The devil lies in the details: how variations in polysaccharide fine-structure impact the physiology and evolution of gut microbes

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Abstract

The critical importance of gastrointestinal microbes to digestion of dietary fiber in humans and other mammals has been appreciated for decades. Symbiotic microorganisms expand mammalian digestive physiology by providing an armament of diverse polysaccharide degrading enzymes, which are largely absent in mammalian genomes. By out-sourcing this aspect of digestive physiology to our gut microbes, we maximize our ability to adapt to different carbohydrate nutrients on time scales as short as several hours, due to the ability of the gut microbial community to rapidly alter its physiology from meal-to-meal. Because of their ability to pick up new traits by lateral gene transfer, our gut microbes also enable adaption over time periods as long as centuries and millennia by adjusting their gene content to reflect cultural dietary trends. Despite a vast amount of sequence-based insight into the metabolic potential of gut microbes, the specific mechanisms by which symbiotic gut microorganisms recognize and attack complex carbohydrates remain largely undefined. Here, we review the recent literature on this topic and posit that numerous, subtle variations in polysaccharides diversify the spectrum of available nutrient niches, each of which may be best filled by a subset of microorganisms that possess the corresponding proteins to recognize and degrade different carbohydrates. Understanding these relationships at precise mechanistic levels will be essential to obtain a complete understanding of the forces shaping gut microbial ecology and genomic evolution, as well as devising strategies to intentionally manipulate the composition and physiology of the gut microbial community to improve health.

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Introduction

Humans consume a broad range of polysaccharide-rich foods, mostly in the form of plant material (cell walls and storage polymers), but also as animal connective tissue, food additives and even microbial and fungal products. Our intrinsic ability to digest the available repertoire of complex carbohydrate molecules remains limited to just starch, lactose and sucrose.[1] This metabolic decrement is due to a paucity of fiber degrading enzymes encoded in the genomes of humans and other animals (for a recent overview see [ref. 1]). Fortunately, we have co-evolved with a dense consortium of symbiotic distal gut microorganisms (microbiota), many of which have adapted to target these polysaccharides for their own nutrition. In return, we reap the benefits of these gut symbionts' largely fermentative metabolism, which produces short-chain fatty acids (SCFA) and other products that are absorbed in the colon as nutrients.[2]

Of the dozens of different phyla of bacteria and archaea that exist on Earth, less than 10 are abundant in the guts of humans.[3; 4; 5] The Gram-positive Firmicutes are typically most numerous, followed by Gram-

negative Bacteroidetes. Other common, but less numerically abundant phyla include Actinobacteria, Verrucomicrobia and Proteobacteria, among others. The selection for a few taxonomic groups was probably ancient, since these same phyla are shared among other mammals and many invertebrates.[4] Moreover, at finer taxonomic levels (genus and species) the microorganisms found in human and animal guts are typically not present in environmental reservoirs, leading to the hypothesis that we have co-evolved with many of these organisms and provide their only habitats.[6]

The genomes of sequenced human gut bacteria, and the metagenomes of the communities they compose, reveal that our microbial symbionts have much more extensive armaments of polysaccharide degrading enzymes than we do.[1; 5; 7; 8; 9] This is evident in both the numbers of enzymes present and the diversity of catalytic activities.[1] As a particularly striking example, the recently published 7.1 Mbp genome of *Bacteroides cellulosyliticus* WH2 contains a total of 424 glycoside hydrolases, polysaccharide lyases and carbohydrate esterases, which is ~25 times the number of human genome-encoded enzymes that are thought to be secreted into the gastrointestinal tract.[10] Of the 76 different carbohydrate-active enzyme (CAZyme) families (as defined in the Carbohydrate-Active Enzymes Database[11]) present in *B. cellulosyliticus* WH2, 56 are not represented in the human genome, highlighting the amount of metabolic expansion that even a single gut bacterium adds. Without this help from symbiotic bacteria, humans and other animals, ranging from termites to ruminants, would simply be incapable of assimilating nutrients from a substantial portion of dietary polysaccharides.

Despite a vast – and expanding – amount of sequence-based insight, precise mechanistic relationships between the enormous diversity of polysaccharides that enter our digestive system and the microbes that degrade them have been slower to develop. In this review, we consider several emerging facets of how symbiotic gut microorganisms assist humans and other animals with polysaccharide digestion. We focus first on the evolutionary benefit of this digestive symbiosis, subsequently outline the sensory and enzymatic mechanisms employed by various gut bacteria to distinguish these nutrients, and conclude by discussing some recent data that imply the presence of finely adapted and niche-specific microbe-polysaccharide interactions in the gut, some of which are being driven by the lateral transfer of genes involved in polysaccharide degradation.

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Old questions still in need of detailed answers

The critical role of intestinal microorganisms in polysaccharide degradation became appreciated around the early 1940's when Robert Hungate, a pioneer in the field of anaerobic microbiology, explored the phenomenon of microbial cellulose degradation in the bovine rumen and termite gut.[12] With the advent of more facile anaerobic culturing techniques – including the development of the anaerobic chamber by Freter and colleagues in the 1960's[13] – pioneers in human gut microbiology, such as Freter, Holdeman, and Moore, reported substantial viable recoveries (up to 46-93%) of the bacterial cells observable by direct microscopic counts.[13; 14] As a testament to the experimental skill of these scientists, the lists of most abundant human gut bacterial species that they found in early cultivation studies share substantial overlap with taxon lists generated using more modern techniques, such as 16S rDNA amplicon sequencing and metagenomics.[14; 15; 16] A much more recent study, which compared large scale anaerobic culturing to direct molecular ecology-based community enumerations, also supports the idea that most human gut bacteria can be readily grown outside of the host.[17] Taken together, these observations reinforce the imperative for continued culturing of microorganisms from the human gut so that their physiology, both alone and in communities, can be studied and understood in great depth.[18]

Bacteriological studies into the abilities of human gut bacteria to degrade dietary fiber and mucin polysaccharides did not initiate in earnest until the 1970's, catalyzed by the seminal efforts of Salyers, Wilkins and colleagues, which involved fermentation studies on a large collection of cultured human gut bacteria.[15; 16] Such analyses continue to be extended to a range of polysaccharides, as well as oligosaccharide components accessible through specific enzymatic treatment and fractionation.[19; 20; 21; 22; 23] These *in vitro* surveys have highlighted that the Bacteroidetes possess notably broad abilities to digest a diverse array of mostly soluble polysaccharides. In contrast, more recent work by Flint and co-workers has suggested that members of the Firmicutes, which demonstrated more limited catabolic breadth in early studies, possess the ability to attack more insoluble substrates that are characteristic of the natural plant fibers in our diets and may serve as "keystone" polysaccharide degraders.[24; 25]

Moving beyond descriptive growth studies, several researchers have provided molecular insight into the enzyme-based strategies through which human gut bacteria process complex carbohydrates, such as starch, inulin and many other polysaccharides.[26; 27; 28; 29; 30; 31] These paradigms extend to various Gram-negative and Gram-positive bacteria and have provided a framework for understanding the molecular processes involved. Still, however, numerous questions remain, including *i*) which species compete most efficiently for each available polysaccharide?, *ii*) to what extent, and how, do the species present cooperate during polysaccharide degradation?, and *iii*) are dominant rumen bacterial strategies, such as deployment of cellulosomes by Gram-positive species, at work in the human colon? *An additional question that serves as a central focus for this review is: how finely tuned are the relationships between members of our gut microbiota and the myriad chemical differences present in the polysaccharides that are so important to their biology?*

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Why do we rely on gut microorganisms for polysaccharide digestion?

Before considering more detailed mechanistic aspects of gut microbiota function, it is worth reflecting on this fundamental question. As discussed above, the human genome, and indeed the genomes of most animals, does not inherently encode a plethora of carbohydrate digestive enzymes; animal genes that encode plant cell wall glycosidases are particularly exceptional.[32; 33] *Why is this so?* By out-sourcing complex carbohydrate metabolism to our symbiotic gut microbes, we greatly enhance our ability to respond to nutritional changes on time scales both short (minutes to hours) and long (centuries to millennia). Since the amounts and types of polysaccharides in our omnivorous diet vary from meal-to-meal, our gut symbionts must be capable of shifting their metabolism to accommodate the diversity that is offered within this limited period. The presence of "generalists", such as *B. cellulosilyticus* (*vide supra*), *B. thetaiotaomicron* and *B. ovatus*, highlight short-term adaptability by individual species. These organisms each possess the ability to degrade over a dozen different polysaccharides[10; 29] and therefore have the option to switch to metabolism of different nutrients as they become available. Metabolic re-organization by these species in complex nutrient conditions has been validated both *in vitro*[34] and *in vivo* in gnotobiotic mice.[10; 35] However, it is likely that much more complex, community-driven metabolic changes also occur on short time scales. Although it remains to be explored at functional levels – or on the very short time scales (hours) that reflect meal-to-meal variation – short-term diet change experiments in humans and mice clearly show that the composition of the gut community changes frequently and within a day after diet switch.[10; 36; 37; 38] These rapid changes likely reflect transient proliferation of the species that are best equipped to metabolize components of new dietary items and, upon introduction of a different meal, are likely to shift again.

Despite the presence of some generalist species noted above, no one microorganism that can "do it all" has yet been identified, suggesting that a fitness trade-off exists between the cost of encoding a diverse array of metabolic responses and the advantage of possessing additional metabolic options. Indeed, as the metabolic abilities of more cultivated gut microorganisms are systematically interrogated, it will be interesting to determine which polysaccharides are targeted by metabolic specialists that have more limited abilities. Such nutrients, for example host mucin glycoproteins, likely represent the most consistent resources that fuel gut microbial growth, and may therefore be most important with respect to supporting community stability.

Our symbiotic microbes also enable adaptation to dietary changes over much longer time periods. Different human cultures adopt unique dietary habits that may exist in geographically restricted populations and persist on time scales of centuries or millennia. These protracted periods may still be too short for the human genome to evolve appropriate enzymes, but given the genomic plasticity of bacteria, adaptation by members of the gut microbiota is more easily achieved. A striking example of such adaptation is thought to have occurred in bacterial members of the Japanese microbiota. In Japan and surrounding Asian cultures the consumption of red algae (*e.g.*, nori in sushi) has been commonplace for over a thousand years. Likely as a consequence of this availability in the gut, one cultured member of the healthy Japanese microbiota, *Bacteroides plebeius*, has obtained the capacity to degrade porphyran, a sulfated polymer of galactose that is abundant in nori.[39; 40] Further molecular analysis suggests that such plasticity is a common feature of gut microbial genomes, as will be elaborated below.

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The benefits (and limitations) of "omics"

The advent of the sequencing era has significantly bolstered studies of the content and dynamics of the microbiota. Culture-independent approaches, such as 16S rDNA and metagenomics, have provided a less biased and more comprehensive view of the microbial taxa that are present. In turn, this data can guide the isolation of organisms not yet represented in pure culture.[18] Sequencing of individual microbial genomes has provided hundreds of reference blueprints for human gut bacteria[41], which can be analyzed using bioinformatics approaches to predict functionality. The degradation of most complex polysaccharides necessarily requires the concerted expression of multiple carbohydrate-active enzymes (CAZymes)[11; 42] and other proteins. However, precisely predicting the phenotypic abilities of gut bacteria to degrade and utilize carbohydrates based on sequence data alone remains difficult.

A key issue is that we currently have much less biochemical data *vis a vis* sequence data– and this gap is only widening.[11; 42] Our ability to predict the function of gene products is therefore perilously stretched in light of the broad sequence diversity exhibited by families of CAZymes and carbohydrate-binding proteins. Additionally, these families are often "polyspecific" and include members with variations in glycosidic linkage specificity. Given the subtle evolutionary changes involved in altering specificity, many glycosidase families are composed of members with non-absolute preferences for related monosaccharides, e.g. epimers (glucose vs. galactose) or substituent variants (galactose vs. *N*-acetylgalactosamine). Subfamily classification has been employed to further refine specificity prediction for a handful of families, but even this is not absolute.[43; 44; 45] Thus, an individual gut enzyme may be hypothetically associated with degradation of multiple polysaccharides, leading to ambiguous functional predictions.

Since many bacteria tightly control the expression of enzyme systems that target various nutrients, whole genome transcriptional analyses of bacteria grown in pure carbohydrates can be very useful to guide identification of gene products that work together.[10; 28; 29; 46; 47] Such functions are often grouped together –

especially in Bacteroidetes – into genomically-linked polysaccharide utilization loci (PULs, Figure 1)[48]. *Empirical delineation of these gene clusters, together with reverse-genetic and biochemical studies, provides a powerful route to the discovery of new metabolic pathways, especially those for complex carbohydrate utilization*. Given the ever-expanding breadth of sequence-based data, such comprehensive findings can be particularly useful because they can be rapidly fit into a much larger biological/ecological picture using comparative- and meta-genomics.

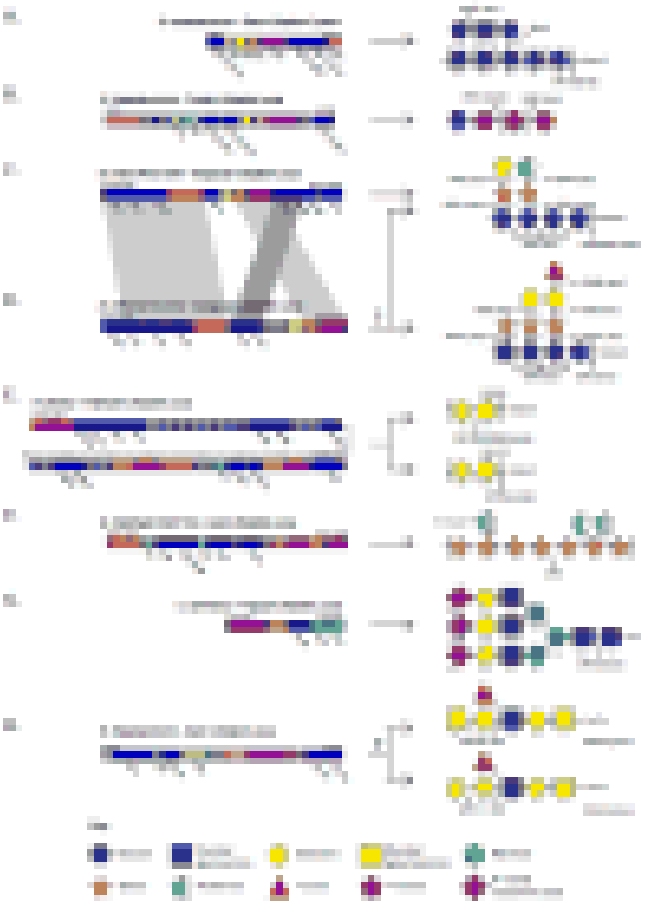

Figure 1
Polysaccharide Utilization Loci (PULs) and their substrates
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Gut symbionts have specialized sensory and enzymatic toolkits for polysaccharide digestion

Substrate specificity is a key facet of microbial responses to complex carbohydrates. It might be expected that an organism such as *B. cellulosilyticus*, which harbors 424 different carbohydrate-degrading enzymes, would carefully regulate expression of these enzymes and their associated non-enzymatic factors, or risk needlessly expending energy for superfluous protein synthesis in the competitive gut environment. Indeed, this is the case in *B. cellulosilyticus* and other gut and rumen Bacteroidetes that have been studied.[29; 31; 49] In these organisms, expression of gene clusters ranging in size from 6 to several dozen ORFs are regulated via inducible promoters that are usually activated by transcription factors present within or adjacent to the responsive gene cluster. A common theme that has emerged in the Gram-negative Bacteroidetes is that the sensor proteins extend outside of the cytoplasm to interact with

enzymatically released saccharides in either the periplasm or during transport across the outer membrane.[29; 31; 48; 50] By sensing carbohydrate cues before degradation to individual monosaccharides, these organisms are able to integrate additional information about the polysaccharide that has been encountered, including sugar sequence, linkage stereochemistry, and linkage regiochemistry. This strategy allows bacteria to couple a particular sensor to the expression of a more specific set of polysaccharide degrading enzymes and may account for much of the apparent redundancy in genes encoding some enzyme families that are heavily represented in the genomes of symbiotic gut bacteria. When over-expressed and analyzed *in vitro* (away from their normal regulatory control and associated enzymatic partners) such enzymes may display overlapping activities on a range of substrates that harbor a particular target linkage.[51] However, in the context of a living cell, deployment of these overlapping enzymes in response to specific substrates becomes much more specialized.

Generalized polysaccharide degradation schemes for Gram-negative and -positive gut bacteria are illustrated in Figure 2, based on knowledge of several different catabolic systems in Bacteroidetes and Firmicutes/Actinobacteria, respectively (see also recent reviews[52; 53]). Aside from the need for sensory specificity, an additional pre-requisite is access to the target substrate in order to initiate enzymatic attack and release cues that activate a corresponding response. For some bacteria, substrate solubility may create a substantial barrier to access. *B. thetaiotaomicron* and *Eubacterium rectale* can both grow on soluble forms of starch but not on insoluble "resistant starch".[15; 16; 25] In contrast, one Firmicute, *Ruminococcus bromii*, has been shown to degrade resistant starch *in vitro*, revealing that it possesses an alternative mechanism of substrate access relative to other bacteria.[25] At least two other Ruminococci, *R. champenellensis* and *R. torques*, possess the ability to utilize insoluble cellulose and carbohydrate components of human colonic mucin, respectively.[54; 55] These species stand in contrast to *Bacteroides* that have the ability to use soluble portions of these same substrates, such as cellobiose and chemically released mucin *O*-linked glycans.[29; 55] Future elucidation of the molecular mechanisms that mediate these phenotypes will undoubtedly provide novel insight into the first crucial step in insoluble fiber digestion.

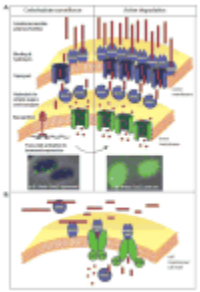

Figure 2

Generic models for polysaccharide acquisition by gut bacteria

When accessible polysaccharides are encountered, initial attack presumably proceeds via constitutively produced enzymes that are present at low "surveillance" levels.[46] Indeed, without such enzymes, the bacterium may very well be incapable of generating the required cues to amplify a catabolic response in the first place. This phenomenon of oligosaccharide substrate-induced up-regulation is readily observed in *B. thetaiotaomicron*. In the presence of glucose, a monosaccharide substrate that does not trigger activation of the Starch Utilization System (Fig. 1A), a punctate pattern of the key SusD protein can be observed at the cell surface (inset micrographs in Fig. 2A). In cells exposed to starch for just 30 minutes, expression of these proteins is dramatically upregulated, due to the production of malto-oligosaccharides that bind the sensor/regulator SusR.[34] Thus, in the model shown in Fig. 2., each polysaccharide substrate can initiate a positive feedback loop in which small amounts of surveillance enzymes produce a substrate-specific cue(s) to amplify expression of more enzymes until the bacterial surface is either saturated or the substrate wanes in abundance.

An additional facet of microbial competition for available nutrients, which has remained largely unexplored for most symbiotic gut bacteria beyond *E. coli*, is the propensity of many bacteria to prioritize some nutrients with respect to others. This idea is predicated on the classic example of repression of *E. coli* lactose utilization, discovered by Monod and co-workers.[56] A recent study on *B. thetaiotaomicron* revealed that this generalist engages in metabolic prioritization of the more than 12 different polysaccharides that it is equipped to consume.[34] Since this species is just one of many hundred that are common to the human gut, an important frontier in understanding the behavior of these organisms will be to understand the programming of different nutrient prioritization schemes. Indeed, many organisms may exhibit similar or identical metabolic potential *in vitro* on pure polysaccharide substrates, but deploy these traits in very different ways in the complex and dynamic nutrient mixtures that are likely to be the norm in the gut.

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Closely related polysaccharides harbor variations that impact their availability to gut microbes

From the discussion above, it follows that detailed knowledge of polysaccharide structure is central to understanding substrate prioritization and the potential catabolic niches that are available to the microbiota. In particular, variations in polysaccharide linkage and sidechain branching affect physical properties such as solubility and solution rheology,[57] which are important in distinguishing "soluble" and "insoluble" forms of dietary "fiber".[58] For example, it is readily appreciated that cellulose from plant cell walls [β(1-4)-glucan], amylose from starch [α(1-4)-glucan], and mixed-linkage β(1-3)/β(1-4)-glucans from cereal endosperm have widely differing solubilities and susceptibilities to biological degradation due to differences in secondary structure, yet all are "simple" homopolymers of glucose [59; 60; 61]. Even the relative abundance and distribution of β(1-3) and β(1-4)-linkages in mixed-linkage glucans has a significant effect on solubility.[62] The effect of substituents on polysaccharide physical and chemical properties is likewise considerable: Amylopectin has significantly greater water solubility than amylose, due to numerous α(1-6) branch points extended with α(1-4)-glucan chains.[59; 63] Likewise, the xyloglucan family of plant cell wall matrix glycans can be considered as soluble cellulose derivatives, in which the common β(1-4)-glucan backbone is highly substituted with diverse monosaccharides extended from α(1-6)-xylosyl branches (Fig. 1).[64; 65] Numerous other such examples of branched polysaccharides (with an equally diverse range of solubilities) are well known.[57; 66; 67] An additional level of structural complexity, which impacts physical-chemical properties of many polysaccharides, is the presence of ether (e.g., methyl) and ester (e.g., acetyl, ferulate, sulfate) substituents.[39; 67; 68] Indeed, the large repertoire of monosaccharide building blocks and the manifold possible stereo- and regiochemical linkage variations combine to generate a tremendous diversity of saccharide structures in terrestrial and marine biomass relevant to the microbiota; this includes polysaccharides, proteoglycans/glycoproteins, and glycolipids.[57; 69; 70; 71] A key corollary is that a similarly large cohort of saccharide-specific carbohydrate-active enzymes, binding proteins, transporters, and sensor-regulators is required by gut microbial communities to address this diversity.

Functional genomics and enzymological studies of the microbiota have necessarily been guided – and likely limited – by our current knowledge of the structural diversity of complex carbohydrates in Nature (as well as the general availability of these substrates for experimentation).[19; 20; 21; 22; 29; 46; 72] Given the intrinsic technical difficulties in determining oligo- and polysaccharide sequences, *vis a vis* polypeptides or polynucleotides, accurate structural data is challenging to obtain and generally limited to abundant, readily extracted representatives of a given polysaccharide family. Polysaccharide biosynthesis is often not tightly regulated, with the result that variations in composition and degree of polymerization arise

across individual tissues and growth conditions.[62; 66] This is as true for plant cell wall polysaccharides as it is for animal glycoproteins, such that all “xylan” and all “mucin” is not created equal (to name just two examples).[62; 73] This heterogeneity poses a significant challenge to the fractionation of pure compounds for structural determination, with the result that monosaccharide and linkage compositions most often represent average values. As such, particular substructures may escape detection, either due to technical limitations or due to source-sampling bias. Fortunately for microbiota research, insight into complex carbohydrate structure continues to increase, buoyed by a rising global biotechnological interest in functional foods/prebiotics and biomass saccharification for fuel and chemical production.

Work on the xyloglucan family provides one example in which both strictly analytical and combined genetic/analytical approaches continue to uncover novel polysaccharide motifs. Indeed, the structural variation in this family across plant species has been probably the most widely and precisely explored of all the matrix polysaccharides.[64; 65; 74] Nonetheless, recent detailed analysis of xyloglucans from the Ericaceae, notably including the edible bilberry fruit (*Vaccinium myrtillus* L), revealed an unusual di-xylosylated motif [β-d-Xyl*p*-(1-2)-α-d-Xyl*p*-(1-6)-β-d-glucan] as a dominant structural component.[75; 76] Likewise surprising, previously unknown galacturonic acid (Gal*p*A)-containing branches [β-d-Gal*p*A-(1-2)-α-d-Xyl *p*-(1-6)-β-d-glucan and α-l-Fuc*p*-(1-2)-β-d-Gal*p*A-(1-2)-α-d-Xyl*p*-(1-6)-β-d-glucan] have been revealed recently in root tissues of the model dicot *Arabidopsis thaliana*. Moreover, the glycosyltransferase family 47 (GT47) enzyme responsible for adding the Gal*p*A residue has been identified, [77] which may in turn facilitate the possible discovery of this type of xyloglucan in other plant species, including food crops. Although such structural differences may seem subtle, on one hand they may impart significant differences in solubility and accessibility by the microbiota to a given polysaccharide, as outlined above. On the other hand, each novel glycosidic linkage in a polysaccharide or family of polysaccharides will also further complicate attack by a given microbiota species or consortium, due to the requirement for an additional glycosidase (and possibly substitution-tolerant sensor/regulators, transporters, and substrate-binding proteins) in the microbiome.

The concept that enzyme cohorts must increase in complexity in step with polysaccharide fine-structure is exemplified by a number of recent biochemical studies of specific PULs. As a point of reference, the archetypal PUL, the Starch Utilization System of *B. thetaiotaomicron*, encodes three glycoside hydrolases sufficient to cleave all the linkages in starch: a GH13 *endo*-amylase [*endo*-α(1-4)-glucanase], a GH13 α(1-6)-glucosidase, and a GH97 *exo*-α(1-4)-glucosidase (Fig. 1A). This PUL also encodes one transcriptional regulator, one TonB-dependent receptor/transporter, and three outer-membrane starch-binding proteins. In addition to the genetics and biochemistry of this system, the structural biology of all these starch-binding proteins and enzymes, except for the GH13 α(1-6)-glucosidase, has been elucidated through a detailed series of studies.[48; 78; 79; 80; 81; 82] The selected examples shown in Fig. 1 further highlight the correlation between polysaccharide and PUL complexity for a number of systems in which functional predictions have been supported by rigorous reverse genetic and biochemical analyses. [30; 31; 40; 83; 84]

An overarching problem in generating robust functional predictions for novel PULs is our general inability to precisely map putative specificities of enzyme cohorts (e.g., deduced from CAZy family membership) one-to-one with known polysaccharide sub-structural motifs. The *B. plebeius* Porphyran Utilization Locus[40] and the *B. intestinalis* Xylan Utilization Locus[83] are both complex loci, which despite partial characterization of their enzyme complements, encode gene products whose correlation to polysaccharide structure is not immediately obvious. Observations of this type highlight gaps in our current knowledge of both enzyme function and polysaccharide structural diversity. These systems provide opportunities to leverage information from bacterial genomic sequences (i.e., groups of co-regulated enzymes with partially unknown targets) to inform detailed studies of new polysaccharide sub-structures. In contrast to these complex loci, other PULs are less extensive and clearly lack the complete

enzyme cohort necessary to full degrade their cognate substrates (Fig. 1G,H),[84; 85] suggesting that they may work together with other systems during catabolism.

Recent studies have demonstrated how a "change in scale" of PUL characterization, to encompass functional characterization of most, if not all, of the gene products can bring a more holistic understanding of PUL function in the context of organism and ecosystem.[86] One of the first examples of this approach elegantly revealed the molecular basis of levan [β(2-6)-fructan] specificity of *B. thetaiotaomicron*, which otherwise grows poorly on the isomeric inulin [β(2-1)-fructan] polysaccharide. Here, functional characterization of the GH32 *endo*-levanase and the SusD-like carbohydrate-binding protein encoded by a Fructan Utilization Locus (Fig. 1B), both of which are located on the cell surface, revealed a strict specificity for levan polysaccharide. In striking contrast, neither the hybrid two-component system sensor regulator nor the three GH32 *exo*-fructosidases (including one encoded outside of this PUL) discriminated against either linkage.[31]

A similarly extensive study from our own collaborative work recently revealed the functions of all eight glycoside hydrolases and two carbohydrate-binding proteins encoded by a unique Xyloglucan Utilization Locus (XyGUL) in *Bacteroides ovatus* (Fig. 1C).[30] The ensemble of enzymes encoded by this locus is consistent with a particular specificity for solanaceous (arabinogalacto)xyloglucan (as found in tomatoes, peppers, eggplants, olives, etc.),[64; 87; 88; 89] due to the presence of two α-L-arabinofuranosidases from GH43. Notably, the *B. ovatus* XyGUL lacks a fucosidase, as would be required to fully degrade the (fucogalacto)xyloglucan widespread among other dicot plants (e.g., leafy vegetables). In contrast, partially homologous XyGULs in other prevalent human- and termite-gut Bacteroidetes encode *both* a GH43 member, as well as predicted fucosidase from GH95 or GH29, which indicates the evolution of these XyGULs to address both types of xyloglucans from dietary plants (Fig. 1D). Strikingly, analysis of metagenomic data revealed that at least 92% of the humans sampled (n = 250) across N. America, Europe, and Japan carry at least one or more homologous XyGULs, underscoring the particular importance of xyloglucan metabolism in our diet.[30]

Together, the levan and xyloglucan PUL studies highlight the comprehensive insight that a combination of biochemical, enzymological, genetic, and structural analyses can bring. We anticipate that similar multidisciplinary approaches will bring significant new insight into PUL functional diversity in future studies. Further, as the polysaccharides studied become more complex or variable (arabinoxylans, pectins, mucin glycoproteins, etc.), it is likely that similar or greater levels of fine-structural adaptations by the cognate bacterial enzyme systems will be revealed.

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How are gut bacterial genomes upgraded to target new polysaccharides?

Moving beyond the effective deployment of existing enzyme and protein complements, the evolution or acquisition of genes enabling the degradation of alternate polysaccharides can allow access to new or unused nutrient niches. In some cases, the mechanism of gene acquisition is apparent, while in others this evolution is less clear. For example, delineation of the genes required for porphyran degradation by *B. plebeius* and analysis of the surrounding genomic region revealed that a mobile element belonging to the conjugative transposon (cTn) family, a family of mobile elements long associated with transfer of antibiotic resistance among gut bacteria[90], likely mediated transfer of this ability into *B. plebeius* (Fig. 1E).[40] Moreover, similar genes involved in porphyran degradation are present in the fecal metagenomes of Japanese subjects, but are less abundant or absent in the microbiota of westerners.[39] Additional studies

that have initially focused on the enzymatic abilities of marine Bacteroidetes to degrade the red and brown algal polysaccharides agarose and alginate have revealed, using comparative genomics, that enzymes for degrading these polysaccharides are present in the genomes of some gut *Bacteroides*. While putative mechanisms for these transfer events remain unclear, these observations lend further support for the idea that genetic material can be transferred, perhaps along with foods containing the target polysaccharides, into gut bacteria to enable catabolism of novel nutrients. Interestingly, a cTn that is related to the one implicated in transfer of porphyran utilization into the *B. plebeius* genome is also present in the sequenced type strain of *B. thetaiotaomicron*, but carries genes that are expressed during degradation of *Saccharomyces cerevisiae* cell wall α-mannan instead of porphyran.[40; 46]

Taken together, the observations of porphyran and α-mannan degrading abilities associated with mobile elements suggest that dietary polysaccharides that are novel to regional human diets or increase historically as a result of new dietary customs can elicit adaptive genetic changes in members of the gut microbiota. To explore this point further in the context of this review, we searched fully sequenced or draft genomes for conjugative elements related to the two that are present in the genomes of *B. plebeius* and *B. thetaiotaomicron*, which revealed that numerous additional site-specific cTns exist and that some of these newly identified elements harbor genes likely to be involved in bacterial polysaccharide degradation (Figure 3). Inspection of a subset of cTns related to that conferring porphyran degradation further revealed that all of the related elements are found in gut isolates and are separate from sequences found in environmental isolates (compare blue branch highlighted in light green with dark green branches in Figure 3A). This latter observation suggests that transfer of these genes, which are ordinarily found in phylogenetically more distant marine bacteria[39], may have been more complex than simple transfer of a single cTn from a marine organism to one residing in the gut. Interestingly, another common "genetic cargo" that we found located in cTns are gene clusters predicted to be involved in capsular polysaccharide synthesis, suggesting that both catabolic and anabolic functions related to polysaccharides are being mobilized by this mechanism.

Figure 3

Sampling of Bacteroidetes conjugative transposon (cTn) diversity in sequenced genomes

Notably, our recent investigation of xyloglucan utilization in gut Bacteroidetes[30] revealed marked heterogeneity in carriage of this PUL among closely related strains of *B. ovatus* and *B. xylanisolvens*. In this case, appearance of the gene cluster required for xyloglucan utilization was not associated with any discernable mobile element that might have been responsible for its transfer. Rather, it appeared to be precisely inserted, with only a few hundred basepairs or less of flanking sequence, into a single homologous region of the *B. ovatus* genome that also contained additional PULs in some isolates. The order and specificity of enzymes contained in other *B. ovatus* xyloglucan utilization PULs were different in several cases, revealing that enzyme encoding genes are likely being duplicated, re-arranged and probably diversified via mutations within closely related gene clusters (Fig. 1C,D). Such phenomena remain to be studied in detail. However, the presence of new and re-arranged genes within closely related PULs suggests that, once a gene cluster is acquired, it can subsequently be subjected to additional, fine-level tailoring to modify or refine its function.

In the future, it will be interesting to determine if the polysaccharide utilization abilities associated with other Bacteroidetes cTns and other divergent PULs enable metabolism of polysaccharides that have been introduced into some human diets on relatively recent timescales or those that are fine-level variants of more commonly consumed plant and animal polysaccharides. Some of these items exist uniquely in other regional foods, such as seeds from *Salvia hispanica* (chia), which have been cultivated and consumed in Mexico and Guatemala since pre-Columbian times and contain a unique polymer composed of xylose, glucose and 4-*O*-methyl glucuronic acid.[91] Other novel polysaccharides such as xanthan gum (approved for human consumption in 1968) are much more recent introductions to the human food supply and are often used as food additives or thickeners.[92] In the context of dietary red seaweed galactan utilization, it is interesting to note that a screen of nearly 300 common gut *Bacteroides* strains revealed only two, *B. uniformis* NP1 and *B. thetaiotaomicron* VPI-3731, that were able to grow on agarose and kappa-carrageenan, respectively.[40] These capacities stand in contrast to the strict porphyran specificity of *B. plebeius*,[40] which implies that each strain harbors a unique ensemble of sensory proteins and enzymes to address these distinct, complex polysaccharides (see ref. [57] for a structural summary). Indeed, several families of enzymes specific for agars (comprising agarose and porphyrans) and the diverse types of carrageenans have already been characterized from marine bacteria[93] and it will be interesting to ascertain to what extent these may have been acquired and further evolved by the human gut microbiota in parallel with dietary habits.

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Prospectus: How fine is the level of polysaccharide niche adaptation by gut microbes?

The interactions between our symbiotic gut microorganisms and dietary polysaccharides play important roles in several aspects of health.[52; 53; 94] Polysaccharides represent the major class of nutrients that escape digestion in the proximal intestine, illuminating dietary fiber or purified polysaccharide prebiotics as promising non-invasive avenues to intentionally manipulate the gut community. In order to achieve a highly sophisticated ability to employ such manipulations, we must first understand the precise connections between the many different dietary and endogenous mucosal carbohydrates and the microorganisms that directly degrade them.

Beginning in the 1950's Freter and colleagues began formulating the nutrient-niche theory of gastrointestinal colonization by microorganisms[95; 96; 97], which postulates that a diverse, but finite, number of niches are present in the gut, each defined by availability of a subset of particular nutrients. When these niches are filled by endogenous symbionts, invasion of the intestine by outside species, including

pathogens, is more difficult and leads to the phenomenon of colonization resistance. When nutrient niches are opened, due either to large-scale loss of microbial colonization during antibiotic use, or perhaps due to smaller perturbations such as diet shift, the gut environment may become transiently susceptible to invasion by pathogens, such as *Clostridium difficile*, *E. coli*, *Salmonella enterica* or *Vibrio cholera*. While most of these pathogens may not be metabolically equipped to directly attack polysaccharides (a notable exception is *Yersinia enterocolitica*, which has been shown to target plant cell wall pectin[98; 99]), it is likely that they compete for other limiting nutrients that are used or released by polysaccharide-degrading organisms. Thus, the presence of endogenous bacteria filling most available niches reinforces the ability of the community to exclude pathogens. Indeed, this is a likely mechanism behind the remarkable success of fecal transplant therapy and "repopulation" to combat chronic *C. difficile* infection.[94; 100; 101] In light of the points discussed above, connecting specific gut symbionts with certain fiber polysaccharides, understanding the specific details of nutrient-microbe relationships in the gut, and determining downstream interactions between microbes, may represent a pinnacle step in comprehending Freter's theory. Such knowledge may reveal how many niches actually exist to be both filled by endogenous microbes and subsequently competed for when the gut microbial ecosystem is perturbed and potential pathogens have an opportunity to gain foothold.

Our central hypothesis is that many polysaccharide-gut microbe relationships have evolved to be highly specific, involving sensory and enzymatic adaptations by particular microbes that best equip certain species or strains to utilize fine-level variations that exist in polysaccharides. Support for this idea has been born out in the literature with a few rigorously investigated examples[30; 31] but more are needed. As the catalog of common gut microorganisms expands along with knowledge of these species' genetic potential, defining precise relationships between individual organisms and exogenous forces such as dietary polysaccharides will be an essential part of understanding the behavior of this complex ecosystem. Such knowledge will be required to design strategies to manipulate the impact of gut microbes on aspects of our health ranging from nutrition to mitigation of diseases like inflammatory bowel disease, colon cancer, obesity, and diabetes.[102]

Research highlights

1. Bacteria expand animals' digestive ability by providing polysaccharide-degrading enzymes
2. Dietary and mucosal polysaccharides vary greatly in their fine-level structures
3. Fine-level structural variations diversify available "nutrient niches" for bacteria
4. Expression of microbiota enzymes is dependent on cues contained in polysaccharides
5. Nutrient-microbiota relationships provide concepts to manipulate health.

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Footnotes

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