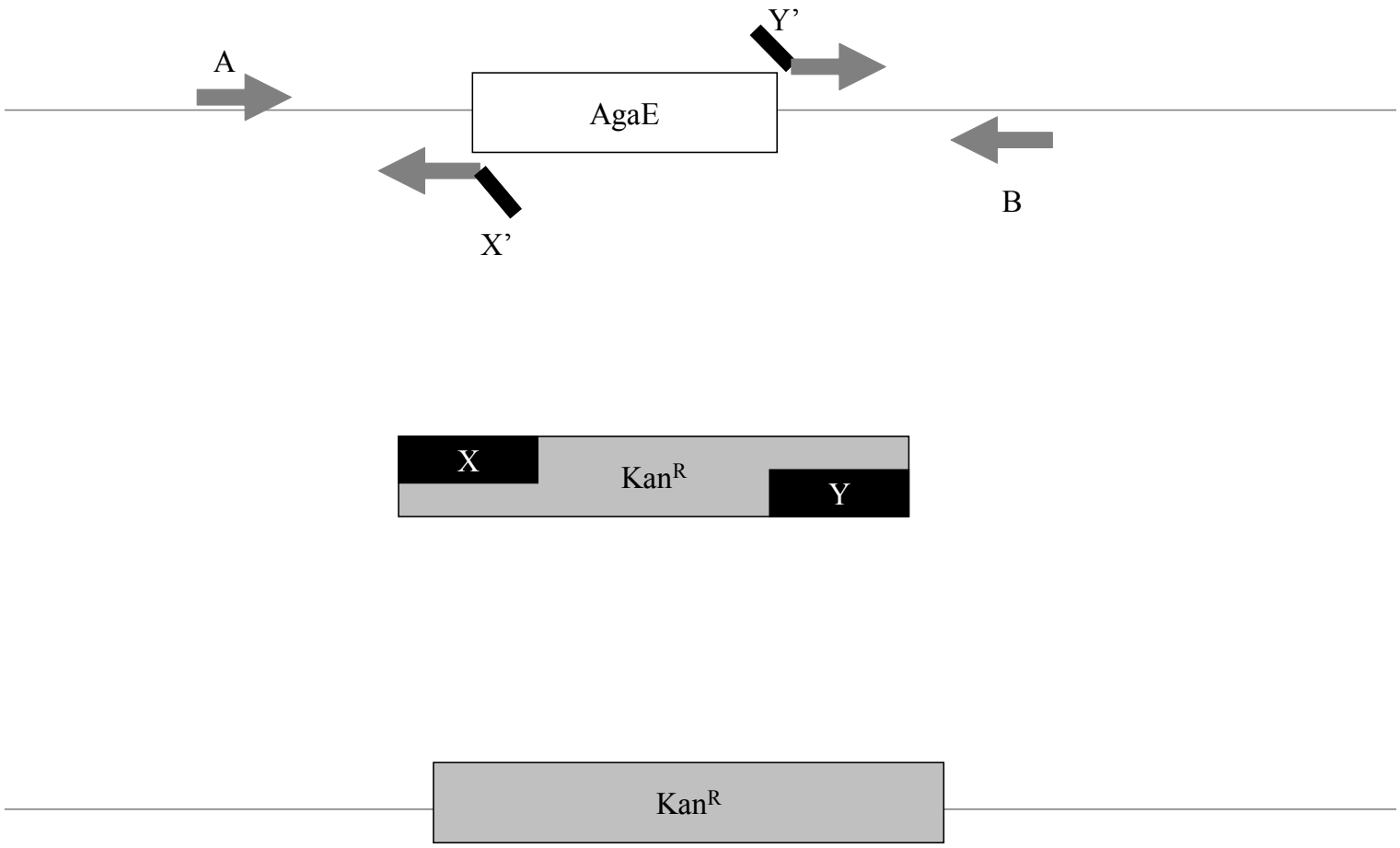
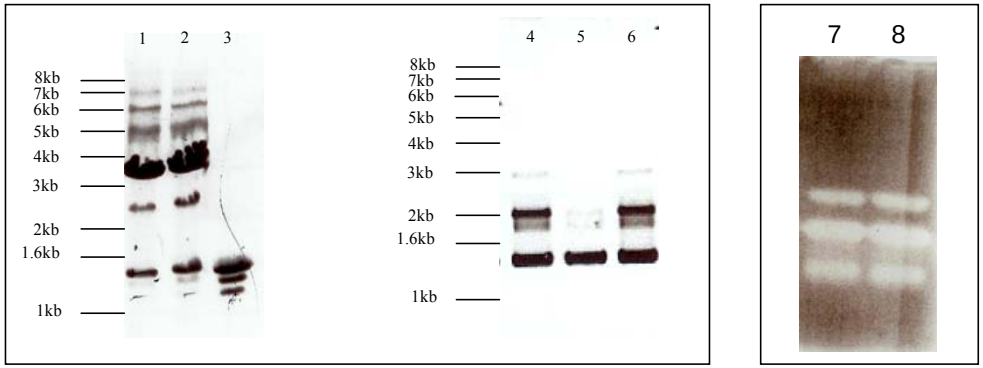

A
Y'
AgaE
B
X'
X
Kan^R
Y
Kan^R

Figure 3.11

Splicing PCR reaction to replace *agaE* and confirmation of the *agaE* and *agaA* deletion mutants

Splicing PCR reactions were performed and the resulting products fractionated by agarose gel electrophoresis (left). The entire mixture was used to transform a culture of *S. degradans*. The resulting kanamycin resistant colonies were screened by PCR for the correct construct using primers Y' and B. The splicing reaction (left) contains multiple fragments including the desired 3.2kb product. Lane: 1 and 2 splicing PCR reaction; 3, splicing PCR reaction lacking the kanamycin resistance cassette. The confirmatory PCR detected the desired 2kb product for positive recombinants and an apparent false priming site for both negative and positive yielded a 1.5kb product. Lanes: 4 and 6 positive recombinant for *agaA:kan*; 5 negative recombinant. Lanes: 7and 8 Cell lysates of the *agaA* (lane 7) and *agaE* deletion mutants (lane 8) were fractionated on an agarose zymogram. Similar patterns of degradation were noted for both.


1
2
3
8kb
7kb
6kb
5kb
4kb
3kb
2kb
1.6kb
1kb
4
5
6
8kb
7kb
6kb
5kb
4kb
3kb
2kb
1.6kb
1kb

7
8

Table 3.5

Frequency of recombination

Gene	Kan^R Frequency	Spontaneous Kan^R Frequency	Recombinants/ Spontaneous	Null Mutation Generated
agaA	$6.5x10^{-6}$	$3.4x10^{-6}$	2:1	+
agaB	$6x10^{-6}$		2:1	-
agaC	$3x10^{-6}$		1:1	-
agaD	$3x10^{-6}$		1:1	-
agaE	$6x10^{-6}$		2:1	+

Splicing reactions were used to transform *S. degradans*. Cultures were plated in the absence of selection to titer each transformation mixture. Kanamycin colonies were scored. Kan^R frequency = Kan^R colonies/titer. Spontaneous Kan^R frequency was generated by plating and scoring 5 transformation cultures lacking linear DNA plated onto selective or non-selective media. Spontaneous Kan^R frequency = Kan^R resistant CFU/ total CFU on non-selective media. Recombinants/spontaneous is the approximate ratio between Kan^R resistant CFU/ spontaneous mutants. Null mutants generated describes whether mutants were isolated and confirmed by PCR.

Discussion

Previous data suggested that *S. degradans* degrades agar using multiple secreted hydrolytic proteins (140). The data presented in this chapter identified for the first time five agarases encoded by *S. degradans*. Upon the availability of the genomic sequence it was noted that the agarases encoding genes were found in two distinct regions of the genome. These regions appeared to correspond with inverted and direct repeats suggestive of areas of lateral gene transfer (Ilya Bororvich, personal communication). Further study into the conserved regions of agarase or other hydrolytic enzymes in other organism is needed to further support this observation. The agarases encoded by *S. degradans* contained newly established catalytic domains, GH16 GH50 and GH86. These domains were identified using multiple amino acid sequence alignments. Since this initial observation similar reports have been made by other research groups (5, 6, 76). *S. degradans* is the only organism known to encode these three GH domains. This suggests that this organism utilizes structurally distinct catalytic domains to degrade agarose. This may be advantageous for the utilization of the heterogeneous forms of agar found in nature. Biochemical analyses were used along with proteomics and a newly described mutagenic technique to categorize the five predicted agarases encoded by *S. degradans*. This has led to a model of agarase activity utilizing a cascade of enzymes.

The first agarase identified in *S. degradans* was AgaB. This protein was expressed in *E. coli* and degraded agarose in zymograms and *in vitro* yielding neoagarotetraose and

neoagarohexaose. This activity was consistent with that of a β-agarase I. Furthermore, a collaborative study with Dr. Alisdair Boraston at the University of Victoria and Dr. Harry Gilbert at the University of Newcastle upon Tyne has shown that the CBM6 domains from AgaB bind neoagarohexaose. This is the first report demonstrating the ability of CBM6 to bind neoagaro-oligosaccharides.

AgaE also degraded agarose in zymograms releasing neoagarobiose from the degradation of agarose *in vitro*. This indicated β-agarase II activity. As other sugars were not detected in these reactions it is likely that AgaE is an exo-lytic enzyme. The preferred substrate for this enzyme may, in fact, be smaller neoagaro-oligosaccharides that are, unfortunately, no longer commercially available. AgaE was also detected in culture supernatants by mass spectrometry indicating that it is secreted by *S. degradans*. A null mutant of *agaE* could not grow in the presence of solely agar, but was still capable of degrading agarose. This mutant, however, did pit agar plates when grown along with glucose indicating that *agaE* was not solely responsible for agar degradation but more likely the metabolism of the released neoagarotetraose. Furthermore the zymographic profile of secreted agarases for the *agaE* null mutant was similar to that of wild-type *S. degradans*. This suggested that AgaE was required for growth on agarose but not its depolymerization and likely is the sole β-agarase II encoded by *S. degradans*. This protein also contained CBM6 that shared high similarity to the CBM6 of AgaB. This suggests that the CBM6 of AgaE may also bind neoagaro-oligosaccharides.

Similar to AgaE, AgaC also contained a GH86 domain. AgaC was detected by mass spectrometry in an agarase active protein gel slice. AgaC was the only protein detected from this region that contained a GH domain suggestive of agarase activity. This suggested that AgaC is produced by *S. degradans* and is capable of degrading agarose. To date three GH86 domains, including that from AgaE, have been identified in functional agarases. This further supports the annotation of this protein as an active agarase.

The results presented in this chapter also suggested that AgaA is an active agarase. *agaA* appeared to be divergently transcribed from a common promoter region as *agaB* suggesting the possible co-regulation of these genes. AgaA contained a GH50 domain that has only been observed in agarases. Furthermore, an *S. degradans* null mutant of *agaA* was not able to grow on agarose as a sole carbon source, but was able to pit agar plates if glucose was present. This suggested that the activity of AgaA is not redundant with that of the other agarases produced by *S. degradans*. Furthermore, when analyzed by zymography the agaA mutant had similar pattern of agarase activity as wild type. For these reasons AgaA could represent the single neoagarobiose hydrolase enzyme of this system. Because this enzyme was not detected in supernatant of *S. degradans* it is likely to be a periplasmic or cytoplasmic constituent of this system.

AgaD also contained a GH50 domain suggesting agarase activity. However, multiple attempts to characterize AgaD including expression in *E. coli*, mass spectrometry, and mutagenesis were not successful. It is currently unknown whether AgaD is expressed by

S. degradans and, if so, what its role is in the degradation of agar. It is possible that AgaD does not degrade agarose but perhaps a substituted form of this polysaccharide such as porphyran.

A model of agar degradation by *S. degradans* is proposed based on the presented data. The agarose depolymerases of this system are most likely AgaB and AgaC. AgaB appears to be a freely secreted enzyme. This protein could travel away from the cell and attach to the agarose polymer by means of its CBM6 domains. AgaC, alternatively, contains a lipobox suggestive of outer-membrane attachment. This agarase may be transiently embedded in the outer membrane of *S. degradans* then released into the extracellular milieu. A similar model of attachment was proposed for the lipoprotein pullulanase from *K. pneumoniae* (116). It appears that *S. degradans* has addressed the limitations and advantages of secreted versus cell associated enzymes by producing both classes enzymes enabling the utilization of both near-by and distant sources of agar. Selective pressure on the localization of each β-agarase may indicate how both have been maintained.

After the initial degradation of agarose by AgaB and AgaC the released sugars could then be degraded by AgaE. This protein appears to produce neoagarobiose solely. AgaE appears to be the only β-agarase II of this system. This enzyme is likely a secreted enzyme as it was detected in culture supernatants. The neoagarobiose produced by AgaE can then be degraded by either AgaA or perhaps AgaD to galactose and 3,6, anhydro-L-galactose. It is unknown whether this final step occurs extracellularly, but due to the fact

that neither has been detected in concentrated culture supernatants a periplasmic or cytoplasmic localization for each protein may exist. As described, the cascade of enzymes utilized by *S. degradans* to degrade agarose is similar to that previously described for *P. atlantica*.

In this manner the agarase system appears similar in strategy to the chitinase system of *S. degradans*. Both systems appear to contain freely secreted and membrane bound enzymes active against the polymeric substrate. In both cases one enzyme from the system is a predicted lipoprotein: AgaC in the agarase system and ChiB in the chitinase system (68). The final product of the AgaB, AgaC and AgaE extracellular degradation is most likely neoagarobiose which may diffuse into the periplasm similar to the dimer, chitobiose, produced exocellularly in the chitinase system (68). Once in the periplasm neoagarobiose could be imported into the cytoplasm or degraded to monosaccharides. The same ambiguity exists with the chitinase system in which perhaps both may occur with chitobiose (70). Considering that *S. degradans* appears to encode putative secreted β-galactosidases, such as GalA, to degrade, presumably, lactose to glucose and galactose, the α-neoagarobiose hydrolase enzyme may also be secreted into the periplasm to produce galactose. This would eliminate the need for a specific neoagarobiose inner-membrane transporter. The final products of the agarase and chitinase system are soluble monosaccharides, which are further metabolized. Unlike the chitinase system the agarase system yields a sugar, 3,6,-anhydro-L-galactose, whose biochemical fate remains unknown. Although L-conformation sugars are not typically metabolized reports exist of the metabolism of L conformation sugars, such as L-fucose, by *E. coli*. Furthermore, a

mutant of *E. coli* has been shown to utilize L-galactose as a carbon source (149). This suggests that it is possible that 3,6 anhydro-L-galactose is further metabolized by *S. degradans.*

Chapter 4: Surface Localization of a β-agarase II in *Saccharophagus degradans*

Introduction

Most microorganisms degrade complex polysaccharides (CPs) by secreting extracellular enzymes. The products, soluble sugars, are then imported. Using this strategy, enzyme and substrate diffusion away from the cell constitutes a disadvantageous bioenergetic loss. To minimize such loss, certain species of terrestrial fungi and gram-positive bacteria localize hydrolytic proteins to the cell surface in supra-molecular structures termed cellulosomes (12, 13, 21, 120). These structures have both substrate binding and hydrolytic modules to complex cell, substrate, and enzyme (12, 13, 17, 19, 21). Analogous systems have not, as yet, been described for marine microorganisms where, tidal currents can disperse macromolecules even more rapidly than in terrestrial environments.

As on land, communities of gram-negative bacteria and fungi degrade CPs in marine environments (24, 28, 100) studies of which have been mostly descriptive. Few mechanisms of cell surface (exocellular) enzyme attachment in gram-negative bacteria have been described. One such mechanism uses a recognized amino-terminal sequence motif termed a lipobox (116). This site allows the protein to be transiently embedded in

the outer-membrane via a post-translational modification whereby a cysteine residue is modified with a diacylglycerol group. The secretion signal is cleaved exposing the cysteine. This residue is then acylated with a fatty acid and embedded into the outer-membrane exposing the hydrolytic protein to the extracellular environment (73, 86). This mechanism is exemplified by PulA from *Klebsiella pneumoniae* (34-37, 116).

Other motifs for cell surface display of proteins in gram-negative bacteria exist. One example, found in integral membrane proteins, consists of β-barrels that form multiple transmembrane regions (132). Such a structure is found in the outer-membrane receptor LamB that functions in the uptake of maltose and maltodextrins. Another, less frequently, reported structure is the GPI anchoring motif. These regions are post-translationally modified with a glycosyl phosphatidylinositol moiety which is then embedded in the outer membrane. An example is the ice–nucleation protein (Inp) from *Pseudomonas syringae*. The carboxy-terminal site of modification has been determined and used for display of multiple fusion proteins (78, 79).

Saccharophagus degradans strain 2-40^{T} (*S. degradans*) is a rod-shaped aerobic bacterium isolated from the surface of decomposing saltwater cord grass, *Spartina alterniflora*, in the lower Chesapeake Bay (7). *S. degradans* is related to a group of marine gamma-proteobacteria adept at degrading complex polysaccharides, a critical function in the marine food web (48, 56). *S. degradans* can utilize at least 10 complex polysaccharides as a sole carbon source including agar, alginate, chitin, cellulose, β-glucan, laminarin, pectin, pullulan, starch, and xylan (7, 50, 70, 83). It is thought to do so through the use of

multiple cell surface hydrolytic enzymes. In fact, a recent survey indicates that 32 of the 112 predicted carbohydrases encoded by *S. degradans* are predicted lipoproteins (L. Taylor, unpublished data).

The *S. degradans* β-agarase system consists of five enzymes designated AgaA, AgaB, AgaC, AgaD and AgaE. AgaB and AgaE have been cloned and biochemically characterized. Initially, agarose is degraded by the β-agarase I, AgaB, to yield neoagarotetraose. AgaC also appears to degrade agarose, yielding unknown oligomers. AgaE, an exolytic β-agarase II, yields neoagarobiose. Deletion analysis indicates that AgaE could be the sole β-agarase II. Lastly, according to deletion analysis, neoagarobiose may be degraded to D-galactose and 3,6, anhydro-L-galactose by AgaA.

Previous evidence from immunoelectron micrographs and activity assays (126, 139), suggested that *S. degradans* synthesized exocellular agarases. Subsequent genomic analysis revealed that one, AgaC, was a lipoprotein. The size of AgaC (approximately 90kDa) was similar to the agarase previously observed in micrographs. It was unknown, however, if other agarases encoded by *S. degradans* were also exocellular.

The present study investigated the possibility that AgaB and AgaE are also exocellular. AgaB contains a glycoside hydrolase (GH) 16 domain and two, type six carbohydrate binding modules (CBM6), however, no obvious surface anchoring motifs. AgaE contains a GH86 domain, three CBM6, and four thrombospondin type three (Tsp-3) repeats. These domains bind calcium and mediate protein-protein interactions of the adhesive

glycoprotein thrombospondin(87). The function of these repeats in prokaryotic proteins is unknown, but could, arguably, mediate the exocellular attachment of proteins via a protein-protein interaction. This report describes, for the first time, the cell surface localization of the β-agarase II, AgaE. A model for the cell surface localization of AgaE is discussed.

Materials and Methods

Bacterial growth media and conditions: *E.coli* Tuner (Novagen, Madison, WI) was grown at 37C on LB agar or broth + 1% glucose and 50µg/ml ampicillin and 30µg/ml chloramphenicol. *S. degradans* strain 2-40^T (ATCC43961) was maintained on ½ strength Marine Agar: 18.7g/L Difco Marine broth 2216. Agar (Sigma) was added to solid media to a concentration of 1.5%. For growth analysis *S. degradans* was grown on minimal medium consisting of 2.3% Instant Ocean artificial sea salts (Aquarium Systems, Mentor OH), 0.1% yeast extract (Difco), 50mM Tris buffer pH7.4 and 0.05% NH_4Cl. Minimal media (MM) was supplemented with carbon sources, either glucose or agarose, to a final concentration of 0.2%. All *S. degradans* cultures were incubated at 27°C on a rotary shaker at 225rpm.

Culture and membrane fraction preparation: Broth cultures containing agarose were modified to facilitate the separation of cells and agarose. Flasks containing 10 ml of 2% agarose were autoclaved and allowed to solidify at room temperature. Sterile MM was pipeted on top of the agarose gel to a final concentration of agarose of 0.2%. Samples

were inoculated. During growth the agarose gel typically broke into many small pieces. Cells were harvested at an OD_{600}=0.3 corresponding to logarithmic growth. To harvest the cells the agarose was allowed to settle and the liquid portion was decanted into sterile 50ml centrifuge bottles. The sample was centrifuged at 2,000 x g to settle residual agarose gel and the supernatant decanted to a sterile centrifuge bottle and centrifuged at 10,000 x g for 15 minutes to pellet the cell portion. The resulting supernatant was filtered through 0.2μM filters (Millipore; Billerica, MA) to remove residual cells. The cell free supernatants were concentrated approximately 100 fold using a centricon filtering device with a 15kDa cutoff (Millipore). Portions of the cell pellet were resuspended in 50mM Tris buffer pH 7.6 containing 2.3% sea salts (SS buffer), washed 3 times to remove residual supernatant then resuspended in Tris buffer pH6.8, boiled for 5 minutes and frozen at –20°C. The remaining cell pellet was used for membrane preparation.

The pellets were washed five times with SS buffer and resuspended in ice-cold SS buffer. The suspension was pipeted into a screw cap tube with nearly equal volume of glass beads. The suspension was lysed using a Mini Bead Beater (Glen Mills) following the manufacture's protocol. The cell lysate was decanted into a clean centrifugation tube and centrifuged at 10,000 x g for 10 min at 4°C. The resulting supernatant was centrifuged at 150,000 x g for 2 hours at 4°C to pellet the total crude membrane preparations. The preparation was washed with SS buffer 3 times. These preparations yielded a clear-blue gel along with insoluble material and termed the crude membrane preparation. The crude membrane preparations were resuspended in buffer with 0.5% SDS, boiled for five minutes, and centrifuged (16,000xg) for five minutes. The resulting supernatant was the

total soluble membrane preparation. This sample was viscous and appeared very hydrophobic. The resulting insoluble material was resistant to increased amounts of SDS (up to 10%), the inclusion of reducing agents, prolonged boiling and sonication. This material was discarded. The remaining samples were tested for agarase activity.

Zymograms: Samples were fractionated by SDS-PAGE in an 8% polyacrylamide gel supplemented with 0.1% agarose. Gels were washed twice in 20ml of 20mM PIPES buffer pH6.8 containing 2.5% Triton X-100 (P-T buffer) for 20 minutes at room temperature and then incubated in P-T buffer overnight at 4°C. Gels were washed twice with 20 ml 20mM PIPES buffer pH 6.8 and incubated at 42°C for 2 hours. Zymograms were stained with an iodine solution (23) to identify agarase activity.

Generation of anti-AgaB and anti-AgaE antiserum: Antibodies were raised against approximately 5mg of purified recombinant AgaE-His (rAgaE-His) and rAgaB-His purified as described previously in Chapter 3. The resulting sample was fractionated on an 8% SDS-PAGE gel and stained with Coommassie blue. A 150kDa band corresponding to rAgaE-His and a similar band of 65kDa corresponding to rAgaB-His were excised from separate gels and washed to remove residual acetic acid. Antibodies were raised by Sigma Genosys in two New Zealand white rabbits. Preimmune sera, four production bleeds and a post bleed-out samples were acquired.

Immunoblot Detection of AgaB and AgaE: Cultures containing agarose as a sole carbon source were inoculated (0.1% inoculum/ culture) with an overnight culture of *S.*

degradans grown in MM+glucose. Samples were taken once the cultures appeared turbid at approximately an OD_{600}= 0.2. The cultures were monitored for growth by OD_{600}. Cell lysates and supernatants were harvested by centrifugation at 10,000 x g, washed three times in SS buffer and resuspended in Tris buffer pH6.8. Culture supernatants were collected, filtered through a 0.2µm filter (Millipore) and concentrated. All samples were standardized with respect to protein. Equal protein amounts were fractionated on an 8%SDS-PAGE gel, blotted on 0.2µm nitrocellulose and blocked with 0.1% hydrolyzed casein (Novagen), probed with the specific primary antibody then a donkey raised anti-rabbit secondary antibody (Amersham). The immunoreactive proteins were visualized using an ECL detection kit (Amersham).

Immuno-fluorescence: Cells from an exponentially growing culture (OD_{600}=0.3-0.5) were smeared on a glass slide, allowed to air dry then heat fixed. The samples were probed with a 1/10 dilution in PBS of either anti-AgaB or anti-AgaE for 10minutes then washed twice in a fresh beaker of PBS for 5 minutes. The sample was incubated with 1/32 dilution of a goat raised anti-rabbit FITC conjugated secondary antibody for 5 minutes then washed. Samples were observed with a Zeiss Axiophot microscope.

Immunogold transmission electron microscopy: Cultures were grown to specific culture conditions then fixed in 0.5% glutaraldehyde for at least 2 hours. Carbon- and Formvar-coated 200-mesh nickel TEM grids were floated on top of drops of samples for 10 minutes to adsorb cells. Grids were incubated in 0.05 M glycine for 20 minutes then transferred to drops containing a blocking solution consisting of 5% BSA, 5% goat

serum, 0.1% cold water fish skin gelatin (Aurion, Costeweg, The Netherlands) and PBS pH7.4 for 30 minutes. Grids were washed twice for 5 minutes in incubation buffer (IB) consisting of 0.15% Aurion BSA-c^{TM} and PBS pH 7.4. Grids were transferred to drops containing primary antibody or preimmune serum diluted 1/20 in PBS pH 7.4 for 1 hour and washed six times in IB for 5 minutes each. Grids were transferred to drops of goat raised anti-rabbit 10nm gold conjugated antibody diluted 1/30 for 1 hour. Grids were washed six times for 5 minutes each in IB then washed twice for 5 minutes each in PBS pH7.4. Samples were post fixed in 2% glutaraldehyde for 5 minutes then washed twice in double distilled water for 5 minutes each. Samples were contrasted with 2% uranyl acetate and blotted dry.

Thin Sectioning: Cells were collected by centrifugation and chemically fixed in 0.5% glutaraldehyde in SS buffer for at least 2 hours. Samples were rinsed 3 times with PBS for 5 minutes each and dehydrated in an ethanol series of 50% for 15min, 75% for 15min, 95% for 15min, 100% for 15min, 100% for 20 min and 100% for 30 min then exchanged for propelene oxide for 15min and 30 min. The samples were incubated with propelene oxide and a Eponate 12 resin (50:50) overnight then embedded in fresh Eponate 12 resin for at least 48 hours. Eponate 12 resin mixture consisted of 45.3% Eponate 12, 27.9% DDSA, 24.4% NMA and 2.4% BDMA. The embedded samples were trimmed and mounted onto metal slug mounts. The mounted samples were planed and semi-thin sections were taken and stained with 2% methylene blue to ensure sample was present. The planed surface was hand trimmed with a razor blade to a raised trapezoid 1mm x 2mm. Ultra-thin sections (gold or silver inference colors) were made with a glass knife,

exposed to chloroform vapors and collected onto 200 mesh nickel grids. Samples were immunoprobed and contrasted as described for the whole cell mounts.

Results

Biochemical Evidence of Membrane Associated Agarase Activity

Cell free culture supernatants, washed cell pellets, and crude membrane preparations were collected from cultures grown with agarose as a sole carbon source and analyzed by zymography to detect active agarases (Figure 4.1). Culture supernatants contained five active protein bands: two predominant bands at of 85kDa and 65kDa and two lesser bands at approximately 120kDa and 39kDa. This pattern of activity was distinct from that observed in the whole cell lysates, which contained two agarase active bands at 85kDa and 100kDa, but not at 65kDa. Total membrane preparations revealed three agarases of 150kDa, 100kDa, and 85kDa. The 150kDa and 100kDa proteins were only detected in membrane fractions. In fact, agarase active AgaE has only been detected at this molecular weight in membrane fractions.

The active agarases were identified using the predicted molecular weights from the available genomic sequence annotation (Table 4.1). The 65kDa band, observed solely in the supernatant sample was thought to be AgaB, a β-agarase I with a predicted molecular weight of 64kDa. AgaB was not detected in the membrane preparations. AgaC, the predicted 86kDa lipoprotein, was active in all samples including cell lysates,

supernatants, and membrane fractions. The active band at approximately 150Kd, solely detected in the membrane preparations, corresponded to the predicted size of AgaE. The 120kDa, 100kD and 39kDa proteins did not correspond to the size of any predicted agarase. These proteins could be proteolytic degradation fragments of an agarase. It is unlikely that they are unidentified agarases since the genome has been closed and the GH domains of agarases are conserved.

Antibody tools to probe AgaE and AgaB

Anti-serum specific for AgaE (α-AgaE) was prepared against recombinant AgaE-His. It was shown by immunoblot analysis to cross-react with a single protein of approximately 146kDa in whole cell lysates and concentrated culture supernatants of *S. degradans* (Figure 4.2a) Cross-reactive proteins were not observed using preimmune sera. Likewise α-AgaE did not cross-react with whole cell lysates of the expression host E. coli Tuner or recombinant AgaB-His.

Figure 4.1

Zymogram analysis to detect agarase active proteins in culture and cell fractions of *S. degradans*

Concentrated culture supernatants, cell lysates and crude membrane preparations were collected from a culture grown with agarose as the sole carbon source and fractionated on an agarose-zymogram. The proteins were refolded *in gel*, incubated and stained with an iodine solution to detect agarase depolymerizing proteins. Prestained molecular weight standards (left and right) were used and marked on the gel before iodine staining. Lanes: A, concentrated culture supernatant; B, cell lysate; C, membrane preparation. Agarase activity attributed to AgaE is noted by an arrow

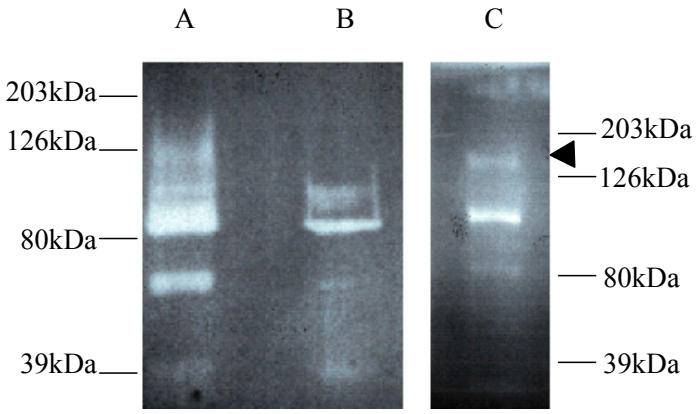

A
B
C
203kDa
126kDa
80kDa
39kDa
203kDa
126kDa
80kDa
39kDa

Table 4.1

Agarases identified by zymography in culture and cell fractions of *S. degradans*

Sample	Observed MW (kDa)	Predicted Agarase	MW Predicted Agarase (kDa)
Culture Supernatants	65	AgaB	64
	85	AgaC, AgaA, AgaB, AgaD, AgaE	86
	120	AgaE	
	39	Unknown	
Cell lysates	85	AgaC	86
	100	Unknown	
Membrane Preparations	150	AgaE	150
	85	AgaC	86
	100	Unknown	

Observed MW, determined by zymography; **Predicted agarases**, determined by the predicted molecular weights of the noted agarases. Multiple agarases are listed for regions where ambiguity exists. **Note**: AgaB is expressed as two derivative of 64 and 86 kDa;

The AgaB anti-serum (α-AgaB) was prepared from recombinant AgaB-His, and shown by immunoblot analysis to be specific for AgaB (Figure 4.2b). The α-AgaB serum cross reacted with AgaB in whole cell lysates and concentrated culture supernatants from *S. degradans* while the preimmune sera did not. Furthermore, α-AgaB did not cross-react with recombinant AgaE-His or whole cell lysates of *E. coli* Tuner.

Expression and localization of AgaE and AgaB during the growth cycle

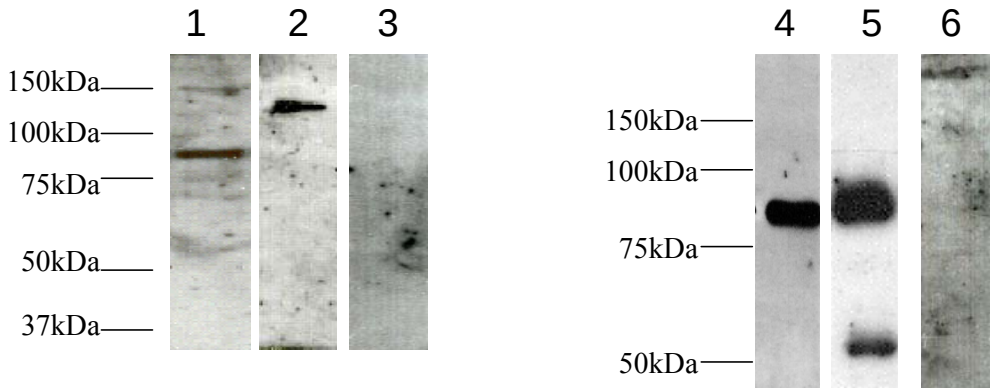
AgaE and AgaB were monitored in cell lysates and culture supernatants by immunoblot assays to determine when and where each was present in broth cultures (Figure 4.3). The cultures maintained exponential growth for approximately five hours, followed by a characteristic decrease in growth rate when the culture reached OD_{600}=0.8 and 0.9. Afterward, the culture entered the stationary phase growth.

In cultures grown with agarose as the sole carbon source, the highest concentrations of AgaB were detected in cell lysates during early exponential growth (Figure 4.3b). Thereafter, concentrations steadily declined until they were nearly undetectable as the cultures entered the stationary phase of growth (OD_{600}=0.8). Conversely, AgaB was barely detected in the supernatant during the early exponential growth (OD_{600}=0.2) when

Figure 4.2

Immunoblot detection to determine the specificity of anti-rAgaB (α-AgaB) and anti-rAgaE (α-AgaE)

Antibodies were raised against purified rAgaE-His or rAgaB-His. The resulting anti-sera were used to probe cell lysates of *S. degradans* or purified recombinant protein. Samples were fractionated on an 8% SDS-PAGE gel, blotted onto nitrocellulose and probed with either anti-His (α-His 1/1000), α-AgaB (1/1000 dilution), preimmune sera (1/1000 dilution), α-AgaE (1/100 dilution) or preimmune sera (1/100 dilution). Molecular weight standards are marked to the left or right of the gels. Lanes 1. rAgaE-His purified from *E. coli* and probed with α-AgaE; 2. An *S. degradans* cell lysate probed with α-AgaE the expected 146kDa protein is detected. 3. An *S. degradans* cell lysate probed with preimmune sera. 4. rAgaB-His purified from *E. coli* and probed with α-His; 5. An *S. degradans* cell lysate probed with α-AgaB; both expected derivatives of AgaB were detected. Later analysis demonstrated that the 65 and 85 kDa forms of AgaB can be immunoprecipitated from culture supernatants using α-AgaB (see Appendix II). 6. An S. degradans cell lysate probed with preimmune sera.


1
2
3
150kDa
100kDa
75kDa
50kDa
37kDa

4
5
6
150kDa
100kDa
75kDa
50kDa

its concentration continued to increase until the culture reached stationary phase (OD_{600}=0.8). As stationary phase progressed, concentrations of AgaB in the supernatant declined. It was concluded that AgaB was steadily secreted during exponential growth, with concentrations accumulating in the supernatant. Thereafter, secreted protease activity (M. Howard, unpublished data) may reduce its concentration.

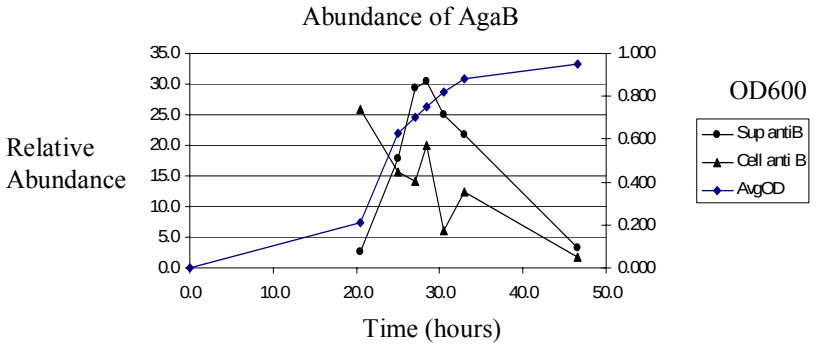
The expression pattern of AgaE was different (Figure 4.3). AgaE was detected in cell lysates at an OD_{600}= 0.2. However, unlike the case for AgaB, the concentration of AgaE in the cell lysates increased until the culture entered early stationary phase, suggesting that either the induction of AgaB and AgaE were independent of each other or AgaE remained cell associated. Since AgaE acts on polymers too large to enter the outer membrane, these considerations hinted that AgaE is exocellular during logarithmic growth and is thought to be membrane bound. As the culture progressed into stationary phase, however, the amount of cell-associated AgaE decreased as the concentration in culture supernatants increased. It is noted that the increasing amounts of AgaE observed in culture supernatants of stationary phase cultures could be due to autolysis and not only secretion.

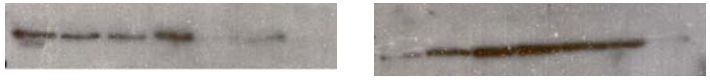
AgaB and AgaE were not detected in glucose-grown cultures until the later phases of growth, (e.g. stationary phase), in cell lysates (Figure 4.4). This result suggested that an exponentially growing culture using glucose as a sole carbon source could be used as a negative control in immuno-localization experiments.

Figure 4.3

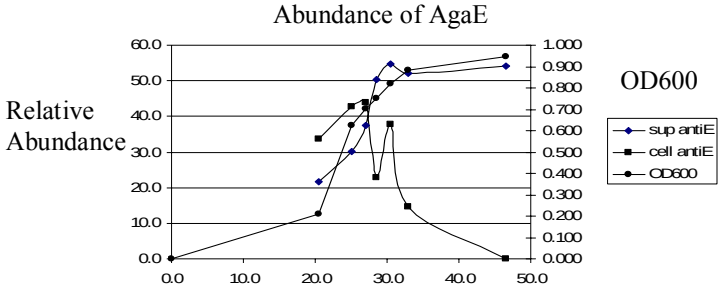
Immunoblot detection to determine the abundance of AgaE and AgaB in cell lysates and supernatants of *S. degradans* during growth with agarose as the sole carbon source

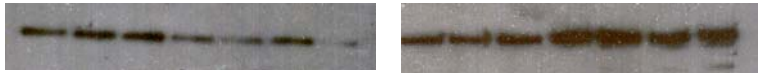
Cells were harvested from cultures monitored by absorbance as OD_{600}. Samples were standardized to protein amounts. Two μg of each protein was fractionated on an 8% SDS-PAGE gel and probed with either α-AgaB or α-AgaE. The signal intensity of the cross reactive bands was quantified by the NIH ImageJ software package and plotted as relative abundance (primary y-axis) versus time (x-axis) and OD_{600} (secondary y-axis) for both AgaE (Top) or AgaB (Bottom) Legend, Diamonds, average culture OD_{600} for two independent cultures; circles, culture supernatants; triangles, cell lysates. The corresponding Western Blots for both cell lysates and supernatants are shown below each chart. The blots correspond to the following time points in hours (left to right): 20.5, 25, 27, 28.5, 30.5, 33, 46.5.





Cell Supernatant



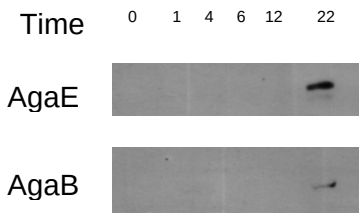


Cells Supernatants

Figure 4.4

Immunoblot of AgaE and AgaB present in cell lysates of *S. degradans* during growth with glucose as the sole carbon source

Cells were harvested from cultures and equal protein amounts (2μg) were fractionated on an 8% SDS-PAGE gel then probed with either α-AgaB or α-AgaE. The time post inoculation is shown above in hours: Top panel, cells probed with α-AgaE; Bottom panel, cells probed with α-AgaB. Only cells from stationary state cultures (22 hours post inoculation) cross-reacted with either anti-sera.


Time
0
1
4
6
12
22
AgaE
AgaB

Immuno-epifluorescence indicates AgaE is cell associated

Logarithmically growing cells in agarose fluoresced brightly in the presence of α-AgaE and FITC conjugated secondary antibody (Figure 4.5). Alternatively, cells, grown in glucose, fluoresced only slightly and controls using FITC conjugated secondary antibody only or pre-immune serum plus secondary antibody did not fluoresce at all (Figure 4.5). When the cells in phase contrast micrographs were compared with those in identical superimposed epifluorescence micrographs it was determined that 34% fluoresced (+/- 8%; N=400). Among cells grown in glucose, only 2% fluoresced (+/- 1.5%; N=400). Supporting the previous biochemical data, agarose grown cells, exposed to α-AgaB and FITC conjugated secondary antibody also did not fluoresce suggesting that AgaE is exocellular while AgaB is not.

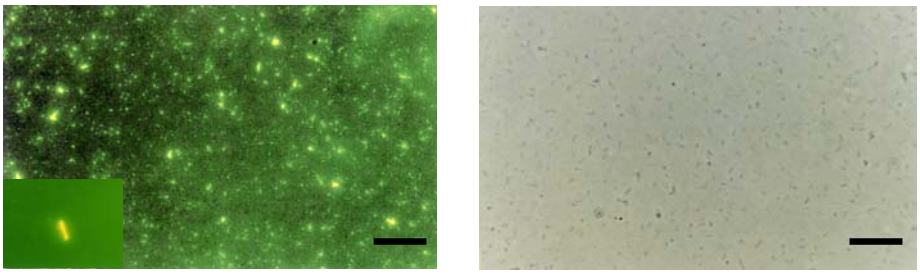
Cellular localization of AgaE and AgaB by immunogold electron microscopy

Logarithmically growing cells in agarose were probed with α-AgaE or α-AgaB along with immunogold-conjugated secondary antibody. The following negative controls were not labeled: AgaE deletion mutant strain *S. degradans* Δ*agaE*::*kan*, wild type *S. degradans* probed with secondary antibody only, and wild type *S. degradans* probed with preimmune sera and secondary antibody. Cultures grown with glucose as the sole carbon source were labeled, but at a much lower frequency. The density and distribution of gold

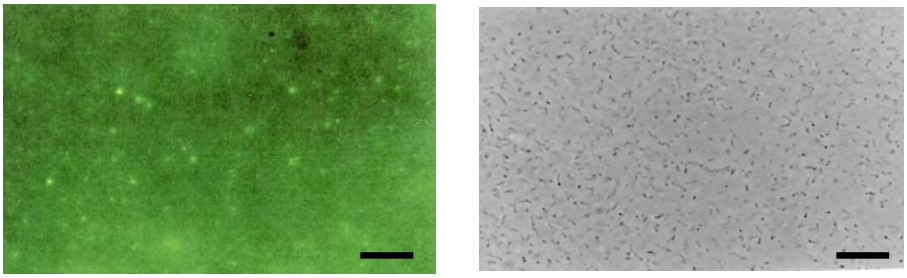
Figure 4.5

Immuno epi-fluorescence detection of AgaE on *S. degradans* grown with agarose or glucose as the sole carbon source

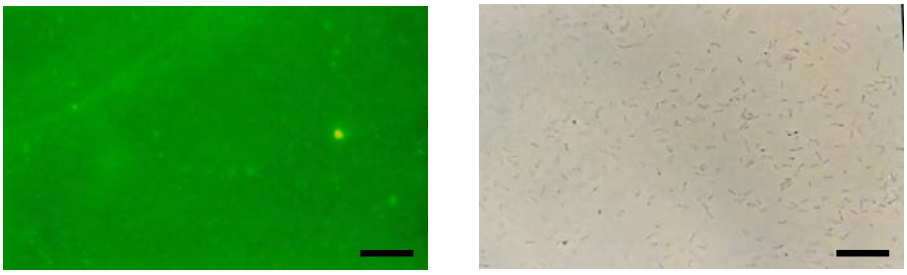
Cells harvested from mid-logarithmic (OD_{600}=0.3) cultures of *S. degradans* were heat fixed to glass slides and probed with α-AgaE and a corresponding FITC-labeled secondary antibody. Both fluorescence and phase contrast image are shown. Magnifications for all images are 4000x. The exposure time for each image is noted. Panels top, *S. degradans* grown with agarose; middle, *S. degradans* grown with glucose; Bottom *S. degradans* grown with agarose and probed with preimmune sera in place of α-AgaE. A single fluorescing cell is shown as an insert in the bottom left corner of the top right panel.



S. degradans Agarose, anti-AgaE, 10s exposure



S. degradans Glucose, anti-AgaE, 45s exposure



S. degradans Agarose, preimmune sera, 45s exposure

labeling were tabulated (Table 4.2), showing that agarose-grown cells were labeled at a higher frequency and density than glucose-grown cells.

The fine-structure immunoprobing suggested AgaE was exocellular and arrayed on the cell surface differently depending on the phase of growth. Among cells grown with agarose as a sole carbon source, gold labeling patterns appeared to correspond to four distinct stages. The first was a linear arrangement of gold along the outer-membrane of cells with typical gram negative morphology (Figure 4.6a). The outer membrane adjacent to these particles appeared intact. In the second stage, gold labeled cell surface material was present. The regions were discreet areas of lightly contrasted material that did not appear to be surrounded by an outer-membrane (Figure 4.6b). They typically contained multiple gold particles labeled on with the inclusion of AgaE. In the third stage, this material was released from the cell and, again, was positively probed with anti-AgaE within the lightly contrasted cell material (Figure 4.6c). This correlates with the biochemical evidence of the shift of AgaE from the cell fraction to the supernatant fraction during the later growth phases. Gold labeled anomalous cell surface structures were also observed. These included a large surface bleb that was surrounded by inner and outer membranes. The embedded gold particles were observed attached to its outer-membrane (Figure 4.7d).

Cultures grown with glucose as a sole carbon source had reduced levels of labeling at slightly above that observed for the control samples (Table 4.2). The labeling in these samples appeared restricted to cell surface arrangements with epicellular structures not

observed. Furthermore, the lightly contrasted cell surface material observed on cells grown with agarose as a sole carbon source were not apparent in these samples.

Immunogold electron microscopy of thin sections shows exocellular arrangements of AgaE

Using α-AgaE, positively probed ultra-thin sections of *S. degradans*, grown with agarose as the sole carbon source were observed. Gold particles were observed embedded within the membrane (Figure 4.7a) and on the cell surface in areas suggestive of cell surface structures (Figure 4.7b). Also, as in the whole cell samples, gold labeled exocellular aggregates, attached to lightly contrasted material were also apparent (Figure 4.7c).

These results were different from those observed in cultures grown with glucose as a sole carbon source. These samples were rarely labeled and, then, limited to a linear cell surface arrangement (Figure 4.7d). Negative controls including *S. degradans* treated with secondary antibody only or *S. degradans* treated with preimmune and secondary antibody were not labeled.

Taken together, these observations suggest that AgaE is initially embedded in the outer-membrane and then later released in aggregates. These results were consistent with those from whole cell mounts and correlated with three patterns of cell associated AgaE: a] in a linear arrangement, b] bound to cell surface material, and c] secreted in aggregates.

Table 4.2

Frequency and density of labeling observed in immuno-electron microscopy experiments

Strain / medium	Probe	% Labeled Cells	Mean Gold Particles per cell
S. degradans wild type / agarose	α-AgaE	85% (p>95%)	10 +/- 3
	α-AgaB	40%	2.3 +/-1.2
	Preimmune sera	50%	3.1+/-3
	Secondary Antibody Only	2%	0.17 +/- 4
S. degradans wild type/ glucose	α-AgaE	64%	4+/-2
	α-AgaB	45%	2.4 +/- 1.5
S. degradans Δ*agaE*::kan/ glucose and agarose	α-AgaE	47%	2.8+/-1.2
	Preimmune sera	10%	2+/-1
	Secondary Antibody Only	2%	1 +/-1

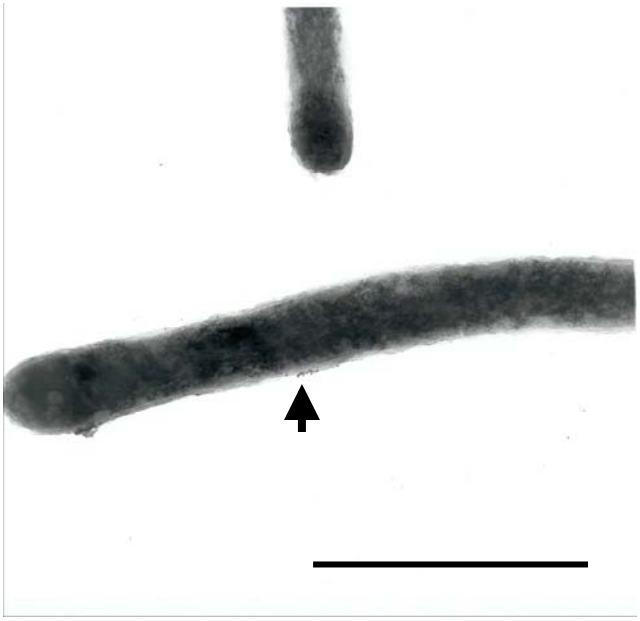
% labeled cells = 100 randomly chosen cells containing at least 1 gold particle contacting the cell surface at a magnification of 16,000x. Mean gold particles per cell = number of gold particles/ labeled cells. Error calculated for mean gold particles per cell indicate a range of particles observed per cell. Note: Only *S. degradans* grown with agarose as a sole carbon source contained numerous clusters containing greater than 5 particles. P value represent a confidence interval (p-value) based upon the labeling of wild type *S. degradans* and the *S. degradans agaE* deletion mutant with α-AgaE. The equation was derived from Brown and Hollander 1977 (27).

Figure 4.6

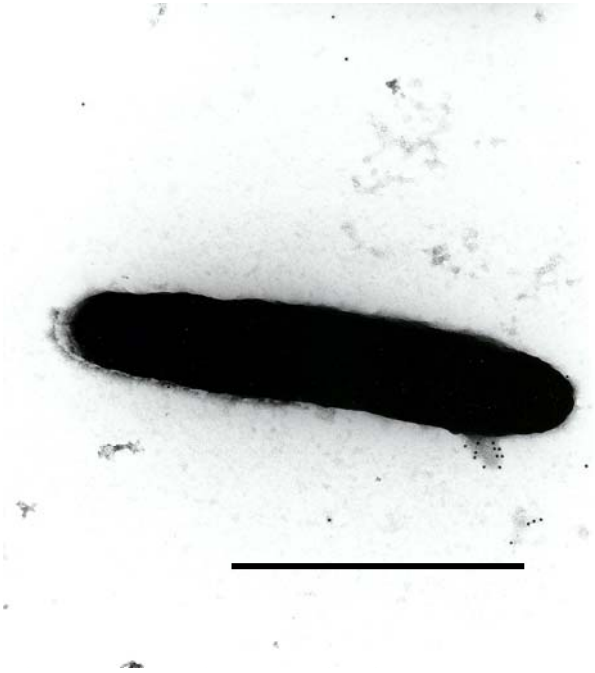
Detection of AgaE on the cell surface of *S. degradans*

Cells harvested from cultures growing exponentially were fixed, adsorbed to nickel grids, probed with α-AgaE (1/10 dilution) and a corresponding 10nm gold conjugated secondary antibody. The preparations were contrasted with uranyl acetate and viewed by TEM as described in the Materials and Methods. Panels A, *S. degradans* cell showing linear pattern of AgaE on its cell surface (94,500 x; scale bar=1μm); B, *S. degradans* cell with a AgaE containing cell surface protuberance (94,500 x, scale bar= 1μm); C. *S. degradans* cells showing multiple stages of AgaE including attached o the cell surface or freely secreted in aggregates (94,500 x scale=1μm); D, *S. degradans* cell with anomalous cell surface structure containing AgaE on its surface. The structure is surrounded by an outer membrane. Structure is labeled. (94,500 x, scale bar = 1μm); E, *S. degradans* ΔagaE::kan probed with α-AgaE (31,500 x, scale bar = 1μm); F, *S. degradans* probed with preimmune sera in place of α-AgaE (31,500 x, scale bar 1μm). Arrows correspond to text.

4.6A



4.6B



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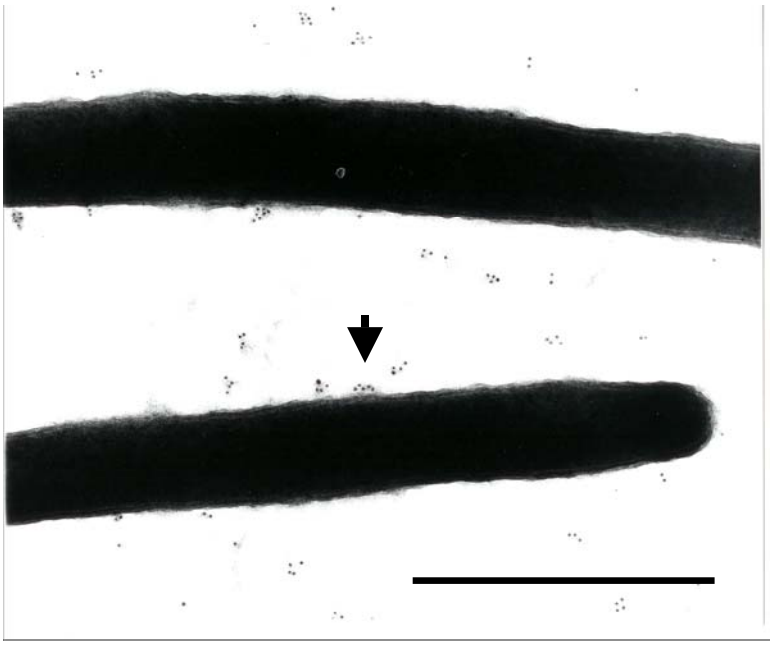
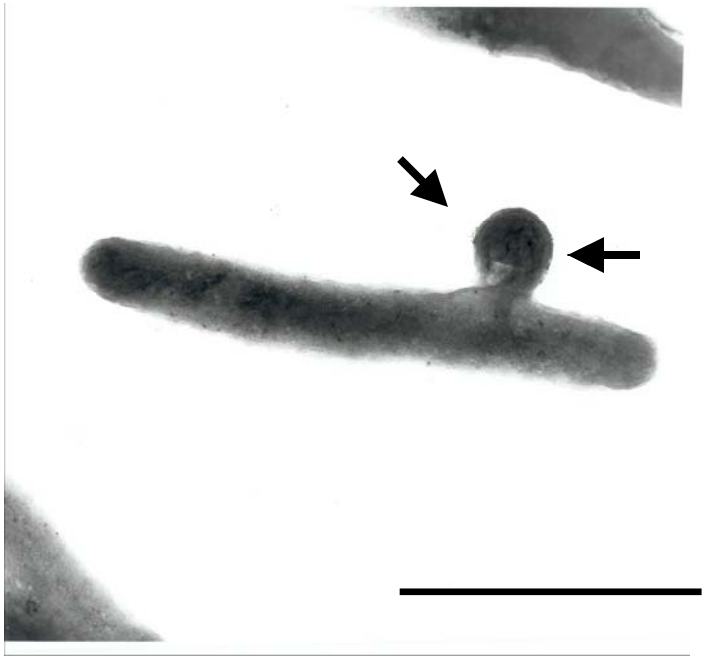
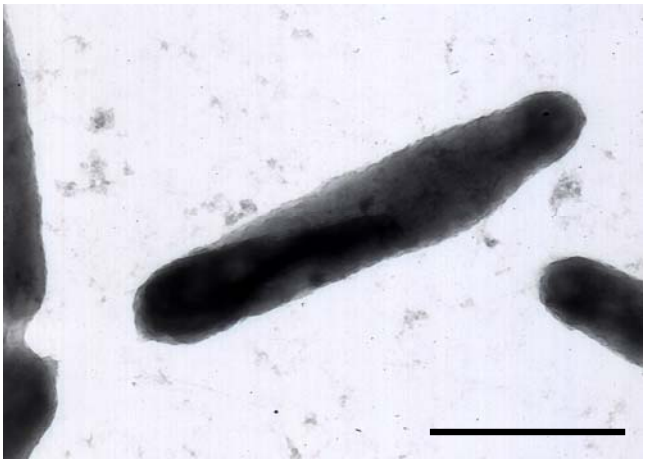


4.6D



4.6E



4.6F

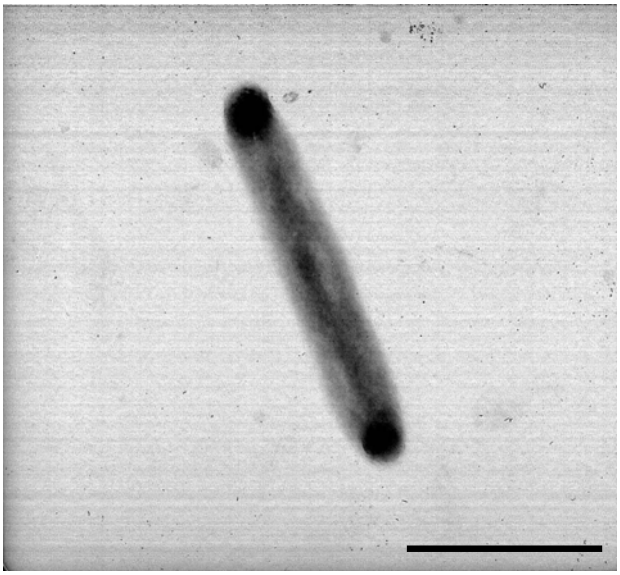
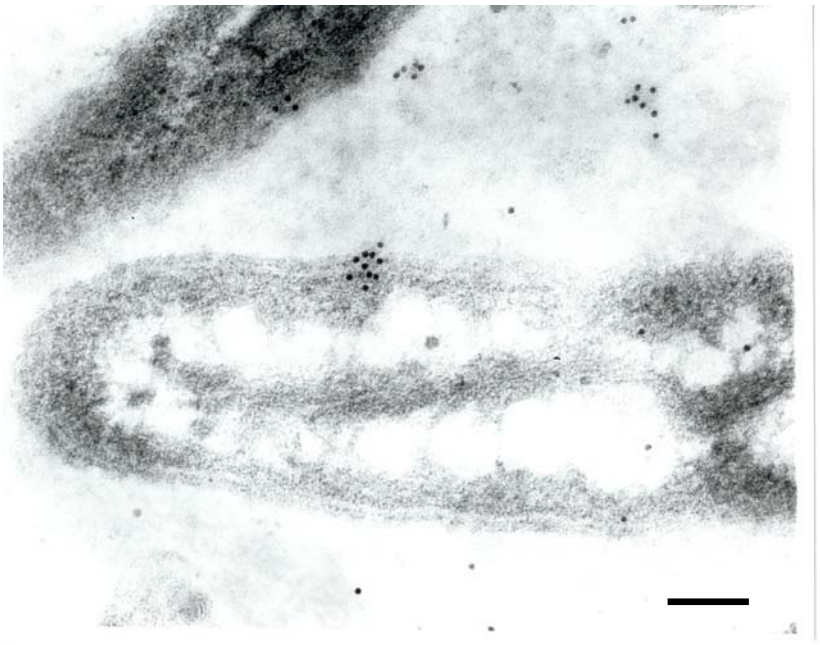


Figure 4.7

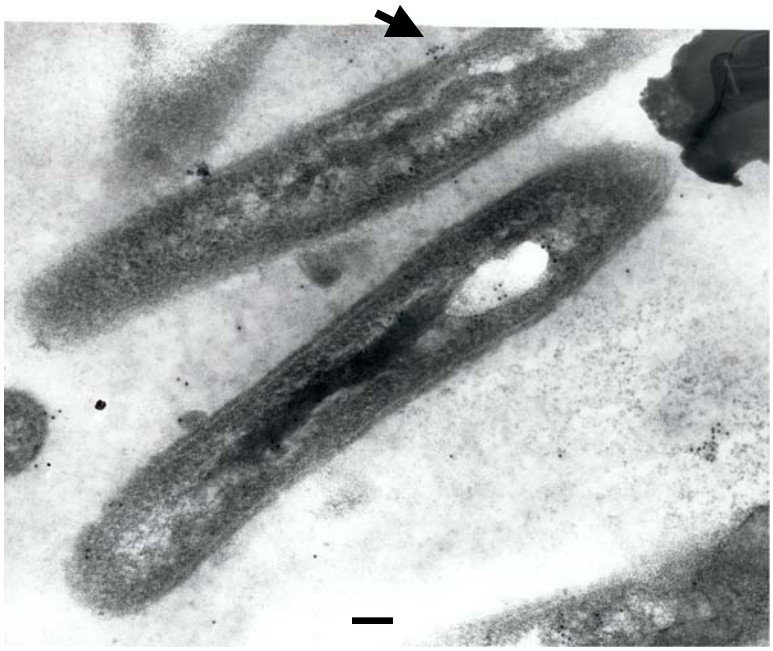
Detection of AgaE by immunogold transmission electron microscopy in ultra thin sections of *S. degradans* grown with either agarose or glucose as the sole carbon source

Cells were fixed, dehydrated and embedded in an EPON 812 resin. Ultra thin sections were taken with a glass knife, collected on nickel grids and probed with α-AgaE and a corresponding gold conjugated secondary antibody. Sections were contrasted described in the Materials and Methods. Panels: A, *S. degradans* grown with agarose showing AgaE embedded in its outer membrane and perhaps periplasm (240,000 x, scale bar 0.1μm); B, *S. degradans* grown with agarose showing AgaE on or surrounding the outer-membrane (120,000x, 0.1μm); C, *S. degradans* grown in agarose demonstration the detachment of AgaE from the cell surface in the form of aggregates within lightly contrasted material (120,000x, 0.1μm); D, *S. degradans* grown with glucose showing a linear arrangement of AgaE gold on its outer membrane 150,000x, μm.

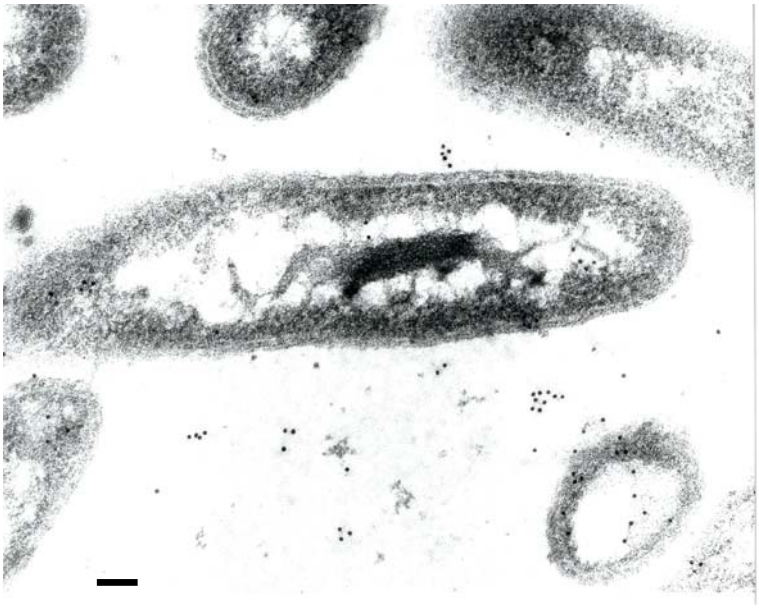
4.7A



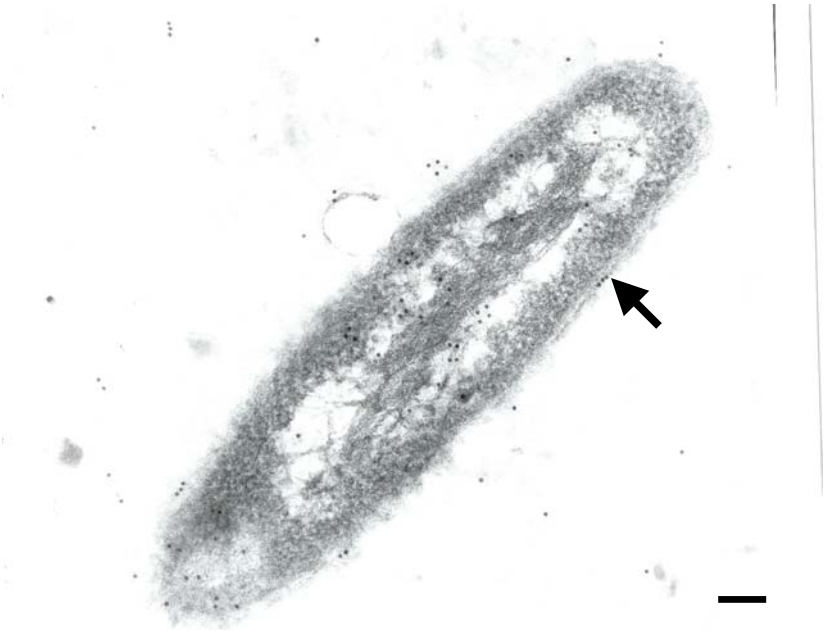
4.7B



4.7C



4.7D



Similar experiments with AgaB including FITC detection and immunogold probing indicated that it was not cell surface associated and, most likely, freely secreted during growth with agarose as a sole carbon source.

Discussion

The degradation of complex polysaccharides (CPs) is a critical function in the carbon cycle that returns recalcitrant carbon to a soluble state. In terrestrial environments a large portion of this material is degraded by characterized microorganisms that array hydrolytic proteins upon their cell surface (19). The organisms that degrade CPs in marine environments are much less well known. Recently, multiple studies into the decay system of *Spartina alterniflora* have uncovered large communities of both fungi and bacteria that degrade the plentiful supply of available CPs (100). Although these organisms have been characterized pylogenetically, the means by which they degrade CPs remain unknown. One organism, *S. degradans*, isolated from such an environment, can degrade multiple CPs including agar, through the use of some cell associated hydrolytic enzymes.

Cell surface associated agarases have been alluded to in several organisms, including *Cytophaga* sp. (44, 46), *Cytophaga flevensis* (136), *Zobellia galactanivorans* (6, 76)and *Pseudoalteromonas atlantica* (101, 103). The β-agarase I from *P. atlantica* was a freely secreted protein (101, 103) while its β-agarase II was purified from cellular lysates (59). However, while these reports suggest the localization of these agarases, little firm data was presented and the mechanisms of exocellular attachment remain unknown.

Two membrane associated agarases were detected and identified as active regions in agarose zymograms of cellular fractions of *S. degradans*, an expected 85kDa protein thought to be the predicted lipoprotein AgaC and a 146kDa agarase, genomically predicted to be AgaE. Unlike AgaB, the amounts of cell-associated AgaE increased during logarithmic growth followed by an increase in freely secreted AgaE as the cultures entered stationary phase. This apparent shift suggests the release of AgaE from a predominantly cell associated form to a freely secreted state.

Using immuno-epi fluorescence and immuno-gold electron microscopy, AgaE was observed on the cell surface of *S. degradans* during logarithmic growth. Several hypotheses could be tested to explain this association. First, AgaE could be bound to agarose appearing as lightly stained aggregates. This association could occur via the CBM6 modules of AgaE binding cell surface or extracellular agarose. If this were the case, however, AgaB should bind since it also contains CBM6 that bind agarose (H. Gilbert, personal communication). Notably, surface associated AgaB was not detected by epifluorescence or immunogold labeling. This hypothesis could be more rigorously tested by repeating the epifluorescence and immunogold labeling with an *E. coli* host expressing AgaE-His grown in the presence of agarose. If AgaE-His is observed on the cell surface of *E. coli* then this would suggest that AgaE does bind agarose at a detectable level which could explain the interaction observed with *S. degradans* and α-AgaE.

A second hypothesis is that the α-AgaE antibodies randomly associate with a cell surface substance such as extracellular polysaccharide (EPS) or another cell constituent and that

this interaction could be amplified in the immunogold labeling experiment due to the high titer of antisera used. However, this hypothesis is refuted by data showing the *S. degradans* deletion mutant is not labeled with α-AgaE antisera. This supports the observation that α-AgaE is bound to the cell surface of *S. degradans*.

Multiple lines of evidence suggest that AgaE is specifically bound to the cell surface of *S. degradans*. The mechanism, indeed, of the selective and discreet labeling by anti-AgaE of wild type *S. degradans* grown in agarose, but not glucose is not known. From its domain analysis we know that AgaE contains a GH86 and three CBM6 modules, a slightly hydrophobic amino terminal domain and four Tsp-3 repeats. The hydrophobic amino terminus is composed of 15 residues that overlap a type two secretion signal. Secretion signal sequences can be confused for single pass transmembrane domains by some prediction software and so it remains likely that this region is solely responsible for secretion and the short hydrophobic region is cleaved upon export to the periplasm.

Tsp-3 repeats are present in a variety of prokaryotic cell surface proteins. A survey of the PFAM database revealed a total of 179 total proteins that contain Tsp-3 repeats, with 117 of them being prokaryotic. Of these, 103 contain outer membrane protein A (OmpA) domains. The Tsp-3 repeats occur between the predicted functional modules in proteins. Aside from the Omp proteins, there are five hydrolytic proteins including four cellulases and one α-agarase that contain Tsp-3 repeats. The PFAM database lists many of the Tsp-3 containing proteins as calcium binding or adhesin proteins. This survey suggests that these repeats are typically found on cell surface proteins. In *S. degradans,* two enzymes

have been genomically identified that contain Tsp-3 repeats, AgaE and a laminarinase (Lam 16A). Lam16A has four Tsp-3 repeats between the GH domains and CBM modules. These repeats could mediate a cell surface interaction perhaps in a similar fashion as that described for cellulosomes.

Cellulosomes are high molecular weight cell surface protuberances capable of hydrolyzing complex polysaccharides including cellulose, pectin, and xylan (43). These structures are produced by a number of species of cellulose degrading *Clostridia* and fungi (21). Cellulosomes array carbohydrases upon cell surfaces via a well characterized protein-protein interaction. Each cellulosome can range from 1-3 mDa and contain multiple non-catalytic high molecular weight scaffold proteins termed scaffoldins (21). Multiple enzymes attach to the scaffoldin which also usually has a cellulose binding domain. The cellulosome is anchored to the cell envelope where it interfaces the environment (18, 19). The interaction between hydrolytic enzymes and scaffoldin proteins are well understood. Scaffoldin proteins contain cohesion domains (cohesins) that bind to repetitive docking sequences (dockerins) within hydrolytic enzymes in a calcium dependent manner(17).

Interestingly, Tsp-3 repeats first identified in the adhesive glycoprotein, thrombospondin, also complex calcium ions (87). Furthermore, they share structural and, perhaps, functional characteristics with dockerin domains. Both Tsp-3 and dockerin repetitive sequences are aspartic acid rich regions containing the motif, DXDXDX(X_{4-5})DX, where the second aspartic acid residue may be absent in dockerin domains. Both domains bind

calcium and mediate adhesion to other proteins. Furthermore, both domains are found in hydrolytic proteins. As in the case of dockerin-scaffoldin interactions, a pair of Tsp-3 repeats on one protein could bind a pair of Tsp-3 repeats on another protein.

If the Tsp-3 repeats of AgaE function in protein-protein interactions then the cognate interactive protein may be present on the *S. degradans* surface, either anchored to the outer membrane or envelope (possibly S layer protein). This interactive protein could be a scaffoldin analog. In fact, 18 candidate scaffoldin/surface attachment proteins encoded by *S. degradans* exist, identified based on their very high molecular weights, low pI's, secretion signal and modules that annotate as protein/protein interaction mediators (L. Taylor, unpublished data). For example CabA has a molecular weight of 1,568kDa, 14,609 amino acids, 35 Tsp-3 modules, seven E-F hand-like modules, a calculated pI of 4.18 and a secretion signal. A 692 residue fragment from CabA annotates most closely ($e=10^{-19}$) to the *C. thermocellum* scaffoldin protein (R. Weiner, unpublished data). Therefore, it is distinctly possible that CabA is an S-layer protein, binding AgaE in a calcium dependent manner similar to the assembly of cellulosomes in the cellulolytic *Clostridia* such a model is shown in figure 4.8.

At least four enzymes, AgaA, AgaB, AgaC and AgaE, synthesized by *S. degradans* are active against neoagaro-oligosaccharides. A model of the enzymology and spatial arrangement of these enzymes is presented. Polymeric agarose would be degraded by AgaB, a freely secreted β-agarase I, producing neoagarotetraose as the major product. Another β-agarase, AgaC, anchored to the cell surface by a lipobox would be

depolymerizing agarose. In this way *S. degradans* can degrade both proximal and distant agarose. AgaE, a cell surface attached β-agarase II, would further hydrolyze the neoagarotetraose product of AgaB and AgaC. The CBM6 of AgaE would bind to soluble neoagaro-oligosaccharides and retain them on the cell surface. The degradation of these substrates would create a high local concentration of neoagarobiose in close proximity to the cell surface. Cells entering stationary phase of growth appear to release AgaE into the exocellular environment. This may be a response to decreased concentrations of available carbon and may represent an attempt to detect and utilize agar that is not in close proximity to the cells. Neoagarobiose would then be imported into the periplasm where it would be degraded by AgaA to D-galactose and 3,6 anhydro-L-galactose. D-galactose would be imported into the cytoplasm and metabolized via glycolysis. The metabolic fate of the L-galactose derivative has not been determined.

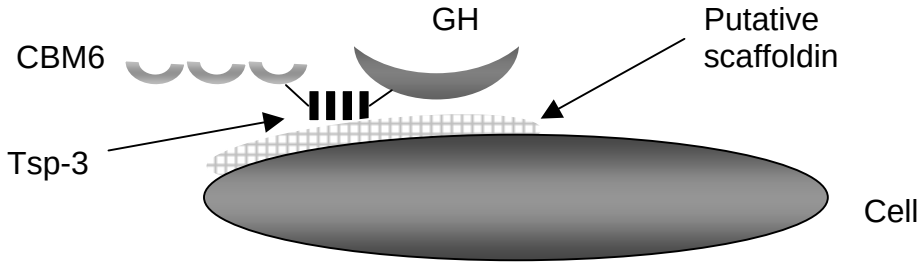
Figure 4.8

Model of cell surface attachment by AgaE

Top alignment of Tsp-3 repeats from AgaE compared to selected dockerin domains from various organisms. Note that both contain similar aspartic acid repeats; Below, diagram of AgaE attachment to the cell surface of *S. degradans.* The Tsp-3 repeats are located between both the catalytic GH86 domains and the carbohydrate binding domains, CBM6. Binding at the Tsp-3 repeats would allow the functional domains to be arrayed on the cell surface. Binding is shown to an unknown protein, perhaps a putative scaffold protein, which contains numerous Tsp-3 repeats. This interaction is hypothesized to be calcium dependent analogous to the cohesin-dockerin interaction observed in cellulosomes.

```
Tsp-1 DGDNDGVPDTSDNC
Tsp-2 DTDEDGINDKIDQC
Tsp-3 DGVLNGADQCGNTP
Tsp-4 DADNDGVANSEDTC

EngE  DVDGNDVVNSLDFE
G.vi  DVDGNGVVDSLDIT
G.vi  DIDGDGRVGAGDIQ
ManA  DVDANGKVNAIDLA
ManK  DVDNDTLISAIDLA
AgaA  DVDGDQQITALDFS
ScaA  DVDNDGNVDSDDYA
```



Chapter 5: Future Directions and Proposed Experiments

Background

The agarase system of *Saccharophagus degradans* illustrates what is hypothesized to be a common thread in the degradation of complex polysaccharides (CPs) by this, and perhaps, related organisms. This system is comprised of multiple apparently redundant enzymes some of which appear to be surface localized. In particular, this work describes five enzymes involved in agar degradation and the cell surface localization of a β-agarase II, AgaE. The interaction between AgaE and the cell surface of *S. degradans* deserves further study as it appears to occur by a heretofore unknown mechanism that may also occur with other hydrolytic enzymes produced by *S. degradans*. This chapter proposes additional experiments to examine further the interaction of AgaE with the cell surface of *S. degradans*.

As stated previously the degradation of CPs is a crucial function in the biome converting recalcitrant carbon to soluble sugars. In terrestrial habitats CP degradation is largely mediated by characterized bacteria and fungi; however, the marine microorganisms, which degrade CPs are largely unknown. Chapter Two describes a related group of gamma proteobacteria, defined by conserved 16s rDNA sequences that, surprisingly, share the ability to degrade CPs. These organisms are currently classified as belonging to the *Saccharophagus*, *Microbulbifer* or *Teredinibacter* genera (49). The data from this

chapter suggest that there may be sufficient selective pressure for these bacteria to maintain similar hydrolytic proteins. In fact, preliminary data suggested that at least some of the chitinases produced by *S. degradans* have homologs produced by *Microbulbifer hydrolyticus* (M.Howard, unpublished data). Although further study into the relatedness of these enzymes is needed, it is possible that distinct hydrolytic protein lineages may also correspond to 16s rDNA lineages.

Five proteins involved in the degradation of agar by *S. degradans* were identified: AgaA, AgaB, AgaC, AgaD and AgaE. These proteins contain three distinct glycoside hydrolase (GH) domains, including GH16, GH50 and GH86. *S. degradans* is the only known organism that encodes these three catalytic domains. Unusual for agarases, AgaB and AgaE contain multiple type-six carbohydrate binding modules (CBM6). Further analyses, by collaborators Dr. Harry Gilbert and Dr. Alisdair Boraston, have determined that the CBM6 of AgaB bind neoagaro-oligosaccharides. When published, this will be first report of agar-binding domains.

The hydrolytic capabilities of the agarases produced by *S. degradans* were determined using multiple techniques. Two agarases, AgaB and AgaE, were expressed and purified from *E. coli.* These proteins were found to be active against polymeric agarose yielding soluble neoagaro-oligosaccharides. AgaB is a β-agarase I producing predominantly neoagarotetraose while AgaE is a β-agarase II producing solely neoagarobiose. Difficulties in the purification of other agarases led to the development of alternative methods for their characterization. For example, AgaC was identified in an agarase active

protein band by mass spectrometry. This analysis was performed by excising agarase active protein bands from an SDS-PAGE gel suggesting that AgaC is a β-agarase. Furthermore, using a newly described method, site specific null mutations were created in *S. degradans*. Equally as important, this demonstrated for the first time the ability of *S. degradans* to import linear DNA and the capability to create site specific null mutations in this organism.

The identification of two membrane associated agarases, AgaC and AgaE, suggested both proteins contained domains or sequence motifs to mediate this interaction. In fact, one of these proteins AgaC was a predicted surface associated lipoprotein; however, AgaE did not contain a domain suggestive of outer-membrane attachment. Using an AgaE specific antiserum both epifluorescence microcopy and immuno-gold transmission electron microscopy suggested the cell surface association of AgaE. In some samples it was attached to the cell surface by what appeared to be lightly stained material. This material was also detached from the cell surface appearing as aggregates with AgaE embedded within it.

It is hypothesized that the four Tsp-3 repeats of AgaE mediate the cell surface attachment of this protein. These domains, identified in human thrombospondin, are aspartic acid rich predicted calcium-binding sequences that mediate protein-protein interactions (87). The hypothesis that Tsp-3 repeats mediate the cell surface attachment of AgaE suggests a structural similarity to the cellulosomes produced by many terrestrial microorganisms. Cellulosomes are characterized as protein complexes that array hydrolytic proteins upon

the cell surface of some gram-positive bacteria and fungi(19). These protein arrays are characterized by calcium dependent interactions between docking (dockerin) repeats found on hydrolytic proteins and cohesion (cohesin) domains found on large cell surface scaffold proteins (scaffoldins) (19). This assembly allows for a CP-enzyme-cell interaction producing soluble sugars in close proximity to the cell and reducing excessive diffusion of product or enzyme. An analogous system has not been identified in marine bacteria while the challenges of CP degradation in these environments remain. This chapter proposes future directions and experiments to determine if the interaction of AgaE is structurally similar to that observed in cellulosomes.

Proposed Research

a. The central question: Do Tsp-3 repeats mediate cell surface protein binding? Cell surface display of AgaE appears to utilize a previously uncharacterized mechanism. The proposed function of the Tsp-3 repeats of AgaE is to mediate a calcium dependent anchoring interaction with a putative scaffoldin protein. If this is the case then other hydrolytic proteins that contain Tsp-3 repeats should also be found on the cell surface of *S. degradans*.

b. A functional model of the interaction of Tsp-3 domains from hydrolytic proteins and candidate scaffoldin proteins. The previous chapter proposed a model of AgaE interaction similar to the assembly of cellulosomes. The following

experiments attempt to identify and preliminarily characterize the scaffoldin analogs encoded by *S. degradans*.

Hypothesis 1:

H1: Tsp-3 domains are specific sequences that mediate cell surface binding.
H1a: Tsp-3 domains are random sequences that do not have a function.

This question can be approached by analyzing another hydrolytic protein produced by *S. degradans* that contains Tsp-3 repeats, termed Lam16A. This protein is a laminarinase identified in the genomic sequence annotation (L. Taylor personal communication). Antibodies specific for this enzyme can be used for immunolocalization experiments similar to those performed in the previous chapter with AgaE and anti-AgaE (α-AgaE). Anti-sera specific for Lam16A (α-Lam) can be generated in either goat or mouse hosts. *S. degradans* grown with laminarin as a sole carbon source can be probed with α-Lam and observed by either immuno-fluorescence and/or immunogold electron microscopy. Cell surface localization of Lam16A would strongly suggest the interaction of Tsp-3 domains with the outer-membrane of *S. degradans*.

This interaction can be analyzed further by culturing *S. degradans* in the presence of both agarose and laminarin. Cells prepared from these cultures could be probed with α-AgaE and α-Lam and observed for co-localization of the antisera. It is anticipated that the localization of Lam16A and AgaE occurs on the cell surface perhaps at similar cell

surface structures. This result would suggest the production of a cell surface anchoring protein that mediates the binding of multiple proteins in a scaffoldin analogous mechanism.

If α-Lam does not localize to the cell surface of *S. degradans* it suggests that Tsp-3 repeats do not mediate cell surface attachment of hydrolytic proteins. This result would warrant further analysis of other modules within AgaE that could mediate cell surface attachment, in particular, the CBM6. AgaE could be, however unusual, bound to a cell surface polysaccharide by the CBM6. This interaction could be tested using specific deletions of AgaE constructed with the mutagenic technique described in Chapter 3 (Figure 5.1) Recombinants could be selected for by antibiotic resistance and screened in immunoblots with α-AgaE. Detection of a smaller molecular weight derivative of AgaE would indicate the proper construct.

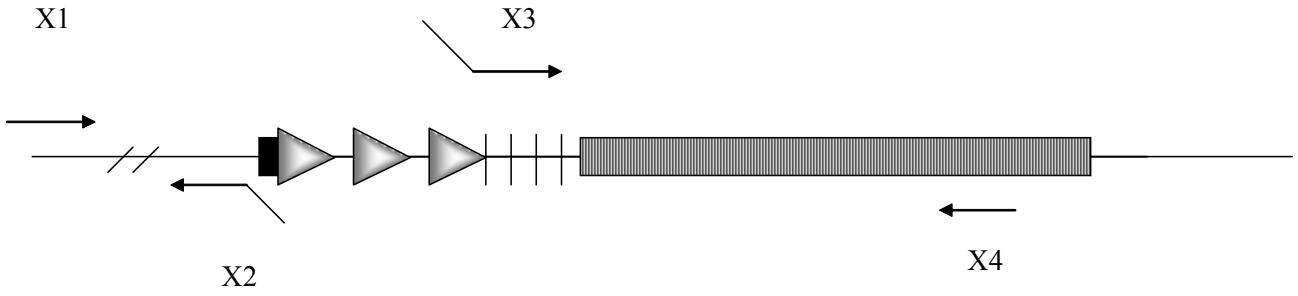
Using a similar mutagenic technique described above the Tsp-3 repeats of AgaE could be selectively deleted. Immunolocalization experiments using α-AgaE could be performed. If AgaE derivatives are not observed on the cell surface of *S. degradans* it would suggest that the Tsp-3 repeats of AgaE are involved in cell surface localization of this protein.

Figure 5.1

Proposed means to selectively delete the CBM6 of AgaE

The proposed primers, X1, X2, X3, X4 are shown (arrows). The coding sequence of AgaE is shown (strain line) with an overlapping model of the encoded modules. Key, solid box, secretion signal; triangles, CBM6; striped box, GH86 domain; vertical lines, Tsp-3 repeats. By using a method similar to that performed in Chapter 2, site specific deletions may be constructed. Primers X1 and X2 would amplify a 1kb fragment upstream of *agaE*. Primer X2 would contain a sequence tail complementary to the 5' region of a kanamycin resistance cassette described below. Primers X3 and X4 would amplify a 1kb fragment, which includes the GH86 domain of AgaE. Primer X3 would contain a sequence tail complementary to the 3' region of the kanamycin resistance cassette. The X3 sequence complementary to *agaE* would begin directly after the CBM6. This construct can be transformed into *S. degradans* as previously described (Chapter 3).

This approach would require the use of a kan^R cassette (below) containing a stop codon (TAA) followed by a start codon (ATG) and a secretion signal (black box). The secretion signal sequence can be derived from *agaE*. This cassette could be constructed using standard cloning procedures. The resulting product could be used to make in frame fusions resulting in a truncated secreted proteins.


X1
X3
X2
X4


Kan^R
TAA ATG

Immunolocalization experiments could be repeated for the presence of the truncated derivative of AgaE. This technique could determine the role of other unforeseen interactions between AgaE and the cell surface of *S. degradans*.

Hypothesis 2

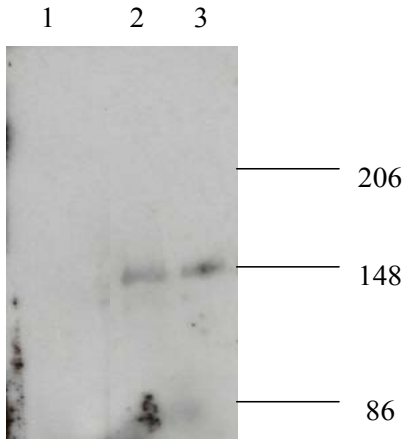
H2: AgaE utilizes Tsp-3 domains to attach to a cell surface bound protein.
H2a: AgaE utilizes Tsp-3 domains to attach directly to the outer-membrane of *S. degradans.*

Candidate scaffoldin proteins encoded by *S. degradans* have been identified. These proteins are characterized by their high molecular mass, in excess of 1.5mDa, and the presence of multiple Tsp-3 repeats (L. Taylor personal communication). Unfortunately, the large size of these protein precludes them from traditional methods of detection and analysis i.e. SDS-PAGE, immunoblot analysis, expression and purification from *E. coli*. In fact, previous attempts to identify AgaE-His interacting proteins may have been hindered by this fact (Figure 5.2). The presumed scaffoldin function of these proteins can be determined, however, in a relatively straightforward manner by creating null mutations of the encoding genes.

Figure 5.2

Far-Western immunoblot detection of AgaE binding proteins in cell lysates of *S. degradans*

Cell lysates of either glucose grown or agarose grown *S. degradans* were fractionated on an 8% SDS-PAGE gel. The Far-Western blot was performed by standard methods (118). Briefly, the gel was blotted onto nitrocellulose and the proteins refolded. The blot was probed with AgaE-His overnight at 4°C, washed, probed with Hrp conjugated anti-His, incubated with chemiluminescent substrate and exposed to film. Lanes 1, glucose cells; 2 &3, agarose cells. This blot solely detected a protein of similar molecular weight to AgaE-His (146kDa) and may indicate that AgaE-His is bound to AgaE. Large proteins such as the putative scaffoldins would be excluded from this analysis because of their inability to enter the polyacrylamide gel.



1. Glucose

2&3 Agarose

Due to the extreme size of the putative scaffoldin encoding genes, it is unlikely that the entire coding sequence could be removed. Mutagenic constructs could contain the 5' promoter region and the start codon of each gene and an antibiotic resistance cassette flanked by 1000 nucleotides of homologous sequence. The resulting recombinants can be probed with either α-AgaE or α-Lam to observe whether cell surface localization of either protein occurs. If so, sequential mutations of the other putative scaffoldin proteins can be performed using various antibiotic resistance cassettes. In this manner the function of these large proteins can be inferred by specific null mutations.

An interaction of AgaE or Lam16A with a scaffoldin protein can be tested using truncated derivatives of the scaffoldin proteins expressed and purified from *E. coli*. Scaffoldin proteins can be affinity purified using a specific epitope tag, i.e. GST tag. Upon purification these fragments could be used in various protein affinity column chromatography experiments. Specific elution of either AgaE-His or Lam16A-His from a column containing immobilized scaffoldin-GST would indicate an interaction between these proteins. Controls for these experiments could include the following: 1. non-Tsp-3 containing hydrolytic proteins in place of AgaE-His or Lam-His 2. a Tsp-3 deletion of AgaE or Lam constructed and purified from *E. coli* 3. a region of the scaffoldin protein that does not contain Tsp-3 repeats. Bound proteins from these columns could be eluted using various conditions including increased pH, SDS or, perhaps, EGTA. The eluted proteins can be analyzed in immunoblots using either anti-His or anti-GST. If such an interaction is detected, the use of EGTA can be used to determine if it occurs in a calcium dependent manner such as the cohesin and dockerin interactions of cellulosomes. The

interaction of these proteins can be assessed in buffers not containing calcium or in buffers containing calcium and increasing concentrations of EGTA.

If the localization of AgaE or Lam16A is not affected by the deletion of the putative scaffoldin proteins it would suggest that the proteins are directly anchored to the cell surface. This could be due to an unidentified hydrophobic interaction between AgaE and cell surface lipids. This model could be tested by constructing spheroplasts of *E. coli* and incubating them in the presence of AgaE. The resulting spheroplasts could be collected onto nickel grids and immuno probed with α-AgaE and a gold conjugated secondary antibody and observed by TEM. The immunolocalizaton of α-AgaE to spheroplasts of *E. coli* would indicate that the interaction of AgaE to the cell surface of *S. degradans* either occurs non-specifically or by a conserved cell surface protein.

Conclusions

The study of the interaction of AgaE with the cell surface of *S. degradans* has various significant implications. First and foremost, a specific AgaE-scaffoldin interaction would demonstrate a novel means by which gram-negative bacteria display cell surface proteins. If Tsp-3 repeats mediate this interaction then they represent short amino acid sequences that could be used for microbial cell surface protein display. Libraries of these surface proteins could be constructed and used analogously to phage display protein libraries. These microbial display libraries could be used to identify novel proteins; for example, a

library of cell surface displayed hydrolytic proteins could be probed with fluorescently labeled saccharides (68) and screened for their ability to bind such a target.

Furthermore, if similarities between the cell surface attachment of AgaE and cellulosomes are found it would imply convergent evolution directed possibly from the challenges of CP degradation. It is surprising that some terrestrial bacteria and fungi produce functionally and structurally similar cellulosome complexes, the characteristics of each varying only slightly. A functionally analogous system may be produced by other organisms, such as *S. degradans.* The need for such a strategy may be selected for by the challenges of CP degradation most notably the advantage of retaining substrate, enzyme and released products in close proximity to the cell.

Appendices

Appendix I

Phenotypic Instability of *E. coli* EPI300 (pNE10)

Phenotypic variation was observed in clones of *E. coli* EPI300 (pNE10) resulted in a mixed population of pitting (agarase positive) and non-pitting (agarase negative) clones. Colonies of *E. coli* EPI300 (pNE10) appeared to become agarase negative after growth in broth cultures. This was observed by inoculating a broth culture with an agarase positive clone and observing the frequency of agarase negative colonies upon plating. Of the 179 colonies screened, three were agarase negative (1.7%). All agarase positive colonies were half the diameter (1mm) of agarase negative clones (2-3mm). This experiment was repeated using a single agarase positive or agarase negative colony. From the agarase positive culture 587 colonies were screened 146 of which were agarase negative (25%). 987 colonies from the agarase negative culture were screened all of which remained agarase negative. The phenotype was confirmed by flooding the plates with iodine solution. The agarase positive colonies were surrounded by large unstained halos indicating depolymerized agar while agarase negative colonies were not surrounded by halos. Once the sequences of *agaA* and *agaB* were available the agarase positive and

agarase negative clones were tested for their presence. Cellular lysates of these clones were analyzed by agarose zymography (Figure AI.1).

The presence of *agaA* and *agaB* in both agarase positive and agarase negative colonies was determined by PCR using primers specific for each full-length gene (Figure AI.2). Agarase positive colonies yielded PCR products indicative of their presence while selected agarase negative clones did not. Agarase positive clones revealed three agarase positive proteins while the agarase negative clones were entirely agarase negative. Plasmid preparations from agarase positive and negative clones indicated that both populations contained fosmid DNA (Figure AI.2). These results suggested the instability of the agarase phenotype of *E. coli* EPI300 (pNE10) was due to the deletion or interruption of *agaA* and *agaB*. The exact nature of this instability remains unknown.

Figure AI.1

Phenotypic variation of *E. coli* ECI300 (pNE10)

Pitting and non-pitting colonies of *E. coli* ECI300 (pNE10) were resuspended in lysis buffer and analyzed by zymography. The pitting colony revealed three bands of agarase activity (Lane 1) while the non-pitting colony was agarase negative (Lane2). These results suggested that a deletion or interruption had occurred in *agaA* and *agaB*. This also indicated that a significant potion of the agarase activity encoded by pNE10 was due to *agaA* and *agaB*.

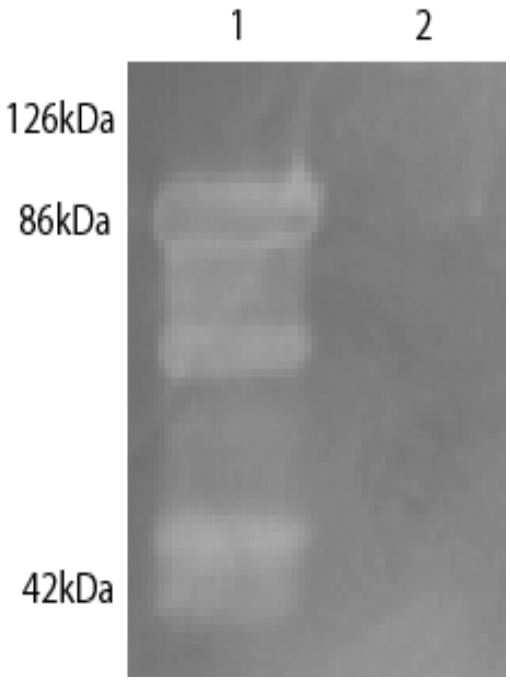
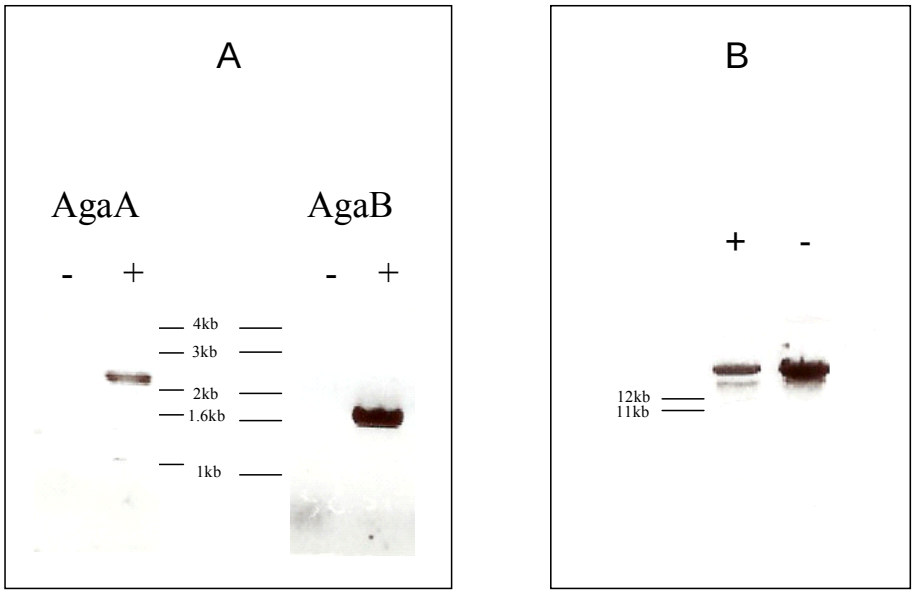

1
2
126kDa
86kDa
42kDa

Figure AI.2

Analysis of the apparent *agaA* and *agaB* deletion of pNE10

The presence of *agaA* and *agaB* in agarase positive and agarase negative *E. coli* pNE10 was determined using PCR. Primers were constructed to amplify the full length coding region of both agaA and *agaB*. Agarase positive (+) and agarase (-) colonies of *E. coli* pNE10 were used as templates. *agaA* and *agaB* were detected in agarase positive colonies, but not in agarase negative colonies (Panel A). Similar sized plasmids indicative of fosmid DNA (40kb) were isolated from either agarase positive or agarase negative colonies (Panel B).


A
AgaA
AgaB
-
+
-
+
4kb
3kb
2kb
1.6kb
1kb
B
+
-
12kb
11kb

Appendix II

Variability of the molecular weight of AgaB

As discussed in Chapter 3 the agarase activity detected in zymograms of AgaB-His preparations did not solely migrate at the expected molecular weight. The agarase activity of *E. coli* Tuner (pAgaB) was associated with the 85kDa protein. Various molecular weight standards including Kaleidoscope, Multimark, and Precision Plus were used with similar results obtained. It has been reported that multiple forms of post-translational modification of proteins, such as glycosylation and phosphorylation, may cause a higher than expected molecular weight derivatives. To determine whether the shift in molecular weight was due to a specific post-translational modification AgaB-His was analyzed for the presence for a glycosylated derivative. These results of this test did not indicate that AgaB-His was a glycosylated derivative. Later AgaB-His was also tested for phosphorylation this test as well did not indicate the presence of a post-translationally modified derivative.

It was still unknown whether this apparent modification occurred at a certain region of AgaB-His. Truncated derivatives of AgaB-His were constructed to investigate whether the shift in molecular weight of AgaB was due to a certain region of the protein. The derivative were isolated from *E. coli*, fractionated on an SDS-PAGE gel, and detected by

Western blot analysis. Each of the derivatives tested were detected at molecular weights that deviated from the expected molecular weight (Figure AII.1). It appeared that each derivative was approximately 20kDa greater than expected. Furthermore, multiple proteins derived from *S. degradans* and expressed in *E. coli* were later observed at higher than expected molecular weights (L. Taylor and M. Howard, unpublished observation). As expected two agarolytic proteins were immunoprecipitated from *S. degradans* culture supernatants using α-AgaB (AII.2). It is currently thought that this shift in molecular weight may be due to a non denatured region of this protein that causes AgaB to aberrantly resolve in SDS-PAGE gels.

Figure AII.1

Molecular weight analysis of AgaB-His.

AgaB-His (lane 1) is typically purified as an 86kDa protein and a 65kDa protein other degradation products may be present especially in native protein preparations (lane1). Three derivatives of AgaB (left) were constructed. The coding regions included in these derivatives are noted below with the predicted molecular weight of each derivative is noted. The truncated products were analyzed in immunoblots using anti-His antibody. Full length AgaB-His (lane 1) had an approximate molecular weight of 87kDa. Multiple smaller derivatives were also detected in this preparation. Derivative 2 (lane2) had an expected molecular weight of 36kDa, but was detected at approximately 50kDa. Derivative 3 (lane3) was an expected 30kDa product but was detected at approximately 48kDa. Derivative 4 (lane4) had an expected molecular weight of 35kDa but was detected at 48 kDa. Each derivative ran at a higher than expected molecular weight. The cause of this is unknown but has been observed in other proteins from *S. degradans.*

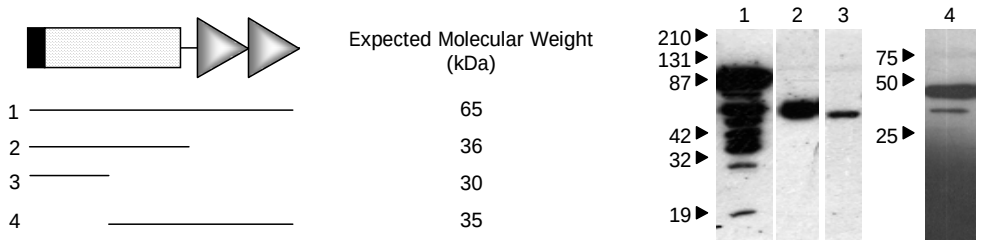
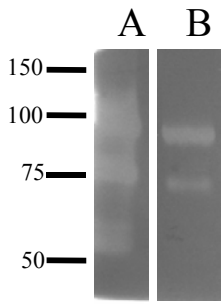

Expected Molecular Weight
(kDa)
1
2
3
4
65
36
30
35
1
2
3
4
210
131
87
42
32
19
75
50
25

Figure AII.2

Immunoprecipitation of both derivatives of AgaB from supernatants of *S. degradans*

Concentrated culture supernatants were exposed to anti-AgaB antisera and allowed to incubate overnight at 4°C. To the mixture, protein A coated acrylamide beads were added. The precipitate was collected by centrifugation, washed and boiled. The supernatant was collected and fractionated on an agarose zymogram (lane B) and compared to an *S. degradans* concentrated culture supernatant (lane A).


A
B
150
100
75
50

Bibliography

1. 1982. Carcinogenesis Bioassay of Agar (CAS No. 9002-18-0) in F344 Rats and B6C3F1 Mice (Feed Study), p. 121, National Toxicology Program. U.S. Department of Health and Human Services, Research Triangle Park.

2. **Abdel-Hafez, A.** 2003. Production and Genomics of Microbulbifer degradans alginases. Ph.D. University of Maryland, College Park.

3. **Ahsan, M., M. Matsumoto, S. Karita, T. Kimura, K. Sakka, and K. Ohmiya.** 1997. Purification and characterization of the family J catalytic domain derived from the *Clostridium thermocellum* endoglucanase CelJ. Biosci Biotechnol Biochem **61:**427-31.

4. **Ahsan, M. M., T. Kimura, S. Karita, K. Sakka, and K. Ohmiya.** 1996. Cloning, DNA sequencing, and expression of the gene encoding *Clostridium thermocellum* cellulase CelJ, the largest catalytic component of the cellulosome. J Bacteriol **178:**5732-40.

5. **Allouch, J., W. Helbert, B. Henrissat, and M. Czjzek.** 2004. Parallel substrate binding sites in a beta-agarase suggest a novel mode of action on double-helical agarose. Structure (Camb) **12:**623-32.

6. **Allouch, J., M. Jam, W. Helbert, T. Barbeyron, B. Kloareg, B. Henrissat, and M. Czjzek.** 2003. The three-dimensional structures of two beta-agarases. J Biol Chem **278:**47171-80.

7. **Andrykovich, G., and I. Marx.** 1988. Isolation of a new polysaccharide digesting bacterium from a salt marsh. Appl. Microbiol. Biotechnol **54:**1061-1062.

8. **Andrykovitch, G., and I. Marx.** 1988. Isolation of a New Polysaccharide-Digesting Bacterium from a Salt Marsh. Appl Environ Microbiol **54:**1061-1062.

9. **Aoki, T., T. Araki, and M. Kitamikado.** 1990. Purification and characterization of a novel beta-agarase from *Vibrio* sp. AP-2. Eur J Biochem **187:**461-5.

10. **Araki, T., M. Hayakawa, Z. Lu, S. Karita, and T. Morishita.** 1998. Purification and characterization of agarases from a marine bacterium, *Vibrio* sp. PO-303. Journal of Marine Biotechnology **6:**260-265.

11. **Aurilia, V., J. C. Martin, S. I. McCrae, K. P. Scott, M. T. Rincon, and H. J. Flint.** 2000. Three multidomain esterases from the cellulolytic rumen anaerobe *Ruminococcus flavefaciens* 17 that carry divergent dockerin sequences. Microbiology **146 (Pt 6):**1391-7.

12. **Bayer, E. A., J. P. Belaich, Y. Shoham, and R. Lamed.** 2004. The cellulosomes: multienzyme machines for degradation of plant cell wall polysaccharides. Annu Rev Microbiol **58:**521-54.

13. **Bayer, E. A., H. Chanzy, R. Lamed, and Y. Shoham.** 1998. Cellulose, cellulases and cellulosomes. Curr Opin Struct Biol **8:**548-57.

14. **Bayer, E. A., P. M. Coutinho, and B. Henrissat.** 1999. Cellulosome-like sequences in *Archaeoglobus fulgidus*: an enigmatic vestige of cohesin and dockerin domains. FEBS Lett **463:**277-80.

15. **Bayer, E. A., and R. Lamed.** 1992. The cellulose paradox: pollutant par excellence and/or a reclaimable natural resource? Biodegradation **3:**171-88.

16. **Bayer, E. A., and R. Lamed.** 1986. Ultrastructure of the cell surface cellulosome of *Clostridium thermocellum* and its interaction with cellulose. J Bacteriol **167:**828-36.

17. **Bayer, E. A., E. Morag, and R. Lamed.** 1994. The cellulosome--a treasure-trove for biotechnology. Trends Biotechnol **12:**379-86.

18. **Bayer, E. A., E. Setter, and R. Lamed.** 1985. Organization and distribution of the cellulosome in *Clostridium thermocellum*. J Bacteriol **163:**552-9.

19. **Bayer, E. A., L. J. Shimon, Y. Shoham, and R. Lamed.** 1998. Cellulosomes-structure and ultrastructure. J Struct Biol **124:**221-34.

20. **Beguin, P., and P. M. Alzari.** 1998. The cellulosome of *Clostridium thermocellum*. Biochem Soc Trans **26:**178-85.

21. **Beguin, P., and M. Lemaire.** 1996. The cellulosome: an exocellular, multiprotein complex specialized in cellulose degradation. Crit Rev Biochem Mol Biol **31:**201-36.

22. **Belaich, J. P., C. Tardif, A. Belaich, and C. Gaudin.** 1997. The cellulolytic system of *Clostridium cellulolyticum*. J Biotechnol **57:**3-14.

23. **Belas, R.** 1989. Sequence analysis of the *agrA* gene encoding beta-agarase from *Pseudomonas atlantica*. J Bacteriol **171:**602-5.

24. **Benner, R., M. A. Moran, and R. E. Hodson.** 1986. Biogeochemical cycling of lignocellulosic carbon in marine and freshwater ecosystems: relative contribution of prokaryotes and eukaryotes. Limnology and Oceanography **31:**89-100.

25. **Bibb, M. J., G. H. Jones, R. Joseph, M. J. Buttner, and J. M. Ward.** 1987. The agarase gene (*dag A*) of *Streptomyces coelicolor* A3(2): affinity purification and characterization of the cloned gene product. J Gen Microbiol **133:**2089-96.

26. **Bingle, W. H., J. F. Nomellini, and J. Smit.** 1997. Cell-surface display of a *Pseudomonas aeruginosa* strain K pilin peptide within the paracrystalline S-layer of *Caulobacter crescentus*. Mol Microbiol **26:**277-88.

27. **Brown, B., and M. Hollander.** 1977. Statistics: A Biomedical Introduction. John Wiley & Sons, Inc, New York.

28. **Buchan, A., S. Y. Newell, M. Butler, E. J. Biers, J. T. Hollibaugh, and M. A. Moran.** 2003. Dynamics of bacterial and fungal communities on decaying salt marsh grass. Appl Environ Microbiol **69:**6676-87.

29. **Chaleff, R. S., and P. S. Carlson.** 1974. Somatic cell genetics of higher plants. Annu Rev Genet **8:**267-78.

30. **Chen, H., X. L. Li, and L. G. Ljungdahl.** 1995. Biomass degrading enzymes from anaerobic rumen fungi. SAAS Bull Biochem Biotechnol **8:**1-6.

31. **Chenna, R., H. Sugawara, T. Koike, R. Lopez, T. J. Gibson, D. G. Higgins, and J. D. Thompson.** 2003. Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Res **31:**3497-500.

32. **Chiovitti, A., A. Bacic, D. J. Craik, G. T. Kraft, and M. L. Liao.** 2004. A nearly idealized 6'-O-methylated iota-carrageenan from the Australian red alga *Claviclonium ovatum* (*Acrotylaceae, Gigartinales*). Carbohydr Res **339:**1459-66.

33. **Craigie, J.** 1990. Chapter 10 Cell Walls, p. 221-258. *In* S. R (ed.), Biology of the Red Algae. Cambridge Unversity Press, New York.

34. **d'Enfert, C., C. Chapon, and A. P. Pugsley.** 1987. Export and secretion of the lipoprotein pullulanase by *Klebsiella pneumoniae*. Mol Microbiol **1:**107-16.

35. **d'Enfert, C., and A. P. Pugsley.** 1987. A gene fusion approach to the study of pullulanase export and secretion in *Escherichia coli*. Mol Microbiol **1:**159-68.

36. **D'Enfert, C., and A. P. Pugsley.** 1989. *Klebsiella pneumoniae pulS* gene encodes an outer membrane lipoprotein required for pullulanase secretion. J Bacteriol **171:**3673-9.

37. **d'Enfert, C., A. Ryter, and A. P. Pugsley.** 1987. Cloning and expression in *Escherichia coli* of the *Klebsiella pneumoniae* genes for production, surface localization and secretion of the lipoprotein pullulanase. Embo J **6:**3531-8.

38. **Devillard, E., D. B. Goodheart, S. K. Karnati, E. A. Bayer, R. Lamed, J. Miron, K. E. Nelson, and M. Morrison.** 2004. *Ruminococcus albus* 8 mutants defective in cellulose degradation are deficient in two processive endocellulases, Cel48A and Cel9B, both of which possess a novel modular architecture. J Bacteriol **186:**136-45.

39. **Ding, S. Y., M. T. Rincon, R. Lamed, J. C. Martin, S. I. McCrae, V. Aurilia, Y. Shoham, E. A. Bayer, and H. J. Flint.** 2001. Cellulosomal scaffoldin-like proteins from *Ruminococcus flavefaciens*. J Bacteriol **183:**1945-53.

40. **Distel, D. L., W. Morrill, N. MacLaren-Toussaint, D. Franks, and J. Waterbury.** 2002. *Teredinibacter turnerae* gen. nov., sp. nov., a dinitrogen-fixing, cellulolytic, endosymbiotic gamma-proteobacterium isolated from the gills of wood-boring molluscs (*Bivalvia: Teredinidae*). Int J Syst Evol Microbiol **52:**2261-9.

41. **Doi, R. H., and A. Kosugi.** 2004. Cellulosomes: plant-cell-wall-degrading enzyme complexes. Nat Rev Microbiol **2:**541-51.

42. **Doi, R. H., J. S. Park, C. C. Liu, L. M. Malburg, Y. Tamaru, A. Ichiishi, and A. Ibrahim.** 1998. Cellulosome and noncellulosomal cellulases of *Clostridium cellulovorans*. Extremophiles **2:**53-60.

43. **Doi, R. H., and Y. Tamaru.** 2001. The *Clostridium cellulovorans* cellulosome: an enzyme complex with plant cell wall degrading activity. Chem Rec **1:**24-32.

44. **Duckworth, M., and J. R. Turvey.** 1969. The action of a bacterial agarase on agarose, porphyran and alkali-treated porphyran. Biochem J **113:**687-92.

45. **Duckworth, M., and J. R. Turvey.** 1968. The extracellular agarase from a *Cytophaga* species. Biochem J **109:**6P.

46. **Duckworth, M., and J. R. Turvey.** 1969. The specificity of an agarase from a *Cytophaga* species. Biochem J **113:**693-6.

47. **Duckworth, M., and W. Yaphe.** 1970. Thin-layer chromatographic analysis of enzymic hydrolysates of agar. J Chromatogr **49:**482-7.

48. **Ekborg, N. A., J. M. Gonzalez, M. B. Howard, L. E. Taylor, S. W. Hutcheson, and R. M. Weiner.** 2005. *Saccharophagus degradans* gen. nov., sp. nov., a versatile marine degrader of complex polysaccharides. Int J Syst Evol Microbiol **55:**1545-9.

49. **Ekborg, N. A., J. M. Gonzalez, M. B. Howard, L. E. Taylor, S. W. Hutcheson, and R. M. Weiner.** 2005. *Saccharophagus degradans* gen. nov., sp.

nov., a versatile marine degrader of complex polysaccharides. Int J Syst Evol Microbiol **55:**1545-9.

50. **Ensor, L., S. Stosz, and R. Weiner.** 1999. Expression of multiple complex polysaccharide-degrading enzyme systems by marine bacterium strain 2-40. J Ind Microbiol Biotechnol **23:**123-126.

51. **Felix, C. R., and L. G. Ljungdahl.** 1993. The cellulosome: the exocellular organelle of *Clostridium.* Annu Rev Microbiol **47:**791-819.

52. **Felsenstein, J.** 1988. Phylogenies from molecular sequences: inference and reliability. Annu Rev Genet **22:**521-65.

53. **Gerwig, G. J., J. P. Kamerling, J. F. Vliegenthart, E. Morag, R. Lamed, and E. A. Bayer.** 1992. Novel oligosaccharide constituents of the cellulase complex of *Bacteroides cellulosolvens*. Eur J Biochem **205:**799-808.

54. **Glockner, F. O., M. Kube, M. Bauer, H. Teeling, T. Lombardot, W. Ludwig, D. Gade, A. Beck, K. Borzym, K. Heitmann, R. Rabus, H. Schlesner, R. Amann, and R. Reinhardt.** 2003. Complete genome sequence of the marine planctomycete *Pirellula* sp. strain 1. Proc Natl Acad Sci U S A **100:**8298-303.

55. **Gonzalez, J. M., F. Mayer, M. A. Moran, R. E. Hodson, and W. B. Whitman.** 1997. *Microbulbifer hydrolyticus* gen. nov., sp. nov., and *Marinobacterium georgiense* gen. nov., sp. nov., two marine bacteria from a lignin-rich pulp mill waste enrichment community. Int J Syst Bacteriol **47:**369-76.

56. **Gonzalez, J. M., and R. M. Weiner.** 2000. Phylogenetic characterization of marine bacterium strain 2-40, a degrader of complex polysaccharides. Int J Syst Evol Microbiol **50 Pt 2:**831-4.

57. **Gorska, E., B. Tudek, and S. Russel.** 2001. Degradation of cellulose by nitrogen-fixing strain of *Bacillus polymyxa*. Acta Microbiol Pol **50:**129-37.

58. **Gram, L., H. P. Grossart, A. Schlingloff, and T. Kiorboe.** 2002. Possible quorum sensing in marine snow bacteria: production of acylated homoserine lactones by *Roseobacter* strains isolated from marine snow. Appl Environ Microbiol **68:**4111-6.

59. **Groleau, D., and W. Yaphe.** 1977. Enzymatic hydrolysis of agar: purification and characterization of beta-neoagarotetraose hydrolase from *Pseudomonas atlantica*. Can J Microbiol **23:**672-9.

60. **Grossart, H. P., T. Kiorboe, K. Tang, and H. Ploug.** 2003. Bacterial colonization of particles: growth and interactions. Appl Environ Microbiol **69:**3500-9.

61. **Ha, J. C., G. T. Kim, S. K. Kim, T. K. Oh, J. H. Yu, and I. S. Kong.** 1997. beta-Agarase from *Pseudomonas* sp. W7: purification of the recombinant enzyme from *Escherichia coli* and the effects of salt on its activity. Biotechnol Appl Biochem **26 (Pt 1):**1-6.

62. **Hantke, K., and V. Braun.** 1973. Covalent binding of lipid to protein. Diglyceride and amide-linked fatty acid at the N-terminal end of the murein-lipoprotein of the Escherichia coli outer membrane. Eur J Biochem **34:**284-96.

63. **Hassairi, I., R. Ben Amar, M. Nonus, and B. B. Gupta.** 2001. Production and separation of alpha-agarase from *Altermonas agarlyticus* strain GJ1B. Bioresour Technol **79:**47-51.

64. **Haygood, M. G., and S. K. Davidson.** 1997. Small-subunit rRNA genes and in situ hybridization with oligonucleotides specific for the bacterial symbionts in the larvae of the bryozoan *Bugula neritina* and proposal of "Candidatus Endobugula sertula". Appl Environ Microbiol **63:**4612-6.

65. **Henrissat, B., and A. Bairoch.** 1993. New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. Biochem J **293 (Pt 3):**781-8.

66. **Henrissat, B., and A. Bairoch.** 1996. Updating the sequence-based classification of glycosyl hydrolases. Biochem J **316 (Pt 2):**695-6.

67. **Hosoda, A., M. Sakai, and S. Kanazawa.** 2003. Isolation and characterization of agar-degrading *Paenibacillus* spp. associated with the rhizosphere of spinach. Biosci Biotechnol Biochem **67:**1048-55.

68. **Howard, M. B., N. A. Ekborg, L. E. Taylor, 2nd, R. M. Weiner, and S. W. Hutcheson.** 2004. Chitinase B of "*Microbulbifer degradans*" 2-40 contains two catalytic domains with different chitinolytic activities. J Bacteriol **186:**1297-303.

69. **Howard, M. B., N. A. Ekborg, L. E. Taylor, S. W. Hutcheson, and R. M. Weiner.** 2004. Identification and analysis of polyserine linker domains in prokaryotic proteins with emphasis on the marine bacterium Microbulbifer degradans. Protein Sci **13:**1422-5.

70. **Howard, M. B., N. A. Ekborg, L. E. Taylor, R. M. Weiner, and S. W. Hutcheson.** 2003. Genomic analysis and initial characterization of the chitinolytic system of *Microbulbifer degradans* strain 2-40. J Bacteriol **185:**3352-60.

71. **Howard, M. B., N. A. Ekborg, R. M. Weiner, and S. W. Hutcheson.** 2003. Detection and characterization of chitinases and other chitin-modifying enzymes. J Ind Microbiol Biotechnol **30:**627-35.

72. **Imamura, N., M. Nishijima, T. Takadera, K. Adachi, M. Sakai, and H. Sano.** 1997. New anticancer antibiotics pelagiomicins, produced by a new marine bacterium *Pelagiobacter variabilis*. J Antibiot (Tokyo) **50:**8-12.

73. **Inouye, S., S. Wang, J. Sekizawa, S. Halegoua, and M. Inouye.** 1977. Amino acid sequence for the peptide extension on the prolipoprotein of the *Escherichia coli* outer membrane. Proc Natl Acad Sci U S A **74:**1004-8.

74. **Ivanova, E. P., I. Y. Bakunina, O. I. Nedashkovskaya, N. M. Gorshkova, Y. V. Alexeeva, E. A. Zelepuga, T. N. Zvaygintseva, D. V. Nicolau, and V. V. Mikhailov.** 2003. Ecophysiological variabilities in ectohydrolytic enzyme activities of some *Pseudoalteromonas* species, *P. citrea*, *P. issachenkonii*, and *P. nigrifaciens*. Curr Microbiol **46:**6-10.

75. **Ivanova, E. P., E. A. Kiprianova, V. V. Mikhailov, G. F. Levanova, A. D. Garagulya, N. M. Gorshkova, M. V. Vysotskii, D. V. Nicolau, N. Yumoto, T. Taguchi, and S. Yoshikawa.** 1998. Phenotypic diversity of *Pseudoalteromonas citrea* from different marine habitats and emendation of the description. Int J Syst Bacteriol **48 Pt 1:**247-56.

76. **Jam, M., D. Flament, J. Allouch, P. Potin, L. Thion, B. Kloareg, M. Czjzek, W. Helbert, G. Michel, and T. Barbeyron.** 2005. The endo-beta-agarases AgaA and AgaB from the marine bacterium *Zobellia galactanivorans*: two paralogue enzymes with different molecular organizations and catalytic behaviours. Biochem J **385:**703-13.

77. **Jindou, S., S. Karita, E. Fujino, T. Fujino, H. Hayashi, T. Kimura, K. Sakka, and K. Ohmiya.** 2002. alpha-Galactosidase Aga27A, an enzymatic component of the *Clostridium josui* cellulosome. J Bacteriol **184:**600-4.

78. **Jung, H. C., J. M. Lebeault, and J. G. Pan.** 1998. Surface display of *Zymomonas mobilis* levansucrase by using the ice-nucleation protein of *Pseudomonas syringae*. Nat Biotechnol **16:**576-80.

79. **Jung, H. C., J. H. Park, S. H. Park, J. M. Lebeault, and J. G. Pan.** 1998. Expression of carboxymethylcellulase on the surface of *Escherichia coli* using *Pseudomonas syringae* ice nucleation protein. Enzyme Microb Technol **22:**348-54.

80. **Kang, N. Y., Y. L. Choi, Y. S. Cho, B. K. Kim, B. S. Jeon, J. Y. Cha, C. H. Kim, and Y. C. Lee.** 2003. Cloning, expression and characterization of a beta-agarase gene from a marine bacterium, *Pseudomonas* sp. SK38. Biotechnol Lett **25:**1165-70.

81. **Kazemi-Pour, N., G. Condemine, and N. Hugouvieux-Cotte-Pattat.** 2004. The secretome of the plant pathogenic bacterium *Erwinia chrysanthemi*. Proteomics **4:**3177-86.

82. **Kelly, S., V. Coyne, D. Sledjeski, W. Fuqua, and R. Weiner.** 1990. Identification of a tyrosinase from a periphytic marine bacterium. FEMS Microbiol Lett **67:**275-280.

83. **Kelly, S., V. E. Coyne, S. DD, F. WC, and W. RM.** 1990. Identification of a tyrosinase from a periphytic mairne bacterium. FEMS Lett **67:**275-280.

84. **Kiorboe, T., H. P. Grossart, H. Ploug, and K. Tang.** 2002. Mechanisms and rates of bacterial colonization of sinking aggregates. Appl Environ Microbiol **68:**3996-4006.

85. **Kiorboe, T., K. Tang, H. P. Grossart, and H. Ploug.** 2003. Dynamics of microbial communities on marine snow aggregates: colonization, growth, detachment, and grazing mortality of attached bacteria. Appl Environ Microbiol **69:**3036-47.

86. **Lai, J. S., and H. C. Wu.** 1980. Incorporation of acyl moieties of phospholipids into murein lipoprotein in intact cells of *Escherichia coli* by phospholipid vesicle fusion. J Bacteriol **144:**451-3.

87. **Lawler, J., and R. O. Hynes.** 1986. The structure of human thrombospondin, an adhesive glycoprotein with multiple calcium-binding sites and homologies with several different proteins. J Cell Biol **103:**1635-48.

88. **Lee, S. Y., J. H. Choi, and Z. Xu.** 2003. Microbial cell-surface display. Trends Biotechnol **21:**45-52.

89. **Lemaire, M., H. Ohayon, P. Gounon, T. Fujino, and P. Beguin.** 1995. OlpB, a new outer layer protein of *Clostridium thermocellum*, and binding of its S-layer-like domains to components of the cell envelope. J Bacteriol **177:**2451-9.

90. **Li, L., J. Guezennec, P. Nichols, P. Henry, M. Yanagibayashi, and C. Kato.** 1999. Micobial diversity in Nankai Trough sediments at a depth of 3,843m. J. Oceanography **55:**635-642.

91. **Li, L., C. Kato, and K. Horikoshi.** 1999. Bacterial diversity in deep-sea sediments from differnet depths. Biodivers. Conserv. **8:**659-677.

92. **Lim, G. E., and M. G. Haygood.** 2004. "*Candidatus* Endobugula glebosa," a specific bacterial symbiont of the marine bryozoan *Bugula* simplex. Appl Environ Microbiol **70:**4921-9.

93. **Malmqvist, M.** 1978. Purification and characterization of two different agarose-degrading enzymes. Biochim Biophys Acta **537:**31-43.

94. **Marx, I.** 1986. Isolation and characterization of an agar-degrading bacterium. Master's Thesis. George Mason University, Fairfax, VA.

95. **Metzgar, D., J. M. Bacher, V. Pezo, J. Reader, V. Doring, P. Schimmel, P. Marliere, and V. de Crecy-Lagard.** 2004. *Acinetobacter* sp. ADP1: an ideal model organism for genetic analysis and genome engineering. Nucleic Acids Res **32:**5780-90.

96. **Meulen, H. J., and W. Harder.** 1976. The regulation of agarase production by resting cells of *Cytophaga flevenis*. Antonie Van Leeuwenhoek **42:**277-86.

97. **Mishima, Y., K. Momma, W. Hashimoto, B. Mikami, and K. Murata.** 2003. Crystal structure of AlgQ2, a macromolecule (alginate)-binding protein of *Sphingomonas* sp. A1, complexed with an alginate tetrasaccharide at 1.6-A resolution. J Biol Chem **278:**6552-9.

98. **Momma, K., W. Hashimoto, O. Miyake, H. J. Yoon, S. Kawai, Y. Mishima, B. Mikami, and K. Murata.** 1999. Special cell surface structure, and novel macromolecule transport/depolymerization system of *Sphingomonas* sp A1. J Ind Microbiol Biotechnol **23:**425-435.

99. **Momma, K., M. Okamoto, Y. Mishima, S. Mori, W. Hashimoto, and K. Murata.** 2000. A novel bacterial ATP-binding cassette transporter system that allows uptake of macromolecules. J Bacteriol **182:**3998-4004.

100. **Moran, M. A., and R. E. Hodson.** 1989. Bacterial secondary production on vascular plant detritus: relationships to detritus composition and degradation rate. Appl Environ Microbiol **55:**2178-89.

101. **Morrice, L. M., M. W. McLean, W. F. Long, and F. B. Williamson.** 1983. Beta-agarases I and II from *Pseudomonas atlantica*. Substrate specificities. Eur J Biochem **137:**149-54.

102. **Morrice, L. M., M. W. McLean, W. F. Long, and F. B. Williamson.** 1983. Porphyran primary structure. An investigation using beta-agarase I from *Pseudomonas atlantica* and 13C-NMR spectroscopy. Eur J Biochem **133:**673-84.

103. **Morrice, L. M., M. W. McLean, F. B. Williamson, and W. F. Long.** 1983. beta-agarases I and II from *Pseudomonas atlantica*. Purifications and some properties. Eur J Biochem **135:**553-8.

104. **Murashima, K., A. Kosugi, and R. H. Doi.** 2002. Synergistic effects on crystalline cellulose degradation between cellulosomal cellulases from *Clostridium cellulovorans*. J Bacteriol **184:**5088-95.

105. **Murphy, K. C.** 1998. Use of bacteriophage lambda recombination functions to promote gene replacement in *Escherichia coli*. J Bacteriol **180:**2063-71.

106. **Newell, S.** 2001. Multiyear patterns of fungal biomass dynamics and productivity within naturally decaying smooth cordgrass shoots. Limnology and Oceanography **46:**573-583.

107. **Ohara, H., S. Karita, T. Kimura, K. Sakka, and K. Ohmiya.** 2000. Characterization of the cellulolytic complex (cellulosome) from *Ruminococcus albus*. Biosci Biotechnol Biochem **64:**254-60.

108. **Ohta, Y., Y. Hatada, S. Ito, and K. Horikoshi.** 2005. High-level expression of a neoagarobiose-producing beta-agarase gene from *Agarivorans* sp. JAMB-A11 in *Bacillus subtilis* and enzymic properties of the recombinant enzyme. Biotechnol Appl Biochem **41:**183-91.

109. **Ohta, Y., Y. Hatada, M. Miyazaki, Y. Nogi, S. Ito, and K. Horikoshi.** 2005. Purification and characterization of a novel alpha-agarase from a *Thalassomonas* sp. Curr Microbiol **50:**212-6.

110. **Ohta, Y., Y. Hatada, Y. Nogi, Z. Li, S. Ito, and K. Horikoshi.** 2004. Cloning, expression, and characterization of a glycoside hydrolase family 86 beta-agarase from a deep-sea *Microbulbifer*-like isolate. Appl Microbiol Biotechnol **66:**266-75.

111. **Ohta, Y., Y. Hatada, Y. Nogi, M. Miyazaki, Z. Li, M. Akita, Y. Hidaka, S. Goda, S. Ito, and K. Horikoshi.** 2004. Enzymatic properties and nucleotide and amino acid sequences of a thermostable beta-agarase from a novel species of deep-sea *Microbulbifer*. Appl Microbiol Biotechnol **64:**505-14.

112. **Ohta, Y., Y. Nogi, M. Miyazaki, Z. Li, Y. Hatada, S. Ito, and K. Horikoshi.** 2004. Enzymatic properties and nucleotide and amino acid sequences of a thermostable beta-agarase from the novel marine isolate, JAMB-A94. Biosci Biotechnol Biochem **68:**1073-81.

113. **Olabarria, G., J. L. Carrascosa, M. A. de Pedro, and J. Berenguer.** 1996. A conserved motif in S-layer proteins is involved in peptidoglycan binding in *Thermus thermophilus*. J Bacteriol **178:**4765-72.

114. **Pell, G., L. Szabo, S. J. Charnock, H. Xie, T. M. Gloster, G. J. Davies, and H. J. Gilbert.** 2004. Structural and biochemical analysis of *Cellvibrio japonicus* xylanase 10C: how variation in substrate-binding cleft influences the catalytic profile of family GH-10 xylanases. J Biol Chem **279:**11777-88.

115. **Potin, P., C. Richard, C. Rochas, and B. Kloareg.** 1993. Purification and characterization of the alpha-agarase from *Alteromonas agarlyticus* (Cataldi) comb. nov., strain GJ1B. Eur J Biochem **214:**599-607.

116. **Pugsley, A. P., C. Chapon, and M. Schwartz.** 1986. Extracellular pullulanase of *Klebsiella pneumoniae* is a lipoprotein. J Bacteriol **166:**1083-8.

117. **Ryu, S. K., S. J. Cho, S. R. Park, W. J. Lim, M. K. Kim, S. Y. Hong, D. W. Bae, Y. W. Park, B. K. Kim, H. Kim, and H. D. Yun.** 2001. Cloning of the *cel9A* gene and characterization of its gene product from marine bacterium *Pseudomonas* sp. SK38. Appl Microbiol Biotechnol **57:**138-45.

118. **Sambrook, J., and R. D.** 2000. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press.

119. **Schroeder, D. C., M. A. Jaffer, and V. E. Coyne.** 2003. Investigation of the role of a beta(1-4) agarase produced by *Pseudoalteromonas gracilis* B9 in eliciting disease symptoms in the red alga *Gracilaria gracilis*. Microbiology **149:**2919-29.

120. **Schwarz, W. H.** 2001. The cellulosome and cellulose degradation by anaerobic bacteria. Appl Microbiol Biotechnol **56:**634-49.

121. **Shieh, W. Y., and W. D. Jean.** 1998. *Alterococcus agarolyticus*, gen.nov., sp.nov., a halophilic thermophilic bacterium capable of agar degradation. Can J Microbiol **44:**637-45.

122. **Silliman, B. R., and M. D. Bertness.** 2002. A trophic cascade regulates salt marsh primary production. Proc Natl Acad Sci U S A **99:**10500-5.

123. **Solano, F., and A. Sanchez-Amat.** 1999. Studies on the phylogenetic relationships of melanogenic marine bacteria: proposal of *Marinomonas mediterranea* sp. nov. Int J Syst Bacteriol **49 Pt 3:**1241-6.

124. **Steenbakkers, P. J., A. Freelove, B. Van Cranenbroek, B. M. Sweegers, H. R. Harhangi, G. D. Vogels, G. P. Hazlewood, H. J. Gilbert, and H. J. Op den Camp.** 2002. The major component of the cellulosomes of anaerobic fungi from the genus *Piromyces* is a family 48 glycoside hydrolase. DNA Seq **13:**313-20.

125. **Steenbakkers, P. J., X. L. Li, E. A. Ximenes, J. G. Arts, H. Chen, L. G. Ljungdahl, and H. J. Op Den Camp.** 2001. Noncatalytic docking domains of cellulosomes of anaerobic fungi. J Bacteriol **183:**5325-33.

126. **Stosz, S.** 1994. An Agarase System from a Periphytic Procaryote. Ph.D. University of Maryland, College Park.

127. **Sugano, Y., T. Matsumoto, H. Kodama, and M. Noma.** 1993. Cloning and sequencing of *agaA*, a unique agarase 0107 gene from a marine bacterium, *Vibrio* sp. strain JT0107. Appl Environ Microbiol **59:**3750-6.

128. **Sugano, Y., T. Matsumoto, and M. Noma.** 1994. Sequence analysis of the *agaB* gene encoding a new beta-agarase from *Vibrio* sp. strain JT0107. Biochim Biophys Acta **1218:**105-8.

129. **Sugano, Y., I. Terada, M. Arita, M. Noma, and T. Matsumoto.** 1993. Purification and characterization of a new agarase from a marine bacterium, *Vibrio* sp. strain JT0107. Appl Environ Microbiol **59:**1549-54.

130. **Swartz, M. N., and N. Gordon.** 1959. Agarase from an agar-digesting bacterium. J Bacteriol **77:**403-9.

131. **Tamaru, Y., and R. H. Doi.** 1999. Three surface layer homology domains at the N terminus of the *Clostridium cellulovorans* major cellulosomal subunit EngE. J Bacteriol **181:**3270-6.

132. **Tamm, L. K., A. Arora, and J. H. Kleinschmidt.** 2001. Structure and assembly of beta-barrel membrane proteins. J Biol Chem **276:**32399-402.

133. **Tanaka, T., L. Yan, and J. G. Burgess.** 2003. *Microbulbifer arenaceous* sp. nov., a new endolithic bacterium isolated from the inside of red sandstone. Curr Microbiol **47:**412-6.

134. **Usov, A. I., and M. D. Martynova.** 1970. [Detection of agarase in molluscs of the genus *Littorina*]. Dokl Akad Nauk SSSR **194:**455-7.

135. **Usov, A. I., and L. I. Miroshnikova.** 1975. Isolation of agarase from *Littorina mandshurica* by affinity chromatography on Biogel A. Carbohydr Res **43:**204-7.

136. **Van der Meulen, H. J., and W. Harder.** 1975. Production and characterization of the agarase of *Cytoplaga flevensis*. Antonie Van Leeuwenhoek **41:**431-47.

137. **Vera, J., R. Alvarez, E. Murano, J. C. Slebe, and O. Leon.** 1998. Identification of a marine agarolytic pseudoalteromonas isolate and characterization of its extracellular agarase. Appl Environ Microbiol **64:**4378-83.

138. **Voget, S., C. Leggewie, A. Uesbeck, C. Raasch, K. E. Jaeger, and W. R. Streit.** 2003. Prospecting for novel biocatalysts in a soil metagenome. Appl Environ Microbiol **69:**6235-42.

139. **Whitehead, L. A.** 1997. Complex Polysaccharide Degrading Enzyme Arrays Synthesized by a Marine Bacterium. Ph.D. University of Maryland, College Park.

140. **Whitehead, L. A., S. K. Stosz, and R. M. Weiner.** 2001. Characterization of the agarase system of a multiple carbohydrate degrading marine bacterium. Cytobios **106 Suppl 1:**99-117.

141. **Xu, Q., E. A. Bayer, M. Goldman, R. Kenig, Y. Shoham, and R. Lamed.** 2004. Architecture of the Bacteroides cellulosolvens cellulosome: description of a cell surface-anchoring scaffoldin and a family 48 cellulase. J Bacteriol **186:**968-77.

142. **Xu, Q., W. Gao, S. Y. Ding, R. Kenig, Y. Shoham, E. A. Bayer, and R. Lamed.** 2003. The cellulosome system of *Acetivibrio cellulolyticus* includes a novel type of adaptor protein and a cell surface anchoring protein. J Bacteriol **185:**4548-57.

143. **Yaphe, W.** 1957. The use of agarase from *Pseudomonas atlantica* in the identification of agar in marine algae (*Rhodophyceae*). Can J Microbiol **3:**987-93.

144. **Yoon, J. H., H. Kim, K. H. Kang, T. K. Oh, and Y. H. Park.** 2003. Transfer of *Pseudomonas elongata* Humm 1946 to the genus *Microbulbifer* as *Microbulbifer elongatus* comb. nov. Int J Syst Evol Microbiol **53:**1357-61.

145. **Yoon, J. H., I. G. Kim, T. K. Oh, and Y. H. Park.** 2004. *Microbulbifer maritimus* sp. nov., isolated from an intertidal sediment from the Yellow Sea, Korea. Int J Syst Evol Microbiol **54:**1111-6.

146. **Yoon, J. H., I. G. Kim, D. Y. Shin, K. H. Kang, and Y. H. Park.** 2003. *Microbulbifer salipaludis* sp. nov., a moderate halophile isolated from a Korean salt marsh. Int J Syst Evol Microbiol **53:**53-7.

147. **Yoshizawa, Y., A. Ametani, J. Tsunehiro, K. Nomura, M. Itoh, F. Fukui, and S. Kaminogawa.** 1995. Macrophage stimulation activity of the polysaccharide fraction from a marine alga (*Porphyra yezoensis*): structure-function relationships and improved solubility. Biosci Biotechnol Biochem **59:**1933-7.

148. **Zhong, Z., A. Toukdarian, D. Helinski, V. Knauf, S. Sykes, J. E. Wilkinson, C. O'Bryne, T. Shea, C. DeLoughery, and R. Caspi.** 2001. Sequence analysis of a 101-kilobase plasmid required for agar degradation by a *Microscilla* isolate. Appl Environ Microbiol **67:**5771-9.

149. **Zhu, Y., and E. C. Lin.** 1987. Loss of aldehyde dehydrogenase in an *Escherichia coli* mutant selected for growth on the rare sugar L-galactose. J Bacteriol **169:**785-9.