

**INVESTIGATION OF THE STRUCTURE AND DIGESTIBILITY OF
FLUORESCENTLY LABELED CARBOHYDRATES USING GLYCOSIDE
HYDROLASES**

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Dedication

This work is dedicated to my husband Dr. Uchenna Anele- thank you for your love, support, encouragement and understanding; my daughter Ada- ever cheerful and encouraging in your own way; as well as my mother Mrs. 'Sola Ogundimu for your encouragement, prayers and support; and my siblings- Kola and Femi for their support and encouragement as well. Thank you all for your love, for giving the tangible and intangible, anything and everything and supporting me unconditionally and unreservedly all through this program. I also dedicate this in loving memory of my father, Prof. Bamidele Ogundimu- an erudite scholar.

Above all, I dedicate this work to the Lord, my strength and ability - I cannot thank you enough. “ Now to Him who is able to do exceedingly, abundantly, more than we could ask or think according to the power at work in us, to Him be all the glory ...” (Ephesians 3:20).

I love you all.

Abstract

Developing alternatives to the use of antimicrobials is essential to maintaining the sustainability of animal production and lowering the prevalence of antimicrobial resistance. Prebiotics have not been readily adopted due to limited knowledge of the mechanisms driving prebiotic-bacteria-host interactions. Presented here are preliminary insights into the utility of three prebiotic compounds: neoagarooligosaccharides (NAOS) mannoooligosaccharides (MOS), and pectic oligosaccharides (POS). The activity of three agarases from *Bacteroides uniformis* NP1: *BuGH86* (an endolytic enzyme that produces NAOS from agarose), as well as *BuGH2C* and *BuGH117B* (exolytic enzymes that remove 3,6-anhydro-L-galactose from the non-reducing end of NAOS and D-galactose from the non-reducing end of agarooligosaccharides, respectively) were determined. NAOS, MOS and POS were then used as substrates to generate fluorescent probes for evaluating interactions with bacterial enzymes. For NAOS and MOS, the digestion of ANTS-labeled oligosaccharides was similar to that of native versions; however, digestion of ANTS-labeled POS was considerably hindered.

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List of Abbreviations

AGP- Antimicrobial growth promoter
ANTS- 8-Aminonaphthalene-1,3,6-trisulfonic acid
AOS- agarooligosaccharide(s)
BtGH76- enzymes of the glycoside hydrolases GH76 of *Bacteroides thetaiotaomicron*
BtGH125- enzymes of the glycoside hydrolases GH125 of *Bacteroides thetaiotaomicron*
BT3781- exolytic cell periplasm *BtGH125s*
BT3782- endolytic cell periplasm *BtGH76s*
BT3792- endolytic cell outer membrane *BtGH76s*
BuGH2C- enzymes of the glycoside hydrolases GH2 of *Bacteroides uniformis* NP1 origin
BuGH86- enzymes of the glycoside hydrolases GH86 of *Bacteroides uniformis* NP1
BuGH117B- enzymes of the glycoside hydrolases GH117 of *Bacteroides uniformis* NP1
DP- Degree of polymerization
FACE- Fluorophore assisted carbohydrate electrophoresis
Gal- D-galactose
GalA- galacturonic acid
GalA₂- digalacturonic acid
GalA₃- trigalacturonic acid
GH- Glycoside hydrolase(s)
HG- Homogalacturonan
IMAC- Immobilized metal ion affinity chromatography
Kan- Kanamycin
LB- Luria Broth
LMP- Low melting point agarose
LS- Loading sample
MALDI-TOF- Matrix assisted laser desorption/ionization-time of flight mass spectrophotometry
MOS- Mannoooligosaccharide(s)
NAOS- Neoagarooligosaccharide(s)
NRE- Non reducing end
OD- Optical density
POS- Pectic oligosaccharide(s)
RE- Reducing end
RhGH28- enzymes of the family GH28 of *Rhizopus sp.* origin
SDS-PAGE- Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sus- Starch Utilization System
TLC- Thin layer chromatography
3,6-LGal- 3,6-anhydro-L-galactose
YeGH28- enzymes of the family GH28 of *Yersinia enterocolitica* ATCC 9610D

Chapter 1

Literature review

1.1 Antimicrobial use in livestock production

Since the 1950's, antimicrobial agents have been used widely in livestock production for therapeutic use, prevention of infections, and growth promotion [1]. Recently, the use of antimicrobial agents (which consist of antibiotics, antivirals and antifungals of both synthetic and natural origin) has become a public health concern, particularly because of the documented increase in resistance genes occurring in microbes, which are found in environmental reservoirs such as livestock [2, 3]. For example, as a result of the broad use of fluoroquinolones, tetracyclines and cephalosporins there has been an increase in the reported prevalence of bacteria resistant to these antimicrobial agents, such as *Salmonella enterica*, *Staphylococcus aureus*, *Neisseria gonorrhoeae*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Mannheimia haemolytica* and *Campylobacter jejuni* [4-8]. Antibiotic resistance in bacteria can be acquired by horizontal gene transfer or *de novo* mutation. Horizontal gene transfer usually results from genes associated with efflux pumps, drug modification and target protection [9]. *De novo* mutations typically lead to gene amplification, alterations in the antibiotic target, reduced expression of the target, alteration of drug modification enzymes and increases in drug efflux [9].

The World Health Organization stated its objections to the administration of antibiotics as growth promoters and later provided a structure for cautious use of antibiotics for agricultural purposes [10]. The Food and Drug Administration has amended the veterinary feed directive regulation, which now dictates that, the sub-therapeutic administration of antibiotics for animal production is to be phased out and veterinary

oversight of antibiotics that are prescribed for therapeutic purposes is to be phased in for the US beef industry [11] and livestock production in North America [12, 13]. Similarly in Canada, there has been progress in the elimination of antibiotic growth promoters with relevance to human medicine and increased regulation of veterinary drugs [14, 15]. The risks of developing resistance from the sub-therapeutic application of antibiotics are thought to be higher than the risks emanating from therapeutic antibiotic use because of their prolonged usage at low concentrations [16]. Animal husbandry is a major contributor to the increase in antimicrobial resistance worldwide [17]. Not all countries have a legislative framework to control the misuse of antibiotics in agriculture. In Canada, these regulations are lagging partly because there is a lack of suitable alternatives to antimicrobial growth promoters (AGPs). Consequently, there is a sense of urgency for the development of economically competitive alternatives to AGPs that do not compromise end-product quality. The effects of administering alternatives to AGPs have been inconsistent [18-20] and research on finding alternatives in cattle production continues to get traction with increasing public concerns regarding the use of antimicrobials in livestock production [5, 21]. While there has been substantial research exploring the potential of many alternative AGPs, such as probiotics, prebiotics, and synbiotics, the mechanisms by which these compounds function are not well understood.

1.2 Probiotics, Prebiotics and Synbiotics

1.2.1 Probiotics

Probiotics are live organisms that provide health benefits to the host [22]. For a live organism to be considered a probiotic it must be non-pathogenic, resistant to stomach acids and bile, free of antibiotic resistance genes or have reduced gene transfer

functionality, and be able to release nutrients to the host [23]. Some of the most commonly used probiotic microorganisms are *Lactobacilli* (such as *Lactobacillus plantarum*, *Lactobacillus acidophilus*, and *Lactobacillus bulgaricus*) and *Bifidobacteria* (such as *Bifidobacterium bifidum* and *Bifidobacterium infantis*) [22, 24]. *Lactobacilli* are gram-positive non-spore forming rod-like bacteria [25]. *Bifidobacteria* are gram-positive, anaerobic, rod-like bacteria with a Y- or V-shaped cell morphology [26]. Some of the mechanisms by which probiotics are believed to elicit a health effect in the host include competitive exclusion of pathogens (e.g. block adhesion sites or compete for nutrients), inhibition of pathogens (by production of metabolites such as lactic acid and bacteriocins), immune modulation and reduced inflammation, maintenance of normal large intestine pH, enhancement of bowel motility, cholesterol lowering, suppression of toxin production, and detoxification of dietary carcinogens [27].

1.2.2 Prebiotics

Foods that are recalcitrant to digestive enzymes of host origin, and which selectively stimulate the growth and activity of one or more beneficial bacteria species thus effecting health benefits to the host are known as prebiotics [22, 28, 29]. Such compounds include dietary fibres (e.g. inulin) and oligosaccharides (e.g. fructooligosaccharides, galactooligosaccharides, and maltooligosaccharides) [23, 30].

Prebiotic applications and efficacy varies between monogastric and ruminant animals. Since ruminants possess digestive compartments prior to the large intestine, prebiotics are likely to be chemically modified and / or fermented by the complex microbial community in the rumen. These transformations may influence the downstream impacts that a prebiotic may have on the large intestine microbiota. Therefore, further insight into the

spatial and temporal relationships that exist between prebiotics, probiotics and the host microbial community is needed. In particular defining the mechanisms that drive these interactions, is required to inform the selection or design of next-generation prebiotics that will influence bacteria community dynamics in different livestock.

1.2.3 Synbiotics

Synbiotics are the combined use of prebiotics and probiotics to promote “synergistic” health benefits to the host. Typically, synbiotics aim to increase the survival and colonization of proven probiotics *in vivo*, as well as stimulating indigenous bacterial species [22, 28]. Studies have shown that synbiotics exhibit synergistic effects, such as fostering the rapid growth of beneficial bacteria and boosting the perpetuation of newly introduced probiotics in non-ruminants [31] and ruminants [32]. For example, a combination of a prebiotic (Celmanax: manooligosaccharides (MOS) from *Saccharomyces cerevisiae*) and probiotic (DairyMan’s Choice: *Bacillus subtilis*, *Bacillus licheniformis*) has been shown to eliminate morbidity and mortality losses resulting from Shiga toxin-producing *Escherichia coli* infections in dairy calves [33]. A second example reported that cellooligosaccharides or a combined use of dextran and *Lactobacillus casei* ssp. *casei* led to increased weight gain and feed efficiency in dairy calves [34]. Despite the demonstration of the beneficial effects of synbiotics, the mechanisms by which synbiotics function within the complex ecosystem of the host large intestine remains to be established. In particular, more research is required to define how prebiotic-probiotic-host interactions occur.

1.3 Microbial communities in the digestive system

Humans are host to diverse and populous communities of microorganisms in the large intestine. Microbial load in the proximal large intestine has been quantified with numbers as high as 10^{11} microorganisms per ml, and metagenomes have been described that consist of 2 to 4 million genes [35]. Although there is marked diversity in the microbiota at the species and strain levels between individuals, more than 90% of the sequenced bacteria in human, equine, porcine large intestines and rumen in ruminants belong to the Bacteroidetes (e.g. *Bacteroides thetaiotaomicron*, *Bacteroides ovatus*, and *Bacteroides cellulosilyticus*) and Firmicutes (e.g. *Roseburia intestinalis*, *Roseburia inulinivorans*, *Ruminococcus bromii*, and *Eubacterium rectale*) phyla [36-40]. Other less abundant phyla present in the human large intestine include Actinobacteria, Verrucomicrobia and Proteobacteria [40]. In ruminants, the predominant bacteria in the rumen are the Bacteroidetes (e.g. *Prevotella ruminicola* and *Prevotella bryantii*), Firmicutes (e.g. *Butyvirio fibrisolvens*, *Ruminococcus flavefaciens*, *Ruminococcus albus*, and *Eubacterium cellulosolvens*), Proteobacteria (e.g. *Enterobacter cloacae*) and *Fibrobacter* (e.g. *Fibrobacter succinogens* and *Fibrobacter intestinalis*) [36, 41].

1.4 Bacteroides spp.

The *Bacteroides* genus contains anaerobic, non-spore forming gram negative bacilli. These microorganisms are most often mutualistic intestinal bacteria that maintain a complex and beneficial relationship with their hosts [42]. Along with other members of the large intestinal microbiota, *Bacteroides* spp. metabolize recalcitrant dietary carbohydrates for their own nutrition. In this regard, *Bacteroides* spp. commonly possesses an impressive toolkit of carbohydrate active enzymes (CAZymes) that release

simple sugars from complex glycans. These pathways have been described to be “selfish” (*Bacteroides thetaiotaomicron* and yeast mannan utilization) [43, 44] or distributive (*Bacteroides ovatus*, *Bifidobacterium adolescentis* and a number of Bacteroidales) that result in the release of “public goods” [44-46]. This research project investigates the use of enzymes from two species of *Bacteroides*- *Bacteroides thetaiotaomicron* and *Bacteroides uniformis* to generate oligosaccharides that will be fluorescently labeled in order to develop fluorescent bioactive probes.

1.4.1 *Bacteroides thetaiotaomicron*

Bacteroides thetaiotaomicron is a member of the human large intestine (others include *Bacteroides ovatus*, and *Bacteroides cellulosilyticus*) community and is proficient at utilizing a wide array of glycans [47]. This metabolic ability enables *B. thetaiotaomicron* to occupy numerous “nutrient niches”, a strategy that classifies it as a ‘generalist’ [44, 48]. A hallmark of generalists is that their genome contains a large number of diverse genes involved in the metabolism of carbohydrates. These genes can be involved in sensing, transporting, and modifying carbohydrates [48]. The genes involved in carbohydrate modification, otherwise referred to as “carbohydrate active” are crucial for the metabolism of defined species of carbohydrates. For example, the genome of *B. thetaiotaomicron* VPI-5482, contains 271 glycoside hydrolases (GHs), 91 glycosyltransferases (GTs), 18 polysaccharide lyases (PLs), 20 carbohydrate esterases (CEs) and 31 carbohydrate binding modules (CBMs) [49]. Alternatively, nutritional ‘specialists’ are more selective in their substrate utilization, and correspondingly, have more limited enzyme resources for harnessing energy from carbohydrates [48]. Intriguingly, there can also be an overlap in the nutritional pathways of related and

unrelated species [50]. Therefore, further understanding of how carbohydrate active enzymes (CAZymes) and transporters are regulated and function in the metabolism of carbohydrates is required. This will enable informed steps taken in the direction of creating ‘designer prebiotics’ tailored to fit different livestock digestive systems for a desired health benefit.

1.4.2 *Bacteroides uniformis*

Bacteroides uniformis (*B. uniformis*) is a member of the human large intestine microbial community with the ability to utilize dietary fibres and glycans such as inulin, fructan, and xyloglucan [40, 45, 51, 52]. The utilization of marine polysaccharides by *B. uniformis* has not been intensely investigated and has only recently generated research interest as reported in the strains *B. uniformis* L8 [53] and *B. uniformis* NP1 [54].

B. uniformis NP1 is a member of the human large intestine microbiota which has the ability to grow on the marine polysaccharides agar and agarose [54]. In previous work, a pathway containing a cluster of genes believed to be involved in agarose metabolism (GH86, GH117, GH2) was identified [54]; unpublished work; see Appendix 1). Investigating relationships between carbohydrate structure and the enzymes that modify them will be central to establishing the role of *B. uniformis* NP1 mediated agarolysis *in vivo*.

1.5 Carbohydrates

The major classes of carbohydrates include monosaccharides, oligosaccharides and polysaccharides [55]. Monosaccharides are single sugar aldehyde or ketone derivatives of straight chain alcohols, which contain at least three carbon atoms. Examples are D-glucose the repeating units of cellulose, D-galactose (Gal) (Figure 1.1A) and 3,6-anhydro-

L-galactose (3,6-LGal) (Figure 1.1B), the repeating subunits of agarose (Figure 1.1C); D-mannose (Figure 1.1D), the primary repeating subunit of yeast mannan (Figure 1.1E); and D-galacturonic acid (GalA) (Figure 1.1F), the repeating subunit of the homogalacturonan (HG) (Figure 1.1G). Oligosaccharides (i.e. 'few') and polysaccharides (i.e. 'many') are combinations of monosaccharides that are covalently linked by condensation reactions forming glycosidic bonds. Polysaccharides can either be branched such as in yeast mannan or linear such as in agarose and HG.

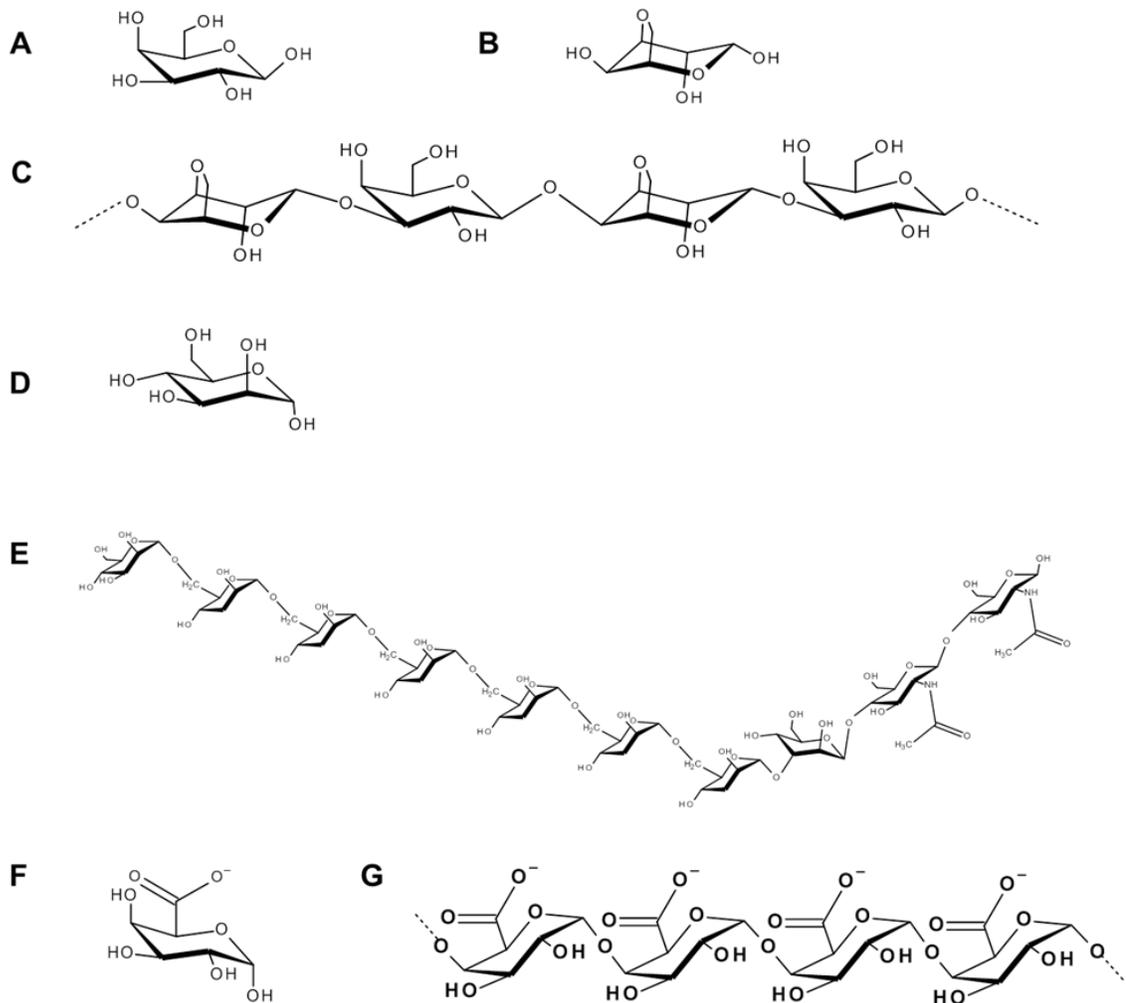


Figure 1.1: Schematic depiction of the relationship between monosaccharides and polysaccharides. (A) D-galactose (B) 3,6-anhydro-L-galactose (C) Agarose, a polysaccharide made up of repeating units of the monosaccharides in **A** and **B** (D) D-mannose (E) α 1,6 yeast mannan backbone, a polysaccharide made primarily of units of D-mannose and N-acetylglucosamine at the core (F) D-galacturonic acid (G) homogalacturonan, a polysaccharide made up of repeating units of D-galacturonic acid.

Many plant cell wall polysaccharides and oligosaccharides and storage polysaccharides cannot be digested by host enzymes. These sources of carbohydrates, referred to as dietary fibre, represent nutrients for chemoorganotrophic members of the large intestine microbiota [36]. Simple sugars released during these reactions are fermented into small chain fatty acids (e.g. acetate, propionate, butyrate) [56]. In humans this symbiosis accounts for approximately 10% of their daily caloric uptake [57]. In ruminants, this relationship is even more critical for host nutrition as 70% of energy derived from diet depends on symbiotic carbohydrate deconstruction and fermentation [58].

1.6 Polysaccharide Utilization Loci (PUL)

In Bacteroidetes, dietary and host glycan metabolism is performed by clustered pathways called Polysaccharide Utilization Loci (PULs) [59]. PULs encode functions for sensing, transporting, and degrading carbohydrates; and are found in Bacteroidetes that colonize environments as diverse as the large intestine, ocean and soil [54]. The model system for studying PUL function is the *B. thetaiotaomicron* starch PUL. In the presence of starch, the single eight-gene locus *susRABCDEFG* encodes factors for binding and saccharifying into oligosaccharides which are transported into the periplasm for further degradation into simpler sugars [60] (Figure 1.2).

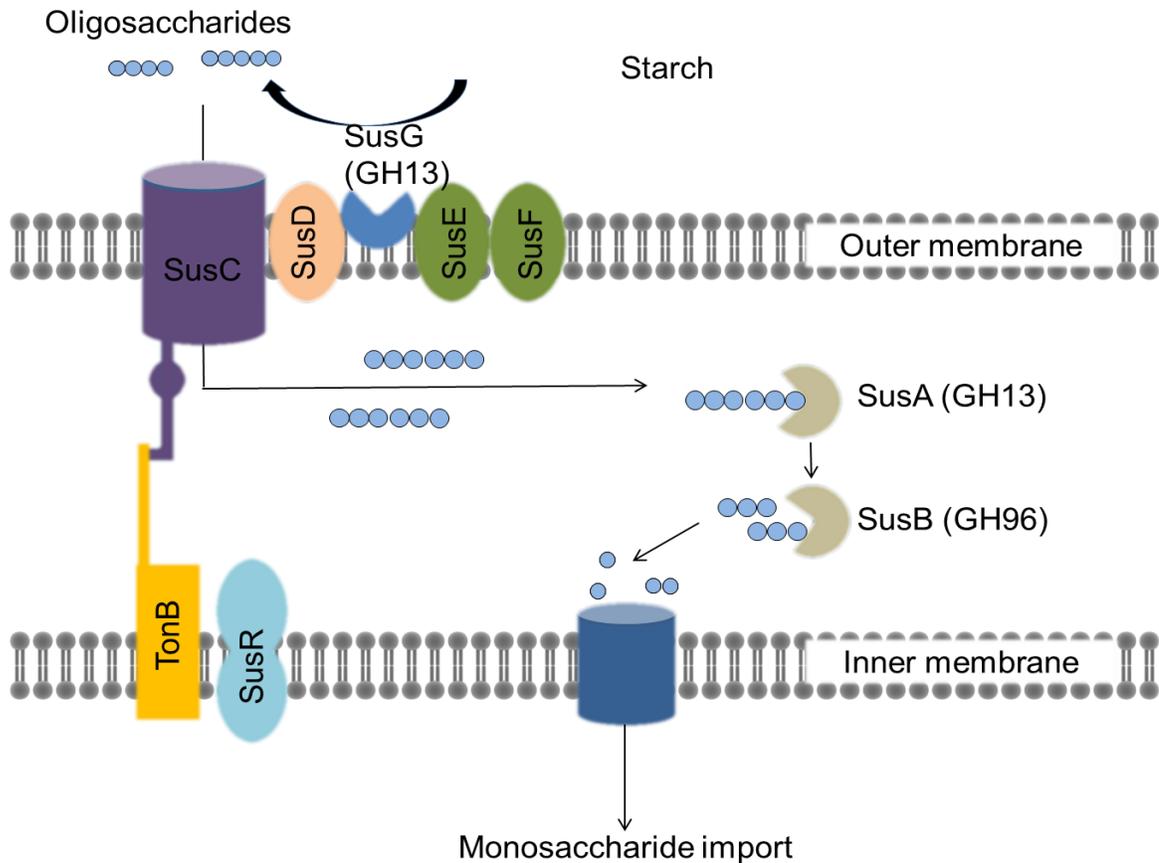


Figure 1.2 Cartoon representation depicting the starch utilization system (Sus) from *B. thetaiotaomicron*. SusD, SusE and SusF initiate starch binding after which SusG initiates degradation. The released extracellular oligosaccharides are then transported into the periplasm through the SusC outer membrane channel in a TonB-driven reaction. Maltooligosaccharides are further cleaved into glucose by the SusA and SusB. SusR, the inner-membrane-spanning-regulator senses the release of maltose in the periplasm and upregulates the expression of other Sus pathways. Glucose is imported into the cell through an unidentified inner membrane permease. While the SusC-SusD pair is the hallmark of a Sus-like system, the architecture of individual PULs can be diverse, including SusE, SusF and specialized enzymes (figure adapted from [60]. Stock images taken from www.somersault1824.com and modified for use).

The current hypothesis is that an individual PUL encodes a pathway that is specific for the metabolism of a single glycan or a group of related glycans [50]; however, this may not be a universal principle as highly branched (e.g. yeast mannan) or chemically complex carbohydrates (e.g. rhamnogalacturonan-II) have been linked to activities encoded within multiple PULs [43, 50].

1.7 Glycoside Hydrolases

The hydrolysis of glycosidic bonds between two or more carbohydrates, or a carbohydrate and non-carbohydrate component leading to the generation of a new reducing end and the corresponding aglycon (leaving group) is catalyzed by enzymes known as glycoside hydrolases (Figure 1.3A) [61]. Hydrolysis is catalyzed by two amino acid residues of the enzyme, namely a general acid, which is the proton donor, and a nucleophile/base. The reaction can occur with either inversion (Figure 1.3B) or retention (Figure 1.3C) of the anomeric configuration at C1 depending on the spatial position of the catalytic residues at the active site [61].

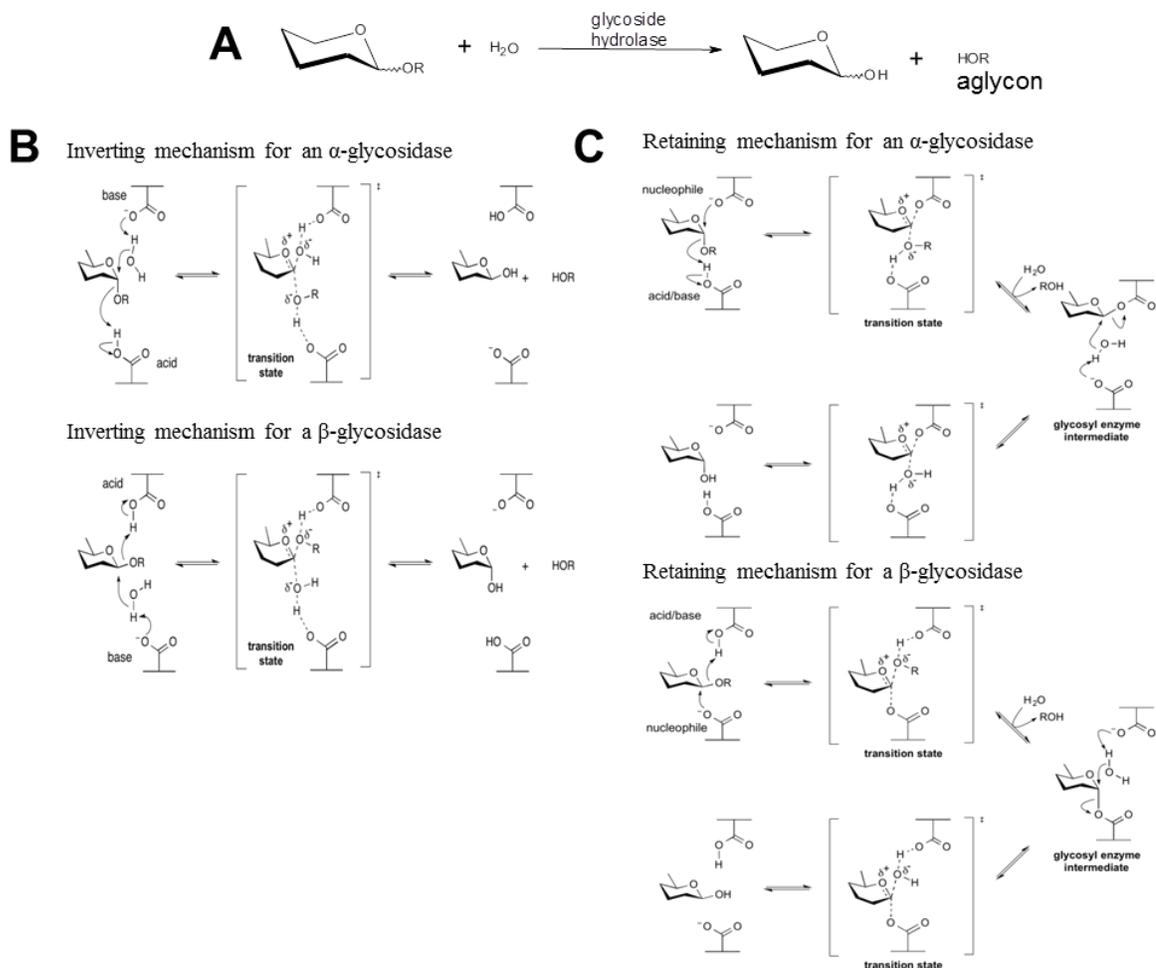


Figure 1.3: Schematic depiction of glycoside hydrolases. (A) General model of hydrolysis by glycoside hydrolases (B) catalytic mechanism for double displacement retaining mechanism (C) catalytic mechanism for single displacement inverting mechanism (Source: [61]; permission taken for use of figures).

The carboxylic acid residues are commonly located either ~5.5 or ~11 Å apart, for retaining or inverting enzymes respectively [62]. Both of these mechanisms involve oxocarbenium ion-like transition states [63]. Hydrolysis resulting in inversion of the anomeric configuration (i.e. $\alpha \rightarrow \beta$ or $\beta \rightarrow \alpha$) is carried out by inverting glycoside hydrolases. Inverting glycoside hydrolases carry out hydrolysis of the glycosidic bond by a single-displacement mechanism in one step (Figure 1.3B) involving a general acid and general base [62, 63]. Retaining glycoside hydrolases carry out hydrolysis through a double-displacement mechanism in two steps (Figure 1.3C) where one residue acts as a general acid and general base whilst the other residue acts as the nucleophile [62]. Products of these reactions maintain the anomeric configuration of the substrate (i.e. $\alpha \rightarrow \alpha$ or $\beta \rightarrow \beta$). Hence the starting substrate before glycosidic hydrolysis has the same anomeric configuration as the final product.

Glycoside hydrolases are classified into families based on amino acid sequence similarity, conservation of catalytic machinery, and structural fold [49]. Glycoside hydrolases of a particular enzyme family can be abbreviated based on the details of the family they originate from and/or the bacteria species. For example, glycoside hydrolases of family 86 can be described as GH86, and GH86 enzymes originating from *B. uniformis* can be described in the short form *BuGH86*.

1.8 Pathways for the Controlled digestion of Agarose, Yeast Mannan, and Homogalacturonan Modification

Industry uptake of alternatives to AGPs, such as prebiotics, has been hampered by inconsistent results [18, 20] and perhaps more importantly, because the mechanisms of prebiotic-probiotic-host interactions are not well understood. To address these issues,

prebiotic carbohydrates with known structures can be generated by enzymes to help define interactions with responsive bacteria. Such products represent next-generation prebiotics as new tools for research applications. Towards this end, three promising prebiotic sources: agarose, HG and yeast mannan, have been selected as candidates for generating defined oligosaccharides by enzymatic digestion. The structures of these carbohydrates and the enzymes used to generate them will be introduced in the following sections.

1.8.1 Agarose

1.8.1.1 Agarose structure

Agarose is a polysaccharide found in the cell wall of marine red algae such as *Gracilaria* spp. and *Gelidium* spp.[54, 64]. Unlike crystalline polysaccharides common in terrestrial plants, polysaccharides such as agarose found in the cell walls of marine macroalgae form flexible gels which are crucial for preventing the desiccation of the plant during periods of dehydration as well as providing support to withstand the mechanical turbulence of waves and tides [65]. Agarose is a neutral sugar comprised of a linear chain with alternating β -1,3- Gal and α -1,4-3,6-LGal units [53, 66] (Figure 1.4A-D). Individual chains form a parallel double helix ultrastructure, which is stabilized by hydrogen bonds [54, 65, 66]. The bridged bicyclic 3,6-LGal is particularly crucial to this helix formation, and is ultimately responsible for the high gel strength of agarose [65]. This attribute of agarose has wide applications in microbiology, molecular biology and the food industry [67]. In molecular biology laboratories, low melting point agarose and solid agarose (also known as normal melting point agarose or standard agarose) are commonly used. Low melting point agarose has increased methylations, which lower the melting and gelling

points of agarose. This increases the ease of manipulations of agarose for experiments requiring lower temperature ranges (e.g. for 1.5% concentrations of low melting point agarose, the gelling point is at 24-28 °C and melting point at ≤ 65 °C). The temperature ranges for solid agarose are higher (e.g. for 1.5% concentrations of agarose, gelling point is at 36-39 °C and melting point at ≥ 87 °C).

1.8.1.2 Agarases

Agarases can act on agarose in an exolytic or endolytic manner (Figure 1.4). Endolytic enzymes cleave the polysaccharide indiscriminately along the length of a polysaccharide; whereas, exolytic enzymes cleave exclusively at the termini of polysaccharide chains to release products of a uniform size. The alternating linkages between the core repeating disaccharide subunits within agarose require, at minimum, two different enzyme activities to saccharify agarose. β -agarases cleave β -linkages to release neoagarooligosaccharides (NAOS), products, which are defined by having 3,6-LGal at their non-reducing end (Figure 1.4E). β -agarases belong to GH50, GH16, GH86 and GH118 [65]. GH16 and GH86 agarases carry out hydrolysis of β 1,4 linkages in agarose by a retaining mechanism releasing a tetramer as the predominant product [49]. Agarases belonging to GH2 are β -galactosidases, which carry out exolytic hydrolysis via a retaining mechanism [49].

α -Agarases belong to families GH96 and GH117 and release agarooligosaccharides (AOS), which are characterized by a Gal at their non-reducing end (Figure 1.4F). Family GH96 is a small family of endolytic α -agarases, whereas, GH117 enzymes are the only known enzymes to release a single 3,6-LGal unit from NAOS [65]. The mechanism by which GH96 and GH117 carry out hydrolysis is not yet confirmed experimentally; however, GH117 is believed to use an inverting mechanism [65].

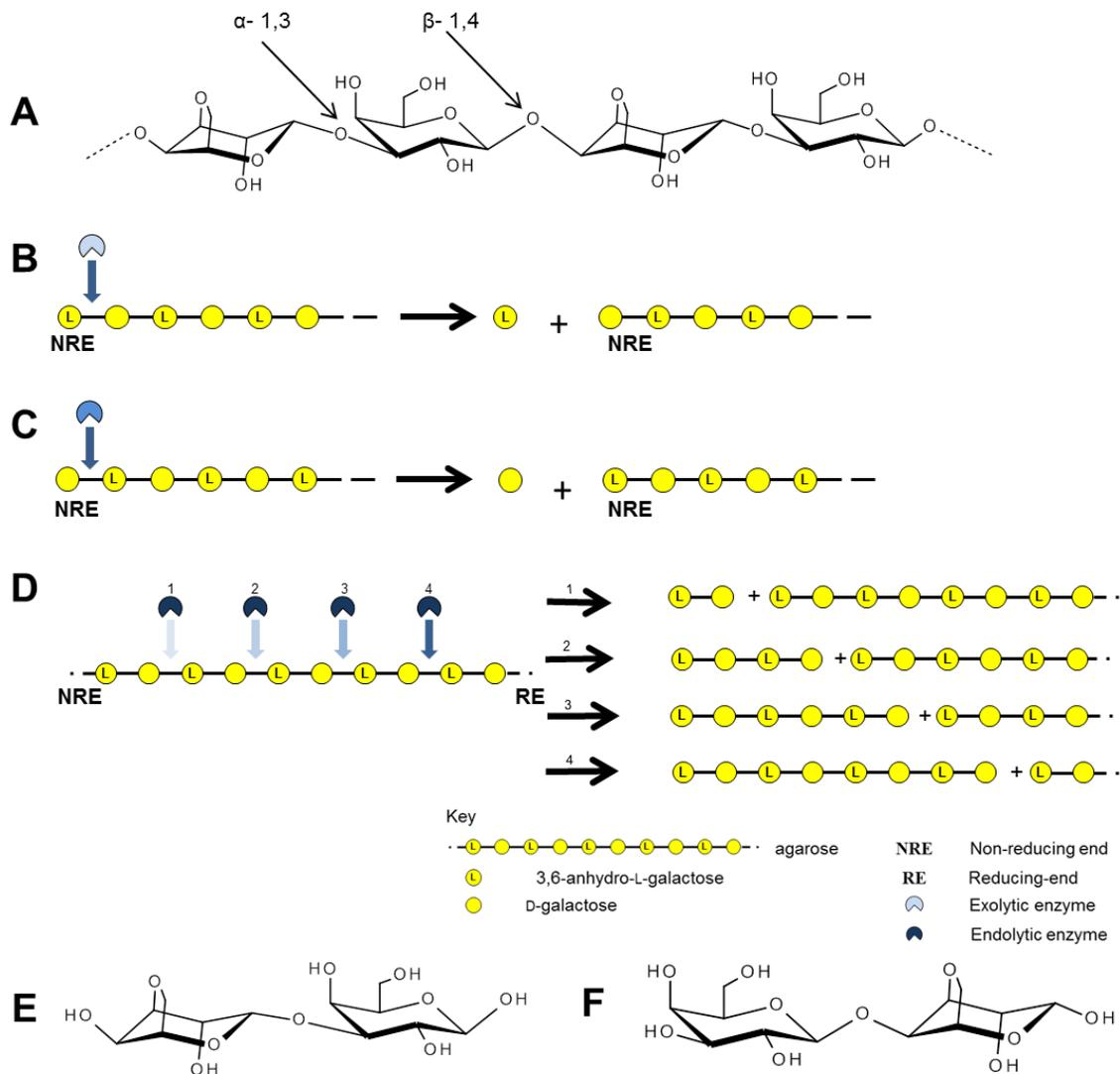


Figure 1.4: Agarose structure showing the predicted activity of exolytic and endolytic agarases. (A) Agarose polysaccharide and its two different linkages. (B) Exolytic cleavage of 3,6-anhydro-L-galactose from agarose by GH117 enzyme at the non-reducing end (C) Cleavage of D-galactose from agarose by exolytic agarases at the non-reducing end (D) Reactions 1, 2, 3 and 4 show possible enzymatic reactions of endolytic agarases and their resulting end products (E) Neoagarobiose with 3,6-anhydro-L-galactose at the non-reducing end (F) Agarobiose with D-galactose at the non-reducing end.

In this research, one predicted endolytic β -agarase (*BuGH86*) cloned from *B. uniformis* NP1 will be used to generate neoagarooligosaccharides (NAOS). Also, two predicted exolytic agarases (*BuGH2C* and *BuGH117B*) cloned from *B. uniformis* NP1 will be used to generate monosaccharides sequentially from the non-reducing end of NAOS. *BuGH2C*

is a predicted β -galactosidase while *BuGH117B* is a predicted α 1,3-L-neoagarooligosaccharide hydrolase.

1.8.1.3 Neoagarooligosaccharides as prebiotics

NAOS are highly resistant to enzymes of the upper gastrointestinal tract making them excellent candidates as prebiotics [68]. These have been found to be favourable for the growth of *Bifidobacteria* and *Lactobacilli* in mice [68]; however, the genomes of these two genera are not known to contain agarases, and they have not been determined to be agarolytic. It is likely, that these observations may result from syntrophic effects with agarolytic intestinal bacteria, or inhibitory effects of NAOS on competing species. These conclusions necessitate the further study of the interactions between agarose and intestinal agarolytic bacteria.

1.8.2 Yeast mannan

1.8.2.1 Yeast mannan structure

Yeast mannan is an extracellular cell wall polysaccharide found in yeasts such as *Saccharomyces cerevisiae* (Figure 1.5A), *Candida albicans* (Figure 1.5B) and *Schizosaccharomyces pombe* (Figure 1.5C) [43]. It is comprised of an α 1,6-mannan backbone that is decorated by side-chains that display species-specificity [44] (Figure 1.5D). For example, in *S. cerevisiae*, side-chains consist of α 1,2-linked mannosyl side-chains that are capped with α 1,3-linked mannose residues. Some side-chains can be further branched through phosphodiester linkages. Intriguingly, some MOS decorations have shown to be immunogenic, and are linked to the exacerbation of intestinal inflammatory disease in humans [44].

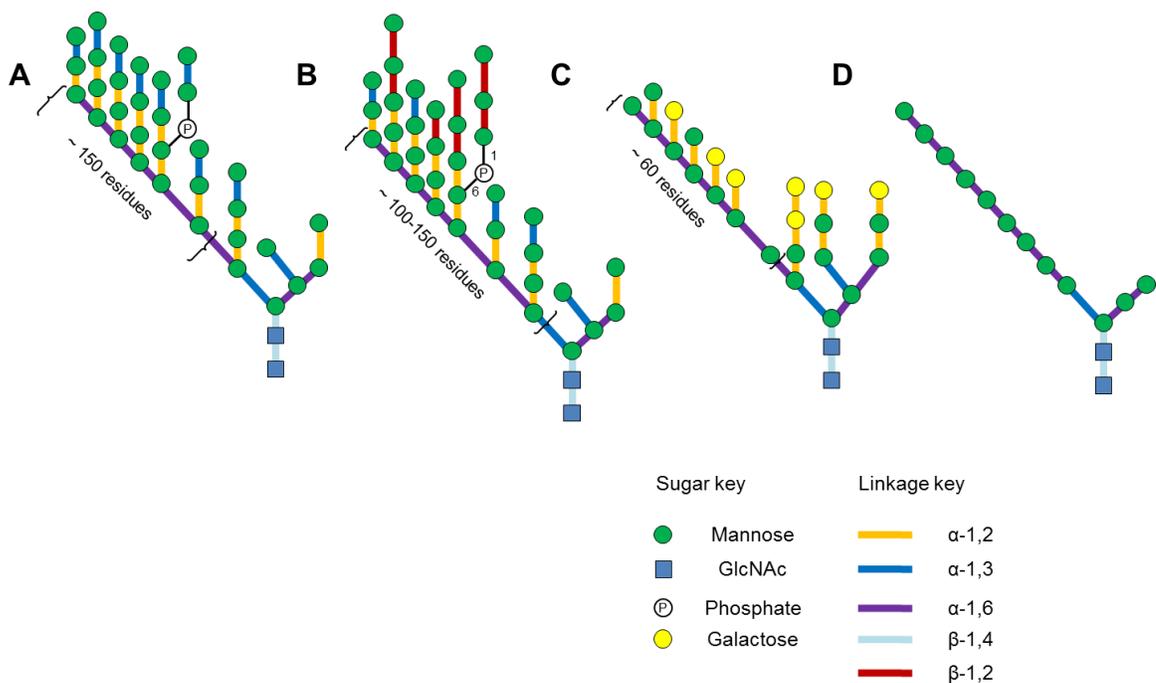


Figure 1.5: Cartoon representation showing various yeast mannan structures, differing in sugar composition, spatial arrangement of sugars and sugar linkages and the α 1,6 yeast mannan backbone. (A) *S. cerevisiae* yeast mannan (B) *C. albicans* yeast mannan and (C) *S. pombe* yeast mannan. (D) α 1,6 yeast mannan backbone produced by *S. cerevisiae* mannan mutant 2 strain (figures adapted from [43]).

1.8.2.2 Yeast mannan hydrolytic enzymes

Deconstruction of a highly complex polysaccharide such as *S. cerevisiae* yeast mannan requires the combinatorial action of many different enzymes, including α -mannanases, α -mannosidases, and sugar phosphatases. During metabolism of yeast mannan by *B. thetaiotaomicron*, the α 1,6-mannan backbone is made more accessible to *B. thetaiotaomicron* by the sequential action of surface enzymes that process the large substrate into smaller branched fragments that are imported into the periplasm [43]. The periplasm is the primary site of saccharification as α 1,2-mannosidases, α 1,3-mannosidases, mannose-6-phosphatases, endolytic α 1,6-mannanases and the exolytic

α 1,3-mannosidases and α 1,6-mannosidases convert imported substrates into mannose [43]. To simplify the purification of α 1,6-mannooligosaccharides (MOS) from yeast mannan, two endolytic *Bt*GH76 α 1,6-mannanases, (BT3782 and BT3792) and one α 1,6-mannosidase (BT3781) from this pathway will be used in this research. Although both perform hydrolysis via a retaining mechanism, they yield different sized products [43]. The BT3781 α 1,6-mannosidase, a member of GH family 125, releases mannose from the non-reducing end of α 1,6-MOS using an inverting mechanism [43, 69].

1.8.2.3 Mannooligosaccharides (MOS) as prebiotics

Yeast mannan is one of the most studied sources of prebiotics for livestock. Yeast mannan products have been shown to improve nursery performance in supplemented pigs [70] and weight gain and dry matter intake in dairy and beef cattle [71, 72]. Intriguingly, MOS bolsters the immune responses of piglets [73] and cows to rotavirus [74]. Also, MOS is believed to prevent adhesion and effacing of pathogens to the large intestine epithelium thereby improving the integrity of the large intestine mucosal layer [75]. In order to generate pure MOS at high yields, α 1,6-MOS will be generated from the debranched backbone of yeast mannan (Figure 1.5D).

1.8.3 HG and Pectic polysaccharides

1.8.3.1 Pectic polysaccharide structure

Plant cell wall polysaccharides are essential for plant growth, development and structure. Both primary and secondary walls of the plants are composed of structural polysaccharides such as cellulose, hemicellulose and pectin [76]. Pectin is the most complex carbohydrate found in nature having a high GalA content [77] (Figure 1.1G).

This polysaccharide interlocks the structural fibres in the primary cell wall and is also present in the interstitial space of the middle lamella of terrestrial plants [78]. HG accounts for more than 60% of pectin and possesses the simplest structure [77, 79].

1.8.3.2 Pectinases

Pectinolytic bacteria often possess multiple copies of pectinases suggesting differential regulation and preferential activity on heterogeneous substrates, which vary in degree of polymerization, esterification and carbohydrate composition [77]. The enzymes capable of cleaving glycosidic linkages in pectic polysaccharides by hydrolysis are referred to as polygalacturonases and belong to GH family 28. GH28 enzymes hydrolyze their substrates via a single displacement mechanism inverting the anomeric carbon from the alpha to beta configuration [77]. This study will use two GH28 enzymes, including a commercial endolytic polygalacturonase from *Rhizopus* sp and an exopolygalacturonase from *Yersinia enterocolitica* ATCC 9610D (*Y. enterocolitica*) [80]. The endolytic GH28 (*RhGH28*) from *Rhizopus* sp indiscriminately hydrolyzes α 1,4 galacturonyl linkages in HG to generate pectic oligosaccharides (POS). The exolytic GH28 enzyme from *Y. enterocolitica* (*YeGH28*) releases digalacturonic acid (GalA₂) from POS and HG [80].

1.8.3.3 Pectin and POS as prebiotics

Pectin is a component of dietary fibre and transits to the large intestine where it is depolymerized and fermented by resident bacteria [81]. Due primarily to its gel-forming and water holding capacity, diets rich in pectin lead to delayed gastric emptying, hypocholesteromic effects, and interaction with the intestinal metabolism of ions [81]. Pectic oligosaccharides (POS) have been suggested as candidate prebiotics [82] which can stimulate the bloom of *Bifidobacteria* and inhibit the growth of pathogenic bacteria

[81, 83]. Pectin and POS have not been widely studied as prebiotics in livestock, thus necessitating more extensive studies in this regard.

1.9 Labeling and fluorophore-assisted carbohydrate electrophoresis

Currently, the study of how prebiotics modulate intestinal communities uses indirect methods to correlate changes in community structure. Although these approaches can provide insight to enhancing host health and production, they are limited in their scope and ability to define mechanisms of function. One of the objectives of this research is to provide tools that will substantiate health claims through empirical validation of prebiotic-probiotic interactions. In this regard, the primary goal of my research is to develop labeled carbohydrates with defined structures to be used as probes in bacterial communities. To enhance sensitivity for detection of an oligosaccharide, fluorescent labeling can be carried out by tagging of an aromatic amine at the reducing end of the oligosaccharide (Figure 1.6) [84].

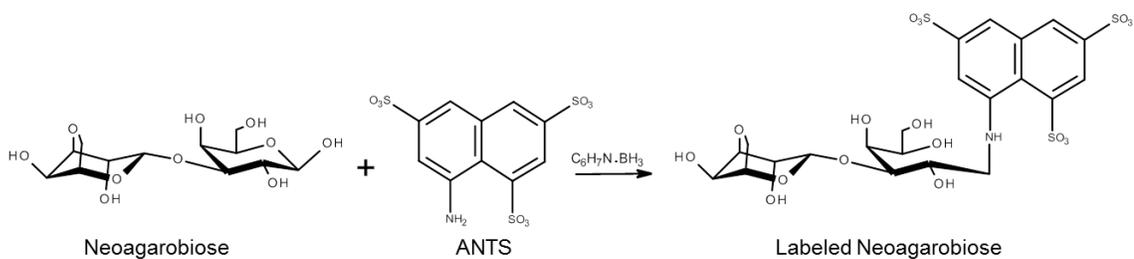


Figure 1.6: Labeling of NAOS with ANTS. ANTS is covalently added to the reducing end of neogaroibiose in the presence of a reducing agent (e.g. 2-picoline borane).

In addition to increased sensitivity of detection, fluorescent approaches have the advantage of being non-invasive [85]. Additionally, fluorescent intensity and decay can also be used as a tool to evaluate biological activity of bacteria with labeled

oligosaccharide [85]. In this research the retention of bioactivity on fluorogenic carbohydrates will be assessed using diagnostic glycoside hydrolases. Fluorescence will be detected or measured using techniques, including fluorophore assisted carbohydrate electrophoresis (FACE) and fluorescent microscopy. FACE is a straightforward, sensitive, polyacrylamide gel electrophoresis method useful for characterizing and determining the presence and abundance of oligosaccharides in a sample using low concentrations of the oligosaccharide and obtaining high quality resolution [84].

1.10 Hypotheses

The hypotheses are:

- i. Oligosaccharides (i.e. NAOS, MOS, and POS) can be fluorescently labeled with ANTS
- ii. Exolytic enzymes will retain their bioactivity on fluorophore labeled carbohydrate products generated by the enzymatic digestion of agarose, α 1,6-mannan, and HG

1.11 General objective

The general objective of this study was to:

Develop ANTS-labeled carbohydrates to be used as probes for detecting and characterizing carbohydrate-bacteria interactions.

1.11.1 Specific objectives

In order to carry out the above general objective, the following objectives were specifically taken:

- i. Production and purification of glycoside hydrolase enzymes (GH) from the following classes: *BuGH86* (to produce NAOS), *BtGH76* (to produce MOS), and *RhGH28* (to produce POS).
- ii. Production of NAOS, MOS, and POS.
- iii. Fluorescent labeling of NAOS, MOS, and POS digests.
- iv. Scale up of production of NAOS, MOS, and POS for fluorescent labeling to generate fluorescent oligosaccharide substrates that can be used in enzymatic digestion.
- v. Perform *in vitro* enzymatic digestion assays with fluorescent oligosaccharide substrates.

Chapter 2

Materials and Methods

In order to investigate the utility of three promising prebiotic oligosaccharides as fluorescent oligosaccharide substrates, enzymes known or predicted to produce these oligosaccharides from the parent polysaccharide were expressed (section 2.1). Next the oligosaccharides used in this project were generated by enzymatic hydrolysis of parent polysaccharides. The predicted endolytic activity of *BuGH86* was first investigated (sections 2.2.1-2.2.3) in order to generate NAOS (sections 2.2.4-2.2.6). The predicted exolytic activity of *BuGH2C* and *BuGH117B* on NAOS was subsequently investigated (section 2.3). Appropriate endolytic enzymes were used to generate MOS (section 2.4) and POS (section 2.5) based on conditions modified from literature. All generated oligosaccharides were subsequently labeled with ANTS to enhance sensitivity of the oligosaccharide to detection (section 2.6). Finally, the fluorescently labeled oligosaccharides were tested in enzymatic assays using exolytic enzymes characterized in this project and from literature (section 2.7).

2.1 Recombinant protein production

In order to produce enzymes to generate NAOS, MOS and POS for further studies, constructs of *BuGH86* (from *B. uniformis* NP1), BT3782 (*BtGH76*) and BT3792 (*BtGH76*) (from *B. thetaiotaomicron* VPI-5482) were expressed (Table 2.1). Also for further studies of exolytic hydrolysis on generated NAOS, constructs of the predicted exolytic enzymes *BuGH2C* and *BuGH117B* were expressed (Table 2.1). Exolytic enzymes BT3781 (*BtGH125*) from *B. thetaiotaomicron* VPI-5482 and *YeGH28* from *Y.*

enterocolitica ATCC 9610D were also expressed for further exolytic hydrolysis on MOS and POS respectively.

Expression plasmids were transformed into chemically competent *E. coli* BL21 (DE3) cells (EMD Millipore, Cat #69450-3, Karmstadt, Germany) that were grown subsequently on Luria Broth (LB) agar containing 50 µg/ml of kanamycin (Kan, Amresco, Cat# E710) and incubated at 37°C overnight. The following day, LB containing Kan (50 µg/ml) was inoculated with cells from overnight colonies and cultures were grown with agitation (200 rpm) at 37°C to an optical density (600 nm) between 0.8 to 1. At this density, the temperature was dropped to 16°C for an hour after which the culture was induced with isopropyl β-D-1-thiogalactopyranoside (Gold Biotechnology, Cat# 12481C50) at a concentration of 0.2 mM and shaken overnight [86]. Cells were collected from overnight cultures by centrifugation and disrupted by enzymatic lysis (for constructs *BuGH86*, *BuGH2C*, *BuGH117B*, BT3782, BT3792, and BT 3781). The cell pellet was first resuspended in 25% sucrose (Amresco, Cat# M117), 50 mM Tris (pH 8, Amresco, Cat# 0497). The cells were then lysed in 10 mg/l chicken egg white lysozyme (Sigma Aldrich, Cat# L7651) and two times volume of lysis buffer: 1% sodium deoxycholate (Sigma Aldrich, Cat# D6750), 1% triton X-100 (Sigma Aldrich, Cat# T8787), 20 mM Tris-HCl (pH 7), 100 mM NaCl (Amresco, Cat# 0241) and 200 µg/l DNase (Amresco, Cat# 0649). In the case of *YeGH28*-transformed *E. coli* cells, induced overnight cells were lysed by sonication in 20 ml of binding buffer with medium intensity sonic pulses (for 2-2.5 mins of 1 sec intervals) on ice at a power setting of 4.5 (Heat systems ultrasonics Model W-225 and probe). *YeGH28* was expressed by Dr. Darryl Jones, Agriculture and Agri-Food Canada, Lethbridge.

All cell lysates were clarified of cellular debris by centrifugation at 17,500 x g for 45 min at 4°C and filtration through 0.45 µM membrane (Pall Acrodisc, Cat# 28143-954). Protein was purified from the lysate by applying to a Ni²⁺ immobilized metal affinity column that was equilibrated in binding buffer (0.5 M NaCl, 20 mM Tris-HCl, pH 8). After washing the column to remove non-specific proteins with 5 column volumes of binding buffer, protein was eluted using a stepwise gradient of imidazole in binding buffer (5, 10, 50, 100, 250 and 500 mM). The elution profile was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and fractions containing pure and appreciable amounts of protein were pooled and concentrated by nitrogen pressurized stirred cell ultrafiltration using a filter membrane with a molecular weight cut-off (MWCO) of 5 kDa (EMD Millipore, Cat # PLCC04310 and PLCC02510). The purified enzymes were buffer exchanged by dialysis against 4 l of experimentally optimized buffers at 4°C to maximize solubility. Dialysis conditions for each protein are as described in Table 2.1. All proteins were dialyzed using a dialysis membrane (Spectrum, Biotech CE, Cat # 08-670) with a MWCO of 6,000 - 8,000 Da. The dialyzed proteins were quantified at OD_{280nm}. Extinction coefficients for all proteins were determined using ProtParam analysis tool (<http://web.expasy.org/protparam/>) based on the protein sequence of each construct.

Table 2.1: Sources of expression vector for enzymes and buffer conditions of recombinant proteins

| Construct | Expression vector | Dialysis buffer | Source of constructs |
|-------------------------|--------------------------|---------------------------------------|---|
| <i>BuGH2C</i> | pET28a | 20 mM Tris-HCl (pH 8) and 150 mM NaCl | Dr. Alisdair Boraston, University of Victoria, Canada |
| <i>BuGH117B</i> | pET28a | 20mM Tris-HCl (pH 8) | Dr. Alisdair Boraston, University of Victoria, Canada |
| <i>BuGH86</i> | pET28a | 20mM Tris-HCl (pH 8) and 300mM NaCl | Dr. Alisdair Boraston, University of Victoria, Canada |
| <i>YeGH28</i> | pET28a | 20mM Tris-HCl, pH 8 | Dr. Wade Abbott, Agriculture and Agri-Food Canada |
| <i>BtGH76 (BT3782)</i> | pET28a | 20mM Tris-HCl, pH 8 | Prof. Harry Gilbert, University of Newcastle, UK |
| <i>BtGH76 (BT3792)</i> | pET28a | 20mM Tris-HCl, pH 8 | Prof. Harry Gilbert, University of Newcastle, UK |
| <i>BtGH125 (BT3781)</i> | pET28a | 20mM Tris-HCl, pH 8 | Prof. Harry Gilbert, University of Newcastle, UK |

2.2 Production of NAOS from agarose

2.2.1 Optimizing of enzyme concentration and assay incubation time of *BuGH86* on low melting point and solid agarose

To determine optimal conditions for digesting low melting point (LMP) agarose (IBI Scientific, Cat# IB70057), enzyme concentrations of 20 nM, 200 nM and 2 μ M were used to digest 1 mg/ml of LMP agarose at 37°C in 20 mM Tris-HCl buffer at pH 7. Aliquots of the degradation products were taken at time intervals of 0 min, 5 min, 10 min, 30 min, 1 h, 2 h and 21 h. Digestion was halted at each of these time points by heating samples to 100°C for 5 min. After initial experiments with LMP, it was determined that agarolysis products were contaminated with gelled agarose. In order to circumvent this problem,

digestion in subsequent experiments was carried out with solid agarose in solid phase while other components were maintained in the liquid phase. The lowest concentration at which agarose could be gelled and maintained in solid phase at digestion temperatures during reactions was determined to be 8 mg/ml. Hence, parallel reactions were performed for solid agarose (Invitrogen Life Technologies, Cat# 16500-500) using 8 mg/ml agarose gelled in reaction tubes. Product profiles of each of these samples were analyzed by thin layer chromatography (TLC). The products of each gradient (6 μ l) were loaded onto a silica gel 60 TLC plate (Merck, Germany, Cat #1055530001) and mobilized in a butanol-water-acetic acid (2:1:1 by volume) solvent (Butanol: Sigma Aldrich, Cat# 34867). The plate was dried to completion, stained with 1% orcinol monohydrate (Sigma Aldrich, Cat# 01875) dissolved in a solution of 70:3 of acetic acid and sulfuric acid, and then air dried to completion. The plate was subsequently heated in an oven at 105°C for 5 min to visualize the digest products.

2.2.2 Effect of pH on BuGH86 activity on LMP and solid agarose

A pH range of 4 to 8 was tested. An enzyme concentration of 2 μ M was used to digest 1 mg/ml of LMP agarose or 8 mg/ml solid agarose at 37°C in 20 mM Tris-HCl. Aliquots of the degradation products were taken over a time course, digestion halted, and product profiles of each of the samples analyzed by TLC as described in section 2.2.1.

2.2.3 Optimization of LMP agarose concentration for digestion by BuGH86

LMP agarose (1 mg/ml, 2 mg/ml and 5 mg/ml) was digested at 20 mM Tris-HCl (pH 5) and enzyme concentrations of 2 μ M. Aliquots of the degradation products were taken over a time course, digestion halted, and product profiles of each of the samples analyzed by TLC as described in section 2.2.1.

2.2.4 Large scale digest of LMP agarose by BuGH86 and purification of digest products

Based upon the above experimentally determined conditions, *BuGH86* (200 nM) was used to digest 100 ml of 2 mg/ml of LMP agarose in 20 mM Tris-HCl (pH 5), at 37°C for 20 h. The digestion was frozen and lyophilized. The lyophilized digest was resuspended in 2 ml of dH₂O and fractions of the digest purified by size exclusion chromatography through Bio-gel P2 resin (Bio-Rad Laboratories, Cat# 1504118) equilibrated in dH₂O at a flow rate of 0.25 ml/min (column dimensions: 2.5 cm x 120 cm). Samples were collected in 2 ml fractions, and a volume of ~600 ml was used to elute the loaded digestions. Elution peaks were detected by spotting 6 µl of every third fraction on a silica gel 60 plate, air drying, staining and visualized using the method for TLC described above. Samples containing sugars were then fully developed by TLC to determine differences in digestion products. Similar NAOS populations were pooled and lyophilized.

2.2.5 Mass spectrophotometry of NAOS

Purified NAOS from the large scale LMP agarose digestion and purification was analyzed by matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) spectrophotometry. 1 µl of matrix (20 mg/ml 2,5-dihydroxybenzoic acid in 1:1 water:methanol) was mixed with 1 µl of aqueous oligosaccharide sample, and allowed to air dry. MALDI-TOF mass spectra were collected on N₂-laser equipped MicroFlex LT instrument (Bruker) in the positive mode. These experiments were carried out by Dr. Sami Tuomivaara at the Complex Carbohydrate Research Centre, Athens, Georgia.

2.2.6 UV-Sulfuric assay

An aliquot of each sample (25 μ l) was transferred to a microplate well (Corning, Cat # 07-200-91) and the absorbance measured at 315 nm. Concentrated sulfuric acid was added to each well (75 μ l), shaken for 1 min, cooled on ice and another absorbance measured at 315 nm. The final absorbance was calculated from both readings. These experiments were carried out by Dr. Sami Tuomivaara at the Complex Carbohydrate Research Centre, Athens, Georgia.

2.3 Screening of enzymes for saccharification of agarose

To determine the optimal saccharification conditions of agarose, *BuGH2C*, *BuGH117B* and *BuGH86* from *B. uniformis* were used to saccharify agarose. These reactions were designed to include all potential combinations of the three endolytic and exolytic agarases, including single, double and triple enzyme digests. In addition, due to methylation digestion products detected with LMP agarose in previous experiments, solid agarose was used in subsequent experiments.

2.3.1 Single and double agarose digests

Gelled solid agarose (8 mg/ml) in a 50% reaction volume was digested in 20 mM Tris-HCl, pH 7 with a single enzyme at a concentration of 1 μ M of *BuGH86*. Double enzyme digests with 1 μ M of *BuGH86* and 1 μ M of *BuGH117B* in separate 300 μ l reactions were performed at 37°C with shaking at 300 rpm. Aliquots of the agarooligosaccharide (AOS) degradation products were collected after digestion at 21 h without disturbing the undigested gelled agarose. Digestion was halted at this time by heating samples to 100°C for 5 min. Product profiles of each of AOS samples were analyzed via TLC as previously described in section 2.2.1.

2.3.2 Gradient digestion of BuGH2C on AOS products

Gelled solid agarose (8 mg/ml) in a 50% reaction volume was digested in 20 mM Tris-HCl, pH 7 and using a double enzyme digest at a concentration of 2 μ M for both *BuGH86* and *BuGH117B* at 37°C and 300 rpm in a 300 μ l reaction. Digestion was halted at 21 h and products analyzed by TLC as previously described. Subsequently, these digest products were used as a substrate for an enzyme gradient of *BuGH2C* at 0 nM (negative control), 20 nM, 200 nM and 2 μ M. Time points were taken at 5 min, 10 min, 30 min, 1 h, 2 h and 21 h. Digestion was halted at these time points by heating samples to 100°C for 5 min and products analyzed by TLC as previously described in section 2.2.1.

2.3.3 Test of activity of triple digests

Gelled solid agarose (8 mg/ml) in a 50% reaction volume was digested in 20 mM Tris-HCl, pH of 7 and using a triple enzyme digest at a concentration of 2 μ M for *BuGH2C*, *BuGH86* and *BuGH117B* at 37°C and 300 rpm in a 300 μ l reaction. Aliquots were taken at time points of 5 min, 10 min, 30 min, 1 h, 2 h and 21 h. Digestion was halted at these time points by heating samples to 100°C for 5 min and products analyzed by TLC as previously described in section 2.2.1.

2.3.4 Matrix optima assay of individual, double and triple enzymes with agarose

Digestion was carried out on gelled solid agarose (8 mg/ml) in 20 mM Tris-HCl, pH 7 at 37°C with shaking at 200 rpm in separate 300 μ l reactions. *BuGH2C*, *BuGH86* and *BuGH117B* single reactions were performed at enzyme concentrations of 1 μ M. Double digests with *BuGH2C* and *BuGH86*; *BuGH2C* and *BuGH117B*; and *BuGH86* and *BuGH117B* and a triple digest of *BuGH2C*, *BuGH86* and *BuGH117B* were carried out similarly using equivalent enzyme concentrations. To identify the rate limiting

concentrations for each enzyme within the mixed reactions, titrations were performed for each possible reaction condition. Firstly, *BuGH2C* and *BuGH86* (1 μ M) were used to supplement a *BuGH117B* gradient from 10 nM, 20 nM, 50 nM, 100 nM, and 2 μ M. Similar digestion conditions were also performed for *BuGH2C* and *BuGH86*. Next, single enzymes were kept constant and the complementary enzyme pairs were titrated at increasing concentrations. For example, *BuGH86* (1 μ M) was incubated with *BuGH2C* and *BuGH117B* at concentrations of 10 nM, 20 nM, 50 nM, 100 nM, and 2 μ M. These conditions were repeated using *BuGH2C* and *BuGH117B* at 1 μ M, which was supplemented with the complementary enzyme pairs. Digestion was halted at 21 h by heating samples to 100°C for 5 min and products analyzed by TLC as previously described in section 2.2.1.

2.4 Production of MOS from α 1,6 yeast mannan

2.4.1 Yeast mannan production

The *S. cerevisiae* mannan mutant 2 strain (which produces debranched yeast mannan; Figure 1.5D) was graciously provided by Dr. Harry Gilbert (University of Newcastle, UK). The yeast mannan was extracted using the citrate buffer-autoclaving method [43, 87]. Yeast strains were grown at 30°C in yeast extract-peptone-dextrose (YPD) broth (pH 6.7) with shaking (150 rpm) for 4 d to stationary phase. The yeast cells were harvested by centrifugation at 12,000 x g for 10 min. Yeast cells were resuspended in 10 ml of 20 mM citrate, pH 7 per 1 l of cell culture harvested and autoclaved at 121°C and 15 psi for 90 min to release sugars on the yeast cell wall surface into solution. The autoclaved cells were pelleted by centrifugation as above and the supernatant collected (supernatant 1). Recovered cell pellets were resuspended in 1.5 X original resuspension volume of 20 mM

citrate buffer, pH 7, and autoclaved again as described above. The yeast cells were pelleted again as described above. The supernatant obtained from this (supernatant 2) was also kept and all yeast cells discarded. Supernatant 1 and supernatant 2 were added slowly to an equal volume of active Fehling's reagent with frequent agitation to avoid precipitation. Active Fehling's reagent is a 1:1 ratio of Fehling's reagent I and II. Fehling's reagent I is a blue, aqueous solution of 0.0692 g/ml of copper (II) sulfate pentahydrate (Sigma Aldrich, Cat# 209198). Fehling's reagent II is a clear, aqueous solution containing 0.346 g/ml potassium sodium tartarate tetrahydrate (Rochelle's Salt) (Sigma Aldrich, Cat# 217255) and 0.1 g/ml of NaOH (Sigma Aldrich, Cat# 221465). Both were prepared fresh prior to the extraction of cells. The supernatants 1 and 2/active Fehling's reagent mixture was incubated at 40°C for 2 h with stirring at 150 rpm. After incubation, the precipitate was recovered by centrifuging at 5,000 x g for 10 min and the supernatant discarded. The recovered pellet was dissolved in 3 M hydrochloric acid (Sigma Aldrich, Cat# H1758) to obtain a light green solution. A minimal volume of HCl was used to dilute the precipitate until the resuspension turned light green. The resuspensions were pooled together and added to 100 ml of 8:1 methanol (Sigma Aldrich, Cat# 494291)/acetic acid (Sigma Aldrich, Cat# A6283) with gentle stirring. This was allowed to incubate for 15 min. The thick, white precipitate obtained in this process was recovered by centrifugation at 5,000 x g for 10 min and the supernatant discarded. The recovered pellets were resuspended in 100 ml of 8:1 methanol/acetic acid mixture, vortexed briefly and centrifuged at 385 x g for 5 min. This wash step was repeated at least three times and until the pellets lost their green color. The supernatant was disposed after each wash. Wash steps were repeated three more times using 100% methanol. After the final 100% methanol wash, pellets were left to dry overnight in a fume hood. The dried

pellets were resuspended in ultra-pure H₂O and dialysed in H₂O. The resuspension was then lyophilized to obtain purified α 1,6 mannan.

2.4.2 Enzyme assay on yeast mannan

Optimization of enzyme reactions were carried out on extracted yeast mannan using *BtGH76* enzymes from loci BT3782 or BT3792 in different reactions as previously described [43] with the following modifications. Briefly, 10 mg/ml purified α 1,6 yeast mannan was digested with 2 μ M of either BT3782 or BT3792 in 50 mM sodium phosphate buffer (pH 7) at 37°C over a time course of 5 min, 10 min, 30 min, 1 h, 2 h and 20 h at 300 rpm. Enzyme reactions were stopped by incubating samples at 100°C for 5 min and the products analyzed as described above for NAOS (section 2.2.1).

2.5 Production of pectic oligosaccharides (POS) from HG

2.5.1 Generation of POS from HG

POS was generated from HG using *RhGH28* purified from *Rhizopus* sp. (Sigma Aldrich, Cat# P2401). To determine optimal digestion conditions, 0.1, 1 and 5 units of enzyme were applied to 10 mg/ml of HG (Megazyme, Cat# P-PGACT) at 25°C, 300 rpm in sodium citrate buffer (pH 4) according to manufacturer's specifications. Aliquots of the degradation products were taken at time intervals of 10 min, 1 h and 2 h. Digestion was halted at each of these time points by heating samples to 100°C for 5 min and the products analyzed as described above for NAOS (section 2.2.1). GalA (Sigma Aldrich, Cat# 48280), GalA₂ (Sigma Aldrich, Cat# D4288), and trigalacturonic acid (GalA₃; Sigma Aldrich, Cat# T7407) were used as standards.

2.6 Fluorescent labeling of NAOS, MOS and POS

2.6.1 Fluorescent labeling of NAOS

Lyophilized NAOS (2 mg) were derivatized in 5 μ l of 8-aminonaphthalene-1,3,6-trisulfonate (ANTS) solution (Sigma Aldrich, Cat# 08658): 0.15 M ANTS in 15% acetic acid (Sigma Aldrich, Cat# A6283) v/v with an equal volume of sodium cyanoborohydride (Sigma Aldrich, Cat# 156159) solution: 1.0 M in DMSO (Sigma Aldrich, Cat# D-5879) at 37°C for 18 hours. The derivatized product was dried under vacuum and dissolved in 5 μ l of water. An aliquot of this solution was added to an equal volume of 2 X Laemmli Buffer without SDS (0.5M Tris-HCl (pH 6.8), Glycerol (Sigma-Aldrich, Cat# 56-81-5), 0.2% (w/v) Bromophenol Blue (Sigma Aldrich, Cat# B-0126)) and run on 28% or 30% polyacrylamide gels made from 40% acrylamide/bis-acrylamide (Bio-Rad Laboratories, Cat# 1610144). The gel was analyzed by FACE using modified methods [88, 89] in an electrophoresis tank with pre-chilled 1 X electrode running buffer (0.192M glycine (Amresco, Cat# 0167), 0.025M Tris, pH 8.3) which was run at 300V for ~ 1 hour at 4°C in an ice bath. Bands were visualized with a Bio-Rad Gel Doc XR+ gel imager equipped with a 580AF120 ethidium bromide filter and controlled by Image Lab software. The ethidium bromide filter was used as recommended by the manufacturer for imaging fluorescence. In order to produce probes using less toxic reagents, an alternative labeling reaction was performed using ANTS as the fluorescent label in the presence of 2-picoline borane ([88] Sigma Aldrich, Cat# 654213) as a reducing agent using the same conditions already described with sodium cyanoborohydride.

2.6.2 Fluorescent labeling of MOS

Lyophilized MOS was derivatized as described earlier in section 2.6.1. Derivatized MOS was dried under vacuum, resuspended in 5 μ l of water and analyzed by FACE as described previously in section 2.6.1.

2.6.3 Fluorescent labeling of POS

Lyophilized POS was derivatized as described earlier in section 2.6.1 with some differences. Labeling with ANTS and appropriate reducing agents as discussed before was carried out at 40°C for 16 hours [90]. Derivatized POS was dried under vacuum, resuspended in 5 μ l and analyzed by FACE as described previously.

2.7 Enzymatic assays on fluorescently labeled NAOS, MOS and POS

Lyophilized NAOS and MOS were labeled as previously described in section 2.6.1. Lyophilized POS was labeled as previously described in section 2.6.3. To test activity of enzymes on the labeled and modified sugars, a 2 μ M enzyme concentration of appropriate exolytic enzymes was used to digest 2 mg/ml of the labeled sugar under experimentally determined conditions: at 37°C, pH 7 with shaking (300 rpm), for 1 and 24 h. Exolytic enzymes and buffers applicable to each labeled substrate are described in Table 2.2. Enzyme hydrolysis was halted by heating samples to 100°C for 5 minutes. After digestion, both controls and treatments were concentrated by 50 X under vacuum at 35°C. FACE was used to profile the labeled sugar digestions.

Table 2.2: Buffer conditions for enzyme assay on labeled sugars

| Substrate | Enzyme | Buffer conditions | pH |
|-------------------|--------------------------------------|--------------------------|-----------|
| ANTS-labeled NAOS | <i>BuGH2C</i> and <i>BuGH117B</i> | Tris-HCl (20 mM) | 7 |
| ANTS-labeled MOS | <i>BtGH125</i> (BT3781) | Sodium phosphate (50 mM) | 7 |
| ANTS-labeled POS | <i>YeGH28</i> | Citric acid (20 mM) | 7 |

Chapter 3

Results

3.1 Production of neoagarooligosaccharides (NAOS) and the retention of ANTS-labeled NAOS bioactivity.

3.1.1 Optima of LMP and solid agarose saccharification for NAOS generation

Recombinant *BuGH86* was purified using IMAC (Figure 3.1; Table 3.1). Enzyme assays were performed to evaluate the activity of *BuGH86* on both LMP and solid agarose. TLC analysis of the digestion products demonstrates that *BuGH86* produces a ladder of oligosaccharides ranging from neoagarobiose (G_1A_1 , DP2 = 306.1 g/mol) to large oligosaccharides (Figure 3.2). This profile is representative of an enzyme with endolytic activity. The optimal enzyme concentration on both forms of agarose was determined to be 2.0 μ M (24 hours). The enzyme concentrations were used to test the

Table 3.1: Summary of enzymes used for oligosaccharide generation and diagnostic digests of ANTS-labeled substrates

| Enzyme | Species | Family | MW (kDa) | Products |
|-------------------------|--|--------|----------|-----------------|
| <i>BuGH2C</i> | <i>B. uniformis</i> NP1 | GH2 | 94 | Gal |
| <i>BuGH117B</i> | <i>B. uniformis</i> NP1 | GH117 | 43 | 3,6-LGal |
| <i>BuGH86</i> | <i>B. uniformis</i> NP1 | GH86 | 72 | NAOS |
| <i>RhGH28</i> | <i>Rhizopus</i> sp. | GH28 | ND | POS |
| <i>YeGH28</i> | <i>Y. enterocolitica</i> ATCC9610D | GH28 | 55 | Digalacturonate |
| <i>BtGH76</i> (BT3782) | <i>B. thetaiotaomicron</i> VPI-5482 | GH76 | 43 | MOS |
| <i>BtGH76</i> (BT3792) | <i>B. thetaiotaomicron</i> VPI-5482 | GH76 | 42 | MOS |
| <i>BtGH125</i> (BT3781) | <i>B. thetaiotaomicron</i> VPI-5482 | GH125 | 53 | Mannose |

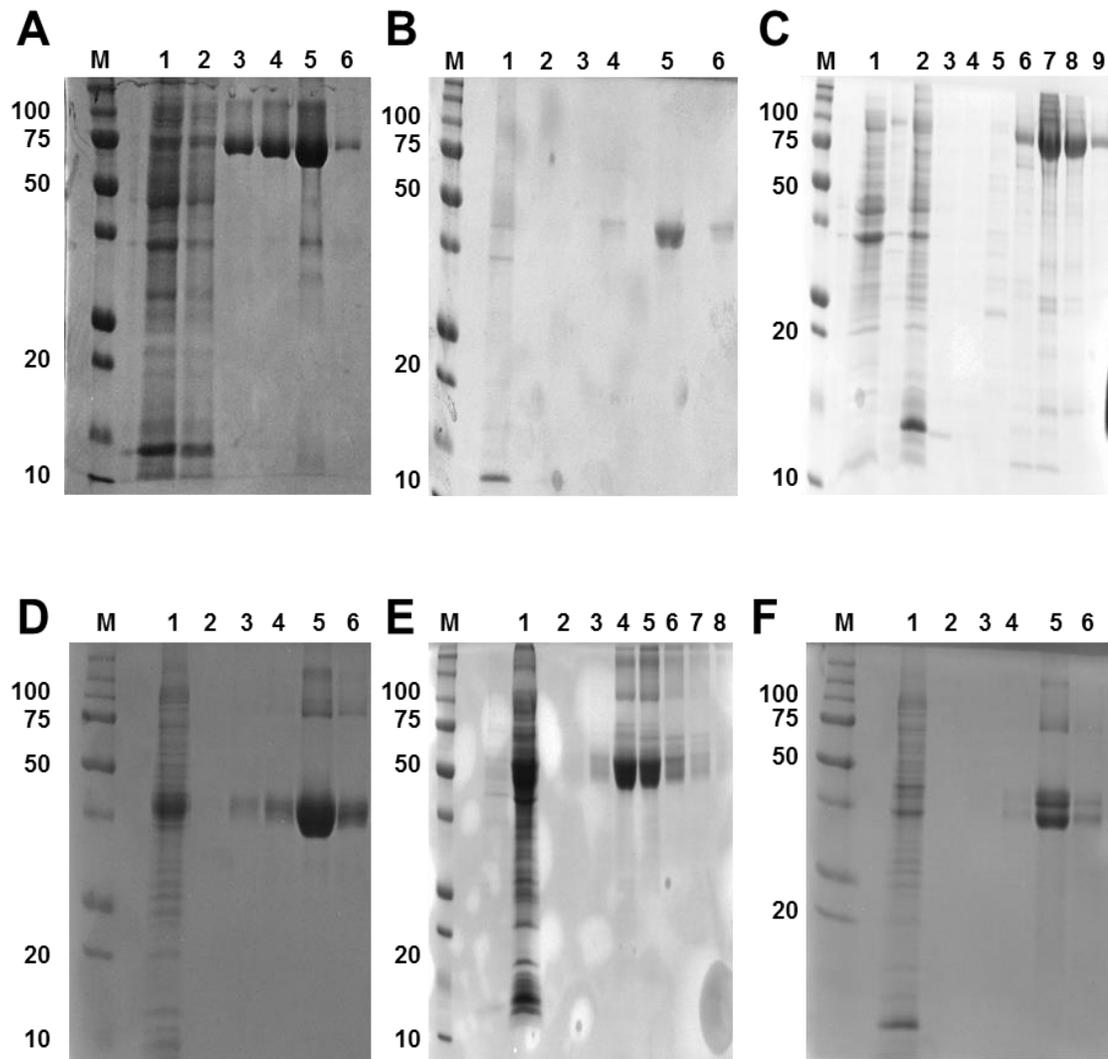


Figure 3.1: SDS-PAGE gels of IMAC purification of recombinant proteins. (A) *BuGH86*, (B) *BuGH117B*, (C) *BuGH2C*, (D) BT3782, (E) BT3781, and (F) BT3792. For all gels, M = marker (Biorad, Cat # 1610363), 1 = cell lysate flow through, 2 = binding buffer wash and 3 = 5 mM imidazole buffer elution, 4 = 10 mM imidazole buffer elution, 5 = 50 mM imidazole buffer elution, 6 = 100 mM imidazole buffer elution. In C and E, 7 = 250 mM imidazole buffer elution, 8 = 500 mM imidazole buffer elution and in (C) 9 = 2nd 500 mM imidazole buffer elution.

effect of other conditions which were most efficient at: pH of 5, time (21 hours), and substrate concentration (5 mg/ml); however, a substrate concentration of 2 mg/ml was ultimately selected due to difficulties associated with gelation of agarose substrate and NAOS observed at higher concentrations. A pH of 7 was also subsequently used on solid agarose so that experimental pH conditions were similar to that of the large intestine.

3.1.2 Large scale digest of LMP agarose and generation of NAOS

Following optimization of agarose digestion by *BuGH86* using pilot reactions a large scale digest of 200 mg LMP agarose (100 ml of 2 mg/mL) was performed (Figure 3.2A). The reaction was heat killed, cleared by centrifugation, and the products were lyophilized and collected. Following resuspension, the soluble products were loaded onto a P2 Biogel size exclusion column in dH₂O. About 200 fractions (2 mL) were collected. The fractions were screened by TLC to identify fraction boundaries. Samples containing similar sized NAOS were pooled and analyzed by TLC for purity. In total seven NAOS populations were pooled. Pool 1 consisted of fractions 90-99 (20 ml), pool 2 = 100-104 (10 ml), pool 3 = 106-109 (8 ml), pool 4 = 120-131 (24 ml), pool 5 = 135-154 (40 ml), pool 6 = 156-164 (18 ml) and pool 7 = 171-182 (24 ml). Further after SEC, a sulfuric acid-UV assay (Figure 3.2A) was carried out to produce a chromatogram. The NAOS species were also analyzed by FACE (Figure 3.2B).

3.1.3 Mass spectrophotometry of NAOS released by BuGH86

To determine the size and structure of NAOS products released by *BuGH86* digestion, the seven purified NAOS pools were analyzed by MALDI-TOF (Figure 3.2C-I, Table 3.2). The NAOS species ranged in size from G₁A₁ (DP2) to a 22-mer (DP22) (Figure 3.2C).

Table 3.2: Relationship between NAOS from LMP agarose and sizes determined by MALDI-TOF analysis. LS* = *BuGH86* digest used as loading sample.

NOTE: In the NAOS populations there are different methylation profiles per peak as indicated in a close up of each peak (see appendix 2). For example in G₄A₄ from the entire digest, some of the populations have 0 methylations (peak 1) while others have 1 (peak 2), 2 (peak 3), 3 (peaks 4 and 5), 5 (peak 6) and 6 (peak 7) methylations per G₄A₄. G = Gal, A = 3,6-LGal, Me = methyl group. Subscripts indicate the number of the sugar, residues or methylations present.

| Sample | NAOS | MW | DP | NAOSMe | MW | Number of methylations |
|--------|---------------------------------|--------|----|---|----------------|------------------------|
| LS* | G ₃ A ₃ | 918.3 | 6 | G ₃ A ₃ Me ₂ | 946.3 | 2 |
| | G ₄ A ₄ | 1224.4 | 8 | G ₄ A ₄ Me- G ₄ A ₄ Me ₆ | 1238.4-1308.5 | 0, 1, 2, 3, 5, 6 |
| | G ₅ A ₅ | 1530.5 | 10 | G ₅ A ₅ Me- G ₅ A ₅ Me ₈ | 1544.5-1642.6 | 1, 2, 4, 5, 6, 8 |
| | G ₆ A ₆ | 1836.6 | 12 | G ₆ A ₆ Me- G ₆ A ₆ Me ₇ | 1850.6-1934.7 | 1, 3, 4, 5, 6, 7 |
| | G ₇ A ₇ | 2142.7 | 14 | G ₇ A ₇ Me ₄ | 2198.7 | 4 |
| | G ₈ A ₈ | 2448.8 | 16 | G ₈ A ₈ Me ₅ | 2518.8 | 5 |
| | G ₉ A ₉ | 2754.9 | 18 | G ₉ A ₉ Me ₄ | 2810.9 | 4 |
| | G ₁₀ A ₁₀ | 2898.9 | 20 | - | - | 0 |
| | G ₁₁ A ₁₁ | 3205 | 22 | - | - | 0 |
| 1 | G ₄ A ₄ | 1224.4 | 8 | G ₄ A ₄ Me ₃ , G ₄ A ₄ Me ₅ | 1266.4, 1294.5 | 3, 5 |
| | G ₅ A ₅ | 1530.5 | 10 | G ₅ A ₅ Me ₂ - G ₅ A ₅ Me ₅ | 1558.5-1600.6 | 2, 3, 4, 5 |
| | G ₆ A ₆ | 1836.6 | 12 | G ₆ A ₆ Me ₃ - G ₆ A ₆ Me ₅ | 1878.6-1906.6 | 3, 4, 5 |
| | G ₇ A ₇ | 2142.7 | 14 | G ₇ A ₇ Me ₂ - G ₇ A ₇ Me ₅ | 2170.7-2212.7 | 2, 3, 4, 5 |
| | G ₈ A ₈ | 2448.8 | 16 | G ₈ A ₈ Me ₂ | 2476.8 | 2 |
| 2 | G ₃ A ₃ | 918.3 | 6 | G ₃ A ₃ Me ₄ | 974.3 | 4 |
| | G ₄ A ₄ | 1224.4 | 8 | G ₄ A ₄ Me ₂ - G ₄ A ₄ Me ₃ | 1252.4-1266.4 | 2, 3 |
| | G ₅ A ₅ | 1530.5 | 10 | G ₅ A ₅ Me ₂ | 1558.5 | 0, 2 |
| 3 | G ₃ A ₃ | 918.3 | 6 | G ₃ A ₃ Me ₂ , G ₃ A ₃ Me ₄ | 946.3-974.3 | 2, 4 |
| | G ₄ A ₄ | 1224.4 | 8 | G ₄ A ₄ Me ₂ , G ₄ A ₄ Me ₃ | 1252.4-1266.4 | 0, 2, 3 |
| 4 | G ₂ A ₂ | 612.2 | 4 | G ₂ A ₂ Me ₂ - G ₂ A ₂ Me ₃ | 640.2-654.2 | 2, 3 |
| | G ₃ A ₃ | 918.3 | 6 | G ₃ A ₃ Me | 932.3 | 0, 1 |
| 5 | G ₂ A ₂ | 612.2 | 4 | G ₂ A ₂ Me, G ₂ A ₂ Me ₈ | 626.2, 724.3 | 0, 1, 8 |
| | G ₃ A ₂ | 774.2 | 5 | G ₃ A ₂ Me, G ₃ A ₂ Me ₂ | 788.3, 802.3 | 1, 2 |
| 6 | G ₂ A ₁ | 468.1 | 3 | G ₂ A ₁ Me ₃ | 510.2 | 3 |
| 7 | G ₁ A ₁ | 306.1 | 2 | G ₁ A ₁ Me ₂ | 334.1 | 2 |

Using MALDI-TOF MS, it was determined that subpopulations within each NAOS pool contained varying degrees of methylation. In some instances, a population of NAOS consists of a mixture of methylated and unmethylated products (Table 3.2).

The presence of methylated products demonstrates that *BuGH86* is active on methylated agarose and to my knowledge is the first report of an agarolytic enzyme from a bacterium

native to the large intestine; however, this activity has been well-recognized in marine bacteria [91, 92]. Since methylations also occur in natural agarose but to a lower extent than in LMP, this shows that agarolytic activity acquired by *B. uniformis* NP1 is not hindered by methylations.

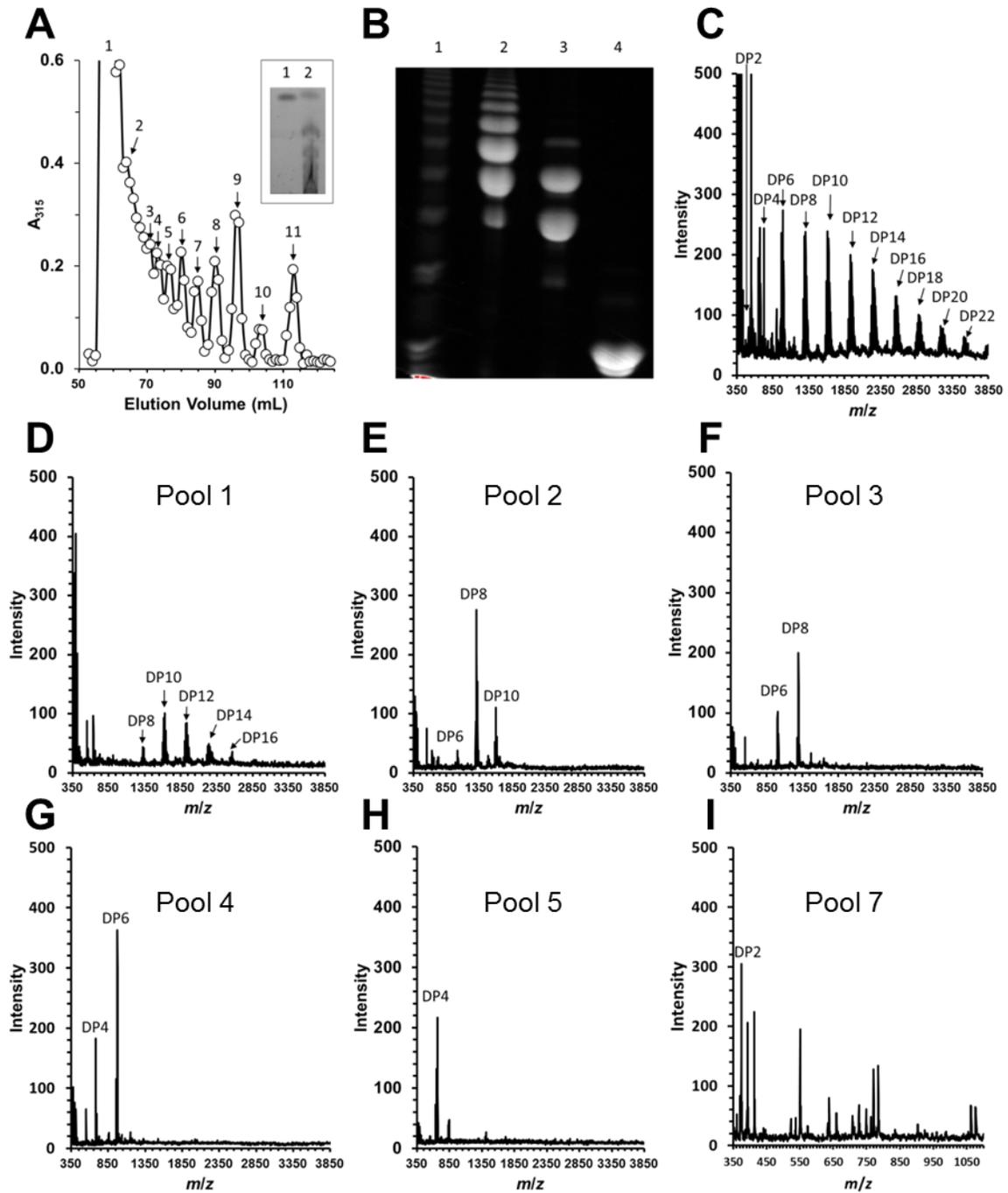


Figure 3.2: Structural analysis of NAOS released from LMP agarose. (A) Sulfuric acid-UV assay of NAOS generated from LMP by *BuGH86*. Black arrows indicate fraction peaks generated by SEC. Inset: Lane 1 = Gal, the marker: 2 = digestion of LMP agarose by *BuGH86* that was used as loading sample for column (NAOS standard). (B) ANTS-labeling of NAOS. FACE gel contains ANTS-labeled NAOS generated by *BuGH86* (lane 1) and different populations of NAOS separated by size exclusion chromatography (SEC) (lanes 2-4). 1 = *BuGH86* digestion end products lane 2 = NAOS of DP 8-16 shown in D, lane 3 = NAOS of DP 6-8 shown in F, and lane 4 = NAOS of DP

4 shown in H. **(C)** MALDI-TOF analysis of *BuGH86* digestion end products. DP2-22 represents the sizes of released NAOS fragments. **(D-I)** Purified fractions of NAOS fragments from the digestion of LMP by *BuGH86*. D = Pool 1, E = Pool 2, F = Pool 3, G = Pool 4, H = Pool 5, I = Pool 7. DP was determined using MALDI-TOF MS (Table 3.2).

3.1.4 Screening of enzymes for optimal saccharification of agarose

Consistent with LMP agarose, single enzyme digests of *BuGH86* on solid agarose led to the release of NAOS of different degrees of polymerization (\geq DP2; data not shown), which is indicative of endolytic activity. To explore if NAOS products are further digested by other glycoside hydrolases in the *B. uniformis* NP1 agarose PUL, single enzyme digests with *BuGH2C* or *BuGH117B* (Figure 3.3A, Table 3.1) were performed.

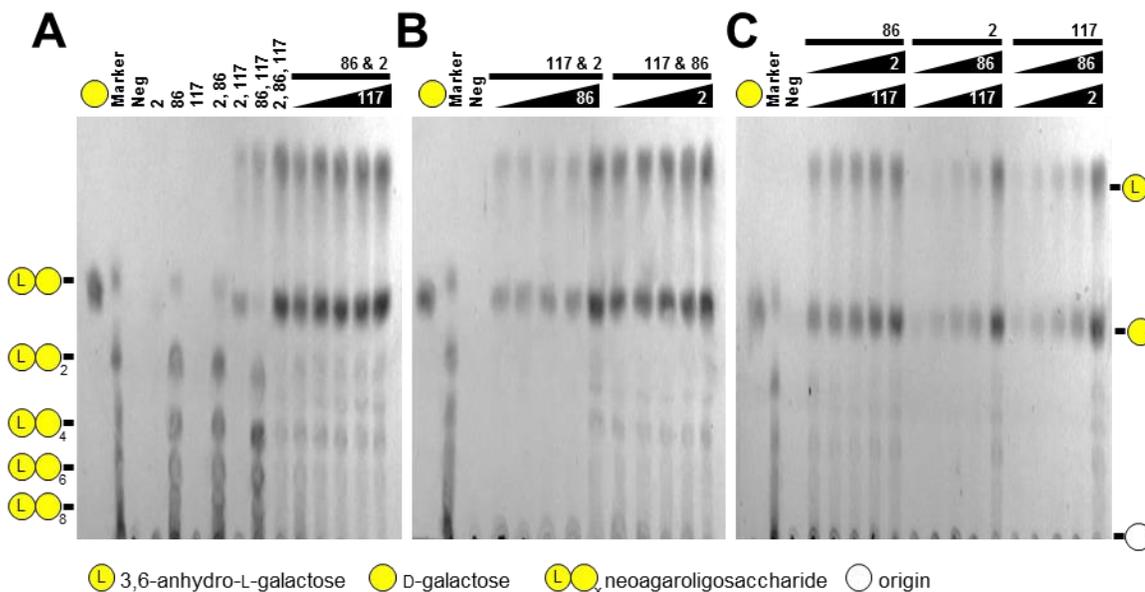


Figure 3.3: Generation of NAOS from solid agarose in an enzyme pathway. TLC of enzyme matrix assay for the optimal saccharification of solid agarose using *BuGH86*, *BuGH117B* and *BuGH2C*. (A) The first six lanes show Gal, the marker (NAOS standard), no enzyme (negative control) and single enzyme digests of *BuGH2C*, *BuGH86*, and *BuGH117B* (1 μ M). These are followed by three lanes containing double digest mixtures. Next a triple digest with all three agarases (1 μ M) is shown. The last five lanes are digestions with same concentrations of *BuGH86* and *BuGH2C* (1 μ M; indicated with a black bar) and an increasing gradient of *BuGH117B* (10 nM, 20 nM, 50 nM, 100 nM, and 2 μ M). (B) The first three lanes show Gal, the marker, and a no enzyme (negative control). The next five lanes have triple digests of consistent levels of *BuGH117B* and *BuGH2C* (1 μ M) with an increasing gradient of *BuGH86* (10 nM, 20 nM, 50 nM, 100 nM, and 2 μ M). The last five lanes show triple digests with consistent levels of *BuGH117B* and *BuGH86* (1 μ M), and an increasing gradient of *BuGH2C* (10 nM, 20 nM, 50 nM, 100 nM, and 2 μ M). (C) The first three lanes show Gal, the marker, and a no enzyme (negative control). The next five lanes show triple digests with consistent levels of *BuGH86* (1 μ M) with an increasing gradient of *BuGH2C* and *BuGH117B* (10 nM, 20 nM, 50 nM, 100 nM, and 2 μ M). This is followed by five lanes showing triple digests with consistent levels of *BuGH2C* (1 μ M) and an increasing gradient of *BuGH86* and

BuGH117B (10 nM, 20 nM, 50 nM, 100 nM, and 2 μ M). The last five lanes show triple digests with consistent levels of *BuGH117B* (1 μ M) and an increasing gradient of *BuGH86* and *BuGH2C* (10 nM, 20 nM, 50 nM, 100 nM, and 2 μ M).

Initially, the activity of these enzymes was tested on pure agarose as a substrate. Individual digests did not produce any appreciable products. Double enzyme digests with *BuGH2C* and *BuGH117B*; however, releases Gal and 3,6-LGal monosaccharides (Figure 3.3A). The observation that two distinct monosaccharides are released, and that it is contingent on the presence of both *BuGH2C* and *BuGH117B*, indicates that both enzymes are exolytic and that they work interdependently to hydrolyze the two distinct linkages in agarose.

In the presence of *BuGH86* and solid agarose, *BuGH117B* releases 3,6-LGal (Figure 3.3). In the absence of *BuGH86*; however, *BuGH117B* has no discernable activity. This suggests that *BuGH117B* is active on the 3,6-LGal at the non-reducing end of NAOS that is exposed following cleavage of the β 1,4-linkage by *BuGH86*. When *BuGH86* and *BuGH2C* are incubated together on solid agarose, the end product profile of enzymatic hydrolysis appeared to be similar to that of *BuGH86* alone (Figure 3.3A). This indicates that *BuGH2C* is unable to recognize and depolymerize NAOS generated by *BuGH86* from solid agarose. When *BuGH2C* is paired with *BuGH117B*; however, Gal is released from the GH86 products. This suggests that *BuGH117B* is required to prune the 3,6-LGal and expose Gal at the non-reducing end, which is the natural substrate for *BuGH2C*. This relationship is confirmed when reactions of *BuGH86*, *BuGH2C* and *BuGH117B* with solid agarose result in a more pronounced release of Gal and 3,6-LGal and the enhanced depletion of NAOS (Figure 3.3). Hence via this enzyme pathway, *BuGH2C* and

BuGH117B work synergistically to release the contiguous monosaccharides from NAOS that are generated by *BuGH86*. While matrix assays (Figure 3.3) confirmed that a mix of all *BuGH86*, *BuGH2C* and *BuGH117B* enzymes are needed for the efficient release of the monomeric units of agarose and efficient saccharification of agarose, enzyme concentration titrations of all three enzymes suggest that the endolytic *BuGH86* must be present in high concentrations at all times with respect to the other two enzymes (Figure 3.3B, C). This is evident where Gal and 3,6-LGal are not released from agarose when *BuGH86* is present in lower concentrations with respect to one of or both of *BuGH2C* and *BuGH117B*.

3.1.5 Fluorescent labeling of NAOS

ANTS is a fluorescent dye that can be covalently attached to the reducing end of a sugar by reductive amidation (Figure 1.6). It has previously been shown to make NAOS fluorescent and enable its visualization by FACE [67, 68]. FACE is more sensitive than TLC, and enables the high-resolution detection of carbohydrates. Additionally, electrophoretic separation of fluorescent NAOS allows for distinguishing between oligosaccharides of different degrees of polymerization. In this manner, ANTS was observed to efficiently label NAOS generated from solid agarose (Figure 3.4A), a mixed population of NAOS ranging in size from DP 2-22, and is equally efficient on purified fractions (DP 8-16, DP 6-10, and DP 4-5) when analyzed by FACE (Figure 3.2B, Table 3.2).

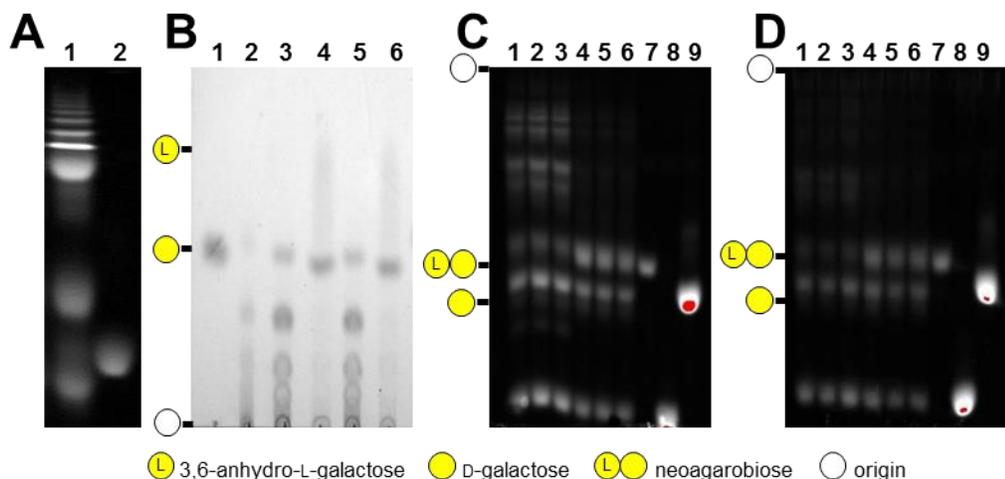


Figure 3.4: Generation of NAOS from solid agarose and labeling for enzymatic assays. (A) FACE gel of ANTS labeled NAOS generated from *BuGH86* digestion of solid agarose (lane 1) and a G_1A_1 standard (lane 2). NAOS was labeled with ANTS in the presence of 2-picoline borane (B) TLC of unlabeled NAOS controls. Lane 1 = Gal, 2 = NAOS standard, 3 = *BuGH86* generated NAOS, 4 = *BuGH2C* and *BuGH117B* treated NAOS for 1 hour, 5 = *BuGH86* generated NAOS, 6 = *BuGH2C* and *BuGH117B* treated NAOS for 24 hours. FACE gels of a 1-hour (C) and 24-hour (D) digestion of ANTS-labeled NAOS with *BuGH2C* and *BuGH117B*. Lanes 1-3 = ANTS labeled NAOS negative controls in triplicate; 4-6 = ANTS labeled NAOS treated with both *BuGH2C* and *BuGH117B* in triplicate; 7 = G_1A_1 (DP2) standard; 8 = free ANTS; 9 = ANTS-labeled Gal. Samples were labeled with ANTS in the presence of 2-picoline borane.

3.1.6 *BuGH117B* and *BuGH2C* digestions of ANTS-labeled NAOS

As determined above, both exolytic enzymes *BuGH117B* and *BuGH2C* are required for efficient agarolysis of NAOS generated by *BuGH86*. To determine if the addition of ANTS to the reducing end of NAOS compromised the efficiency of exolysis from the non-reducing end, complementary enzyme digests were performed on ANTS-labeled substrates. As expected, *BuGH117B* and *BuGH2C* were effective at hydrolyzing unlabeled NAOS (Figure 3.4B). In the presence of ANTS the larger fragments of NAOS were hydrolyzed, but the digestion of G_1A_1 (DP2) was inhibited (Figure 3.4C, D). This result was not altered by increasing the time of enzyme treatment (Figure 3.4C, D). This suggests that when ANTS is positioned at the reducing end of NAOS it creates a steric or

chemical hindrance to prevent the digestion of G_1A_1 (DP2), thereby preventing the complete saccharification of *BuGH86* products.

3.2 Production of manno oligosaccharides (MOS) and analysis of ANTS-labeled MOS bioactivity.

3.2.1 Optima of α 1,6 yeast mannan saccharification for MOS generation

Recombinant α 1,6 mannanases from GH family 76 (BT3782 and BT3792) were purified by IMAC (Figure 3.1; Table 3.1). To confirm their activity on α 1,6 mannan, digestions were performed using α 1,6 yeast mannan as a substrate (Figure 1.5D). As anticipated, BT3782 and BT3792 yielded different product profiles (Figure 3.5A; [43]). BT3792 predominantly generated MOS with a DP of >3 while BT3782 predominantly generated oligosaccharides with a DP of 1- 3. The presence of multiple products demonstrates the endolytic properties of these enzymes (Figure 3.5A, B). As previously described, [43] these enzymes are not active on the fully branched wild-type yeast mannan (Figure 1.5A) but do digest the yeast mannan backbone which is a homopolysaccharide of α 1,6 linked mannose. This observation suggests that GH76s cannot accommodate the branch points or extended sidechains within their active site, which is confirmed by the cleft-structure and subsite architecture of GH76s with known structures [43].

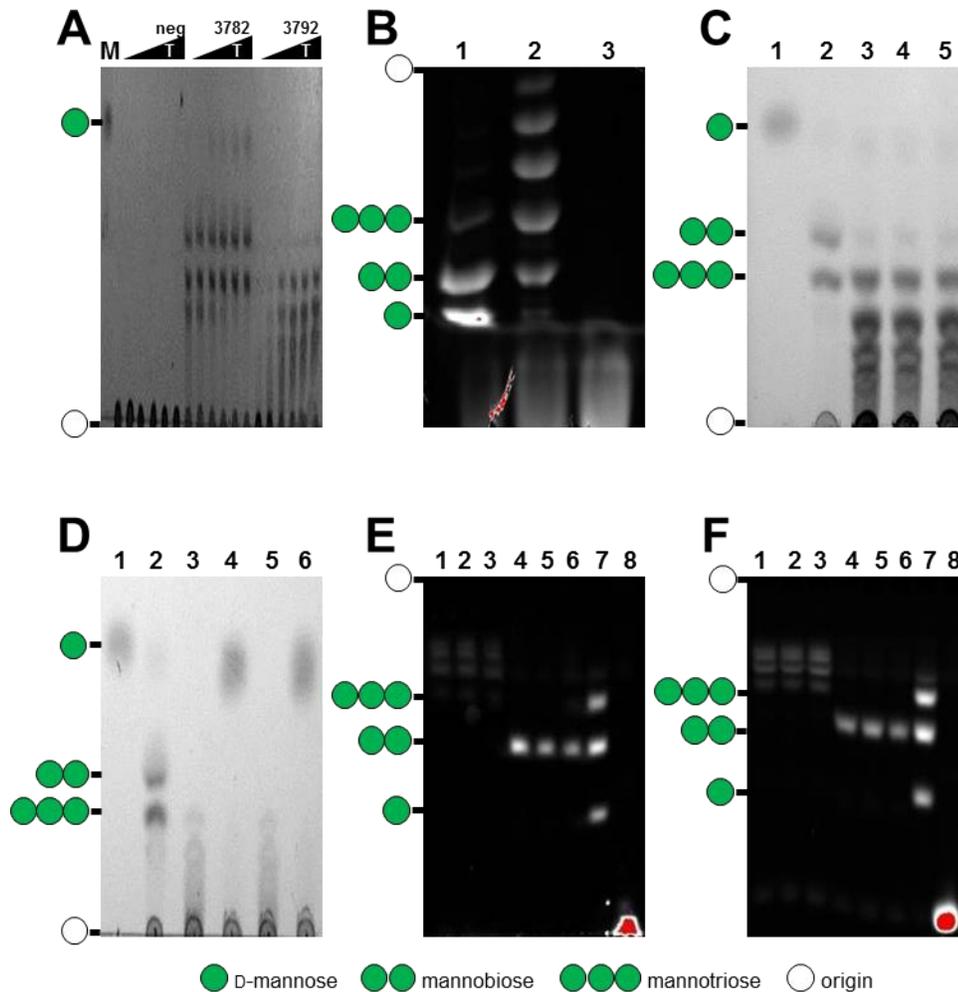


Figure 3.5: Generation of MOS from α 1,6 mannan and ANTS-labeling for enzymatic assays. (A) MOS generation over a time course. M = D-mannose marker, the next five lanes show negative control over time, the next five lanes show digestion of MOS from α 1,6 mannan in the presence of BT3782 over time, and the last five lanes show digestion of MOS from α 1,6 mannan in the presence of BT3792 over time. Increasing gradient is indicated by a black triangle over respective lanes where T= time (B) FACE gel contains labeled MOS generated from BtGH76. Lane 1 = MOS generated by the hydrolysis of α 1,6 mannan in the presence of BT3782, 2 = MOS generated by the hydrolysis of α 1,6 mannan in the presence of BT3792, and 3 = free ANTS. MOS was labeled with ANTS in the presence of 2-picoline borane (C) TLC of MOS generation from BT3792 and α 1,6 mannan for labeling and enzymatic assays. Lane 1 = D-mannose and 2 = MOS standard (i.e. D-mannose, mannobiose, mannotriose). 3-5 are generated MOS (D) TLC of unlabeled MOS controls. Lane 1 =D-mannose, 2 = MOS standard, 3 = BT3792 generated MOS, 4 = BT3781 treated MOS for 1 hour, 5 = BT3792 generated MOS, 6 = BT3781 treated MOS for 24 hours. FACE gels of a 1-hour (E) and 24-hour (F) digestion of ANTS-labeled MOS with BT3781. Lanes 1-3 = ANTS labeled MOS negative controls in triplicate; 4-6 = ANTS labeled MOS treated with BT3781 in

triplicate; 7 = MOS standard; 8 = free ANTS. Samples were labeled with ANTS in the presence of 2-picoline borane.

3.2.2 Fluorescent labeling of MOS with ANTS

In order to generate MOS for labeling and bioactivity experiments, large scale digests were carried out with BT3782 (not shown) and BT3792 (Figure 3.5C). Products were comparable to the MOS generated in small scale digests. MOS was labeled with ANTS as previously described [93] and visualized by FACE (Figure 3.5B). The labeling of different populations of MOS generated from both *BtGH76* α 1,6 mannanases were distinguishable. ANTS-labeled MOS generated from BT3782 showed MOS with a DP 1-3 as well as trace amounts of DP 4 (Figure 3.5B). FACE of BT3792 generated MOS labeled with ANTS showed fluorescently labeled oligosaccharides of larger DP in the range of 2-6 (Figure 3.5B).

3.2.3 Enzyme activity on labeled MOS

To test the bioactivity of ANTS-labeled MOS, digestions with *BtGH125*, an exolytic α 1,6-mannosidase that releases pure mannose from α 1,6-MOS (Figure 3.5D; [43]), were performed. On the fluorescent substrates, *BtGH125* displayed a more limited activity, and behaved in a similar manner to what was observed with the exolytic agarases on ANTS-labeled NAOS. *BtGH125* was unable to hydrolyse MOS smaller than a disaccharide, which suggests that ANTS creates a steric or chemical hindrance that prevents labeled-mannobiose from being correctly positioned within the active site (Figure 3.5E, F). This observation is consistent with what has been described for the structure of GH125 active site [43, 69].

3.3 Production of pectic oligosaccharides (POS) and analysis of ANTS-labeled POS bioactivity.

Digestion of HG by the endolytic GH28 from *Rhizopus* sp. (*RhGH28*) resulted in the release of POS with a DP range of 2-4 (Figure 3.6A). The efficiency of the release of these products increased with time and enzyme concentration. Small scale digests were scaled up to generate preparative amounts of POS for labeling and bioactivity experiments.

3.3.1 Fluorescent labeling of POS with ANTS

Previously, POS was successfully labeled with ANTS using a modified protocol from what is commonly performed on neutral carbohydrates [90]. These conditions were reproduced, and the efficiency of labeling was monitored using FACE. POS with a DP ranging from 1-4 was detected (Figure 3.6B). The inherent charge of GalA appears to hinder the resolving power of different sized ANTS-labeled POS (Figure 3.6B; lane 1) when compared to the neutral carbohydrates mannose, Gal, and 3,6-LGal. Neutral carbohydrates such as mannose, Gal, and 3,6-LGal appear to resolve better than the charged GalA and POS residues.

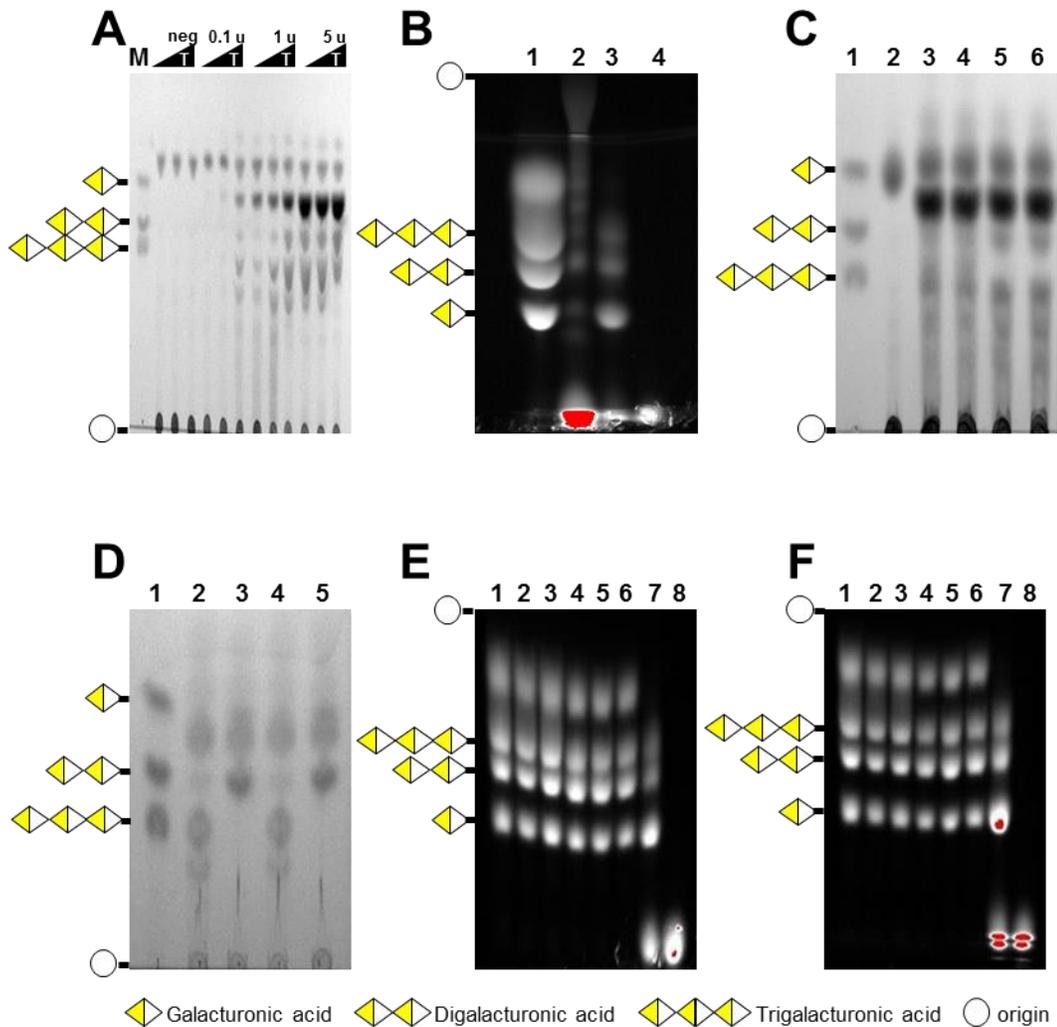


Figure 3.6: Generation of POS from HG and labeling for enzymatic assays. (A) POS generation over a time course. M = GalA, GalA₂, and GalA₃ trisaccharide marker (i.e. POS standard), the next three lanes show negative control over time, the subsequent three lanes show digestion of HG in the presence of 0.1 unit of *RhGH28* over time, the next three lanes show digestion of HG in the presence of 1 unit of *RhGH28* over time, and the last three lanes show digestion of HG in the presence of 5 units of *RhGH28* over a time course of enzyme. Increasing gradient is indicated by a black triangle over respective lanes where T= time (B) FACE gel contains labeled POS generated from *RhGH28*. Lane 1 = POS, 2 = HG, 3 = GalA, GalA₂, and GalA₃ trisaccharide marker (i.e. POS standard), 4 = free ANTS. POS was labeled with ANTS in the presence of 2-picoline borane (C) TLC of POS generation from HG in the presence of *RhGH28* for labeling and enzymatic assays. Lane 1 = POS standard, 2 = negative control, 3-6 = POS generated for enzymatic assays. (D) TLC of unlabeled POS controls. Lane 1 = POS standard, 2 = POS, 3 = *YeGH28* treated POS for 1 hour, 4 = POS, 5 = *YeGH28* treated POS for 24 hours. FACE gels of a 1-hour (E) and 24-hour (F) digestion of ANTS-labeled POS with *YeGH28*. Lanes 1-3 = ANTS labeled POS negative controls in triplicate; 4-6 = ANTS labeled POS

treated with *YeGH28* in triplicate; 7 = POS standard; 8 = free ANTS. Samples were labeled with ANTS in the presence of 2-picoline borane.

3.3.2 Exopolygalacturonase digestion of ANTS-labeled POS

To test the relative digestibility of ANTS-labeled POS compared to unlabeled POS, fluorescent products were digested with the GH28 exopolygalacturonase from *Yersinia enterocolitica* ATCC 9610D [80] and visualized by FACE (Figure 3.6D-F). The digestion pattern was not as pronounced as what was observed for NAOS and MOS; however, a subtle shift was detected for a subpopulation of tetragalacturonic acid. It should be noted that this enzyme characteristically releases disaccharides from POS oligosaccharides and HG (Figure 3.6D). This result suggests that *YeGH28* is not as sensitive for evaluating the release from ANTS-labeled products as the monosaccharide releasing exolytic enzymes *BuGH117B* and *BuGH2C* were for NAOS, and *BtGH125* was for MOS. It might also be more sensitive to the presence of the fluorophore compared to the other investigated enzymes. Regardless, the diagnostic digests of ANTS-labeled POS indicate that the addition of a chemical fluorophore to the reducing end impedes substrate recognition and / or modification of POS (Figures 3.6E and F) by *YeGH28*. Based on enzyme assays of labeled MOS and NAOS, it is predicted that a monosaccharide releasing exopolygalacturonase, with a smaller requirement for subsite recognition (<4 subsites), may be a more sensitive assay for the digestions of ANTS-labeled POS.

Chapter 4

Discussion

An understanding of the mechanisms driving prebiotic-bacterial interactions will be essential for validating their efficiency and establishing them as growth promoters in livestock health. Currently, most investigations of prebiotic effects are limited to indirect observations such as measuring the effects of prebiotics on animal weight or growth rate; and changes to microbial community structure. Indeed, research tools for directly studying prebiotic-bacterial interactions within complex communities are lacking. To address this need, purified oligosaccharides that represent promising prebiotics for animal agriculture, NAOS, MOS, and POS, were generated using determined enzyme pathways and then successfully labeled with ANTS so they could be evaluated as bioactive probes. These compounds may represent valuable first-in-class probes for characterizing interactions with bacteria that colonize the large intestine of animals. In the following sections I will first discuss the enzyme reactions that I developed to produce defined oligosaccharide substrates for ANTS-labeling, and then discuss their potential for downstream applications by evaluating their retention of bioactivity.

4.1 Pathways for the generation of structurally defined NAOS, MOS, and POS

4.1.1 Characterization of a novel agarolytic pathway from the intestinal bacterium B. uniformis NP1 and the design of an enzymatic pathway for generating NAOS from agarose.

Previously, it has been suggested that microorganisms colonizing the large intestine of terrestrial animals are not agarolytic [94]. This was recently proven otherwise [54] where it was demonstrated that *B. uniformis* NP1 is able to grow on agar. The genes responsible

for agarolysis; however, were not identified nor their activity investigated. Here *BuGH86*, *BuGH2C*, and *BuGH117B*, three agarases from the putative *B. uniformis* NP1 agarolytic pathway, have been cloned and characterized.

4.1.1.1 BuGH86 is a keystone agarase for the initial deconstruction of agarose

The enzyme *BuGH86* was observed to be an endolytic agarase that generates NAOS ranging in size from neoagarobiose (DP2) to large oligosaccharides (Table 3.1; Table 3.2; Figure 3.2). Significantly, this is the first agarolytic enzyme from the GH86 family which originates from a bacterium colonizing the human large intestine. *BuGH86* appears to have an analogous role to *BpGH86A* from the human symbiont *Bacteroides plebeius* DSM17135, which endolytically cleaves the β 1,4 glycosidic bond in porphyran [54, 65]. *BuGH86*, therefore, is likely a key enzyme for the release of initial fragments from agarose for further hydrolysis by other members of the *B. uniformis* agarolytic pathway (Figure 4.1A).

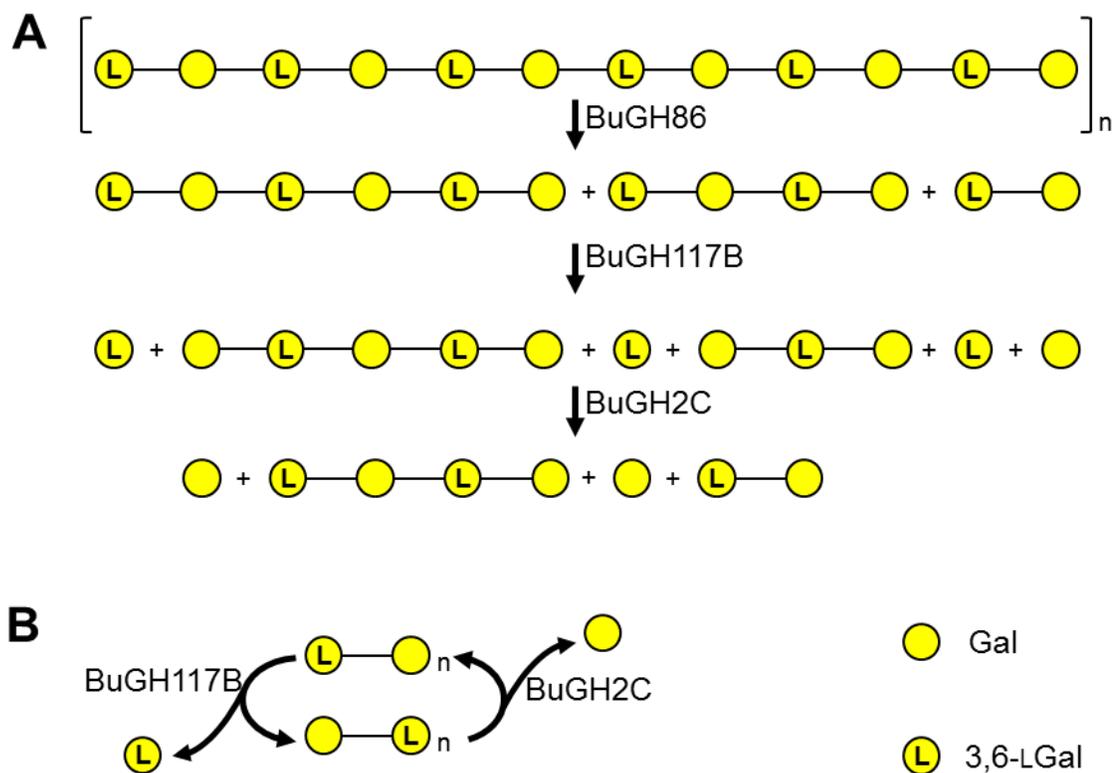


Figure 4.1: Model depicting an enzyme pathway for efficient agarose saccharification using three recombinant enzymes expressed from *B. uniformis* NP1.

(A) Agarolysis initiates with the activity of the endolytic *BuGH86*. NAOS released by *BuGH86* are substrates for the exolytic *BuGH117B* and *BuGH2C*, which systematically remove 3,6-LGal and Gal respectively. (B) The activities of *BuGH117B* and *BuGH2C* are cyclical. Each enzyme continuously generates oligosaccharides which are natural substrates for the other enzyme for efficient deconstruction.

All the products generated by *BuGH86* are present in equal stoichiometry without any clear preference for the accumulation of a main product, which is also comparable to the profile of *BpGH86A*, but is not consistent with agarases originating from marine bacteria. For example, AgaO from *Microbulbifer thermotolerans* JAMB-A94 generates a wide range of oligosaccharides in the first thirty minutes of digestion. The longer oligosaccharides are then used as substrates and further cleaved into oligosaccharides of

DP 2-6 and with NAOS of DP 6 as the main product over longer duration [95]. Alternatively, NAOS generated by a GH86 agarase from a non-marine *Cellvibrio* sp. OA-2007 had a DP of 2-6 without the generation of any intermediate larger oligosaccharides [96]. It should be noted that reaction conditions for enzymatic hydrolysis of agarose by *BuGH86* and these other enzymes varied. For example in this study, digestion was carried out with 2% agarose, at pH 7 and 37°C. Alternatively in the other studies, digestion was carried out with 1% agarose, pH 6.5 and 42.5°C for a GH86 agarase from a non-marine *Cellvibrio* sp. OA-2007 [96] and with 1% agarose, pH 7.5 and 40°C for AgaO [95].

4.1.1.2 BuGH2C and BuGH117B are exolytic agarases that release monosaccharides from agarose fragments

The digestion of agarose with *BuGH86* generates a NAOS ladder. This observation suggests that other enzymes are required to saccharify these products into their monosaccharide constituents. *BuGH2C* and *BuGH117B* were predicted to perform these exolytic functions based upon their assignment into GH family 2 and 117, respectively [49]. Firstly, their activity was tested on gelled agarose. Independently, both *BuGH2C* and *BuGH117B* released very low or undetectable amounts of products from agarose (Figure 3.3A); however, when combined together both Gal and 3,6-LGal were efficiently released in stoichiometric amounts (Figure 3.3A-C). This indicates that the two linkages in agarose are being digested systemically by these two enzymes. Stepwise digests of NAOS by *BuGH117B* revealed that this enzyme cleaves 3,6-LGal from the non-reducing end to generate AOS products (Figure 3.3; Figure 4.1A), which is consistent with the described functions of other GH117s including *BpGH117*[65], *ZgAhGA* [97], and an α -NAOS hydrolase from *Bacillus* sp. MK03 [98] on other marine polysaccharides with

similar repeating structures to agarose. This confirms that there is at least one enzyme in the *B. uniformis* NP1 agarose degradation pathway (Appendix 1) that is responsible for the cleavage of α -glycosidic bonds.

Subsequently, *BuGH2C* cleaves the exposed Gal residue at the non-reducing end of AOS (Figure 3.3; Figure 4.1A). Significantly, this is the first agarolytic activity assigned to the GH2 family, but is consistent with the common theme of GH2, which is a family enriched in galactosidases that are active on diverse substrates, including lactose, lactulose, galactosylarabinose, *o*-nitrophenyl- β -D-galactopyranoside, *p*-nitrophenyl- β -D-galactopyranoside and 5-bromo-4-chloro-indoyl- β -D-galactopyranoside (X-gal) [99-101]. The removal of Gal from AOS results in the regeneration of a NAOS substrate that has been reduced by one 3,6-LGal-Gal disaccharide (Figure 3.3; Figure 4.1). This finding validates that *BuGH117B* and *BuGH2C* are monosaccharide releasing agarases, which together with the demonstrated endolytic activity of GH86, provide a fully functional pathway for the saccharification of agarose by *B. uniformis* NP1 (Figure 4.1B).

4.1.1.3 The role of methylation in agarolysis

The observation that both solid and LMP forms of agarose are digested by GH86, GH2 and GH117 reveals that variations in the levels of methylation do not impede agarolysis. In nature these modifications disrupt inter-chain bonding and are prohibitive to gelation, which keeps agarose in solution at lower temperatures. Methylations are not unique to agarose. In porphyran, a structural marine polysaccharide similar to agarose, the 3,6-LGal is replaced by L-galactose-6-sulfate with C-6 methylations occurring on the Gal residue [54]. Evidence for how these carbohydrates are accommodated within the active site of *BpGH86A* has been provided [54]. A small pocket in the active site of *BpGH86A* is able

to accommodate the C-6 methylations on the Gal residue in porphyran but which is lacking in the GH16 porphyranases [54].

4.1.1.4 *B. uniformis* NPI may play a direct or indirect role in large intestine health

The observation that agarolysis is performed by a bacterial strain that colonizes the intestine of terrestrial animals is intriguing, and much could be speculated on the origin and function of this pathway. In the case of *B. plebeius* DSM17135, the metabolism of porphyran was hypothesized to have been acquired through horizontal gene transfer from marine agarolytic species that had contaminated marine foods ingested by the host, such as *Porphyra* (nori; used to prepare sushi), *Undaria* (wakame), and *Ulva* (sea lettuce), which contain porphyran in their cell walls. Similar to porphyran, agars (which consists of agarose and agaropectin. Agaropectin is structurally similar to agarose including substitutions of the hydroxyl groups of some of the 3,6-LGal with sulfoxy, methoxy and pyruvate residues) are found in the cell wall of marine red algae such as *Gracilaria* spp. and *Gelidium* spp. [64, 67, 95]. *Gracilaria* spp are eaten as Ogonori in Japanese cuisine and the Chinese Feng food. Historically, agar has been used as a gelling agent in foods such as bakery products, desserts, sweets, ice cream, jelly, dairy products and traditional Japanese foods such as Yokan, Mitsumame, and Tokoroten made from *Gelidium* spp. [53, 102, 103]. It is possible that these innovations for the food industry have provided both a source of agarolytic DNA from contaminating marine microorganisms and the appropriate selective pressure to facilitate the transfer of this pathway into a terrestrial bacterium.

Hence the health implications of agarolysis products for the host are of great importance.

It has been suggested that products of agar metabolism have pharmaceutical effects and

health benefits [104]. For example, NAOS exhibit prebiotic effects for the growth of bifidobacteria and lactobacilli in mice [68]; although, such effects likely result from syntrophic relationships with undefined agarolytic intestinal bacteria. Also, AOS possess anti-oxidative properties for scavenging hydroxyl free radicals and superoxide anion radicals which can be damaging to the host cells [105]. Since these effects were elicited by dietary oligosaccharides that were generated *in vitro* by exogenous enzymes, it would be of value to determine whether these effects are also induced by agarolytic bacteria within the large intestine when supplied with dietary agarose. In addition, it would be useful to determine if such metabolism of NAOS and release of fermentation by-products by agarolytic bacteria could help modulate the large intestine microbiota and host responses.

4.2 Generating ANTS-Labeled NAOS, MOS and POS for use as biological probes

A primary goal of this research project was to generate ANTS-labeled oligosaccharides that could be used as biological probes for studying bacteria-carbohydrate interactions within complex microbial communities. In order for such probes to have uptake, they must perform similar functions to the unlabeled, native versions of the carbohydrate – i.e. the addition of the chemical label must not significantly alter the bioactivity of the carbohydrate. For this research project, the utility of ANTS-labeled NAOS, MOS, and POS to function in this capacity were evaluated using exolytic enzymes to compare digestion patterns between labeled and unlabeled versions of the same substrates.

4.2.1 Digestion patterns differ in labeled NAOS, MOS and POS

Generally, the covalent addition of ANTS did not inhibit the digestion patterns of larger oligosaccharides for NAOS and MOS (Figure 3.4, 3.5 and 3.6); however, the digestion of

small NAOS and MOS was consistently affected. For example, for ANTS-labeled NAOS, oligosaccharides were readily cleaved down to DP2 by *BuGH2C* and *BuGH117B*. This is contrary to unlabeled NAOS, which were completely digested into 3,6-LGal and Gal (Figure 3.4). Similarly to ANTS-labeled MOS, oligosaccharides of higher DP are also cleaved down to DP2 by the monosaccharide releasing α 1,6-mannosidase BT3781; whereas, unlabeled versions of MOS is completely hydrolyzed into mannose (Figure 3.5).

The observation that small ANTS-labeled oligosaccharides are refractory to digestion suggests that the chemical label interferes with binding in the core subsites of the enzymes (Figure 4.2).

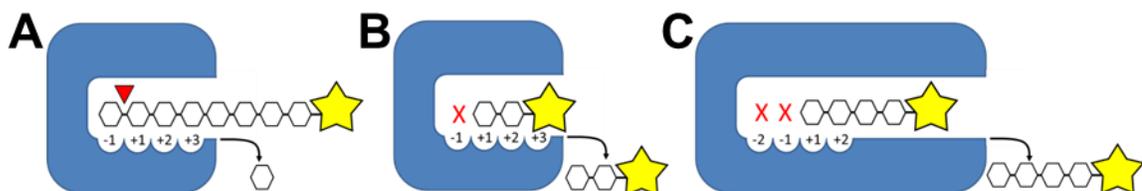


Figure 4.2: Schematic models of the exolytic digestion of carbohydrate substrates.

(A) Model for how ANTS-labeled NAOS and MOS are modified initially. Exolytic enzymes digest carbohydrates from the non-reducing end. The scissile bond (indicated by red arrow) must be properly positioned by interactions between subsites and substrate residues and monomers. In this example, a monosaccharide is released following hydrolysis. The presence of a fluorophore at the reducing end of the substrate can prevent substrate recognition. (B) Model for how ANTS-labeled disaccharides restrict access to the catalytic machinery of BT3781 and exolytic agarases. When the substrate in A has been modified up to the disaccharide, further hydrolysis is hindered (C) Model for how ANTS-labeled POS are not modified by *YeGH28*.

Individual exolytic enzymes have different requirements of binding energy that are provided by interactions with subsites in the minus (-1, -2, -3, etc) direction (i.e. towards the non-reducing end) and plus (+1, +2, +3, etc) direction (i.e. towards the reducing end) [106], Fig. 4.2A). CAZymes that can process substrates completely to monosaccharides

typically have a minimum requirement of two primary subsites: the -1 and +1 subsites, which interact with the sugar residue on either side of the scissile bond. For example, BT3781 systematically cleaves α 1,6 MOS to monosaccharides (Figure 3.5). In the case of BT3781 with labeled MOS, BT3781 is able to release mannose until only labeled manno- α 1,6 mannobiose remains (Figure 3.5). Even though the GH125 active site is suited for accommodating α 1,6 manno- α 1,6 mannobioses [69], further hydrolysis is impeded by the steric obstruction of the ANTS fluorophore at the reducing end of the α 1,6 manno- α 1,6 mannobiose. Although this does not provide any insight into the rate of the reaction, it does indicate that the enzyme will cleave a Man- α 1,6-Man bond as long as the -1 and +1 subsites are occupied and the glycosidic bond is properly positioned. Therefore, the observation that BT3781 only digests ANTS-labeled MOS to manno- α 1,6 mannobiose suggests that the chemical label occludes interactions of manno- α 1,6 mannobiose with the -1 and +1 subsites (Fig. 4.2B).

Similarly to MOS, NAOS were digested to small oligosaccharides by the combinatorial reactions catalyzed by *Bu*GH2 and *Bu*GH117. The structure of *Bp*GH117 has been reported, which is predicted to be similar to *Bu*GH117B (81% amino acid sequence identity), and illuminates how the fluorophore may interfere with digestion of neoagarobiose. *Bp*GH117 possesses a pronounced pocket which is typical for exolytic glycoside hydrolases [65]. The pocket of *Bp*GH117 is able to accommodate neoagarobiose with Gal at the +1 subsite and 3,6-LGal at the -1 subsite [65]. The modification of Gal at the reducing end by ANTS would prevent the accommodation of the labeled neoagarobiose residue.

Unlike the digestion profiles of NAOS and MOS, *Ye*GH28 does not digest ANTS-labeled POS to small oligosaccharides. This was in contrast to its efficient digestion of unlabeled

POS (Figure 3.6), [80]. It appears that only a small percentage of ANTS-labeled POS, ranging in size from a DP4 to DP6, was hydrolyzed by *YeGH28* (Figure 3.6). Based upon these results, it appears that when ANTS is covalently attached to the reducing end of POS it presents a steric or chemical obstacle that is detrimental to *YeGH28* activity (Fig 4.2C). Although further experiments could be performed, including the characterization of potential interactions of ANTS-labeled POS with POS transporters and other classes of pectinases (e.g. polysaccharide lyases), it appears that ANTS-labeled POS may not be an effective probe for investigating the interactions of pectic carbohydrates with *YeGH28*. Importantly, these findings suggest that the utility of fluorescent probes is dependent upon the carbohydrate species in question, and each should be considered independently.

4.2.2 Applications of ANTS-labeled polysaccharides for improving the bioactivity of fluorescent carbohydrate probes

The digests with exolytic enzymes performed here suggest that ANTS-labeled oligosaccharides may have limited applications as probes for studying prebiotic-bacteria interactions. Using polysaccharides for these types of interactions may provide an alternative to these limitations. With larger substrates, the chemical modification at the reducing end would be more distal to the active site residues, which reduces the potential for steric or chemical clashes. Furthermore, the SusD-like and SusE-like binding proteins on the surface of Bacteroidetes are designed to bind larger carbohydrates with high affinity due to avidity effects [107]. Importantly, based upon the examples that have been studied to date, these classes of proteins do not interact with the reducing-end, rather recognize the internal residues within extended polysaccharide chains [40]. These observations imply that ANTS-labeled polysaccharides may retain a higher level of

bioactivity than oligosaccharides. Increasing the DP of the carbohydrate lowers the fluorophore:sugar ratio, however, which may lower the potency of the probes.

Chapter 5

Conclusions and Future Directions

5.1 Conclusions

Antimicrobial use is currently an important component of modern livestock production. The cost of this practice has been detrimental, however, as antibiotic resistance in bacteria found in human and animals, and the environment have been documented. The increased presence of antimicrobial resistance factors is likely to lead to the reduced effectiveness of antimicrobial agents used to treat human and animal infections. In order to maintain the delicate balance between sustainable livestock production and protecting human health, alternatives to antibiotics must be developed. Understanding how prebiotics are utilized in the large intestine of livestock will be foundational for evaluating their potential benefits and applications, developing new prebiotics, and ultimately fostering uptake by the industry.

In order to gain some insight into the potential of NAOS, MOS and POS as prebiotics, I have developed a two-step approach for generating and evaluating the bioactivity of fluorescent oligosaccharides. The first step was to enzymatically digest polysaccharides (i.e. agarose, α 1,6-mannan, and HG) with endolytic hydrolases that produce ladders of oligosaccharides. The products were then fluorescently labeled and digested with exolytic enzymes to evaluate their retention of bioactivity in comparison to their unlabeled counterparts. The outcomes for each carbohydrate species are summarized below.

NAOS was successfully generated from both solid agarose and LMP using the endolytic agarase *BuGH86* from *B. uniformis* NP1. The oligosaccharides generated from both forms of agarose yielded NAOS of DP ranging from 2-22, as confirmed by mass

spectrometry. Consistently, NAOS generated from LMP was found to be decorated with various levels of methylation, which is known to increase the solubility of this polysaccharide at lower temperatures. *BuGH2C* and *BuGH117B* were characterized as exolytic agarases that systematically remove 3,6-L-Gal from the non-reducing end of NAOS and Gal from the non-reducing end of AOS, respectively. This is the first report of enzymes from GH families 2, 86 and 117 found within the genomes of bacteria that colonize the large intestine of terrestrial animals being capable of using agarose as a substrate. ANTS-labeled NAOS was digested to a DP of 2 using *BuGH2C* and *BuGH117B*, which indicates that bioactivity, is retained for larger NAOS fragments (DP >2).

MOS was generated from α 1,6-yeast mannan by the enzymes BT3782 and BT3792 [43]. Both are endolytic members of GH76 from *B. thetaiotaomicron* *BtGH76* that are specific for the α 1,6-mannan backbone of yeast mannan. BT3782 generates MOS of smaller DP (1-3) than BT3792 generated MOS (DP \geq 3). Both MOS forms were further processed by *BtGH125* which releases mannose from the α 1,6-MOS of various DPs [43]. Similar to NAOS, ANTS-labeled MOS was digested to a DP of 2 using *BtGH125*, which indicates that bioactivity is retained for larger MOS fragments (DP >2).

POS was generated from HG by the endolytic polygalacturonase *RhGH28*. The product sizes at completion were of DP >4. *RhGH28* products were successfully digested by *YeGH28*, a disaccharide releasing exopolygalacturonase, to final size of DP2 [80]. In contrast to NAOS and POS, the addition of ANTS to the reducing end of POS appears to inhibit the modification of the substrate by *YeGH28*.

The salient findings of this research can be summarized as follows, (i) *B. uniformis* NP1 possesses enzymes tailored for agarolysis, (ii) *BuGH86* is an endolytic enzyme from *B. uniformis* NP1 involved in a primary step of generating NAOS for refining in a concerted and simultaneous effort with other enzymes of *B. uniformis* NP1, (iii) *BuGH117B* is an exolytic enzyme necessary for cleaving 3,6-LGal from the non-reducing end of NAOS, (iv) *BuGH117B* generates AOS, which is a substrate for *BuGH2C*, (v) *BuGH2C* is the first exolytic CAZyme necessary for cleaving Gal from the non-reducing end of AOS, (vi) *BuGH2C* generates NAOS, which is a substrate for *BuGH117B*, (vii) *BuGH2C* and *BuGH117B* work synergistically and systematically to saccharify NAOS generated by endolytic enzymes of *B. uniformis* NP1, (viii) *B. uniformis* NP1 enzymes can digest both LMP and solid agarose, (ix) *B. uniformis* NP1 enzymes are active on methylated agarose and NAOS, (x) NAOS, MOS and POS can be fluorescently labeled with ANTS, (xi) enzymes expressed from select intestinal bacteria and pathogenic bacteria can interact with and modify ANTS-labeled NAOS and MOS, whereas labeled POS is refractory to digestion, (xii) the utility of ANTS-labeled probes is carbohydrate specific, and (xiii) ANTS-labeled substrates can be used to probe the subsite architecture of CAZymes, which is a complementary assay to other biochemical and structural methods of subsite determination.

In conclusion, based upon the findings of this thesis, ANTS-labeled NAOS and MOS could be investigated as *in situ* and *in vivo* probes for detecting interactions with intestinal bacteria. As these compounds are substrates for CAZymes derived from intestinal bacteria it appears likely that they should retain their bioactivity within microbial communities.

5.2 Future directions

The ability of *BuGH86*, *BuGH2C* and *BuGH117B* to utilize LMP agarose despite the presence of methylations is an important observation. Future work could focus on how the methylation density of agarose influences the activity of these enzymes as compared to solid agarose. The effect of abundance of methylations present in LMP and solid agarose on the intestinal bacteria community structure would also be of interest. Also the possibility of beneficial effects for host health by agarolytic bacteria within the large intestine when supplied with dietary NAOS, AOS or agarose merits further study.

The enzymes of *B. uniformis* NP1 work in a concerted manner to deconstruct agarose into its Gal and 3,6-LGal monomers. Another important area of future research would be to characterize the other putative enzymes within the agarolytic pathway (Appendix 1). It would also be important to determine the relevance of both monomers as carbon sources for *B. uniformis* NP1 and for potential syntrophic relationships with other members of the microbiota.

Initial attempts to use ANTS-labeled POS and HG as fluorescent bioactive probes on *B. thetaiotaomicron* VPI-5482 was unsuccessful; examination of bacteria cells with the probes generated in this project using confocal scanning light microscopy failed to detect any difference in fluorescence between *B. thetaiotaomicron* VPI-5482 and $\Delta B. thetaiotaomicron$ [4108-4124] (negative control strain deficient in HG metabolism). The success of the bioactivity enzymatic assays, however, indicates that the labeled probes do interact with biologically relevant proteins. Further efforts towards optimizing the labeling conditions for microscopy using pure cultures and extracted bacterial

communities should be investigated. Screening of fluorophores with increased sensitivity may also be a promising possibility in this regard.

References

1. Mathew, A.G., R. Cissell, and S. Liamthong, *Antibiotic resistance in bacteria associated with food animals: a United States perspective of livestock production*. Foodborne Pathogens and Disease, 2007. **4**(2): p. 115–133.
2. Allen, H.K., *Antibiotic resistance gene discovery in food-producing animals*. Current Opinion in Microbiology, 2014. **19**: p. 25–29.
3. Schmidt, J.W., Agga, G.E., Bosilevac, J.M., Brichta-Harhay, D.M., Shackelford, S.D., Wang, R., Wheeler, T.L., Arthur, T.M. , *Occurrence of anti-microbial resistant Escherichia coli and Salmonella enterica in the beef cattle production and processing continuum*. Appl. Environ. Microbiol, 2015. **81**: p. 713-725.
4. Hooper, D., *Emerging Mechanisms of Fluoroquinolone Resistance*. Emerging Infectious Diseases, 2001. **7**(2): p. 337–341.
5. Inglis, G.D., et al., *Effects of Subtherapeutic Administration of Antimicrobial Agents to Beef Cattle on the Prevalence of Antimicrobial Resistance in Campylobacter jejuni and Campylobacter hyointestinalis*. Applied and Environmental Microbiology, 2005. **71**(7): p. 3872–3881.
6. Donaldson, S.C., et al., *Molecular Epidemiology of Ceftiofur-Resistant Escherichia coli Isolates from Dairy Calves*. Applied and Environmental Microbiology, 2006. **72**(6): p. 3940–3948.
7. Klima, C.L., et al., *Genetic characterization and antimicrobial susceptibility of Mannheimia haemolytica isolated from the nasopharynx of feedlot cattle*. Veterinary Microbiology, 2011. **149**(3-4): p. 390–398.
8. Otto, S.J.G., et al., *Estimating the Number of Human Cases of Ceftiofur-Resistant Salmonella enterica Serovar Heidelberg in Quebec and Ontario, Canada*. Clinical Infectious Diseases, 2014. **59**(9): p. 1281–1290.
9. Andersson, D.I. and D. Hughes, *Antibiotic resistance and its cost: is it possible to reverse resistance?* Nat Rev Micro, 2010. **8**(4): p. 260-271.
10. WHO. *World Health Organization. WHO global principles for the containment of antimicrobial resistance in animals intended for food*. 2000 [cited 2014 June 16]; Available from: www.who.int/salmsurv/links/en/GSSGlobalPrinciples2000.pdf
11. FDA. *Food and Drug Administration. Veterinary Feed Directives: FDA Final Rule and Draft Guidance*. 2015 [Access from FDA website on October 28, 2015].
12. Duff, G.C. and M.L. Galyean, *BOARD-INVITED REVIEW: Recent advances in management of highly stressed, newly received feedlot cattle*. Journal of Animal Science, 2007. **85**(3).

13. Wu, G.D., et al., *Linking Long-Term Dietary Patterns with Gut Microbial Enterotypes*. *Science*, 2011. **334**(6052): p. 105–108.
14. HC. *Health Canada*. 2013 [cited 2015 December 19]; Available from: <http://www.hc-sc.gc.ca/ahc-asc/performance/eval/vdp-evaluation-pmv-eng.php>.
15. CAHI. *Canadian Animal Health Institute*. 2014 [cited 2016 January 06]; Available from: <http://www.cahi-icsa.ca/uploads/UserFiles/files/CAHI%20Press%20Release%20Veterinary%20Antimicrobials%20April%2011.pdf>.
16. Khachatourians, G.G., *Agricultural use of antibiotics and the evolution and transfer of antibiotic-resistant bacteria*. *CMAJ: Canadian Medical Association Journal*, 1998. **159**(9): p. 1129-1136.
17. WHO. *World Health Organization. The evolving threat of antimicrobial resistance: options for action*. 2012 [cited 2014 16 June]; Available from: whqlibdoc.who.int/publications/2012/9789241503181_eng.pdf
18. Ribble, C.S., Stitt, T., Iwasawa, S., Toews, L., Stephen, C. . *A review of alternative practices to antimicrobial use for disease control in the commercial feedlot*. 2010 [cited 2015 October 19]; Available from: www.nccid.ca
19. Costa, E., et al., *Non-therapeutic administration of a model antimicrobial growth promoter modulates intestinal immune responses*. *Gut Pathogens*, 2011. **3**(1): p. 14.
20. Stover, M.G., Watson, R.R., Collier, R.J. , *Pre- and Probiotic Supplementation in Ruminant Livestock Production*, in *Probiotics, Prebiotics, and Synbiotics: Bioactive Foods in Health Promotion*, R.R.a.P. Watson, V.R., Editor. 2016, Academic Press: Cambridge, MA, USA. p. 938.
21. McAllister, T.A., et al., *Review: The use of direct fed microbials to mitigate pathogens and enhance production in cattle*. *Canadian Journal of Animal Science*, 2011. **91**(2): p. 193–211.
22. Quigley, E.M.M., *Prebiotics and probiotics; modifying and mining the microbiota*. *Pharmacological Research*, 2010. **61**(3): p. 213–218.
23. Allen, H.K., et al., *Treatment, promotion, commotion: antibiotic alternatives in food-producing animals*. *Trends in Microbiology*, 2013. **21**(3): p. 114–119.
24. PICARD, C., et al., *Review article: bifidobacteria as probiotic agents - physiological effects and clinical benefits*. *Alimentary Pharmacology and Therapeutics*, 2005. **22**(6): p. 495–512.

25. Jafarei, P., Tajabadi, E.M. , *Lactobacillus acidophylus cell structure and application*. Afr. J. Microbiol, 2011. **5**: p. 4033-4042.
26. Moubareck, C., Gavini, F., Butel, M.J., Doucet-Populaire, F. , *Antimicrobial susceptibility of bifidobacteria*. J. Antimicrob. Chemother, 2015. **55**: p. 38-44.
27. Kumar, M., Nagpal, R., Hemalatha, R., Yadav, H., Marotta, F. , *Probiotics and prebiotics for promoting health: through gut microbiota*, in *Probiotics, Prebiotics, and Synbiotics: Bioactive Foods in Health Promotion*, R.R.a.P. Watson, V.R., Editor. 2016, Academic Press: Cambridge, MA, USA. p. 938.
28. Vieira, A.T., M.M. Teixeira, and F.S. Martins, *The role of probiotics and prebiotics in inducing gut immunity*. Frontiers in Immunology, 2013. **4**: p. 445.
29. Walker, A.W., Lawley, T.D. , *Therapeutic modulation of intestinal dysbiosis*. Pharmacol. Res., 2013. **69**: p. 75-86.
30. Gaggia, F., P. Mattarelli, and B. Biavati, *Probiotics and prebiotics in animal feeding for safe food production*. International Journal of Food Microbiology, 2010. **141 Suppl 1**: p. S15-28.
31. Montagne, L., J.R. Pluske, and D.J. Hampson, *A review of interactions between dietary fibre and the intestinal mucosa, and their consequences on digestive health in young non-ruminant animals*. Animal Feed Science and Technology, 2003. **108**(1-4): p. 95–117.
32. Crittenden, R., Playne, M.J., *Prebiotics*, in *Handbook of probiotics and prebiotics*, Y.K. Lee, Salminen, S., Editor. 2009, John Wiley & Sons: Hoboken, New Jersey.
33. Baines, D., et al., *Aflatoxin, Fumonisin and Shiga Toxin-Producing Escherichia coli Infections in Calves and the Effectiveness of Celmanax®/Dairyman's Choice™ Applications to Eliminate Morbidity and Mortality Losses*. Toxins, 2013. **5**(10): p. 1872–1895.
34. Hasunuma, T., et al., *Effect of cellooligosaccharide or synbiotic feeding on growth performance, fecal condition and hormone concentrations in Holstein calves*. Animal Science Journal, 2011. **82**(4): p. 543–548.
35. Xu, J., Bjursell, M.K., Himrod, J., Deng, S., Carmichael, L.K., Chiang, H.C., Hooper, L.V., Gordon, J.I. , *A Genomic View of the Human-Bacteroides thetaiotaomicron Symbiosis*. Science, 2003. **299**(5615): p. 2074–2076.
36. Flint, H.J., et al., *Polysaccharide utilization by gut bacteria: potential for new insights from genomic analysis*. Nature Reviews Microbiology, 2008. **6**(2): p. 121–131.

37. Jeffery, I.B., et al., *Categorization of the gut microbiota: enterotypes or gradients?* Nat Rev Micro, 2012. **10**(9): p. 591-592.
38. Spor, A., O. Koren, and R. Ley, *Unravelling the effects of the environment and host genotype on the gut microbiome.* Nat Rev Micro, 2011. **9**(4): p. 279-290.
39. Walter, J. and R. Ley, *The Human Gut Microbiome: Ecology and Recent Evolutionary Changes.* Annual Review of Microbiology, 2011. **65**(1): p. 411-429.
40. Martens, E.C., et al., *The Devil Lies in the Details: How Variations in Polysaccharide Fine-Structure Impact the Physiology and Evolution of Gut Microbes.* Journal of Molecular Biology, 2014. **426**(23): p. 3851–3865.
41. Kim, M., M. Morrison, and Z. Yu, *Status of the phylogenetic diversity census of ruminal microbiomes.* FEMS Microbiology Ecology, 2011. **76**(1): p. 49-63.
42. Wexler, H.M., *Bacteroides: the Good, the Bad, and the Nitty-Gritty.* Clinical Microbiology Reviews, 2007. **20**(4): p. 593–621.
43. Cuskin, F., et al., *Human gut Bacteroidetes can utilize yeast mannan through a selfish mechanism.* Nature, 2015. **517**(7533): p. 165–169.
44. Abbott, D.W., et al., *Coevolution of yeast mannan digestion: Convergence of the civilized human diet, distal gut microbiome, and host immunity.* Gut Microbes, 2015. **6**(5): p. 334–339.
45. Rakoff-Nahoum, S., M.J. Coyne, and L.E. Comstock, *An Ecological Network of Polysaccharide Utilization among Human Intestinal Symbionts.* Current Biology, 2014. **24**(1): p. 40–49.
46. Rogowski, A., et al., *Glycan complexity dictates microbial resource allocation in the large intestine.* Nat Commun, 2015. **6**.
47. Koropatkin, N.M., et al., *Starch Catabolism by a Prominent Human Gut Symbiont Is Directed by the Recognition of Amylose Helices.* Structure, 2008. **16**(7): p. 1105–1115.
48. Xu, J., et al., *Evolution of symbiotic bacteria in the distal human intestine.* PLoS Biology, 2007. **5**(7): p. e156.
49. Lombard, V., et al., *The carbohydrate-active enzymes database (CAZy) in 2013.* Nucleic Acids Research, 2014. **42**(Database issue): p. D490-D495.
50. Martens, E.C., et al., *Recognition and degradation of plant cell wall polysaccharides by two human gut symbionts.* PLoS Biology, 2011. **9**(12): p. e1001221.

51. Sonnenburg, E.D., et al., *Specificity of polysaccharide use in intestinal Bacteroides species determines diet-induced microbiota alterations*. Cell, 2010. **141**(7): p. 1241-1252.
52. Kaoutari, A.E., et al., *The abundance and variety of carbohydrate-active enzymes in the human gut microbiota*. Nat Rev Micro, 2013. **11**(7): p. 497-504.
53. Li, M., et al., *Isolation and Characterization of an Agaro-Oligosaccharide (AO)-Hydrolyzing Bacterium from the Gut Microflora of Chinese Individuals*. PLoS One, 2014. **9**(3): p. e91106.
54. Hehemann, J.-H., et al., *Bacteria of the human gut microbiome catabolize red seaweed glycans with carbohydrate-active enzyme updates from extrinsic microbes*. Proceedings of the National Academy of Sciences, 2012. **109**(48): p. 19786–19791.
55. Cummings, J.H. and A.M. Stephen, *Carbohydrate terminology and classification*. European Journal of Clinical Nutrition, 2007. **61**: p. S5-S18.
56. Sonnenburg, E.D. and J.L. Sonnenburg, *Starving our Microbial Self: The Deleterious Consequences of a Diet Deficient in Microbiota-Accessible Carbohydrates*. Cell Metabolism, 2014. **20**(5): p. 779–786.
57. McNeil, N.I., *The contribution of the large intestine to energy supplies in man*. Am J Clin Nutr, 1984. **39**: p. 338-342.
58. France, J., Dijkstra, J., *Volatile fatty acid production*, in *Quantitative aspects of ruminant digestion and metabolism*, J. Dijkstra, Forbes, J.M., France, J. , Editor. 2005, CABI Publishing: Wallingford, UK. p. 157–175.
59. Lee, S.M., et al., *Bacterial colonization factors control specificity and stability of the gut microbiota*. Nature, 2013. **501**(7467): p. 426–429.
60. Koropatkin, N.M., E.A. Cameron, and E.C. Martens, *How glycan metabolism shapes the human gut microbiota*. Nature Reviews Microbiology, 2012. **10**: p. 323-335.
61. Withers, S., and Williams, S. . *Glycoside hydrolases in CAZypedia*. 2013 [cited 2015 December 28]; Available from: <http://www.cazypedia.org>.
62. McCarter, J.D. and G. Stephen Withers, *Mechanisms of enzymatic glycoside hydrolysis*. Current Opinion in Structural Biology, 1994. **4**(6): p. 885–892.
63. Rye, C.S. and S.G. Withers, *Glycosidase mechanisms*. Current Opinion in Chemical Biology, 2000. **4**(5): p. 573–580.

64. Allouch, J., et al., *The Three-dimensional Structures of Two α -Agarases*. Journal of Biological Chemistry, 2003. **278**(47): p. 47171–47180.
65. Hehemann, J.-H., et al., *Analysis of Keystone Enzyme in Agar Hydrolysis Provides Insight into the Degradation (of a Polysaccharide from) Red Seaweeds*. Journal of Biological Chemistry, 2012. **287**(17): p. 13985–13995.
66. Pluvinage, B., J.-H. Hehemann, and A.B. Boraston, *Substrate Recognition and Hydrolysis by a Family 50 α -Agarase, Aga50D, from the Marine Bacterium *Saccharophagus degradans**. Journal of Biological Chemistry, 2013. **288**(39): p. 28078–28088.
67. Hehemann, J.-H., et al., *Biochemical and Structural Characterization of the Complex Agarolytic Enzyme System from the Marine Bacterium *Zobellia galactanivorans**. Journal of Biological Chemistry, 2012. **287**(36): p. 30571–30584.
68. Hu, B., et al., *Prebiotic effects of neoagaro-oligosaccharides prepared by enzymatic hydrolysis of agarose*. Anaerobe, 2006. **12**(5-6): p. 260–266.
69. Gregg, K.J., et al., *Analysis of a new family of widely distributed metal-independent α -mannosidases provides unique insight into the processing of N-linked glycans*. The Journal of Biological Chemistry, 2011. **286**(17): p. 15586–15596.
70. Miguel, J.C., Rodriguez-Zas, S.L., Pettigrew, J.E. , *Efficacy of a mannan oligosaccharide (Bio-Mos®) for improving nursery pig performance*. J Swine Health Prod, 2004. **12**: p. 296-307.
71. Wohlt, J.E., T.T. Corcione, and P.K. Zajac, *Effect of Yeast on Feed Intake and Performance of Cows Fed Diets Based on Corn Silage During Early Lactation*. Journal of Dairy Science, 1998. **81**(5): p. 1345–1352.
72. Finck, D.N., et al., *Yeast supplementation alters the performance and health status of receiving cattle*. The Professional Animal Scientist, 2014. **30**(3): p. 333-341.
73. Nochta, I., et al., *Effect of different levels of mannan-oligosaccharide supplementation on some immunological variables in weaned piglets*. Journal of Animal Physiology and Animal Nutrition, 2009. **93**(4): p. 496–504.
74. Franklin, S.T., et al., *Immune Parameters of Dry Cows Fed Mannan Oligosaccharide and Subsequent Transfer of Immunity to Calves*. Journal of Dairy Science, 2005. **88**(2): p. 766–775.
75. Loddi, M.M., Moraes, V.M.B., Nakaghi, L.S.O., Tucci, F.M., Hannas, M.I., Ariki, J. Bruno, L.D.G. *Mannan oligosaccharide and organic acids on intestinal*

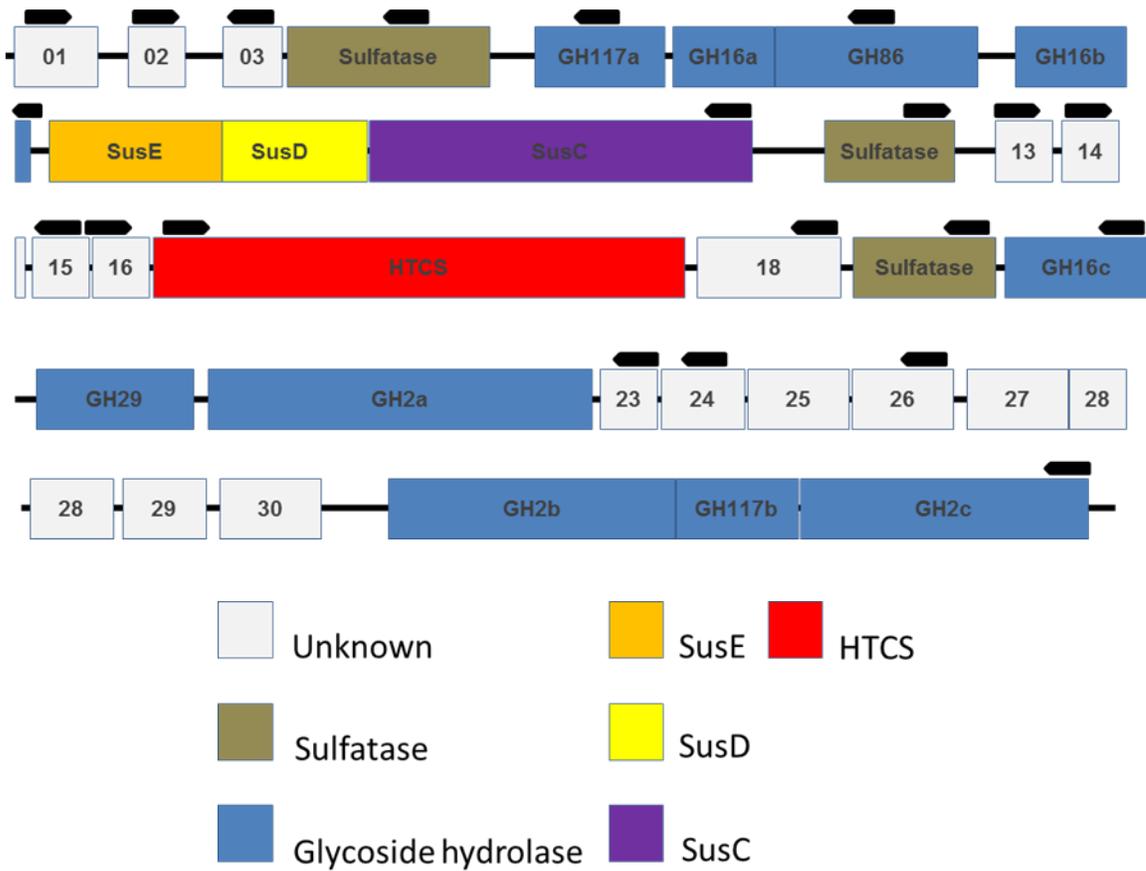
morphology integrity of broilers evaluated by scanning electron microscopy. in *Proceedings of 11th European Poult. Sci.* 2002. Bremen.

76. Gilbert, H.J., *The Biochemistry and Structural Biology of Plant Cell Wall Deconstruction.* Plant Physiology, 2010. **153**(2): p. 444–455.
77. Abbott, D.W., Farnell, B.J., and Yamashita, J.W. , *Pectins: structure, function, and the human distal gut microbiota,* in *Pectin: Chemical Properties, Uses and Health Benefits.* 2014, Nova Publishers.
78. Caffall, K.H. and D. Mohnen, *The structure, function, and biosynthesis of plant cell wall pectic polysaccharides.* Carbohydrate Research, 2009. **344**(14): p. 1879–1900.
79. Abbott, D.W., et al., *An Ancestral Member of the Polysaccharide Lyase Family 2 Displays Endolytic Activity and Magnesium Dependence.* Applied Biochemistry and Biotechnology, 2013. **171**(7): p. 1911–1923.
80. Abbott, D.W. and A.B. Boraston, *The Structural Basis for Exopolygalacturonase Activity in a Family 28 Glycoside Hydrolase.* Journal of Molecular Biology, 2007. **368**(5): p. 1215–1222.
81. Olano-Martin, E., G.R. Gibson, and R.A. Rastall, *Comparison of the in vitro bifidogenic properties of pectins and pectic-oligosaccharides.* Journal of Applied Microbiology, 2002. **93**(3): p. 505–511.
82. Arland, T.H., et al., *Pectic Oligosaccharides as Prebiotics,* in *Oligosaccharides in Food and Agriculture.* 2003, American Chemical Society. p. 54-62.
83. Hotchkiss, A.T., et al., *Pectic Oligosaccharides as Prebiotics,* in *Oligosaccharides in Food and Agriculture.* 2003, American Chemical Society. p. 54-62.
84. Anumula, K.R., *Advances in fluorescence derivatization methods for high-performance liquid chromatographic analysis of glycoprotein carbohydrates.* Analytical Biochemistry, 2006. **350**(1): p. 1-23.
85. Pickup, J.C., et al., *Fluorescence-based glucose sensors.* Biosensors and Bioelectronics, 2005. **20**(12): p. 2555–2565.
86. Gräslund, S., et al., *Protein production and purification.* Nat Meth, 2008. **5**(2): p. 135-146.
87. Kocourek, J. and C.E. Ballou, *Method for Fingerprinting Yeast Cell Wall Mannans.* Journal of Bacteriology, 1969. **100**(3): p. 1175-1181.

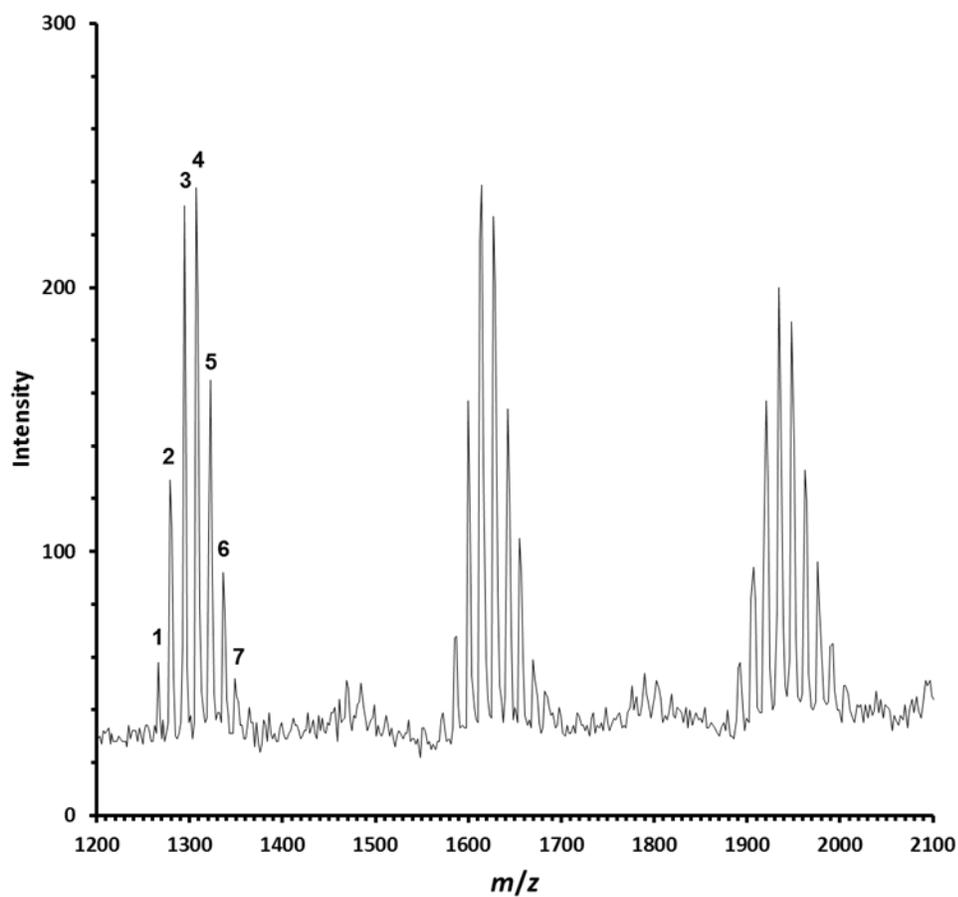
88. Ruhaak, L.R., et al., *2-picoline-borane: a non-toxic reducing agent for oligosaccharide labeling by reductive amination*. *Proteomics*, 2010. **10**(12): p. 2330-6.
89. Gao, N., *Fluorophore-assisted carbohydrate electrophoresis: a sensitive and accurate method for the direct analysis of dolichol pyrophosphate-linked oligosaccharides in cell cultures and tissues*. *Methods*, 2005. **35**(4): p. 323-7.
90. Sun, J.L., Huang, J., Zhongfu, L.W., *Studies on Pectic Oligosaccharides Analysis by Fluorophore-assisted Carbohydrate Electrophoresis*. *Acta Chim. Sinica*, 2009. **67**(9): p. 951-956.
91. Zhang, W.-w. and L. Sun, *Cloning, Characterization, and Molecular Application of a Beta-Agarase Gene from Vibrio sp. Strain V134*. *Applied and Environmental Microbiology*, 2007. **73**(9): p. 2825–2831.
92. Yun, E.J., et al., *Production of 3,6-anhydro-l-galactose from agarose by agarolytic enzymes of Saccharophagus degradans 2-40*. *Process Biochemistry*, 2011. **46**(1): p. 88–93.
93. Masuoka, J.H., K.C., *Effect of monosaccharide composition, glycosidic linkage position and anomericity on the electrophoretic mobility of labeled oligosaccharides*. *Electrophoresis*, 2006. **27**(2): p. 365-372.
94. Salyers, A.A., et al., *Fermentation of mucins and plant polysaccharides by anaerobic bacteria from the human colon*. *Applied and Environmental Microbiology*, 1977. **34**(5): p. 529–533.
95. Ohta, Y., et al., *Cloning, expression, and characterization of a glycoside hydrolase family 86 α -agarase from a deep-sea Microbulbifer-like isolate*. *Applied Microbiology and Biotechnology*, 2004. **66**(3): p. 266–275.
96. Ariga, O., et al., *Cloning of agarase gene from non-marine agarolytic bacterium Cellvibrio sp.* *Journal of Microbiology and Biotechnology*, 2012. **22**(9): p. 1237–1244.
97. Rebuffet, E., et al., *Discovery and structural characterization of a novel glycosidase family of marine origin*. *Environmental Microbiology*, 2011. **13**(5): p. 1253–1270.
98. Suzuki, H., et al., *Purification and characterization of an extracellular alpha-neoagarooligosaccharide hydrolase from Bacillus sp. MK03*. *Journal of Bioscience and Bioengineering*, 2002. **93**(5): p. 456–463.
99. McCarter, J.D., M.J. Adam, and S.G. Withers, *Binding energy and catalysis. Fluorinated and deoxygenated glycosides as mechanistic probes of Escherichia coli (lacZ) β -galactosidase*. *Biochemical Journal*, 1992. **286**(3): p. 721–727.

100. Hall, B.G., *Experimental evolution of Ebg enzyme provides clues about the evolution of catalysis and to evolutionary potential*. FEMS Microbiology Letters, 1999. **174**(1): p. 1–8.
101. Juers, D.H., B.W. Matthews, and R.E. Huber, *LacZ β -galactosidase: Structure and function of an enzyme of historical and molecular biological importance*. Protein Science, 2012. **21**(12): p. 1792–1807.
102. Saha, D. and S. Bhattacharya, *Hydrocolloids as thickening and gelling agents in food: a critical review*. Journal of Food Science and Technology, 2010. **47**(6): p. 587–597.
103. Shin, M.H., et al., *Global metabolite profiling of agarose degradation by Saccharophagus degradans 2-40*. New Biotechnology, 2010. **27**(2): p. 156–168.
104. Kim, H.T., S. Lee, D. Lee, H. S. Kim, W. G. Bang, K. H. Kim and I. G. Choi *Overexpression and molecular characterization of Aga50D from Saccharophagus degradans 2-40: an exo-type beta-agarase producing neoagarobiose*. Appl Microbiol Biotechnol, 2010. **86**(1): p. 227-234.
105. Wang, J., et al., *Anti-oxidation of agar oligosaccharides produced by agarase from a marine bacterium*. Journal of Applied Phycology, 2004. **16**(5): p. 333–340.
106. Davies, G.J., K.S. Wilson, and B. Henrissat, *Nomenclature for sugar-binding subsites in glycosyl hydrolases*. Biochemical Journal, 1997. **321**(Pt 2): p. 557-559.
107. Karunatilaka, K.S., et al., *Superresolution Imaging Captures Carbohydrate Utilization Dynamics in Human Gut Symbionts*. mBio, 2014. **5**(6): p. e02172-14.

Appendix



Appendix 1: Agarose-PUL for *B. uniformis* NP1. Genes with known or predicted functions are coloured while genes with unpredicted function are uncoloured. Black arrows indicate directionality of genes.



Appendix 2: Methylated peaks on an NAOS population generated from LMP. Semi-peaks highlighted on the close up to a peak representing a G₄A₄ population from *BuGH86* digest (Table 3.2). Each semi-peak contains varying degrees of methylation.