Bacteria of the human gut microbiome catalyze red seaweed glycans with carbohydrate-active enzyme updates from extrinsic microbes

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Humans host an intestinal population of microbes—collectively referred to as the gut microbiome—which encode the carbohydrate active enzymes, or CAZymes, that are absent from the human genome. These CAZymes help to extract energy from recalcitrant polysaccharides. The question then arises as to if and how the microbiome adapts to new carbohydrate sources when modern humans change eating habits. Recent metagenome analysis of microbiomes from healthy American, Japanese, and Spanish populations identified putative CAZymes obtained by horizontal gene transfer from marine bacteria, which suggested that human gut bacteria evolved to degrade algal carbohydrates—for example, consumed in form of sushi. We approached this hypothesis by studying such a polysaccharide utilization locus (PUL) obtained by horizontal gene transfer by the gut bacterium Bacteroides plebeius. Transcriptomic and growth experiments revealed that the PUL responds to the polysaccharide porphyran from red algae, enabling growth on this carbohydrate but not related substrates like agarose and carrageenan. The X-ray crystallographic and biochemical analysis of two proteins encoded by this PUL, BACPLE_01693 and BACPLE_01693_3 showed that they are β-porphyranases belonging to glycoside hydrolase families 16 and 86, respectively. The product complex of the GH86 at 1.3 Å resolution highlights the molecular details of porphyran hydrolysis by this new porphyranase. Combined, these data establish experimental support for the argument that CAZymes and associated genes obtained from extrinsic microbes add new catabolic functions to the human gut microbiome.

Two phyla dominate the bacterial community of the adult distal gut in humans and in other mammals: the Firmicutes and the Bacteroidetes (1). Metagenomic studies and experiments with isolated strains revealed that members of the genus Bacteroides are broadly adapted to the degradation of host glycans and terrestrial plant material (2). This adaptation is exemplified by the enzyme systems devoted to starch, pectin, hemicelluloses, and other plant carbohydrates as well as host glycans (2, 3); the wide variety of genes encoding enzymes for the degradation of these carbohydrates dominate carbohydrate active enzyme, or CAZyme, arsenals in human gut metagenome datasets. Hence, the evolution of this carbohydrate catabolizing machinery is key for host and microbiome mutualism. An important question, which has thus far only been addressed by bioinformatic approaches, regards how members of this community evolve—for instance, by horizontal gene transfer (HGT) (4, 5)—to process new carbohydrate resources (6).

In Bacteroides, the genes encoding CAZymes that target a specific carbohydrate or related groups of carbohydrates are often found in gene clusters termed polysaccharide utilization loci (PUL) (7). These systems use a generally conserved strategy to sense, degrade, bind, and import carbohydrates encountered in diverse environments like the gut, ocean, and soil (7, 8). The CAZymes (www.cazy.org) (9) cleave high-molecular-weight glycans into oligosaccharides that can be bound by the Sus-like proteins, imported into the bacteria, and ultimately catabolized (10). Using a bioinformatic approach, we recently identified a putative PUL in the gut bacterium Bacteroides plebeius; the PUL contains possible CAZymes encoding genes that appear to have been acquired by HGT from marine microbes and which may target carbohydrates from red seaweeds (4).

The major matrix polysaccharides in the cell walls of red algae—which are the most common dietary red seaweed polysaccharides consumed by humans and present in many processed foods (11, 12)—are carrageenans (13), agars, and porphyran (14), and all contain sulfate esters that are absent in terrestrial plants. Furthermore, the sugar backbones can contain unique mono- and disaccharides, such as the 3,6-anhydro-β-galactose present in the carrageenan of Chondrus crispus (Irish moss) and the 3,6-anhydro-D-galactose (LA) found in agars of Gelidium and Gracilaria spp. In both cases, the 3,6-anhydro-galactose is α-1,3 linked to D-galactose (G) and the resulting disaccharide is connected with β-1,4 linkages (SI Appendix, Fig. S1). Porphyran from Porphyra spp. belongs to the agar-family of polysaccharides, although the majority of the LA is replaced by D-galactose-6-sulfate (LOS), and C-6 methylations of G are frequent (14). The unique structural properties of these red algal galactans requires a distinct set of enzymes that are predominantly encoded in genomes of marine microbes and are far less frequent or absent in bacteria that break down terrestrial polysaccharides (4, 8). Thus, genetic and functional tracking of CAZymes with these rare specificities holds the potential to dissect carbohydrate resource partitioning by human gut microbes. For example, putative agars, porphyranases, and alginate lyases have recently been identified in the intestinal microbiomes of Japanese (4), Spanish (15), and American (16) people, suggesting that their gut microbes may have adapted to novel algal carbohydrates in the modern diet. However, all of these previous studies focused on bioinformatic analysis and lacked functional characterizations of gut microbes and their enzymes, raising the question of whether these HGT events conferred active pathways to the human microbiome or simply introduced cryptic genes.

In this study, we used seaweed polysaccharides as substrates and established that a horizontally acquired integrative and conjugative element (ICE) expanded the catabolic repertoire of B. plebeius allowing it to use porphyran. Furthermore, through functional screening with additional algal substrates, two other gut Bacteroides, B. uniformis NPI and B. thetaotaomicron VP1-3731, were identified, which grew on agar and on carrageenan, contrasting previous reports that suggested such activities are lacking in


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Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.pdb.org (PDB ID codes 4W97 and 4W90).

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intestinal microbes (17). Using *B. plebeius* as a model organism, we show that when the bacterium was grown using porphyran as a sole carbon source, the genes of the PUL were up-regulated. Consistent with this result is the presence of active β-porphyranases, the crystal structures of which are presented, including a structural analysis of a family 86 glycoside hydrolase. Our results show that gut bacteria catabolize red seaweed galactans, a feature that may influence health (18) and refines our understanding of the evolutionary plasticity of the human gut microbiome.

**Results**

**Screening of Gut Bacteria for Growth on Red Algal Galactans.** *B. plebeius* contains a putative porphyran degrading PUL covering up to 40 ORFs from BACPLE_01667 to BACPLE_01706. Within this set, the products of two genes (BACPLE_01670 and BACPLE_01689) share high amino acid sequence identity with GH16 homologs from marine seaweed-degrading microbes; these homologs have previously demonstrated activity on porphyran. This finding suggested that agarose, porphyran, or even carrageenan may be nutrients for this gut microbe. Thus, we used these three galactans as substrates and observed that *B. plebeius* grew specifically on porphyran (SI Appendix, Fig. S2A). To test whether this result was an isolated case or if these common algal food galactans are catabolized by other human gut microbes, we screened an additional collection of 291 human gut Bacteroidetes (see SI Appendix for details) and identified several carrageenolytic and agarolytic bacteria. One isolate, *B. uniformis* NP1, grew on agarose (SI Appendix, Fig. S2B) and showed reduced growth on porphyran, whereas another, *B. thetaiotaomicron* VPI-3731, exhibited growth specificity for carrageenan (SI Appendix, Fig. S2C). Together, these growth experiments showed that human gut bacteria catabolize seaweed carbohydrates and that each of these strains is specific for a certain type of red algal galactan. To further understand the molecular mechanism of the degradation of such galactans, we focused on the PUL of *B. plebeius* for which the whole genome sequence is available.

**B. plebeius PUL Is Activated by Porphyran.** The 40 genes that comprise the putative *B. plebeius* PUL include 12 putative glycoside hydrolases, 2 SusC/SusD-like protein pairs (encoded by BACPLE_01697–01698 and BACPLE_01704–01705), and a hybrid two-component system (HTCS) sensor/regulator (ref. 19; BACPLE_01699) (Fig. 1A and SI Appendix, Table S2). Using quantitative PCR (qPCR), the expression of 35 genes in the PUL was examined in response to porphyran as the only carbohydrate source in the growth medium (growth in galactose served as a reference). Compared with galactose, porphyran promoted expression of all 35 examined genes (Fig. 1B). A notable feature is the mosaic profile in which the expression of genes BACPLE_01692–01699, measured by fold-change analysis, appears lower. We quantified transcript abundance of the low-fold-change susC/susD genes (BACPLE_01697–10698) and compared them to the high-fold-change susC/susD genes (BACPLE_01704–01705), using genomic DNA that contained equal copies of each gene as a standard. Our results indicated that the susC/susD genes (BACPLE_01697–10698) that exhibited low fold change had 16- and 18-fold higher

![Fig. 1. Expression of genes when *B. plebeius* is grown in porphyran. (A) Synteny between the adjacent genomic region surrounding the porphyran PUL to a fungal α-mannan–specific PUL in *B. thetaiotaomicron*. (B) The expression of 35 genes within the PUL was monitored by RT-PCR upon incubation with porphyran compared with the control incubated with galactose. Five additional genes ranging in size from 126 to 489 bp (BACPLE_01681, 01687, 01688, 01690, and 01691) are present within this locus but were not probed by qPCR. Data in B are mean and SD of independent biological replicates.](http://www.pnas.org/content/109/48/19787.full)
Fig. 2. B. plebeius contains two endo-acting porphyranases, BpGH16B and BpGH86A, and an agarase, BpGH16A. (A) Activity test on agar plate revealed similar agarase activity of BpGH16A to the previously characterized ZgAgaA from Z. galactanivorans (positive control). BpGH16B and GH86A were inactive on this substrate. Heat-inactivated enzymes served as control. (Scale bar: 1 cm.) (B) Relative activity of BpGH16B and BpGH86A with porphyran as substrate measured by reducing sugar assays showed porphyranolytic activity. (C) The reaction products of BpGH16B and BpGH86A, incubated with native porphyran, were analyzed by fluorophore-assisted PAGE showing a ladder-like pattern typical for endo-acting glycoside hydrolases. (D) Comparative hydrolysis of native and pure porphyran by B. plebeius enzymes and ZgAgaA showing that BpGH16B and BpGH86A have higher activity than the agarases. (E) TLC analysis of degradation products reveals that BpGH16B releases similar reaction products to the previously characterized ZgPorA (positive control), which releases L6S-G– and L5S-G–LS6-G– as major products (14), whereas BpGH86A releases mainly the larger oligosaccharide L6S-G–L6S–G–. Notably, these products are not released by the previously characterized agarase ZgAgaA (negative control). BpGH16A has an agarase-like reaction pattern similar to ZgAgaA. Data in D are mean and SD of independent enzymatic replicates.
For product profiling, porphyrin was treated with BpGH16B or BpGH86A, and the reaction products were labeled with the fluorophore 2-aminoacridone (AMAC) and analyzed by carbohydrate polyacrylamide gel electrophoresis (PAGE). Both BpGH86A and BpGH16A successively degraded the high-molecular-weight polysaccharide into smaller oligosaccharides of random size, apparent in the ladder-type pattern that is typical for endo-acting glycoside hydrolyses (Fig. 2C). One well-resolved degradation product from both enzymes corresponded to a band that had the same mobility as an AMAC-labeled tetra-oligosaccharide standard derived from porphyran (L6S-G-L6S-G-AMAC), which is consistent with cleavage of the β-glycosidic linkage (for sugar residue nomenclature, see SI Appendix, Fig. S1). We further used TLC to analyze the reaction products of the different enzymes (Fig. 2E). BpGH16B showed a reaction profile similar to the β-porphyranase ZgPorA from Z. galactanivorans, with L6S-G~ as a major end product (14). BpGH86A produced predominantly larger oligosaccharides, the smallest of which was the tetra-oligosaccharide L6S-G-L6S-G~. Consistent with its agarolytic activity, BpGH16A had a different reaction profile than the porphyranases, yet a similar reaction profile to the β-agarase ZgAgaA; the major reaction products showed the typical blue color of neoagarooligosaccharides as observed with the resorcinol staining procedure (23). Combined, these results support the assignment of BpGH16A as a β-agarase, whereas BpGH16B and BpGH86A are β-porphyranases.

Crystal Structure of the Porphyrainase BpGH86A. After establishing that BpGH86A is a porphyrainase, we determined its structure to 1.3-Å resolution. The structure of BpGH86A, comprising the catalytic domain (residues 25-599) (Fig. 3A), revealed a multidomain architecture consisting of an N-terminal (β/α)7 barrel and two C-terminal β-sandwich domains, which align via their convex faces to the exterior of the (β/α)8 barrel and connect with two N-terminal β-strands that become part of these C-terminal β-sandwich domains (Fig. 3B; data collection and refinement statistics are summarized in SI Appendix, Table S1). The (β/α)8 barrel fold, or TIM-barrel, is found in glycoside hydrolyses belonging to clans GH-A,D,H,K, and it generally forms a closed ring structure (24), the center of which harbors the catalytic residues. In BpGH86A, the distal part of the barrel lacks the helix α2 in the outer ring and parts of the surface loops, which form the rim in the canonical (β/α)8 barrel fold. The absence of this distal α-helix and associated loops shapes the structure of the enzyme into an open toroid, further accentuating an already pronounced cleft of ~40-Å length (SI Appendix, Fig. S4A), which is longer than in BpGH16B (~32 Å; SI Appendix, Fig. S5A) and consistent with the longer oligosaccharides produced by BpGH86A.

Product Complex of BpGH86A with a Hybrid Porphyrain-Agarose-Oligosaccharide. The crystal structure revealed unambiguous electron density for the six sugar rings of an oligosaccharide bound in the pronounced cleft of the enzyme. The electron density showed that this bound oligosaccharide, likely a product from the hydrolysis of the porphyrin added during crystallization, was a hybrid-hexamer of structure L6S-G-LA-G-L6S-G~ occupying six minus subsites (25) (Fig. 3C and SI Appendix, Fig. S3B). A series of hydrogen bonds and hydrophobic interactions with the enzyme stabilized the carbohydrate.

The reducing end of the oligosaccharide is a G residue that fits into a subsite whose architecture, namely, the presence of the...
putative catalytic residues Glu-152 and -279 (see below), suggest it is the -1 subsite. Cleavage at this site of the polysaccharide is consistent with the classification of BpGH86A as a β-galactanase that cleaves the β-1,4 glycosidic bond in porphyran (Fig. 3C). In this -1 subsite, the sugar ring is situated above and stabilized by the hydrophobic platform formed by the residues Tyr-237 and Phe-324, and its C6-OH projects toward a small pocket in the active site (Fig. 3C and D). This pocket may accommodate the methyl substitutions that are frequently found on the C6 of G residues in porphyran; such substitutions inhibit productive binding in the GH16 porphyranases because they lack such a pocket (SI Appendix, Fig. S5 E and F) (14).

The L6S bound in subsite -2 is surrounded by His-53, Phe-77, Phe-324, and Tyr-324, and these side chains form the cavity that harbors the sugar ring and the sulfate substitution on C6 (Fig. 3C). This sulfate group points toward the interior of the substrate binding cleft and is accommodated in a pocket similar to BpGH16B (SI Appendix, Fig. S5F) and previously described GH16 β-porphyranases (4). Furthermore, His-53, located below the sugar ring, stabilizes the sulfate group by an ionic interaction.

The G unit bound in subsite -3 is clamped between Tyr-331 and Phe-77, which align with their β- and α-faces, respectively, with the sugar ring. The axial hydroxyl group on C4 of the sugar makes a hydrogen bond to His-114. Compared with the three sugar rings that are bound in subsites -1, -2, and -3, the LA in subsite -4 is far less engaged by the enzyme and makes a single hydrogen bond with Lys-76. This bicyclic sugar is in the C4 conformer compared with the C4 conformation of the L6S. This arrangement leads to all equatorial glycosidic bonds between the LA and adjacent G sugars and an almost coplanar arrangement of these three sugar rings, highlighting the conformational and chemical heterogeneity introduced by varying anhydro- or C6 sulfate modifications in natural agars and carrageenans (SI Appendix, Fig. S1). The paucity of interactions in the -4 subsite allows binding of either L6S or LA, and therefore hydrolysis close to interspersed LA-G motifs in porphyran. However, because the bound hexasaccharide was not further degraded to L6S-G and LA-G-L6S-G or L6S-G-LA-G~, we assume that BpGH86A does not accept LA in subsites -2 or +1. The G residue bound in subsite -5 stacks with its β-face to Trp-78, and the L6S bound in subsite -6 is stabilized by the side chains of Trp-83 and the Lys-87, which form an ion bridge to the sulfate group on C6 (Fig. 3C).

Active-Site Architecture of BpGH86A Supports a Retaining Catalytic Mechanism. The G in subsite -1 is present in an undistorted C4 conformation, and both the β- and α-anomer have been modeled at the C1 atom (Fig. 3E). The G interacts with the putative acid/base residue Glu-152, which was modeled in a double conformation. One conformation makes a hydrogen bond to the equatorial hydroxyl group (Fig. 3F), and the other conformation coincides with the axial hydroxyl group at the C1 of the sugar ring (Fig. 3G).

Hydrogen bonds with two histidine residues stabilize each of the two conformations of Glu-152. His-235 forms a hydrogen bond (2.8 Å) to the conformer of Glu-152 that approaches the equatorial hydroxyl, in this position, Glu-152 is appropriately positioned to protonate the β-1,4 glycosidic bond (Fig. 3F), whereas Glu-279 attacks the anomic carbon from below at a distance of 3.2 Å, supporting its role as the catalytic nucleophile. Thus, His-235 may reduce the pKₐ of Glu-152, which becomes poised to act as an acid for protonation of the glycosidic bond. The second conformer of Glu-152 further approaches the C1 with its new axial C1–OH and comes to rest almost at plane of the equatorial hydroxyl group of the β-anomer (Fig. 3G). This conformer is stabilized by a hydrogen bond to His-235 (2.5 Å), and we suggest that this second conformation may protect the C1 of the bound intermediate from the back reaction with the aglycone in a transglycosylation reaction. Thus, the deglycosylation step may be initiated when Glu-152 turns back to its interaction with His-235 and now as a base activates the incoming water that attacks at C1 and releases the glycon. It is further possible that the dynamic range of interactions with both histidines modulates pKₐ cycling of the Glu-152 during the retaining mechanism, according to its dual roles as an acid and a base (26). However, as an alternative or complementing scenario, we propose that the role of this interaction, between Glu-152 and His-235, may be a molecular ratchet favoring hydrolysis over transglycosylation (27), which may be beneficial in cases where strong binding between products and enzyme are encountered.

Discussion

The collective group of CAZymes in the human gut microbiome is large and diverse, allowing it to extract energy and carbon from a wide variety of carbohydrates. Using B. plebeius as a model microbiome species that possesses a rare catalytic ability, we were able to associate a 40-gene PUL with the catabolism of porphyran, a structurally unique algal polysaccharide. The biochemical analysis revealed that the encoded agarase (BpGH16A) and porphyranases (BpGH86A, BpGH16B) concertedly degrade porphyran, consistent with the heterogeneous nature of this galactan.

The porphyran PUL displayed a mosaic expression profile in which its central part, including genes BACPLE_01692–01699, showed lower relative induction by porphyran because of their higher basal expression in galactose. The high basal expression of these genes, even in the absence of their polysaccharide substrate, suggested the production of “surveillance” levels of their respective enzymes. This common theme in gut Bacteroides delivers small amounts of each PUL-encoded system to initially sense and begin the degradation of a specific polysaccharide (21). In particular, we postulate that BpGH86A, located within the surveillance set of genes, with its longer substrate binding cleft compared with GH16 porphyranases, may play a key role by performing the initial production of larger oligosaccharides, allowing the HTCS protein and Sus-like system to sense and respond to their presence (2). The release of oligosaccharides from porphyran, and not agarose, which failed to induce growth of the bacterium, subsequently triggers the production of the porphyranases (BpGH16B), agarases [BpGH16A, BpGH117 (28)], and additional CAZymes and accessory proteins [sulfatase (29)] that can further deconstruct these larger oligosaccharides (21).

The acquisition of the B. plebeius porphyran PUL is likely to have originated from a marine Bacteroidete because similar gene clusters are present in oceanic species (8, 30–32). Because the B. plebeius PUL is harbored at a similar location on an otherwise very similar ICE that is present in B. thetaiotaomicron, this finding suggests that HGT via conjugative elements is the mechanism of transfer and that similar ICE vehicles are capable of acquiring new cargo (20). The observation that both the B. plebeius and B. thetaiotaomicron elements were observed to excise and circularize from the genome via site-specific recombination events supports our conclusion that we have identified the extent of these ICEs involved in PUL transfer. The apparently low excision rate, which was only detected with 36–70 additional cycles of PCR, suggests that these two example ICEs do not undergo efficient excision in the growth conditions tested, which included the cognate substrates that trigger expression of the RteA/B homologs. The frameshift mutation in the B. plebeius RteB homolog indicates that this ICE has lost the ability to excise and mobilize by the same mechanism as that of Bacteroides conjugative transposons with similar structure (20).

The degradation of seaweeds by intestinal microbes may have health implications because red algal galactans, including porphyran, have been associated with a wide range of pharmacological activities, such as antiviral, anticancer, anti-inflammatory, anti-oxidative, and anticoagulative effects (see ref. 33 for review). These activities largely depend on the molecular weight of the polymer (34) and the amount of sulfations along the polysaccharide chain,
both factors which are modulated with specific microbial CAZymes and sulfatases (29). Indeed, health implications and concerns have been associated with the low-molecular-weight degradation products of carrageenan, which elicit ulcerative colitis in animal models (11, 35–37). Our screening for red algal galactan degrading gut microbes revealed B. thetaiotaomicron VPI-3731, which presented strong growth on carrageenan and must therefore contain one or more carrageenases. Future studies are needed to characterize the enzymes involved in these red algae degrading pathways and to test whether they are distributed in human gut microbiomes and form harmful oligosaccharides in vivo.

Overall, this work demonstrates that B. pelleus, a member of the gut microbiome, has an active PUL that degrades the marine red algal polysaccharide porphyran. This finding supports the hypothesis that the gut microbiome coevolves with host diet through HGT from extrinsic microbes enabling the catalolism of new carbohydrates.

**Materials and Methods**

Gut isolates were grown as described (21), and for qPCR of B. pelleus PUL genes, B. pelleus DSM 17135 was grown in triplicate on either galactose or porphyrin as a sole carbon source, and cells were harvested during exponential growth (A600 values of 0.41, 0.42, 0.43 for galactose and 0.57, 0.56, 0.57 for porphyrin). The catalytic domains of the porphyranases and agarase were cloned from B. pelleus DSM 17135 genomic DNA (28). Crystallization screenings were carried out in sitting-drop experiments by using commercial screens and optimizations in hanging-drop setups by grid screen expansion around initially successful hits. Biochemical characterization of the enzymes was carried out as described (4, 14). The structure of BpGHI16B was solved by molecular replacement, and the structure of BpGH86A was solved by the single-wavelength anomalous dispersion method. The data collection and refinement statistics are listed in SI Appendix, Table S1. (For additional details, see SI Appendix, SI Materials and Methods.)

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