Engineering agarolytic enzymes into *Bacteroides thetaiotaomicron* for the development of a drug delivery system with algal polysaccharide-derived capsules

by

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Abstract

The microbiota of the distal gastrointestinal tract (GIT) of monogastric animals plays a vital role in maintaining host health, such as releasing energy and nutrients from dietary polysaccharides that are otherwise indigestible by human enzymes. These dietary polysaccharides can be coupled to the metabolism and proliferation of defined bacteria in order to achieve an improved health outcome, which is known as synbiotics. Agarose is a marine polysaccharide that is well suited to act as a selective nutrient in a designer synbiotic system because it is resistant to digestion by the vast majority of microorganisms residing in the distal GIT of terrestrial animals. Agarose can be completely saccharified into its monosaccharide substituents by three agarases: GH16, GH117, and GH2, found in a polysaccharide utilization locus from a terrestrial bacterium *Bacteroides uniformis* NP1. The first objective of this research project aimed to engineer an agarolytic strain of the commensal gut bacteria, *B. thetaiotaomicron*, by introducing intrachromasomal copies of the three agarases with extracellular-directed signal peptides on the N-terminal domain. The transgenic agarases will be expressed to the outside of the cell in order to access the agarose substrates and import the released D-galactose into the cell to use as a carbon source. The second area of my research focused on developing an assay to measure release of cargo from algal-polysaccharide-derived capsules after digestion from the engineered strain. The agarase genes were successfully introduced into the *B. thetaiotaomicron* genome through homologous recombination, and all three agarases were produced by the bacterium in detectable amounts on western blots. Notably, enzymatic products were observed using thin layer chromatography following incubation with the engineered strains indicating the transgenic agarases are functional and able to hydrolyse agarose substrates. Only the GH16 enzyme was confirmed to be active on the outer membrane of the cell. Supplementation of the GH117 and GH2 into the medium on the

GH16 producing strain was able to confer growth on agarose. The second objective was completed by measuring the oxidation activity of released HRP from porphyran- and carrageenan-derived capsules. The capsules were leaky and optimization of the integrity of the capsules as well as the cargo used would be important to confirm the polysaccharides efficacy of capsules to release therapeutic molecules within the distal GIT. Importantly, oligosaccharides observed from capsules digested with purified GH16 enzymes as well as GH16 enzymes produced from both *B. uniformis* NP1 and engineered *B. thetaiotaomicron* support the development of a drug delivery system using algal polysaccharide-derived capsules and engineered agarolytic bacteria to deliver therapeutic molecules within the distal GIT.

Preface

This thesis is an original work by Stephanie M. Monteith. This thesis is part of a collaborative grant titled “a novel non-antibiotic innovation to treat inflammatory enteric diseases and promote growth in mammalian livestock” from the Alberta Livestock and Meat Agency (ALMA).

The initial design of Ag-Cassette from Chapter 2 was performed by Dr. Julie Grondin. Chapter 3 was a collaborative effort by myself and Paul Moote. Paul helped design and generated the algal-polysaccharide-derived capsules. I developed the assay used to measure the amount of HRP released from capsules and analyzed the supernatants after enzymatic and bacterial digest of capsules.

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List of Abbreviations

AHG	3,6-anhydro-l-galactose
ALMA	Alberta Livestock and Meat Agency
ANOVA	analysis of variance
AOS	agarooligosaccharides
AT	alimentary tract
B. theta	*Bacteroides thetaiotaomicron*
BCR	B-cell receptor
BHI	brain heart infusion
BSA	bovine serum albumin
CAZyme	carbohydrate-active enzyme
CBM	carbohydrate binding module
CD	Crohn's disease
CFU	colony forming unit
CGN	carrageenan, when constituting a capsule
CTL	cytotoxic T lymphocytes
DGM	distal gut microbiota
DNA	deoxyribonucleic acid
DEX	dextran
EMP	empty capsule, lacks HRP
FUdR	fluor-2-deoxyuridine
GAL	galactose
GH	glycoside hydrolase
GIT	gastrointestinal tract
GLC	glucose
GRAS	generally recognized as safe
HGT	horizontal gene transfer
HRP	horse radish peroxidase
IBD	inflammatory bowel disease
L6S	L-galactose-6-sulfate

LMPA	low melting point agarose
LPS	lipopolysaccharide
MHC	major histocompatibility complex
MM	*Bacteroides* minimal medium
N2	neoagarobiose
NAOS	neoagarooligosaccharide
NBU2	non-replicating Bacteroides unit 2
NPOS	neoporphyrooligosaccharide
PAMP	pathogen associated molecular pattern
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pINT	integrate plasmid
POR	porphyran, when constituting a capsule
PRR	pattern recognition receptors
PUL	polysaccharide utilization locus
PVDF	polyvinylidene fluoride
SACCHARIS	Sequence Analysis and Clustering of CarboHydrate Active enzymes for Rapid Informed prediction of Specificity
SCFA	short chain fatty acid
SGBP	surface glycan-binding protein
Sus	starch utilization system
TBDT	TonB-dependent transporter
TBS-T	tris-bufferd saline with Tween
TCR	T-cell receptor
Th	T helper
TLC	thin layer chromatography
TLR	toll-like receptor
TYG h/h	tryptone yeast glucose with histidine/hematin solution
UC	ulcerative colitis

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Chapter 1: Literature Review

1.1. Introduction

The alimentary tract (AT) of monogastric animals is a complex organ system responsible for many important functions including: the mastication and digestion of food, absorption of nutrients, excretion of waste, and protection between the environment and host physiological functions [1]. The gastrointestinal tract (GIT) is a subdivision of the AT and is comprised of the stomach, and small and large intestines. Within the distal GIT there is a large, heterogeneous community of microorganisms that is referred to as the distal gut microbiota (DGM) [2]. The DGM consists of trillions of organisms that collectively have a 150-fold larger number of genes as compared to the host. It has been suggested that the microbiota may even represent an additional organ that performs a very important role in contributing to intestinal health and host well-being [2]. The impact of the DGM on host health is dependent on the establishment and maintenance of homeostatic interactions between the microbiota and the host, and importantly, this homeostatic relationship develops and progresses throughout the host's life. An adverse alteration in the DGM, resulting from a variety of factors including changes in diet, the long-term use of antibiotics, and on occasion acute intestinal injury, can lead to an imbalance in the community structure. This type of change in community structure is known as dysbiosis and can induce serious intestinal injury and substantive harm to the host [3]. In addition to changes to diet and antibiotic use, induction of dysbiosis has also been associated with a wide range of diseases and disorders in people and livestock. For instance, in humans; dysbiosis has been linked to incidence of inflammatory bowel diseases, Type 2 diabetes, obesity, and cancer [4, 5], which collectively can incur a substantive financial burden on the health care system [6]. In monogastric livestock species such as pigs, dysbiosis is often associated with increased susceptibility to intestinal infection by enteric

pathogens and severe inflammatory diseases within the intestine [7]. Importantly, enteritis in pigs can also have considerable economic costs for the livestock sector as it can reduce feed intake, lower weight gain, and increase morbidity and mortality [8]. Providing in-feed antibiotics to animals is a strategy to treat enteritis in livestock species; however, the risk of enteric pathogens developing antibiotic resistance is a major public health concern, and as such, the long term use of antibiotics as feed additives is considered a contributing factor of antibiotic resistance [9]. Therefore, strategies that reduce intestinal inflammation and improve intestinal health in livestock and are independent of antibiotic use are important areas of research currently being investigated.

Alternative approaches to mitigate dysbiosis in livestock include the addition of probiotics, prebiotics, or the combination of probiotics and prebiotics (synbiotics) as feed additives [10, 11]. Probiotics are defined as live microorganisms that when administered in adequate amounts confer a health benefit on the host [11]. The administration of probiotics can help restore the microbial community balance by potential competitive exclusion of pathogens, promotion of mucosal immunity, and improvement of digestion; all factors that can enhance both weight gain, and animal performance [11]. As such, engineering bacteria with attributes capable of restoring a balanced microbiota is a promising strategy to mitigate intestinal-related diseases. Indeed, bacterial engineering has made incredible advancements in the recent years as there is currently the potential to engineer bacteria to both colonize the gut to re-establish a healthy microbiota as well as to release therapeutic agents within the GIT [12]. Prebiotics are dietary components that are often composed of non-digestible fibres that provide a source of energy to promote the growth of intestinal bacteria needed for the maintenance of a healthy microbial community [11]. In general, prebiotics are commonly plant derived dietary fibres from cereal grains, fruits, vegetables, nuts, and legumes that are resistant to digestion by host enzymes [13]. Synbiotics are combinations of

select prebiotics and probiotics that are administered together to improve intestinal health within the host [14]. Synbiotics couple the consumption of specific prebiotic dietary carbohydrates to the metabolism and proliferation of select beneficial bacteria. A synbiotic system designed to contain a unique engineered bacterium, such as a member of *Bacteroides* spp., with the ability to metabolize a rare or exclusive prebiotic may provide a strategy to colonize the distal gut with a microorganism that releases bioactive agents for treatment of enteric disease.

The literature review will provide background information on the following major topics: i) structure and function of the AT, ii) the intestinal microbiota, host immunity and host inflammatory responses, iii) mitigation of dysbiosis with prebiotics, probiotics, and synbiotics, iv) intestinal bacterial species: *Bacteroides* spp. and bacterial engineering, and v) the structure of algal polysaccharides and enzymatic functions of carbohydrate-active enzymes (CAZymes) on algal polysaccharides. Notably some topic areas (i.e. immunology, alimentary tract, microbiota) are highly complex and broad fields of study and for the scope of this thesis, only information that is needed to provide adequate background material and is relevant to the research project will be provided.

1.2. The Alimentary Tract

The AT is a tubular structure that begins at the mouth and terminates at the anus. This organ system is critical for the mechanical, chemical and enzymatic digestion of food, nutrient uptake, waste excretion, immune induction and immune tolerance, and acts as a physical barrier between the environment and host. Food enters the AT at the mouth which masticates and moistens the ingesta, the ingesta is subsequently swallowed and passes through the oesophagus to the stomach. The stomach continues the mechanical and chemical digestion of the ingesta by mixing the food and exposing the food to stomach enzymes and strong stomach acids. The resulting chyme enters

the small intestine where pancreatic enzymes and bile from the gallbladder further digest the food components into simple carbohydrates, amino acids, and lipids, which are then absorbed. Undigested dietary nutrients travel to the large intestine where the microbiota ferments the dietary fibres releasing products such as volatile short chain fatty acids (SCFA). The large intestine also absorbs water, electrolytes, and micronutrients that traverse the GIT. Finally, the remaining digesta is excreted as waste through the rectum [1, 15].

1.2.1. Tissues of the Alimentary Tract

In general, the AT is comprised of four tissue layers based on histological structures: the mucosa, submucosa, muscularis externa, and serosa or adventitia. The mucosa is the inner most layer that communicates with the lumen of the AT and plays a large role in the secretion of mucus, absorption of nutrients, and protection against infections. The mucosa layer is further sub-divided into three tissue layers: the epithelium, lamina propria, and muscularis mucosa. The epithelium is responsible for most of the digestive, absorptive, and secretory processes [15]. The epithelial cell lining throughout the AT have different morphological characteristics that aid in specific cell function. Epithelial cells include simple and stratified cell layers, cuboidal or columnar and squamous morphology, and produce variable amounts of keratin that contribute to the structural integrity of the cells [16]. Stratified epithelial cells are present in the oral cavity and oesophagus and provide a protective barrier from injury associated with the passage of rough food particles. Moreover, squamous epithelial cells are flattened epithelial cells and are important for absorption and transportation of metabolic products and cells into the blood and lymphatic vessels. Cuboidal or columnar cells facilitate absorption of nutrients and are associated with secretion of enzymes, mucus, water, ions, and other small molecules within the GIT [17]. The lamina propria is comprised of loose connective tissue that contain capillaries that supply the epithelium with

nutrients and oxygen. The lamina propria also contains numerous leukocytes and many of these cells (T cells, B cells, macrophages, and dendritic cells) are associated with mucosa-associated lymphoid tissue and importantly mucosal immunity. The submucosa is a dense, irregular layer of connective tissue with large blood vessels, lymphatic vessels, and nerves. The submucosa also connects the mucosa layer to the bulk of the underlying smooth muscles of the muscularis externa [18]. Patches and isolated lymphoid follicles, and secretory glands to aid in nutrient enzymatic digestion. The muscularis mucosa is a thin layer of smooth muscle that separates the mucosa from the submucosa and may help with localized movement of products within the mucosa [18].

The muscularis externa is composed of smooth muscle cells and is responsible for segmental contractions and peristaltic movements of the intestine within the GIT. These coordinated contractions propel food along the AT and prevent food from retrograde transportation within the intestine. In several regions along the AT, the circular muscle layer thickens forming the cardiac, peritoneal, and ileocecal sphincters; these structures function as valves to control food passage between various segments of the intestine. The muscularis externa layer of the colon is thick because significant muscular force is required to move feces towards the rectum for excretion. The serosa outer tissue layer of the intestinal wall consists of an epithelial layer that secretes serous fluid, to lubricate the organs and a thick connective tissue layer that functions as a partition between the AT and the surrounding structures (i.e. peritoneum). This reduces friction between the AT and other structures during muscle contractions and peristalsis. In contrast, the oesophagus is surrounded by underlying connective tissue known as the adventitia and this helps to stabilize the oesophagus within the neck, and thorax [15].

1.2.2. The Gastrointestinal Tract

The GIT is a subsection of the AT that consists of the stomach, small intestine, and large intestine which serve as a site for digestion, and nutrient absorption; it is also a major site for the induction of the immune response within the AT. The small intestine has a markedly folded epithelial cell monolayer that contains villus crypt units—alternating multifunctional villi extensions and deep proliferative crypt compartments [19]. Villi are columnar cell folded extensions of the mucous membrane that can substantively increase the surface area for nutrient absorption. The surface area is further increased by additional extensions from villi known as microvilli. The crypts are populated by stem cells that are required for cell turnover [19]. Indeed, the intestinal crypts have the highest rate of cell turnover in the mammalian GIT, it has been estimated that 10^8 cells are lost per day in the average human, and the crypt stems are needed to replace the cells during cell turnover [16]. In addition to the epithelial cells, the small intestine contains goblet cells that secrete mucus, and specialized enteroendocrine cells that are crucial for regulating intestinal functions including the secretion of intestinal products and controlling motility [20]. Another specialized cell within the GIT are M cells. These cells are involved with the GALT and function as an interface between the luminal contents and the underlying immune competent cells. The large intestine is involved in the absorption of water, electrolytes, minerals, and vitamins and is needed in the excretion of remaining digesta. The colon and cecum contain the largest and most diverse community of microorganisms within the AT and is the primary region for the fermentation of dietary fibre and the production and absorption of volatile SCFAs. Morphologically, the large intestine is sacculated, lacks villi, is comprised of simple columnar epithelial cells, and contains more mucus secreting goblet cells than other regions of intestines. The mucous layer coats the mucosa and is much thicker compared to the small intestine. This

mucous is thought to have two distinct layers: a thin, dense inner layer with only small numbers of bacteria and a thick, loosely structured outer layer that contains the majority of bacteria within the intestine [21].

1.3. The Immune System

The immune system is an integrated system of tissues, cells, proteins, and chemicals that function to protect the host from infection and disease. As mentioned, the AT acts as a barrier between the external environment and the host and contains large populations of bacteria that can be potentially harmful. As such the host has developed an immune system that is classified into two forms: innate immunity and adaptive immunity. These systems work in concert to protect the host from enteric infections and tissue injury. The innate immune system is composed of physical-chemical defenses as well as cellular and acellular immune processes [22]. Importantly, the innate immune response is rapid, non-adaptive, and considered the first response directed against invading microorganisms [23, 24]. The innate immune system also plays a key role in initiating and coordinating the second line of defense against microbial infections. The adaptive immune response is considered the second line of defense as it requires a longer period to become active and functional, and provides highly specific immune protection against pathogens and can develop immunological memory—a hallmark function of the adaptive immune system.

1.3.1. Innate Immunity

The innate response is the first line of defense to protect the host from infections and includes physical and chemical barrier function as well as cellular and acellular components. A major function of innate immunity is the physical prevention of antigens and pathogens from entering host tissue layers. The epithelial lining of the GIT mucosa is bound together by tight junctions, layered by mucus, and secretes enzymes and peptides that help maintain the epithelial

barrier and prevent antigens and pathogens from breaching the surface layer. Chemical barriers and acellular components further protect the host as part of innate immune function [24]. For instance, it has been shown that increasing stomach pH following the ingestion of antacids increases the survival of pathogenic bacteria, such as *Vibrio cholerae*, within the stomach and subsequently can cause disease [25]. Acute phase proteins are produced in response to inflammatory signals that can aid in the opsonization, agglutination, and elimination of pathogens by the initiation of complement and coagulation cascades, and secretion of protease inhibitors [26]. The innate immune system also recognizes conserved molecular patterns derived from foreign pathogens (Pathogen Associated Molecular Patterns; PAMPs), such as lipopolysaccharide (LPS), peptidoglycan, flagellin, and microbial nucleotides using Pattern Recognition Receptors (PRRs) [27]. PRRs are expressed on innate effector cells, such as macrophages and dendritic cells, and epithelial cells. The PRRs are needed for the release of a suite of cytokines and chemokines involved in the induction and progression of antigen-specific adaptive immune responses. Toll-like receptors (TLRs) are membrane-bound and intracellular receptors that can initiate intracellular signaling cascades including the MyD88 dependent and independent pathways. These pathways are involved in the activation of transcription factors, and required to promote expression of numerous proinflammatory cytokines [27]. Innate immune cells including natural killer cells, macrophages, neutrophils, and dendritic cells recognize foreign antigens and, with the exception of natural killer cells, also recognize opsonized pathogens. These cells phagocytize the antigens and pathogens which are degraded within phagosomes containing oxidative reactive species, antimicrobial peptides/proteins, and lytic enzymes. Some innate cells also present antigens to lymphocytes and importantly, link the innate immune system to the adaptive immune system—a key function for coordinated communications between the systems [24]. Finally, other cells, such

a mast cells and basophils are part of the innate immune system and these cells are often involved in the initiation of an inflammatory response by causing localized vasodilatation, accumulation of tissue edema, and chemotaxis of leukocytes [24].

1.3.2. Adaptive Immunity

The adaptive immune system consists of highly specialized leukocytes with the capacity to develop responses that are antigen and pathogen specific. The adaptive immune system requires longer periods of time to respond as compared to the innate immune response and is considered the second line of defense against invading pathogens [28]. The two primary cell types that form the adaptive response are T cells and B cells which are derived from lymphoid progenitor cells. Both T cells and B cells are major histocompatibility complex (MHC) restricted and notably capable of recognizing a multitude of foreign antigens [28]. Importantly, the antigen receptors on B and T cells distinguish foreign antigens from "self-antigens"–a requirement to develop immunotolerance and to prevent autoimmunity. Moreover, lymphocytes are capable of developing immunological memory and provide robust responses to additional challenges with the same antigen; a process that is not observed in innate immune function. The specificity of the adaptive response is associated with the variation in the T-cell receptors (TCRs) and B-cell receptors (BCRs). T cells are classified as CD4+ T-helper (Th) cells or CD8+ cytotoxic T lymphocytes (CTLs), and their classifications are determined by the expression of the CD4 or CD8 surface glycoproteins, which are also co-receptors for the T-cell receptor (αβTCR). These surface glycoproteins determine MHC class restriction and as such CD4+ generally interacting with MHC class II and CD8+ interacting with MHC class I [29]. T-helper cells are divided into five major subpopulations, including Th1, Th2, Th17, Threg and Tfh, and each T-helper cell subpopulation is characterized by signature cytokine cascades produced following activation. Each T-helper cell

subpopulation corresponds with a specific immune response developed and is tailored to eliminate the specific invading pathogen or induce immune quiescence. CTLs recognize specific antigens expressed on MHC class I and produces cytolytic factors to be released on the target cell [30]. These cytolytic factors are often produced in response to target cell injury or cells infected with intracellular pathogens such as viruses. B cells are responsible for antibody production, and each B cell produces an antibody that is specific to a unique antigen. Similar to the αβTCR, antibodies have very high specificity for antigens and produce antigen specific antibodies that assist antigen neutralization, activation of complement system, and opsonisation of antigen for phagocytosis [28].

1.4. The Distal Gut Microbiota

The heterogeneous composition of the microbiota of mammals varies in both the amount and the diversity of microorganisms present, with the most complex microbial community residing within the distal GIT. The distal GIT has a neutral pH, anaerobic environment, and a slower transit time compared to the small intestines. These factors along with a slower rate of cell turnover and the absence of many host enzymes are factors that contribute to the high percentage (~70%) of the total DGM residing in this region [31]. Although the DGM is diverse, the main bacterial species identified in the mammalian DGM are members of the Bacteroidetes and Firmicutes phyla [32]. The majority of these bacteria obtain their nutrients from non-digestible dietary fibre and host mucin-glycans, and diet is considered the primary factor that drives community diversity. Bacteroidetes are well-suited to colonize the large intestines of mammals as these bacteria contain tightly regulated gene clusters known as a polysaccharide utilization locus (PUL) and notably these clusters encode for enzymes that metabolize recalcitrant polysaccharides that cannot be digested by host enzymes [33]. *Bacteroides* spp. have tailored PULs and these gene clusters enable the

bacteria to colonize different regions of the large intestine with distinct metabolic niches. This allows these bacteria to adapt to changes in intestinal environment, host physiological responses, and nutrient content, thereby enabling *Bacteroides* spp. to colonize the GIT throughout the life of the host [34, 35].

Commensal bacteria within the distal GIT contain a repertoire of enzymatic processes that aid in the saccharification and fermentation of both non-digestible fibre and digestible polysaccharides that were not hydrolysed by host small intestinal enzymes [35, 36]. Fermentation of these polysaccharides can be beneficial to the host; for instance, fermentation of the polysaccharides results in the production of significant amounts of the SCFAs: butyrate, propionate, and acetate, which can provide energy to intestinal epithelial cells via β-oxidation. Indeed, SCFAs provide 60-70% of the caloric energy to colonic epithelial cells [3]. In addition to their nutrient role, SCFAs also have antimicrobial and anti-inflammatory properties and are involved in the regulation of the immune system [36, 37]. As an example, SCFAs have been shown to induce the activation of Treg cells in the distal GIT. Butyrate can also regulate gene expression by inhibiting histone deacetylases, which result in enhanced anti-inflammatory activity within the GIT [35, 37]. Finally, SCFAs can also inhibit the colonization of pathogenic organisms [37]. A recent study showed that fermentation of non-digestible fibre by *Bacteroides* produced adequate amounts of propionate within the gut to inhibit the growth of *Salmonella* bacteria [38]. It is apparent from these observations that regulatory and protective physiological responses induced by SCFAs following the fermentation of non-digestible polysaccharides are crucial for the maintenance of both a stable microbiota and the homeostatic relationship between gut microbial communities and the host.

1.4.1. Commensal Bacteria and Immune Function in GIT

As indicated previously, the immune system plays an important role in the control and regulation of microbiota bacteria within the GIT, including protecting the host from enteric pathogens. Moreover, the immune system helps maintain a homeostatic balance and regulates populations of commensal organisms, which are defined as bacteria that receive nutrients from the host but neither directly benefit or harm the host, within the GIT. This is an important consideration since the immune system can indirectly affect the competitive exclusion of pathogens, by altering community structure of commensal bacteria. Indeed the commensal microorganisms within the microbiota of the GIT can prevent pathogens from colonizing the intestines by outcompeting pathogenic bacteria for similar ecological niches, an ecological principle known as competitive exclusion [35]. Conversely, commensal microorganisms have a direct effect on the host immune system, as commensal bacteria are required to direct the development of immune system and assist in maintaining an immunologically quiescent environment within a healthy gut. As examples, the microbiota is important for the development of organized lymphoid follicles within the intestines [39]. A study by Pabst, *et al.* (2006) [40] showed that lymphoid follicles within the GIT only fully developed in the presence of commensal bacterial communities and in contrast, mice devoid of intestinal bacteria (i.e. germ free) had poorly formed and undeveloped lymphoid structures—highlighting the importance of intestinal bacteria for lymphoid follicle development. Commensal bacteria also help to regulate MyD88 dependant pathway responses that are needed in epithelial repair, induction of epithelial antimicrobial proteins, and production of cytokines following binding of LPS, and flagellin to TLR 4 and 5, respectively. The DGM also induce the production of inflammatory cytokines that improves host defence. For instance, commensal bacteria enhance IL-1β production by inducing the expression of the inactive pro-cytokine, that is then rapidly

converted into the active form enabling a faster inflammatory response to enteric infection [35]. Conversely, other commensal bacteria have been shown to enhance anti-inflammatory responses through the production of IL-10 and directing the differentiation of T cells into Treg cells. Indeed, *Bacteroides fragilis* produces a capsular polysaccharide, PSA, that induces IL-10 production and prevents expansion of pro-inflammatory Th17 responses. Interestingly, production of PSA in *B. fragilis* is considered to be instrumental for the bacteria to successfully colonize the distal GIT [41]. From the information provided, it is evident that the establishment and maintenance of stable commensal bacterial communities within the intestinal microbiota is important for maintaining good intestinal health.

1.5. Dysbiosis and Dysbiosis-Associated Intestinal Diseases

1.5.1. Dysbiosis

Dysbiosis is defined as a compositional and functional alteration in the microbiota which is generated by a suite of environmental and host-related factors that disrupt the intestinal microbial ecosystem. Notably, these changes need to exceed the resistance and resilience capabilities of the microbial community [3, 42]. Dysbiosis can be divided into three main categories and include increases in the numbers of pathogenic organisms, loss in the numbers of commensal organisms, and loss in the diversity of the microbiota which disrupts the homeostatic balance within the bacterial community structure [42]. Pathogenic bacteria are typically present in low numbers within the GIT bacterial population, but a change in the intestinal milieu, such as an intestinal inflammatory event, can lead to an overgrowth of pathogenic bacteria. For example, inflammation can influence growth of facultative anaerobic bacteria. These bacteria grow more proficiently within the hypoxic environment of inflamed tissues as compared to obligate anaerobic commensal bacteria that grow preferentially in healthy tissue [43]. Indeed, it has been observed

that there is an increase in the abundance of the facultative anaerobes, *Enterobacteriaceae* bacteria, in enteric infections and this increase in bacterial numbers is present in patients with Inflammatory Bowel Disease (IBD) [42, 44]. Similar observations have been associated with *Citrobacter rodentium* and *Salmonella enterica*, infections, and the presence of these pathogens has also caused marked dysbiosis in various mammals [45, 46]. Dietary products and antibiotics can also alter microbial diversity within the GIT. It has been shown that the microbiota within a healthy colon can quickly change following the ingestion of diets with a significant modification of the nutrient composition [47, 48]. Most certainly, mice fed low-fibre diets are shown to have a progressively reduced bacterial diversity in their microbial communities compared to mice fed high fibre diets [48]. The long-term use of antibiotics can also reduce large numbers of gut commensal bacteria, consequently diminishing the abundance and diversity of bacteria within the intestinal microbiota. As such, the application of antibiotics in the livestock industry as both therapeutic agents and antimicrobial growth promoters used to enhance performance has led to higher prevalence of enteritis and the increase on of antibiotic resistant microorganisms [49]. From the information provided, it is evident that loss of commensal bacteria and disruption of a homeostatic bacterial community structure are key elements to the reduction of colonization resistance and induction of dysbiosis-associated enteric disease.

1.5.2. Inflammatory Bowel Disease

Inflammatory Bowel Disease (IBD) is a chronic debilitating and often reoccurring GIT disorder, that includes both Crohn's Disease (CD) and Ulcerative Colitis (UC), and is characterized by chronic intestinal inflammation and intermittent periods of unregulated activation of the intestinal immune system [39, 50]. At present, the etiologies for the induction and progression of IBD is not fully known. Studies into the development of IBD, however, have shown that host

genetics, immune responses, and the interactions between the host and the microbiota of the GIT are important predisposing factors in the development of the disease [51]. Most certainly, studies have shown that dysbiosis has often been associated with this disease and disruptions in microbial communities play an important role in dysregulation of immune function and onset of intestinal inflammation. In a dysbiotic state within the gut, antigens from microbes activate Th1 and Th17 cells and these cells can induce intestinal inflammation and tissue injury. These T cells also produce bioactive molecules that decrease mucus production and therefore reduce the thickness of the mucous layer resulting in enhanced microbial invasion of the mucosa, and induction of severe IBD [39]. A reduction in bacterial community diversity in an inflamed gut has been shown in numerous studies. For example, patients with CD have reduced α-diversity in the fecal bacteria as compared to healthy individuals [52]. Similarly, a reduction in bacterial diversity and bacterial numbers was observed in people with a propensity to develop IBD as compared to individuals with no familial history of IBD [53].

1.6. Prebiotics, Probiotics, and Synbiotics

1.6.1. Prebiotics

Prebiotics are nutrients that stimulate the growth or activity of advantageous autochthonous bacteria and typically prebiotics are non-digestible dietary carbohydrates, such as fibre, fructans, or oligosaccharides [11]. Diet has been shown to have the biggest and most direct effects on colonization of the DGM because many bacteria use fermentation products as energy sources for bacterial growth and colonization [54]. As a result, diet is closely related to the alteration of bacterial communities within the DGM, and therefore, has potential to affect intestinal health of the host. Prebiotics may also improve health of the GIT by mechanisms that are independent of assisting in growth of intestinal bacteria. For instance, some prebiotics can exert a direct

antimicrobial effect on bacteria as the prebiotics adhere to the binding sites of bacteria on enterocytes, and thus competitively block the adhesion of pathogenic bacteria to intestinal epithelial cells [55].

One of the challenges in studying selective nutrients as prebiotics is that prebiotics may not stimulate growth of desirable taxa as many commensal bacteria have the potential to metabolize the same nutrient substrate [54]. In this regard, chemically selective prebiotics obtained from rare dietary ingredients, such as seaweed, may provide a unique strategy to overcome these challenges. The majority of bacteria within the DGM of terrestrial animals lack the enzymes and metabolic pathways required to digest carbohydrates present in seaweeds. In addition, the major polysaccharides present in seaweed are anionic with unique chemical linkages, and therefore, resistant to modification by stomach acid and enzymatic degradation in terrestrial animals. Thus, these polysaccharides traverse to the colon and are available for use by competent bacteria as selective energy sources. Therefore, designing commensal bacteria with the propensity to hydrolyse seaweed polysaccharides would allow these bacteria to preferentially grow in the GIT.

1.6.2. Probiotics

The administration of probiotic bacteria is another possible method to mitigate the development of intestinal dysbiosis, as probiotic bacteria can restore the proper balance of bacterial populations within the intestinal microbiota. Probiotics are live microorganisms that when administered in adequate amounts confer a health benefit for the host [11]. Most bacterial strains that are considered as potential probiotic bacteria, are members of *Bifidobacterium* and *Lactobacillus* genera of bacteria. Paralleling the effects of a balanced and healthy microbiota, the introduction of selective bacterial species into the GIT may occupy nutritional niches that could otherwise be exploited by invasive pathogens, and thereby reduce disease. In addition to

competitive exclusion, these administered bacteria can produce vitamins and other metabolites as well as ferment recalcitrant carbohydrates to produce SCFAs and aid in the promotion of intestinal health. In addition, these beneficial bacteria may also directly block the mucosal invasion of intestinal pathogens by producing antibacterial substances that prevent the attachment of pathogen to host cells. Moreover, probiotics could help mitigate intestinal inflammation by stimulating appropriate anti-inflammatory immune responses with the host [11, 56]. One of the biggest disadvantages of employing probiotics to mitigate intestinal disease is that many currently used probiotic bacterial species are 'transient' within the host and do not effectively colonize the colon. Therefore, using probiotics to treat enteric disease often requires the continuous administration of the bacteria to provide prolonged beneficial effects within the intestinal microbiome.

1.6.3. Synbiotics

Individual administration of either prebiotics or probiotics has had limited success in the treatment of inflammatory bowel diseases and other diseases related to intestinal dysbiosis [51]. Designing a treatment strategy that co-administers probiotic bacteria and prebiotic nutrients to work in synergy offers a possible better method to mitigate intestinal dysbiosis and IBD. The combination of probiotics and prebiotics administered to augment host intestinal health is defined as synbiotics [11]. One method to positively select for beneficial bacteria within the GIT is to couple specific dietary carbohydrates to the metabolism and proliferation of select beneficial bacteria. In theory, synbiotic systems can be genetically engineered to employ desired biochemical traits (ie. select enzymatic digestion of nutrient) into a recipient probiotic bacterium in order to facilitate the positive selection of the engineered bacterium in the presence of rare dietary carbohydrates (prebiotic). Furthermore, synbiotics could be engineered to produce bioactive

molecules, such as immunomodulating agents in response to the presence of selective prebiotic nutrients, thus regulating the release of products and improving treatment of enteric disease.

1.7. Engineering Bacteria to Release Bioactive Agents

Advancements in the understanding of processes, functions, and applications of synthetic biology in medical and biological sciences have greatly increased over the last several decades [12, 57]. These advancements have extended into the use of engineered bacteria as potential therapeutic agents by their ability to release immunomodulatory products, colonize specific environments within tissues, and use specific nutrients as growth activators [58, 59]. Bacteria have evolved to respond to environmental changes as well as produce biomolecules at quantities that elicit physiological responses. Using this ecological adaptation, bacteria could be designed to survive and colonize the distal GIT and produce targeted therapeutic agents in a local environment. Importantly, this strategy could circumvent some challenges faced by probiotic bacteria, such as poor intestinal colonization, and possibly reduce the amount of agent required to induce the desired physiological response to treat disease [12]. As an example, *Lactococcus lactis* has been engineered to produce IL-10 to treat mice with experimentally induced IBD-like diseases. It was shown that the localized IL-10 produced from *L. lactis* strain reduced enteric inflammation with a much lower dose of IL-10 as compared to other mice treated systemically administered IBD therapeutic agents [60]. Bacteria have also been engineered to elicit specific physiological responses that involve immune function. *L. monocytogenes* has been designed to produced tumour-associated antigens that induce innate and adaptive immune responses in tumour challenge models [61]. In addition, bacterial circuits that express gene products involved in a specific biological response and act as a biomarker signal can be designed as either indicators of disease and used for diagnostic applications or used as tightly controlled therapeutic agent delivery systems. As an

example, an engineered strain, *E.coli* NGF-1, is capable of sensing the production of tetrathionate, a by-product of inflammation, and produce β-galactosidase through a bacterial memory circuit. [62]. The resulting production of β-galactosidase by these bacteria after sensing inflammatory signals can be used as a diagnostic indicator of disease.

Engineering bacteria to deliver targeted therapeutic agents has the potential to circumvent problems that can occur in the treatment of disease, such as the use of large quantities of drugs that occurs with the systemic administration of medications. One possibility is creating circuits to identify specific biological signals and producing therapeutic agents in response. These memory circuits can also be designed to stop producing therapeutic agents following the elimination of the stimulus, thus preventing potential adverse effects to the host from prolonged release and subsequent exposure of therapeutic agents [12]. An additional consideration when choosing engineered bacteria would be using ones that are capable of selectively colonizing distant regions of the intestine. Regions such as the proximal and distal colon can be occasionally difficult to treat with orally administered drugs due to the size and location of the organ within the host [63]. Most certainly, orally administered therapeutic agents may be denatured by stomach acids, bound to bile acids, or modified by digestive enzymes; all processes that can alter the drug therapeutic potential prior to reaching the distal GIT. Other biochemical, physiological, and microbiological considerations for using targeted engineered bacteria to treat enteric disease include: i) the potential toxic side effects following inadvertent release of the therapeutic agents at unexpected regions such as the small intestine when targeting the colon, ii) the additional energy required from the host to support bacterial growth, iii) the impact of the inflammatory mediators on colonization of engineered bacteria, and iv) effects of genetic circuits on bacterial community structure [12]. Some progress has been achieved for colonization of bacteria in the inflamed gut tissue with

subsequent release of therapeutic agents. A study using engineered *Salmonella enterica* ser. Typhimurium showed that the bacterium could both grow and colonize within the hypoxic environment of tumours and deliver select anti-tumour agents [43].

Another challenge with genetically engineering bacteria is the additional energy demand required for the expression of the inserted genes. Engineered bacteria with gene insertions into their genomes require energy to transcribe and translate these proteins, and as such, energy can be redirected from the bacteria's abilities to proliferate and colonize within the GIT. Certainly, this would further burden the bacteria to grow and colonize in an already competitive environment [12]. As a result, there is potential for selective pressures to inadvertently cause point mutations within the inserted engineered genes and as such render the genes inactive. Solutions to overcome these challenges could include adding retention mechanisms to incorporated plasmids, or refining control of gene expression to reduce the total energy burden of transcription on the bacteria. As an example, a CRISPR-Cas9 based system has shown the ability of engineered *E. coli* to restrict gene transcription when it senses a challenge to the bacterium's energy sources and yet still maintains the ability to produce good levels of protein [64]. It would appear that engineering bacteria to colonize specific areas of the GIT and release bioactive products may be a good strategy to treat enteric disease, and although there have been examples of engineered strains of bacteria surviving for long periods within the gut, this process has not been well established. As such designing a different bacteria that can utilize a highly selective prebiotic, such as a seaweed cell wall polysaccharide, and survive and colonize the colon would be an excellent tool for developing a delivery system of intestinal targeted therapeutic agents.

1.8. Algal Cell Wall Polysaccharide Structure

Algal cell wall polysaccharides are complex non-digestible carbohydrates that are present in the amorphous matrix of the cell walls of Rhodophyta macroalgae (i.e. red algae), and these complex macroalgae polysaccharides include porphyran, agarose, and carrageenan [65]. Algal polysaccharides are remarkably different from terrestrial plant cell wall polysaccharides as they can be highly sulfated, contain anhydrous monosaccharides, and are enriched in L-galactose, an uncommon enantiomeric form of galactose. Moreover, the complexity of these marine polysaccharides can be further increased with the addition of other covalent modifications such as methylesterification and methoxylations [65]. The algal polysaccharides have a linear backbone structure and are composed of repeating disaccharide units consisting of 3-linked-β-D-galactopyranose and 4-linked-α-galactopyranose sugars. The spatial orientation of the 4-linked-α-galactopyranose can vary as D or L configurations and are present in carrageenan and agarose, respectively. The unique structural physical-chemical properties of these algal polysaccharides enable the polysaccharides to form aqueous gels, and as such these polysaccharides are also known as phycocolloids and have been applied to many industrial manufacturing processes [66].

The structure of agarose is mainly composed of α-3,6-anhydro-L-galactose (L-AHG) and β-D-galactose (D-GAL) monosaccharides [65, 66]. These two monosaccharides are linked by α-1-3 glycosidic bonds to form the basic repeating disaccharide unit: neoagarobiose (N2) with L-AHG on the non-reducing end, and two individual N2 disaccharides are linked together with β-1,4 glycosidic bonds (Figure 1.1.). Agarose can also have varying amounts of methyl esters on the C6 carbon, and these methylations can change the physical properties of the polysaccharide; including the alteration of the melting temperature of the carbohydrate. Porphyran has a similar structure to agarose but differs by having increased numbers of L-galactose-6-sulfate (L6S) subunits and fewer

L-AHG subunits; porphyran also has an increased frequency of C-6 methylations on GAL subunits. Conversely, carrageenan differs from porphyran and agarose as carrageenan polysaccharides have α-3,6-anhydro-D-galactose monosaccharides (D-AHG) instead of L-AHG and a higher level of sulfations as compared to agarose (Figure 1.1.). Carrageenans are characterized into subgroups based on the degree of sulfations and other decorations on the monosaccharide backbone. Kappa (κ)- and iota (ι)-carrageenan both contain α-D-AHG and D-GAL monosaccharides with a sulfate ester group on the D-GAL C4. Kappa (κ)-carrageenan has a higher prevalence of the anhydrous monosaccharide as well as another sulfate ester on the anhydrous subunit's C2. In contrast, lambda (λ)-carrageenan is not sulfated on the C4 of the GAL and does not contain anhydrous monosaccharides. λ-carrageenan is the most sulfated carrageenan with a total of three sulfate groups on the repeating disaccharide unit including both monosaccharides' C2 atoms and the C6 of the D-GAL subunit [65]. Importantly, the amount of sulfations and the presence of the 3,6-anhydrous bridge on the monosaccharides can change the carrageenan physical-chemical properties. For example, more sulfate ester decorations on the monosaccharides result in lower gel forming capabilities. In this regard, (κ)-carrageenenan can form rigid gels, ι-carrageenan forms soft gels, and λ-carrageenan has limited ability to form any stable gel structure, and consequently λ-carrageenan is used as a thickening agent in dairy products [67].

Notably, the structural complexity of these non-terrestrial red algal polysaccharides requires that bacteria have a distinct set of enzymes to hydrolyse carbohydrate modifications and linkages, which enable the bacteria to use the carbohydrate as a carbon source. The genes that encode these specialized hydrolytic enzymes are predominantly present in marine bacterial genomes and are either found to a lesser extent or more commonly absent in the genomes of bacteria present in the intestinal microbiota of terrestrial animals [68].

1.9. Carbohydrate Active Enzymes

Complex dietary carbohydrates consist of monosaccharide subunits joined together by glycosidic linkages and can be modified by methylations, acetylations, and sulfations, and glycan branching to create structural complex macromolecules with distinct conformations and physicochemical properties. Therefore, dismantling this rich source of glycan structures and chemistries requires bacteria to express a large suite enzymes with saccharolytic capabilities. Several bacteria within the microbiota, including members of the Bacteroidetes phylum, are known for their ability to saccharify a wide variety of complex carbohydrates, and by association, express an impressive repertoire of carbohydrate active enzymes (CAZymes), enzymes responsible for the synthesis and degradation of carbohydrates [69]. In comparison, the human genome contains nearly one hundred CAZymes and only a fraction of these enzymes are involved in the metabolism of dietary carbohydrates, primarily starch and sucrose [70]. In contrast, an intestinal bacterium, *Bacteroides thetaiotaomicron,* devotes 18% of its genome to express enzyme systems containing CAZymes that can facilitate the modification and complete saccharification-–hydrolysis of a complex polysaccharide into single monosaccharide subunits [33]. Glycoside hydrolases (GHs) are the largest class of CAZymes, and these enzymes are subdivided sequence-related enzyme families [71]. As such, the GH class of enzymes display conserved secondary structural folds, active site residues, and catalytic mechanisms. In general, GHs hydrolyse glycosidic bonds between two carbohydrate residues, but the substrate specificity varies between GH families and these variations are associated with the pronounced structural diversity of complex dietary carbohydrates.

1.9.1. CAZymes used for Algal Polysaccharide Saccharification

The ability of certain marine microorganisms to efficiently saccharify algal polysaccharides depends on the presence of specialized CAZymes in their genomes. Signature GH families involved in these reactions include GH50, GH86, and GH117 [72]. The GH50 family of enzymes contains specific β-agarase activity that release neoagarobiose or neoagarotetraose following hydrolysis [71]. Structural characterization of a GH50 enzyme from *Saccharophagus degradans* showed the exo-β-agarase Aga50D adopts an elaborated $(\alpha/\beta)_8$ barrel fold and also contains a CBM-like β-sandwich domain (Figure 1.2A) [73]. The tunnel-like fold contains the catalytic machinery and suggests that the enzyme works in a processive mode of action allowing the product to be released from an opening near the minus (-) active sites of the enzyme. This allows the enzyme to stay bound to the shortened agarose chain [73]. The GH86 family of enzymes are classified into β-agarases and β-porphyranases and have the same structural fold as GH50 family enzymes [71]. Hehemann *et al.* (2012) characterized the first specific β-porphyranase GH86 from *Bacteroides plebeius* and structural analysis of the enzyme revealed important structural formations for porphyran-specific enzyme activity (Figure 1.2B). The minus one (-1) subsite of this GH86 contains a hydrophobic pocket not present in the GH16 family of porphyranases. The hydrophobic pocket is able to accommodate methyl substitutions on the C6 of the D-GAL residues. At present, within the GH117 family of CAZymes, only a small number of enzymes have been characterized, and all of the characterized enzymes within this family have specific α-1,3-L-neoagarooligosaccharide hydrolase activity [71]. This family of enzymes is found in many bacteria with agarolytic mechanisms and it is one of the few hydrolases that cleaves α-1,3 linkages in agarose. Interestingly, GH117 enzymes are only active in a dimer form, unlike GH86 and GH50 enzymes, which are active as monomers [68].

GH16 is a 'polyspecific' family of enzymes and generally not considered a member of the classical family of agar-hydrolysing enzymes. Nevertheless, some GH16 enzymes have been characterized as endo-acting β-agarases, β-porphyranases, and κ-carrageenases and play an important role in the saccharification of algal polysaccharides [71]. These enzymes maintain a β-jelly roll configuration and can hydrolyse β-linkages throughout the polysaccharide backbone to produce smaller oligosaccharides that can be subsequently hydrolysed into individual monosaccharides by other enzymes. Hehemann *et al*. (2010) characterized two *Zobellia galatanivorans* β-porphyranases with distinct activities that differed from β-agarase characterized GH16 enzymes (Figure 1.2C). Structural analysis of these two porphyranases showed structural site accommodation for the sulfate group on the L6S monosaccharide in the minus two (-2) subsite, which is one of the primary monosaccharides in porphyran. The minus two (-2) subsite contains a pocket that accommodates the L6S sugar, which in β-agarase GH16 enzymes, is occupied by a tyrosine residue. The pocket also contains residues (H53 and R133) that neutralize the negative charge of the sulfate group by forming salt bridges [75]. The GH2 family of enzymes is another 'polyspecific' family that has β-galactosidase activity to hydrolyse GAL monosaccharides off of the non-reducing end [71]. GH2 have been found in *Vibrio* spp. and *Bacteroides* spp. that have tailored-agarose activity as the plus 1 (+1) subsite can better accommodate anhydrous bridge from the L-AHG structure compared to other GH2 β-galactosidases [68].

1.9.2. Mechanisms of Agarolysis by CAZymes

The digestion of complex marine polysaccharides into monosaccharide units requires a coordinated alignment of enzymatic activity. Indeed, the enzymatic activities of these agarase families work in concert to saccharify marine polysaccharides into their monosaccharides which enter bacterial metabolic pathways. A pathway for agarose saccharification by marine bacteria has

been determined [76, 77]. The main pathway includes: *endo*-acting enzymes from GH16 and GH86 families that hydrolyse the β-1,4 bonds within the polysaccharide backbone of agarose into neoagarooligosaccharides (NAOS) of different sizes, a function that is dependent on the specificity of the enzyme (Figure 1.3A). For example, *B. uniformis* NP1 produces both GH16 and GH86 enzymes, and the GH16B agarase, primarily produces tetra-NAOS (N4) and hexa-NAOS (N6), while GH86 primarily produces neoagarobiose (N2) [68]. The NAOS products are then hydrolysed by the *exo*-acting GH50 or GH86 family resulting in the production of N2. N2 is further hydrolysed into the monosaccharides L-AHG and D-GAL by the GH117B α-1,3-L-neoagarooligosaccharide hydrolase. An alternative pathway for the saccharification of agarose, has been recently described in the marine bacteria *Vibrio spp.* (Figure 1.3B). This mechanism uses a specialized GH2 β-galactosidase that recognizes AOS (products of GH117 family enzyme activity) and hydrolyse the GAL residue from the non-reducing end of these carbohydrates [78].

1.10. Polysaccharide Utilization Loci

Bacteroides spp. organize genes related to carbohydrate break down into PULs. PULs are clusters of co-localized, co-regulated chromosomal genes with expression products involved in the recognition, sequestration, enzymatic digestion, and transport of complex carbohydrates within the bacteria [79]. Traditionally, PULs are defined by the presence of at least one pair of adjacent *susD* and *susC* genes. The *susD* gene encodes an N-terminally lipidated surface glycan-binding protein (SGBP), while the *susC* gene encodes an outer membrane TonB-dependent transporter (TBDT). These genes are important in the recognition and transport of oligosaccharides into the periplasm of the bacteria. In addition, PULs encode a suite of CAZymes required for the complete saccharification of a target carbohydrate that is specific to each PUL. Finally, PULs also encode bacterial carbohydrate receptors and transcriptional regulators. The first PUL described is

comprised of eight genes involved in starch saccharification and metabolism and as such this PUL is often referred to as the starch utilization system (Sus). The Sus system is very well described and is considered the archetypal cellular model for the study of PUL activities within diverse groups of bacteria [80].

Marine Bacteroidetes express PULs with CAZymes required to metabolize marine polysaccharides. Interestingly, CAZymes with similar enzymatic capabilities have been discovered in the genomes of terrestrial microorganisms, including microorganisms present in the soil and mammalian GIT [75, 81]. Indeed, the genome of *Bacteroides plebeius*, a bacterium found within the human gut, contains PULs that hydrolyse algal polysaccharides such as porphyran [75]. Further analysis showed gene products from these human gut symbionts have enzymatic activity similar to the marine bacterium *Zobellia galactanivorans.* This discovery led to the "Sushi Factor Hypothesis", which proposed that *B. plebeius*, a human gut symbiont, received a set of genes from other marine bacteria through horizontal gene transfer (HGT). This HGT endowed the bacterium with the ability to metabolise dietary porphyran. In support of this hypothesis, a metagenomic analysis of the intestinal microbiota of Japanese and North American individuals demonstrated that porphyranase genes (genes present in marine bacteria) were only observed in coastal Japanese populations, which further supported the hypothesis of HGT. It is possible that the intestinal bacteria of Japanese individuals were introduced to marine bacteria from ingesting seaweed. This resulted in a human gut symbiont that was then able to occupy a previously unavailable ecological niche within the gut microbiome of the Japanese individuals [75, 82].

Another human intestinal bacteria, *B. uniformis* NP1, was isolated from humans and was identified as an agarolytic gut bacterial species based on its ability to cause pocking on blood agar cultured plates [74]. Annotation of the genome of this species revealed the presence of a PUL

containing CAZymes with activities consistent with agarose utilization (Ag-PUL), namely members of the GH2, GH16, GH86 and GH117 enzyme families [68]. Detailed structural and functional analysis of these enzymes revealed a mechanism for the complete saccharification of agarose, similar to the mechanism described above using a GH16, GH117, and GH2 family enzyme found in *Vibrio sp.* The *B. uniformis* NP1 mechanism for agarose saccharification was determined and is as follows: Firstly, GH16B and GH86 (endo-β-agarases) cleave β-1,4 glycosidic linkages between D-GAL and L-AHG, producing neoagarooligosaccharides (NAOS) of varying lengths. Secondly, the exo-α-1,3-L-neoagarooligosaccharide hydrolase GH117B hydrolyses the exposed L-AHG on the non-reducing end of the NAOS, resulting in the production of agarooligosaccharides (AOS) with D-GAL residues at the non-reducing end of the oligosaccharide backbone. Finally, D-GAL is removed by the exo-β-galactosidase GH2C. In as such, complete saccharification occurs by GH117B and GH2C in a coordinated manner by hydrolysing L-AHG and D-GAL from the non-reducing ends of NAOS and AOS, respectively [68].

PULs are the primary enzymatic systems for the metabolism of nutrients in *Bacteroides* spp., and as such, are intrinsically linked to *Bacteroides'* carbohydrate-dependent colonization of the microbiota [79]. Bacteria within the microbiota are considered to be either carbohydrate 'specialists' or 'generalists' with either a narrow- or broad-spectrum carbohydrate utilization mechanism, respectively. Glycan generalists, such as *Bacteroides* spp., are equipped with PULs that hydrolyse a diverse variety of diet- and host-derived polysaccharides, enabling their survival in the highly competitive gut environment as they can colonize unique nutritional niches. In contrast, specialist species are well-suited to use a specific carbohydrate within its environment to colonize the distal GIT [83].

1.11. Bacterial Engineering

1.11.1. Engineering Carbohydrate Utilization into Bacteria

Only a few species of intestinal bacteria have been found to encode genes involved in algal polysaccharide metabolism [75]. This could be perceived as either an advantage or disadvantage for employing algal polysaccharides as a source of prebiotics as they would only stimulate the growth of small numbers of autochthonous bacteria within the gut microbiota. An alternative to using only prebiotics would be employing a synbiotic system. As mentioned, synbiotics are the combination and application of probiotic bacteria and prebiotic nutrients to provide beneficial effects to the host [11]. One method to positively select for beneficial microbes within the GIT is to couple the requirement of bacterial growth and colonization to specific dietary carbohydrates. By using this strategy, monogastric intestinal bacteria that contain specific genes for the metabolism of marine polysaccharides could be used to digest dietary algal polysaccharides and importantly allow the bacteria to colonize and thrive within the intestine. As an example, Shepherd *et al*. (2018) [58] were able to modify the microbial community in a gnotobiotic murine model by positively selecting bacteria through the administration of rare nutrients in the mouse-diet. Using a human gut symbiont, *B. ovatus,* genetically engineered to contain a porphyran-specific PUL, the investigators showed that *B. ovatus* was able to produce the enzymes necessary for porphyran digestion and metabolism. The researchers were able to modulate the population size of *B. ovatus* within the GIT and as such overcome isogenic self-exclusion from the host intestine. Moreover, this group engineered the porphyran PUL into other human gut *Bacteroides* spp. and showed that control of the engineered bacteria's growth and colonization can be tightly regulated by varying amounts of porphyran present within the diet [58].

1.11.2. Engineering *Bacteroides* spp. to Release Bioactive Agents

Bacteroides spp. are gram negative, rod-shaped, obligate anaerobes that reside in the human colon and are capable of fermenting recalcitrant non-digestible carbohydrates, primarily into acetate. These organisms are present in both the distal GIT and environment and can be considered as both commensal microorganisms and opportunistic pathogens. Indeed, *Bacteroides* spp. have been associated with incidence of septicemia in humans and pododermatitis in cattle [84], although these events are commonly associated with compromised gut barrier function and predisposing disease. The *Bacteroides* spp. composes a large percentage of the bacteria within the human microbiota and currently members of the species are being investigated as potential candidates for engineered probiotics. In particular, these microorganisms can form stable bacterial communities within the intestinal microbiota, unlike other commercially available probiotic bacteria such as *L. lactis*—a bacteria that is transient and does not effectively colonize the GIT [11]. *Bacteroides* spp. are known for their abilities to digest a wide variety of complex polysaccharides, and therefore, are able to adapt to the presence of fluctuating levels of dietary non-digestible carbohydrates and host-derived glycans present within the GIT [85]. *B. ovatus* has been previously engineered to produce a variety of immunomodulatory molecules intended to prevent and treat IBD. This bacterium colonizes within the distal large intestine and has been engineered to effectively produce interleukin-2 [86], trefoil factor 3 [87], transforming growth factor β [88], and keratinocyte growth factor [89] under regulation of a xylan promoter system. As an example, mice administered engineered *B. ovatus* that secreted keratinocyte growth factor in the presence of xylan had a reduction in tissue injury in dextran-sodium sulfate challenged mice [89].

Bacteroides thetaiotaomicron is a prominent member of the microbiota of the human and pig GIT [90]. *B. theta* devotes 18% of its genome to encode 88 different PULs [33]. In addition,

to having the ability to colonize the GIT, molecular tools have been developed to modify the genome of *B. theta*, making this bacterium a promising candidate for genetic engineering. Two different mutagenesis systems exist for *B. theta*: i) pExchange plasmid is a counter-selectable system that uses homologous recombination to make site-directed chromosomal insertions and unmarked deletions with single base pair resolution [91-93]; and ii) the pNBU2 plasmid, which contains a mobile transposon element known as the non-replicating *Bacteroides* unit 2 (NBU2) [92, 94]. TheNBU2 integrates the entire plasmid sequence into one of two serine tRNA sites within the chromosome of *B. theta* for rapid screening of heterologous gene expression. Promoter elements and ribosomal sites have also been defined and optimized for the expression of heterologous proteins within *B. theta* [91, 95]. Recently, both the pExchange and pNBU2 plasmids have been modified to provide a targeted insertion or deletion of a specific gene under the regulation of constitutive or inducible *B. theta* promoters using a deletion strain of *B. theta*. It was shown that the strains produced detectable amounts of heterologous protein and this did not affect the growth of *B. theta* [96].

1.12. Conclusion

The microbiota of the GIT is a large, dynamic, and diverse community of bacteria that plays a vital role in maintaining host health. The microbiota is important for the regulation of the host immune system, promoting maturation and proliferation of gut epithelial cells, and occupying a diverse range of nutritional niches. The impact of the microbiota on the host is dependent on the establishment and maintenance of a dynamic composition of species over the lifespan of the individual. Many host and microorganism-associated mechanisms are needed to maintain a homeostatic balance between the host innate and adaptive immune responses, and a stable intestinal microbial community structure. Notably, dysbiosis can develop following a disruption

in the microbial community structure; a consequence of acute dietary changes, prolonged antibiotic use, and tissue injury. Importantly, this can lead to significant disease in both people and farmed animals, placing a significant burden on the health care system and livestock industry. Various strategies could be employed to reduce dysbiosis and intestinal injury and this could include adding probiotic bacteria, prebiotic nutrients, or a combination of both probiotics and prebiotics (ie. synbiotics) to the diet.

Studies have shown probiotics and prebiotics have some success restoring the balance of the intestinal microbiota by the administration of 'beneficial' bacterial species to the GIT or by selecting for the growth of commensal bacteria with non-digestible dietary fibres [11, 56, 97]. In this regard, algal polysaccharides could be employed as a selective prebiotic. Algal polysaccharides are found in the cell walls of red algae and contain unique structural composition, such as the presence of 3,6-anhydrous bridges and a high degree of sulfate esters and methylations on individual monosaccharides. These modifications require a suite of specialized enzymes to completely saccharify the polysaccharide backbone. Marine bacteria have a repertoire of such specialized enzymes that belong to the GH50, GH86, and GH117 enzyme families. Recently, it has been discovered that some human gut symbionts have acquired algal polysaccharide-specific PULs through HGT. This discovery along with the advancements of bacterial engineering techniques for using *Bacteroides* spp. as potential delivery systems for therapeutic agents, suggests there could be new strategies for engineering algal polysaccharide saccharification mechanisms into human gut symbiont *B. theta*. Such engineered strains would be well-suited to saccharify rare, chemically complex substrates and long-term colonization of the intestine. Importantly, engineering these bacteria would be the first step in developing a delivery system that could enable the targeted release of bioactive molecules following the co-administration of engineered with *B*

theta with recalcitrant carbohydrates. This symbiotic system could therefore be used to mitigate enteric disease and improve gut health in monogastric mammals and people.

1.13. Hypotheses

The literature review demonstrates the need to develop new strategies to mitigate intestinal inflammation and enteric diseases in humans and livestock. The review also demonstrates the possibility of designing bacteria that colonize the intestinal microbiota, and in the presence of specific dietary products, such as agarose found in marine seaweed, have potential to be used to release either therapeutic agents or other cargo from algal polysaccharide formulated capsules. The initial step needed to employ this strategy for treatment of intestinal inflammation, is the development of specialized bacteria that can easily grow and colonize the mammalian GIT in the presence of agarose as a selective prebiotic.

The hypotheses tested in this thesis were:

1) The genome of *B. theta* can be engineered to encode an Ag-Cassette regulated by either an inducible or constitutive promoter that contains three functional agarases that together completely saccharify agarose.
2) Development of a colourimetric assay can measure the release of bioactive molecules encapsulated in algal-polysaccharide derived capsules after digestion with purified enzymes or human gut bacteria producing these enzymes.

1.14. Figures

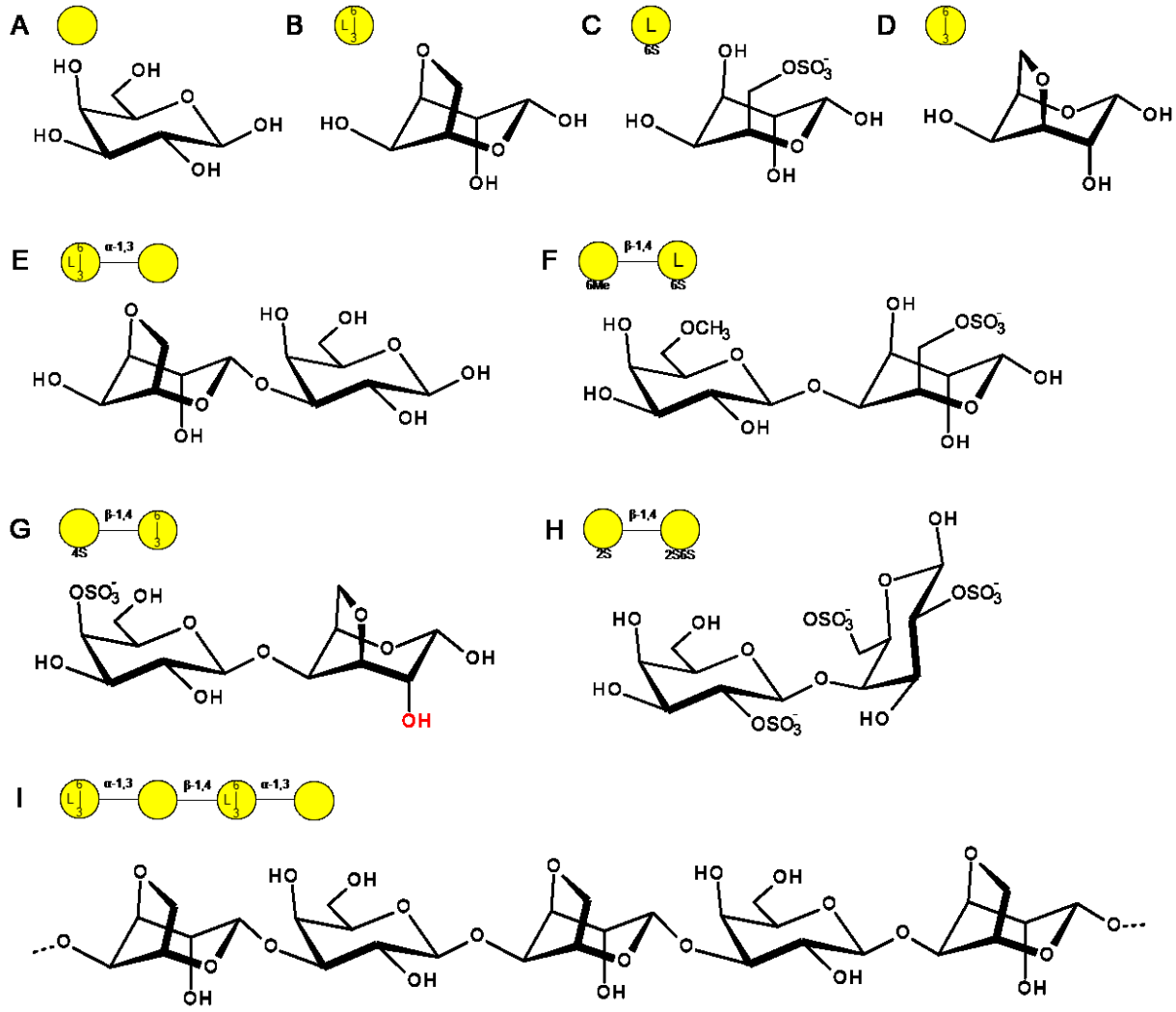


Figure 1.1. Structural representation of monosaccharide and disaccharide units of red algal polysaccharides. A) D-galactose, B) 3,6-anhydro-L-galactose, C) 6-*O*-sulfo-L-galactose, D) 3,6-anhydro-D-galactose, E) neoagarobiose (N2), F) porphyrobiose, G) κ-carrabiose, if the C2 hydroxyl group (red) is sulfated then ι-carrabiose H) λ-carrabiose, I) agarose.

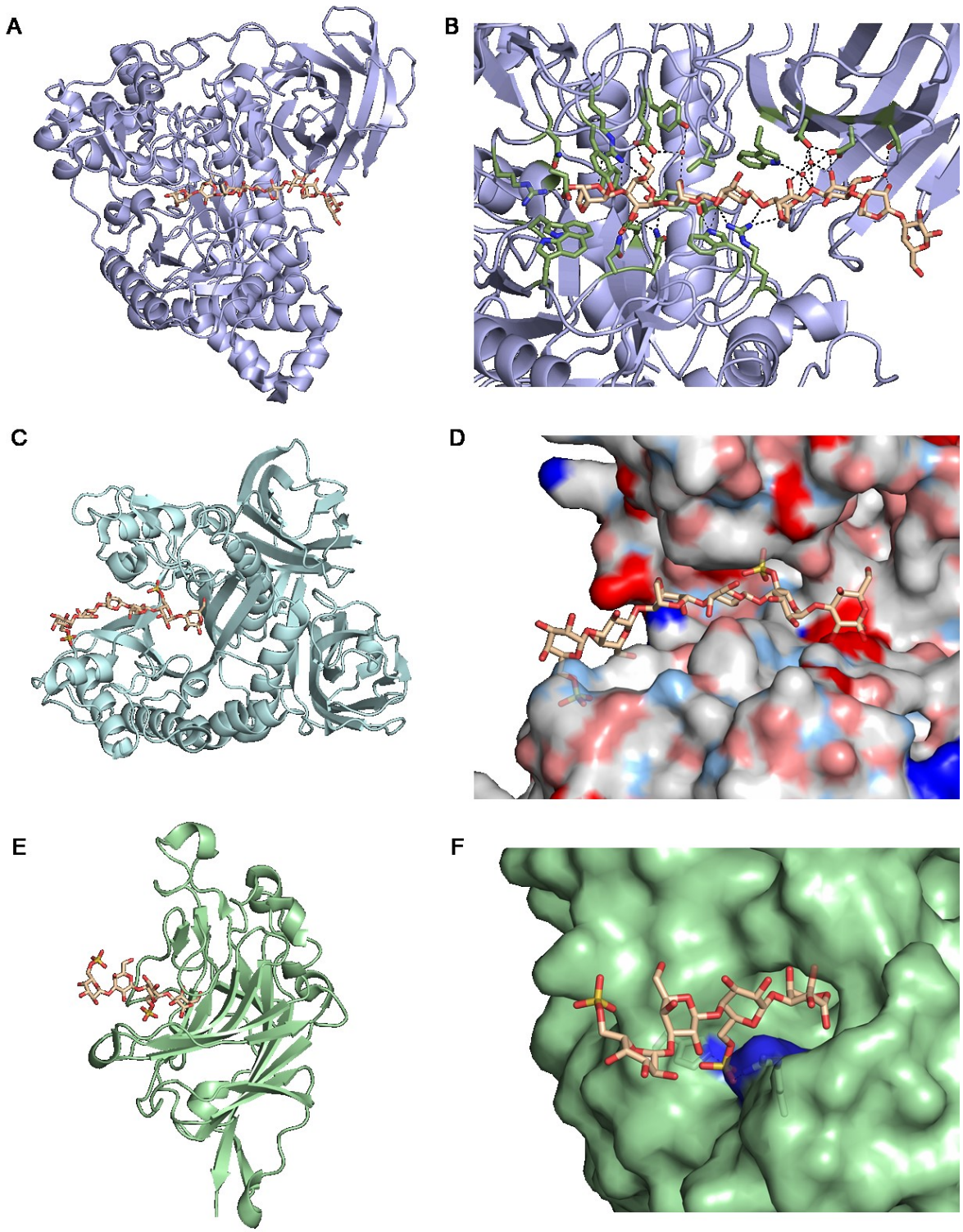


Figure 1.2. Protein structures of characterized CAZymes, highlighting active site structure to accommodate algal polysaccharide substrates. A) Schematic of the crystal structure of Aga50D

from *Sacharophagus degradans* [PDB:4BQ5], showing its tunnel-like fold that allows the enzyme to work in a processive order to hydrolyse the agarose substrate. B) Neoagarooctaose bound in the mutant E534Q GH50 active site, active site residues are stick structures and the hydrogen bond interactions between residues and substrate are represented by dashed lines. C) Schematic of crystal structure of GH86A from *Bacteroides plebeius* [PDB:4AW7] fitted with neoporphyrohexaose D) Surface representation coloured by atom type: blue is nitrogen, red is oxygen, and white is carbon to show polarity of active site fitted with a neoporphyrohexaose. Polar residues are represented by bright colours. E) Schematic of crystal structure of PorA GH16 [PDB:3ILF] in complex with neoporphyrotetraose. F) Surface representation of active site pocket with positive charge of H53 and R133 that neutralize negative charge of sulfate group on L6S.

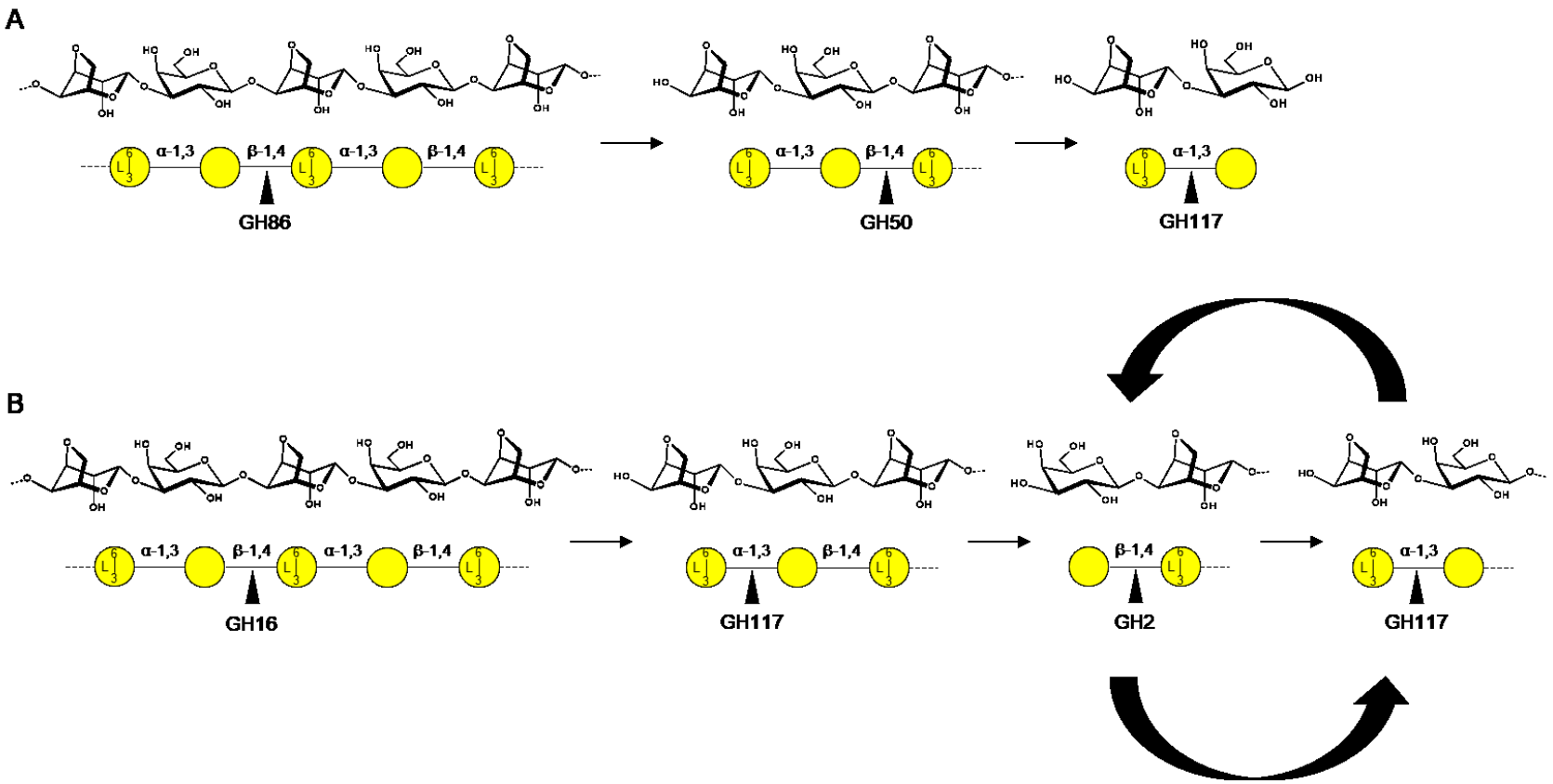


Figure 1.3. Agarose saccharification mechanisms into monosaccharide subunits by different agarase families. A) An endo-β-agarase begins hydrolysing the agarose polysaccharide into neoagarooligosaccharides (NAOS), next a GH50 exo-β-agarase releases neoagarobiose (N2) from the non-reducing end of the NAOS. GH117 α-1,3-neoagarooligosaccharide hydrolase hydrolyses the α-1,3 bond of N2 releasing the two monosaccharides. B) An endo-β-agarase hydrolyses the agarose polysaccharide into neoagarooligosaccharides (NAOS), GH117 then hydrolyses the α-1,3 linkage from the non-reducing end of the NAOS releasing 3,6-

anhydro-L-galactose and agarooligosaccharide (AOS) units. Next, a GH2 exo-β-galactosidase hydrolyses the β-1,4 bond on the non-reducing end releasing D-galactose and NAOS. GH117 and GH2 work in a cyclical procedure to continually cleave the non-reducing end monosaccharide from the other's oligosaccharide products.

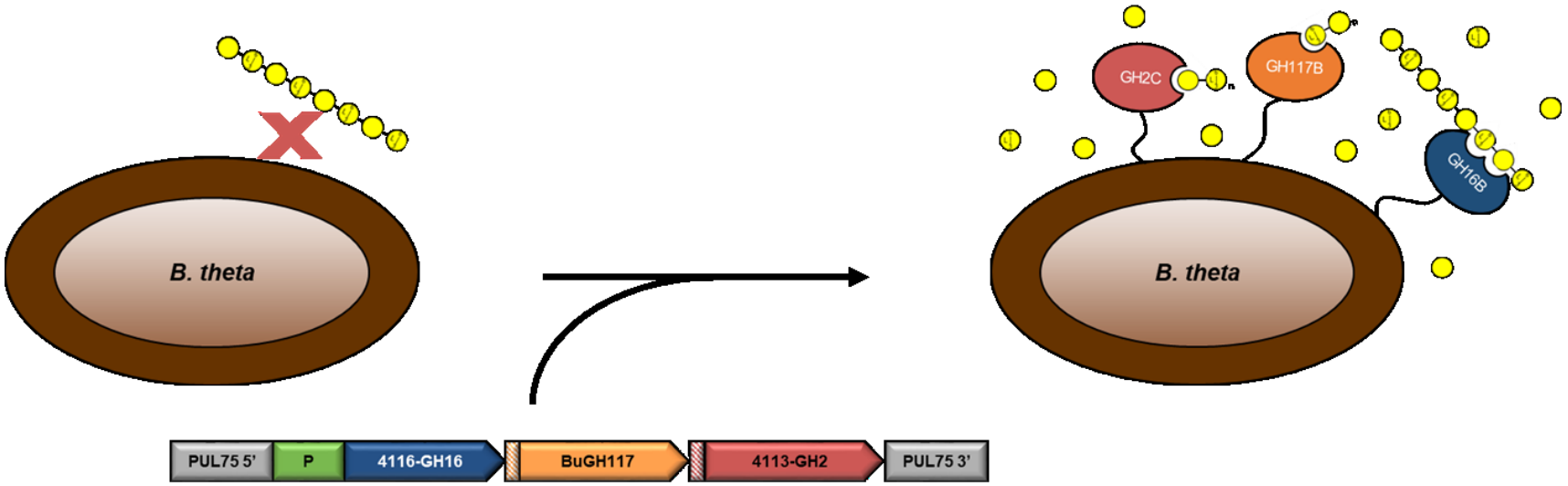


Figure 1.4. Genetic engineering of B. theta to produce three surface active agarases that hydrolyse agarose into individual monosaccharide units.

Chapter 2: Engineering agarases into human gut symbiont *Bacteroides thetaiotaomicron*

2.1. Introduction

The distal gut microbiota (DGM) plays an important role in regulating immune function, preventing pathogen colonization, as well as providing an energy source by fermenting dietary fibres [35, 47]. The impacts of a healthy DGM are dependent on the establishment and maintenance of homeostatic interactions between the DGM and the host [35]. An adverse change in the DGM resulting from a variety of factors including changes in diet, prolonged use of antibiotics, or acute intestinal injury can lead to an imbalance in the community structure, which is known as dysbiosis [3]. Dysbiosis has been implicated in many diseases such as inflammatory bowel disease, cancer, and metabolic disorders including obesity and Type 2 diabetes [3, 4], which collectively incur a large financial burden on the Canadian health care system [6]. As a result, methods to mitigate dysbiosis in the DGM are important areas of research.

Current methods to mitigate dysbiosis include the addition of probiotics and prebiotics to diets, which are thought to restore the balance of beneficial bacteria within the DGM of the distal gastrointestinal tract (GIT) [11]. Prebiotics predominantly act as selective energy sources for bacteria in the DGM. In addition, prebiotics can have indirect effects, following bacterial fermentation that include production of SCFAs, and anti-inflammatory activities that stimulate the immune system in the GIT, in addition, prebiotics can have direct effects on the DGM, such as having antimicrobial properties [36, 37]. To date, common probiotics often comprise beneficial bacteria that are transient (eg. *Lactobacillus* spp.) and do not colonize the host intestine. Therefore, these bacteria do not have long-term effects within the gut and must be continuously administered. The combination of probiotics and prebiotics, or “synbiotics”, could augment the effects of both probiotics and prebiotics. Synbiotics would select for the proliferation of beneficial bacteria that

ferment the administered selective prebiotics, with the goal to increase the duration of the beneficial effects (ie anti-inflammatory responses) within the intestine.

Autochthonous bacteria are promising candidates for use as probiotics as they can adapt to the host intestinal environment and have the ability to colonize the GIT, thereby providing long lasting effects within the GIT and importantly without the need for repeated administration of the bacteria. These bacteria can colonize the gut within nutrient niches developed by the addition of rare or selective carbohydrates within the diet of the host. Fermentation of these prebiotics can result in the production of SCFAs and other bioactive molecules, such as immunomodulatory peptides and secondary metabolites that can improve GIT health. *Bacteroides* spp. can effectively colonize the mammalian GIT and are considered carbohydrate generalists as they devote a large percentage of their genome to the metabolism of diet- and host-derived carbohydrates [83]. *Bacteroides* spp. organize genes responsible for utilization of a specific carbohydrate into a co-localized and co-regulated PUL [79]. PULs encode proteins responsible for carbohydrate depolymerisation, transportation, and gene regulation. These pathways are very adaptive and can exchange genomic information between donor and receipt strains of Bacteroidetes [68, 75]. As a result, members of this phylum are highly adaptable within their local environments and can modify their metabolic functions in the presence of fluctuating levels of complex carbohydrates within the colon and other environmental ecosystems [79]. In addition to fermenting dietary glycans, *Bacteroides* spp. are capable of digesting the host-derived glycans as an energy source present on the surface of the luminal epithelium when the supply of dietary glycans is limited [98].

B. theta is an obligate anaerobic bacterium that is present within the human DGM. *B. theta*, a highly studied member of *Bacteroides* spp., devotes 18% of its genome to carbohydrate metabolism which is separated into a minimum of 88 different PULs [99]. Molecular tools have

been developed to modify the genome of *B. theta*, making this bacterium a promising candidate to act as an engineered synbiotic to improve gut heath. One tool is the pExchange-*tdk* plasmid, a counter-selectable system that uses homologous recombination for site-directed chromosomal insertions and unmarked deletions with single base pair resolution [91, 92]. In addition, promoter elements and ribosome binding sites have also been defined and optimized for heterologous protein expression in *B. theta* [91, 95]. Recently, this pExchange-*tdk* system has been modified to provide streamlined, targeted insertion or deletion of a specific gene under the regulation of constitutive or inducible *B. theta* promoters. This is accomplished by using a PUL75 knock-out strain of *B. theta, B. theta ΔtdkΔPUL75* [96]. Genome modifications made with this exchange system have been successful in modifying heterologous protein expression and does not appear to adversely affect the growth or viability of the organism within a bacterial culture. As such, this molecular tool can be used towards engineering bacteria to incorporate new carbohydrate utilization strategies to metabolize rare polysaccharides into *B. theta*.

Polysaccharides that comprise the cell walls of Rhodophyceae (red algae) are complex, non-digestible agars that markedly differ in structure from terrestrial plant cell wall polysaccharides. Agars are enriched in chemically diverse galactans that can contain L-galactose monosaccharides and anhydrous monosaccharides, and are highly sulfated. One of the primary galactans in agar is agarose. The chemical structure of agarose consists of repeating disaccharide subunits of 4-*O*-α-3,6-anhydro-L-galactose (L-AHG) and 3-*O*-β-D-galactose (D-GAL) (Figure 2.1A) [65]. In order to saccharify agarose, a collection of enzymes that digest 4-β-D-GAL and 3-α-L-AHG are required. Hallmark carbohydrate active enzyme (CAZyme) glycoside hydrolase (GH) families involved in the hydrolysis of β–linkages are typically found in GH86, GH50, GH16,

and GH2 families. In contrast, α-agarases that hydrolyse the α-linkages are present in GH117 and GH96 families [71, 75].

Agarases are mainly found in marine bacterial species, such as *Vibrio* spp. and *Zobellia* spp., but some bacteria present in the DGM have also acquired agarase genes as a result of HGT [75]. *Bacteroides uniformis* NP1 is an agarolytic human gut bacterium that was discovered by pocking of blood agar plates [74]. Further annotation of the genome of *B. uniformis* NP1 revealed the presence of a PUL containing CAZymes with activities consistent with agarose utilization (Ag-PUL), including three GH2 enzymes, three GH16 enzymes, two GH117 enzymes, and a single GH86 enzyme. A mechanism for the saccharification of agarose using these CAZymes has recently been defined [68]. GH16B and GH86 are endo-β-agarases that cleave the β-1,4 glycosidic linkage between D-GAL and L-AHG, producing neoagarooligosaccharides (NAOS) of varying lengths. The exo-α-1,3-L-neoagarooligosaccharide hydrolase, GH117B hydrolyses an exposed AHG unit from the non-reducing end of NAOS, resulting in the production of agarooligosaccharides (AOS) with D-GAL at the non-reducing end. D-GAL is then removed by the exo-β-galactosidase GH2C. As such, GH117B and GH2C in a coordinated manner remove L-AHG and D-GAL from the non-reducing ends of NAOS and AOS, respectively [68].

The acquisition of the Ag-PUL by *B. uniformis* NP1 enhances the capacity of this species to utilize agarose as a carbon source for energy. In non-agarolytic bacteria, the addition of Ag-PUL to the bacterial genome enables these bacteria to use agarose as a carbon energy source and occupy a rare nutritional niche. Moreover, agarose is a good candidate for use as a prebiotic, as it is anionic and has unique chemical linkages that are resistant to acid hydrolysis in the stomach and resistant to modification with host digestive enzymes. Agarose and other seaweed algal polysaccharides also contain anti-oxidant and anti-microbial properties thought to further benefit

the health of the host [100]. Indeed, coupling agarose utilization with an engineered synbiotic system that could be positively selected in the presence of agarose would improve bacterial fitness within the DGM. Furthermore, engineered agarolytic bacteria have the potential to hydrolyse agarose-derived capsules and release encapsulated bioactive products, such as immunomodulatory and antimicrobial agents, and would provide a unique targeted release system for the treatment of intestinal disease within the distal GIT.

This chapter explores the possibility of engineering agarases from the agarolytic human gut symbiont *B. uniformis* NP1 into the non-agarolytic symbiont *B. theta.* This engineered bacterium would become a designer probiotic that specifically hydrolyses the rare dietary polysaccharide, agarose. Further characterization of the Ag-PUL GHs showed that only three agarases (GH16B, GH117B, and GH2C) are required to completely saccharify agarose into the individual monosaccharide substituents: D-GAL and L-AHG [68]. By engineering N-terminal signal peptides that traffic the proteins to the outer surface of the bacterium, the agarases will be able to access and saccharify the agarose in the environment without the need for additional mechanism to transport the agarases outside the bacterium. The three agarase genes were sequentially added in to the genome of *B. theta Δtdk ΔPUL75* under regulation of either a single inducible or a constitutive promoter using the modified pExchange-*tdk* system, pIntegrate (pINT) [96]. The resulting engineered *B. theta* strains were characterized to determine if the transgenic agarases were effectively secreted outside the bacterium and retain enzyme activity.

2.2. Materials and Methods

2.2.1. Bacterial genomic mutagenesis

2.2.1.1. Cloning gene inserts into E. coli

Donor vectors that contain the sequence to be inserted into *B. theta's* genome were built by inserting PCR amplified gene sequences into the pINT vector by restriction digest [96]. Agarase genes were amplified along with a 750 bp flanking region from the recipient strain upstream of the site of gene insertion. Overlapping PCR was used to insert the N-terminal signal peptide and a 10 bp intragenic region between each agarase gene. Four N-terminal signal peptides characterized as outer surface SPII peptides from genes that are encoded in *Bacteroides* spp. were used in this study and include, *bt4116* (4116), *bt4113* (4113), *bt3698* (Anc), and *np1_32* (Bu) (Table 2.1) [68, 101, 102]. After the gene insertion vector plasmids were engineered, the plasmids were transformed into S17λpir *E.coli* cells and sequence validated. The 4116-GH16B gene sequence was amplified and inserted in to the pINT-DX vector using NcoI and XhoI restriction digest enzymes. The BuGH117B and 4113-GH2C agarase genes were each amplified with a 750 bp overlap of GH16 or GH117, respectively, to form the 5' flank to ensure correct orientation and placement agarase gene within the Ag-Cassette. Unsuccessful cloning attempts with overlapping PCR for the GH117B and GH2C vectors required the vectors to be synthesized by BioBasic, which were then subsequently amplified and ligated into the pINT backbone (Table 2.2).

2.2.1.2. Homologous recombination of agarase genes into *B. theta*

2.2.1.2.1. Uptake of donor vector into *B. theta*

Double displacement chromosomal mutagenesis was performed as previously described [103]. Briefly, donor S17-λpir *E.coli* cultures were inoculated into Luria-Bertani broth (LB) (B244620, Fischer Scientific) medium supplemented with 100 μg/mL ampicillin and grown

aerobically overnight at 37°C and continuously shaken at 300 rpm. Recipient strains of *B. theta Δtdk ΔPUL75* were inoculated in Tryptone Yeast extract Glucose (TYG) medium (1% Bacto Tryptone (B211705, Thermo Fisher, Waltham, MA), 0.5% yeast extract (J850, VWR, Radnor, PA), 4.1 mM L-cysteine, 0.2% glucose, 0.1 M KPO_4 pH 7.2, 2.2 µM vitamin K_3, 40 µL/ mL TYG salts (2 mM $MgSO_4 \cdot 7H_2O$, 119 mM $NaHCO_3$, and 34.2 mM NaCl), 28.8 µM $CaCl_2$, 1.4 µM $FeSO_4$) supplemented with 1 uL/mL (v/v) of histidine/hematin solution (1.9 mM hematin and 200 mM L-histidine) (TYG h/h) were grown overnight at 37°C in a vinyl anaerobic chamber (Coy Lab Products, Grass Lake, MI) with an atmosphere of 85% N_2, 10% CO_2, 5% H_2. Dense ($OD_{600\ nm}$ ~ 1.0) cultures were subsequently subcultured (200 uL culture into 5 mL medium) into fresh media and incubated for an additional 6 hours. Bacterial cultures were pelleted by centrifugation at 5,000 rpm for 5 minutes. The pellets of recipient and donor cultures were combined and resuspended together in 1 mL of TYG h/h medium and added onto Brain and Heart Infusion (BHI) agar and incubated aerobically for 20 hours at 37°C. Bacteria were then resuspended in 5 mL of TYG h/h and 100 µL of the resuspension and a 1:10 serial dilution of the resupended culture were plated on BHI agar supplemented with 200 µg/mL gentamycin and 25 µg/mL of erythromycin to select for *Bacteroides* and incorporation of the donor vector. These plates were incubated in anaerobic conditions at 37°C for 24 – 48 hours. Eight bacterial colonies were replated and single colonies were cultured anaerobically for 16 hours in TYG h/h medium. This was the first selection step of the double displacement recombination conjugation process.

2.2.1.2.2. Insertion of target gene into B. theta genome

The second selection step for pINT vector conjugations includes combining 1 mL from each bacterial culture and plating in 1:10 serial dilution on BHI agar plates supplemented with 200 µg/mL of 5-fluoro-2-deoxyuridine (FUdR) (F0503, Millipore Sigma) and incubated in anaerobic

conditions at 37°C for 24-48 hours. Eight colonies were selected, replated, and single colonies were cultured in anaerobic conditions for 16 hours at 37°C in TYG h/h medium. Bacterial cultures inoculated from single colonies were cryoprotected in glycerol (30% v/v) solution and stored at -80°C. Sequence validation of the inserted genes required genomic DNA purification (K0512, Thermo Fisher, Waltham, MA) and subsequent PCR amplification of the inserted genes to be sequenced using Sanger sequencing.

2.2.2. Transgenic Protein Production in *B. theta*

B. theta strains were inoculated from -80°C glycerol stocks and cultured overnight in TYG h/h medium at 37°C in anaerobic conditions. Dense (OD_{600nm}~1.0) cultures were then pelleted by centrifugation (5000 rpm for 5 minutes at room temperature) and washed in 2.5 mL of 2x *Bacteroides* minimal medium (MM), pH 7.2: 200 mL/L (v/v) 10X *Bacteroides* salts solutions pH 7.2 (999 mM KH_2PO_4, 30 mM NaCl, 17 mM $(NH_2)_2SO_4$), 20 mL/L (v/v) Balch's Vitamins pH 7.0 (36.5 µM p-aminobenzoic acid, 4.5 µM folic acid, 8.2 µM biotin, 40.6 µM nicotinic acid, 10.5 µM calcium pantothenate, 13.3 µM riboflavin, 14.8 µM thiamine HCl, 48.6 µM vitamin B_6, 73.8 nM vitamin B_{12}, and 24.2 mM thioctic acid), 20 mL/L (v/v) Amino Acid Solution (5 mg/mL of L-amino acids: alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine), 20 mL/L (v/v) Purine/Pyrimidine Solution pH 7.0 (1 mg/mL adenine, guanine, thymine, cytosine, and uracil), 20 mL/L (v/v) Trace Mineral Solution pH 7.0 (1.7 M ethylenediaminetetraacetic acid, 12.2 M $MgSO_4 \cdot 7H_2O$, 3 M $MnSO_4 \cdot H_2O$, 17.1 M NaCl, 359.7 mM $FeSO_4 \cdot 7H_2O$, 901.1 mM $CaCl_2$, 347.7 mM $ZnSO_4 \cdot 7H_2O$, 40.1 mM $CuSO_4 \cdot 5H_2O$, 161.7 mM H_3BO_3, 41.3 mM $Na_2MoO_4 \cdot 2H_2O$, and 84.1 mM $NiCl_2 \cdot 6H_2O$), 4.4 µM vitamin K_3, 2.9 µM $FeSO_4 \cdot 7H_2O$, 14.4 µM $CaCl_2$, 2 mM $MgCl_2 \cdot 6H_2O$, 7.4 pM vitamin B_{12}. 16.5 mM L-cysteine,

and 2 μL/mL (v/v) histidine/hematin solution. The cells were pelleted again and resuspended in 5 mL of 2X MM. These 5 mL bacterial cultures were used to determine heterologous protein production and enzyme activity.

Bacterial strains containing the DX promoter (P_{DX}) were induced with 0.5% dextran to promote expression of the engineered agarase genes. Bacterial cultures in 2.5 mL of 2X MM were added to 2.5 mL of either 1.0% (w/v) dextran or glucose and incubated for 6 hours at 37°C in anaerobic conditions. Cultures were then pelleted, washed, and resuspended in 5 mL of 2X MM as described above.

2.2.3. Agarase Production and Function Validation in *B. theta*

2.2.3.1. Western Blot Analysis

A 2 mL aliquot of each bacterial culture in 2X MM was pelleted at 5,000 rpm for 5 minutes at room temperature, and resuspended in 100 μL of BugBuster® (70923, Millipore Sigma) to lyse the cells. Cell lysates were mixed 1:1 with 2X SDS loading dye (125 mM Tris-HCl pH 6.8, 20% (v/v) glycerol, 140 mM SDS, 60 μM bromophenol blue, 10% (v/v) β-mercaptoethanol) and denatured at 95°C for 10 minutes. Samples were then loaded on Bolt™ 4-12% Bis-Tris Plus SDS gels (NW04125, Invitrogen, Carlsbad, CA) and developed in 1X MES SDS running buffer (B0002, Novex) at 200 V for 22 minutes at room temperature. Protein was transferred on to polyvinylidene fluoride (PVDF) membrane (162-0184, BioRad) in a transfer apparatus at 4°C and 30 V for 16 hours. Membranes were incubated in blocking buffer (5% (w/v) skim milk powder, TBS-T: 50 mM Tris pH 7.2, 150 mM NaCl, and 0.1% (v/v) Tween 20) at 4°C for 1 hour. The membranes were washed with TBS-T, and incubated with 1:10 000 of rabbit anti-6-his IgG antibody conjugated to horseradish peroxidase (A190-114P, Bethyl, Montgomery, TX) in TBS-T at 4°C for 6 hours. After this incubation, the membranes were again washed three times in TBS-T, and the

Opti-4CN™ substrate kit (1708235, Bio-Rad, Hercules, CA) was used to visualize protein bands on the membranes.

2.2.3.2. Agarase Assays

Agarase assays and supernatant analysis were performed as described by Pluvinage et al (2018) [68]. Briefly, samples of whole cells and bacterial lysates were used. Whole cell agarase assays were performed by adding 100 µL of bacterial cells (OD_{600nm}~1.0) in 2X MM to 100 µL of 0.8% (w/v) agarose (16500, Invitrogen) and incubated overnight at 37°C in anaerobic conditions. Whole cell lysate agarase assays were performed by adding 15 µL of cell lysate to 100 µL of 0.8% (w/v) agarose, 20 mM Tris pH 7.2, and 500 mM NaCl and incubating overnight at 37°C in anaerobic conditions. After incubation supernatants were collected and cell debris was pelleted at 5,000 rpm for 5 minutes. The supernatants were collected and heat killed at 95°C for 10 minutes and then cooled to room temperature.

Analysis of supernatants was performed using Thin Layer Chromatography (TLC). 6 µL of each supernatant was loaded on a silica TLC plate (105553, EMD Millipore, Darnstadt, Germany) and run in a 2:1:1 (v/v) solution of butanol:acetic acid:water. Plates dried at room temperature were stained with a solution consisting of 1 part 0.2% (w/v) dihydroxynaphthalene in ethanol to 2 parts 3.75:1 (v/v) ethanol: sulfuric acid. After drying, the stained plates were incubated at 150°C for 5 minutes.

2.2.4. Agarase Localization Assays

2.2.4.1. Osmotic shock to fractionate bacterial cells and determine agarase localization

Gram-negative bacteria can secrete proteins to the external environment, onto the LPS surface, or into the periplasm. Therefore, osmotic shock was performed on engineered *B. theta* strains to determine the location of produced proteins. Bacteria cells were fractionated and

analyzed by immunodetection in a method adapted from McLean *et al.* (2017) [102]. Bacterial cultures inoculated from glycerol stocks were grown for 16 hours in TYG h/h medium as described in Section 2.2.2. Before washing the bacterial cells, a 100 µL aliquot of supernatant from each pelleted culture was taken for the media sample. After washing the cultures in 2X MM, the cultures were separated into two tubes of 2 mL aliquots and these aliquots were pelleted by centrifugation at 13,000 x g for 3 minutes. One aliquot of cells was subsequently lysed with 100 µL of BugBuster® (70923, Millipore Sigma) to be used as a control. The second cell pellet was gently resuspended in 500 µL of 4°C STE solution (10% (w/v) sucrose, 20 mM Tris pH 8.0, 1 mM EDTA) and then incubated on ice for 10 minutes. After incubation, the cells were pelleted by centrifugation at 13,000 x g for 3 minutes and the supernatants were aspirated. The bacterial pellets were then gently resuspended in 100 µL of ice cold 100 mM $MgCl_2$ solution and incubated on ice for 10 minutes. After incubation the cells were again pelleted by centrifugation at 13,000 x g for 3 minutes and the supernatant was collected and retained as the periplasmic fraction of the bacterial sample. The remaining pellet was lysed with 100 µL of BugBuster® (70923, Millipore Sigma) and incubated at room temperature for 1 minute. The suspension was then pelleted by centrifugation at 13,000 x g for 3 minutes and the supernatant was collected as the soluble cytoplasmic fraction of the bacteria and the pellet was resuspended in 100 µL of water and this represented the insoluble fraction of the bacterial sample. It was important to gently resuspend cell pellets as this limited unintentional lysing of the cell fractions throughout the protocol. The media, periplasm, soluble and insoluble fractions, as well as the whole cell lysate samples were subsequently mixed 1:1 with 2X SDS loading dye and then heat denatured at 95°C for 10 minutes. These samples were loaded onto a SDS gel and detection protein was determined by western blot analysis as described in section 2.2.3.1.

2.2.4.2. Proteinase K Digestion of Bacteria to determine outer surface proteins

To determine if proteins were trafficked to the cell surface of the bacteria, whole-cell proteinase K digestions were performed [102]. Bacteria that were resuspended in 2 mL of 2X minimal media were digested with 11 active units of proteinase K to remove cell surface proteins and compared to untreated samples to identify bacterial samples with expressed agarases on the outer surface. A 1 mL aliquot of each cell culture was separated into two tubes and pelleted. One pellet was suspended in 100 µL of 1X phosphate buffered saline (PBS) solution (10.1 mM Na_2HPO_4, 1.74 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl pH 7.4), the other was resuspended in 100 µL of 100 µM proteinase K in 1X PBS solution. Both samples were incubated at 56°C, and continuously shaken at 300 rpm for 16 hours. After incubation, both cell samples were pelleted by centrifugation at 13,000 x g for 3 minutes, supernatants were aspirated and the pellets were resuspended in 100 µL of 1X PBS solution. This washing step was repeated two additional times and resuspended to a final 100 µL volume in 1X PBS solution. The cell samples were subsequently added to PVDF membranes. In this process, cells were loaded on the membrane by first adding 10 µL of 100% methanol to the membrane immediately followed by pipetting 10 µL of the cell sample to the wet membrane. Following the addition of the samples to the membrane, the membrane was dried at 37°C and then was immersed in 100% methanol and then incubated in 5% (w/v) skim milk powder blocking solution for 1 hour at 4°C. The membrane was then incubated with anti-his IgG antibody (A190-114P, Bethyl, Montgomery, TX) in TBS-T solution and developed for protein detection as described in section 2.2.3.1.

2.2.5. Cell Incubation on Agarose

2.2.5.1. Purification of agarases for supplementation in growth medium

To help augment the extracellular agarase activity of *B. theta* strains, purified recombinant agarases were added to the bacterial cultures. The enzymes were purified as described by Pluvinage *et al.* 2018 [68]. Briefly, expression vectors were transformed into *E. coli* BL21 Star (DE3) cells and grown in LB broth supplemented with kanamycin (50 µg/mL) at 37°C until an OD_{600nm} ~0.8 was reached. Protein production was induced by the addition of 0.5 mM final concentration of isopropyl-D-1-thiogalactopyranoside and incubated overnight at 16°C and 200 rpm. Cells were then harvested by centrifugation and lysed by sonication using 1 second medium intensity sonic pulses for 2 minutes. Cell lysate separated by centrifugation and protein was purified from the supernatant using Ni^{2+}-immobilized metal affinity chromatography. Purified proteins in 20 mM Tris-HCl pH 8.0, 500 mM NaCl were concentrated using (tube) with a 10,000 Da molecular weight cut-off. Protein concentration was determined by measuring Abs_{280nm} and using the following extinction coefficients: GH16B: 84,465 M^{-1} cm^{-1}, GH117B: 117,145 M^{-1} cm^{-1}, and GH2C: 210,340 M^{-1} cm^{-1}.

2.2.5.2. Optical Density measurements

To determine if the engineered strains of bacteria had transformed metabolic potential, monocultures were incubated with agarose provided as a sole carbon source, *B. uniformis* NP1, *B. theta Δtdk ΔPUL75*, and the engineered strains ON-GH16, ON-AG, and ON-ANC, were inoculated from glycerol stocks into BHI media overnight cultures under anaerobic conditions. The overnight cultures (OD_{600nm} 0.5-1.0) were then diluted to an OD_{600nm} 0.05 in 2X MM supplemented with 10 mg/mL beef extract powder (0114, Amresco, Solon, OH) to form the inoculant. Wells in a sterile 300 µL 96-well microtiter plate (Falcon) were filled with 100 µL of

sterilized, anaerobic, and warm 1% (w/v) low melting point agarose or galactose with 80 uL of inoculant (n=4). Wells were then supplemented with 20 µL of 10 µM stock agarase solution in 2X MM with 10 mg/mL beef extract powder. Agarase solution was supplemented with 1 µM final concentration of purified GH2C, GH117B, GH16B enzymes, and an equivalent mass of bovine serum albumin (BSA) (0.87 mg). Plates were sealed with Breathe-Easy gas-permeable membranes (Sigma Z390059) and absorbance at 600 nm was read every 10 minutes for 50 hours with a Eon microplate reader and recorded on Biotek Gen5 software (BioTek Instruments, Winooski, VT). Blanks were prepared in the same way as reaction wells, except 2X minimal media with beef extract did not contain bacteria. The mean (+/- the standard deviation) of each condition (n=4) was visualized using GraphPad Prism 6. Supernatants after incubation were used for product profile analysis with TLC as described in section 2.2.3.2.

2.3. Results

2.3.1. Constructing Ag-cassette constructs

The first iteration of the Ag-Cassette, containing the three *B. uniformis* NP1 agarases (GH16B, GH117B, and GH2C) with 6-10 bp of intergenic space between each gene, was designed as a proof-of-concept, to demonstrate *B. theta* can produce transgenic agarases. Each agarase was affixed with a unique N-terminal SPII signal peptide in order to direct the enzyme to the outer membrane of the bacterium (Table 2.1), and a C-terminal 6xHis tag to be used for immunodetection of the enzymes. All three genes were placed downstream of a dextran inducible promoter, P_{DX}, which drives expression of the SusC-like gene in PUL48 of *B. theta* [34, 96]. The genes were inserted sequentially by homologous recombination as the entire cassette was too large to directly clone into a single vector. The order in which the genes were inserted into the *B. theta* chromosome was: GH16B, GH117B, and then GH2C (Figure 2.1B), and this enabled the

sequential evaluation of enzyme activities using standard agarase assays (Fig. 2.1B). GH16B is an endo-β-agarase that hydrolyses the β-1,4 linkages within the agarose backbone producing NAOS that can be visualized using TLC and a differential colour stain that colours the bands blue-purple. GH117B is an exo-α-neoagarooligosaccharide hydrolase that hydrolyses the non-reducing end of the NAOS products from GH16B into AOS and L-AHG. AOS bands stain purple and migrate like NAOS, near the bottom of the TLC, while AHG is represented by a blue smear that migrates to the top of the TLC. This demonstrates the decreased polarity of the AOS and L-AHG as compared to D-GAL and NAOS. Finally, the addition of the exo-β-agarase GH2C, hydrolyses the non-reducing end of AOS from GH117B digestion to produce smaller NAOS relative to the substrates and D-GAL units. The D-GAL band in the TLC migrates to the middle of the TLC and stains pink (Figure 2.1B).

Based upon the evaluation of enzyme activities, several modifications were introduced to optimize the function of the *Bt* DX-AG cassette. First, since it was not possible to validate whether *Bt* DX-AG could use agarose as a sole carbon source as dextran is also required to induce agarase gene expression, the DX promotor was replaced with the constitutive *B. theta* ribosomal promotor P_{ON}, in a constitutively active Ag-Cassette strain (*Bt* ON-AG). Second, the signal peptide used for the GH117B was the endogenous tag from the *B. uniformis* NP1 Ag-PUL (Bu). However, based on a subsequently elucidated mechanism of agarolysis in *B. uniformis* NP1, it was found that GH117B is tethered to the inner lipid bilayer of the outer membrane, and is presented to the periplasm instead of the intended outer cell surface. As such, this SPII tag from *B. uniformis* NP1 (Bu) was successfully replaced with the SPII of SusG from the canonical *B. theta* starch utilization PUL, resulting in the formation of DX-ANC and ON-ANC cassettes. Therefore in total, four Ag-Cassettes were successfully engineered for this project. These are illustrated in Figure 2.2.

2.3.2. Dextran-Inducible Protein Production and Activity

Once the strains were engineered, protein production was confirmed by Western blot and enzyme activity assays shown in Figure 2.3. Cultures were grown in 0.5% (w/v) final concentration of dextran (DEX) to induce expression of the agarase genes or in glucose (GLC) to be used as a control because it is the monosaccharide substituent of dextran. The *B. theta* recipient strain, *Bt Δtdk ΔPUL75*, was used as a negative control, as it does not contain any inherent agarase activity. Individual agarase *B. theta* strains displayed corresponding transgenic agarases detected on Western blots (Figure 2.3A) and the enzymes migrated with the expected mobility based upon molecular masses: GH16B (36.6 kDa), GH117B (40 kDa), and GH2C (94.1 kDa). Importantly, the proteins were only visible when induced by DEX and no protein expression was observed in cultures supplemented with GLC. Similar results were observed with the Ag-Cassette bacterial strains as only strains induced with DEX had visible protein production. In addition to the individual agarase strains, all three proteins were visible in the triple mutant *Bt* DX-AG and *Bt* DX-ANC strains (Figure 2.3A). Notably, the GH117B band migrated less in the *Bt* DX-ANC strain compared to the *Bt* DX-AG strain. This migration pattern observed by the *Bt* DX-ANC strain may be a result from an increase in mass following the addition of the Anc N-terminal signal peptide onto GH117B.

TLC was used to analyze the product profiles generated by whole cell lysates of each strain after incubation with agarose (Figure 2.3B). Consistent with the Western blot results, products were only produced when the bacteria were induced with dextran. Minimal oligosaccharides were produced by the *Bt* 4113-GH2 and *Bt* BuGH117 strains. This was expected as there would be little substrate available for these enzymes on unhydrolysed agarose [68]. In addition to the differences in band separation patterns, the staining colours varied between NAOS and AOS products. In *Bt*

4116-GH16 only blue bands were visible, highlighting the generation of NAOS products. In contrast, the *Bt* 4116-GH16-BuGH117 strain had a shift in the banded product profile with a slight colour change to purple, and a blue smear near the top of the gel, which correlates to the production of AOS and AHG, respectively. Both the *Bt* DX-AG and *Bt* DX-ANC strains had similar product profiles. There were bands representing NAOS, L-AHG, and D-GAL, which were expected products of agarose digestion by bacteria encoding the full complement of enzymes. The negative control *Bt Δtdk ΔPUL75* showed no oligosaccharides, suggesting that the agarose digestion products are a result of the addition of the three agarases in *B. theta*.

2.3.3. Constitutive Protein Production and Activity Profiles

Constitutive strains *Bt* ON-GH16, *Bt* ON-AG, and *Bt* ON-ANC were grown in TYG h/h medium and used directly from the overnight cultures, as agarase gene expression did not require induction in these strains. All three strains showed protein bands corresponding to the molecular weights of the agarases (Figure 2.3A), and these migrated similarly to inducible DX-cassette strains agarases (Figure 2.4A). Consistent with the *Bt* DX-ANC strain, the AncGH117 agarase band in the *Bt* ON-ANC strain also migrated less than the BuGH117B on the *Bt* ON-AG strain, which likely results from the addition of different N-signal peptides. Importantly, the corresponding negative control showed no protein band. The product profiles observed with TLC are also consistent with products from the inducible strains (Figure 2.4B). The *Bt* ON-GH16 shows production of the NAOS in purple and both the *Bt* ON-AG and *Bt* ON-ANC have NAOS, L-AHG, and D-GAL bands indicating functional agarase activity.

2.3.4. Cellular Localization of Agarases

Following confirmation that the enzymes were produced and active, the cellular location of each agarase was determined. All agarases contained an SPII signal peptide (Table 2.1) and it was

expected that these enzymes would be trafficked to the outer surface of the cell. To determine the cellular localization of the enzymes, the osmotic shock method was performed on intact cells [102]. This method fractionated the cells into periplasmic, insoluble, and soluble fractions (Figure 2.5A), and these fractions can be compared to whole cell digests to confirm enzyme localization (Figure 2.5B). *Bt* ON-GH16, *Bt* ON-AG, and *Bt* ON-ANC engineered strains were assessed for agarase protein production using Western blot analysis (Figure 2.5C). The whole cell lysate fraction was the control sample used to show total protein produced in the cells. As expected, there was no observed protein production for the *Bt ΔtdkΔPUL75* control lysate sample. Protein bands corresponding to agarase production were observed in the periplasmic, cytoplasmic soluble, and insoluble fractions for all three strains. This was expected as proteins are translated in the cytoplasm and need to traverse through the periplasm to reach the outer membrane. In addition, although the three recombinant agarases are soluble, the N-signal peptides anchor the proteins to the membrane in the engineered strains, which would result in the presence of protein in the insoluble fraction as well. Medium fractions collected after cells were pelleted did not contain visible protein bands for the GH117B and the GH2C, as these proteins were not secreted outside the cell and into the medium. There was a slight band visible in the medium GH16-containing fractions which could be a result of some protein cleavage occurring at the surface, but this was not observed in the *Bt* ON-AG and *Bt* ON-ANC medium fractions (Figure 2.5C). To determine which agarases were attached to the cell surface, whole cells were treated with and without proteinase K, blotted onto PDVF membranes, and probed with antibody (Figure 2.5B). Only the *Bt* ON-ANC strain had a positive signal for its untreated sample visualized on the dot blot. All other samples had similar signals to the proteinase K treated samples and the *Bt ΔtdkΔPUL75* negative control. This result suggested that an agarase in the *Bt* DX-ANC strain was present on

the outer surface of bacterial strain and was detected by the antibody. This signal could be suggestive of the AncGH117B agarase, another agarase constitutively expressed by the *Bt* DX-ANC strain that has a different signal peptide (Anc). Dilineation of the signal between the enzymes for each bacterial strain is difficult as all the heterologous enzymes contain a similar His6 epitopes used for antibody detection.

Cellular location was also tested using whole cell agarose digestion assays. Whole cells that display the agarases on the outer surface of the cell should be able to access and digest agarose in the medium. As anticipated, the *B. theta* strains expressed GH16B under control of the DX and ON promoters and generated NAOS product profiles (Figure 2.6). Surprisingly, the engineered strains that contained all three agarases: *Bt* ON-AG, *Bt* DX-AG, *Bt* ON-ANC, and *Bt* DX-ANC, had similar product profiles as the *Bt* DX-GH16 and *Bt* ON-GH16 profiles that had NAOS blue bands (Figure 2.6). These results indicated that only GH16B was functioning on the outer surface of the cell, despite GH16B not being detected in the whole cell dot blot assay (Figure 2.5B). Interestingly, the *Bt* DX-ANC and *Bt* ON-ANC strains did not have product profiles consistent with GH117B activity as no L-AHG or AOS was produced.

2.3.5. Engineered *B. theta* incubation on agarose

To determine if engineered strains *Bt* ON-AG and *Bt* ON-ANC could use agarose as a sole carbon source, both strains were cultured on low melting point agarose (Figure 2.7). Although each strain grew on the D-GAL control, only the NP1 strain grew on agarose. Therefore, bacterial strains were supplemented with exogenous agarases by adding combinations of recombinant GH117B and GH2C to determine if the strains could grow on the D-GAL released from the agarose (Figure 2.7). Cultures supplemented with GH117B (blue) or GH2C (yellow) did not grow on agarose; however, when both GH117B and GH2C (green) were added the strains demonstrated

similar growth curves that were comparable to *B. uniformis* NP1 (red) reaching a final OD_{600nm} of 0.4 after 20 hours. When all three agarases GH16B, GH117B, and GH2C were supplemented in the medium (purple), growth of all engineered strains outperformed *B. uniformis* NP1, reaching an OD_{600nm} of 0.6 in less than 10 hours. This was similar to *B. theta* growth on monosaccharides as demonstrated by Martens E. *et al*. (2011) [83].

Analysis of the supernatants from the bacterial cultures showed product profiles consistent with the growth curves (Figure 2.7). Engineered strains *Bt* ON-AG, *Bt* ON-ANC, and *Bt* ON-GH16 had band migration patterns indicative of NAOS bands and GH16B activity on LMPA, similar to what was shown in preliminary studies (Figure 2.6). Following the addition of GH117B to the medium, there was an observed change in band colour from NAOS blue bands to pink AOS bands as well as a faint band near the top of the TLC, which indicated L-AHG was being produced (Figure 2.7). Supplementing the engineered strains with only GH2C (Figure 2.7) observed no band change from no enzyme controls. Supplementing with both GH117B and GH2C enzymes to the medium (Figure 2.7), supported growth, and resulted in similar product band migration patterns as the *B. uniformis* NP1 control strain. Concurrent with growth of the engineered bacterial strains, the disappearance of D-GAL within the supernatants was observed in *Bt* ON-AG, *Bt* ON-ANC, and *Bt* ON-GH16 strains when both GH117B and GH2C agarases were added to the medium, indicating D-GAL was being utilized by the bacteria. Supernatants from *Bt Δtdk ΔPUL75* control strain cultures, showed no oligosaccharide formation in all conditions but one and matched the product profiles of controls that lacked bacteria. When all three agarases were supplemented in the medium, *Bt Δtdk ΔPUL75* was observed to grow and the observed banding pattern on the was similar to *B. uniformis* NP1, but prominent L-AHG and D-GAL bands were present. This result suggests that the increased production of NAOS by augmented GH16B activity from

supplementation of GH16B into the medium, could enhance the hydrolysis and subsequent release of D-GAL from agarose.

2.4. Discussion

This study was directed at engineering three functional *B. uniformis* NP1 agarases into the human gut symbiont, *B. theta.* Recent studies that have shown heterologous protein production in *Bacteroides* spp. have typically produced reporter proteins as a method to help deepen the understanding of expression systems within the bacterial species. Previously, heterologous protein production has been verified by measuring relative luminescence of NanoLuc or fluorescence of GFP to determine whether genes have been properly introduced into bacterial expression systems [95, 96, 104]; however, the introduction of these proteins serve no functional advantage to the bacteria. In this study, transgenic agarases that target agarose were selected in an attempt to transform the metabolic potential of *B. theta*. Using homologous recombination, GH16B, GH117B, and GH2C from *B. uniformis* NP1 were successfully introduced into the genome of *B. theta* (Figure 2.2) and were produced together under constitutive and induced regulation (Figures 2.3A and 2.4A). The transgenic proteins were shown to be functional when product profiles from cell lysate incubations on agarose were analysed for expected agarase activity (Figures 2.3B and 2.4B).

The agarolytic function of the engineered *B. theta* strains is reliant on the agarases being trafficked to the outer surface of the bacterium by the addition of SPII signal peptides to each protein. SPII signal peptides are located on the N-terminal end of proteins and have conserved sequences containing an important cysteine residue that forms a “lipobox”, which are recognized by signal peptidase II proteins [105]. After translocation to the periplasm, the prolipoproteins are recognized by signal peptidase II proteins with the cysteine residue in the +1 site and are

subsequently cleaved resulting in the protein becoming lipid membrane bound to the outer membrane of the cell [105]. In this study, two different SPII predicted peptides derived from *B. theta* genes were cloned on to the N-terminal of GH16 and GH2: *bt4116* from a PL1 and *bt4113* from the SUS D-like binding protein both from the PUL responsible for homogalacturonan metabolism [101]. These sequences were selected because they were taken from the deleted PUL75 in *B. theta*, and therefore, should not create issues such as untargeted recombination events. Importantly, GH117 family of enzymes are only active in a dimer conformation [106], which posed a design problem for fusing a signal peptide to the N-terminal end because the N-terminal domain of one protomer interacts with the C-terminal domain of the other protomer [68]. Unfortunately, as demonstrated by cell fractionation (Figure 2.5C) and whole-cell assays (Figures 2.5B and 2.6), it was determined that BuGH117B was not trafficked to the outer surface of the cell.

The 4116-GH16B endo-agarase is likely located on the outer surface of the cell and appears to be functional because NAOS product profiles were observed in supernatants from whole cells of the engineered *B. theta* strains digestion of agarose (Figure 2.6). Moreover, engineered strains that contain the ON-GH16 have repeatedly shown the agarolytic phenotype of pocking on agar plates. Unfortunately cellular localization assays did not support the 4116-GH16B being located to the outer surface of the cell. Although protein bands appear in all but the media fractions from osmotic shock samples (Figure 2.5C), no signal was observed on the whole cell untreated sample on the dot blot (Figure 2.5B). This issue may be a result from antibody detection as the antibody might not have access to the GH16B C-terminal epitope when the lipidated enzyme is anchored to the cell membrane. The crystal structure of the GH16B shows the N-terminal and C-terminal ends on the same side of the protein and therefore the His tag epitope would be in proximity of the N-

terminal SPII signal peptide that is attached to the outer membrane [68]. This would only be an issue when immunodetection was used with whole cells as the antibody's interaction with the epitope would be impeded because it is anchored to the membrane (Figure 2.5B). Lysed cells that were used in Western blot detection were not be affected as the GH16B was not be fixed to the cell membrane (Figures 2.3A and 2.4A).

To address the BuGH117B trafficking issue, a second secretion peptide was selected to enable flexibility in GH117B oligomerization and attachment during secretion. The endogenous signal peptide on the BuGH117B in *Bt* DX-AG was replaced with a *B. theta* canonical *susG* amylase gene, *bt3698*, and resulted in the *Bt* DX-ANC construct (Figure 2.2) [102]. This change to a *B. theta* signal peptide would increase the likelihood that *B. theta* would traffic the protein correctly while also increasing the chances of proper protein oligomerization because there is more unstructured protein between the signal peptide and the N-terminal domain of the GH117B (Table 2.1). Similar to the endogenous tag, the alternate tag did not change the production or activity of the GH117B in cell lysates incubated with agarose (Figures 2.3B and 2.4B). On Western blots the GH117B band migrated less than the GH117B with the endogenous tag (Figure 2.3). This is most likely a result of the signal peptide being larger (44 residues compared to 25 residues) and having a larger unstructured region making it bulkier which would affect the migration of the agarase through the acrylamide gel (Table 2.1). Protein signal from the *Bt* ON-ANC strain on the dot blot shown in Figure 2.5B, which is missing in the same sample treated with proteinase K, suggested that AncGH117B is locating to the outer surface. The GH117B signal peptide is the only difference compared to the *Bt* ON-AG strain that did not have any detected signal on the dot blot. However, this result does not coincide with the whole cell activity assay shown in Figure 2.6, as it would be expected that AOS and L-AHG products would be detected with the *Bt* DX-ANC and *Bt* ON-ANC

supernatants, but only NAOS are observed. Although suggested that the AncGH117B is the cause of the signal observed in Figure 2.5B, this can not be determined as all three agarases contain the same C-terminal epitope so there is no way to distinguish between the agarases on whole cell dot blots.

There are limitations to the cellular localization assays developed in this study and these limitations could be improved upon. Firstly, the His tag epitope used for the three agarases is not very strong, and therefore, could be inefficiently binding to the proteins on the whole cell dot blots as well as non-specific binding to histidine rich sequences within endogenous *B. theta* proteins. Indeed, background signal was observed for the control *Bt Δtdk ΔPUL75* dot blot samples (Figure 2.5B) as well as a band was observed in every cell lysate sample that migrated in the gels corresponding with a molecular weight of ~20 kDa (Figures 2.3A and 2.4A). Changing the epitopes to stronger, robust epitopes such as c-myc or FLAG peptides may increase the antibodies signal detection capacity as they are not derived from bacterial proteins [107, 108]. However, increasing the epitopes affinity typically results in using larger sized peptides which may affect the target proteins function [109]. Therefore, designing antibodies specific to each of the three agarases would not only increase epitope affinity, lower background detection, and not require engineering bulky peptides on the C-terminal of the target protein, but also provide specific agarase information in the strains containing all three agarases. Certainly, specific agarase detection would also confirm where the transgenic proteins are localizing in the cell. Using secondary-fluorescent conjugated antibodies that recognize the primary antibodies would enable cell fluorescent imaging to visualize each cell and the outer surface bound agarases.

Growth of *B. uniformis* NP1 is dependent on the coordinated function of enzymes and transporters specific for agarose and its released products [68]. The engineered strains developed

here were alternatively designed to grow on D-GAL released from agarose outside the cell, and growth would be conferred by D-GAL metabolism, which is inherent in *B. theta*. Therefore, for the engineered strains to grow on agarose, all three agarases would need to be produced by and trafficked to the outer surface of *B. theta*. Although GH117B and GH2C were shown to be functional in cell lysates, they were not detected on the cell surface Figure 2.6) and appeared to be blocked in the periplasm (Figure 2.5). In an attempt to clarify these issues, the engineered constitutive strains were tested for their ability to grow on agarose when the medium was supplemented with recombinant agarases to assess what enzymes were active on *B. theta's* outer surface and to confirm that *B. theta* was able to use D-GAL released from agarose for energy. Only the constitutive strains: *Bt* ON-GH16, *Bt* ON-AG, and *Bt* ON-ANC were used for this experiment as they did not need to be induced by a carbohydrate and therefore agarose was the only carbon source available for the bacteria to use to proliferate.

Supplementation of GH2C, which cleaves the terminal D-GAL exposed by GH117B, confirmed that BuGH117B and AncGH117B were not functional on the outer surface of the cell in the *Bt* ON-AG or *Bt* ON-ANC strains as no growth was observed (Figure 2.7, yellow). Similarly, addition of GH117B to the medium for the ON-AG and ON-ANC strains did not confer growth on agarose suggesting that GH2C was not functional on the outer surface of the cell (Figure 2.7, blue). It is likely that the GH117B and GH2C were located within *B. theta* and could not access their respective substrates, NAOS and AOS, because *B. theta* lacks transport proteins to transport oligosaccharides into the cells, unlike *B. uniformis* NP1. When both GH117B and GH2C were supplemented in the medium (Figure 2.7, green), all three *B. theta* strains grew, confirming that 4116-GH16B was located and functioning on the cell surface and *B. theta* is able to proliferate on the D-GAL products (Figure 2.6).

The growth phenotype for all three engineered bacterial strains supplemented with GH117B and GH2C were similar to the *B. uniformis* NP1 control growth phenotype and product profile; however, the optical density was ~0.6-fold less (Figure 2.7). This may be a result from the surface enzyme, 4116-GH16B, reaching saturation or not having access to similar amounts of cleavable substrate in agarose. When recombinant GH16B was added there was a much shorter lag phase and the curves look similar to *B. theta* grown on monosaccharides rather than *B. uniformis* NP1 (Figure 2.6). This was likely a result of the three agarases being present in excess to saccharify agarose, and therefore, monosaccharide production was independent of the amount of cells producing enzyme. Furthermore, this result indicates that 4116-GH16B produced by engineered *B, theta* strains is the rate limiting enzyme. In conclusion, *B. theta* was able to grow on products released from agarose; but in order to successfully engineer agarolysis into *B. theta*, the expression and secretion of both exo-agarases will need to be optimized to ensure that these enzymes are trafficked to the outer surface.

Analysis of the product profiles in the bacterial growth supernatants correlated with the growth curve results (Figure 2.6). The supernatants of the engineered strains supplemented with both exo-agarases had less galactose than the no cell control triple digest, suggesting that the engineered *B. theta* strains are importing D-GAL for metabolism. Conversely, the AHG monosaccharide band was still present in the *B. theta* growth supernatants which suggests *B. theta* could not use this monosaccharide because of its L-conformation and 3,6 anhydrous bridge (Figure 1A). The *B. uniformis* NP1 supernatants do not have any L-AHG, D-GAL, or smaller oligosaccharides product bands as a result of the surface binding (SusD-like) and transport (SusC-like) proteins encoded within the AgPUL that bind and transport larger oligosaccharides into the cell where exo-agarases completely saccharify them into monosaccharides [68, 79]. This

mechanism is referred to as a "selfish" mechanism and is employed by *B. theta* in the metabolism of some complex polysaccharides, such as yeast mannan [110]. Selfish metabolism minimizes product loss to other members of the community. In contrast, bacteria can also employ "distributive" mechanisms, in which carbohydrates are depolymerized extracellularly to generate "public goods" for the community [111, 112]. Distributive mechanisms can increase fitness of the primary and secondary feeders. Indeed, it has been shown that *B. ovatus* contains two outer membrane enzymes that function to break down inulin which were unnecessary for the metabolism by *B. ovatus,* but had a reciprocating benefits to increase its fitness when co-cultured with another bacterial species, *B. vulgatus* [111].

The engineered feeding mechanism attempted here represents a reorganization of the selfish mechanism in *B. uniformis* NP1 into a distributive mechanism in *B. theta*. The agarolytic cassettes were designed to produce three agarases to the outer surface that could saccharify agarose into D-GAL to provide a metabolizable monosaccharide from a rare, chemically complex substrate. A possible limitation of this engineered mechanism is that in a community other bacteria will also be able to use D-GAL produced by *B. theta*, which represents a zero sum game. This may result in an evolutionary pressure to repress gene expression as it is unclear if this would provide a cooperative advantage with other species. However, the engineered strain coupled with agarose would still contribute non-specific synbiotic benefits because it is still providing enzymatic activities that are lacking in the community. In this light, the design issues with exo-agarase secretion may offer a solution that is a truncated representation of the selfish pathway in *B. uniformis* NP1. SusC-like and SusD-like proteins are hallmark proteins required in PUL systems that consist of an outer membrane TBDT and an N-terminal lipidated surface binding glycan protein (SBGP) that when deleted cause loss or reduced function in carbohydrate metabolism [33, 79]. Engineering the SusC-

like and SusD-like transporters from the agarolytic pathway in *B. uniformis* NP1 may enable the intracellular transport of NAOS, which would provide localized substrates to GH117B and GH2C in the periplasm. This strategy may overcome the problems associated with the cellular location of the exo-agarases and provide a fitness advantage for engineered *B. theta* as the D-GAL present within agarose would be released within the cell and prevent metabolic loss as a public good.

Previous work has shown that introduction of an isogenic strain of *Bacteroides* spp. into an already established community will not colonize the DGM [113]. By introducing rare nutrient utilization into *B. theta,* it may be possible to overcome this dilemma by using the nutrient as a positive selection tool. Previously, *B. theta* has been engineered with the capacity to metabolize the algal polysaccharide porphyran by inserting a portion of a PUL from *B. ovatus* NB001 into *B. theta.* This not only allowed for *B. theta* to grow in the presence of porphyran as a sole carbon source, but its proliferation could be tightly controlled by the amount of porphyran administered *in vivo* [58]. This study underpinned the capacity of using rare dietary carbohydrates as selection tools for engineered bacteria within the GIT.

In this project, I was able to engineer four strains of *Bt Δtdk ΔPUL75* to contain intrachromosomal copies of three agarases from *B. uniformis* NP1 under either a dextran inducible promoter, P_{DX}, or a constitutively active promoter, P_{ON} (Figure 2.2). All agarases were produced and detected via a C-terminal His-tag using Western blots and an anti-His antibody. Agarases were shown to be functional through agarase assays but only the endo-β-agarase, 4116-GH16B was confirmed to be localized to and functional on the outer surface of the cell. The engineered strains containing 4116-GH16B all showed agarolytic activity through pocking on agar plates but could not grow solely on agarose because the exo-agarases were not functional on the outer surface of the bacteria. Optimization of the signal peptides on the GH117B and GH2C may not be enough to

have functional outer surface lipidated proteins because GH117B must form a dimer structure. Therefore, the addition of the AgPUL TBDT and SGBP encoded genes to the engineered bacteria could shuttle NAOS products from GH16B hydrolysis into the periplasm where the GH117B and GH2C are located. This design strategy would provide the *B. theta* with a selfish mechanism for agarose utilization. A disappearance of D-GAL in the product profiles of these strains underpins that *B. theta* is able to grow on the D-GAL present within agarose, but not L-AHG. Therefore, successful design of an engineered agarolytic system could provide a platform for engineering of synbiotic-biologic delivery systems to help mitigate dysbiosis and dysbiosis-associated diseases within the DGM.

2.5. Tables

Table 2.1. N-terminal signal peptide protein sequences used in this study.

Underlined, red letters indicate the signal peptide "lipobox" containing the cysteine residue important for peptidase recognition, red letters indicate the unstructured region of the gene separating the signal peptide from the beginning of the agarase gene, and black letters indicate the N-terminal unstructured region from np1_32.

Label	Gene	Peptide Sequence	Ref.
4116	*bt4116*	MNKTFLGAFLASVFISFTACSEENLEQDTNPPIEQPGDS	[101]
Bu	*np1_32*	MLLKNVLTIVGGIALFASCVNQPSATVSTDDSAYDQRKADSLGIPKGNKLSAAMKRAMEWPQRDNSW	[68]
Anc	*bt3698*	MNKHLHFLSLLWLSMLMAFMTACSDDKNITDPAPEPEPPVEGSATVSTDDSAYDQRKADSLGIPKGNKLSAAMKRAMEWPQRDNSW	[102]
4113	*bt4113*	MKKLLYTVIAAMPFCGSSLLMTSCDDLFDTKSPSSMDDSNIFSIYD	[101]

Table 2.2. Donor and recipient strain constructs used to engineer agarases into *B. theta Δtdk ΔPUL75.*

Construct	***E. coli S17λpir DONOR***			***B. theta Δtdk ΔPUL75 RECIPIENT***
	Vector	**Gene Insert**	**Res.Enzymes**	
DX-AG	pINT DX	4116-GH16 3' flank -BuGH117 *	PstI/NheI	*B. theta Δtdk ΔPUL75* DX 4116-GH16
	pINT DX	BuGH117 3' flank - 4113 GH2 *	PstI/XhoI	*B. theta Δtdk ΔPUL75* DX 4116-GH16-BuGH117
ON-AG	pINT DX	1311-4116-GH16 5' flank	BglII/NotI	*B. theta Δtdk ΔPUL75* DX 4116-GH16
	pINT DX	1311-4116-GH16 5' flank	BglII/NotI	*B. theta Δtdk ΔPUL75* DX Ag-Cassette
DX-ANC	pExchange-tdk	GH16 3' flank - Anchor tag - GH117 5' flank	PstI/XbaI	*B. theta Δtdk ΔPUL75* DX 4116-GH16-BuGH117
	pINT DX	BuGH117 3' flank - 4113 GH2	PstI/XhoI	*B. theta Δtdk ΔPUL75* DX 4116-GH16-AnchGH117
ON-ANC	pINT DX	1311-4116-GH16 5' flank	BglII/NotI	*B. theta Δtdk ΔPUL75* DX Anch-Cassette

* Indicates that target was synthesized by BioBasic and reamplified with cut sites for ligation

2.6. Figures

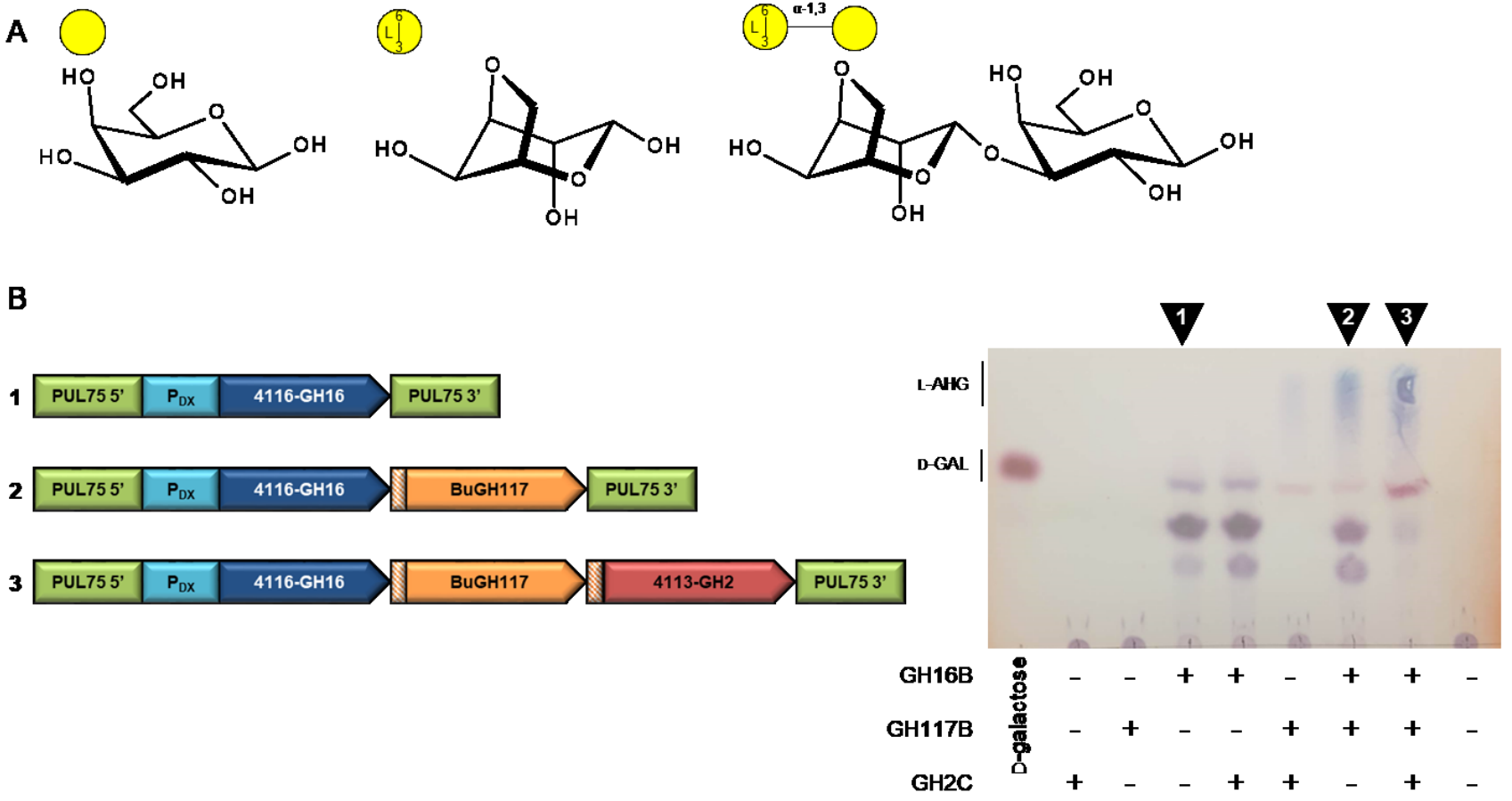


Figure 2.1. Structures of agarose subunits and diagram showing agarase insertion in to B. theta and their resulting product profiles after digestion with agarose. A) Structural representation of D-GAL, L-AHG, and neoagarobiose (N2) from left to right, respectively. B) Schematic of agarase insertion into *B. theta* and specific product profiles observed after agarose digestion by purified agarases:

GH16B, GH117B, and GH2C. For the TLC analysis: Lane 1 uses a previously constructed strain of *B. theta* containing GH16B and produces NAOS after incubation with agarose. Lane 2 shows the addition of GH117B to the construct and purified GH16B and GH117B together produce AOS and L-AHG following incubation with agarose. Lane 3 shows the addition of GH2 to complete the agarose cassette. All three agarases, GH16B, GH117B, and GH2C, are able fully saccharify agarose into individual D-GAL and L-AHG monosaccharides.

Name	Regulation	SPII peptide + agarase	Schematic Representation
Bt DX-GH16	Dextran-inducible	4116-GH16B	
Bt ON-GH16	Constitutive	4116-GH16B	
Bt DX-GH117	Dextran-inducible	BuGH117B	
Bt DX-GH2	Dextran-inducible	4113-GH2C	
Bt DX-GH16-GH117	Dextran inducible	4116-GH16B BuGH117B	
Bt-DX AG	Dextran-inducible	4116-GH16B BuGH117B 4113-GH2	
Bt-DX ANC	Dextran-inducible	4116-GH16B Anc-GH117B 4113-GH2	
Bt-ON AG	Constitutive	4116-GH16B BuGH117B 4113-GH2	
Bt-ON ANC	Constitutive	4116-GH16B Anc-GH117B 4113-GH2	

Figure 2.2. Table of engineered *B. theta* strains used in this study delineating the name of the construct, the type of regulation, and the transgenic agarases with N-terminal signal peptides engineered into the strain, along with a schematic for each construct's constituents.

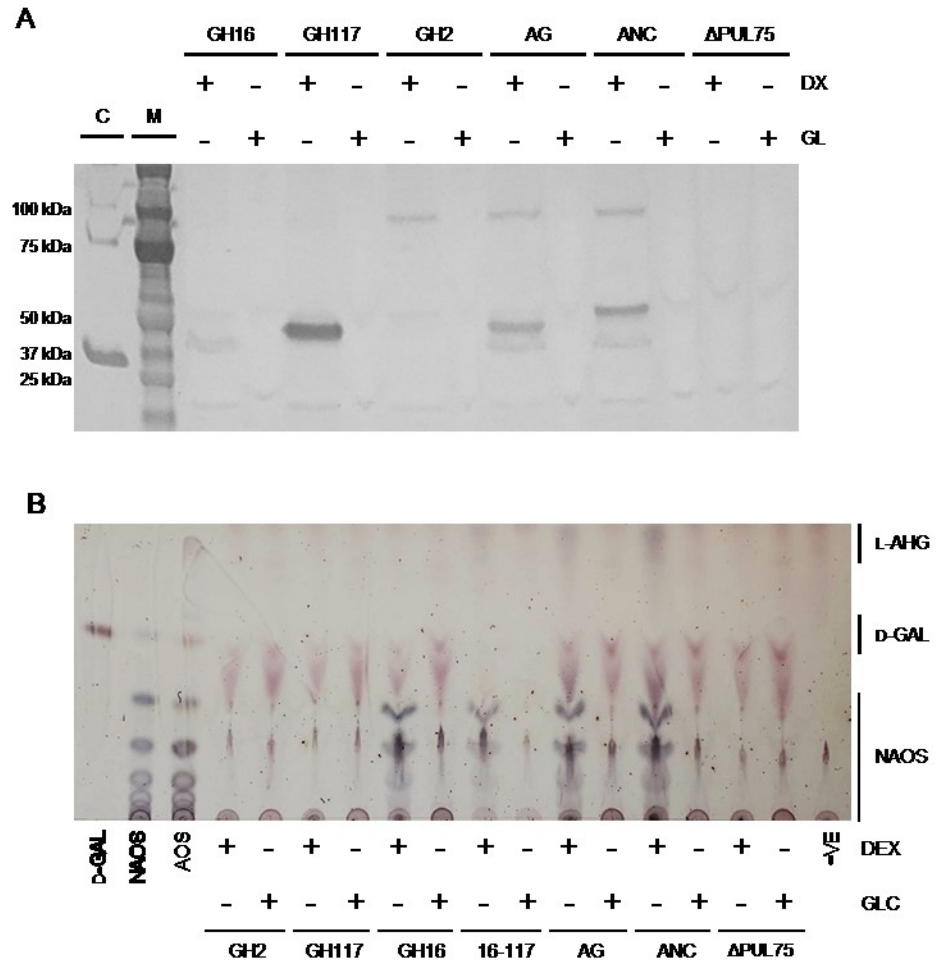


Figure 2.3. Western blot and TLC analysis of dextran-inducible engineered *B. theta* strains. A) Western blot showing agarase protein bands from DX-cassette lysates after cell incubation in 0.5% (w/v) dextran or glucose and *Bacteroides* 1X MM. C represents GH16 purified protein as an antibody control, and M represents a molecular weight marker (1610374, BioRad) with corresponding molecular weights labeled on left side of Western. B) TLC analysis of oligosaccharide products in supernatant from agarase assays performed with cell lysates incubated on 0.8% agarose. Standards on left side of TLC include D-galactose (D-GAL), neoagarooligosaccharides from β-agarase hydrolysed agarose (NAOS), and

agarooligosaccharides produced from GH117 hydrolysed neoagarooligosaccharides (AOS). Labels on right side of TLC align with the migration of NAOS, D-GAL, and L-AHG up the TLC.

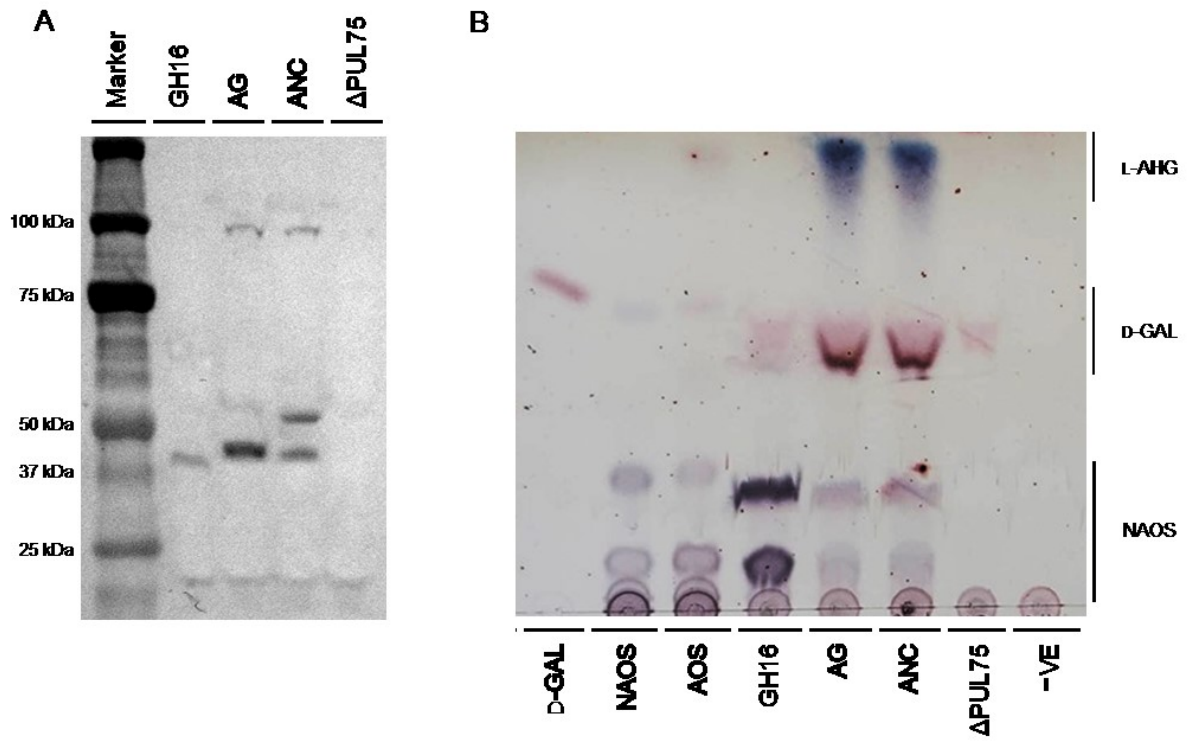


Figure 2.4. Western blot and TLC analysis of constitutively active engineered B. theta strains. A) Western blot showing agarase protein bands from ON-cassette lysates. Marker represents a molecular weight marker (1610374, BioRad) with corresponding molecular weights labeled on left side of Western. B) TLC analysis of oligosaccharide products in supernatants from agarase assays performed with engineered constitutive *B. theta* cell lysates incubated on 0.8% agarose. Standards on left side of TLC include D-galactose (D-GAL), neoagarooligosaccharides from β-agarase hydrolysed agarose (NAOS), and agarooligosaccharides produced from GH117 hydrolysed neoagarooligosaccharides (AOS). Labels on right side of TLC align with the migration of NAOS, D-GAL, and L-AHG up the TLC.

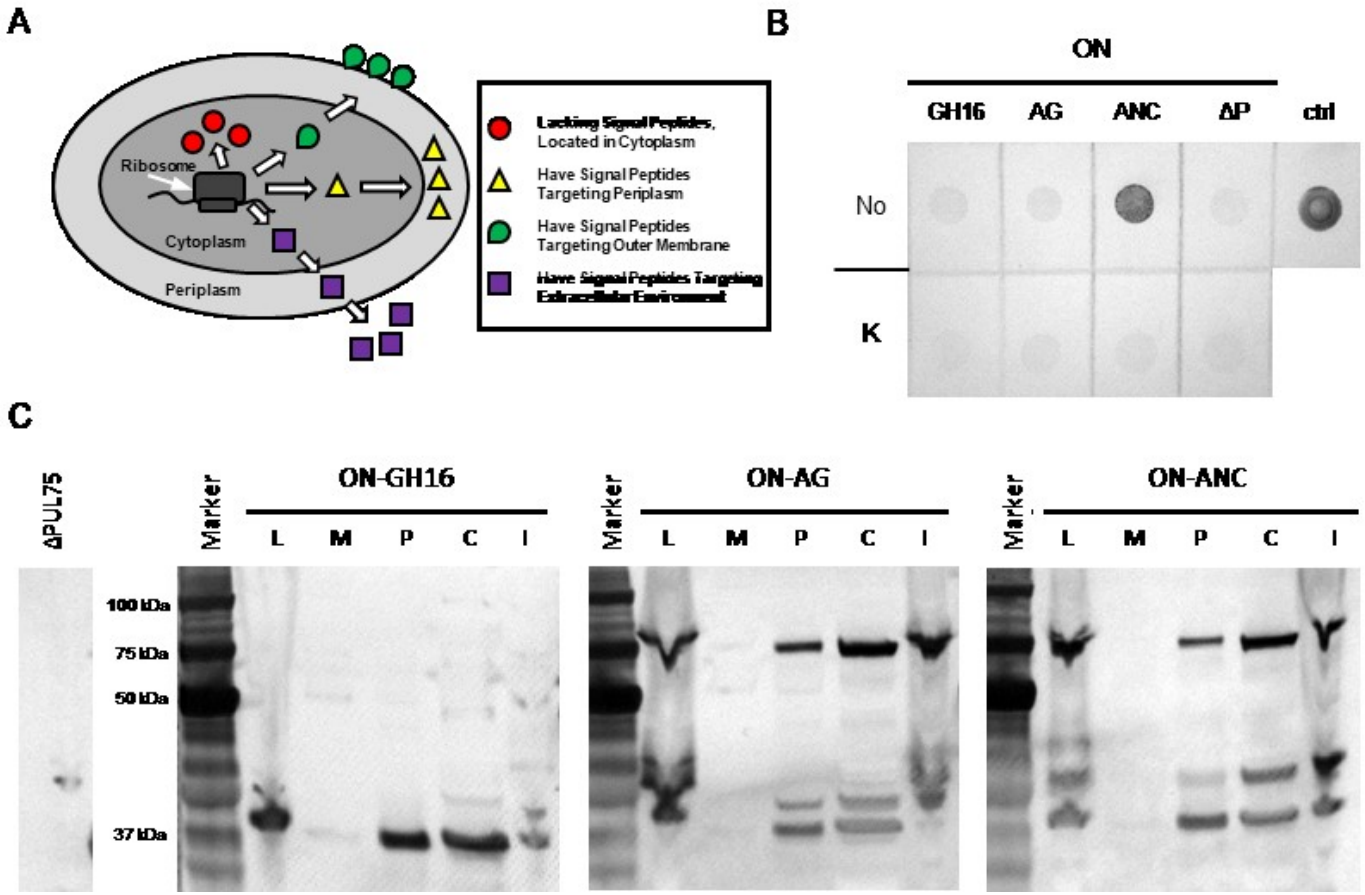


Figure 2.5. Cellular location assays of agarases in engineered *B. theta* strains. A) Schematic of proteins being translated by ribosome with peptides that target the proteins to different cellular locations in a Gram-negative bacterium adapted from McLean R. *et al*. (2017) [102]. Red circles indicate proteins without signal peptides; yellow triangles have signal peptides that traffic them to the periplasm; green tear drops indicate proteins with signal peptides that traffic them to the outer membrane; and purple squares have

signal peptides that secrete the proteins into the extracellular environment. B) Dot blot analysis of untreated and proteinase K treated whole cell samples of constitutive *B. theta* strains developed with anti-His antibody. C) Western blot analyis of osmotic shock treated constitutive *B. theta* strains. Molecular weight marker is labelled as Marker, L represents the cell lysate fraction, M represents the media fraction, P represents the periplasmic fraction, C represents the soluble cytoplasmic fraction, and I represents the insoluble fraction.

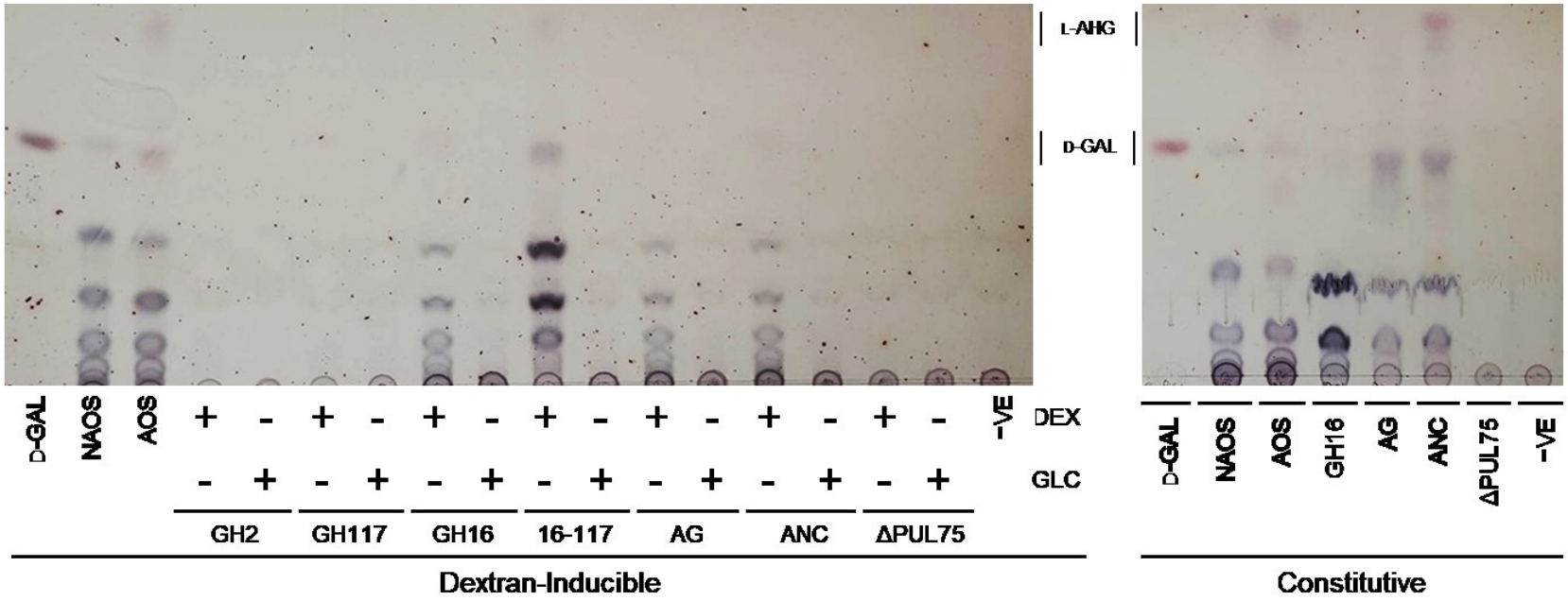


Figure 2.6. TLC supernatant analysis of dextran-inducible and constitutive engineered B. theta strains after whole cell incubation on agarose. A) Dextran-inducible strains were incubated in 0.5% (w/v) dextran or glucose before incubating with 0.8% agarose. B) Constitutive strains were cultured in rich medium before incubating on 0.8% agarose. Standards on left side of TLCs include D-galactose (D-GAL), neoagarooligosaccharides from β-agarase hydrolysed agarose (NAOS), and agarooligosaccharides produced from GH117 hydrolysed neoagarooligosaccharides (AOS). Labels in middle of TLCs align with the migration of D-GAL, and L-AHG up the TLC.

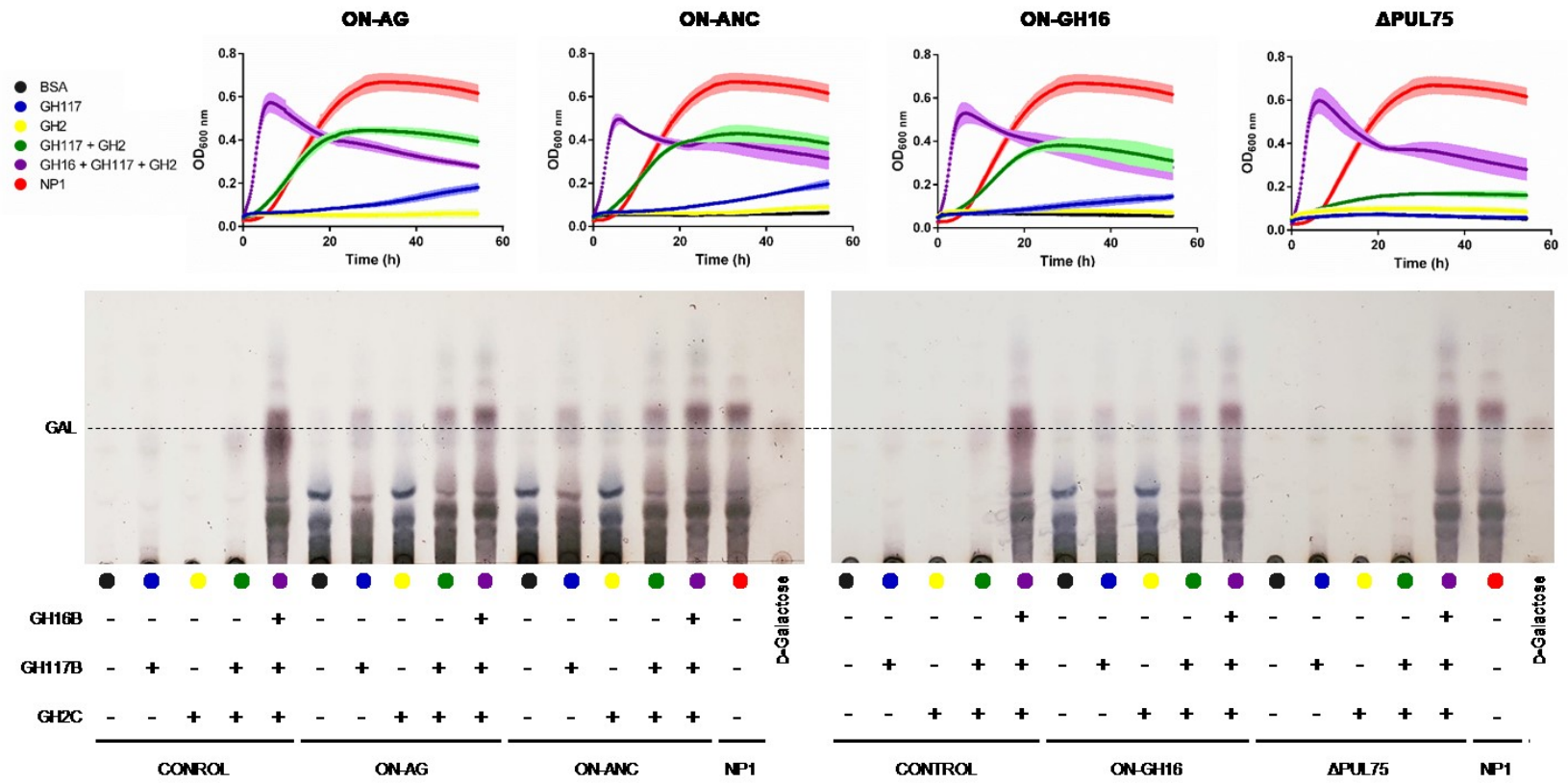


Figure 2.7. Growth curve and supernatant analysis of constitutive engineered strains grown on LMPA with supplemented agarases. Black represents media supplemented with non-catalytic BSA protein. The agarolytic positive control, *B. uniformis* NP1 supplemented with BSA is represented by red circles. Media supplemented with only GH117B is represented with blue circles, media supplemented with only GH2C is represented with yellow circles, media supplemented with both GH117B and GH2C agarases is represented by

green circles, and media supplemented with GH16B, GH117B, and GH2C agarases is represented by purple circles. Black dashed line on TLCs represents the band migration pattern of D-GAL.

Chapter 3: Development of an assay to quantify the release of horse radish peroxidase from protamine-algal galactan capsules

3.1. Introduction

The AT of monogastric animals is a complex organ system responsible for many important functions, including the mastication and digestion of food, absorption of nutrients, excretion of waste, and protection between the environment and host physiological functions [1]. The AT also has a highly regulated intestinal immune system, which helps maintain a homeostatic environment within in the gut. Indeed, the intestinal immune system is required to modulate pro- and anti-inflammatory responses as the host is exposed to dietary antigens and environmental toxins and contains diverse populations of bacteria within the DGM [37]. Additionally, the DGM also has a direct effect on the host immune system and is important in establishing intestinal immunological quiescence as well as inducing intestinal inflammatory responses–components that will affect homeostasis within the gastrointestinal tract (GIT) [39]. As such, an adverse alteration in intestinal bacterial populations, resulting from a variety of factors, including changes in diet, long-term use of antibiotics, and on occasion acute intestinal injury, can lead to an imbalance in the community structure, a process known as dysbiosis [3]. Dysbiosis can induce serious intestinal injury and substantive harm to the host and has been implicated in the inflammatory bowel diseases: Crohn's Disease and Ulcerative Colitis [51].

Although dysbiosis has been shown to have an impact on the development of inflammatory bowel disease, the etiology of the disease is still unknown [51]. As a result, general strategies used to treat inflammatory bowel disease are directed towards treating dysbiosis to restore a balanced bacterial community structure within the DGM or attenuating physiological responses, such as inflammation [63]. Current therapeutic modalities for reducing intestinal injury associated with

dysbiosis include: administering drugs such as metronidazole an antimicrobial that reduces intestinal anaerobic bacteria and protozoa load, glucocorticoid steroids to decrease inflammation, or a sulfa drug, sulfasalazine, a compound with immunosuppressive, antibacterial, and anti-inflammatory properties [63]. Probiotics and prebiotics, although only partially successful, have also been used to treat dysbiosis, and it is thought that probiotics and prebiotics may help restore the balance of 'beneficial' commensal bacterial populations within the DGM [11, 51].

An important consideration for the mitigation of inflammatory bowel diseases, is the need of the therapeutic compounds to reach the proximal and distal GIT, at concentrations that achieve an effective response. These segments of the AT, can be difficult to treat with orally administered drugs due to the size of the organ, location of the tissue, and site of intestinal inflammation [63]. Most certainly, orally administered therapeutic agents must traverse the upper AT, where the compounds can be inactivated by stomach acids, bile acids, or host digestive enzymes. Therefore, to circumvent this problem, a number of different colon-specific drug delivery systems are used to treat IBD. These include: 1) administration of delayed, timed, or extended release capsules, 2) treatment with prodrugs which are inactive derivatives of therapeutic drugs, which become active forms of the drug following the hydrolysis of chemical linkages by enzymes expressed by the DGM, or 3) colon-specific degradable delivery systems that rely on the expression of enzymes by enteric bacteria that digest the capsules and subsequently release the encapsulated drug [63, 114]. Targeted delivery systems comprised of 'Generally Recognized as Safe' (GRAS) [115, 116] polysaccharide capsules, represent a promising method of delivery as they are easy to modify, stable within the intestinal tract, and can be enzymatically hydrolysed by bacteria commonly found within the DGM [114].

Algal polysaccharides are complex indigestible galactans that comprise the amorphous matrix of Rhodophyta (i.e. red algae) cell walls [65]. Algal polysaccharides are remarkably different from terrestrial plant cell wall polysaccharide, as these can be highly sulfated, contain anhydrous monosaccharides, and are enriched in L-galactose, an uncommon enantiomeric form of galactose. The structures of porphyran and agarose are mainly composed of repeating disaccharide units of 4-*O*-α-L-galactose (L-GAL) and 3-O-β-D-galactose (D-GAL) monosaccharides (Figure 3.1A), and 4-*O*-α-L-3,6-anhydrogalactose (L-AHG) and 3-linked D-GAL (Figure 3.1B), respectively. The L-GAL of porphyran also contains a high degree of sulfation (L6S) (Figure 3.1C), which provides the polysaccharide a negative charge [65, 66]. [117]. Carrageenans are considered a different subgroup of sulfated galactans as these are composed of 3-linked GAL and 4-linked α-3,6-anhydro-D-galactose (D-AHG) monosaccharides (Figure 3.1D). Algal polysaccharides readily form gels and are naturally resistant to acid hydrolysis due to the incorporation of negative charges on sulfated sugars, and therefore are good candidates for developing drug delivery systems for the GIT [67].

The structural complexity of these non-terrestrial red algal polysaccharides requires bacteria to produce a distinct set of enzymes that hydrolyse glycosidic linkages in these complex carbohydrates to produce monosaccharides to be used as a carbon source for energy. The genes that encode these specialized hydrolytic enzymes are predominantly present within marine bacterial genomes and are either found to a lesser extent or more commonly absent in the genomes of bacteria present in the intestinal microbiota of terrestrial animals [68]. GH16 is a 'polyspecific' family of enzymes and generally not considered a member of the classical family of agar-degrading enzymes. Nevertheless, some GH16 enzymes have evolved to specifically accommodate the anhydride bridge and sulfate esters associated with algal polysaccharides. Thus, these enzymes

have been characterized as endo-acting β-agarases, β-porphyranases, and κ-carrageenases and play an important role in the saccharification of algal polysaccharides [71, 76].

The physical-chemical properties of the algal polysaccharides can limit the capsular disruption and enzymatic degradation of the encapsulated drug delivery systems within the upper digestive tract of a host species. In this regard, development of an algal polysaccharide-based capsule could be an excellent drug delivery system that increases the efficiency of drug delivery to the colon. Designing algal polysaccharide capsules that are resistant to digestion within the upper AT, would be the initial step in developing a drug delivery system for targeted release of bioactive molecules to the distal GIT. Co-administering algal polysaccharide-derived capsules with bacteria engineered to produce enzymes that depolymerizing algal polysaccharides within the capsule could increase cargo release within the proximal and distal colon. With this anticipated goal, capsules have been made by mixing sulfated algal polysaccharides, porphyran or ι-carrageenan (Figure 3.1E and F) with a small, arginine-rich protein, protamine. The positive charge from the protamine forms ionic bonds with the sulfate groups on the algal polysaccharides and this increases the structural integrity of the capsules.

This study focused on developing an effective assay to quantitatively measure the amount of cargo released into the medium from capsules supplemented with agarases and porphyranases or bacterial cells that produce these algal polysaccharide-active enzymes on their outer surface. Horseradish peroxidase (HRP), an enzyme that oxidizes a variety of molecules in the presence of hydrogen peroxide (Figure 3.2A), was used as a reporter enzyme to measure the amount of cargo released. This enabled the development of an indirect reporter assay that measured the relative amount of HRP released from algal polysaccharide capsules after enzymatic digestion with two GH16 family enzymes expressed from *B. uniformis* NP1. In addition to quantitatively measuring

the amount of cargo released, the product profiles following enzymatic hydrolysis were analyzed to determine whether GH16 enzymes could effectively depolymerize the capsular algal polysaccharides. Importantly, this study provided a proof of principle that engineered bacterial GHs adequately hydrolyse algal polysaccharides-derived capsules and as such, provides the first steps towards developing a colon-targeted-algal-polysaccharide-based drug delivery system that could administer therapeutic agents to mitigate IBD.

3.2. Materials and Methods

3.2.1. Phylogenetic tree to predict activity of GH16s used in this study

Sequence Analysis and Clustering of CarboHydrate Active enzymes for Rapid Informed prediction of Specificity (SACCHARIS) is an automated pipeline that uses known protein sequences for the discovery of CAZyme or carbohydrate binding module (CBM) specificity from characterized families archived on the CAZy database [118]. Two query GH16 sequences from *B. uniformis* NP1 were run through the SACCHARIS pipeline and compared against previously characterized GH16 sequences retrieved and extracted from the CAZy database. Best-fit model selection using the sequence alignment was performed using Prot Test [119] and the ITOL website was used to generate the phylogenetic tree [120].

3.2.2. Analysis of algal oligosaccharides from GH16 digestion

Enzyme assays and supernatant analysis was performed to determine the enzymatic activity of the GH16s, and proteinase K, as compared to BSA controls on algal polysaccharides used in the capsules in this study: porphyran, ι-carrageenan (C1138, Millipore Sigma), and low melting point agarose (LMPA) (IB70057, IBI Scientific). The methods were adapted from [68]. Briefly, 100 µL of 1% (w/v) carbohydrate solution of LMPA, porphyran, or ι-carrageenan were mixed with 100 µL of 2 µM enzyme in 0.1 M phosphate solution pH 6.0 and incubated at 37°C with shaking

at 200 rpm overnight for 24 hours. After incubation, samples were pelleted by centrifugation at 5,000 rpm for 3 minutes, and supernatants were collected. The supernatants were heated at 95°C for 10 minutes to stop the reaction and then cooled before loading on a TLC plate. Supernatants from capsule digests, as described in Section 3.2.4, were not heat treated to retain HRP enzymatic activity. Supernatants were analyzed using TLC as described in Chapter 2, Section 2.2.3.2.

3.2.3. HRP assay

An HRP assay was developed to measure the amount of encapsulated HRP released into solution following digestion of algal polysaccharide capsules. HRP oxidizes *o*-dianisidine using hydrogen peroxide (Figure 3.2A), which can be measured over time. For the assay, 10 µL of HRP (9003-99-0, BioBasic) in 100 mM phosphate buffer pH 6.0 was mixed with 100 µL of reaction buffer (100 mM phosphate buffer pH 6.0, 0.32 mM *o*-dianisidine (D9154, Millipore Sigma), and 0.88 mM hydrogen peroxide). Reactions were performed in triplicate in a 96-well microtitre plate (267578, Thermo Scientific, Roskilde, Denmark) and absorbances at 450 nm were measured every minute for 20 minutes at 37 °C with an Eon microplate reader and Gen5 software (BioTek Instruments, Winooski, VT). Blanks were prepared without HRP and subtracted from the reaction values to normalize the amount of HRP present within the assay. The standard curve was generated from 1:2 serial dilutions of 500 µM HRP and 50 µM HRP stock solutions added to 100 mM phosphate buffer pH 6.0. Initial rates of reaction were measured by the slope of the line (ΔAbs 450 nm/ time (hours)) and plotted against each HRP amount (nmol).

3.2.4. Algal polysaccharide capsule digestions

A schematic illustration of the assay developed in this study is shown in Figure 3.2B. First, capsules are incubated with two different recombinantly purified (see Section 2.2.5.1) algal-polysaccharide-active GH16 enzymes from *B. uniformis* NP1 to hydrolyse the β-1,4 glycosidic

linkages present in the algal polysaccharide capsules. After incubation, supernatants from the digestions are incubated with hydrogen peroxide and *o*-dianisidine, a molecule that colourizes when oxidized and measurable through absorbance readings, to quantify the amount of free HRP. The oxidation and colour change can only occur in the presence of HRP, and therefore, the change in absorbance is indicative of HRP released from capsule digestion.

Four different capsules were generously provided for the following experiments and include: porphyran:protamine (POR) and ι-carrageenan:protamine (CGN) capsules, either encapsulating HRP cargo (ie. POR HRP or CGN HRP), or lacking HRP cargo (POR EMP or CGN EMP).

3.2.4.1. Enzymatic digestions of algal polysaccharide capsules

Enzymatic digestions of capsules were performed to test whether GH16 enzymes from *B. uniformis* NP1 could hydrolyse algal polysaccharide-formulated capsules and release detectable amounts of HRP. Proteins used for the capsule digestion included GH16B, GH16C, BSA, and proteinase K. Briefly, 50 µL of a 2 µM protein stock solution was mixed with 50 µL of 10 mg/mL of algal polysaccharide capsules (POR HRP, POR EMP, CGN HRP, or CGN EMP), and incubated at 37°C and 200 rpm shaking for 24 hours. After incubation, the samples were pelleted and supernatants were collected for analysis. 10 µL of each sample was loaded on to a 96-well plate and 100 µL of reaction buffer and the reaction was immediately recorded with absorbance readings at 450 nm over time. The initial rates for each sample were calculated by measuring the linear slope, and data were fit to the standard curve to determine the amount of HRP released into solution. Enzyme conditions included four technical replicates per treatment and were normalized to controls that lacked enzymes.

3.2.4.2. Bacterial digestions of algal polysaccharide capsules

Bacterial cells were also tested to determine if cultures enriched with algal carbohydrates, upregulate the expression of enzymes that could hydrolyse algal polysaccharide capsules, releasing encapsulated HRP. The bacterial strains used in this study were selected based on native or engineered algal polysaccharide hydrolytic enzymes to digest the polysaccharide capsules. Thus, capsular digestion assays used bacterial strains *B. theta VPI-5482* (WT), *B. theta Δtdk ΔPUL75* (ΔPUL75), *B. theta* ON-GH16 (ON-GH16), and *B. uniformis* NP1, to hydrolyse the algal polysaccharides that makeup the capsules. The bacteria were cultured as described in section 2.2.2. Briefly, all bacterial strains were cultured on 10% blood-supplemented Columbia agar from glycerol stocks and incubated in a vinyl anaerobic chamber (Coy Lab Products Inc., Grass Lake, MI) with an atmosphere of 85% N_2, 10% CO_2, 5% H_2 and temperature of 37°C for 16 hours. Four colonies were picked from each bacterial strain and inoculated in custom chopped meat broth (CCMB) and incubated anaerobically at 37°C for 16 hours [74]. Once bacterial cell cultures were measured at an OD~1.0, bacteria were subcultured into minimal medium (MM) as previously described in [99], with 0.5% carbohydrate to an OD~0.05. MM and carbohydrate medium was used to induce the production of algal polysaccharide-active enzymes within the bacteria. As such *B. uniformis* NP1 was incubated with porphyran, LMPA, or GAL (G0750, Millipore Sigma) as a control, while *B. theta* was incubated with GAL only. Cells were grown to an OD~0.4 and pelleted. Cell pellets were washed with 1X phosphate-buffered saline (PBS) solution (10.1 mM Na_2HPO_4 (0705, VWR), 1.74 mM KH_2PO_4 (0781, VWR), 137 mM NaCl (S3014, Millipore Sigma), 2.7 mM KCl (P5405, Millipore Sigma) pH 7.4) and resuspended with 5 mL 1X PBS. Cell viability counts were quantified using colony forming unit (CFU) measurements whereby serial dilutions of each culture were plated on Columbia agar in duplicate and incubated for 24 hours at 37°C

anaerobically. Enumerations of bacterial cells were completed by quantifying the number of CFUs per dilution plate then converting to CFU/mL of medium, factoring in the 100 μL plated and dilution factor used.

Cell cultures in 1X PBS were diluted ten-fold, and 50 μL of the culture was added to 50 μL of 100 mg/mL capsules and incubated at 37°C, 200 rpm, anaerobically for 24 hours. After incubation, cells and capsules were pelleted and supernatants were used in duplicate in HRP assay described above to measure relative HRP amounts released from cellular digestion. Cellular assays included four biological replicates and two technical replicates between conditions and were normalized to a no cell control incubation.

3.2.4.3. Statistical analysis of HRP assays

To examine whether or not the relative amount of HRP released from capsule digests was significant between different enzymes and cell types, HRP release assay data were analyzed using GraphPad Prism 7.0. Statistical significance of data were assessed using two-way ANOVA tests between the enzymes and bacterial strains used in the enzymatic digestion and cellular digestion assays, respectively.

3.3. Results

3.3.1. Phylogenetic characterization of *B. uniformis* NP1 GH16s

Two GH16 protein sequences from *B. uniformis* NP1 were used as query sequences and run through the SACCHARIS pipeline to be compared with characterized GH16 members from the CAZy database. The resulting phylogenetic tree was labelled based on subfamilies that have demonstrated activities associated with algal polysaccharide degradation (Figure 3.3) [121]. Specifically, subfamilies 11-17 contain activities of interest relevant to the breakdown of algal cell wall polysaccharides including: β-agarases, β-porphyranases, β-carrageenases, and κ-

carrageenases. Each subfamily contained only one characterized activity; however, multiple subfamilies shared common activities.

The GH16B enzyme from *B. uniformis* NP1 has previously been characterized as a β-agarase [68] and is closely related to members in the largest family of β-agarases, GH16_16 (Figure 3.3), a β-agarase from *B. plebeius* DSM 17135, a member of the human DGM. In comparison, the GH16C β-porphyranase from *B. uniformis* NP1 [68] can be grouped within two small subfamilies of β-porphyranases: GH16_11 and GH16_12 (Figure 3.3). The grouping of the porphyranase subfamilies was not as clearly defined as those of other enzymatic activities, due to limited characterized sequences. Nevertheless, GH16C remains closely related to a characterized β-porphyranase from the marine bacterium, *Z. galactanivorans*, suggesting that GH16C can be similarly classified in the GH16_12 subfamily.

3.3.2. GH16 activity on algal polysaccharides

Before capsule digestion was performed, the enzymes that were used in this study were tested for their activity on the purified algal polysaccharides: low melting point agarose (LMPA), porphyran, and ι-carrageenan. The enzymes GH16B, GH16C, proteinase K, and protein BSA were incubated overnight on the three carbohydrates and their supernatants analysed for product formation (Figure 3.4). As expected, GH16B produced neoagarooligosaccharides (NAOS) from LMPA. The agarase GH16B also was seemingly active on porphyran, as indicated by the production of NAOS, suggesting that the porphyran used in this study contained agarose. Although GH16B produced NAOS from both LMPA and porphyran, the product band pattern observed in the TLC was different (Figure 3.4). LMPA is highly methylated and therefore the resulting NAOS would migrate differently compared to those produced from non-methylated agarose within porphyran. GH16C produced neoporphyranoligosaccharides (NPOS) from incubation on

porphyran but was not active on LMPA or carrageenan. GH16B and GH16C treatment of porphyran resulted in different oligosaccharide products formed (Figure 3.4), as they are active on different parts of the substrate backbone. No enzymes were able to hydrolyse ι-carrageenan as viewed by TLC, likely as a result of the D-AHG present in the polysaccharide backbone. There were no carbohydrate bands present in the TLC for BSA and proteinase K digestion of algal polysaccharides indicating they have no enzymatic activity on algal polysaccharides, as expected.

3.3.3. HRP standard curve

The assay developed in this study used the spectrophotometric properties of *o*-dianisidine to determine if HRP was released from the capsules after enzyme hydrolysis. When oxidized, *o*-dianisidine can be measured for absorbance at 450 nm. The rate at which a fixed amount of *o*-dianisidine is oxidized is dependent on the amount of HRP present to catalyse the reaction. The standard curve was determined by measuring the rate of the change in absorbance at 450 nm over time with increasing known amounts of HRP. The oxidation reaction occurred very quickly and only picomole amounts of HRP were needed for the reaction to have a measurable rate (Figure 3.5), and all HRP assays used this standard curve to determine amount of HRP released from capsules.

3.3.4. Capsule Digestions

3.3.4.1. Enzymatic digestion to release HRP

Enzyme digestions were performed on all four capsules: POR HRP, POR EMP, CGN HRP, and CGN EMP. The first enzyme digest performed only compared the ability of GH16B, GH16C, and the BSA control to hydrolyse the polysaccharide capsules and release HRP. A statistically significant, $p<0.001$ and $p<0.0001$, amount of HRP was measured in the GH16C digest supernatant from POR HRP as compared to the GH16B and BSA on the same capsule, respectively

(Figure 3.6A). The GH16B released a non-significant amount of HRP compared to BSA although it was shown to produce NAOS from porphyran (Figure 3.6A). This may be a result from agarose sections lacking sulfated monosaccharides within the porphyran backbone. The relative amount of HRP released in CGN HRP capsules (~2.3) was more compared to the POR HRP capsules (<2), but there was no significant difference in the amount of HRP released from CGN HRP between the GH16 digests and the BSA (Figure 3.6A). This was indicative that there is no significant hydrolysis activity between the enzymes and the non-catalytic control and may indicate that the integrity of the capsule was poor.

Supernatants of the enzymatic capsule digestion were analyzed by TLC to determine whether enzymes were able to hydrolyse the capsules. Both GH16B and GH16C enzymes have product profiles consistent with hydrolysis of porphyran as NAOS and NPOS products are observed by TLC for each enzyme respectively (Figure 3.6B). Assays containing BSA show no oligosaccharide bands, yet HRP was found to be present; it is likely that the integrity of the capsules are unstable resulting in the release of HRP even without enzymatic degradation of the coating (Figures 3.6B and 3.6C). Furthermore, no product bands were observed by TLC for any of the protein digestions on CGN capsules (Figure 3.6C). As GH16 enzymes are not able to hydrolyse 1% ι-carrageenan (Figure 3.4), the presence of HRP measured (Figure 3.6A) could be a result of leaky capsules.

In the second enzyme digestion assay, proteinase K was used to test if the capsules were vulnerable to protease digestion. Two time points (0 hours and 24 hours) were measured as an attempt to normalize the relative HRP activity accounting for the unstable capsules observed previously (Figure 3.6A). The 0 hour time point normalization could not fully account for the amount of HRP release from non-catalytic controls. Despite this, capsules treated with GH16C

released a statistically significant amount of HRP from POR HRP capsules compared to both proteinase K and BSA controls, while no significant amount of HRP was released by GH16B (Figure 3.7A). These results suggest that GH16C is able to hydrolyse the POR HRP capsules better than GH16B, even though NAOS and NPOS products are observed in the supernatant by TLC from POR capsule digestion (Figure 3.7B). The CGN HRP capsules were susceptible to degradation by both a protease and porphyranase, as both proteinase K and GH16C treatment of the capsules resulted in significant amounts of HRP released (Figure 3.7A). However, supernatant analyses by TLC does not support capsule hydrolysis by GH16C and proteinase K is incapable of producing oligosaccharide product, as seen here (Figure 3.7C).

3.3.4.2. Bacterial digestion of capsules to release HRP

To determine if bacteria that produce the GH16 enzymes are able to digest POR and CGN capsules to release HRP, capsules were incubated with two different sets of cells. *B. uniformis* NP1 was used as this species contains several GH16 enzymes organized into a polysaccharide utilization locus (PUL) known as the AgPUL, which has been recently characterized [68]. As the AgPUL is upregulated in the presence of agarose, cell cultures were enriched in LMPA, porphyran, or galactose solution to produce the secreted GH16 enzymes encoded within the AgPUL. The *B. uniformis* NP1 cells did not release significant amounts of HRP from either POR HRP or CGN HRP capsules compared to the GAL control (Figure 3.8A). This result was unexpected because NP1 contains both GH16B and GH16C and therefore it was thought the HRP release would be similar to the enzymatic digestion (Figure 3.6A). This result suggested the enzymes were not produced at sufficient levels or displayed on the bacterial cell surface. However, TLC analysis of incubations of capsules with *B. uniformis* NP1 did show product bands consistent with NAOS being released from porphyran and LMPA, suggesting that algal polysaccharide enzymes were

produced and active on parts of the capsules (Figure 3.8C). The similarity of the product profiles between the *B. uniformis* NP1 and GH16B digests (Figure 3.6B and 3.8C) suggests the agarase is expressed and active on the bacterial surface but may not be enough to release HRP. The porphyran- and LMPA-enriched cell digests on CGN HRP do have a larger amount of free HRP measured (Figure 3.8A) but when compared to product analysis by TLC (Figure 3.8C), none of the cells were able to produce carrageenan oligosaccharides suggesting that HRP released is not the result of enzymatic activity on the capsules.

A non-agarolytic human gut symbiont, *B. theta*, was previously engineered (strain: *Bt* ON-GH16) and shown to constitutively produce functional GH16B to the outer surface of the cell (Chapter 2). As GH16B was shown to hydrolyse POR HRP capsules (Figure 3.6), *Bt* ON-GH16 was tested for its ability to hydrolyse algal polysaccharide capsules to release HRP. The amounts of HRP calculated to be released by the *B. theta* cells was comparable to those released by *B. uniformis* NP1 cells, and there was no change in the amount released from POR HRP between *Bt* ON-GH16 and the control *B. theta* strains (Figure 3.8B). Surprisingly, a significant amount of HRP was released from CGN HRP when incubated with *Bt* ON-GH16, suggesting *Bt* ON-GH16 is able to hydrolyse CGN HRP capsules (Figure 3.8B). However, although *Bt* ON-GH16 was able release NAOS products from POR capsules (Figure 3.8D) there were no products observed when incubated with CGN capsules.

3.6. Discussion

The objective of this study was to develop an assay to detect the release of HRP from algal polysaccharide-derived capsules when treated with pure enzymes and agarolytic bacterial strains. Two GH16 enzymes from human gut symbiont *B. uniformis* NP1 were chosen because they had been previously characterized as a β-agarase and a β-porphyranase [68]. GH16s are a polyspecific

family of endo-acting enzymes that hydrolyse β-1,4 glycosidic linkages in polysaccharide backbones into small oligosaccharides [71]. Both the GH16B and GH16C enzymes were confirmed to hydrolyse porphyran into NAOS and NPOS, respectively, as porphyran is known to contain up to 30% agarose (Figure 3.4) [117]. Since the enzymes are endo-acting and able to hydrolyse the polysaccharides, it was expected that the enzymes would be active on the porphyran in the capsules. Indeed, it was observed that NAOS and NPOS were present in the supernatants after being incubated with the capsules (Figure 3.6B and 3.7B).

The two GH16 enzymes were unable to digest the ι-carrageenan and the CGN capsules (Figure 3.4, 3.6C, and 3.7C), and therefore, rational design of a system to disrupt CGN capsules will require the deployment of a *bone fide* carrageenase that is found within the DGM. The structure of carrageenan differs substantially from agarose and porphyran as it only contains D-enantiomers of GAL (Figure 3.1). Carrageenases, therefore, must display topological differences in their active sites to accommodate the differentiated repeating structure of CGN. These differences are reflected within their amino acid sequences, as the κ-carrageenases partition as a single clade away from porphyranases and agarases (Figure 3.3). As the GH16s in this study were not ι-carrageenanases, which contains an additional sulfate group on the C2 of the 3,6-anhydro-D-galactose monosaccharide (Figure 3.1F), these substrates are more likely to be degraded by carrageenases found in marine environments [66]. To date, only the genomes of marine bacteria have been found to harbour GH82s; a dedicated ι-carrageenase family [71, 76], and therefore, ι-carrageenan may not be a suitable polysaccharide for capsules to be used for applications within the human DGM. Alternatively, GH16 does contain enzymes with κ-carrageenase activity in two subfamilies (GH16_13, GH16_17) (Figure 3.3) [71, 121], which are encoded within *Bacteroides* spp.. The human gut symbiont, *B. thetaiotaomicron* 3731, for example, has been shown to be

carrageenolytic and grow on κ-carrageenan, which may represent a suitable system for the design of κ-carrageenan capsules [74].

The GH16 phylogenetic tree provided insight into the evolution of enzyme activities responsible for the digestion of agarose and porphyran (Figure 3.3). β-agarases and β-porphyranses are closely related enzymes and target substrates with similar overall structure. However, there are adaptations to accommodate the structural differences between the two substrates. β-porphyranases recognize L6S (Figure 3.1C) monosaccharides in the porphyran backbone and are not active on the parts of the backbones containing L-AHG (Figure 3.1B) [75, 122]. Figure 3.3 shows the porphyranases divide into two closely related subfamilies, GH16_11 and GH16_12, and form a uniform subfamily at lower thresholds [121]. It is unclear what subfamily the GH16C from *B. uniformis* NP1 belongs to as there are limited characterized structures of porphyranases within the CAZy database, and small differences in tertiary structure motifs may thus be misrepresented as much larger variation within the subfamily. β-agarases have the largest number of characterized enzymes and recently evolved from an ancestral progenitor to accommodate L-AHG. There are three subfamilies of β-agarases: GH16_14, GH16_15, and GH16_16, and the GH16B from *B. uniformis* NP1 aligned with GH16_16, which is the largest of these subfamilies. Unlike the β-porphyranase subfamilies, GH16_15 and GH16_16 partition into distinct clades, have different active site residues, and GH16_15 is not associated with CBMs [121]. Surprisingly, both *B. uniformis* NP1 GH16B and GH16C share higher sequence homology with the more distantly related bacterial species *Z. galactanivorans* than those from *B. plebeius*, a bacterium from the same genus. Genetic sequence alignments suggest that unlike the GH16B agarase, the *B. uniformis* NP1 GH16C porphyranase did not co-evolve with the *B. plebeius* β-porphyranase. This may have been caused by selective pressures creating differential roles of *Bacteroides* bacteria in algal

polysaccharide break down: *B. uniformis* NP1 is known to be agarolytic, while *B. plebeius* has demonstrated porphyranolytic activity [75].

Algal polysaccharide capsules were generously provided by a colleague to be used in this study. Capsules used for all experiments were from the same batch to maintain consistency between experiments. Capsules were made through a spontaneous reaction that happens when sulfated carbohydrates are added to protamine, a small, arginine rich protein. The negatively charged sulfate groups can form ionic bonds with the positively charged amine groups on the arginine residues, thus forming matrices that can encapsulate molecules. This process may differ between polysaccharide chemistry and the properties of the cargo. As the reaction is spontaneous, it is unknown whether the capsules were uniformly formed or vary in size and so it was unclear how much HRP was being encapsulated. For the assays, it was assumed all capsules contained the same amount of HRP so that the amount released would correlate with the digestion of the capsules. It was observed, however, that CGN capsules may be larger than the POR capsules as they settled out of solution quicker. This is a possible explanation as to why more HRP was detected to be released from CGN capsules, as larger capsules could accommodate more cargo (Figure 3.6A and 3.7A). Future work should include measuring the capsule size to determine the consistency of the formation of the capsules.

The CGN HRP capsules had a higher level of HRP in all treatments, including the BSA control. This result did not correspond with the appearance of digestion products (Figures 3.6 and 3.7), which is not surprising as the GH16 enzymes used in this experiment are not active on pure CGN (Figure 3.4). These observations suggest HRP release from CGN capsules may be a non-specific event resulting from weaker capsule integrity. It should be noted that the polysaccharide dots on the TLCs are much lighter for the CGN capsules indicating there is less polysaccharide

loaded and therefore products are harder to see, or the colourimetric stain used does not stain carrageenan. Normalizing signal for 0 hour time points did decrease overall amount of HRP results to less than one to lessen the background noise. Normalization to the 0 hour time point did reduce the HRP released in the BSA digestions to similar amounts for both POR HRP and CGN HRP digestions which provides better comparisons between the two capsule assays and therefore should be continued on in future experiments. Although HRP appeared to be released in both enzyme digestion assays that BSA was releasing HRP, no product bands were observed in the supernatants as BSA is non-catalytic, which further contributes to the idea that the capsule integrity is weak (Figure 3.6 and 3.7). Thus, future microscopic work to measure the size and qualify the capsules before and after each digestion would provide further insight as well as analyzing digest capsule products using more sensitive procedures such as high performance liquid chromatography.

To determine if the protamine molecules are susceptible to proteolysis, which is a consideration for technologies that transit through the upper AT, a proteinase K digest was performed to determine its effect on capsule integrity. Compared to POR HRP, CGN HRP capsules digested with proteinase K had a significant amount of HRP released ($p<0.001$) (Figure 3.7). This inconsistency could suggest that either the two polysaccharide capsules formed differently and protamine is more susceptible to proteolysis; or it is an artifact as similar to the GH16 digestions, there could be non-specific disruption of the capsule. Interestingly, the proteinase K treatments did not appear to affect HRP activity, and therefore, it is possible the protease was inactive on both proteins and capsules. Future work should include a protease control to ensure activity as well as a non-protein cargo.

The assay was developed in this study to quantitate cargo release resulting from capsular digestion by pure enzymes and whole-bacterial cells. HRP was chosen as a reporter enzyme

because its activity on certain substrates can be measured by the production of chromogenic products, thus providing insight on the effect of encapsulation of a bioactive protein as well as being able to develop assays towards measuring the HRP released. HRP uses hydrogen peroxide as an electron acceptor to oxidize substrates, in this study oxidized *o*-dianisidine changes colour and can be measured by absorbance at 450 nm readings (Figure 3.2A). The rates of the HRP reaction were measured and plotted against the activity of known amounts of HRP to generate a standard curve. This curve could then be used to calculate amount of HRP released from the capsule digests by interpolation. Results for these assays were difficult to interpret, however, because the integrity of the capsules and the unknown capsule size resulted in different values when the experiments were repeated. In addition, selecting HRP as a reporter molecule confounded the reproducibility of the data as rate of activity is an indirect measurement and less accurate than direct measurement of molecule release. This issue has been addressed in other cargo-capsule systems. For example, fluorescent molecules have been used to directly measure cargo release from magnetic nanoparticles [123], and Ondansetron, an antiemetic drug, was directly detected at 310 nm to measure release from microspheres [124].

In this study, an assay was developed to measure the HRP release from an algal polysaccharide capsule as part of an initial step to developing a colonic drug delivery system. It was determined that β-agarase and a β-porphyranase were able to produce NAOS and NPOS, respectively from porphyran-derived capsules. Interestingly, the POR HRP enzyme digest assay results seemed more consistent overall with only GH16C releasing significant amounts of HRP compared to controls (Figure 3.6A and 3.7A), compared to CGN HRP results which did not show a consistent pattern through both assays (Figures 3.6A and 3.7A). These data also correlated with the TLC analysis where only porphyran capsules had hydrolysis products from both GH16B and

GH16C enzymes. This clearly indicates that porphyran capsules are better suited for designed cargo release. For the whole-cell *B. uniformis* NP1 digests, no significant amounts of HRP released were measured although product bands were observed for both porphyran- and LMPA-enriched cells. Interestingly, product profiles from these reactions are very similar to the *Bt* ON-GH16 strain when incubated with POR capsules, suggesting that native GH16B from *B. uniformis* NP1 as well as the GH16B from *Bt* ON-GH16 are both secreted to the outer surface and active on porphyran capsules. If GH16B is the primary enzyme produced the *B. uniformis* NP1 this may explain why NAOS is released by *B. uniformis* NP1 (Figure 3.8C). This finding gives promise to using engineered and endogenous human gut symbionts as tools to develop strategies as drug delivery systems.

In conclusion, although it is still unclear whether the capsules were able to release bioactive cargo when specifically digested by an algal polysaccharide digestive enzyme or a bacterial strain; enzyme hydrolysis of the POR capsules did occur. Oligosaccharide release underpins that the POR capsules have the potential to be digested by pure enzymes, bacteria engineered with enzymes, and bacteria within the human DGM. Further optimization is needed to validate capsule integrity and stability, and the release kinetics for other types of molecules, such as peptide-based therapeutics and small organic molecules.

3.7. Figures

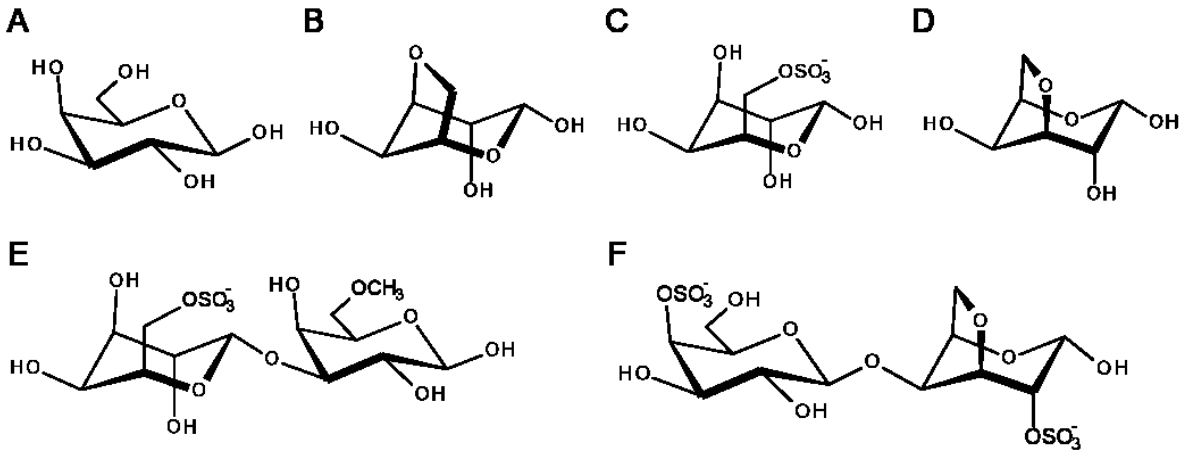
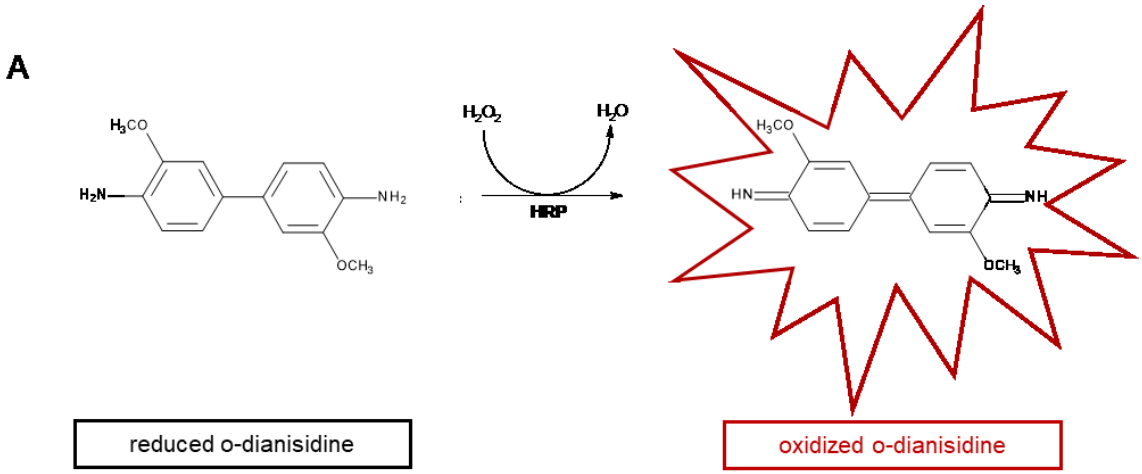


Figure 3.1. Structural representation of mono- and disaccharides comprising the galactans of red algae cell walls. A) D-galactose, B) 3,6-anhydro-L-galactose, C) L-galactose-6-sulfate, D) 3,6-anhydro-D-galactose, E) neoporphryanobiose, F) ι-carrabiose.


A
H3CO
H2N
NH2
OCH3
H2O2
H2O
HRP
HN
NH
reduced o-dianisidine
oxidized o-dianisidine

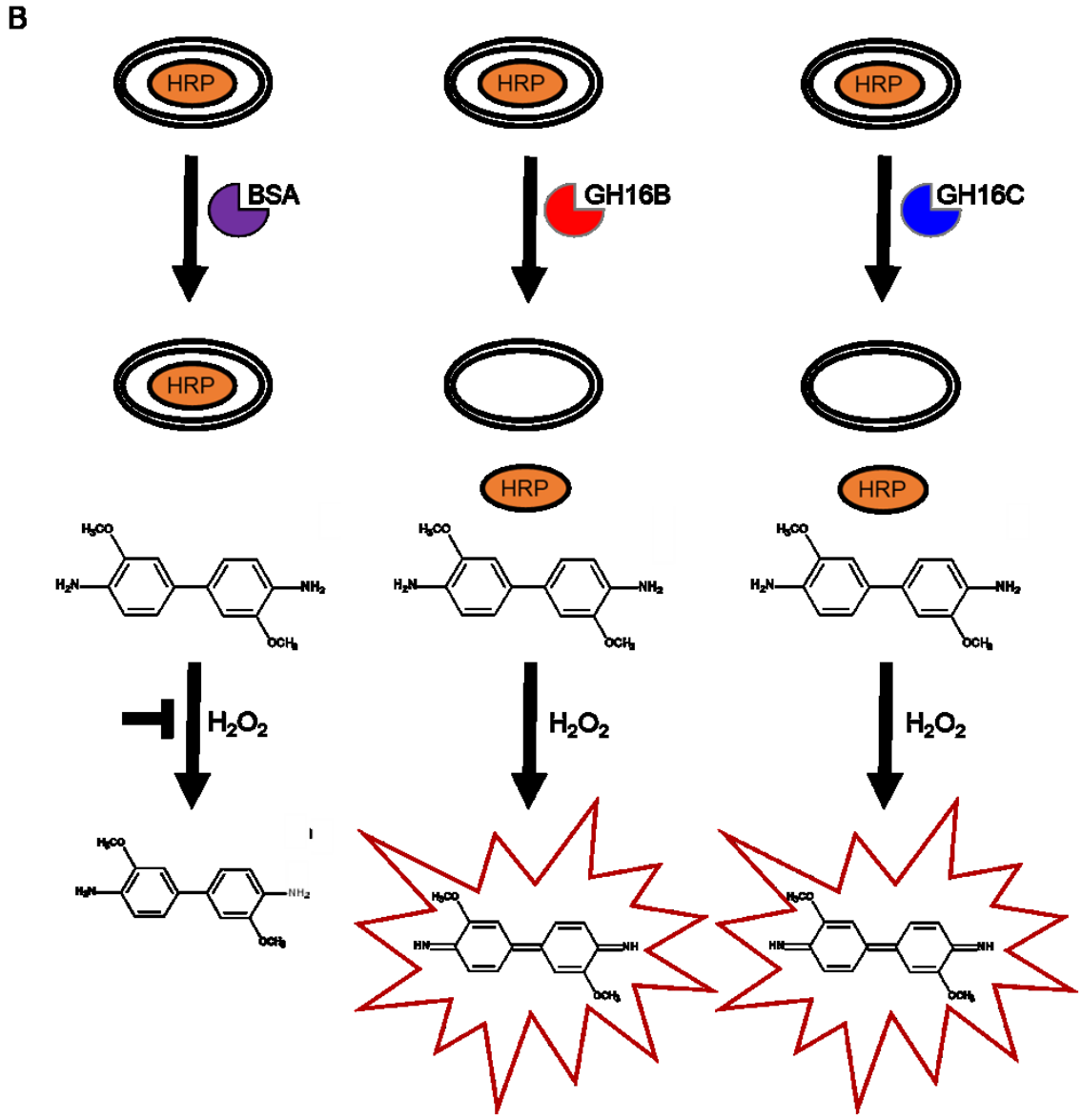

B
HRP
BSA
GH16B
GH16C
H2O2

Figure 3.2. HRP catalyzes oxidation reaction of colourimetric molecule o-dianisidine. A) Oxidation reaction of *o*-dianisidine by HRP and hydrogen peroxidase. B) Schematic representation of HRP release assay using pure proteins. GH16B or GH16C digest the polysaccharide capsule to release HRP that can then oxidize *o*-dianisidine, which can be monitored by reading Absorbance at 450 nm. BSA is a non-catalytic control that is unable to release HRP from the polysaccharide capsule and therefore *o*-dianisidine will remain reduced and uncoloured.

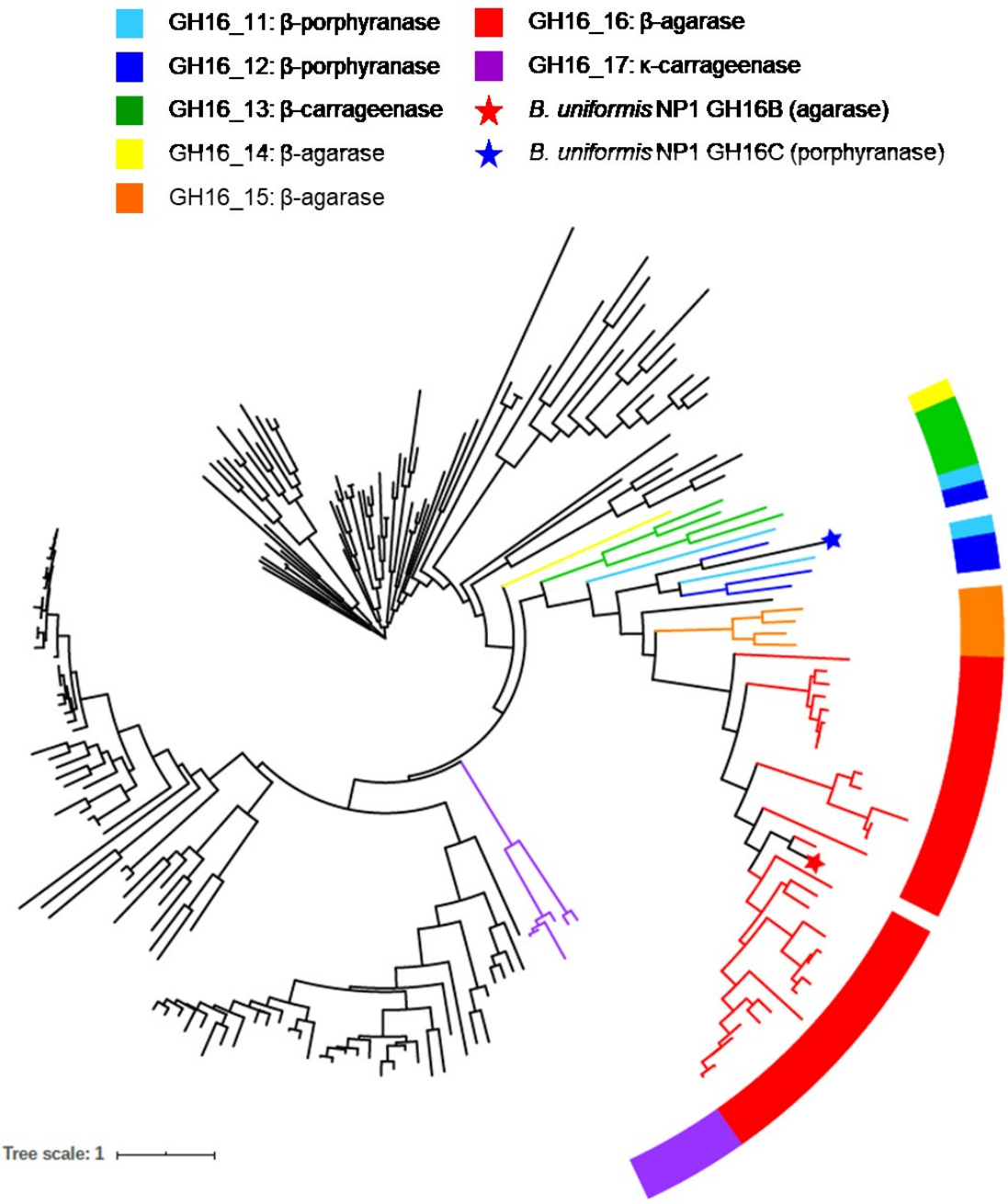


Figure 3.3. Phylogenetic tree of characterized GH16 family agarases, highlighting subfamilies that have activities associated with hydrolysis of red algal cell wall polysaccharides. Two query sequences from *B. uniformis* NP1: GH16B and GH16C are highlighted within their respective clades.

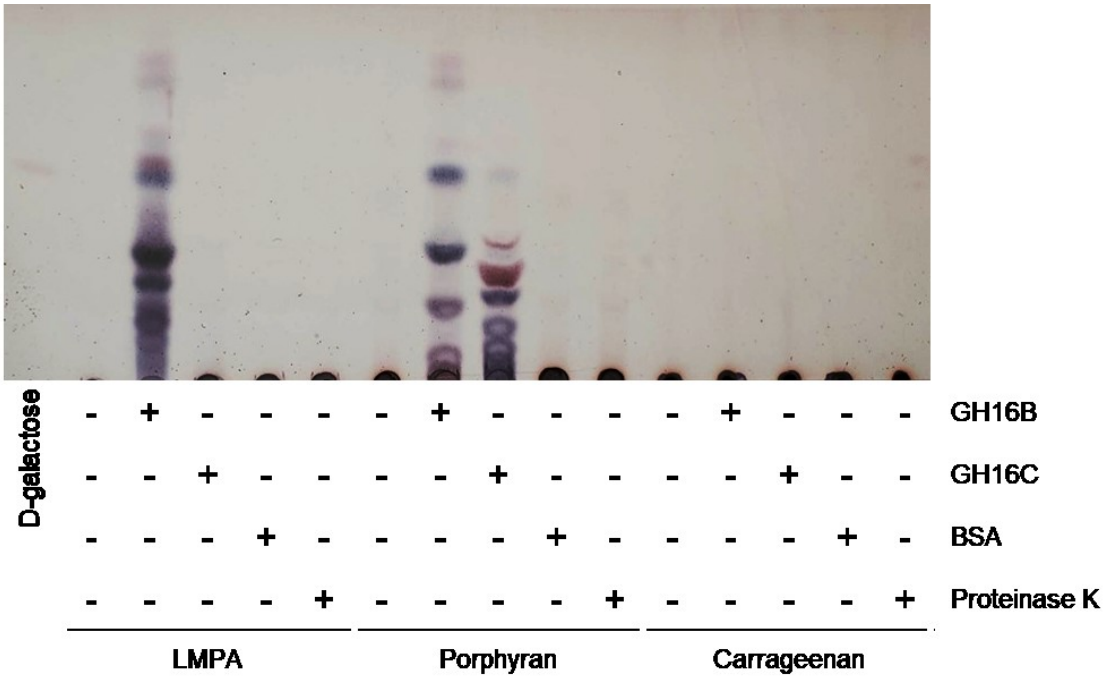


Figure 3.4. Product analysis of agarase: GH16B, porphyranase: GH16C, protease: proteinase K, and non-catalytic control: BSA, after incubation on three red algal cell wall polysaccharides: agarose, porphyran, and ι-carrageenan, developed using TLC.

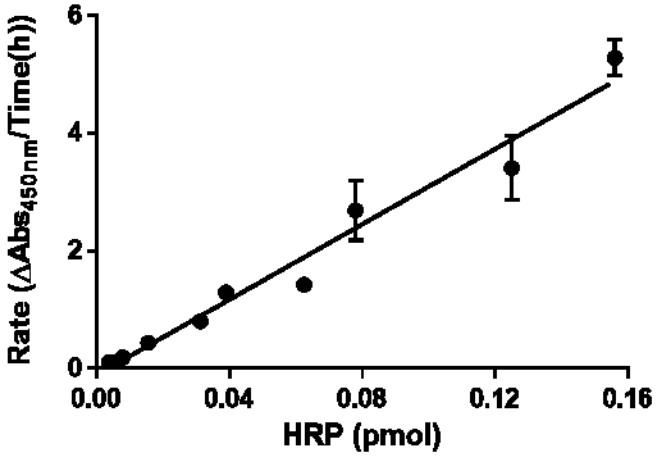


Figure 3.5. Standard curve used to calculate relative amounts of HRP in capsule digest assays. The curve was plotted by measuring the change in absorbance at 450 nm over time (hours) with increasing amounts of HRP (pmol). The line of best fit equation is y=32.05x-0.1091, R^2=0.949.

Four technical replicates were averaged for each point and error bars represent the standard deviation of the mean.

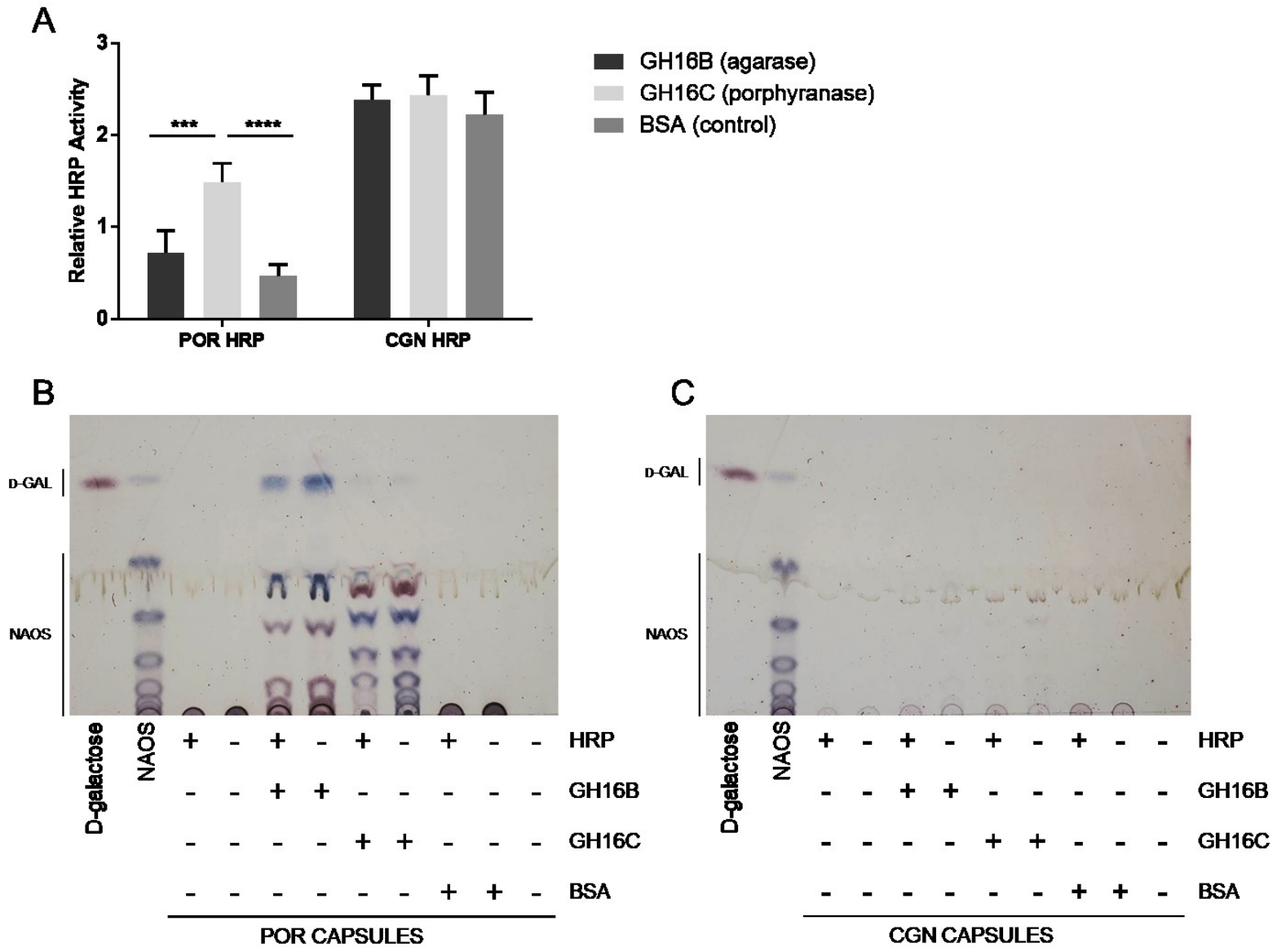

A
Relative HRP Activity
3
2
1
0
***
****
POR HRP
CGN HRP
GH16B (agarase)
GH16C (porphyranase)
BSA (control)
B
D-GAL
NAOS
D-galactose
NAOS
+ - + - + - + - - HRP
- - + + - - - - - GH16B
- - - - + + - - - GH16C
- - - - - - + + - BSA
POR CAPSULES
C
D-GAL
NAOS
D-galactose
NAOS
+ - + - + - + - - HRP
- - + + - - - - - GH16B
- - - - + + - - - GH16C
- - - - - - + + - BSA
CGN CAPSULES

Figure 3.6. GH16 enzyme digestion of POR and CGN capsules. A) Relative HRP amount released by GH16 enzymes after 24 hour incubation. Each calculated HRP amount was normalized to a no enzyme digest blank. Four technical replicates were used and error bars represent standard deviation of the mean. Statistical significance calculated with ANOVA, *** when $p<0.001$, and **** when $p<0.0001$. Supernatant analysis of B) POR capsule and C) CGN capsule digestion products after 24 hour incubation with enzymes using TLC. Standards on left side of TLCs include D-galactose (D-GAL), neoagarooligosaccharides from β-agarase hydrolysed agarose (NAOS) and are indicated with their migration patterns on left side of TLCs.

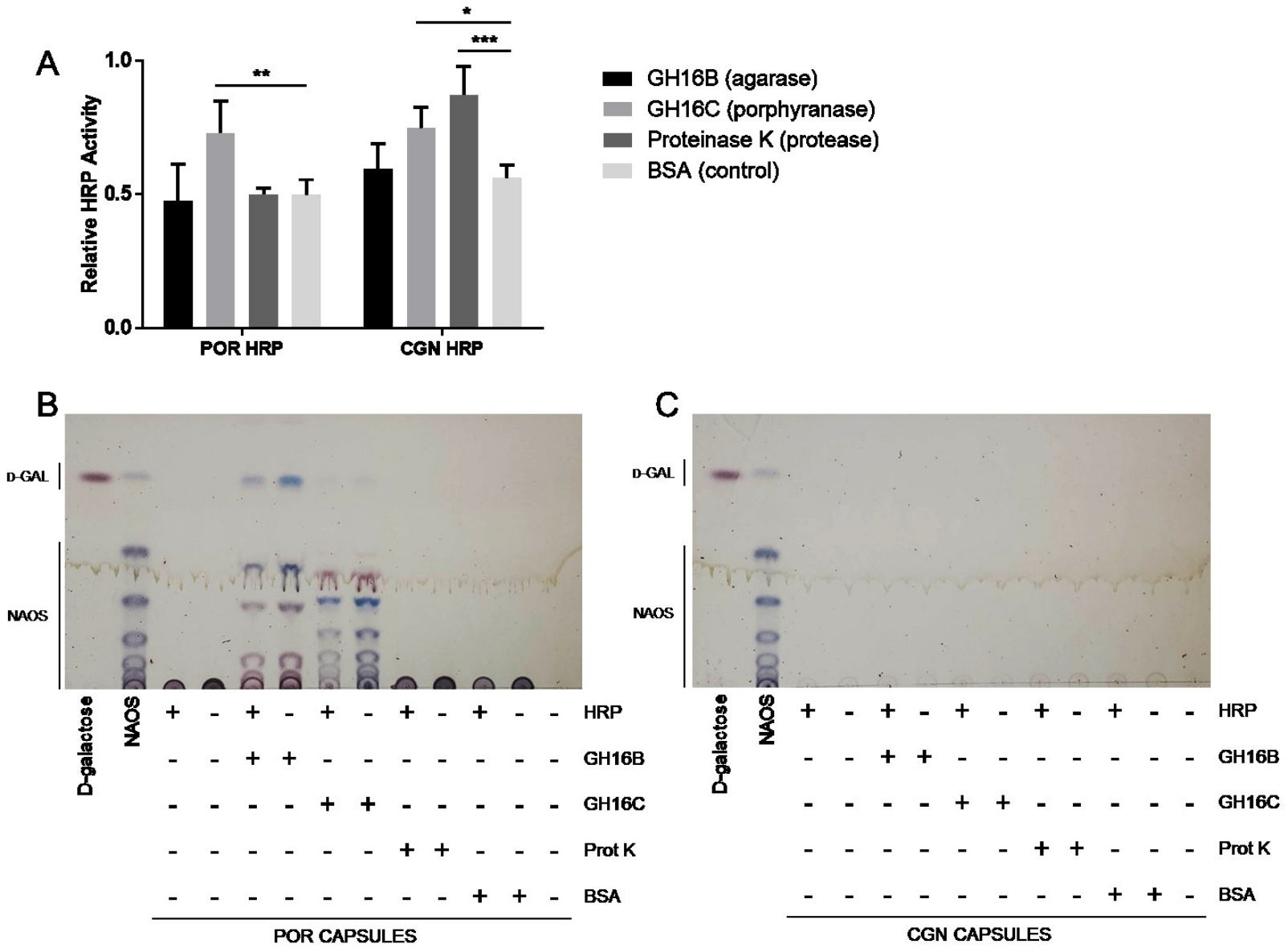

A
Relative HRP Activity
1.0
0.5
0.0
**
*
***
POR HRP
CGN HRP
GH16B (agarase)
GH16C (porphyranase)
Proteinase K (protease)
BSA (control)
B
D-GAL
NAOS
D-galactose
NAOS
HRP
GH16B
GH16C
Prot K
BSA
POR CAPSULES
C
D-GAL
NAOS
D-galactose
NAOS
HRP
GH16B
GH16C
Prot K
BSA
CGN CAPSULES

Figure 3.7. GH16 and proteinase K enzyme digestion of POR and CGN capsules after 24 hours. A) Relative HRP amount released by GH16 enzymes. Each calculated HRP amount was normalized to 0 hour time points. Four technical replicates were used and error bars represent standard deviation of the mean. Statistical significance calculated with ANOVA, * when $p<0.05$ when p ** when $p<0.01$, and *** when $p<0.001$. Supernatant analysis of B) POR capsule and C) CGN capsule digestion products after 24 hour incubation with enzymes using TLC. Standards on left side of TLCs include D-galactose (D-GAL), neoagarooligosaccharides from β-agarase hydrolysed agarose (NAOS) and are indicated with their migration patterns on left side of TLCs.

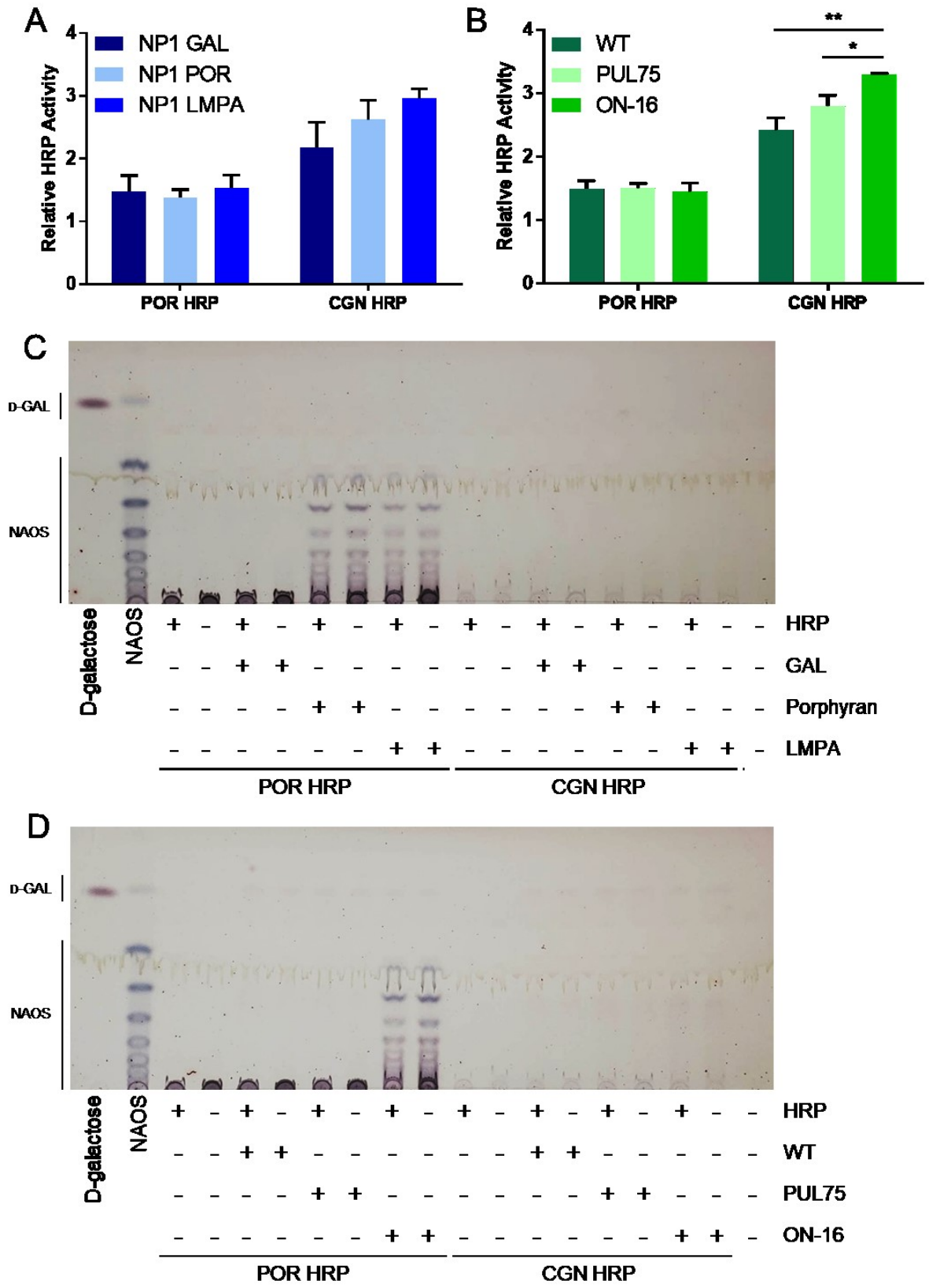

A
NP1 GAL
NP1 POR
NP1 LMPA
Relative HRP Activity
POR HRP
CGN HRP
B
WT
PUL75
ON-16
**
*
Relative HRP Activity
POR HRP
CGN HRP
C
D-GAL
NAOS
D-galactose
NAOS
HRP
GAL
Porphyran
LMPA
POR HRP
CGN HRP
D
D-GAL
NAOS
D-galactose
NAOS
HRP
WT
PUL75
ON-16
POR HRP
CGN HRP

Figure 3.8. Cellular digestion of POR and CGN capsules after 24 hours. Relative HRP amount released by A) *B. uniformis* NP1 bacterial cells enriched on galactose, porphyran, or LMPA or B) *B. theta* wildtype (WT), ΔPUL75, or engineered strain ON-GH16 after POR and CGN incubation for 24 hours. Each calculated HRP amount was normalized to a no cell control. Four biological replicates and two technical replicates were used and error bars represent standard deviation of the mean. Statistical significance calculated with ANOVA, * when $p<0.05$ and ** when $p<0.01$. Supernatant analysis of POR and CGN capsule digestion products after 24 hour incubation with C) *B. uniformis* NP1 and D) *B. theta* bacterial cells using TLC. Standards on left side of TLCs include D-galactose (D-GAL), neoagarooligosaccharides from β-agarase hydrolysed agarose (NAOS) and are indicated with their migration patterns on left side of TLCs.

Chapter 4: Conclusions

Dysbiosis in the distal gut microbiota (DGM) is caused by a variety of factors including changes in diet, the prolonged use of antibiotics, and on occasion, acute intestinal injury. It is defined as a compositional and functional alteration in the microbiota which is generated by a suite of environmental and host-related factors that disrupt the intestinal microbial ecosystem. Notably, these changes need to exceed the resistance and resilience capabilities of the microbial community. Dysbiosis has been implicated in a number of diseases and disorders including cancer, diabetes, and inflammatory diseases. As a result, it is important to study methods that mitigate dysbiosis, and these methods typically work to restore the loss of commensal organisms, decrease the number of pathogenic organisms, or increase the diversity of the DGM. Treatments such as antimicrobial agent administration have become less desirable towards treating dysbiosis as a result of antibiotic resistance developed by pathogenic organisms and as such, have come under public scrutiny for its usage in the livestock sector. Therefore identifying alternatives to antimicrobial use to mitigate dysbiosis is an important area of study.

This Master of Science research project had two objectives: i) to engineer a human gut symbiont, *B. theta* to produce three heterologous agarases that would saccharify agarose into is monosaccharide substituents, and ii) develop an assay to measure the amount of cargo released from an algal polysaccharide capsule after hydrolysis from GH16 enzymes. Collectively, these two objectives could provide a novel system for the mitigation of intestinal dysbiosis and intestinal inflammation. The first objective of my research project, was to engineer *B. theta*, a bacterial species that can colonize the mammalian intestine, to express glycoside hydrolase enzymes that would saccharify agarose—a rare dietary carbohydrate found in red algal cell walls. The co-administration of the engineered *B. theta* with agarose could form an engineered synbiotic system

that could be delivered to the distal GIT. Engineered *B. theta* could populate the intestinal microbial community, and saccharify dietary agarose releasing D-GAL monosaccharides to be used as an energy source for bacterial growth. Furthermore, this objective is the initial step towards developing a targeted drug delivery system by designing bacteria able to digest algal polysaccharide capsules. The second goal of my research was to develop an assay to measure the release of bioactive cargo from an algal polysaccharide capsule. The capsules were digested with either algal polysaccharide-specific GHs or with human gut symbionts to determine the efficacy of releasing cargo from the algal-polysaccharide capsules. The information gained from this research could be applied to develop a site-specific drug delivery system. This system would deliver therapeutic agents to the distal regions of the GIT where these engineered bacteria within the DGM are able to hydrolyse the algal polysaccharide capsules. Conversely, engineered bacteria could be co-administered with the algal-polysaccharide capsules to release therapeutic agents within the distal GIT.

In Chapter 2, I engineered a human gut symbiont to express three heterologous agarase genes under a single promoter, that in concert, completely saccharify agarose into individual monosaccharide units. It was shown that under regulation of two separate *B. theta* promoters, the three agarases were produced and functional as verified on Western blots (Figures 2.3A and 2.4A) and TLC (Figures 2.3B and 2.4B), respectively. Notably, all three agarases were produced and enzymatically active within a single strain of *B. theta,* demonstrating the potential of *Bacteroides* spp, under tight regulation, to express functional enzymes from more than one heterologous gene. Indeed in this study, I engineered the ability to completely saccharify the rare nutrient, agarose, into *B. theta* with only the addition of three agarases from *B. uniformis* NP1: GH16B, GH117B, and GH2C.

Although the three agarases were produced and active, in order for the bacterium to use agarose as a sole carbon source, the agarases contained N-lipidated signal peptides that trafficked the agarases to the outer surface of the bacterium to access the agarose substrate within the medium. Thus, the second part of Chapter 2 examined the localization of the agarases and determined whether these agarases were trafficked to the outer surface of the cell. It was determined that the 4116-GH16B endo-β-agarase was trafficked to the outer surface of the cell as shown by product profiles of whole cell assays (Figure 2.6). This observation was confirmed by optical density measurements on agarose. It was shown that when the bacterial strain *Bt* ON-GH16 was supplemented with both exogenous enzymes, GH117B and GH2C in the medium, the strain grew using agarose as a carbon source (Figure 2.7). Unfortunately, GH117B and GH2C failed to show enzyme activity on the outer surface of *B. theta*, as there were no polysaccharide-hydrolysis product bands in both the whole cell assays (Figure 2.6 and 2.7). The OD measurements further confirmed no enzyme activity on the outer surface of *Bt* ON-AG and *Bt* ON-ANC bacterial strains as both supplemented enzymes, GH117B and GH2C, were required in the medium to grow on agarose. It is unclear from the data whether the GH117B and GH2C enzymes are unable to traffic to the outer surface of the cell or whether these enzymes were inactive when bound to the outer membrane. Further studies that either employ different signal peptides or use agarase specific monoclonal antibodies would help determine the localization of the agarases within the bacterium. Importantly *B. theta* was able to use D-GAL products from agarose saccharification (Figure 2.7), a proof-of-principle that with further optimization of the signal peptides on the agarases within the engineered bacteria, it is possible the bacteria could grow on agarose as a sole carbon source. Nevertheless, *Bt* ON-GH16 strains are able to depolymerize agarose polysaccharides into neoagarooligosaccharides (NAOS), which could have applications in the design of novel drug

delivery systems, such as depolymerisation of agarose-derived capsules to release encapsulated therapeutic agents.

In Chapter 3 of my thesis, I developed an assay that measured the amount of cargo released from algal-polysaccharide-derived capsules, following digestion with individual GHs or human-gut symbiotic bacteria that produce GHs. The algal polysaccharides, porphyran and ι-carrageenan, have sulfate esters decorating the monosaccharides of the polysaccharide backbones (Figure 3.1) and these sulfate esters form ionic bridges with the positively charged small peptide, protamine, resulting in the formation of polysaccharide matrices, referred to as capsules. These capsules contained the enzyme HRP that could be measured by a colorimetric assay following HRP release from the capsule and catalyzing the oxidation of colorimetric molecule, o-dianisidine. In this chapter, I designed an assay to measure the HRP release after hydrolysis of the porphyran:protamine (POR HRP) and carrageenan:protaimine (CGN HRP) glycosidic linkages within the capsules. Two GH16 family enzymes from *B. uniformis* NP1, characterized as an agarase (GH16B) and a porphyranase (GH16C), were incubated with the two different types of capsules and the resulting supernatants were measured for amount of released HRP in the developed assay. GH16C was able to produce significant amounts of HRP as compared to the non-enzymatic control when incubated with the POR HRP capsules at two different time points (Figure 3.6A and 3.7A). The GH16B agarase was also able to release HRP, however the amount of HRP released was less than to controls, and was likely due to the substrate specificity of the agarase as the capsules contain low amounts of agarose. Although the CGN HRP capsules released larger amounts of HRP compared to the POR HRP capsules, the relative amounts of released HRP were inconsistent between the two assays with only a significant amount of HRP being released from capsules in the presence of GH16C and the protease, proteinase K. This suggests that these

enzymes were potentially hydrolysing protamine within the capsules (Figure 3.7A). The inconsistency of the CGN HRP capsule assay results were likely due to the different spatial orientations of the monosaccharides in the backbone of carrageenan and as such GH16B was unable to hydrolyse the glycosidic linkages. Furthermore, the integrity and size of the capsules were uncertain, as non-enzymatic controls released measurable HRP amounts, and there was also more HRP released from CGN capsules following incubation with enzymes. Additional experiments should include characterization of carrageenase activity on CGN capsules. This would determine the ability of the enzymes to hydrolyse the CGN HRP capsules to release cargo and evaluate capsular integrity.

In addition to the development of an assay to examine the release of encapsulated products, supernatants were also analyzed with TLC to determine carbohydrate products produced following enzymatic degradation. Both GH16B and GH16C were able to produce NAOS and NPOS on POR capsules, respectively, (Figure 3.6B and 3.7B) while no hydrolysed product was visualized from the CGN capsule incubation (Figure 3.6C and 3.7C). The oligosaccharide profiles suggest the enzymes, indeed, were capable of hydrolysing polysaccharides within the POR capsules even though HRP measurements were inconsistent. This further supported the suggestion that the integrity of the capsules was poor and therefore permeable. No carbohydrate products were detected from the CGN capsules (Figure 3.6C and 3.7C) as expected because there were no product bands observed in the initial enzyme activity assay on the carrageenan substrate (Figure 3.4). The lack of product bands, however, did not correlate to the measured amount of HRP released from the capsules. From the investigation, it appears POR capsules were better suited to deliver therapeutic agents as expression of different enzymes, not found in microorganisms of the DGM, would be needed by bacteria to hydrolyse the glycosidic linkages of the CGN capsules. In addition,

the HRP colorimetric assay may not be the best system to measure capsular integrity, as this assay is an indirect system that measures the rate of product formation by HRP, rather than the amount of product or HRP. Using a direct measurement, such as absorbance or fluorescence to measure the amount of product made or using a protein concentration assay to measure HRP concentration would be a more sensitive and accurate method to measure cargo release.

As the enzymatic digestion of the two algal polysaccharide capsules showed production of NAOS and NPOS as well as release of some HRP, bacteria present within the human DGM were also used to determine if bacteria producing glycoside hydrolases could release encapsulated cargo. *B. uniformis* NP1 was chosen as it is a human gut symbiont that expresses both GH16B and GH16C enzymes; enzymes that hydrolyse agarose and porphyran, respectively. The engineered strain of *B. theta* described in Chapter 2, was also used in this investigation, as it produces active GH16B. No significant amounts of HRP were released for *B. uniformis* NP1 cell digests of either POR HRP or CGN HRP capsules (Figure 3.8A), but NAOS product bands following incubation were observed in both porphyran and agarose enriched cell samples for POR HRP capsules (Figure 3.8C). Similar results of carbohydrate analysis were observed for POR HRP capsules incubated with engineered *B. theta* expressing GH16B (Figure 3.8B and D). The ability of the *Bt* ON-GH16 strain to hydrolyse the POR HRP capsules into oligosaccharides indicates it may be a good candidate for a drug delivery system employing porphyran-derived capsules. Importantly, this bacterium does not need the induction of agarase gene expression, as these genes are regulated by a constitutive promoter and *B. theta* is a human gut symbiont that readily colonizes within the distal GIT. Further adjustments are needed to improve utility of this novel delivery system. This would include optimization of the POR capsules to improve capsular integrity, as well as

identifying new assay systems to measure cargo release and improving the HRP assay to determine accurate release of encapsulated products.

Advancements in the understanding of processes, functions, and applications of synthetic biology in medical and biological sciences have greatly developed over the last several decades. The engineered bacterial strains from my research project are the initial steps to developing a method to improve intestinal health. In my work, I developed a synbiotic system that couples a human gut symbiont with enzymatic hydrolysis of a complex carbohydrate not present within terrestrial plants to aid in the restoration of a homeostatic and balanced DGM. In addition, and building upon this platform, engineered strains of bacteria could be used as methods to deliver therapeutic agents to the large intestine. Bacteria have been engineered to survive and colonize the distal GIT and produce targeted therapeutic or diagnostic agents in the local environment. This strategy has been shown to reduce the amount of therapeutic compounds required to reduce intestinal disease. One limitation of using bacteria to produce enzymes that release bioactive agents is the extra energy cost in producing these heterologous proteins for the bacterium while providing no added competitive advantage for the microorganism within the DGM. Coupling nutrient utilization to the release of these therapeutic agents could provide a direct benefit to the bacterial strains. The bacteria could encode heterologous genes that express enzymes that hydrolyse rare nutrients not commonly hydrolysed by other DGM bacteria and increase fitness of these bacteria within the DGM. Moreover, coupling the regulation of the production of enzymes that hydrolyse rare carbohydrates to the production of bioactive products within bacteria, could be an effective method for a tightly regulated drug delivery system.

In conclusion, I was able to engineer three transgenic agarases from *B. uniformis* NP1 into *B. theta* under the regulation of two different promoters as well as develop an assay to measure the

release of the bioactive molecule, HRP, from algal polysaccharide capsules. The engineered strains developed were able to produce functional agarases but optimization of trafficking the enzymes to the outer surface of the cells to access and fully saccharify agarose into, D-GAL and L-AHG is required. It was shown that *B. theta* was able to use the GAL product released from agarose saccharification to grow, therefore, *B. theta* has the potential of becoming an agarolytic strain that can hydrolyse agarose as a sole carbon source. Fortunately, the GH16 endo-β-agarase was located on the outer surface of the bacterium and thus able to hydrolyse agarose into NAOS. This bacterial strain was also able to produce NAOS from porphyran:protamine capsules suggesting that co-administering the engineered bacteria with the POR capsules is the initial step towards developing a drug delivery system to the distal GIT. Although further work is needed to generate better, more uniformly produced capsules, this novel system has the potential to be used as a delivery system for therapeutic agents to the distal GIT. Indeed, a therapeutic compound could be encapsulated within algal polysaccharide capsules that traverse the alimentary tract to the distal GIT, where engineered *B. theta* expressing hydrolytic enzymes within the DGM can release the therapeutic agents within a selective area of the distal GIT.

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