

**CAPACITY OF HOOFED ARTIODACTYL DISTAL GUT *BACTEROIDES* SPP. TO
SACCHARIFY BROWN SEAWEED POLYSACCHARIDES**

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CAPACITY OF HOOFED ARTIODACTYL GASTROINTESTINAL TRACT *BACTEROIDES*
SPP. TO SACCHARIFY BROWN SEAWEED POLYSACCHARIDES

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DEDICATION

To my friends, family and partner who have supported me throughout.

ABSTRACT

The ruminant gastrointestinal tract (GIT) contains a highly diverse microbial ecosystem in terms of both species and functional capacity. This community, whose evolutionary history is closely tied to that of their host, facilitates the digestion of recalcitrant plant materials through encoded carbohydrate active enzymes (CAZymes). The ability for ruminants to transform human-indigestible plant material into meat and milk have made them an invaluable asset to humans, with the demand for these products continually increasing in response to human population growth, affluence, and dietary preferences. The composition of the GIT microbiota is consequential for the host in a number of ways, including feed efficiency, health, and enteric methane emission. Thus, manipulating the structure of this community has been an area of interest for the livestock industry. Alternative feedstocks hold great potential in this regard. Macroalgae, or seaweeds, have been an attractive potential feed additive due to some species having anti-methanogenic properties. However, little is still known about the digestion of seaweed cell walls, which are distinct from those of terrestrial plants, by the GIT microbiota and seaweeds are commonly assumed to be non-digestible/non-nutritive. The discovery of CAZymes associated with seaweed polysaccharide saccharification in the human GIT microbiota suggests that these genes penetrated the mammalian gut microbiome via horizontal gene transfer (HGT) from seaweed-associated marine bacteria, and that this acquisition may have been an ancient event. Recent work in the Abbott Lab demonstrated that genes for CAZymes targeting carrageenan, a red seaweed polysaccharide, exist within the GIT microbiomes of geographically and taxonomically diverse ruminant and nonruminant animals. Similarly, cattle and Norwegian White lamb GIT microbiomes have been shown to saccharify alginate, a brown seaweed polysaccharide. These studies have cumulated in the proposal of the “latent traits hypothesis”, suggesting that seaweed-targeting CAZymes are ubiquitous in the animal GIT microbiota and

persist in the absence of substrate, likely carried in the genomes of generalist bacterial members present in low abundance. This work raises questions about the evolution of dietary adaptations, timescales associated with such an acquisition, and the source of relevant GIT microbiota and CAZymes. Currently, it is unknown whether other ruminant species have the same plasticity in adapting to alginate, and if so, how this is accomplished. The thesis presented here investigates whether other ruminants and closely related pseudoruminants from order Artiodactyla harbor microorganisms and CAZymes capable of degrading brown seaweed polysaccharides. Fecal samples were collected from ruminants and pseudoruminants (n=1) at the Wilder Institute/Calgary Zoo and Saskatoon Forestry Farm Park & Zoo, and selectively enriched for microorganisms capable of growth on alginate. Genomic analysis of fecal community metagenomic data and bacterial isolate DNA revealed an alginate utilization pathway that remains conserved amongst all bacterial isolates from 4 different ruminant host species; Masai giraffe, rocky mountain goat, bighorn sheep, and moose. CAZymes identified in this pathway, including BxPLX, a sequence from *Bacteroides xylanisolvens* (Bx) and potential member of a new polysaccharide lyase (PL) family, are predicted to be involved in the saccharification of alginate. The crystal structure of BxPLX was solved in complex with potential substrates. In addition, the activities of the other PLs from this Alginate Utilization Pathway (AUL) were characterized by thin layer chromatography (TLC), high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD), and NMR spectroscopy. The outcomes of this project will expand our knowledge of alginate-specific PLs, as well as provide new evolutionary and functional insights into the ability of ruminants and pseudoruminants to digest seaweed polysaccharides.

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USE OF GENERATIVE AI

Generative AI was used to troubleshoot code scripts during bioinformatics analyses. No part of the following text was written with the assistance of generative AI.

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List of Abbreviations

AL	Alginate lyase
ALG	Alginate
AUL	Alginate utilization loci
BCE	Before common era
<i>Bf</i>	<i>Bacteroides faecium</i>
<i>Bx</i>	<i>Bacteroides xylanisolvens</i>
CAZyme	Carbohydrate-active enzyme
CMB	Chopped Meat Broth
COSY	Correlated Spectroscopy
<i>Csp</i>	<i>Cryptobacteroides</i> sp.
DAPI	4',6-diamidino-2-phenylindole
DEH	4-deoxy-l-erythro-5-hexoseulose uronate
DEHr	4-deoxy-l-erythro-5-hexoseulose uronate reductase
DHF	4-deoxy-d-manno-hexulofuranosidonate
DP	Degree of polymerization
EtOH	Ethanol
FISH	Fluorescence in situ hybridization
GA3P	Glyceraldehyde-3-phosphate
GH	Glycoside hydrolase
GHG	Greenhouse gas
GIT	Gastrointestinal tract
Glu	Glucose
HGT	Horizontal gene transfer
HMBC	Heteronuclear Multiple Bond Correlation
HMM	Hidden Markov model
HPAEC-PAD	High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection
HSQC	Heteronuclear Single Quantum Coherence
KAN	Kanamycin
KDG	2-keto-3-deoxy-d-gluconate
KdgK	2-keto-3-deoxy-d-gluconate kinase
KDPG	2-keto-3-deoxy-phosphogluconate
MAG	Metagenomic assembled genome
MFS	Major facilitator superfamily
MM	Minimal media
NCBI	National Center for Biotechnology Information
NMR	Nuclear magnetic resonance
ORF	Open Reading Frame
PBS	Phosphate buffered saline
PL	Polysaccharide lyase
<i>Psp</i>	<i>Prevotella</i> sp.
PUL	Polysaccharide utilization loci
SP	Signal peptide
TSP-d ₄	3-(trimethylsilyl)propionic-2,2,3,3-d ₄
TLC	Thin-layer chromatography
TOCSY	Total Correlation Spectroscopy

CHAPTER 1: INTRODUCTION

1.1 Ruminant livestock and agriculture; a brief history and current challenges

In the earliest agricultural hubs, the domestication of ruminant livestock, such as sheep (10,050-8,550 BCE), goats (9050 BC-7050 BCE), and cattle (*Bos taurus*, 8550-8050 BCE; *Bos indicus*, 6050–5550 BCE), were major factors aiding the transition of humans from nomadic hunter-gatherer lifestyles to agriculturalist communities (Diamond, 2002; Felius et al., 2014; Marshall et al., 2014). Alongside the cultivation of staple crops, the ability of ruminants to transform recalcitrant plant materials into accessible meat and milk products proved to be an invaluable asset to humankind. This transition was driven in part by decreases in large game populations in the late Pleistocene and the emergence of warmer, stable climate conditions in the Holocene epoch, making both animal husbandry and crop cultivation more productive (Diamond, 2002; Gowdy, 2020). These factors, combined with the possibilities afforded by sedentary living, such as the development of agricultural technologies and shorter intervals between human births, allowed for an explosion in the human population from around 6 million to 100 million over the span of the agricultural revolution (Biraben, 2003; Diamond, 2002). Agricultural production, which both supplied and required a large labor force, necessitated the organization of more complex societal structures, and human cooperation on a larger scale than previously witnessed.

In the present, food production has been optimized to a degree that would be unfathomable to the earliest agriculturalists. Advancements, such as irrigation, heavy machinery and other scientific developments have greatly increased food production efficiency (Ameen & Raza, 2017). Despite this progress, secure food production is challenged by the pace of global human population growth, which is projected to reach ~9.15 billion by 2050 (Alexandratos & Bruinsma, 2012). In particular, the global demand for milk and meat products is expected to

increase by 58% and 73% , respectively by 2050 (Gerber et al., 2013). This trend also reflects changes in dietary preferences towards animal product consumption, rising incomes, and urbanization (Beauchemin et al., 2020; Henchion et al., 2017). With livestock products being a nutrient- and calorie-dense food option, many assert that increasing the production and consumption of livestock products could help alleviate food insecurity (Adesogan et al., 2020)., especially since livestock grazing is possible on land that is unsuitable for crop production (Church, 1993). Additionally, domesticated livestock fulfill some keystone ecological roles, such as top-down control of plant communities and nutrient cycling (Pringle et al., 2023).

Intensification of the livestock sector raises important sustainability considerations, including strain on finite natural resources, such as land and fresh water, and increases in waste production, such as greenhouse gasses (GHG). Since the industrial revolution, atmospheric concentrations of methane, carbon dioxide, and nitrous oxide have increased by 150%, 40%, and 20%, respectively (Pachauri et al., 2014). As of 2013, ~14.5% of total anthropogenic GHG emissions were produced by the livestock supply chain (feed production, land use change, enteric CH₄, manure, and animal product transportation/processing), with ruminant enteric CH₄ alone accounting for 39% of sector emissions (Beauchemin et al., 2020; Gerber et al., 2013). Reducing enteric CH₄ emissions has been an attractive strategy for short-term progress towards climate change mitigation. Compared to CO₂, CH₄ has a shorter half-life but a 28-fold greater warming potential (Beauchemin et al., 2020).

Climate change threatens human and environmental health and safety, with those located in mid to low latitude regions and developing areas facing a disproportionate amount of risk (Stephenson et al., 2010). Associated challenges include extreme temperatures, changes in precipitation patterns, intensification of crop infestations, and more frequent and severe natural disasters (*e.g.*, heatwaves, droughts, floods, forest fires) (Anwar et al., 2013; Schneider et al.,

2022). Without aggressive climate change initiatives, the agricultural sector (which is particularly sensitive to unpredictable climate conditions) will be left increasingly vulnerable, with some insisting that the future of agriculture as a whole is under threat (Gowdy, 2020). This has the potential to negatively impact global food security outcomes (Anwar et al., 2013), making the widespread study and implementation of sustainable agricultural practices imperative.

1.2 Feed additives and alternative feeds as an environmental cost mitigating strategy

The composition of the ruminant gastrointestinal tract (GIT) microbiome is consequential for several critical outcomes in livestock production, such as intestinal health, energy harvesting efficiency, and environmental impacts as a result of production (Beauchemin et al., 2020; Sasson et al., 2017). Given that microbiological processes can influence host phenotype, there has been interest in both understanding and manipulating the GIT community. While several strategies have been employed towards this end including selective breeding, herd management, and administration of antibiotics, dietary modifications in the form of alternative feeds and feed additives have been extensively studied and shown great promise (Beauchemin et al., 2020). Feed additives are especially appealing for their sustainability, contrasted with antibiotics which have been gradually phased out of productivity-associated usage due the emergence of microbial antibiotic resistance. To date, several different feed additives have been investigated for properties related to anti-methanogenic activity, including lipids, nitrates, phytochemicals (*e.g.*, tannins, saponins), and chemical inhibitors (*e.g.*, 3-NOP) (Beauchemin et al., 2020).

In recent years, macroalgae or seaweeds have garnered interest for their antimethanogenic properties, with certain species shown to decrease enteric CH₄ emissions from ruminants when used as a feed additive (Abbott et al., 2020). The most promising example is *Asparagopsis taxiformis* (Rhodophyceae), with an observed reduction of up to 99% at a 2% inclusion rate

(Kinley et al., 2016). This effect is due to the inhibition of the methanogenic pathway by the bromoforms within seaweeds, which notably, is a carcinogen with reported negative impacts on the ozone layer (Abbott et al., 2020). In addition to their antimethanogenic properties, seaweed beds (whether natural or farmed) can provide some environmental benefits, acting as a CO₂ sink, and reducing the requirement for arable land, fresh water, and pesticides/herbicides (Low et al., 2023). Seaweeds also contain an array of bioactive compounds including macromolecules, minerals, and various phytochemicals (Abbott et al., 2020), representing an undervalued supply of nutrients with significantly less competition than terrestrial crop in terms of the “feed, food, fuel” dilemma (Muscat et al., 2020). Emerging research has indicated potential health promoting benefits of macroalgae and associated bioactives (*e.g.*, macromolecules, saponins, alkaloids, and peptides) when used as feed additives for livestock, including prebiotic, antimicrobial, antioxidant, antifungal, and/or anti-inflammatory properties (Abbott et al., 2020; Cheong et al., 2023). Despite promising research on the topic, specific health promoting metrics can be difficult to quantify, given the complexity of the host-GIT microbiota interaction, and the effect of varying substrate inputs underlying this relationship.

1.3 Dietary carbohydrates and the host-microbiota relationship

The mutualistic, obligatory relationship between ruminant hosts and their GIT microbiota is a product of eons of co-evolution. Within this partnership, ruminants are supplied with the rich, microbial enzymatic repertoire required for the fermentation of dietary polysaccharides, which produces short-chain fatty acids that can be absorbed by the host and used for energy (Henderson et al., 2015; Reddy & Hyder, 2023; Sasson et al., 2017). In return, microbes are provided with access to ample resources in the form of exogenous substrates and host glycans, as well as a

habitable anaerobic environment in which to proliferate (Henderson et al., 2015; Sasson et al., 2017).

1.3.1 The ruminant host

Order Artiodactyla (alternatively known as Cetartiodactyla) encompasses a highly diverse group of terrestrial, even-toed ungulates including true ruminants (*i.e.*, suborder Ruminantia), pseudoruminants (suborders Tylopoda and Whippomorpha), and non-ruminants (suborder Suina), as well as large marine mammals, such as dolphins and whales (*i.e.*, infraorder Cetacea; non-ruminants) (Zurano et al., 2019). Artiodactyls are considered highly successful in terms of species diversity (>190 extant species), geographical range, and abundance (Prothero & Foss, 2007). This feat is owed in part to their specialized GIT, which unlike monogastric and hindgut fermenters, is equipped with a multi-chambered stomach that allows for a greater capacity to extract energy from recalcitrant plant cell wall polysaccharides.

All true ruminants (*i.e.*, suborder Ruminantia) are endowed with a rumen, reticulum, omasum, and abomasum, followed by the small and large intestines. Feed materials first enter the oral cavity, where the mechanical degradation of feedstuffs occurs via mastication (Reddy & Hyder, 2023). This material, partially ground and saturated with saliva, travels through the esophagus to the reticulum, a honeycomb textured muscle that traps hard objects such as stones and nails, preventing further advancement into the GIT. The reticulum is separated from the rumen only by the ruminoreticular fold, past which feedstuffs can travel. The rumen is a large anaerobic chamber that facilitates the fermentation of feed materials by a resident microbial community. In adults, the rumen accounts for the largest volume of the four-chambered stomach, holding approximately 100 liters in adult cattle and consisting of ~69-84 % of the stomach (Fregulia et al., 2021; Hofmann, 1993; Karisch, (n.d.); Reddy & Hyder, 2023). Products of microbial enzymatic fermentation, such as short-chain fatty acids (*e.g.*, acetate, propionate,

butyrate) and amino acids, can be absorbed through the walls of the rumen (Moraïs & Mizrahi, 2019), which are lined with papillae that maximize surface area. Saliva entering the rumen contains buffering agents such as HCO_3 and HPO_4 , which maintain the rumen at a pH of ~5.5-7 despite the generation of these fermentation products (Paudyal, 2021; Reddy & Hyder, 2023). Other products such as CH_4 , H_2S , and NH_4^+ are released to the environment through eructation (Beauchemin et al., 2020; Gerber et al., 2013; Morais & Mizrahi, 2019). The reticulum also filters feedstuffs attempting to enter the omasum. If feed digestion is insufficient, then further rumination ensues. Rumination involves several different steps including regurgitation of feed material from the rumen through the contraction of the reticulum, remastication, reinsalivation, and redeglutition (Paudyal, 2021; Reddy & Hyder, 2023). Rumination is often an extended process (10 h a day for cattle on a hay-based diet) (Reddy & Hyder, 2023), with variability dependent on a variety of factors such as feed digestibility (*i.e.*, cellulose, hemicellulose and lignin content), animal health, seasonal variation, stage of reproductive cycle, and cattle breed (Paudyal, 2021). After passing through the reticulum, feed particles enter the omasum, a globe-shaped organ with many tissue folds (often called “many piles” or the “butchers bible”), which absorbs water and nutrients from feed. In the abomasum, the final chamber of the foregut, the dietary and microbial proteins are hydrolyzed by HCl and gastric enzymes, such as pepsin, at a pH of between 3.5-4.0 (Reddy & Hyder, 2023). The abomasum most closely resembles the stomach of a non-ruminant. After travelling through the abomasum, feed materials enter the small intestine (duodenum, jejunum, and ileum), which absorbs hydrolyzed macromolecules, water and nutrients into the bloodstream. The large intestine consists of the cecum, ascending colon, transverse colon, and descending colon, and facilitates stool formation and water absorption. This portion of the GIT also harbours a microbial community that continues fermentation and absorption of feed materials (Zou et al., 2020).

While functionally similar, the GIT of true ruminants from suborder Ruminantia shows several anatomical differences to that of pseudoruminants (Al-Jassim & Hogan, 2012; Reddy & Hyder, 2023). Pseudoruminants possess a three-chambered stomach, labelled C1 (rumen-like), C2 (reticulum-like), and C3 (omasum/abomasum-like) (Al-Jassim & Hogan, 2012). The bottom of the pseudoruminant C1 is lined with glandular sacculles, pockets of tissue increasing the surface area of the rumen and facilitating the buffering of the rumen alongside salivary buffering agents (Al-Jassim & Hogan, 2012). The glandular sacculles fulfill the need for increased surface area, and consequently the C1 is not lined with papillae (Al-Jassim & Hogan, 2012). Pseudoruminants also lack a true differentiated omasum, with this function being adopted by the proximal C3 (Al-Jassim & Hogan, 2012). In the distal C3, gastric enzymes and HCl are released for protein digestion (Al-Jassim & Hogan, 2012).

1.3.2 The ruminant GIT microbiota and encoded CAZymes

The GIT microbial community is highly diverse in terms of both species and functional capacity (Clemmons et al., 2019; Mizrahi & Jami, 2018). The GIT microbiota has been estimated to harbor more than 5,000 species (Cholewińska et al., 2020), consisting of bacteria, protozoa, archaea, fungi, and viruses. These microorganisms occupy proteolytic, fibrolytic, and lipolytic roles, with the community providing up to 70% of daily dietary enzymatic requirements (Cholewińska et al., 2020).

Within the GIT, dietary fibres are an important source of energy for microbial communities, and the ability of members to utilize available resources provides a competitive advantage. Saccharolytic GIT microorganisms depolymerize polysaccharides with carbohydrate-active enzymes (CAZymes). CAZymes are divided into five distinct classes: polysaccharide lyases (PL), glycoside hydrolases (GH), glycosyltransferases, carbohydrate esterases, and

auxiliary activities (Wardman et al., 2022). These classes are assigned according to their inherent catalytic mechanism, which is determined by protein sequence.

In response to diverse carbohydrate substrates, microorganisms from different functional groups occupy distinct dietary “niches.” Some microorganisms are considered “specialists” and only depolymerize a select few polysaccharides. Other groups, such as *Bacteroidota*, employ a rich library of CAZymes targeting an array of different substrates and are considered “generalists.” The CAZymes involved in carbohydrate metabolism are often organized into polysaccharide utilization loci (PUL), which are operonic in structure and tailored to the digestion of discrete substrates.

1.3.3 Carbohydrates

Carbohydrates are one of the four major classes of biological macromolecules essential for life. Defined as polyhydroxy -aldehydes or -ketones, including derivatives of these substances and larger compounds that can undergo condensation during their synthesis (Varki et al., 2015). Carbohydrates are the most abundant, and chemically and structurally diverse group of macromolecules in nature (Low et al., 2023; Sarkar et al., 2024; Varki et al., 2015). Their diversity is reflective of the wide range of biological roles that these macromolecules play. For example, carbohydrates are not only necessary for energy sequestration and storage, but for structural support, cell signaling, immunomodulation, as well as growth and development (Low et al., 2023; Varki et al., 2015).

The heterogeneity of carbohydrates results from their chemical properties. First, the abundance of unique monosaccharides far outnumbers the monomers of amino acids and nucleotides, with some sources suggesting there are hundreds of different species (Cummings, 2024; Varki et al., 2015). These monosaccharides can be linked together or to other molecules via an *O*-glycosidic, *N*-glycosidic, *S*-glycosidic, or *C*-glycosidic linkage (Cummings, 2024), in either

an α or β anomeric conformation, and at one of several different positions on the monosaccharide (Varki et al., 2015). While most monosaccharides in nature appear in the D enantiomeric configuration, some like fucose are found in the L configuration (Varki et al., 2015). The degree of polymerization (DP), or number of monosaccharide units, in oligosaccharides and polysaccharides can vary with some polymers, like cellulose, spanning hundreds to thousands of residues (Béguin & Aubert, 1994). Carbohydrates can also contain branching at multiple positions, undergo non-carbohydrate chemical modifications (*e.g.*, phosphorylation, sulfation, methylation) and be associated with other macromolecules to form glycoconjugates (*e.g.*, glycoproteins, glycolipids) (Low et al., 2023).

Evolutionary adaptation by organisms can often manifest through the production/modification of carbohydrates. One such example are seaweed polysaccharides, and their chemical and structural differentiation in response to a highly ionic marine environment. Seaweeds are divided into three major categories, red (Rhodophyceae), green (Chlorophyceae), and brown (Phaeophyceae), and contain polysaccharides that are unique from terrestrial plants: such as agar, porphyran and carrageenan (Rhodophyceae); ulvan (Chlorophyceae); and laminarin, fucoidan and alginate (ALG; Phaeophyceae) (Kloareg & Quatrano, 1988). These polysaccharides have gelling properties that add both stability and flexibility to the cellular matrix, and are often sulfated which aids in maintaining ionic homeostasis amidst fluctuating salinity in their habitat (Kloareg & Quatrano, 1988).

1.3.4 ALG depolymerization pathway

Due to their chemical and structural differences seaweed cell wall polysaccharides require specific catalytic machinery for their catabolism. For example, PLs are required to accomplish the cleavage of glycosidic bonds within anionic uronic acid-containing polysaccharides, such as ALG (**Figure 1.1**) (Gacesa, 1987). This process occurs via a β -elimination mechanism (**Figure**

1.2). PL substrates can be divided into three groups according to type of uronic acid: 1) galacturonic acid-containing polysaccharides (pectin) (Abbott & Boraston, 2007; Abbott et al., 2010; Charnock et al., 2001); 2) glucuronic/iduronic acid-containing polysaccharides (glycosaminoglycans, xanthan); and, 3) mannuronic acid (ManA)/guluronic acid (GulA)-containing polysaccharides (ALG) (Garron & Cygler, 2014). β -elimination reactions result the cleavage of the 1,4-glycosidic scissile bond between the -1 and +1 subsite (**Figure 1.2**; (Loiodice et al., 2025)). This process, which employs a three-step mechanism, is initiated by a positively charged group, which acts to neutralize the negative charge on the C5 carboxylic acid group in the +1 site (Gacesa, 1987; Garron & Cygler, 2014). The neutralization of the acidic group lowers the pKa of the C5 proton, making it more susceptible to abstraction by a Brønsted base. This abstraction causes the formation of the enolate ion, which is energetically unfavorable. An electron transfer from the carboxylate group will result in an unsaturation located between C4 and C5, followed by the cleavage of the *O*-glycosidic bond through protonation by the Brønsted acid. The result is an unsaturated uronic acid in the +1 site, and the formation of a new reducing end in the -1 subsite. Amongst PLs, there are two reaction landscapes that these catalytic mechanisms generally fit within. The first is a metal ion-assisted neutralization of the carboxylate ion group with Ca^{2+} (less commonly with Mn^{2+} or Ni^{2+} (Abbott & Boraston, 2007)), Lys or Arg acting as the Brønsted base and H_2O as the Brønsted acid. The second is neutralization by Asn/Gln or a protonated Asp/Glu, Tyr or His acting as the Brønsted base, and Tyr as the Brønsted acid.

Through the formation of the 4,5-unsaturated uronate, 4-deoxy-L-erythro-5-hexoseulose uronate (DEH) can be generated (**Figure 1.1**) (Kawai & Hashimoto, 2022). This process can occur either enzymatically through KdgF or spontaneously in solution, resulting in a linearized monomer. DEH is reduced by DEH reductase (DEHr) to become 2-keto-3-deoxy-D-gluconate

(KDG). KDG is targeted by KDG kinase (KdgK) to form 2-keto-3-deoxy-phosphogluconate (KDPG), which can be transformed into pyruvate and glyceraldehyde-3-phosphate (GA3P). Alternatively, DEH can hydrate into one of two forms, either DEH ketoaldo-hydrate or DEH aldo-hydrate (Arntzen et al., 2021). The C5 ketone group in DEH aldo-hydrate, not having reacted with H_2O , can facilitate recyclization into two stable epimers, (5S)-DHF hydrate and (5R)-DHF hydrate (Arntzen et al., 2021).

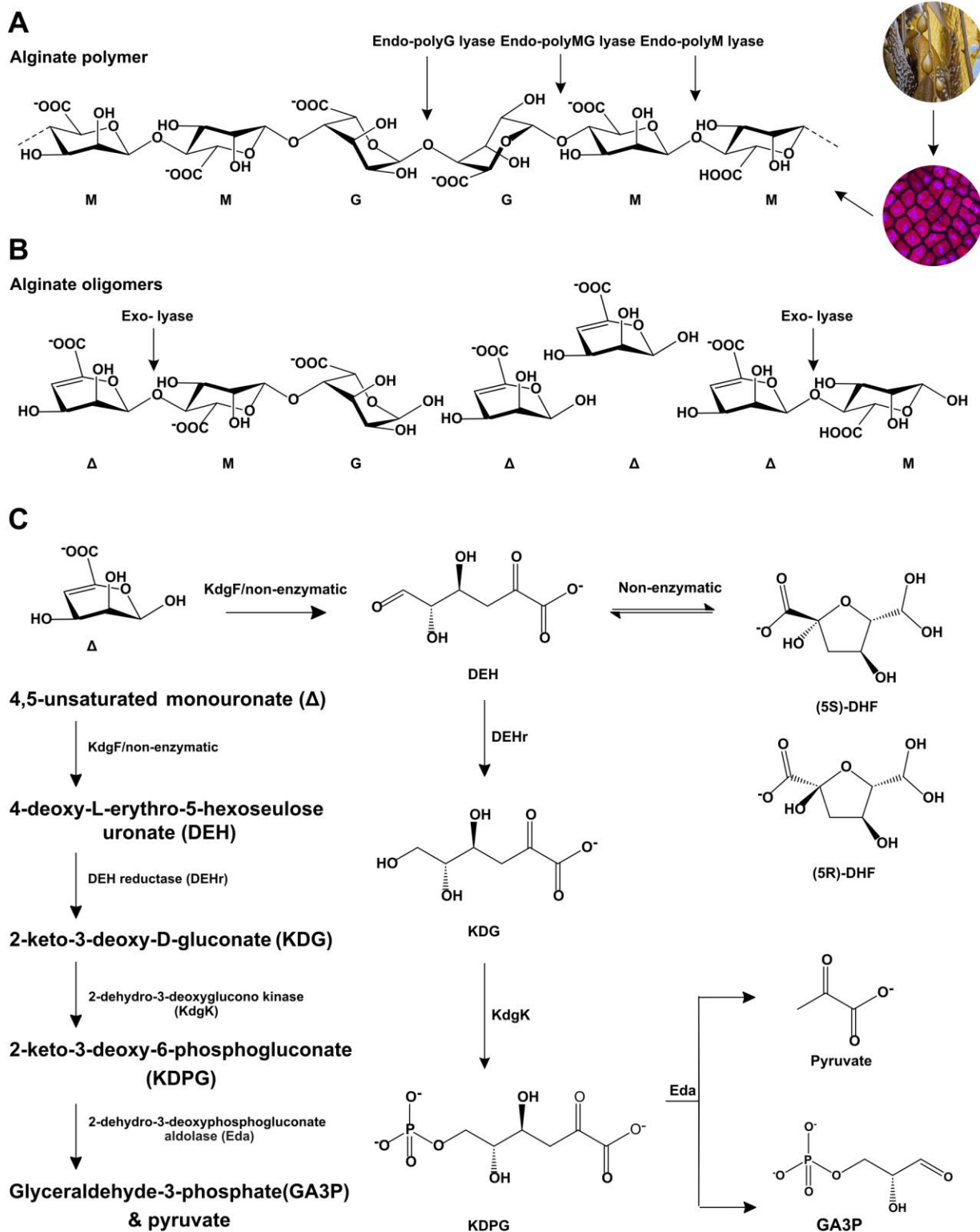


Figure 1.1 ALG catabolism pathway. (A) ALG polysaccharides are depolymerized through a β -elimination mechanism

by endo-acting polysaccharide lyases, forming oligosaccharide products **(B)** Exo-acting polysaccharide lyases further depolymerize ALG oligosaccharides, producing 4,5-unsaturated hexauronates (Δ). **(C)** Catabolism downstream of the 4,5-unsaturated hexauronate (Δ) involves linearization either enzymatically (via KdgF) or spontaneously to form 4-deoxy-l-erythro-5-hexoseulose uronate (DEH). DEH through a series of reactions can transform into pyruvate and glyceraldehyde-3-phosphate (GA3P), relevant to microbial metabolism. In the absence of DEHr and other downstream enzymes, DEH spontaneously recyclize into (5S)-4-deoxy-d-manno-hexulofuranosidionate (DHF) hydrate and (5R)-DHF hydrate. Image of *S. latissima* cell wall stained with DAPI (4',6-diamidino-2-phenylindole) and visualized through confocal microscopy, courtesy of Leeann Klassen.

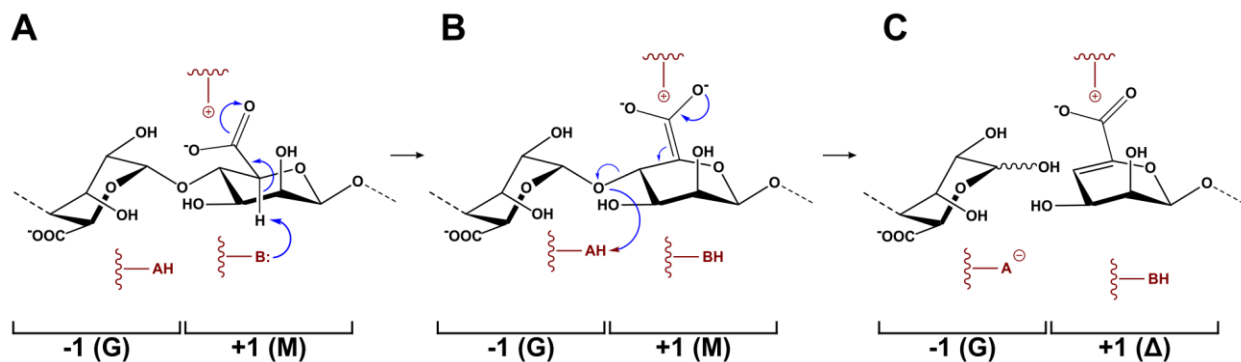


Figure 1.2 β -elimination reaction mechanism on ALG. (A) Enzyme-substrate complex. Neutralization of the negative charge on carboxylate anion by neutralizing group and abstraction of the C5 proton by a Brønsted base. **(B) Intermediate/transition stage.** Formation of the enolate ion at the +1 subsite, and subsequent shifting of electrons towards the bond linking C4 and C5. Cleavage of the glycosidic bond is facilitated by the donation of a proton to the 1,4-glycosidic oxygen by a Brønsted acid. **(C) Product complex.** A new reducing end at the -1 subsite, and 4,5-unsaturated uronate at the +1 subsite resulting from the reaction. Electron path is represented by blue arrows. Brønsted acid, Brønsted base, and neutralizing group are represented in red as A, B, and + attached to wavy bond, respectively.

1.4 Seaweeds as ruminant feeds

Currently, investigation into seaweeds as an alternative feed primarily focuses on seaweeds that contain bromoform and their associated anti-methanogenic properties. The biochemical fate of polysaccharides within the ruminant GIT remains largely unexplored. Due to their chemical differences from typical ruminant feeds that are composed of terrestrial plants and

the absence of seaweeds from modern beef production processes, seaweeds are commonly thought to be non-nutritive and/or non-digestible by the ruminant GIT.

Despite this, seaweeds have an extensive history of being used in livestock production that occurs near the shoreline. In *Bellum Africanum*, a latin text written in 45 BCE, an unknown author describes Ancient Greeks supplementing livestock with seaweeds during times of feed scarcity (Evans & Critchley, 2014; Makkar et al., 2016; Tiwari & Troy, 2015). Similarly, the Icelandic sagas report air-drying and storage *Palmaria palmata* for both human food and animal feed (Evans & Critchley, 2014; Makkar et al., 2016). Throughout the 19th and early 20th centuries, there is ample evidence for seaweed supplementation of livestock feed in France, the Scottish islands, Scandinavian and Nordic countries, and parts of Asia (Abbott et al., 2020; Evans & Critchley, 2014). Today, the Orkney sheep in the North Ronaldsay islands are famous for their seaweed diet that is nearly 100% kelp (Hansen et al., 2003). Also reported recently are the *muusmuus*, a herd of “rewilded” cattle that has been observed grazing green seaweeds off of the beaches of Meares Island, BC, in the traditional territory of the Tla-o-qui-aht First Nation (Tingley et al., unpublished).

Despite its documented history, 20th century nutritional science was not able to explain the benefits of seaweed implementation in livestock production (Abbott et al., 2020; Evans & Critchley, 2014). Nutrient component analysis yielded little insight into the nutritional value of seaweeds, revealing that as compared to other feed ingredients, such as alfalfa or corn, seaweeds had a low theoretical energy value (approximated by a low total digestible nutrient content and low macromolecule content) (Evans & Critchley, 2014). This was undesirable, given that the general understanding within animal nutrition at the time placed little importance on metrics outside of macromolecule, mineral, and vitamin requirements (Evans & Critchley, 2014). Regardless, many continued to supply their livestock with feed additives that fared poorer in

terms of proximate analysis estimations (*e.g.*, citrus peel meal, brewer's yeast, beet pulp), in order to reap the health and productivity benefits that these additions seemed to yield, though unexplained (Evans & Critchley, 2014). Non-nutritive elements, such as recalcitrant complex carbohydrates, were not known at the time to serve any benefit to animals but are now suggested to be important as an energy source for the GIT microbiota (Abbott et al., 2020). Nevertheless, an important stipulation in accessing the benefits of dietary seaweeds is the presence of specific molecular machinery (*i.e.*, CAZymes, transporters, regulatory elements) encoded by the GIT microbiota required to depolymerize their chemically distinct polysaccharides.

1.5 Seaweed polysaccharide saccharification by mammalian gut microbiota

The mechanisms and timescales associated with the development / introduction of CAZymes and other associated machinery within GIT microorganisms for seaweed metabolism is not clear. Seminal work by Hehemann et al., 2010 has suggested that genes conferring the ability to depolymerize seaweed polysaccharides may have been acquired by the distal gut microbiota in humans from seaweed-associated marine bacteria through horizontal gene transfer (HGT). Specifically, a porphyran-targeting PUL found in the marine bacterium *Zobellia galactanivorans* showed high synteny to enzymes within a human gut-associated strain, *B. plebius* DSM 17135 (Hehemann et al., 2010). It was hypothesized that HGT was facilitated by the consumption of seaweed by Japanese people (Hehemann et al., 2010). In 2018, Mathieu and colleagues proposed evidence that the human consumption of seaweeds may not have been the direct cause of a specific HGT event, but the selection pressure that enriched for microbes with this unique dietary capability. This work challenges the timescale at which this HGT event(s) likely occurred (Mathieu et al., 2018).

Recent research by Tingley et al. investigating similar evolutionary processes in the ruminant gut microbiome has proposed the “latent traits hypothesis”, suggesting that seaweed polysaccharide targeting CAZymes are ubiquitous in the animal GIT microbiota at low levels and persist in the absence of substrate (Tingley et al., 2026). The microorganisms carrying these genes are thought to fall below conventional sequencing thresholds and reside within the “dark matter” of the microbiome. Their presence is only confirmed after enrichment with the target substrate (Tingley et al., 2026). This theory relies on the presence of saccharolytic generalists, such as *Bacteroides* spp., that utilize a wide range of substrates as they become available, which prevents their elimination. Concordant with previous work, it is thought that the initial acquisition of these CAZymes may be due to ancient HGT events, early in animal evolutionary history (Tingley et al., 2026). PULs targeting carrageenan have been found to be widespread across the GIT microbiota of geographically and taxonomically distinct ruminants, as well as other animals like humans, gorillas, and seaweed-eating fish (Tingley et al., 2026). Additionally, the first PUL targeting ulvan within terrestrial animal GIT microbiomes was recently identified (Tingley et al., 2026). Within the family *Bacteroidaceae*, several isolates and metagenome-assembled genomes from both cattle and lamb rumen microbiota have been identified that possess ALG utilization loci (AULs) and are detectable after supplementation of dietary seaweed or ALG enrichment of fecal extracts (Ferrillo et al., 2026a). However, it remains unknown if other ruminant species have the same plasticity in adapting to seaweed forages, and if so, how this is accomplished.

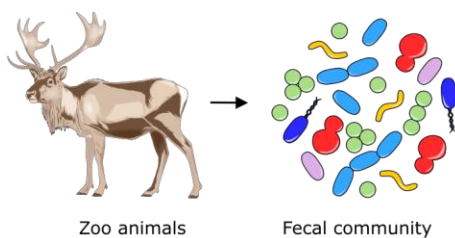
1.6 Hypothesis & objectives

Previous research has described the mechanisms and timescales associated with seaweed polysaccharide-targeting CAZyme acquisition. For this thesis, my hypothesis is; ALG utilization

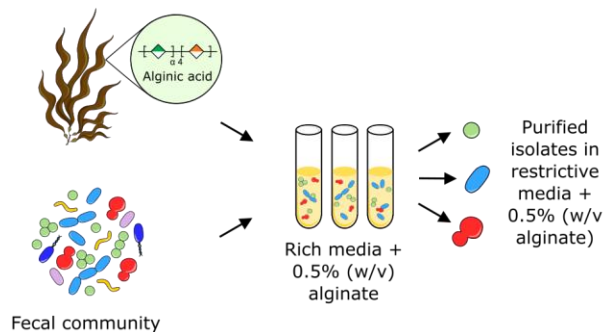
loci (AUL) exist within the gut microbiota of taxonomically distinct ruminant and nonruminant species from order Artiodactyla. The objectives of the study are as follows (**Figure 1.3**):

- 1) Collect fecal material from diverse species of ruminants and pseudoruminants housed at the Wilder Institute/Calgary Zoo and Saskatoon Forestry Farm Park and Zoo.
- 2) Selectively enrich and isolate microorganisms from collected feces collected in objective 1 capable of growth using ALG as the sole carbon source.
- 3) Extract, sequence, and annotate DNA from bacterial isolates and fecal microbial communities generated in objective 2.
- 4) Identify and biochemically characterize CAZymes targeting ALG from annotated sequences in objective 3.

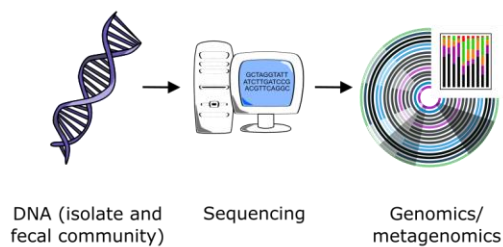
① Sample feces from ruminants and pseudoruminants



② Selectively enrich and isolate microbes



③ Extract DNA and sequence



④ Identify and characterize CAZymes

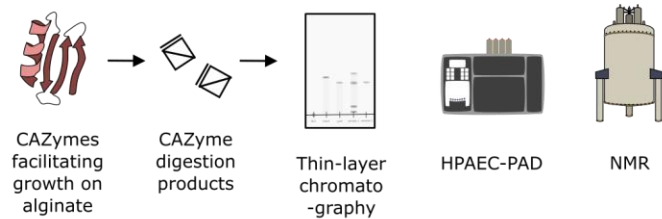


Figure 1.3. Graphical abstract of thesis project objectives. (1) Collect fecal material from diverse species of ruminants and pseudoruminants housed at the Wilder Institute/Calgary Zoo and Saskatoon Forestry Farm Park and Zoo. (2) Selectively enrich and isolate microorganisms from collected feces in objective 1 capable of growth using ALG as the sole carbon source. (3) Extract, sequence, and annotate DNA from bacterial isolates and fecal microbial communities generated in objective 2. (4) Identify and biochemically characterize CAZymes targeting ALG from annotated sequences in objective 3.

CHAPTER 2: METHODOLOGY

2.1 Fecal sample collection

Fresh fecal samples were collected from 13 ruminant and pseudoruminant species at the Wilder Institute/Calgary Zoo (September 23, 2023) and Saskatoon Forestry Farm Park & Zoo (March 24, 2024 & August 24, 2024) (**Table S1**), with the assistance of trained zoo staff. Both facilities are fully accredited by the World Association of Zoos and Aquariums and committed to maintaining a high standard of animal care (World Association of Zoos and Aquariums, 2026). All animal diets are described in **Source Data 1**. Ruminant species included: markhor (*Capra falconeri*), bighorn sheep (*Ovis canadensis*), rocky mountain goat (*Oreamnos americanus*), moose (*Alces alces*), boreal woodland caribou (*Rangifer tarandus caribou*), masai giraffe (*Giraffa tippelskirchi*), siberian musk deer (*Moschus moschiferus*), bison (*Bison bison*), elk (*Cervus canadensis*), and pronghorn (*Antilocapra americana*). Pseudoruminant species included: alpaca (*Vicugna pacos*), bactrian camel (*Camelus bactrianus*), and hippopotamus (*Hippopotamus amphibius*). Fecal material was directly frozen by LN₂ to preserve for metagenomic sequencing and used to perform selective isolations *via* enrichment cultures.

2.2 Selective isolation of ALG consuming bacteria

2.2.1 Selective isolation using commercial sodium ALG

One gram of fecal sample was put into an anaerobic Hungate tube containing 10 ml of anaerobic phosphate buffered saline (PBS) and homogenized. On site, 100 µL of this mixture was inoculated into a modified anaerobic Chopped Meat Broth (CMB; as previously described (Pudlo et al., 2022)) with commercial 0.5% (w/v) sodium ALG, as well as a CMB with 0.5% (w/v)

glucose (Glu) control. Enrichments were incubated at 37.0 °C, anaerobically (atmosphere: CO₂:H₂:N₂, 85:10:5, v/v/v) for 24-48 h until turbid and stored frozen at -70 °C in 20% glycerol. Once turbid, 10 µL of this mixture was used to reinoculated a MM + 0.5% (w/v) ALG media (with MM + 0.5% (w/v) Glu as a control). This was repeated in fresh media, with 10 µL of the MM cultures as inoculants. Pure cultures were isolated from enriched samples, by streaking 5 µL aliquots of culture onto minimal media plates (MM; (Pudlo et al., 2022)) supplemented with 0.5% (w/v) ALG. Single colonies were restreaked on the same media and stored frozen at -70 °C in 20% glycerol.

2.2.2 Selective isolation using *S. latissima* extract

In order to assess the ability of ALG-consuming bacteria to utilize ALG from different sources, the selective isolation process described in section 2.2.1 using commercial sodium ALG was repeated using *S. latissima* extract. From the ruminant species known to produce bacterial isolates capable of growth on commercial sodium ALG, fecal material from rocky mountain goat and bighorn sheep were further assessed on 0.5% (w/v) *S. latissima* extract as the carbon source. This was done to verify that the ability for ALG-degrading bacteria were not limited to commercially sourced ALG but could also extend to ALG within a mixture of other brown seaweed polysaccharides extracted from seaweed cell walls (*i.e.*, laminarin, fucoidan).

2.2.2.1 Extraction of water-soluble polysaccharides from *S. latissima*

Alcohol-insoluble residue preparation of dry *S. latissima* was done in house. First, 120 g of dry material was ball-milled to a fine powder. This material was divided into falcon tubes, with 3 g of powder in each tube. Forty mL of 80% (v/v) EtOH was added to samples and rotated gently overnight at room temperature, centrifuged at 3,000 × g for 30 min, and decanted. This process was repeated twice more with pelleted material. Then, with absolute EtOH the above process was repeated twice. The material was dried completely using a SpeedVac. To each

sample 40 mL of 0.125 M phosphate buffer pH 7.0 was added (0.125 M phosphate buffer; 0.072 M sodium phosphate dibasic heptahydrate, sodium phosphate monobasic monohydrate 0.053 M), incubated at 70 °C without shaking overnight, and cooled to room temperature. The tubes were centrifuged at $3,000 \times g$ for 30 min and decanted into a clean container. The previous step was repeated twice more, decanting each time into the clean container. The extracted supernatant was then dialyzed at 4 °C for 72 h, replacing the water twice each day (MWCO 14,000, flat width 76 mm, Sigma D9402). Dialyzed liquid was then frozen at 20 °C until solid, then freeze-dried completely.

2.3 Fluorescence In Situ Hybridization (FISH)

Fluorescence in situ hybridization (FISH) was used as a preliminary screen of taxa isolated from the enriched fecal samples. The *Bacteroidota*-specific CF968 oligonucleotide probe (5'-GGTAAGGTTCTCGCGTA-3') labelled with the ATTO 594 fluorochrome (Biomers, Germany) was used to identify *Bacteroidota* cells. *Bacteroides thetaiotaomicron* and *E.coli* K12 cells were used as controls for the CF968 and the Gammaproteobacteria-specific probe GAM42a (5'-GCC TTC CCA CAT CGT TT-3'), respectively. Fixed cells were vacuum filtered onto a 0.45 μM filter, and stored at -20 °C. Hybridization buffer (700 μL of 35% formamide, 5M NaCl, 1M Tris-HCl, and 900 μL distilled H_2O , 10% SDS) was added to 8.4 μM μL^{-1} probe solution at a 4:1 ratio. 25 μL of the hybridization buffer-probe mixture was used to saturate each filtered sample, which was left to incubate in a humidity chamber at 46 °C (3 h for GAM42a, and overnight for CF968)(Klassen et al., 2021). All images were generated using a Leica DM RBE epifluorescence microscope at 30% brightness. 4',6-diamidino-2-phenylindole (DAPI; 365 nm) was used for

detection of DNA from all microbial cells at and CF968 (603 nm) for cells belonging to *Bacteroidota*.

2.4 Bacterial growth profiling

Starter isolate cultures were grown anaerobically (atmosphere: CO₂:H₂:N₂, 85:10:5, v/v/v) using 0.5% (w/v) Glu in MM at 37 °C, overnight. Starter cultures (OD₆₀₀ 1.0-1.4) were diluted to an OD₆₀₀ of 0.15 and grown under various conditions. Treatments included: 0.5% (w/v) MM/ALG, 0.5% (w/v) MM/Glu, 0.5% CMB/Glu, and a no carbon MM negative control. This process was also done for bacterial isolates grown on *S. latissima* extract, and were monitored within the following treatments: 0.5% (w/v) MM/*S. latissima* extract, 0.5% (w/v) MM/Glu, 0.5% CMB/Glu, and a no carbon MM negative control. Technical replicates (n=4) at a volume of 200 µL for each reactions) were cultivated in 96 well plates (Corning, 353072), and sealed with polyurethane gas permeable membranes (Sigma-Aldrich, Z390059). UV (600 nm) absorbance readings were taken at 10 min intervals for 48 h in a microplate reader (Cerillo, Stratus) GraphPad Prism 10.3.1 was used to plot the growth curves.

2.5 Whole shotgun genomics and metagenomics

DNA extraction was performed on LN₂ frozen feces and enriched fecal communities, enriched with either ALG or *S. latissima* extract as the carbon source, from ruminant species yielding positive isolates including Masai giraffe (Calgary), bighorn sheep (Saskatoon), rocky mountain goat (Calgary), rocky mountain goat (Saskatoon), and moose (Calgary), as well as bison (Saskatoon) as a negative control. DNA extraction was also performed on purified bacterial

isolates (**Table S5**). All DNA extractions were done using the DNeasy PowerSoil Pro Kit (QIAGEN, 47014). DNA quality and concentration were assessed using both the NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, ND-2000C) and Qubit fluorometer (Thermo Fisher Scientific, Q33326). Whole shotgun genomics and metagenomics DNA sequencing were conducted by Illumina NovaSeq 6000 using 150bp reads at Génome Québec. Illumina reads were trimmed using Trimmomatic (Bolger et al., 2014) and assembled into contigs using SPAdes (Nurk et al., 2017). Open Reading Frames (ORFs) were predicted from assembled contigs using Prodigal (Hyatt et al., 2010), which were further annotated by dbCAN3, a CAZyme annotation tool based on CAZy HMMs (Zheng et al., 2023), for the presence of CAZymes. The function of putative CAZymes were predicted by SACCHARIS v2.0 (Fraser et al., 2024) which seeds phylogenetic trees built with characterized sequences from the CAZy database with user sequences (Drula et al., 2022). Annotated protein data from each bacterial isolate was screened for putative AULs, and plotted through the gggenes package in RStudio (Juggins, 2015).

CAZyme counts according to dbCAN3 HMMs were recorded for PL6, PL17, and PL38 within metagenomics data from fecal and enriched community samples. Since PLX does not have a dbCAN3 HMM, metagenomics data was screened for PLX homologues through a BLAST search against *BxPLX* (E-value $< 1 \times 10^{-3}$, bitscore > 50). To assess the prevalence of the identified AUL amongst other datasets, National Center for Biotechnology Information (NCBI) annotated protein data was downloaded and the presence of the AUL verified. Annotated protein FASTA from each bacterial isolate was collected and screened for the identified AUL. AULs were plotted through the gggenes package in RStudio (Juggins, 2015).

2.6 Purification of ALG lyases

To characterize the catalytic specificities of putative ALG lyases (ALs), identified ORFs of interest were trimmed to exclude signal peptides (predicted by SignalP 6.0), and synthesized into kanamycin (KAN) resistant pET-28a (+) plasmids with an N-terminal His₆-tag. Constructs were transformed into both DH5α and BL21 (DE3) *E. coli*. Single BL21 (DE3) colonies were re-inoculated in LB media with KAN (50 µg mL⁻¹), and grown at 37 °C with shaking at 200 rpm to an OD_{600nm} of 0.6-0.8. Cultures were cooled to 18 °C, induced using isopropyl β-D-1-thiogalactopyranoside at a final concentration of 1 mM, and left overnight at 18 °C and 200 rpm. The next day, cultures were clarified by centrifugation at 6,500 × g for 1 hr. The cell pellet was lysed using ~30 mL of lysis buffer (Lysis buffer: 500mM NaCl, 20 mM Tris pH 8.0, and 0.1 mg mL⁻¹ egg-white lysozyme). Cells were further lysed via sonication, and the resulting mixture was clarified by centrifugation at 17,500 × g for 1 hr. The recombinant protein was purified from the supernatant by Ni-NTA affinity chromatography via the ÄTKA start protein purification system. Bound protein was eluted using an imidazole gradient from 0 to 500mM in 20mM Tris, pH 8.0. Eluate was concentrated and buffer exchanged into 20mM Tris pH 8.0, 500mM NaCl, and 2% glycerol using 30 kDa cut-off Amicon Ultra Centrifugal concentrators (Millipore Sigma, UFC903008). Protein purity and size was assessed by a 12% SDS page gel electrophoresis, and imaged using a gel imaging system (Bio-Rad, Gel Doc XR+ with Image Lab 6.1 software). Protein concentration was determined using the Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific, ND-2000C), using the theoretical molecular weight and extinction coefficient predicted by the Expasy ProtParam tool (Gasteiger et al., 2005).

2.7 Screening of ALG lyase activity

Reactions with ALs were conducted using the following parameters: 1 μ M enzyme, 0.5% (w/v) ALG, in 200 μ L of 0.02 M citrate-phosphate buffer (0.05 M sodium citrate, 0.10 M sodium phosphate), and incubated at 37 °C for 16 h. Reaction was terminated by incubating at 95 °C for 10 min. Digested products were analyzed by TLC and HPAEC-PAD.

2.7.1 Thin-layer chromatography (TLC)

Digest products were spotted onto TLC aluminium plates (Supelco, Z740232) twice in 2 μ L aliquots, allowing sheet to dry completely between applications. Standards included 0.5% (w/v) undigested ALG, ManA, and GulA. Plates were placed in a shallow layer of mobile phase (4:1:1 butanol: acetic acid: H₂O) and allowed to fully saturate twice within a sealed chamber. Plates were dried completely and stained with 1% orcinol, 70:3 EtOH (EtOH) to sulfuric acid solution, and then gently heated at 120 °C in an oven to reveal spotting.

2.7.2 High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD)

Prior to HPAEC-PAD analysis, samples were sterile filtered with 0.2 μ m filters (Thermo Fisher Scientific, 13-1001-08). Samples were diluted 1:5 or 1:10, and 10 μ L was injected onto an analytical (3 $\times$ 150 mm) CarboPac PA20 column (Thermo Scientific) with a CarboPac PA20 guard column (Thermo Scientific) on a Dionex ICS-3000. Samples were eluted in a mobile phase of 100 mM NaOH and an increasing gradient of sodium acetate (0 to 5 min, 0 to 0 M; 5 to 60 min, 0 to 1 M). Column compartment was temperature controlled at 30 °C. Elution was monitored with a PAD detector (standard quadratic waveform for carbohydrates). Peaks were compared to standards of 100 μ M ManA, 100 μ M GulA and H₂O.

2.7.3 UV-Vis (ultraviolet-visible) spectrophotometry

BxPLX was produced and purified as described in section 2.6. 10 nM *BxPLX* was incubated with 0.5% sodium ALG (0.02 M citrate-phosphate buffer, pH 7, 37 °C). Formation of the C4-C5 unsaturation through β -elimination were monitored through the absorption of UV light at 232 nm, on an ID3 Microplate reader for 200 min, with reads at 35 s intervals. Data analysis was done through GraphPad Prism 10.3.1. This process was also performed for *BxPLX* mutants; H122A, K127A, Q125A, R160A, R162A, and Y238A.

2.8 Crystallography of *BxPLX*

2.8.1 Mutant generation

The RCSB Protein Data Bank (Berman et al., 2000) was searched for *BxPLX* structural homologues with crystal structure data using the DALI server (Holm, 2020). *BxPLX* catalytic residues were predicted through the superimposition of structurally homologous PL7 AlyA1 (PDB ID: 3ZPY (Thomas et al., 2013)), PL7 A1-II' (PDB ID: 2Z42 (Ogura et al., 2008)) and PL14 AkAly30 (PDB ID: 5GMT (Qin et al., 2017)) using ChimeraX 1.9 matchmaker tool (Meng et al., 2023). Forward and reverse T7 primers targeting putative *BxPLX* catalytic residues were designed using the Agilent QuikChange Primer Design Program (Agilent Technologies, 2025). Primers were designed to mutate residues H122, K127, Q125, R160, R162, and Y238 to alanine. Mutagenesis of *BxPLX* targets was done with the QuikChange II Mutagenesis Kit (Agilent Technologies, 200524-5). Cycling conditions were as follows; initial denaturation at 95 °C for 30 s, followed by 14 cycles of 95 °C for 30 s, 55 °C for 1 min, and 68 °C for 6 min. Products were evaluated by gel electrophoresis using a 1% agarose gel and visualized through a gel imaging

system (Bio-Rad, Gel Doc XR+ with Image Lab 6.1 software), and NanoDrop 2000c (Thermo Fisher Scientific, ND-2000C). Mutated plasmids were transformed into *E.coli* DH5 α , and clones were confirmed by Sanger sequencing (Eurofins, USA). Verified mutant plasmids were transformed into *E.coli* BL21 (DE3) for expression.

2.8.2 Protein purification and crystallization

Protein production and purification for both *BxPLX* and *BxPLX* Y238A was performed as described in section 2.6, utilizing Ni⁺²-NTA immobilized metal affinity chromatography followed by size exclusion chromatography using a Sephacryl S-200 column (GE Healthcare) pre-equilibrated with 20 mM Tris-HCl pH 8.0 containing 500 mM sodium chloride (Vickers et al., 2025). Purified protein was concentrated to 15 mg ml⁻¹ using a stirred cell ultrafiltration device (Amicon) with a 10 kDa molecular weight cut-off membrane (Millipore) for crystallization. Crystallization screening was conducted with commercially available screens, Index, PEG ION, and Crystal Screen (Hampton Research), and MSCG I, II, III, and IV (Anatrace). Sitting-drop vapour-diffusion at 291 K with 1:1 ratio of protein (15 mg ml⁻¹ in 20 mM Tris-HCl pH 8.0, 500 mM sodium chloride) and crystallization solutions (0.5 μ l each) gave several initial crystals. Optimizations for *BxPLX* and *BxPLX* Y238A using hanging-drop vapour diffusions resulted in the crystals that were harvested and used to collect diffraction data, as summarized in **Table S3**.

2.8.3 Data collection and processing

The crystals were cryoprotected in crystallization solution with 20% (v/v) ethylene glycol and immediately frozen in liquid nitrogen to be stored and shipped. Diffraction data were collected at the Canadian Light Source on the Canadian Macromolecular Crystallography Facility - Insertion Device beamline. Data were integrated, scaled, and merged with XDS and Aimless

(Agirre et al., 2023; Evans & Murshudov, 2013; Kabsch, 2010). Data collection and processing statistics are summarized in **Table S4**.

2.8.4 Structure solution and refinement

The crystal structures of *BxPLX* were solved by molecular replacement using PHASER (McCoy et al., 2007) and a model of *BxPLX* generated by the AlphaFold Server (AlphaFold 3) (Jumper et al., 2021; Varadi et al., 2022). COOT was used to manually correct the models and Phenix.refine was used for the refinement of the experimental models and the addition of water molecules (Emsley & Cowtan, 2004; Terwilliger et al., 2019). Mannuronic acid (ManA) and guluronic acid (GluA) ligands were modelled by superimposing carbohydrate ligands with similar degrees of polymerization from structures previously reported (PDB ID: 7W18 and 7W12, respectively). Refinement procedures were monitored by flagging 5% of all observations as “free” (Brünger, 1992). Model validation was performed with MOLPROBITY (Chen et al., 2010). A summary of model statistics for each structure is shown in **Table S5**.

2.9 Solution-state time-resolved 1D ¹H NMR analysis of sodium alginate degraded by *BxPLX*

2.9.1 Preparation of D₂O solution of sodium alginate

BxPLX (1 mL of 1 μM) was aliquoted into a microcentrifuge tube with a sharp conical bottom and subjected to 3 successive rounds of freezing at -20 °C followed by lyophilization and resuspension in 1 mL of 99% D₂O (Sigma; 151882). The final lyophilization cycle was

performed in an NMR tube and sealed. After each cycle of lyophilization, residual activity was confirmed by TLC analysis of ALG digests.

Autoclaved ALG (1 mL of 3% (w/v)) (Sigma A1112) was subjected to 4 successive rounds of lyophilization and resuspension. After each resuspension, the solution was stirred at 300 rpm for 1 h at room temperature. In the first two cycles, the lyophilized product was resuspended with 1 mL of 99% D₂O (Sigma; 151882). The lyophilized product in the third cycle was resuspended with 500 μ L of 99% D₂O (Sigma; 151882) and 500 μ L of D₂O with 0.75% 3-(trimethylsilyl)propionic-2,2,3,3-d₄ (TSP-d₄) (Sigma; 293040-25G). The final cycle was resuspended in 1 mL of 99.9% D₂O (Sigma; 151882), and sealed. Care was taken to avoid exposure of the sample to contamination by moisture at each step of the process.

2.9.2 Solution-state time-resolved 1D ¹H NMR data collection and analysis

Approximately 0.67 mL of the above-mentioned D₂O solution of sodium alginate was transferred into a 5 mm NMR tube containing 0.7 mL of 1 μ M of *BxPLX* lyase that had been previously lyophilized at the bottom of the tube. The tube was then immediately loaded into a Bruker Avance III HD 700 MHz spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) operating at 700.44 MHz for time-resolved 1D ¹H NMR data acquisition at 310 K using the standard Bruker zg30 pulse sequence (Robb et al., 2022). The first spectrum was acquired after 24 min of incubation, followed by consecutive 15-min back-to-back acquisitions, yielding a total of 156 spectra. Chemical shifts were internally referenced to TSP-d₄ at 0 ppm. For each 1D ¹H NMR experiment, 130 scans were collected with an acquisition time of 5.8555 s, a relaxation delay of 1.0 s, and a spectral width of 11 ppm (7,704.2 Hz). The acquired data contained 45,112 points and were zero-filled to a final spectral size of 65,536 points without linear prediction. Automatic phase and baseline corrections were performed.

2.10 Solution-state 1D and 2D NMR analyses of DEH products generated from sodium alginate by the combined use of four lyases

2.10.1 Preparation of DEH products and their D₂O solution for NMR analysis

BxPL6, *BxPL17*, *BxPL38*, and *BxPLX* were added to a 20 mL solution containing 0.5% (w/v) ALG and millipore H₂O at a final concentration of 1 μ M for each enzyme. The digest was incubated overnight at 37 °C. The reaction was stopped by incubating at 95 °C for 10 min, and clarified by centrifugation for 30 min at 4,000 $\times$ g. The resulting supernatant was frozen at -20 °C, and subsequently lyophilized. EtOH precipitation of lyophilized digest product was done by adding 2 mL of pure EtOH, resuspending and transferring the mixture to a clean glass tube (glass tube 1), to a final EtOH volume of 8 mL. The Falcon tube was rinsed with 2 mL of millipore H₂O, and added to the clean glass tube, resulting in an 80% solution of EtOH (v/v). This solution was stirred magnetically (300 rpm) at ambient temperature overnight.

The stirred mixture (1 mL) was added to a microcentrifuge tube with a sharp conical bottom and centrifuged at 8,000 $\times$ g for 30 min at room temperature. Supernatant was collected and transferred to a clean glass tube (glass tube 2), and evaporated in an air concentrator. This process was repeated in 1 mL aliquots until the entire 10 mL solution was evaporated to dryness. The pellet was then resuspended in 1 mL of H₂O and transferred to ‘glass tube 1’ and magnetically stirred at 300 rpm until fully dissolved. Next, pure EtOH was added to ‘glass tube 1’, to a final concentration of 80% (v/v) solution. This solution was successively evaporated to dryness in 1 mL aliquots in ‘glass tube 2’ as described previously.

To the bottom of ‘glass tube 2’, 1 mL of 99% D₂O (Sigma; 151882) and 100 μ L of D₂O with 0.75% TSP-d₄ (Sigma; 293040) were added and stirred with a magnetic bead at 300 rpm for

1 h at ambient temperature. Care was taken to avoid exposure of the sample to contamination by moisture at each step of the process. The sample was then frozen at -20 °C and lyophilized. Additions of D₂O and subsequent lyophilization were repeated three times. Following the final round of lyophilization, 1 mL of high-purity D₂O was added to dried product and magnetically stirred at 300 rpm overnight. The fully dissolved sample was then transferred into an NMR tube for analysis the following morning.

2.10.2 Solution-state 1D and 2D NMR data collection and analysis

1D and 2D NMR experiments were carried out at 310 K on a Bruker Avance III HD spectrometer operating at 700.44 MHz for ¹H and 176.14 MHz for ¹³C (Bruker BioSpin GmbH, Rheinstetten, Germany). A triple-resonance TBO-Z probe was used, with the outer and inner coils tuned to ¹H and ¹³C, respectively. Chemical shifts for both nuclei were internally referenced to TSP-d₄ at 0 ppm. The sample was analyzed by 1D ¹H, 1D ¹³C, 2D ¹H–¹³C Heteronuclear Multiple Bond Correlation (HMBC), 2D ¹H–¹³C Heteronuclear Single Quantum Coherence (HSQC), 2D ¹H–¹H Total Correlation Spectroscopy (TOCSY), and 2D ¹H–¹H Correlated Spectroscopy (COSY) experiments using the standard Bruker pulse programs zg30, zgpg30, hmbcetgpl3nd, hsqcedetgppsp.3, dipsi2gpphzs, and cosygpmfqq, respectively (Robb et al., 2022; Xing et al., 2026). Spectral processing and interpretation were performed using Mnova version 14.2.1-27684 (Mestrelab Research S.L., Santiago de Compostela, Spain).

The detailed acquisition and processing parameters for each 1D and 2D NMR experiment are described as follows:

For the 1D ¹H experiment, 2,000 scans were collected with an acquisition time of 3.1999 s, a relaxation delay of 1.0 s, and a spectral width of 20 ppm (14,097.7 Hz). The acquired data

contained 45,112 points and were zero-filled to a final spectral size of 131,072 points. Phase and baseline corrections were performed automatically.

The 1D ^{13}C spectrum was recorded with 15,250 scans, an acquisition time of 1.5729 s, a relaxation delay of 2.0 s, and a spectral width of 236 ppm (41,666.7 Hz). A total of 65,536 points were collected and subsequently zero-filled to 131,072 points. Automatic phase and baseline corrections were applied.

For the 2D ^1H - ^{13}C HMBC experiment, 128 scans were collected for each t_1 increment. The relaxation delay was 1.5 s, and the acquisition time was 0.2433 s. Spectral widths were set to 12 ppm (8,417.5 Hz) in F_2 and 200 ppm (35,211.3 Hz) in F_1 . The time-domain matrix consisted of 2,048 points in t_2 and 512 points in t_1 , and the processed spectrum contained 2,048 points along both F_2 and F_1 . The F_1 dimension was zero-filled from 512 to 2,048 points without linear prediction. A sine-squared apodization function with a 90° shift was applied in F_1 , with the first point set to 0.50, whereas a sine-bell function with a 45° shift was applied in F_2 . Phase and baseline corrections were performed automatically, followed by processing with Mnova's Power along F_1 and Power along F_2 functions.

For the 2D ^1H - ^{13}C HSQC experiment, 140 scans were recorded for each t_1 increment. A relaxation delay of 1.5 s and an acquisition time of 0.2433 s were used, with spectral widths of 12 ppm (8,417.5 Hz) in F_2 and 200 ppm (35,211.3 Hz) in F_1 . The acquired matrix comprised 2,048 points in t_2 and 256 points in t_1 . Processing yielded final sizes of 2,048 points in F_2 and 1,024 points in F_1 . The indirect dimension was zero-filled from 256 to 1,024 points with MIST linear prediction applied. In F_1 , a sine-squared apodization function with a 0° shift was applied, whereas a sine-bell function with a 90° shift was applied in F_2 . The first point was set to 0.50 in F_1 and left unadjusted

in F₂. Automatic phase and baseline corrections were followed by processing with Mnova's Power along F₁ and Power along F₂ functions.

For the 2D ¹H–¹H TOCSY experiment, 50 scans were acquired for each t₁ increment. The relaxation delay was 2.0 s, the mixing time was 80 ms, and the acquisition time was 0.2437 s. A spectral width of 12 ppm (8,403.4 Hz) was used in both dimensions. The acquired matrix contained 2,048 points in t₂ and 256 points in t₁, and the final processed spectrum contained 2,048 points in F₂ and 1,024 in F₁, with F₁ dimension zero-filled from 256 to 1,024 points with MIST linear prediction applied. A sine-squared apodization function with a 90° shift was applied in both dimensions. The first point was set to 0.50 in F₁ and left unadjusted in F₂. Phase and baseline corrections were carried out automatically.

For the 2D ¹H–¹H COSY experiment, 50 scans were acquired for each t₁ increment. The experiment used a relaxation delay of 2.0 s and an acquisition time of 0.2433 s. Spectral widths were 12 ppm in both dimensions (8,417.5 Hz in F₂ and 8,403.4 Hz in F₁). The acquired matrix contained 2,048 points in t₂ and 256 points in t₁, and processing produced 2,048 points in F₂ and 1,024 in F₁, with the F₁ dimension zero-filled from 256 to 1,024 points with MIST linear prediction applied. A sine-squared apodization function with a 0° shift was applied in both dimensions, with the first point set to 0.50 in F₁ and left unadjusted in F₂. Automatic baseline correction was applied.

CHAPTER 3: RESULTS

3.1 Taxonomic verification and growth profiling of alginate-utilizing bacterial isolates

3.1.1 Selective isolations on alginate and *S. latissima* extract

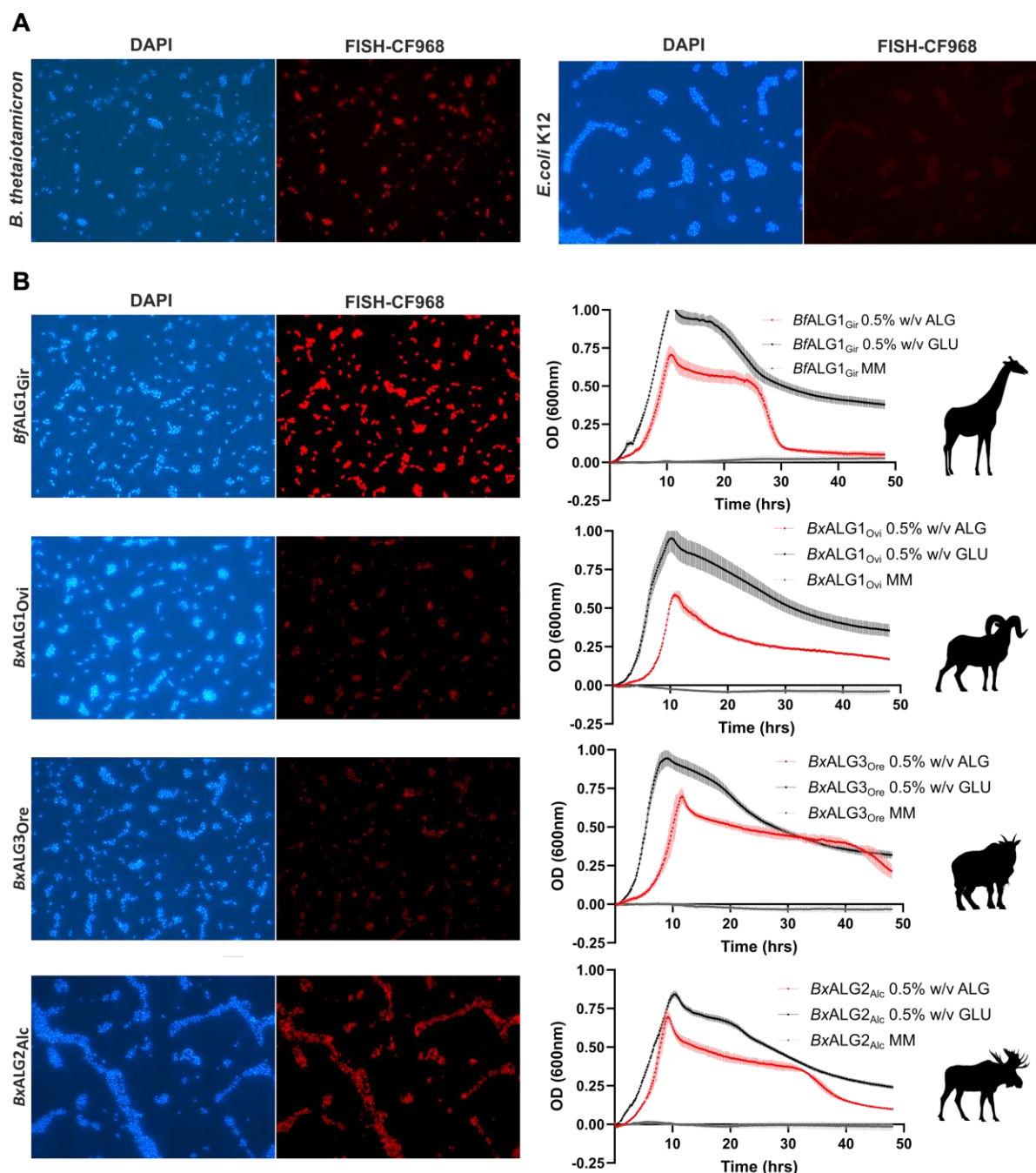
Ruminants and pseudoruminants at the Wilder Institute/Calgary Zoo and Saskatoon Forestry Farm Park and Zoo were maintained on diets by licensed handlers. Dietary information for each animal was screened for ALG-containing ingredients (**Source data 1**). Based on this information, there is no indication of prior ALG consumption by any of the sampled animals.

Selective enrichment of sampled fecal material yielded bacterial isolates from four ruminant species: Masai giraffe, rocky mountain goat, bighorn sheep, and moose (**Table S5**). A total of 20 ALG-enriched bacterial isolates and six *S. latissima* extract-enriched isolates were further investigated. ALG-enriched bacterial isolates included: Masai giraffe (8 bacterial isolates); rocky mountain goat (6 bacterial isolates); bighorn sheep (4 bacterial isolates); and moose (2 bacterial isolates). *S. latissima* extract-enriched bacterial isolates included: bighorn sheep (4 bacterial isolates), and rocky mountain goat (2 bacterial isolates). (**Figure S5; Table S5**). Bacterial isolates were systematically named based on genus/species identifications, followed by enrichment carbon source, bacterial isolate reference number, and host genus (*e.g.*, BxALG1_{Alc} denotes *B. xylanisolvens* bacterial isolate #1 enriched on ALG, sourced from *Alces alces* (moose)).

3.1.2 Fluorescence in-situ hybridization (FISH)

A preliminary taxonomic screen of bacterial isolates was performed via fluorescence in situ hybridization (FISH). The identity of all bacterial isolates were determined to be

Bacteroidota. Fluorescence of cells occurred exclusively when incubated with CF968 and not GAM42a (**Figure 3.1; Figure S1; Figure S2**), indicating successful hybridization of CF968 with the 16S rRNA. Control strains were used to validate probes. *B. thetaiotamicron* VPI-5482 was used as positive control for the *Bacteroidota*-specific probe, CF968, and *E.coli* K12 stained with GAM42a as a negative control for *Bacteroidota*. Notably, no signal was observed when incubated with the opposite probe.



probe, GAM42a, as a control (**Figure S1; Figure S2**). **(A)** Control cells *B. thetaiotaomicron* VPI-5482 and *E. coli* K12 incubated with *Bacteroidota*-targeting probe, CF968. **(B)** FISH images of *Bf*ALG1_{Gir}, *Bx*ALG1_{Ovi}, *Bx*ALG3_{Ore}, and *Bx*ALG2_{Alc}, with corresponding growth profile.

3.1.3 Bacterial growth profiling

The capacity of bacterial strains to metabolize ALG was evaluated by UV monitoring of liquid culture OD₆₀₀ absorbance over 48 h. ALG and *S. latissima* extract were used as the carbon sources. (**Figure 3.1; Figure S3; Figure S4**). Many isolate cultures in the MM + 0.5% (w/v) ALG condition (e.g., *Bf*ALG1_{Gir}) experienced log phase growth peaking at OD_{600 nm} ~0.50-0.75 at 5-10 h, plateaued growth from ~10-30 h, and a drop in absorbance after ~20-30 h. Other isolates within the ALG condition (e.g. *Bx*ALG1_{Alc}) displayed similar log phase growth, followed by a range in profiles for stationary and death phase, showing a very gradually declining OD₆₀₀ shortly following peak growth at ~10 h, extending until the end of the experiment. Others showed less classical growth phases, such *Bf*ALG5_{Gir}, which had a very clear biphasic growth profile peaking once at OD₆₀₀ 0.25 (~10 h), and again at OD₆₀₀ 0.5 (~22 hs), followed by a gradual decline in OD₆₀₀. Growth profiles of bacterial isolates grown in the MM + 0.5% (w/v) Glu and CMB + 0.5% (w/v) Glu (data not shown) conditions were generally more uniform. Bacterial isolates in the Glu condition experienced exponential growth between ~5-10 h, peaking from OD₆₀₀ 0.75-1.00, stationary growth between ~10-20 h, followed by a gradual decline in OD₆₀₀. Bacterial isolates in the CMB + Glu condition (the condition with the richest media) showed the highest OD_{600 nm}, with peak log phase extending to OD₆₀₀ 1.00-1.25 at ~10-15 h, followed by sustained stationary phase until the end of the experiment (data not shown). As expected the MM + no carbohydrate control showed no growth.

3.2 Conserved AUL amongst *Bacteroides* isolate strains

DNA sequencing of genomes from each bacterial isolate DNA confirmed them to be *Bacteroides* spp. (**Figure S5**). Protein sequence data was used to create an ortholog-based species tree (Emms & Kelly, 2019) of all bacterial isolates and the following reference strains: *Bt* VPI 5482 (Xu et al., 2003), *Bf* CBA7301 (Kim et al., 2023), *Bo* ACCT 8483 (Cato & Johnson, 1976; Eggerth & Gagnon, 1933), and *Bx* XB1A (Chassard et al., 2008). The bacterial isolates separated into two distinct clades; *Bacteroides faecium* (*Bf*; sourced from Masai giraffe) and *Bacteroides xylanisolvens* (*Bx*; sourced from rocky mountain goat, bighorn sheep, and moose). In all genomes, an AUL containing 12-13 annotated ORFs, first characterized in human gut *B. ovatus* (Ronne et al., 2023), was identified. The AUL is structured as follows: transcriptional regulator; hypothetical protein (not present in all AULs); SusC-like; SusD-like; IPT/TIG domain protein; PL6, PL17; KdgF; Major Facilitator Superfamily (MFS) transporter; unknown protein 1; PL38; unknown protein 2; and unknown protein 3 (**Figure S5; Table S6**). The sequence similarity of AULs within clades is 100%, with the exception of *Bx*ALG6_{Ore} that shows subtle sequence variation from the other AULs within its clade (>99% sequence similarity) for several ORFs. Across both clades, the ORFs maintain high sequence similarities with their homologs, with most ORFs showing >90% sequence similarity, with the exception of SusC-like, SusD-like, and IPT/TIG domain protein at 88.3%, 84.4%, and 61.5% sequence similarity, respectively. (**Table S6**).

This AUL can be found with high synteny in other *Bacteroides* spp. ortholog data, sourced from human and other ruminant hosts (**Figure 3.2; Table S7**). *B. ovatus* CP926 from the human gut was previously characterized (Ronne et al., 2023). Others include *B. fingoldii* CL09T03C10, *B. faecium* 7301, *B. ovatus* TSDC17.2, *B. ovatus* WH711, *Bacteroides* spp. M10

and *Bacteroides* spp. D2, also sourced from the human gut. *B. ovatus* NLAE-zl-C500 and *B. xylanisolvens* NLAE-zl-G399 were sourced from cattle and goat, respectively. The *B. xylanisolvens* strains from the present study (i.e., sourced from rocky mountain goat, bighorn sheep, and moose) clade with *B. xylanisolvens* NLAE-zl-G399, while the *B. faecium* strains (i.e., sourced from Masai giraffe) clade with *B. faecium* 7301. Sequence similarities between homologous AUL ORFs are listed in **Table S7**.

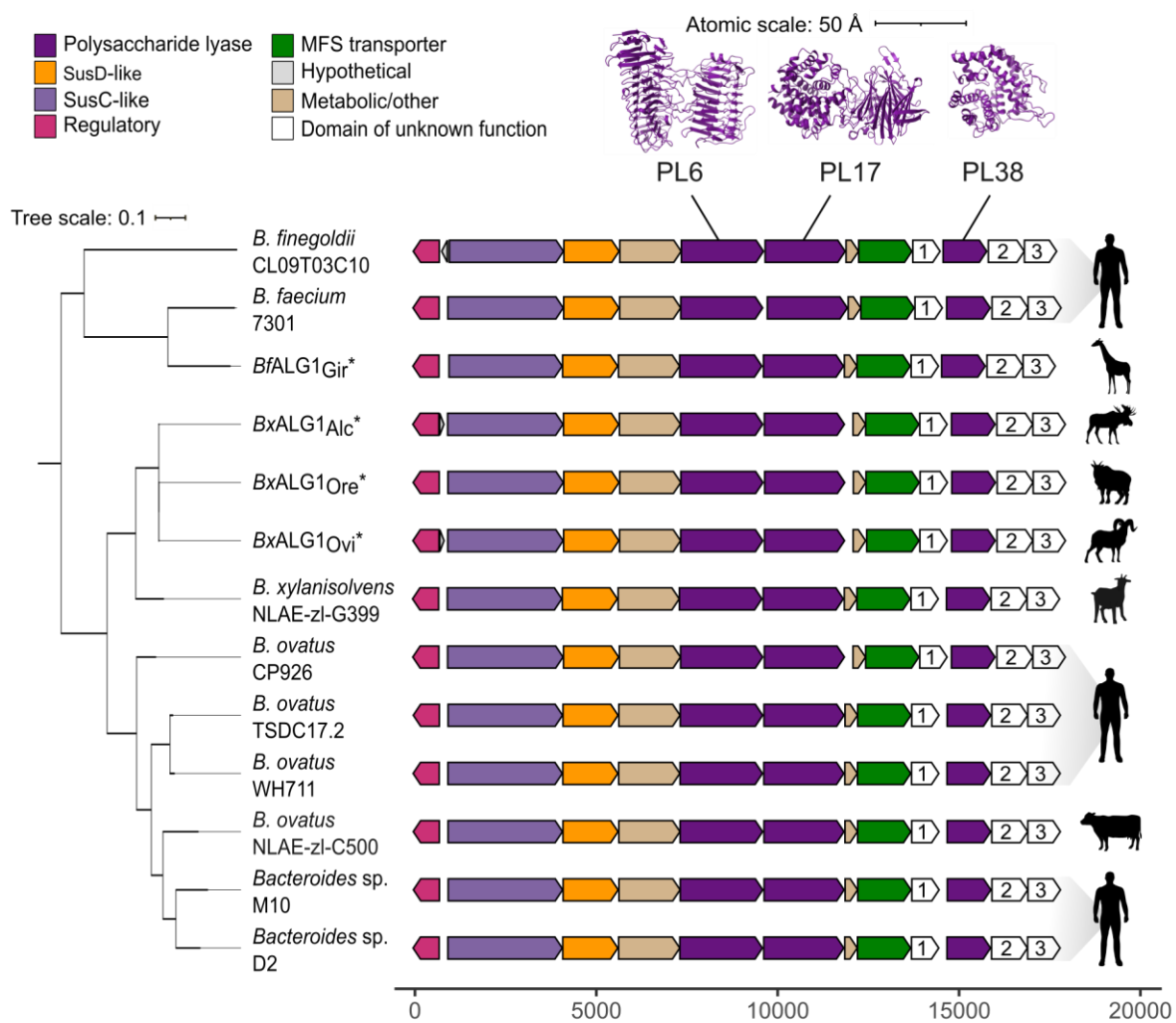


Figure 3.2. Synteny of AULs across different *Bacteroides* spp. and source hosts. The CAZy PULDB was searched for AULs with high synteny to AULs identified in Wilder Institute/Calgary Zoo and Saskatoon Forestry Farm and Park Zoo bacterial isolates. Based on CAZy PULDB hits, NCBI annotated protein data from identified strains were collected and screened for the identified

AUL. Domains of unknown function are numbered 1-3. AULs were plotted through the gggenes R studio package. Bacterial isolates with asterisk (*) are sourced from the present study. Percent identity of ORFs between AULs can be found in supplementary materials (**Table S7**).

3.3 AUL PL activity via TLC and HPAEC-PAD

To determine if the putative PLs were active, representative enzymes were selected from *BxALG1_{Alc}*. These PLs, referred to as *BxPL6*, *BxPL17*, and *BxPL38*, were recombinantly produced, purified, and screened for activity on ALG by TLC and HPAEC-PAD (**Figure 3.3**; **Figure S6**). Similar to previous work on homologs from *B. ovatus* (Ronne et al., 2023), *BxPL6* (80.8 kDa) and *BxPL17* (80.3 kDa) acted as exo-lyases, forming products equal to or smaller in size than ManA and GulA standards (Ronne et al., 2023). *BxPL38* (43.6 kDa) acted as an endo-lyase, generating larger oligosaccharides. HPAEC-PAD analysis of enzyme digestion products corroborated the TLC results (**Figure 3.3**). *BxPL6* and *BxPL17* enzymatic digestion products had a shorter retention time (~5 min) than ManA and GulA standards (~17.5 min), indicating they generate smaller products with a lower binding affinity to the column. In contrast, *BxPL38* products eluted later, with a prominent peak appearing at ~28 min and a ladder of smaller peaks consistent with retention times consistent with larger ALG oligosaccharides (36-65 min). When the enzymes were combined into a signal reaction, the resulting product profile appear to be additive. For example, the *BxPL6/BxPL38* combination shows both smaller (*BxPL6*) and larger ALG products (*BxPL38*) generated. The combination of *BxPL6/BxPL17/BxPL38* leaves the least amount of product at the spotting origin of the TLC plate, with a prominent band above monosaccharide standards, and HPAEC-PAD displays a large peak (~5 min) and resulting from the clearance of larger oligosaccharides with all three enzymes present (**Figure 3.3**).

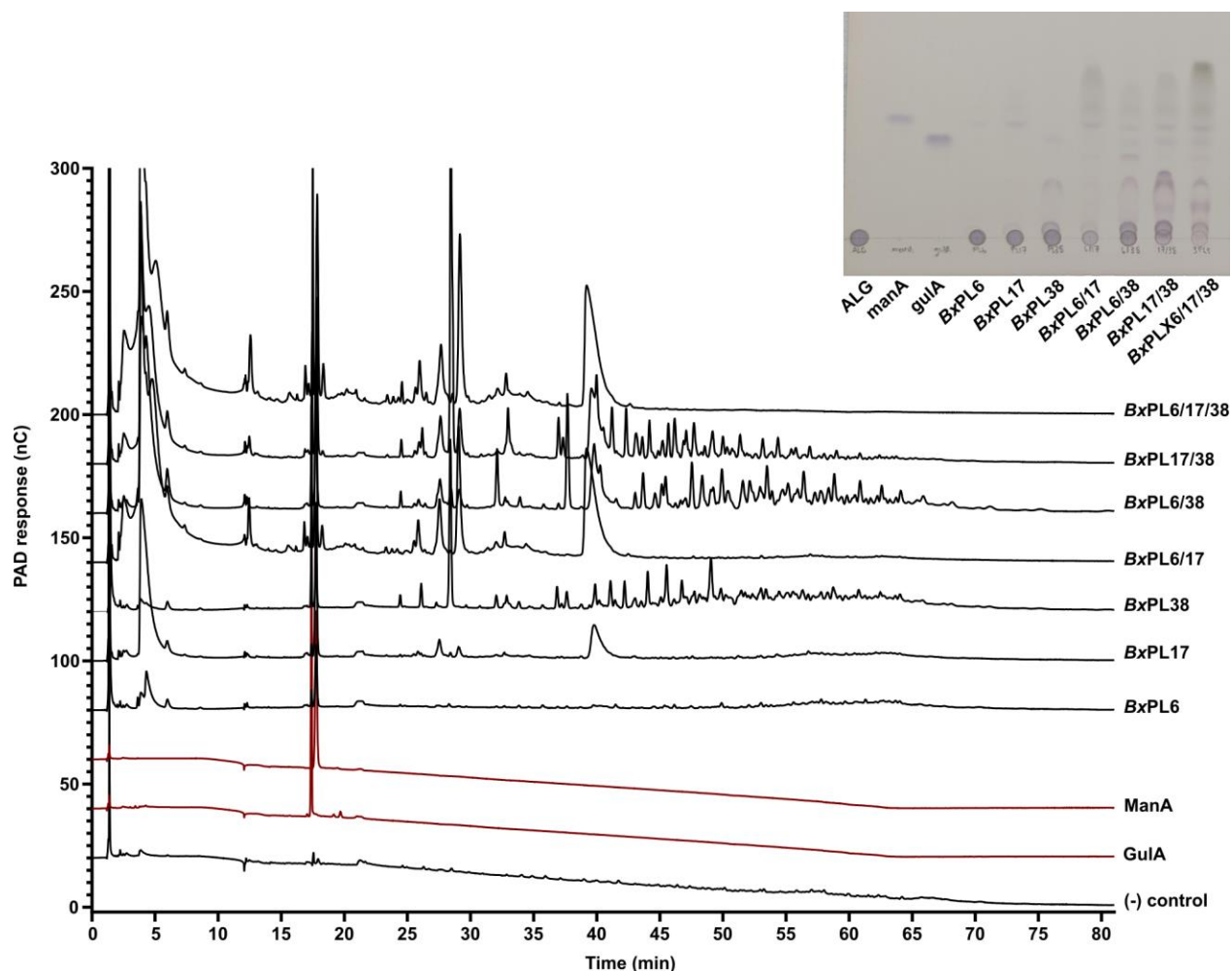


Figure 3.3. HPAEC-PAD analysis of enzyme digestion products generated through incubation with *B. xylanisolvens* AUL PLs. Single and combination enzyme digests of 0.5% (w/v) ALG by AUL PLs (*BxPL6*, *BxPL17*, *BxPL38*) were conducted at an enzyme concentration of 1 μ M (16 hs; 37 $^{\circ}$ C; pH 7). Baseline subtracted PAD response values of elution are shown over time. ManA and GulA standards are shown.

3.4 Discovery of *BxPLX* a new Polysaccharide Lyase family

To explore the potential function of the domains of unknown function with the AUL, the sequences of unknown proteins 1-3 (**Fig. 3.2**) were analyzed for protein sequence, function, and secretion using online bioinformatic tools including NCBI BLASTp database (NCBI Resource Coordinators, 2017), SignalP 6.0 (Teufel et al., 2022), and DALI (Holm, 2020).

Unknown protein 1 lacks a classical signal peptide, and was predicted to be an oxidoreductase/dehydrogenase (**Table S6**). Unknown protein 2 is a putative α/β hydrolase/esterase with a signal peptide (SP) I-type signal peptide. Unknown protein 3 was predicted to encode a lyase-like protein, and will be referred to further as *BxPLX* (**Figure 3.4**). Alphafold 3 and ExPASy ProtParam tools determined *BxPLX* as a ~30.1 kDa jelly-roll (theoretical pI, 6.50; Ext. coefficient: 39, 420) (Gasteiger et al., 2005). Interestingly, using existing dbCAN3 HMMs the gene product was not classified into a characterized PL family (Zheng et al., 2023). Therefore, to determine if *BxPLX* was functional, the gene was synthesized, recombinantly produced and purified. *BxPLX* displayed activity on ALG, producing a ladder of large oligosaccharides, indicating it is endo-acting (**Figure 3.4**). *BxPLX* is predicted to be secreted, possessing a SPII-type signal peptide, which commonly traffics proteins to the outer surface of the bacterial cell.

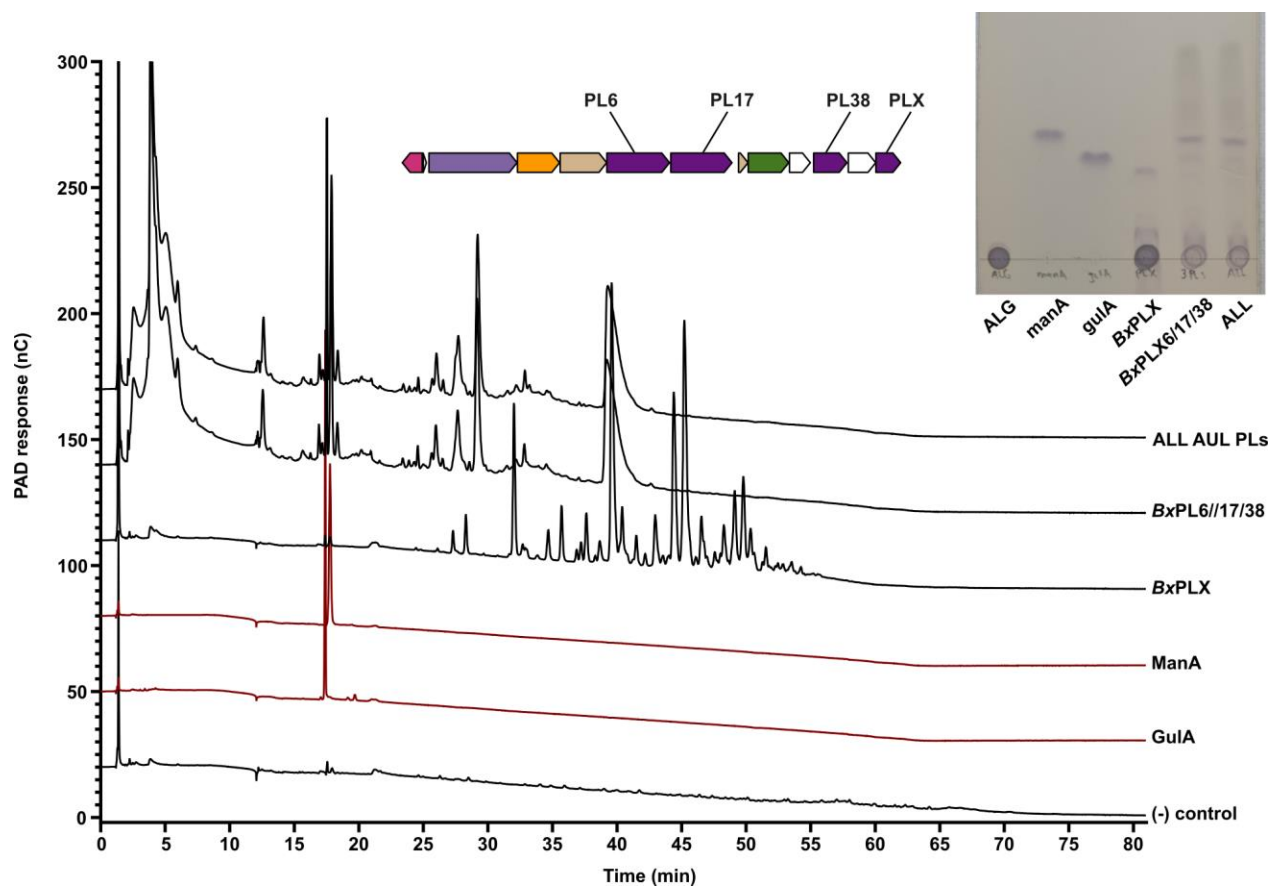


Figure 3.4. HPAEC-PAD analysis of enzyme digestion products generated through incubation with *B. xylanisolvens* AUL PLs including PLX. Single and combination enzyme digests of 0.5% (w/v) ALG by AUL PLs (*BxPL6*, *BxPL17*, *BxPL38*, *BxPLX*) were conducted at an enzyme concentration of 1 μ M (16 hs; 37 $^{\circ}$ C; pH 7). Baseline subtracted PAD response values of elution are shown over time. ManA and GulA standards are shown.

In order to verify presence of ALG-utilizing bacteria within the distal gut of Wilder Institute/Calgary Zoo and Saskatoon Forestry Farm and Park Zoo animals, DNA from fecal material and community enrichments were extracted and sent for whole genome sequencing. Across all samples, the fecal community metagenome yielded more total counts of PL6, PL17, and PL38 than enriched metagenomes (**Table S8**). Since PLX does not have a publically available dbCAN3 HMM to conduct a CAZome screen, a BLAST search against *BxPLX* was done for all samples (**Table S9**). PLX-like proteins were observed at high sequence similarities in

enriched moose (100%), rocky mountain goat (100%), and bighorn sheep (100%) and Masai giraffe (>90%) metagenomes. No hit was found within the bison enriched metagenome, a host species that also did not yield bacterial isolates by selective isolation. Overall the number of hits were greater within all unenriched fecal metagenomes, but sequence similarities were notable lower, between ~30%-50%. *BxPLX* homologues of varying relatedness were also found in MAGs from cattle (**Figure S7**) (**Ferrillo et al., 2026a**).

To investigate the function of PLX, the following ORFs from *Prevotella* sp. (*Psp*) And *Cryptobacteroides* sp. (*Csp*) were chosen for recombinant expression and analyses: *PspPLX1*, *PspPLX2*, *PspPLX3*, and *CspPLX* (**Ferrillo et al., 2026a**). A PLX ortholog tree was constructed, showing relatedness to *BxPLX* with sequence similarities of ranging from 43.7-62.3% (**Figure S7**). TLC and HPAEC-PAD analysis of ALG digestion products revealed the generation of large oligosaccharides by all the PLX homologs (**Figure S7**). Alpha-fold revealed that all enzymes share a jelly-roll structure, with the catalytic domain being approximately 30 kDa in molecular weight. *PspPLX1* and *PspPLX3* proteins contained non-catalytic elements, such as carbohydrate binding modules, which could contribute to the potentiation of enzyme function.

3.5 Crystallography of *BxPLX*

3.5.1 Mutant generation

To define the active site of *BxPLX*, putative catalytic residues were selected based on a superimposition of structurally similar enzymes from PL7 AlyA1 (PDB ID: 3ZPY (Thomas et al., 2013)), PL7 A1-II' (PDB ID: 2Z42 (Ogura et al., 2008)) and PL14 AkAly30 (PDB ID: 5GMT (Qin et al., 2017)), as described in section 2.8.1. Based upon spatial conservation with functional

residues, the following mutations were generated and sequence confirmed in *BxPLX*: Q125A, K127A, Y238A, H122A, R160A, and R162A. Initial analysis of enzyme digestion products by HPAEC-PAD and TLC revealed that H122A, R160A, and R162A mutations did not impede digestion of ALG (**Figure S8**), K127A resulted in a partial loss of activity, and Q125A, and Y238A mutants had a complete loss of β -elimination. This result was also confirmed through absorption spectroscopy at 232 nm (**Figure S8**). Loss of enzyme activity for Q125A, K127A, and Y238A indicated the functional significance of these residues. Based upon structural conversation with PL7 AlyA1 catalytic residues (*i.e.*, Q321, H323, and Y420), they also likely form a catalytic triad. Within the reported PL7 AlyA1 catalytic mechanism, Q321, H323, and Y420 participate in the β -elimination reaction as the neutralizing residue, Brønsted base, and Brønsted acid, respectively.

3.52 *BxPLX* crystal structure

The crystal structures of *BxPLX* (apo), and *BxPLX* Y238A in complex with both ManA (*BxPLX*-ManA) and GulA (*BxPLX*-GulA) oligosaccharides, were determined at 2.6 Å, 1.14 Å, and 2.0 Å, respectively. A summary of all data collection and processing statistics is presented in **Table S2 and S3**. As predicted, the overall structure of *BxPLX* resembles a β -sandwich jelly roll-fold, composed of two stacked anti-parallel β -sheets (**Figure 3.5 A**). The outer and inner sheets composed of 5 and 7 β -strands, are connected by loop regions, which contribute to the formation of a characteristic positively charged catalytic groove. The coordination of one zinc atom was observed, although it does not seem to contribute to the catalytic core. The *BxPLX* Y238A complexes were co-crystallized with penta-ManA and penta-GulA; however, both *BxPLX*-ManA and *BxPLX*-GulA complexes displayed average electron density in the active site that resembled

a higher degree of polymerization. Accordingly, *BxPLX*-ManA and *BxPLX*-GulA were each modelled using two penta-oligosaccharides with partial occupancy to portray hepta-Mannuronic acid and hexa-Guluronic acid ligands to accurately represent all protein-ligand interactions (**Figure 3.5 B**). The catalytic groove of *BxPLX* is highly basic, which is consistent with preference for uronic acid substrates. Several salt-bridges were identified between arginine and the uronate group of both ManA and GulA configured substrates. A total of 7 subsites were identified, spanning from -3 to +4 in *BxPLX*-ManA and +2 to +5 for *BxPLX*-GulA (**Figure 3.5 B-D**). The conformation of GulA prevents crucial interactions with +1 residues and the catalytic tyrosine (**Figure 3.5 F**).

Structural similarity results from a Foldseek server search yielded a diverse set of low sequence identity matches including a PL13 heparanase (PDB ID: 3IKW; sequence identity: 11.2; E-value: $3.18e^{-10}$), and a PL14 alginate lyase (PDB ID: 5GMT; sequence identity: 12.6; E-value: $4.08e^{-11}$). Although these were the top matches, several PL7 alginate lyases portrayed comparable structural similarity and notably, reflect a similar β -sandwich jelly roll-fold to *BxPLX*. Of these, the PL7 AlyA1 shows the highest sequence identity (PDB ID: 3ZPY; sequence identity: 15.7; E-value: $7.72e^{-7}$). Because *BxPLX* does not show significant structural similarity to any one PL family, the conservation of the essential catalytic residues was explored further to investigate the mechanistic details of *BxPLX*, as discussed in **section 4.2.4**.

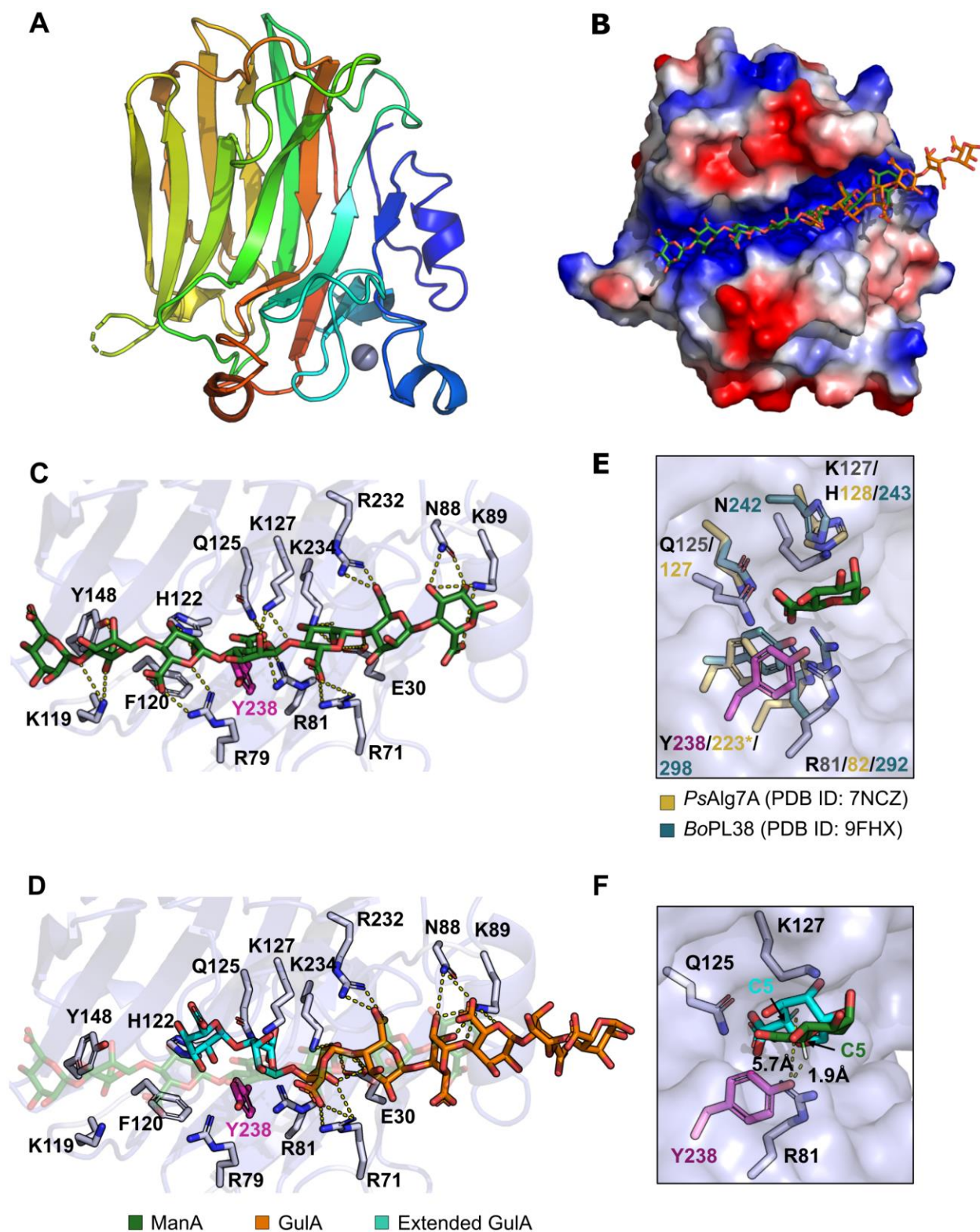


Figure 3.5 Crystal structure of *BxPLX*. (A) Cartoon representation of *BxPLX*. N-terminus is shown in orange and C-terminus is shown in blue. (B) Electrostatic potential surface representation of *BxPLX* displays a highly positive catalytic groove that binds both hepta-

mannuronic and hexa-guluronic acids. **(C)** Residues involved in mannuronic acid recognition in the negative and positive subsites are highlighted. The catalytic tyrosine (modelled by an alignment to the apo structure) is shown in magenta and indicates the site of cleavage and therefore the numbering of subsites. **(D)** Residues involved in GulA recognition in the positive subsites are highlighted. An extension of the ligand (shown in cyan) makes clear the steric clashing subsites -1 and +1, resulting in an unfavourable conformation. **(E)** An alignment of *BxPLX* to *PsAlg7A* (taupe) and *BoPL38* (teal) reveal functional conservation at the +1 subsite. The Y and R residues are strictly conserved. Q125 in *BxPLX* is conserved with *PsAlg7A* and functionally conserved with *BoPL38*. K127 is functionally conserved with both. **(F)** The conformation of GulA prevents crucial interactions with +1 residues and the catalytic tyrosine. Distances to the C5 hydrogens for both ligands are indicated (1.9 Å to ManA and 5.7 Å to GulA). Crystallography and figure images provided by Emily Knudson-Goerner.

3.6 NMR time-resolved analysis of *BxPLX* product profile

The product profile of *BxPLX* was continuously monitored through a series of 1D NMR back-to-back spectrum acquisitions over 39 h, amounting to 156 spectra in total (**Figure 3.6; Figure S9**). Peak assignment of six selected signals (*i.e.*, M α 1, M β 1, G α 1, G β 1, Δ 1M, Δ 4M) corresponding to reducing ends and DEH residues revealed catalytic site substrate preferences by *BxPLX*. M, M α , and M β denote the internal $\rightarrow$ 4)- β -D-ManpA-(1 $\rightarrow$ residue and the reducing-end residues $\rightarrow$ 4)- α -D-ManpA and $\rightarrow$ 4)- β -D-ManpA, respectively. G, G α , and G β denote the internal $\rightarrow$ 4)- α -L-GulpA-(1 $\rightarrow$ residue and the reducing-end residues $\rightarrow$ 4)- α -L-GulpA and $\rightarrow$ 4)- β -L-GulpA, respectively. The number following a residue symbol indicates the position of the ^1H atom in that residue. For example, M α 1 indicates the ^1H signal of H-1 of the M α residue. For diad signals, the underlined residue indicates the residue to which the signal is assigned, and the following number indicates the position of the ^1H atom. For example, Δ 4G denotes the ^1H signal of H-4 of a Δ residue linked to an internal M residue.

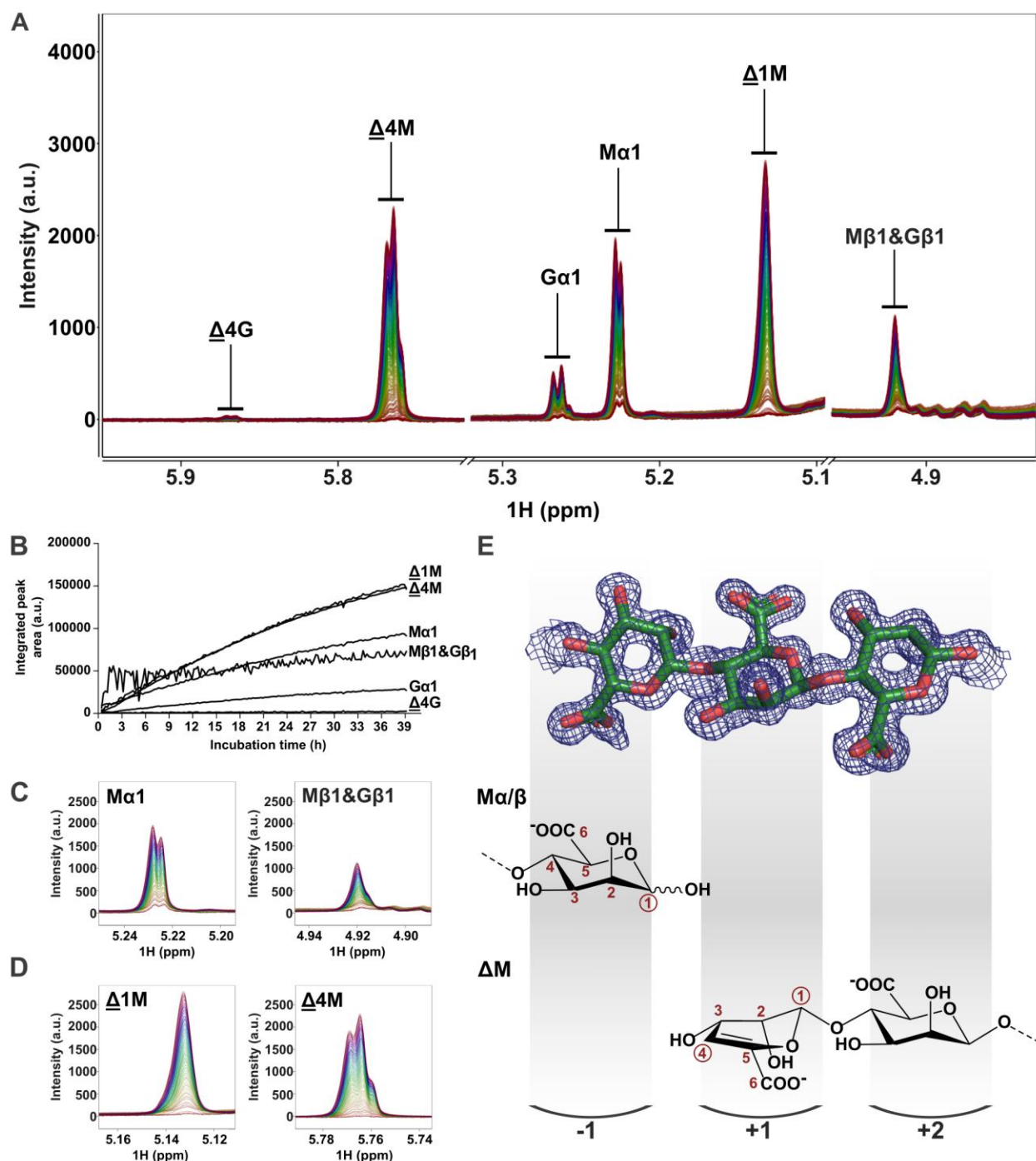


Figure 3.6. Superimposed signals corresponding to reducing ends and 4-deoxy-L-erythro-4-hexenopyranuronate residues (Δ) at the non-reducing ends of ALG lyase degradation products in 156 time-resolved 700 MHz 1D ^1H NMR spectra acquired over 39.15 h at 37°C during the degradation of 20.1 mg of sodium ALG substrate by 700 pmol of lyophilized *BxPLX* lyase from *B. xylanisolvens* AUL in 0.67 mL of D_2O in a standard 5 mm NMR tube. A zoomed-out overview is shown in panel A, while the integrated peak areas of selected signals corresponding to reducing ends and Δ residues at the non-reducing ends of alginate lyase degradation products

are depicted in panel **B**. Enlarged views of select peaks are shown in panels **C** and **D**. Panel **E** depicts the 2Fo-Fc of a mannuronic trisaccharide (courtesy of Emily Knudson-Goerner and Dr. Alisdair Boraston) and interpreted structures within the *BxPLX* -1, +1, and +2 subsites. For each selected peak, 156 overlaid traces are shown from bottom to top, corresponding to the time-dependent generation of degradation products from low to high abundance. Chemical shifts were internally referenced to TSP-d₄ set to 0 ppm. **M**, **Mα**, and **Mβ** denote the internal →4)-β-D-ManpA-(1→ residue and the reducing-end residues →4)-α-D-ManpA and →4)-β-D-ManpA, respectively. **G**, **Gα**, and **Gβ** denote the internal →4)-α-L-GulpA-(1→ residue and the reducing-end residues →4)-α-L-GulpA and →4)-β-L-GulpA, respectively. The number following a residue symbol indicates the position of the ¹H atom in that residue. For example, **Mα1** means the ¹H signal of H-1 of the **Mα** residue. For diad signals, the underlined residue indicates the residue to which the signal is assigned, and the following number indicates the position of the ¹H atom. For example, **Δ4M** denotes the ¹H signal of H-4 of a **Δ** residue linked to an internal M residue. No signals were detected for **Δ** residues linked directly to a reducing end. A 3D colormap surface plot showing the increase in signal intensity with incubation time is presented in Figure S9 in the Supplementary Information. Data collection and analysis conducted by Dr. Xiaohui Xing.

Although the subsite specificity of the +1 subsite cannot be elucidated by product analysis, as unsaturated residues originating from ManA and GulA residues are indistinguishable, compelling evidence was provided for the -1 and +2 subsites. The -1 subsite of *BxPLX* was occupied more frequently by ManA over GulA as determined by a greater combined integrated peak area and intensity (a.u.) of signals for Mα1 with and Mβ1 (**Figure 3.6B-D**). Mα1 and Mβ1 reached intensities of ~2000 a.u. and ~1100 a.u., respectively (**Figure 3.6 B-D**). In contrast, Gα1 and Gβ1 only reached intensities of ~500 a.u. and ~1100 a.u., respectively (**Figure 3.8 B; Figure S9**). The +2 subsite is almost exclusively occupied by ManA, as indicated by the signals **Δ1M** and **Δ4M**. Both signals **Δ1M** and **Δ4M** increased over time to >2000 a.u. The signal **Δ4G** was much less intense, falling below 50 a.u. No signals were detected for **Δ** residues linked directly to a reducing end.

3.7 Formation of (5S)-DHF hydrate and (5R)-DHF hydrate through synergy of AUL PLs

In order to identify the products formed through the combined activities of all PLs from the *Bx*ALG1_{Alc} AUL, a combinatorial digest of *Bx*PL6, *Bx*PL17, *Bx*PL38, and *Bx*PLX was analyzed by 1D and 2D NMR spectroscopy (**Figure 3.7**; **Figure S10**). This assay, which represents the full reconstituted AUL pathway, revealed the formation of two main end products, (5*S*)-DHF hydrate and (5*R*)-DHF hydrate. These metabolites are stable epimers generated from unsaturated GulA and ManA, and the hydration and recyclization of DEH aldo-hydrate (**Figure 1.1**).

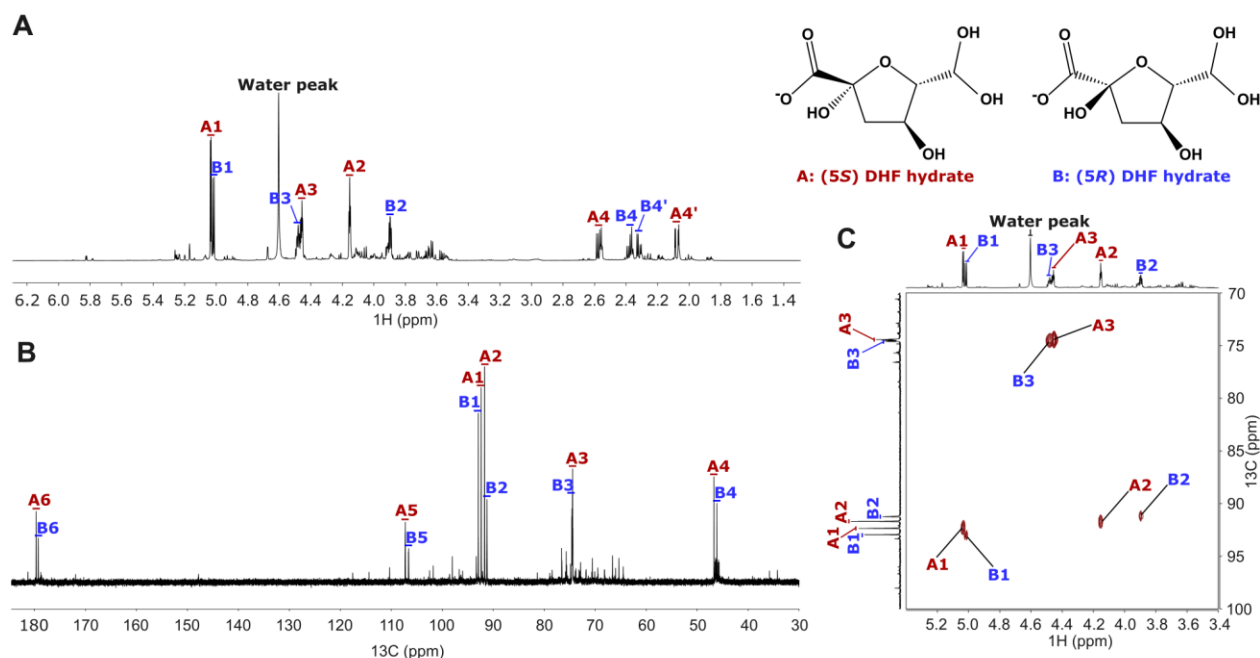


Figure 3.7. Reconstitution of the AUL pathway. ALG was digested by a mixture of *Bx*PL6, *Bx*PL17, *Bx*PL38, and *Bx*PLX, from the *B. xylanisolvens* AUL. Products were analyzed by 700 MHz solution-state 1D and 2D NMR spectra collected at 37 °C in D₂O. 4-deoxy-L-erythro-5-hexoseulose uronic acid (DEH), was assigned from (A) 1D ¹H NMR, (B) 1D ¹³C NMR, and (C) 2D ¹H-¹³C HSQC NMR spectra. Chemical shifts were internally referenced to TSP-d₄ set to 0 ppm. The reaction product is observed predominantly as two hydrated DHF stereoisomers derived from DEH, assigned as residue A, (5*S*)-DHF hydrate, and residue B, (5*R*)-DHF hydrate, labelled in red and blue, respectively. The numbers following A and B correspond to the conventional atom numbering of the DHF hydrate residues. As an example of the

annotation scheme, A1 represents the H-1 signal in the ^1H NMR spectrum, the C-1 signal in the ^{13}C NMR spectrum, and the H-1/C-1 cross-peak in the 2D ^1H - ^{13}C HSQC spectrum of structure A. The same annotation scheme applies to the other numbered positions of structures A and B. The full set of 2D NMR spectra for the product is shown in Figure S7 in the Supplementary Information. Data collection and analysis conducted by Dr. Xiaohui Xing.

CHAPTER 4: DISCUSSION

4.1 ALG-degrading *Bacteroides* spp. isolated from the distal gut of artiodactyls

ALG lyases are ubiquitous within marine ecosystems, with reports in brown seaweeds (Inoue & Ojima, 2019), free-living marine bacteria (Barzkar et al., 2022), marine fungi (Schaumann & Weide, 1990), and marine invertebrate GIT bacteria (*e.g.*, mollusks, gastropods (Hata et al., 2009; Wong et al., 2000)). ALG lyases also play important roles in the biology of terrestrial microorganisms, such as cystic fibrosis-associated *Pseudomonas aeruginosa* (Boyd et al., 1993), soil-inhabiting *Azotobacter* (Ertesvåg et al., 1998), and in some cases, viruses (Suda et al., 1999; Wong et al., 2000). Recent work has revealed ALG lyases within the GIT microbiota of terrestrial animal hosts, including humans (Mathieu et al., 2018; Pudlo et al., 2022), cattle, and Norwegian White lambs (Ferrillo et al., 2026a). The present work adds to existing knowledge on the function of ALG lyases from terrestrial bacteria, reporting for the first time *Bacteroides* spp. ALG-utilizing bacteria isolated from the distal gut of four ruminant species: Masai giraffe, bighorn sheep, rocky mountain goat, and moose (**Table S1; Figure 3.1**). These bacteria were one of two species: *B. faecium*, isolated from Masai giraffe, or *B. xylanisolvens*, isolated from bighorn sheep, rocky mountain goat, and moose.

ALG metabolism was linked to the presence and function of canonical AULs, which were found in cultured strains and enriched fecal metagenomic communities (**Figure S5; Table S7; Table S8; Table S9**). The AUL nearly identical between strains of the same species, very highly conserved between *Bacteroides* spp., and has been found in other GIT bacterial species and mammalian hosts. For example, a homologous AUL was previously characterized in human distal gut-associated *B. ovatus* CP926 (Ronne et al., 2023) (**Figure 3.2**).

It is possible that the presence of this AUL amongst isolates may indicate an important functional purpose involving non-dietary alginate substrate within the host. For example, *P. aeruginosa* is known to synthesize ALG biofilms within the lungs of cystic fibrosis patients, resulting in thick mucus that negatively impacts host lung function and can promote further infection (Boyd & Chakrabarty, 1995; Boyd et al., 1993). It is possible that the ingestion of respiratory mucus contaminated with alginate-containing biofilms could promote the maintenance of these genes within the GIT. Whether these AUL PLs within the distal gut can metabolize bacterial ALG located in the lungs (often acetylated at positions C2 and C3) is a question of both physical access and enzymatic specificity, but this idea may be considered in further research.

An alternate explanation for the presence of this AUL is ALG found within soils, released by soil-inhabiting microorganisms. ALG production is reported in response to desiccation of cells during drought by some strains of soil-inhabiting bacteria belonging to several genera (*e.g.*, *Flavobacterium*, *Erwinia*). It is believed that these secreted biofilms increase cell osmotolerance (Sá et al., 2019). It is possible that ruminants hosts could be directly exposed to ALG in this way during grazing or drinking contaminated water. Alternatively, ALG-utilizing bacteria within the feces of ruminants, shed onto the soil, may come into contact with ALG substrate. These microbes, now present within the environment may be reingested by ruminants.

AULs within other species and with heterogeneity in gene composition and organization have been characterized. *Bacteroides* spp. are often considered “generalists,” known for their diverse saccharolytic abilities. Given this, isolating *Bacteroides* spp. with such homogeneity in AUL is somewhat surprising. The culturing conditions employed here, including growth media, anaerobic environment, and ALG as a carbon source, are partial to isolating *Bacteroides* spp., and less permissive to growth of diverse taxa (Pudlo et al., 2022). Contributing to this, it must be

considered that possession of the pathway, in parts or whole, does not guarantee it is functional, and that a functional PUL is required for bacterial isolations on ALG. For example, *B. eggerthii* was found to possess an AUL encoding three transporters, KdgF, a carbohydrate-binding module, and two PLs (*BePL6* and *BePL17*) (Rønne et al., 2024). However, this strain failed to utilize ALG without other ALG degrading bacteria, relying on the production of keystone enzymes at the cell surface to initiate the first steps of ALG fragmentation. It is possible that other *Bacteroides* spp. with partial AULs were present within the microbiota of Masai giraffe, rocky mountain goat, bighorn sheep, and moose, but they could not be cultured as an independent strain. The fecal metagenomic data supports this possibility as other potential ALG-targeting enzymes were identified, including PL6, PL17, PL38, PLX, as well as PL15 and PL34.

Another surprising finding was that within enriched metagenomic datasets, the total count of ALG-specific PLs were notably lower than in the fecal samples, further suggesting that only strains with a functional AUL become enriched (**Table S8**). This trend was also reflected in the BLAST screening of *BxPLX* against fecal and enriched metagenomic data, with a smaller number of hits with a high percent identity (>90%) in the enriched data, and a larger number of less specific hits in the fecal data above a certain filtering criteria (E-value < 1×10^{-3} , bitscore >50; **Table S9**). The present study has identified the AUL through both whole shotgun sequencing of cultured isolates, as well as through community metagenomic data. The agreement between these two approaches provides further confidence to the presence of this AUL within ruminant distal gut bacteria.

4.2 AULs and their associated polysaccharide lyases

4.2.1 *BxPL6*, *BxPL17*, and *BxPL38* function

BxPL6, *BxPL17*, and *BxPL38* enzymes generated similar products to what was seen previously for the AUL from *B. ovatus* CP926 (i.e., *BoPL6*, *BoPL17*, and *BoPL38* (Ronne et al., 2023)). This was expected as the enzymes displayed high sequence identity with their counterparts, ranging from 99.87-100%, 88.3%, and 94%, respectively (**Table S6**). TLC and HPAEC-PAD data indicate that *BxPL6* and *BxPL17* are exo-acting PLs, primarily generating a large peak with an elution time of ~5 min (**Figure 3.3**). Both enzymes were predicted by SignalP to be secreted into the periplasm (SPI), which is consistent with a downstream role in the enzymatic cascade, processing oligosaccharide fragments into small oligosaccharides and monosaccharides (Kawai & Hashimoto, 2022). *BxPL38* generates a ladder of oligosaccharide products from ALG, suggesting it is endo-acting, and it possesses a SPII signal peptide, which likely targets it to the outer surface. When these enzymes are combined, the effects are additive, resulting in more total end product being formed (**Figure 3.7**). Similar to other endo-acting “keystone” enzymes (Dudek et al., 2020; Tandrup et al., 2025), *BxPL38* increases the abundance of nascent non-reducing ends to which are available substrates for *BxPL6* and *BxPL17*. The combination of three PLs (*BxPL6-BxPL17-BxPL38*) cannot be definitively distinguished from reactions that included *BxPLX* (*BxPL6-BxPL17-BxPL38-BxPLX*), highlighting there is functional redundancy between *BxPLX* and *BxPL38*, and they may provide similar roles within different components of the cell (FIG). Reconstitution of the AUL pathway results in the accumulation of two main products, (5S)-DHF hydrate and (5R)-DHF hydrate, and these metabolites may likely represent the HPAEC-PAD peak with a retention time of ~5 min (**Figure 3.3; Figure 3.7**).

4.2.2 *BxPLX* structure, substrate specificity and activity

Previously, the homologous gene product to *BxPLX* was included as part of the *B. ovatus* CP926 AUL because there was an observed increase in protein abundance in the supernatant (6.4-fold) when grown on ALG as the sole carbon source (Ronne et al., 2023). TLC, HPAEC-

PAD and NMR spectroscopy, determined that *BxPLX* generates large oligosaccharide products, consistent with an endo-acting activity (**Figure 3.4**). In addition to *BxPLX*, other PLX homologues were identified from putative AULs on the NCBI database (**Figure 3.2; Table S7**) and in AULs with notable structural heterogeneity compared to the *B. xylanisolvans* AUL, sourced from cattle MAGs (**Figure S7**). The activity of recombinant PLX-like proteins were shown to generate large oligosaccharide products from ALG similar to *BxPLX* (**Figure S7**). Because *BxPLX* and its homologues could not be detected by existing dbCAN3 HMMs, it is possible that they are representative members of a new PL family.

4.2.3 Time-resolved NMR analysis of *BxPLX* product profile

The catabolism of ALG by *BxPLX* was monitored in real time through 156 time-resolved 700 MHz 1D ^1H NMR spectra acquired over 39.15 h (**Figure 3.6**). The resulting data indicated a notable substrate preference for ManA (specifically, $\text{M}\alpha$ over $\text{M}\beta$) over GulA in the -1 subsite of the enzyme, evidenced by a greater ^1H signal intensity of the $\text{M}\alpha 1$ associated peak. Also, in products associated with the +2 subsite, *BxPLX* showed an overwhelming preference for ManA, evidenced by the $\Delta 1\text{M}$ and $\Delta 4\text{M}$ peaks associated with the ^1H signal of H-1 and H-4 on the Δ residue linked to a ManA residue. The substrate preference of the +1 subsite, though not able to be determined by NMR, is elucidated in the following section through the crystal structure of a catalytic *BxPLX* mutant in complex with ManA and GulA oligosaccharides.

4.2.4 Structure of the *BxPLX* active site

In agreement with the time-resolved NMR analyses, the -1 to +2 subsites play key role in informing the specificity of *BxPLX*. As shown in **Figure 3.5 C & E**, the +2 region can accommodate both ManA and GulA through uronic acid coordination (R71) and hydrogen bonding (K234); however, GulA did not crystallize in the -1 and +1 subsites. Therefore, to

further investigate the interactions at play, a GulA substrate model was superimposed into subsites -1 and +1 and compared with the *BxPLX*-ManA complex (**Figure 3.5 D & F**).

The surface architecture at the -1 and +1 subsites reflects a narrow cavity for binding at the cleavage site and likely requires signature conformations during the reaction landscape. Indeed, while ManA binds at these positions, the extended GulA saccharides pose obvious steric clashing. This is further supported through comparison of key protein interactions in *BxPLX*-ManA (**Figure 3.5 C & F**). Specifically, hydrogen bonding facilitated at the -1 subsite by R79 and H122, in addition to CH- π stacking (F120), seem to favour the linear presentation of ManA. Analogous interactions could not be modelled in the extended GulA disaccharide.

The +1 subsite consists of a constellation of residues (R81, Q125, K127, and Y238) that proved essential for catalysis in structurally conserved residues in PL7s (AlyA1, *PsAlg7A*, and *PsAlg7C*) and *BoPL38* (**Figure 3.5 E**). Accordingly, in *BxPLX* Y238 and R81 are completely conserved between all structures, and Q125 and K127 show functional conservation. This suggests that *BxPLX* employs a similar catalytic mechanism to these enzymes (Rivas-Fernández et al., 2025; Tandrup et al., 2025). The protonation cascade in *PsAlg7A* and 7C has been established, with Y223 (in *PsAlg7A* mutated to F223 in **Figure 3.5 E**) as the catalytic base and general acid. This suggests H128 solely functions to stabilize the ManA through hydrogen-bonding. Somewhat similar, the transition state in *BoPL38* resembles that of *PsAlg7A* for ManA processing, with the +1 saccharide transitioning from a $^4C_1$ to catalytically favourable $^2S_0$ conformation. However, this enzyme utilizes two different mechanisms to process ManA and GulA with Y298 acting as the catalytic base and general acid for ManA processing, and H243 as the catalytic base and Y298 as the general acid for GulA. Both these studies also suggest the importance of R81 in lowering the pKa of the surrounding catalytic microenvironment to allow

for deprotonation of Y238; this interaction is conserved across PL7s, PL38s, and likely *BxPLX* (Xu et al., 2020).

The interactions at the -1 and +1 subsites are crucial for effective β -elimination at C5 on the +1 saccharide by Y238; however, GulA was not able to crystallize in these subsites, suggesting it is not accommodated in this subsite. Importantly, R81, Q125, and K127 orient ManA for optimal interaction with Y238, as shown by the transition state conformation of *PsAlg7A* and *BoPL38*. However, the epimeric orientation of the extended +1 GulA disaccharide clashes with Q125 and K127, and in this conformation, cannot interact with R81 (**Figure 3.5 F**). It is possible that *BxPLX* may employ distortion and partial cleavage of the glycosidic bond to enforce the catalytically favourable ^2S_o TS conformation for GulA, as shown for *BoPL38* (Tandrup et al., 2025).

Due to the clear steric clashing of GulA in the *BxPLX* catalytic groove at the -1 and +1 subsites, GulA is likely not the preferred substrate. It is possible that the detection of some $G\alpha 1$ product formation (though notably less than that of $M\alpha 1$) in the NMR time-resolved experiment, is a result of GulA residue accommodation within a mixed- ALG substrate. As shown in **Figures 3.5 C & D**, the basic residues abundantly present in subsites +2 to +4 are able to bind both ManA and GulA effectively. Specifically, R232 strongly interacts with the uronic acid for both ManA and GulA ligands in the +3 subsite. N88 and K89 also facilitate several hydrogen-bonding interactions that interact with both ligands at the +4 subsite and GulA at +5 subsite. While these interactions may play an important accommodating mixtures of substrates, the precise structural interactions and constriction observed between the -1 to +1 subsites highly suggest that *BxPLX* is preferentially active on ManA substrates.

4.2.5 Spatial reconstruction of the *Bx*ALG AUL

Previous research into ALG catabolism and results from this study, helps to inform a model of *Bx*ALG1_{ALC} AUL function (**Figure 4.1**). Catalysis is initiated at the cell surface by the “keystone” enzyme *Bx*PLX, which converts ALG into large oligosaccharides or small polysaccharide products. Keystone enzymes are characterized by SPII extracellular targeting signal peptides (**Table S7**), and this is supported by proteomics conducted on the *B. ovatus* AUL, which confirmed it was located on outside of the cell (Ronne et al., 2023). Similarly, in the *Zobellia galatanivorans* DsiJ AUL, AlyA1_{PL7} initiates extracellular ALG depolymerization at the cell surface (Dudek et al., 2020). Products of *Bx*PLX reactions are likely imported through the putative SusC/D TonB transport complex that is a feature of different PUL systems in *Bacteroidota* (Reeves et al., 1997). It is likely this AUL utilizes a “selfish” mechanism, similar to described for yeast mannan utilization by *B. thetaiotaomicron* VPI-5482 (Cuskin et al., 2015), preventing loss of ALG oligosaccharides to the community during processing. Within the periplasmic space, endo-acting *Bx*PL38 further digests larger ManA- and GulaA-containing fragments into small oligosaccharides and increases the concentration of substrates for exo-acting *Bx*PL6 and *Bx*PL17. The end products of ALG lyase digestion are 4,5-unsaturated hexauronates, which would be transported into the cell by the MFS carbohydrate transporter. Spontaneous decomposition of 4,5-unsaturated hexauronates or catalysis by KdgF (which is present in the AUL), results in the formation of DEH. These metabolites are then converted into GA3P and pyruvate within the cytoplasm by a series of core metabolic enzymes, including DEHr, KdgK, and Eda (**Figure 1.1**). In their absence, 4,5-unsaturated hexauronates can hydrate into DEH ketoaldo-hydrate or DEH aldo-hydrate, the latter of which can recyclize into (5S)-DHF hydrate and (5R)-DHF hydrate, which was the observed end product by 1D and 2D NMR during the combination digests (**Figure 3.7**).

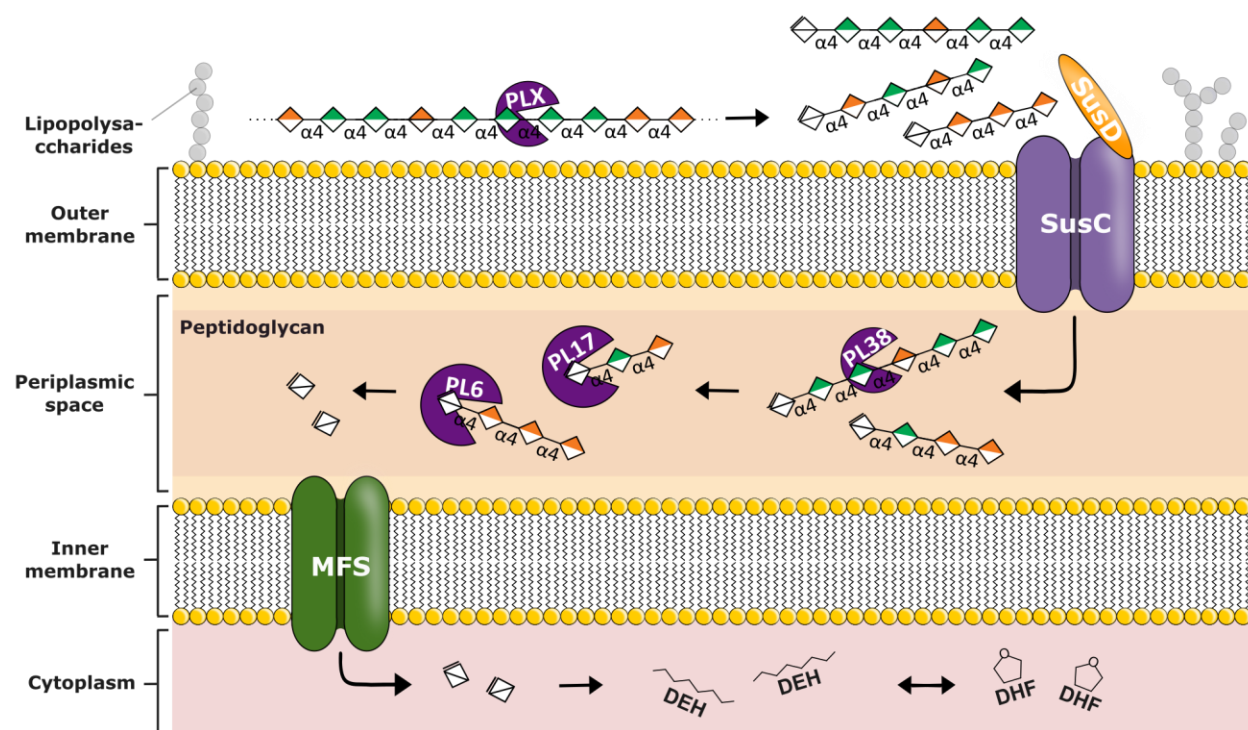


Figure 4.1. Model ALG catabolism by *Bacteroides* spp. ALG polymers are cleaved outside the cell by PLX and the resulting products are transported across the outer membrane through the SusC/D TonB complex. Within the periplasm, large oligosaccharides are further depolymerized by endo-acting PL38, followed by exo-acting PL6 and PL17. Small oligosaccharides and/or 4,5-unsaturated hexouronates are transported into the cytoplasm through the MFS transporter, where ALG catabolism converges with core metabolism (Figure 1C).

Recombinant reconstitution of this AUL has limitations that are important to consider. First, only PLs were produced, explaining the appearance of the final products (5S)-DHF and (5R)-DHF (**Figure 3.7**). *In vivo*, DEH would likely be further acted upon by DEHr, KdgK, and Eda, eventually resulting in GA3P and pyruvate which could be utilized by microbes (**Figure 1.1**). Secondly, within a natural context substrate exposure to ALG lyases would be mediated by intramembrane transporters and enzymes would be compartmentalized into different regions of the cell. While products generated *in vitro* by BxPL6/BxPL17/BxPL38 and BxPL6/BxPL17/BxPL38/BxPLX combination digests are largely indistinguishable, their profiles within the confines of the cell may be noticeably different.

4.3 Latent traits

The adaptation of gut microbiomes to dietary seaweed polysaccharides and their associated timescales is a question that has been investigated in humans (Hehemann et al., 2010; Mathieu et al., 2018; Pudlo et al., 2022), and more recently in animals (Ferrillo et al., 2026a; Tingley et al., 2026). The “sushi factor” hypothesis first introduced by Hehemann et al. (Hehemann et al., 2010) suggests that the gut microbiota of Japanese people adapted to digest seaweed by acquiring enzymes from seaweed-associated marine bacteria through HGT, which was directly facilitated by the human consumption of seaweed. In 2018, the “ancient acquisition” hypothesis by Mathieu and colleagues challenged the timescale for which such acquisitions could occur, providing evidence that the consumption of brown seaweed was likely a selection pressure for gene activation but not the direct cause for acquisition of alginate degrading CAZymes. They argued that the HGT event(s) were more ancient than previously suggested by Hehemann et al. The recently proposed “latent traits” hypothesis suggests that pathways specific for exotic substrates, such as carrageenan, porphyran, and ALG, have been maintained throughout evolution

of terrestrial animals, but often remain undetected in the mammalian GIT microbiota because they persist at levels below the resolution of conventional sequencing depth. Detection requires enrichment with the target substrate (Ferrillo et al., 2026a; Tingley et al., 2026). Persistence of these traits is facilitated in generalists, like *Bacteroidota*, regardless of host diet and lifestyle (Ferrillo et al., 2026a; Pudlo et al., 2022). AULs harbored within the distal gut microbiomes of the four ruminant species described here, including rocky mountain goat, bighorn sheep, moose, and Masai giraffe, supports the latent traits hypothesis, and suggests cryptic seaweed-saccharifying genes are more widespread amongst ruminants than anticipated previously, despite hosts having no perceivable encounters with the substrate (**Source data 1**). It is also possible that bacteria with these seaweed-saccharifying genes may be able to transfer from between hosts or from the environment.

Latent traits are hypothesized to be an evolutionary tool conferring not only competitive advantages to GIT commensals, but also offering host resiliency and dietary flexibility in the face of unpredictable ecological transitions (Tingley et al., 2026). For example, when feedlot cattle were fed a 5% inclusion of *Mazzaella japonica*, the abundance of *Bacteroidota* in the distal gut microbiota saw a significant increase when compared to the control group (Tingley et al., 2026). This population was provided an advantage when exposed to the carrageenan in cell wall of *M. japonica*, a substrate that would be inaccessible to the majority of GIT microbial community. Similarly, the North Ronaldsay sheep are an extreme example of dietary adaptation in the absence of regular feed sources (Hansen et al., 2003). Originally marooned during a storm at sea, this flock has adapted to a primarily seaweed-based diet and are considered to be their own distinct breed. The ability to exclusively survive on kelp was likely facilitated by latent genes maintained within the genomes of generalist GIT microbes.

Currently it is unclear when AULs first penetrated the mammalian GIT microbiota, the underlying mechanism, and how long they are able to persist within the genetic “dark matter” of ruminant GIT microbiomes. However, their presence suggests that any energetic maintenance costs associated with the preservation of these genes is not sufficient to orchestrate their elimination, and there is a competitive advantage experienced by bacterial strains following the addition of the substrate to the diet (Tingley et al., 2026). Given the prevalence of AULs within animal GIT microbiomes, it is possible that an ancient HGT event occurred early within early animal evolutionary history, and has been maintained through repeated vertical transfers between individuals or through predation events (Ferrillo et al., 2026a; Tingley et al., 2026). Though requiring more investigation, the research presented here provides support for the latent traits hypothesis and contributes to our understanding of microbiota-mediated adaptation to dietary seaweeds.

CHAPTER 5: CONCLUSIONS

Ruminant livestock have a longstanding relationship with humankind, due to their ability to transform recalcitrant plant materials into accessible, calorie dense animal products. The acquisition of traits by GIT microorganisms to facilitate adaptations to dietary changes, such as the introduction of seaweed, and timescales associated with these adaptations are not yet elucidated. Previous research has investigated this question in humans (Hehemann et al., 2010; Mathieu et al., 2018), where they proposed that the ability to digest dietary seaweeds was obtained through a HGT event from marine bacteria to GIT bacteria, and this process was selected for by the frequency of seaweed consumption. Conversely, the latent traits hypothesis was recently proposed (Tingley et al., 2026), suggesting that genes targeting seaweed polysaccharides persist within the mammalian GIT microbiota at low abundances, without the requirement of previous perceivable exposure of the host to the target substrate (Ferrillo et al., 2026b; Tingley et al., 2026). My thesis work, presented here, provides further evidence for this hypothesis by showing that ALG-saccharifying bacteria within the distal GIT microbiota were found to be present within ruminants and pseudoruminants that were naïve to dietary seaweed. *Bacteroides* spp. isolated from four ruminant species, rocky mountain goat, bighorn sheep, moose, and Masai giraffe were found to utilize ALG as a sole carbon source. This trait was provided by an AUL, consisting of an enzyme from PL6, PL17, PL38, and PLX, which I am speculating to be the founding member of a new PL family.

To determine the function of the AUL, each PL was recombinantly produced and the pathway reconstituted. When incubated with ALG, the resulting products were monosaccharide-like products DHF-5S-hydrate and DHF-5R-hydrate. Using 1D and 2D NMR, and X-ray crystallography, the molecular determinants of BxPLX catalysis were described and BxPLX was shown to be preferentially active on ManA substrates.

In conclusion, the present study expands on what is currently known regarding alginate-targeting PLs. Future research to determine the status of PLX as an authentic CAZy family and defining features of its HMMs would be of value. This work also supports the latent traits hypothesis by providing evolutionary and functional insights into the acquisition of dietary adaptations by ruminants and pseudoruminants. Research that follows may build on this work by further examining the prevalence of latent traits in other host species, helping to untangle the complex history of seaweed digestion in the gut of terrestrial animals.

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SUPPLEMENTARY MATERIAL

Supplementary Figures

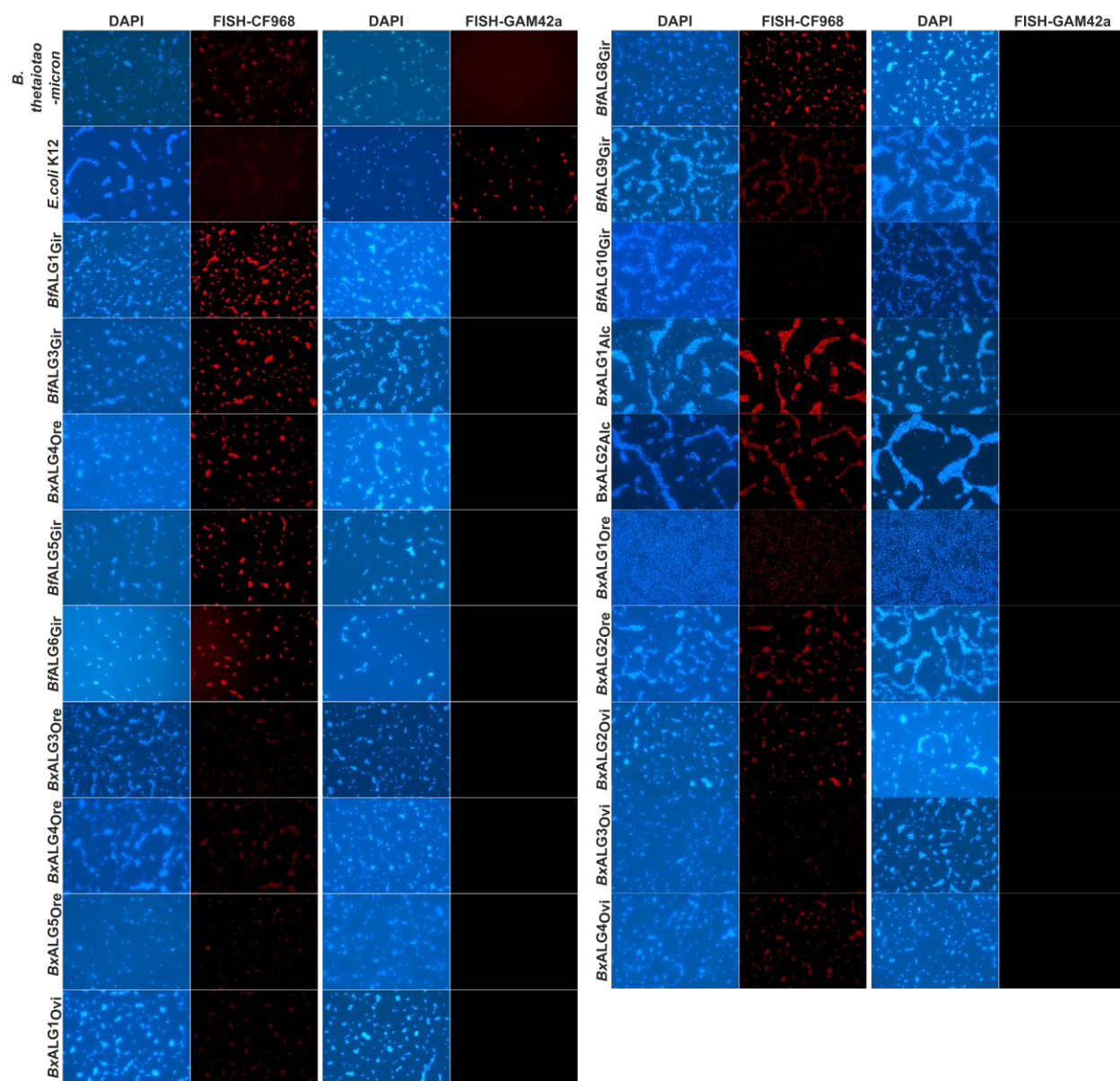


Figure S1. Taxonomic assignment of alginate-utilizing bacterial isolates via fluorescence in-situ hybridization. Alginate-utilizing bacteria isolated from the fecal microbiota of four ruminant host species (Masai giraffe, rocky mountain goat, bighorn sheep and moose) were screened for their taxonomy with the *Bacteroidota*-targeting probe, CF968. DAPI (365 nm) staining of microbial DNA within cells are visualized (365 nm), adjacent to fluorescence of CF968 (603 nm). Isolate cells were also incubated with Gammaproteobacteria-specific probe, GAM42a, as a control. *B. thetaiotaomicron* VPI-5482 and *E. coli* K12 were used as controls.

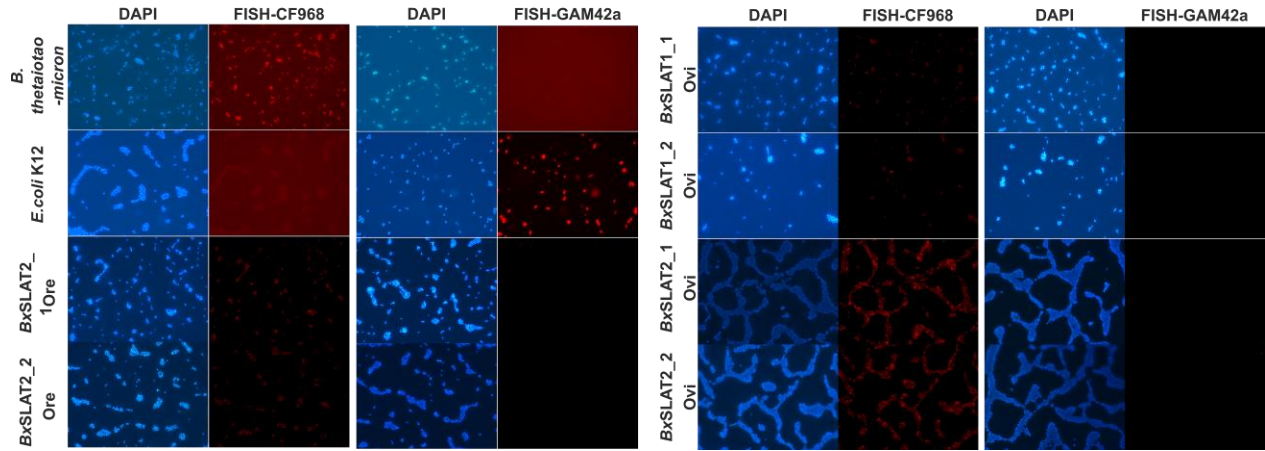


Figure S2. Taxonomic assignment of alginate-utilizing bacterial isolates via fluorescence in-situ hybridization. *S. latissima* extract-utilizing bacteria isolated from the fecal microbiota of four ruminant host species (*i.e.*, Masai giraffe, rocky mountain goat, bighorn sheep and moose) were screened for taxonomy through binding and fluorescence of *Bacteroidota*-targeting probe, CF968. DAPI (365 nm) staining of microbial DNA within cells are visualized (365 nm), adjacent to fluorescence of CF968 (603 nm). Strains were also incubated with Gammaproteobacteria-specific probe, GAM42a, as a control. *B. thetaiotaomicron* VPI-5482 and *E. coli* K12 were used as controls.

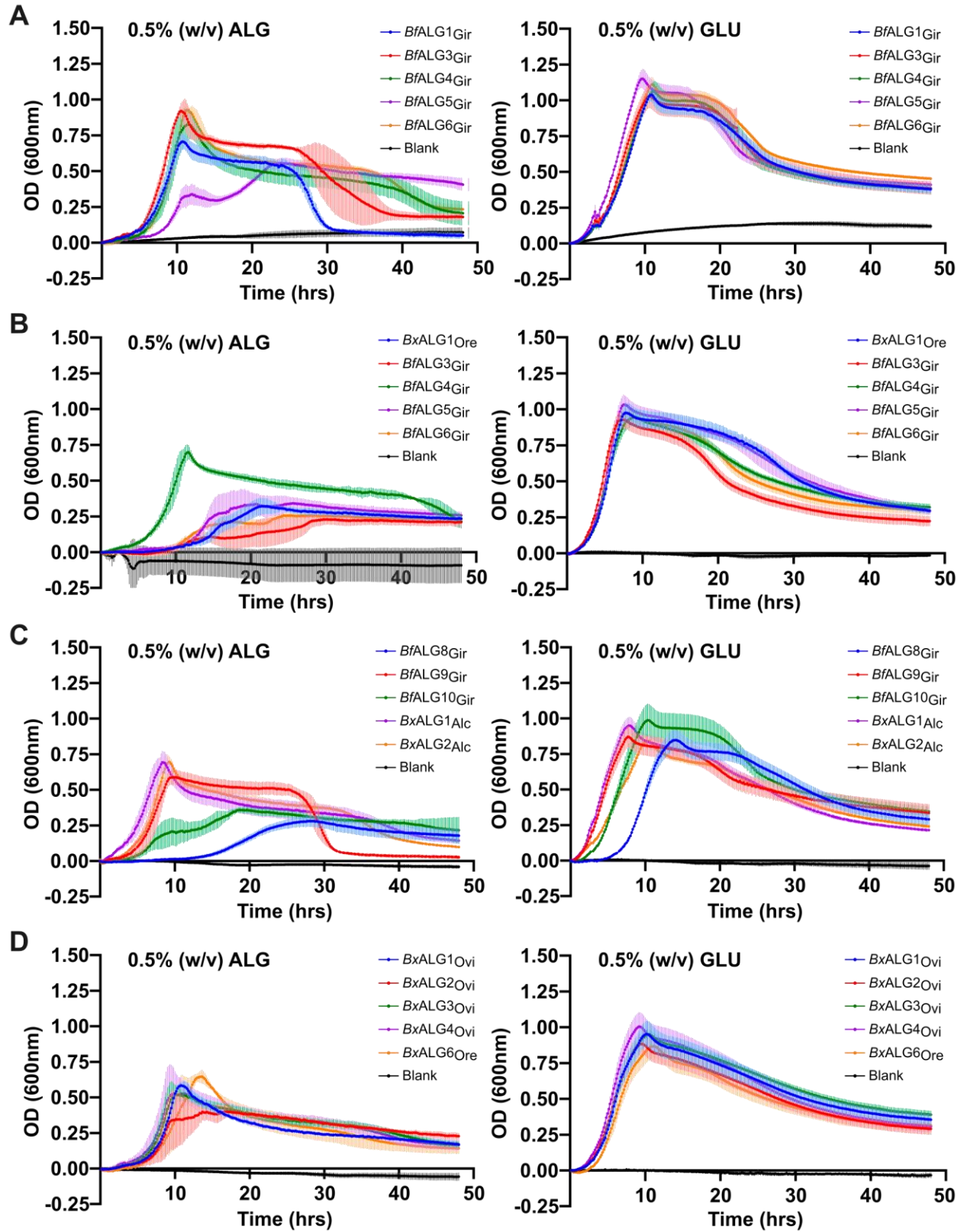


Figure S3. Bacterial growth profiling of alginate-utilizing isolates by OD_{600nm} monitoring in liquid culture. Bacterial isolate growth on 0.5% w/v ALG + MM and 0.5% w/v Glu + MM were recorded over 48 hs. A no carbohydrate control condition was also recorded, marked 'Blank'.

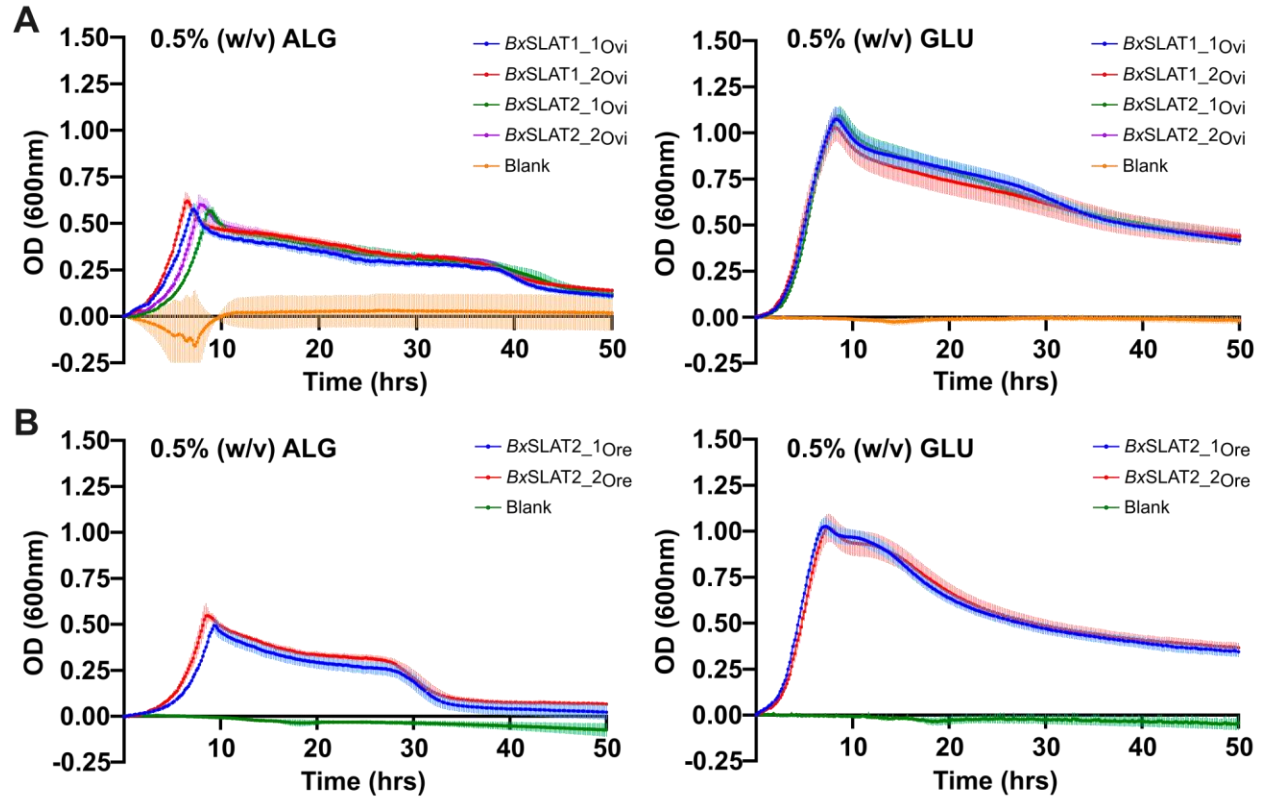


Figure S4. Growth curves of isolated strains cultivated on *S. latissima* extracts. OD measurements (600nm) of isolate culture growth on 0.5% w/v *S. latissima* extract + MM and 0.5% w/v Glu + MM were recorded over 48 h. A no carbohydrate control condition was also recorded, marked 'Blank'. (A) Growth curves of isolates *BxSLAT1_1Ovi*, *BxSLAT1_2Ovi*, *BxSLAT2_1Ovi*, and *BxSLAT2_2Ovi*. (B) Growth curves of *BxSLAT2_1Ore*, and *BxSLAT2_2Ore*.

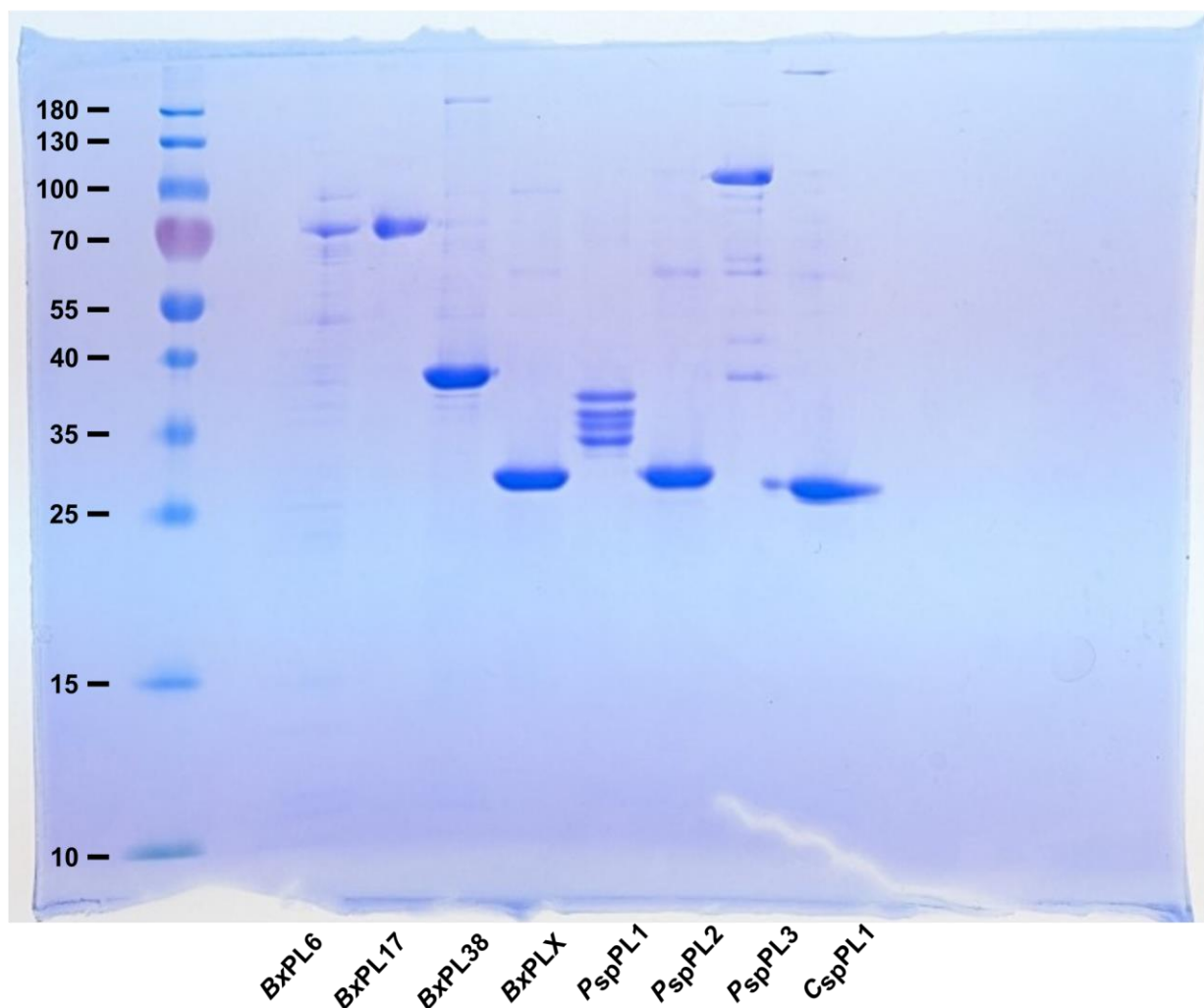


Figure S6. SDS gel of recombinant proteins. SDS samples on 12% acrylamide SDS gel. Extra bands on *PspPLX1* are due to fractioning of a carbohydrate binding module during protein synthesis.

A

	<i>Psp</i> PLX3	<i>Csp</i> PLX	<i>Psp</i> PLX1	<i>Psp</i> PLX2	<i>Bf</i> PLX*	<i>Bx</i> PLX*
<i>Psp</i> PLX3	100.0	46.1	43.7	51.7	47.5	46.6
<i>Csp</i> PLX	46.1	100.0	50.0	51.9	51.9	50.7
<i>Psp</i> PLX1	43.7	50.0	100.0	57.5	52.8	53.1
<i>Psp</i> PLX2	51.7	51.9	57.5	100.0	62.3	62.1
<i>Bf</i> PLX*	47.5	51.9	52.8	62.3	100.0	92.9
<i>Bx</i> PLX*	46.6	50.7	53.1	62.1	92.9	100.0

Tree scale: 1

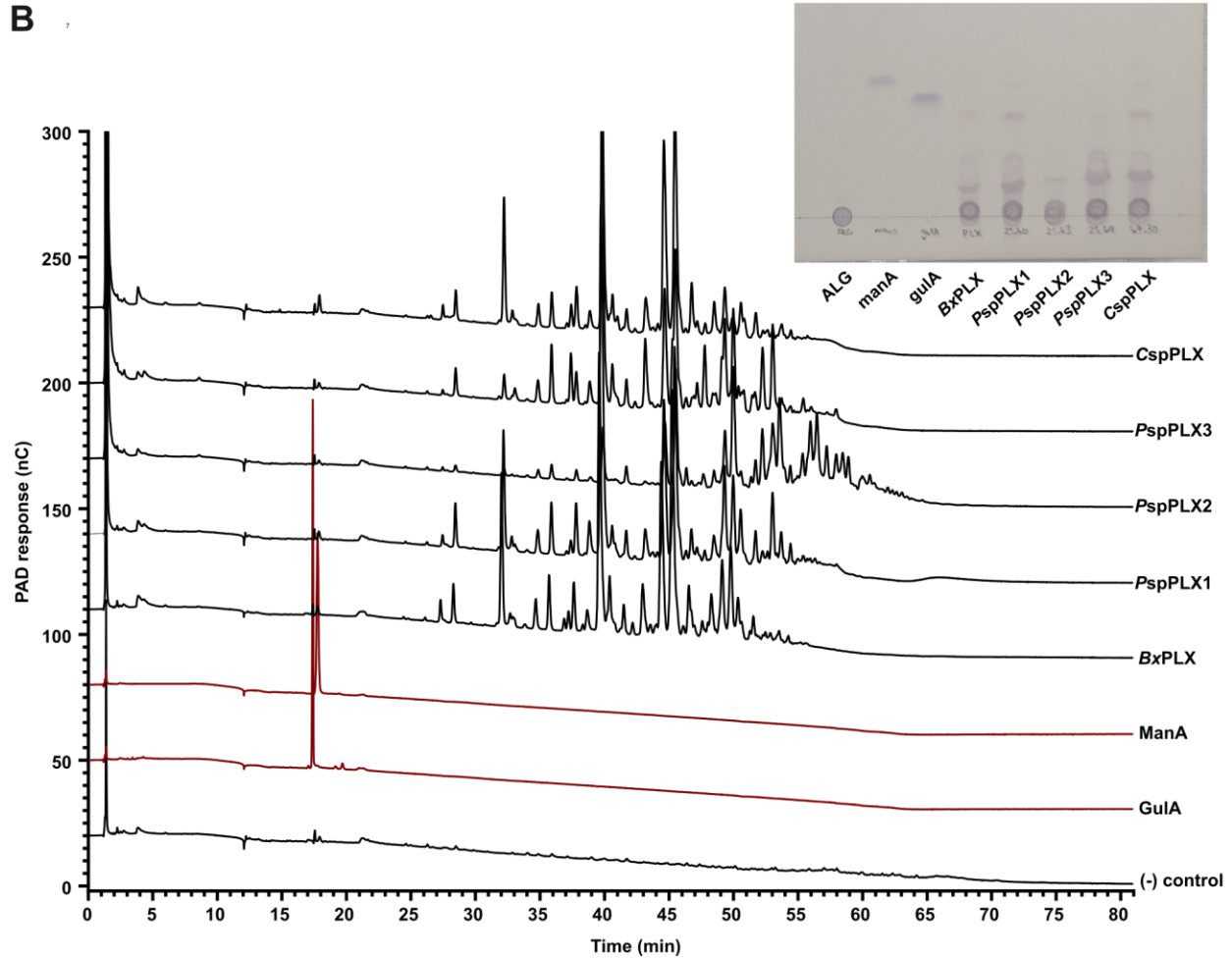
B

Figure S7. Relatedness and activity of *Bx*PLX and its homologues. **A)** Percent identity and phylogenetic tree of *Bx*PLX and homologues (*Bf*PLX, *Psp*PLX1, *Psp*PLX2, *Psp*PLX3, and *Csp*PLX). **B)** HPAEC-PAD analysis of enzyme digestion products. Single enzyme digests of 0.5% (w/v) ALG by *Bx*PLX and homologues (*Psp*PLX1, *Psp*PLX2, *Psp*PLX3, and *Csp*PLX) were conducted at an enzyme concentration of 1 μ M (16 h; 37 $^{\circ}$ C; pH 7). Baseline subtracted PAD response values of elution are shown over time. ManA and GulA standards are shown.

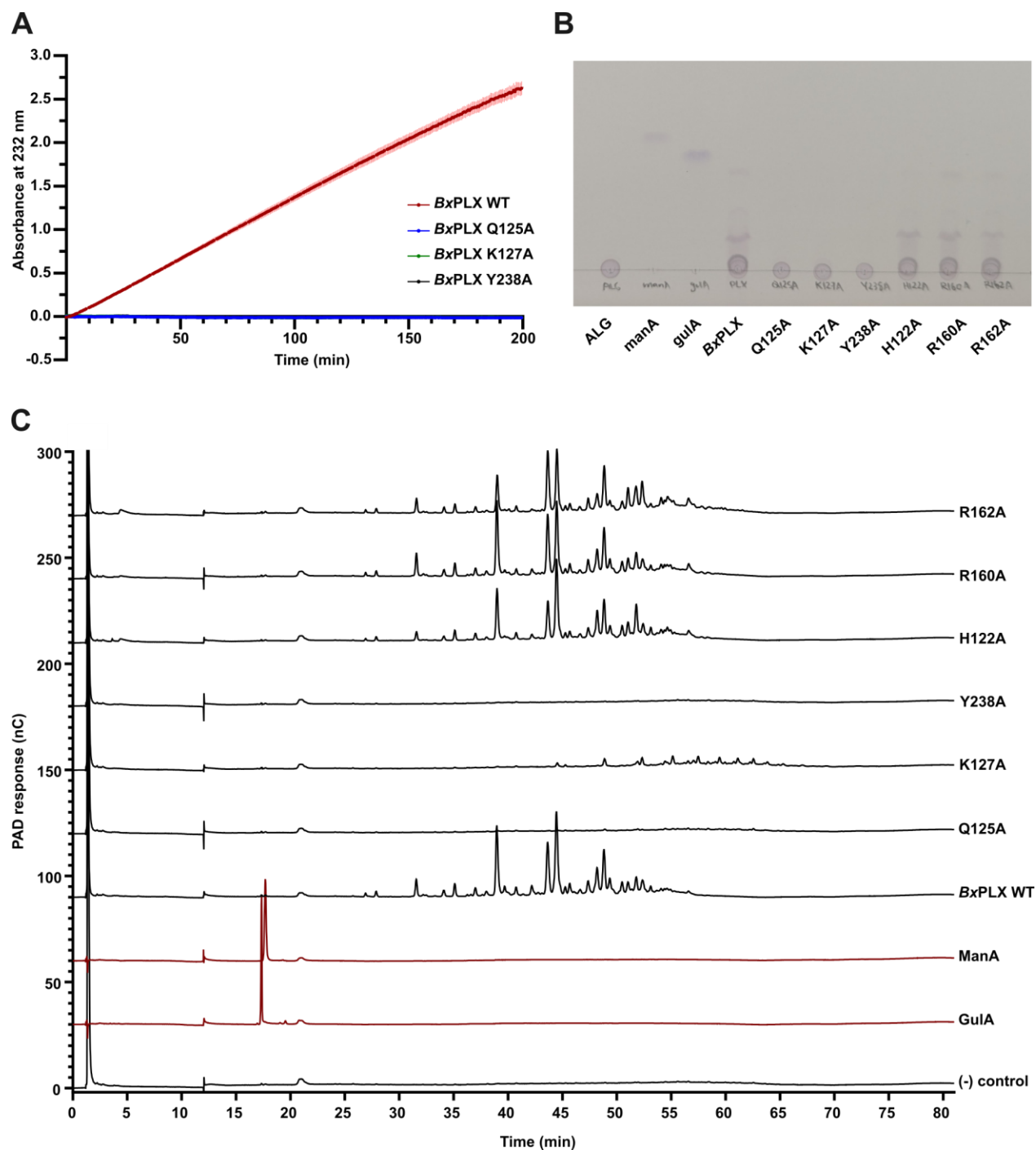


Figure S8. *BxPLX* mutant activity verification. Activity of *BxPLX* mutants (Q125A, K127A, Y238A, H122A, R160A, and R162A) were screened via spectrophotometer absorbance (232 nm), TLC, and HPAEC-PAD. Total ablation of activity was observed for Q125A, and Y238A. K127A showed notable activity loss, with some residual activity remaining. H122A, R160A, and R162A showed no noticeable decreases in *BxPLX* activity.

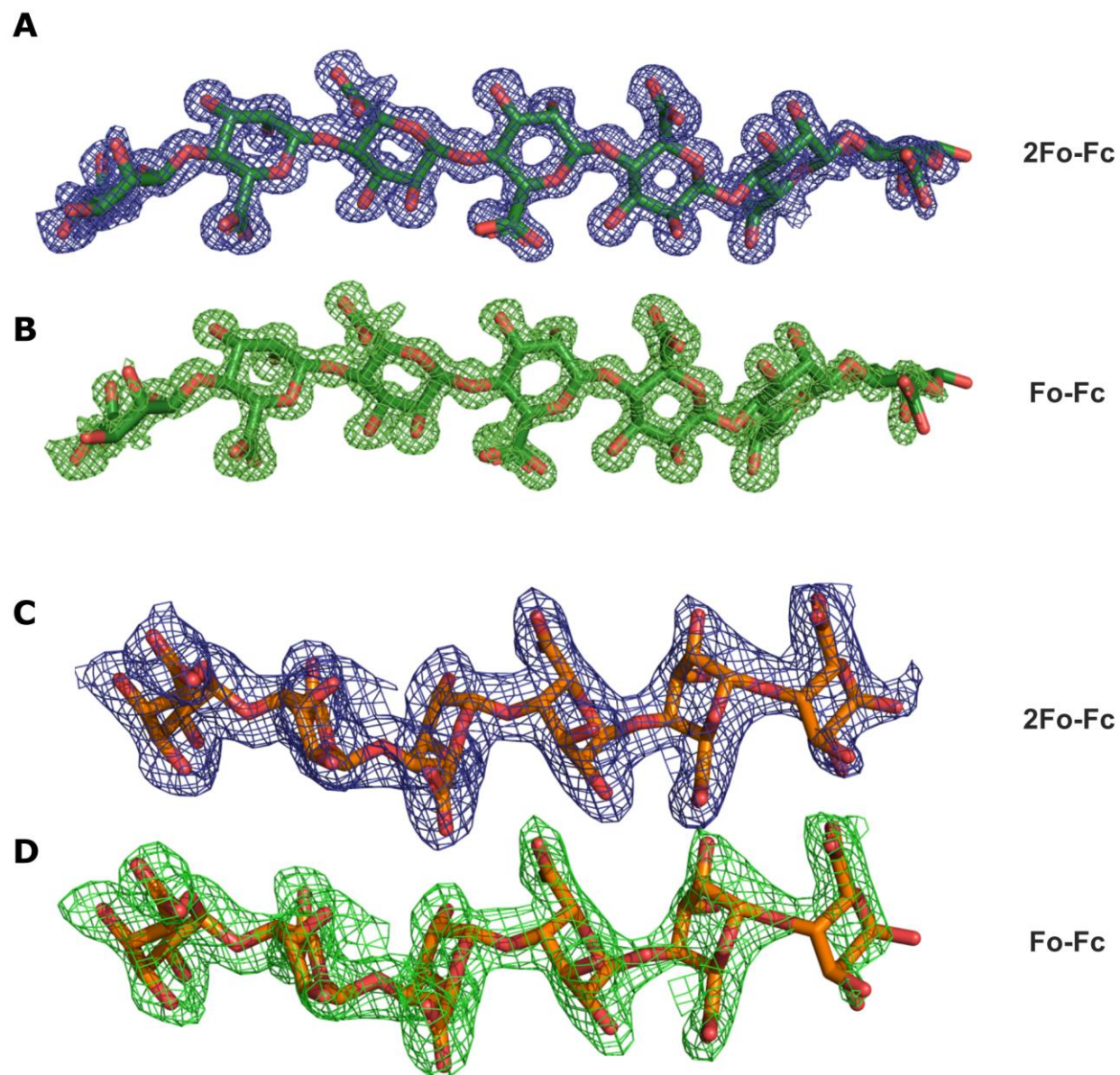


Figure S9 Substrate maps. (A) “Hepta” -ManA 2Fo-Fc (B) “Hepta” -ManA Fo-Fc (C) “Hexa”-GulA 2Fo-Fc (D) “Hexa”- GulA Fo-Fc.

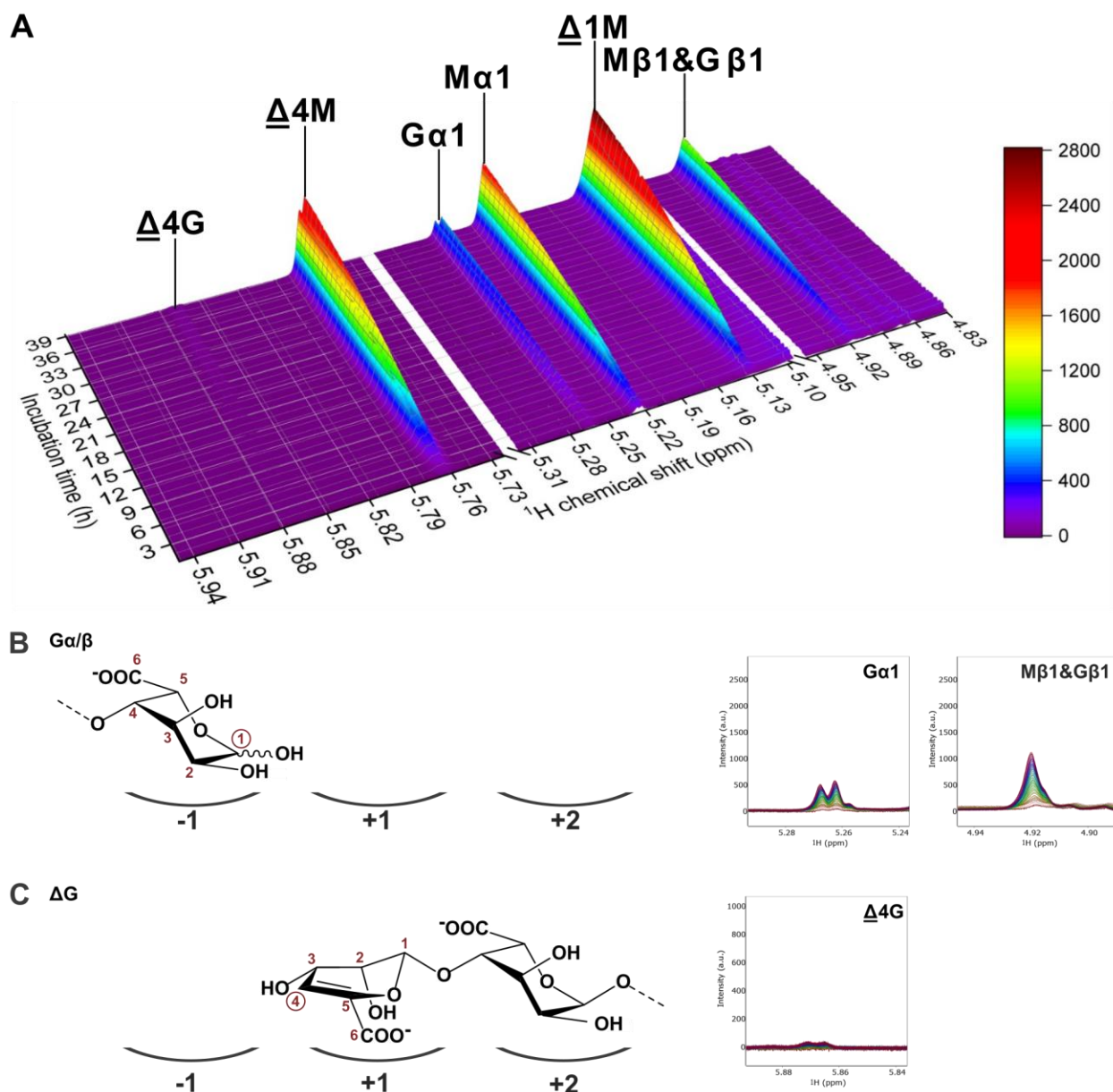


Figure S10 (A) 3D colormap surface plot and (B-C) enlarged views of individual peaks and the interpreted structures of selected signals corresponding to reducing ends and 4-deoxy-L-erythro-4-hexenopyranuronate residues (Δ) at the non-reducing ends of alginate lyase degradation products in 156 time-resolved 700 MHz $1\text{D } ^1\text{H}$ NMR spectra acquired over 39.15 h at 37°C during the degradation of 20.1 mg of sodium alginate substrate by 700 pmol of lyophilized BxPLX lyase from *B. xylanisolvens* AUL in 0.67 mL of D_2O in a standard 5 mm NMR tube. The nomenclature, residue abbreviations, and signal-assignment conventions are the same as those described in the caption to Figure 3.8. Data collection and analysis conducted by Dr. Xiaohui Xing.

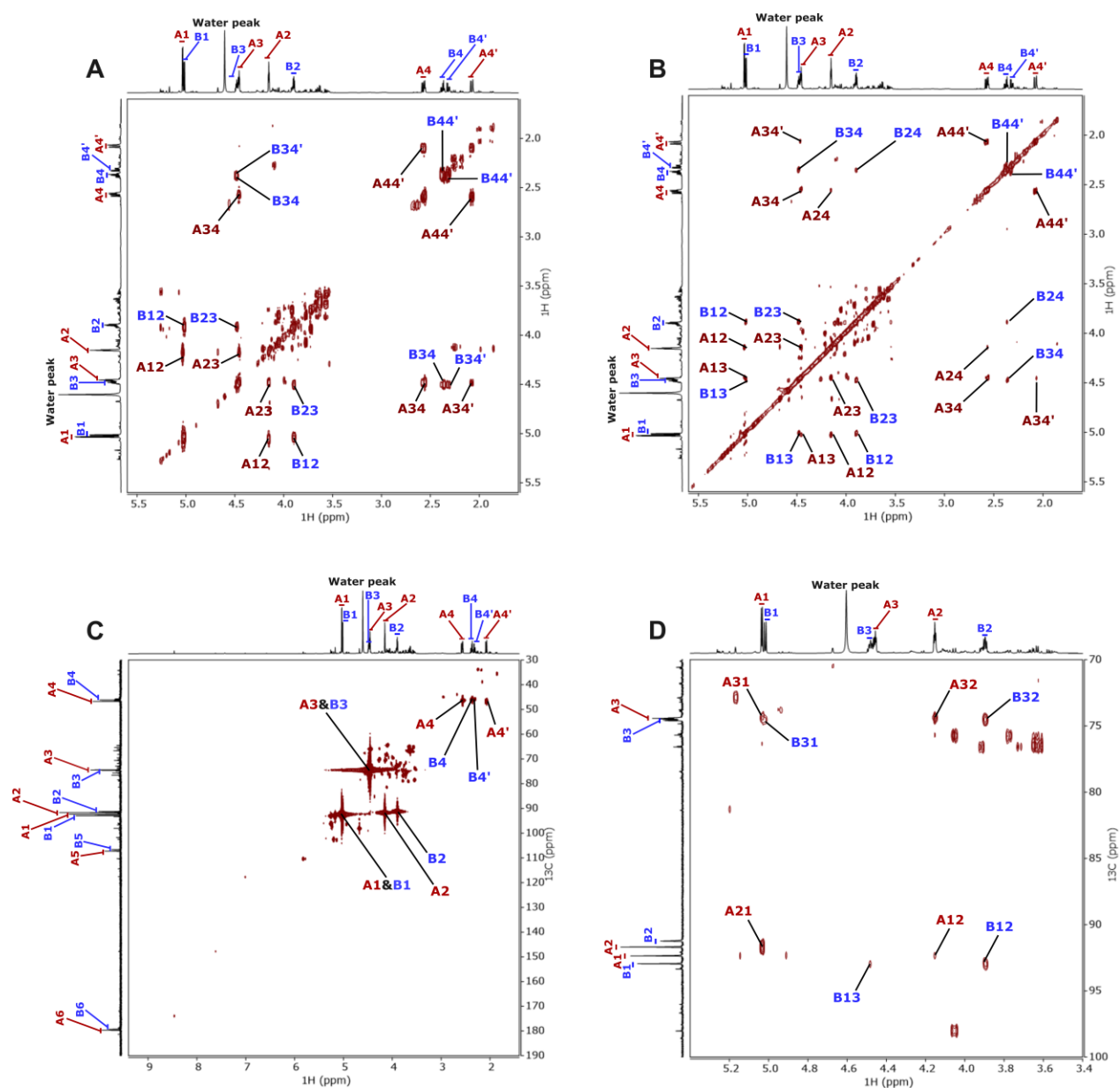


Figure S11. 700 MHz solution-state 2D NMR spectra collected at 37 °C in D₂O from the final products of 4-deoxy-L-erythro-5-hexoseulose uronic acid (DEH) generated by extensive incubation of alginate with a mixture of four polysaccharide lyases, *BxPL6*, *BxPL17*, *BxPL38*, and *BxPLX*, from *B. xylanisolvens* AUL: (A) 2D ¹H–¹H COSY, (B) 2D ¹H–¹H TOCSY, (C) 2D ¹H–¹³C HSQC, and (D) 2D ¹H–¹³C HMBC spectra. Chemical shifts were internally referenced to TSP-d₄ set to 0 ppm. The incubation product was observed predominantly as two hydrated DHF stereoisomers derived from DEH, assigned as residue A, (5*S*)-DHF hydrate, and residue B, (5*R*)-DHF hydrate, labelled in red and blue, respectively. The numbers following A and B indicate the numbered atoms involved in the assigned NMR signals for each residue. As an example of the annotation scheme, A1 denotes the H-1 signal of residue A in the stacked 1D ¹H trace shown along each 2D spectrum, the C-1 signal of residue A in the stacked 1D ¹³C

trace shown along the heteronuclear 2D spectra, and the H-1/C-1 cross-peak of residue A in the 2D HSQC spectrum. A12 denotes the H-1/H-2 cross-peak of residue A in the 2D COSY and 2D TOCSY spectra and the C-1/H-2 correlation of residue A in the 2D HMBC spectrum. Data collection and analysis conducted by Dr. Xiaohui Xing.

Supplementary Tables

Table S1 List of artiodactyls sampled from Wilder Institute/Calgary Zoo and Saskatoon Forestry Farm Park and Zoo. Sampled animals are listed by location and taxonomy (*i.e.*, species, genus, family and suborder). Pseudoruminants are marked with an asterisk (*).

Sampling site	Taxonomy		
	Common name (Latin name)	Family	Suborder
Wilder Institute/Calgary Zoo	Markhor (<i>Capra falconeri</i>)	Bovidae	Ruminantia
	Bighorn sheep (<i>Ovis canadensis</i>)	Bovidae	Ruminantia
	Rocky mountain goat (<i>Oreamnos americanus</i>)	Bovidae	Ruminantia
	Moose (<i>Alces alces</i>)	Cervidae	Ruminantia
	Boreal woodland caribou (<i>Rangifer tarandus caribou</i>)	Cervidae	Ruminantia
	Masai giraffe (<i>Giraffa tippelskirchi</i>)	Giraffidae	Ruminantia
	Siberian musk deer (<i>Moschus moschiferus</i>)	Moschidae	Ruminantia
	Alpaca (<i>Vicugna pacos</i>)*	Camelidae*	Tylopoda*
	Bactrian camel (<i>Camelus bactrianus</i>)*	Camelidae*	Tylopoda*
	Hippopotamus (<i>Hippopotamus amphibius</i>)*	Hippopotamidae*	Whippomorpha*
Saskatoon Forestry Farm Park & Zoo	Bison (<i>Bison bison</i>)	Bovidae	Ruminantia
	Bighorn sheep (<i>Ovis canadensis</i>)	Bovidae	Ruminantia
	Mountain goat (<i>Oreamnos americanus</i>)	Bovidae	Ruminantia
	Markhor (<i>Capra falconeri</i>)	Bovidae	Ruminantia
	Elk (<i>Cervus canadensis</i>)	Cervidae	Ruminantia
	Caribou (<i>Rangifer tarandus</i>)	Cervidae	Ruminantia
	Pronghorn (<i>Antilocapra americana</i>)	Antilocapridae	Ruminantia

Table S2 Crystallization information. Prepared by Emily Knudson-Goerner.

Method	Vapor diffusion, hanging drop
Temperature (K)	291
Protein concentration (mg ml⁻¹)	15
Buffer composition of protein solution	20 mM Tris-HCl pH 8.0, 500 mM sodium chloride
Composition of reservoir solution	
<i>BxPLX</i>	0.05M Sodium Acetate pH 4.5 25% w/v Polyethylene Glycol 3350
<i>BxPLX Y238A Mannuronic acid complex</i>	0.05M Sodium Acetate pH 4.5 24% w/v Polyethylene Glycol 3350 4mM Penta-Mannuronic acid
<i>BxPLX Y238A Guluronic acid complex</i>	0.05M Sodium Acetate pH 4.5 24% w/v Polyethylene Glycol 3350 4mM Penta-Guluronic acid
Volume and ratio of drop	1 µl, 1:1
Volume of reservoir	500 µl

Table S3 Data collection and processing. Values for the outer shell are given in parentheses.

Prepared by Emily Knudson-Goerner

Diffraction source	CLS		
Wavelength (Å)	0.951		
Temperature (K)	100		
Detector	Dectris Eiger X 9M X-ray detector		
Rotation range per image (°)	0.2		
Total rotation range (°)	360		
Exposure time per image (s)	0.5		
Enzyme	<i>BxPLX</i> (Apo)	<i>BxPLX</i> -ManA	<i>BxPLX</i> -GulA
Space group	<i>P</i> ₆ ₁ 22	<i>P</i> ₂ ₁ 2 ₁ 2 ₁	<i>P</i> ₂ ₁
<i>a</i>, <i>b</i>, <i>c</i> (Å)	51.23, 51.23, 373.15	50.60, 75.33, 76.29	56.29, 53.54, 102.97
α, β, γ (°)	90, 90, 120	90, 90, 90	90, 93.68, 90
Mosaicity (°)	0.1		
Resolution range (Å)	28.56-2.60 (9.01-2.60)	28.24-1.14 (6.24-1.14)	28.85-2.00 (8.94-2.00)
Total No. of reflections	92453	596287	156422
No. of unique reflections	9882	106786	41668
Completeness (%)	99.5 (99.5)	98.9 (86.6)	99.8 (100.0)
Redundancy	9.4 (10.5)	5.6 (3.4)	3.8 (3.9)
$\langle I/\sigma(I) \rangle$	13.7 (5.6)	7.5 (2.2)	7.2 (2.4)
<i>R</i>_{pim}	0.033 (0.100)	0.062 (0.304)	0.072 (0.360)
<i>R</i>_{meas}	0.099 (0.326)	0.151 (0.604)	0.143 (0.726)
CC_{1/2}	0.998 (0.995)	0.987 (0.769)	0.992 (0.768)
Overall <i>B</i> factor from Wilson plot (Å²)	47.95	9.78	24.24

Table S4 Structure solution and refinement. Values for the outer shell are given in parentheses.

Prepared by Emily Knudson-Goerner

Enzyme	<i>BxPLX</i>	<i>BxPLX-ManA</i>	<i>BxPLX-GulA</i>
Resolution range (Å)	28.56-2.60	28.24-1.14	28.85-2.00
	(9.01-2.60)	(6.24-1.14)	(8.94-2.00)
Completeness (%)	98.32	98.83	99.76
No. of reflections, working set	9263	100371	39649
No. of reflections, test set	476	5160	1920
Final R_{work}	0.2237	0.1620	0.1792
Final R_{free}	0.2865	0.1746	0.2190
No. of non-H atoms			
Protein	2075	2105	4106
Ligand	N/A	121	145
Metal	1	1	1
Water	59	423	445
Total	2134	2647	4700
R.m.s. deviations			
Bonds (Å)	0.006	0.008	0.007
Angles (°)	0.841	0.969	1.234
Average B factors (Å²)			
Protein	53.56	11.79	31.21
Ligand	N/A	16.33	42.53
Water	51.72	21.42	35.16
Ramachandran plot			
Favoured regions (%)	95.31	98.03	97.25

Allowed (%)	4.69	1.97	2.75
Outliers (%)	0.00	0.00	0.00

Table S5 Summary of animals yielding ALG-metabolizing bacterial isolates. Fecal material from ruminants and pseudoruminants were sampled from the Wilder Institute/Calgary Zoo and Saskatoon Forestry Farm Park and Zoo. Selective enrichment and purification of bacterial isolates were performed using ALG and *S. latissima* extract as carbon sources for fecal community bacteria. Bacterial isolates were one of two species: *B. xylanisolvens* or *B. faecium*. Bacterial isolates were systematically named based on genus/species identifications, followed by enrichment carbon source, bacterial isolate reference number, and host genus (e.g., BxALG1_{Alc} denotes *B. xylanisolvens* bacterial isolate #1 enriched on ALG, sourced from *Alces alces* (moose)). Pseudoruminants are marked with an asterisk (*).

Sampling site	Common name (Latin name)	Bacterial isolates (Y/N)	Bacterial isolate ID
Wilder Institute/Calgary Zoo	Markhor (<i>Capra falconeri</i>)	N	NA
	Bighorn sheep (<i>Ovis canadensis</i>)	N	NA
	Rocky mountain goat (<i>Oreamnos americanus</i>)	Y	BxALG6 _{Ore}
	Moose (<i>Alces alces</i>)	Y	BxALG1 _{Alc} BxALG2 _{Alc}
	Boreal woodland caribou (<i>Rangifer tarandus caribou</i>)	N	NA
	Masai giraffe (<i>Giraffa tippelskirchi</i>)	Y	BfALG1 _{Gir} BfALG3 _{Gir} BfALG4 _{Gir} BfALG5 _{Gir} BfALG6 _{Gir} BfALG8 _{Gir} BfALG9 _{Gir} BfALG10 _{Gir}
	Siberian musk deer (<i>Moschus moschiferus</i>)	N	NA
	Alpaca (<i>Vicugna pacos</i>)*	N	NA
	Bactrian camel (<i>Camelus bactrianus</i>)*	N	NA
	Hippopotamus (<i>Hippopotamus amphibius</i>)*	N	NA
Saskatoon Forestry Farm Park and Zoo	Bighorn sheep (<i>Ovis canadensis</i>)	Y	BxALG1 _{Ovi} BxALG2 _{Ovi} BxALG3 _{Ovi} BxALG4 _{Ovi} BxSlat1_1 _{Ovi} BxSlat1_2 _{Ovi} BxSlat2_21 _{Ovi} BxSlat2_2 _{Ovi}
	Mountain goat (<i>Oreamnos americanus</i>)	Y	BxALG1 _{Ore} BxALG2 _{Ore} BxALG3 _{Ore} BxALG4 _{Ore}

		<i>BxSlat2_1</i> _{Ore}	<i>BxSlat2_2</i> _{Ore}
Markhor	N	NA	
(<i>Capra falconeri</i>)			
Elk	N	NA	
(<i>Cervus canadensis</i>)			
Caribou	N	NA	
(<i>Rangifer tarandus</i>)			
Pronghorn	N	NA	
(<i>Antilocapra americana</i>)			

Table S6 Percent identity of conserved AUL ORFs amongst bacterial isolates. All bacterial isolates from the present study are *B. faecium* or *B. xylanisolvens*. AULs consisting of 12-13 ORFs (predicted by prodigal, BLASTp, dbCAN) were encoded by each bacterial isolate. Localization of each ORF predicted by SignalP 6.0 is listed. The AUL shows high synteny both within and between bacterial species. The 39 bp hypothetical protein is only present in 10 AULs, but maintains 100% sequence identity across all bacterial isolates in which it presents.

Gene	Signal peptide prediction	% identity between species	% identity within species	
			<i>B. xylanisolvens</i>	<i>B. faecium</i>
FadR/GntR family transcriptional regulator,	Other	95	100	100
Hypothetical protein	Other	NA	NA	NA
SusC-like	SPI	88.3	100 (except to <i>BxALG6_{Ore}</i> at 99.6)	100
SusD-like	SPII	84.4	100 (except to <i>BxALG6_{Ore}</i> at 99.6)	100
IPT/TIG domain protein	SPII	61.5	100	100
PL6	SPI	93.3	100 (except to <i>BxALG6_{Ore}</i> at 99.9)	100
PL17	SPI	93.9	100	100
KdgF	Other	98.3	100	100
MFS transporter	Other	97.8	100	100
Unk1 (SDR family NAD(P)-dependent oxidoreductase, glucose 1-dehydrogenase)	Other	99.2	100	100
PL38	SPI	97.0	100	100
Unk2 (Alpha/beta hydrolase)	SPI	96.1	100	100
Unk3 (Heparin lyase I family protein, hypothetical protein, polysaccharide lyase family protein)	SPII	92.3	100	100

Table S7 Homologous AULs present within other *Bacteroides* spp. isolate strains. The CAZy PULDB was screened for AULs with high synteny to those identified in Wilder Institute/Calgary Zoo and Saskatoon Forestry Farm and Park Zoo bacterial isolates (**Figure 3.2**). Based on CAZy PULDB hits, NCBI annotated protein data from identified strains were collected and ORFs from the identified AUL were run through BLAST against the *BxALG1_{Aic}* AUL. Bacterial isolates with an asterisk (*) are sourced from the present study.

Bacterial isolate strain	NCBI RefSeq	Source host	Percent identity of homologous AUL ORFs to <i>BxALG1_{Aic}</i> *											
			Reg	SusC	SusD	IPT/TIG	PL6	PL17	KdgF	MFS	Unk 1	PL38	Unk 2	Unk 3
BfALG1 _{Gir} *	NA	Giraffe	95.0	88.3	84.4	61.5	93.3	93.9	98.3	97.8	99.2	97.0	96.1	92.3
<i>B. xylanisolvans</i> NLAE-zl-G399	GCF_900107825.1	Goat	93.7	87.6	83.8	62.6	91.8	89.0	97.4	95.5	98.8	94.0	92.7	88.3
<i>B. ovatus</i> NLAE-zl-C500	GCF_900102645.1	Cow	93.7	87.6	83.8	62.6	91.9	88.8	97.4	95.5	98.8	94.0	92.7	88.3
<i>Bacteroides</i> sp. M10	GCF_014334015.1	Human	93.7	87.6	83.8	62.5	91.8	89.0	97.4	95.5	98.8	94.5	92.7	87.3
<i>Bacteroides</i> sp. D2	GCF_025147085.1	Human	93.3	87.6	83.6	62.5	91.4	89.1	97.4	95.7	98.8	94.5	92.7	87.3
<i>B. ovatus</i> WH711	GCF_918271665.1	Human	94.1	87.6	83.6	62.3	91.5	88.2	97.4	95.7	98.8	94.0	93.0	86.0
<i>B. ovatus</i> TSDC17.2	GCF_918291015.1	Human	94.1	87.6	83.6	62.3	91.5	88.3	97.4	95.7	98.8	94.0	93.0	86.0
<i>B. finegoldii</i> CL09T03C10	GCF_040687835.1	Human	92.5	97.7	94.7	87.9	91.1	89.8	97.4	96.9	98.0	95.5	89.4	91.6
<i>B. faecium</i> 7301	GCF_012113595.1	Human	99.6	99.6	99.2	98.8	97.7	97.7	100.0	99.0	99.6	98.5	97.9	95.0

Table S8. Comparative analysis of AUL PL counts within whole genome sequencing data on both ruminant fecal and ALG-enriched cultures. CAZome data was screened for ORFs classified by dbCAN3 HMMs to belong to PL families 6, 17 and 38. Classifications were made conservatively, only including definitive hits. ‘Calg’ and ‘Sask’ are used to denote animals located at the Wilder Institute/Calgary Zoo and Saskatoon Forestry Farm Park and Zoo, respectively. Fecal and ALG enriched samples are denoted by (-) and (+), respectively.

Sample	PL6		PL17		PL38	
	-	+	-	+	-	+
Moose (Calg)	14	1	6	1	17	3
Rocky mountain goat (Calg)	9	1	14	1	24	2
Masai giraffe (Calg)	3	2	9	2	20	6
Bighorn sheep (Sask)	7	1	13	1	19	1
Rocky mountain goat (Sask)	13	1	7	1	20	1
Bison (Sask)	45	0	18	0	45	0

Table S9. BLASTp output of fecal and ALG enriched metagenomics data against *BxPLX* query sequence. Since *BxPLX* does not have a defined dbCAN3 HMM, metagenomic data was screened for homologues through BLASTp, with results refined through the following filtering criteria (E-value < 1×10^{-3} , bitscore >50). Fecal and ALG enriched samples are denoted by (-) and (+), respectively.

Sample	Database hit ID	E-value	bitscore	Percent identity	Query coverage
Moose (Calg) +	NODE_16124_length_478_cov_1.815603_1	9.30E-98	284	100.0	45.3
	NODE_50000_length_232_cov_1.627119_1	1.52E-20	83.6	100.0	13.1
Moose (Calg) -	NODE_43_length_217653_cov_39.956410_34	4.91E-88	272	50.4	87.2
Goat (Calg) +	NODE_338_length_49800_cov_288.915027_22	0	621	100.0	100.0
	NODE_295_length_57387_cov_301.063455_13	6.77E-37	135	30.4	94.6
Goat (Calg) -	NODE_30183_length_5079_cov_4.783838_3	2.03E-100	301	51.9	96.6
	NODE_171189_length_1737_cov_5.801427_1	4.46E-100	300	54.7	90.6
	NODE_268_length_95621_cov_38.570307_20	9.51E-100	299	52.4	94.0
	NODE_872_length_52440_cov_21.147523_39	2.95E-94	286	48.7	97.0
Goat (Sask) +	NODE_494_length_47565_cov_594.849484_21	0	621	100.0	100.0
Goat (Sask) -	NODE_286783_length_1266_cov_2.687036_1	2.02E-100	301	51.9	96.6
	NODE_4537_length_17832_cov_30.320527_2	5.79E-100	300	52.7	94.0
	NODE_1821141_length_463_cov_1.566176_1	2.74E-59	191	62.0	49.7
Sheep (Sask) +	NODE_55_length_161276_cov_170.050577_90	0	621	100.0	100.0
Sheep (Sask) -	NODE_21044_length_6320_cov_6.233520_4	4.20E-100	301	54.7	90.6
	NODE_30291_length_4912_cov_5.947293_3	5.53E-100	300	52.7	94.0
	NODE_14628_length_8179_cov_9.515140_5	2.80E-92	281	48.1	97.0
	NODE_37658_length_4265_cov_17.186461_2	6.59E-34	130	34.6	70.5
	NODE_931112_length_652_cov_2.251256_1	8.04E-13	68.6	49.2	20.5
Giraffe (Calg) +	NODE_6_length_565711_cov_2026.324544_211	0	568	92.3	99.3
	NODE_54_length_153608_cov_37.958451_25	0	564	90.9	99.3
Giraffe (Calg) -	NODE_24422_length_5293_cov_11.450935_1	1.33E-33	129	33.5	82.2
	NODE_7529962_length_220_cov_2.327273_1	1.23E-26	103	94.0	16.8

	NODE_6783609_length_231_cov_1.090909_1	1.81E-21	91.3	55.4	24.8
Bison (Sask) +	NA	NA	NA	NA	NA
	NODE_9408_length_9358_cov_5.788455_7	1.31E-92	283	52.2	90.6
	NODE_1137029_length_660_cov_2.725620_1	1.54E-73	229	55.8	68.1
	NODE_2179205_length_482_cov_2.604215_1	4.25E-55	181	56.3	53.7
	NODE_2596456_length_443_cov_2.719072_1	2.96E-44	152	51.7	46.3
	NODE_16643_length_6610_cov_6.085736_3	3.77E-37	139	31.6	91.6
Bison (Sask) -	NODE_1030445_length_692_cov_2.929356_1	7.94E-36	134	36.5	63.4
	NODE_22494_length_5477_cov_15.063261_8	1.86E-34	130	35.1	71.5
	NODE_6012060_length_289_cov_1.641026_1	1.91E-32	120	63.8	31.5
	NODE_3442095_length_387_cov_1.731928_1	7.17E-29	112	44.6	42.3
	NODE_7359214_length_266_cov_1.819905_1	2.13E-13	69.7	52.4	21.1