Unique Organization of Extracellular Amylases into Amylosomes in the Resistant Starch-Utilizing Human Colonic Firmicutes Bacterium Ruminococcus bromii

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ABSTRACT  Ruminococcus bromii is a dominant member of the human gut microbiota that plays a key role in releasing energy from dietary starches that escape digestion by host enzymes via its exceptional activity against particulate “resistant” starches. Genomic analysis of R. bromii shows that it is highly specialized, with 15 of its 21 glycoside hydrolases belonging to one family (GH13). We found that amylase activity in R. bromii is expressed constitutively, with the activity seen during growth with fructose as an energy source being similar to that seen with starch as an energy source. Six GH13 amylases that carry signal peptides were detected by proteomic analysis in R. bromii cultures. Four of these enzymes are among 26 R. bromii proteins predicted to carry dockerin modules, with one, Amy4, also carrying a cohesin module. Since cohesin-dockerin interactions are known to mediate the formation of protein complexes in cellulolytic ruminococci, the binding interactions of four cohesins and 11 dockerins from R. bromii were investigated after overexpressing them as recombinant fusion proteins. Dockerins possessed by the enzymes Amy4 and Amy9 are predicted to bind a cohesin present in protein scaffoldin 2 (Sca2), which resembles the ScaE cell wall-anchoring protein of a cellulolytic relative, R. flavefaciens. Further complexes are predicted between the dockerin-carrying amylases Amy4, Amy9, Amy10, and Amy12 and two other cohesin-carrying proteins, while Amy4 has the ability to autoaggregate, as its dockerin can recognize its own cohesin. This organization of starch-degrading enzymes is unprecedented and provides the first example of cohesin-dockerin interactions being involved in an amyloytic system, which we refer to as an “amylosome.”

IMPORTANCE  Fermentation of dietary nondigestible carbohydrates by the human colonic microbiota supplies much of the energy that supports microbial growth in the intestine. This activity has important consequences for health via modulation of microbiota composition and the physiological and nutritional effects of microbial metabolites, including the supply of energy to the host from short-chain fatty acids. Recent evidence indicates that certain human colonic bacteria play keystone roles in degrading nondigestible substrates, with the dominant but little-studied species Ruminococcus bromii displaying an exceptional ability to degrade dietary resistant starches (i.e., dietary starches that escape digestion by host enzymes in the upper gastrointestinal tract because of protection provided by other polymers, particle structure, retrogradation, or chemical cross-linking). In this report, we reveal the unique organization of the amyloytic enzyme system of R. bromii that involves cohesin-dockerin interactions between component proteins. While dockerins and cohesins are fundamental to the organization of cellulosomal enzyme systems of cellulolytic ruminococci, their contribution to organization of amylases has not previously been recognized and may help to explain the starch-degrading abilities of R. bromii.

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The impact of the human intestinal microbiota upon health is increasingly recognized (1, 2). The dense microbial community within the large intestine depends mainly on the fermentation of nondigestible carbohydrates as its source of energy. For many diets, the single largest source of fermentable carbohydrate entering the colon is estimated to be resistant starch (RS) (3), which is defined as dietary starch that escapes digestion by host enzymes in the upper gastrointestinal tract because of protection provided by other polymers (RS1), particle structure (RS2), retrogradation (RS3), or chemical cross-linking (RS4) (4). Supplementation of diets with resistant starch can confer health benefits, especially in reducing insulin resistance and in protection against colorectal
cancer, that are considered to be mediated mainly by microbial fermentation products (5, 6).

The only starch-degrading enzyme systems from human gut symbionts to have been studied in any detail are those of *Bacteroides thetaiotaomicron* and *Eubacterium rectale*. *B. thetaiotaomicron* relies on a “sequestration” system, encoded by the sus gene cluster, in which outer membrane Sus proteins mediate the binding and transport of partial hydrolysis products of starch into the periplasm, where they are processed further (7–10). The *Firmicutes* species *E. rectale* A1-86 and related *Roseburlia* spp. appear to rely on a large extracellular amylase that is anchored to the cell wall, together with membrane-associated binding proteins and hydrolases that are upregulated by growth on starch (11–13). Although they are able to degrade soluble starches, these species do not show significant ability to degrade and utilize raw particulate starches or even resistant starches that have been pretreated by boiling (14). In contrast, recent investigations have strongly implicated relatives of another *Firmicutes* species, *Ruminococcus bromii*, as an important keystone species in the breakdown of resistant starch in the human large intestine. The populations of this group of bacteria detected in fecal samples are stimulated in individuals given diets enriched in RS2 or RS3 (15–17), while individuals lacking *R. bromii* apparently ferment RS3 inefficiently (16). *R. bromii* is a specialized amylolytic bacterium belonging to the *Ruminococcaceae*, a family of *Firmicutes* that is better known for its role as the preferred rumen species to degrade cellulose (18). *R. bromii* shows high degradative activity against raw or boiled RS2 and RS3 resistant starches in comparison with other amylolytic human intestinal bacteria such as *B. thetaiotaomicron*, *E. rectale*, and *Bifidobacterium adolescentis* (14, 19). Indeed, even non-growing *R. bromii* cells were found to stimulate the growth of those other amylolytic human gut bacteria by releasing soluble sugar from resistant starches (14).

In this investigation, we use genomics, proteomics, and protein-protein interaction studies to reveal the presence of unique enzyme systems in *R. bromii* that are likely to explain its exceptional ability to degrade starches and starch particles. In particular, we demonstrate for the first time the involvement of cohesin (Coh)-dockerin interactions, previously shown to be of importance mainly in lignocellulose-degrading enzyme systems, in the organization of microbial starch-degrading enzyme systems.

**RESULTS**

**GHs of *Ruminococcus bromii* L2-63.** The genome of *R. bromii* L2-63 encodes only 21 glycoside hydrolases (GHs), which compares with much larger numbers of GH enzymes (50 to 150) in other glycan-utilizing human colonic *Firmicutes* and up to 350 in *Bacteroides* spp. (20, 21). Of the 21 *R. bromii* GH enzymes, 15 belong to GH13, a hydrolase family dedicated largely to the degradation of starch, while the single GH31 and GH77 enzymes may also play significant roles in starch degradation (see Table S1 and Fig. S1 in the supplemental material), thus indicating a high degree of nutritional specialization. The remaining four GH enzymes comprise three lysozymes (two GH23 and one GH25) and one ß-glucosidase (GH3). N-terminal signal peptides (SPs) are evident in seven of the GH13s, which are therefore likely to be secreted (Fig. 1).

The relationships of *R. bromii* GH13 sequences were explored using BLASTP searches and phylogenetic comparisons that included three other prominent amylolytic species, *B. thetaiotaomicron*, *E. rectale*, and *B. adolescentis*, and two related nonamylolytic members of the *Ruminococcaceae*, *Ruminococcus champanellensis* and *Faecalibacterium prausnitzii*, from the human colon (Fig. 2). Among the *R. bromii* GH13 enzymes that carry SPs, two (Amy10 and Amy12) group with GH13 subfamily 14, which includes pullulanases (22), and one (Amy5) groups with *E. rectale* ß-amylase EUR_01860 (13), while Amy1, Amy2, Amy4, and Amy9 are not closely related to GH13s from the three other amylolytic human gut species. Amy4 is, however, distantly related to an enzyme from *Bacillus halodurans* that belongs to GH13 subfamily 19 and is known to release maltohexaose from amylose (22, 23) (Fig. 2). The closest relatives of Amy1 and Amy2 were found in the genome of the soil bacterium *Paenibacillus terrae*. The remaining enzymes that lack SPs grouped with a variety of other subfamilies (Fig. 2).
Amy11 and Amy12 are encoded by adjacent genes within the genome, as are Amy8 and Amy15, whereas the genes encoding the remaining 11 GH13 enzymes are unlinked.

**Dockering and cohesin modules and carbohydrate-binding modules (CBMs) in *Ruminococcus bromii* gene products.** Large numbers of proteins carrying dockerin modules have been reported in *Ruminococcus* species that are involved in degrading plant fiber (18, 24, 25). Notably, the rumen cellulolytic species *R. flavefaciens* FD1 encodes over 220 such proteins (18, 24), and those dockerins play a key role in the assembly of a cellulosome complex in this species via specific interactions with cohesin modules (26, 27). It was therefore of interest to discover that the genome of *R. bromii* L2-63 encodes 26 clearly identifiable dockerins, all but one of which are present in proteins that carry N-terminal signal peptides. Four of these dockerin-containing proteins (Amy4, Amy9, Amy10, and Amy12) are associated with GH13 catalytic modules involved in starch breakdown (Fig. 1). Of the remainder, 10 are associated with putative membrane proteins of

![Phylogenetic tree comparing GH13 enzymes from *Ruminococcus bromii* and seven other bacterial genomes.](mbio.asm.org)
unknown function, four with peptidase- or protease-related modules, and one with a putative terpene cyclase (see Fig. S2 in the supplemental material).

In addition, we found four predicted gene products (designated scaffoldin 1 [Sca1], Sca2, Sca3, and Sca4) that include cohesin modules (Fig. 3). Two of these have features that are of particular interest. Sca1 corresponds to the 1,356-amino-acid amylase precursor Amy4 (CBL15040) and carries a cohesin (Coh1) followed by a dockerin at its C terminus. Sca2 (534 amino acids; CBL15370) has a cohesin (Coh2) together with a putative sortase signal motif at its C terminus, reminiscent of the ScaE protein that has been shown to mediate anchoring of the cellulosome complex to the cell surface in R. flavefaciens F3-2 (28). Sca3 carries four repeat “X25” domains. We were therefore interested in determining whether the cohesins found in these four R. bromii proteins could interact with dockerin-containing proteins to help anchor enzymes to the cell surface and to form enzyme complexes, as described below. The R. bromii genome also encodes eight carbohydrate-binding modules (CBMs) that are associated with GH13 enzymes. Amy10 carries two CBM26 modules and one CBM48 module (Fig. 3), while each of five other enzymes carries a single CBM48 module; the CBM26 and CBM48 modules are typically involved in binding to starch (http://www.cazy.org).

Growth and native starch-degrading activity of Ruminococcus bromii. R. bromii strains grow poorly on media lacking rumen fluid, indicating that they have complex growth requirements (14, 29) and fail to grow in the rumen fluid-free medium YCFA (30), indicating that they have complex growth requirements (14, 29). Amy4, Sca2 carries a predicted C-terminal sortase signal (indicated by an arrow). The X25 domains in Sca3 show some similarity to starch-specific CBMs found in the Bacteroides thetaiotaomicron proteins SusE and SusF (10).

**FIG 3** Schematic representation of cohesin-carrying proteins of Ruminococcus bromii L2-63. The four proteins are designated scaffoldins. Scaffoldin 1 (Sca1) contains a GH13 amylase module and is synonymous with the amylase Amy4. Sca2 carries a predicted C-terminal sortase signal (indicated by an arrow). The X25 domains in Sca3 show some similarity to starch-specific CBMs found in the Bacteroides thetaiotaomicron proteins SusE and SusF (10).

**TABLE 1** Impact of growth substrate on amylase activity in R. bromii L2-63

<table>
<thead>
<tr>
<th>Growth substrate</th>
<th>Culture OD₆₅₀ ± SD</th>
<th>Amylase activity (units/mg cell protein ± SD)</th>
<th>Culture supernatant</th>
<th>Cell pellet</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2% fructose</td>
<td>0.52 ± 0.03</td>
<td>0.022 ± 0.007</td>
<td>0.047 ± 0.025</td>
<td></td>
</tr>
<tr>
<td>0.2% potato starch</td>
<td>0.49 ± 0.01</td>
<td>0.027 ± 0.009</td>
<td>0.059 ± 0.039</td>
<td></td>
</tr>
</tbody>
</table>

aData were determined in cultures growing exponentially on RUM medium and are presented as means and SDs of the results of three replicate experiments.

b1 unit = 1 μM glucose/min.
Amy12 were consistent with their predicted molecular sizes, Amy4 sequences were detected in at least two activity bands of higher molecular mass (>250 kDa).

Dockering-cohesin interactions in *Ruminococcus bromii*. Individual dockerin and cohesin modules were expressed as recombinant products in *Escherichia coli* as Xyn (dockerin) or CBM (cohesin) fusion proteins. The resulting recombinant chimeric proteins were purified to allow investigation of interactions between them using an array approach and enzyme-linked immunosorbent assay (ELISA) analysis. By those means, we analyzed interactions among 11 dockerins (including the four that are nosorbent assay (ELISA) analysis. By those means, we analyzed interactions among 11 dockerins (including the four that are thought to be involved in a variety of protein-protein interactions in other bacteria, including interactions with host proteins (33). The cohesins present in two other gene products (Sca3 and Sca4) bound all four of the dockers in GH13 enzymes.

His6-tagged recombinant Amy4-Coh1 was also used to investigate interactions with native *R. bromii* proteins. A pulldown experiment using Coh1 detected the Amy9 and Amy12 proteins, two dockerin-containing membrane proteins of unknown function, and the Amy4 amylase itself (Fig. 6). This confirms that the Amy4 cohesin recognizes several dockerins present in native *R. bromii* proteins, including Amy9 and Amy4. The recovery of Amy12 was not predicted from the studies performed with isolated recombinant dockerin modules, and this might imply that there are other binding mechanisms involving Sca3 or Sca4 or perhaps involving substrate binding. Otherwise, the results obtained with native *R. bromii* proteins agreed well with the interactions seen between the recombinant dockers and cohesins (Table 4).

**TABLE 3** Major proteins identified in *R. bromii* L2-63

<table>
<thead>
<tr>
<th><em>R. bromii</em> protein detected</th>
<th>Protein length (aa)</th>
<th>Cell pellet bit score (% coverage)</th>
<th>Cell pellet bit score (% coverage)</th>
<th>Culture supernatant bit score (% coverage)</th>
<th>Protein ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycosidases</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amy 4</td>
<td>1,356</td>
<td>759 (13)</td>
<td>1,591 (25)</td>
<td>CBL15040.1</td>
<td></td>
</tr>
<tr>
<td>Amy 1</td>
<td>804</td>
<td>510</td>
<td>1,150</td>
<td>CBL14887.1</td>
<td></td>
</tr>
<tr>
<td>Amy 2</td>
<td>751</td>
<td>510</td>
<td>1,135</td>
<td>CBL16180.1</td>
<td></td>
</tr>
<tr>
<td>Amy 9</td>
<td>1,056</td>
<td>250 (4)</td>
<td>1,135 (21)</td>
<td>CBL16180.1</td>
<td></td>
</tr>
<tr>
<td>Pulullanases, type I</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amy 10</td>
<td>1,233</td>
<td>1,190 (19)</td>
<td></td>
<td>CBL15393.1</td>
<td></td>
</tr>
<tr>
<td>Amy 12</td>
<td>1,059</td>
<td>510</td>
<td>1,291 (29)</td>
<td>CBL14812.1</td>
<td></td>
</tr>
<tr>
<td>Hypothetical</td>
<td>548</td>
<td>389 (18)</td>
<td>449 (16)</td>
<td>CBL14592.1</td>
<td></td>
</tr>
<tr>
<td>Pyruvate, phosphate dikinase</td>
<td>875</td>
<td>1,201 (28)</td>
<td>1,291 (29)</td>
<td>CBL14812.1</td>
<td></td>
</tr>
<tr>
<td>Hypothetical</td>
<td>630</td>
<td>1,201 (28)</td>
<td>1,291 (29)</td>
<td>CBL14592.1</td>
<td></td>
</tr>
<tr>
<td>Dockering type I repeat</td>
<td>734</td>
<td>63</td>
<td></td>
<td>CBL15687.1</td>
<td></td>
</tr>
<tr>
<td>Chaperone (DnaK)</td>
<td>718</td>
<td>96 (4)</td>
<td>270 (10)</td>
<td>CBL15021.1</td>
<td></td>
</tr>
<tr>
<td>Archaea/vacuole-type H⁺-ATPase subunit A</td>
<td>584</td>
<td>788 (25)</td>
<td>270 (10)</td>
<td>CBL15964.1</td>
<td></td>
</tr>
<tr>
<td>Chaperonin GroL</td>
<td>542</td>
<td>308 (11)</td>
<td></td>
<td>CBL15709.1</td>
<td></td>
</tr>
<tr>
<td>Aconitase</td>
<td>643</td>
<td>257 (9)</td>
<td>782 (27)</td>
<td>CBL15613.1</td>
<td></td>
</tr>
<tr>
<td>Putative uncharacterized</td>
<td>572</td>
<td>211 (11)</td>
<td></td>
<td>CBL14592.1</td>
<td></td>
</tr>
<tr>
<td>Hypothetical</td>
<td>1,495</td>
<td>1,247 (19)</td>
<td></td>
<td>CBL15066.1</td>
<td></td>
</tr>
<tr>
<td>Carbamoyl-phosphate synthase large subunit</td>
<td>1,350</td>
<td>187 (4)</td>
<td>1,247 (19)</td>
<td>CBL14589.1</td>
<td></td>
</tr>
<tr>
<td>Pyruvate-flavodoxin oxidoreductase</td>
<td>1,186</td>
<td>162 (3)</td>
<td>1,247 (19)</td>
<td>WP_021883784.1</td>
<td></td>
</tr>
<tr>
<td>Translocase subunit SecA</td>
<td>955</td>
<td>564 (14)</td>
<td></td>
<td>CBL15065.1</td>
<td></td>
</tr>
<tr>
<td>Isoeucyl-tRNA synthetase</td>
<td>921</td>
<td>337</td>
<td></td>
<td>CBL15771.1</td>
<td></td>
</tr>
<tr>
<td>Acetaldehyde dehydrogenase/alcohol dehydrogenase AdhE</td>
<td>873</td>
<td>640 (16)</td>
<td>640 (16)</td>
<td>CBL14797.1</td>
<td></td>
</tr>
</tbody>
</table>

### Notes:

- **Table 2** shows the activity of *R. bromii* L2-63 native amylases against different starches.
- **Table 3** lists major proteins identified in *R. bromii* L2-63.

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DISCUSSION

Firmicutes bacteria belonging to the Ruminococcaceae represent 10% to 25% of the microbiota of healthy individuals, according to the results of molecular surveys (34). Certain of these bacteria appear to play important roles in the degradation of insoluble substrates in the human large intestine (21). Ruminococcus relatives were the only bacterial group to be found significantly associated with the fiber fraction of human fecal samples on the basis of 16S rRNA sequence analysis, accounting for 12.2% of fiber-associated but only 3.3% of liquid-phase sequences (35). Furthermore, key roles in the degradation of insoluble substrates have been ascribed recently to two species. The newly defined species R. champenellensis is the only human intestinal bacterium so far reported to degrade microcrystalline cellulose (25, 36) and has

**FIG 4** Detection of major R. bromii amylases by zymogram analysis and sequencing. (a) Zymogram showing activity of amylases against RS3 for R. bromii L2-63 cells grown for 24 h on 0.2% fructose or 0.2% RS3. Values on the left correspond to the molecular masses determined by staining the gel with Coomassie blue, prior to staining the gel with iodine to visualize clear zones of amylase activity. sup, supernatant proteins; cell, cell-associated proteins. Bands a to f, visible active bands. (b) Identification of amylolytic enzymes from excised bands by LC-MS/MS. In addition, a homologue of a Cna (collagen adhesion)-type protein was detected in band a, a hypothetical protein (RBR_05030) in band b, RNA polymerase subunit B in band d, and a hypothetical protein (RBR_07100) in band e.

**TABLE 4** Interactions of recombinant R. bromii dockerin and cohesin modules determined by a microarray approach

<table>
<thead>
<tr>
<th>Dockerin</th>
<th>Interaction</th>
<th>Coh1 (Amy4)</th>
<th>Coh2 (Sca2)</th>
<th>Coh3 (Sca3)</th>
<th>Coh4 (Sca4)</th>
<th>Associated domain(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amy9</td>
<td></td>
<td>++++</td>
<td>+</td>
<td>++++</td>
<td>++++</td>
<td>GH13</td>
</tr>
<tr>
<td>CBL16032.1</td>
<td></td>
<td>++++</td>
<td>+</td>
<td>++++</td>
<td>++++</td>
<td>Peptidase</td>
</tr>
<tr>
<td>CBL14720.1</td>
<td></td>
<td>++++</td>
<td>+</td>
<td>++++</td>
<td>++++</td>
<td>2× LRR</td>
</tr>
<tr>
<td>Amy4</td>
<td></td>
<td>++++</td>
<td>+</td>
<td>++++</td>
<td>++++</td>
<td>GH13, Coh1</td>
</tr>
<tr>
<td>Amy10</td>
<td></td>
<td>++++</td>
<td>+</td>
<td>++++</td>
<td>++++</td>
<td>GH13, CBM26, CBM48</td>
</tr>
<tr>
<td>CBL14834.1</td>
<td></td>
<td>++++</td>
<td>+</td>
<td>++++</td>
<td>++++</td>
<td></td>
</tr>
<tr>
<td>CBL14836.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Cysteine protease</td>
</tr>
<tr>
<td>CBL15647.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amy12</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>++++</td>
<td>GH13, CBM48</td>
</tr>
<tr>
<td>CBL15625.1</td>
<td></td>
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<td></td>
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<tr>
<td>CBL16049.1</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

* Proteins were expressed as recombinant products (CBM fusions [cohesins] and XYN fusions [dockerins]) in E. coli (see Materials and Methods). LRR, leucine-rich repeat.
  
  
  
  ++++, very strong; ++++, strong; ++, moderate; +, weak; –, very weak; –, none.
produce cellulosomes, notably, \textit{R. flavefaciens} (24). Interactions of dockerins with cohesin modules present in scaffolding proteins are responsible for the organization of the members of a diverse set of plant cell wall-degrading enzymes into cellulosome complexes in \textit{R. flavefaciens} (26, 39, 40) and in other \textit{Clostridium}-related cellulytic bacteria (27). A cellulosome complex has also been discovered recently in the human colonic species \textit{R. champanellensis} (25). In contrast, the noncellulytic human colonic species \textit{R. bicirculans} carries only a single dockerin and a single cohesin that are associated with a GH73 enzyme (38); in general, there are few examples of dockerins and cohesins playing a role in protein complexes from noncellulytic species. It was therefore of some surprise to detect 26 dockerin- and four cohesin-carrying proteins in the genome of the specialist starch-degrader \textit{R. bromii} L2-63. We have shown here that some of these modules are involved in the organization of the major extracellular amylases of \textit{R. bromii} into multiprotein complexes, or “amylosomes.”

On the basis of the evidence presented here, the possible arrangements of the major extracellular \textit{R. bromii} amylases were revealed and are summarized in Fig. 7. The Sca2 protein carries a C-terminal motif that predicts attachment to the cell surface via a sortase-mediated mechanism, as previously demonstrated for the ScaEs of \textit{R. flavefaciens} and \textit{R. champanellensis} (25, 26). The Amy9 and Amy4 amylases have the potential to link directly to Sca2 via its C-terminal dockerin. The Sca2 cohesin was also able to bind dockerins from proteins other than amylases, including peptidases, suggesting that it has a more general role as a cell surface-anchoring mechanism. The Amy4 enzyme is unusual in that it carries a cohesin and a dockerin in addition to a catalytic module (Fig. 7). Even more unusual, however, is that the Amy4 cohesin recognizes the dockerin of Amy4 itself; this suggests that Amy4 should be able to form multimeric assemblages. The Amy4 cohesin also provides a partner for the dockerins from Amy10 and Amy9, suggesting that minicomplexes involving two or more amylases are formed. The frequency with which the Amy4 cohesin engages the dockerin of another Amy4 protein, as opposed to the dockerins from Amy9 and Amy10, presumably depends on the relative abundances of the Amy9, Amy10, and Amy4 dockerins and their relative affinities for the Amy4 cohesin. The roles of the other two cohesin-carrying proteins, Sca3 and Sca4, are not yet clear, but both were able to bind dockerins from all four dockerin-carrying GH13 enzymes. The presence of four X25 modules on Sca3 (Fig. 3) is particularly intriguing, since similar modules bind starch in cell surface proteins SusE and SusF of \textit{B. thetaiotaomicron} and have also been observed in a pullulanase of \textit{Bacillus acidopullulolyticus} (10, 41). Dockerin-bearing amylases, i.e., Amy4, Amy9, Amy10, and Amy12, would then be equipped with strong starch-binding properties, through their interaction with Sca3. Binding to starch is also likely to be mediated by the CBM48 modules present in Amy9 and Amy10 when present in the complex. Amy10 also carries two CBM26 modules, which are thought to have a particularly important role in binding to raw starches (23). With regard to enzyme specificity, the predicted pullulanase activity of Amy10 and Amy12 is likely to be complemented by the distinct actions of Amy4 and Amy9, although the specificities of the \textit{R. bromii} enzymes have yet to be determined experimentally. Amy10 and Amy12 also contain additional modules of unknown function that might possibly mediate cell surface attachment. Future work will explore whether other strains of \textit{R. bromii}, notably, the ATCC27255 type strain, which shows activity on RS as well as

been detected mainly in methanogenic individuals (37). A related species from the human colon, \textit{“R. bicirculans,”} shows a more specialized capacity to utilize plant cell wall polysaccharides, including β-glucans, but is unable to degrade cellulose or xylan (38). \textit{R. bromii}, on the other hand, is a remarkably specialized cellulytic bacterium that has no ability to degrade plant cell wall polysaccharides but shows very high degradative activity against resistant starches (14).

Large numbers of dockerin modules are found in proteins from cellulytic \textit{Ruminococcus} species that have been shown to

FIG 5 Interactions of recombinant dockerins and cohesins from \textit{R. bromii} L2-63. Selected cohesin-dockerin interactions were examined by ELISA experiments. (a and b) Amy4 (a) and Amy9 (b) dockerins interact strongly with all four cohesins of \textit{R. bromii}. (c) Strong interaction of the Amy12 dockerin with Coh3, moderate interaction with Coh4, and negligible interaction with Coh1 and Coh2. CohE from \textit{R. flavefaciens} FD1 was included in the experiment as a negative control. Error bars indicate the standard deviations from the means of the results determined for triplicate samples from one experiment.
growth characteristics similar to those seen with \textit{R. bromii} L2-63 (14), produce a similar type of amylosome complex; however, such work depends on genome sequencing, which is currently available only for \textit{R. bromii} L2-63.

\textit{R. bromii} L2-63 apparently produces its major amylases constitutively since we found no significant difference between cultures grown on fructose and those grown on starch in either enzyme assay or zymogram analysis results. This is in marked contrast to the substrate-inducible amylase systems of other human colonic anaerobes such as \textit{B. thetaiotaomicron} (8), \textit{E. rectale} (13) and \textit{Roseburia} spp. (11, 12) and provides further evidence...
that *R. bromii* is extremely specialized in its utilization of carbo-
hydrate substrates.

In conclusion, we have presented evidence that four of the major extracellular starch-degrading enzymes in *R. bromii* are at-
tached to the cell surface and/or assembled into complexes via
cohesin-dockerin interactions. This provides the first example of
the involvement of dockerin and cohesins, best known from their
roles in the celluloses that are responsible for lignocellulose
breakdown (27), in a starch-degrading enzyme system, and we
therefore refer to the complexes formed as “amylosomes.” It
seems likely that this organization will help to explain the excep-
tional degradative activity shown by *R. bromii* against particulate
starches (14).

**MATERIALS AND METHODS**

**Sequence analysis and phylogeny of GH13 enzymes.** The *R. bromii* L2-63
genome was sequenced by the Pathogen Genomics group at the Wellcome
Trust Sanger Institute (United Kingdom) as part of the EU MetaHit pro-
ject (http://www.sanger.ac.uk/resources/downloads/bacteria/metahit/).
Prediction of cohesin and dockerin modular sequences was performed
using the BLASTP and tBLASTn algorithm (42), employing known dock-
erin and cohesin sequences as queries. Analysis of carbohydrate-active
enzymes (CAZymes) was performed using the CAZy database (http://
www.cazy.org), with reference also to the KEGG database (43). Related
GH13 protein sequences were detected using BLASTp. The results were
filtered to exclude all matches with E values of >1e-10, sequence identity
of <35%, or bit scores of <200, and the remaining best-hit annotations
were assigned to the CAZy database sequences. The sequences were then
aligned by ClustalW (44) and used to construct a maximum-likelihood
phylogenetic tree, using MEGA6.0 software (45).

**Growth medium for *R. bromii.*** Isolation of *R. bromii* L2-63 from a human fe-
cal sample using a rumen-fluid-based medium was described by
Ze et al. (14). Semidefined RUM medium, which was developed for the
present work as a modification of YCFA medium (30), consists of (per
100 ml) Casitone (1 g), yeast extract (0.25 g), NaHCO3 (0.4 g), resazurin
(0.1 mg), biotin (1 µg), cobalamin (1 µg), p-aminobenzoic acid (3 µg),
folinic acid (5 µg), pyridoxamine (15 µg), KH2PO4 (0.045 g), K2HPO4
(0.045 g), NaCl (0.09 g), (NH4)2SO4 (0.09 g), MgSO4 7H2O (0.009 g),
and CaCl2 (0.009 g) and the short-chain fatty acids (final concentra-
tions) acetic (33 mM), propionate (9 mM), and isobutyrate, isovalerate,
and valerate (1 mM each). Cysteine (0.1 g/100 ml) was added to the medium
following boiling and was dispersed into Hungate tubes while the tubes
were flushed with CO2. After autoclaving, filter-sterilized solutions were
added to give a final concentration of thiamine and riboflavin of
0.05 µg/ml−1 (each), pantethein and nicotinamide of 1 µg/ml−1,
pantethine of 50 ng·ml−1, and tetrahydrofolic acid of 0.1 µg/ml−1. Addi-
tional trace minerals FeSO4 7H2O (final concentration, 0.4 µg/ml−1),
ZnSO4 7H2O, MnCl2 4H2O (6 ng/ml−1), H3BO3 (60 ng/ml−1), CoCl2 6H2O (40 ng/ml−1), CuCl2 2H2O (2 ng/ml−1),
NiCl2 6H2O (4 ng/ml−1), NaMoO4 2H2O (6 ng/ml−1), and NaSeO3 (15 ng/ml−1) and EDTA (1 µg/ml−1) were also included here, but we have
now shown that these are not required for growth of *R. bromii* L2-63.
Carbohydrate or other energy sources were added as required, and the
final pH of the medium was adjusted to 6.8 ± 0.2. With 0.2% fructose as
the energy source, culture optical densities (ODs) and growth rates for
*R. bromii* L2-63 on RUM medium were very similar to those obtained
on rumen fluid medium (14).

**Proteomic analysis.** Proteomic analysis was performed on culture
supernatants and cell pellets obtained from triplicate biological experi-
ments. *R. bromii* L2-63 was grown anaerobically in 150 ml of RUM me-
dium supplemented with 0.5% soluble potato starch (Sigma) at 37°C to an
OD at 650 nm (OD650) of between 0.7 and 0.8. After centrifugation at
9,000 × g, at 4°C, the culture supernatant was dialyzed (four times with
4 liters of distilled water, at 4°C), freeze-dried, and resuspended in 1.5 ml
of resuspension Tris buffer (50 mM Tris [pH 8.8], 10% glycerol, 0.1% Triton X-100) supplemented with 1× protease inhibitor cocktail (P8465,
Sigma). The cell pellet was washed three times with 1.5 ml phosphate-
buffered saline (PBS) and, following one freeze-thaw cycle, resuspended
in 1.5 ml of resuspension Tris buffer with protease inhibitor. Cell lysis was
achieved by adding 1.2 g of 1-mm-diameter zirconia beads (BioSpec
Products, OK, USA) and beating the cells twice on an MP FastPrep-24
bead beater for 30 s at 6.0 m/s. Nonsolvulosed debris was removed by
centrifugation at 10,000 × g for 5 min. Culture supernatant and cellular
fractions were stored at −70°C until further analysis. Protein concentra-
tions of the supernatant and cellular fractions were measured using
Bradford reagent (Sigma Aldrich, Dorset, United Kingdom). Aliquots of
350 µg of protein were precipitated in 25% trichloroacetic acid (TCA)−
20 mM dithiothreitol (DTT) for 1 h on ice, followed by centrifugation at
10,000 × g for 10 min at 4°C. Pellets were washed four times with 1 ml
ice-cold acetone containing 20 mM DTT. After removal of the acetone,
the protein pellets were resuspended in Rabillou buffer (7 M urea, 2 M
thiourea, 4% CHAPS [3-[3-cholamidopropyl]-dimethylammonio]-1-
propanesulfonate), 0.5% biotinyl Ampholite; pH 3 to 10) and 200 to 250 µg
of protein was separated on two-dimensional gels using 17-cm-long im-
mobilized pH gradient (IPG) strips (pH 4 to 7) as described elsewhere
(46). Gel images were analyzed with PDQuest Advanced 8.0.1 (BioRad,
Hertfordshire, United Kingdom). The densit spots with an apparent
molecular mass of between 49 and 145 kDa were excised from the gels
manually and subjected to trypsinization and protein identification by the
use of Nano LC MS/MS, followed by analysis of the total current ion data
using the MASCOT search engine as described previously (46).

**Investigation of native amylolytic activity.** The starch-mediated in-
ducibility of amylase activity was investigated in cultures of *R. bromii*
L2-63 grown in 100 ml RUM medium supplemented with 0.2% soluble
starch or 0.2% fructose and incubated anaerobically at 37°C to the expo-
nential phase (OD650 of between 0.45 and 0.55) in three independent
experiments. Cells were collected and extracellular/cell-associated
fractions were obtained as described above. Amylase activity was determined
with 0.1% boiled potato starch substrate (Sigma Aldrich, Dorset, United
Kingdom) as the substrate from triplicate incubations in PBS (pH 7) with
CaCl2 (0.1 mM) at 37°C by measuring the release of reducing sugars (47).
Amylase preparations used to investigate activity against different starches
(Table 2) were obtained from stationary-phase cultures grown in RUM
medium containing 0.2% boiled Novelose 330 for 48 h.

**Activity of enzymes in polyacrylamide gels (zymogram analysis).**
Amylolytic enzymes of *R. bromii* were also analyzed using starch
zymograms (11). *R. bromii* was grown on RUM medium containing 0.2%
Novelose (filter sterilized and preboiled for 10 min) (RS3) or 0.2% fruc-
tose and incubated at 37°C for up to 48 h. The cells were then harvested
following centrifugation at 14,000 × g for 10 min at 4°C. Culture sup-
ernatant was collected, dialyzed for 24 h with distilled H2O (3 changes),
frozen overnight at −20°C, and then lyophilized. The cell pellet was
was washed twice and resuspended in 50 mM sodium phosphate buffer
(pH 6.5) before storage at −20°C. Cellular and supernatant protein sam-
plels were denatured by heating at 60°C for 20 min and then subjected
to SDS-PAGE with 0.2% RS3 (Novelose 330) incorporated into the separat-
gel. After the separation was performed, the gel was washed twice with
200 ml of washing solution (10 mM Tris-HCl [pH 7.5], 2 mM DTT, 20%
isopropanol) at room temperature. The proteins were then renatured by
rocking the gel in 200 ml of renaturing solution (50 mM Tris-HCl
[pH 6.8], 2 mM DTT, 1 mM EDTA) overnight at 4°C. The renatured gel
was transferred to 200 ml of sodium phosphate buffer (50 mM, pH 6.8),
soaked for 1 h at 4°C, and then incubated at 37°C for 4 h. The gel was
subjected to Coomassie staining to record the position of protein ladders
(Novagen; EMD Millipore, MA, USA). The gel was neutralized by wash-
ing in Tris-HCl solution (0.1 M, pH 8.0) for at least 4 h with several
solution changes in the first hour. After neutralization, the gel was
stained with 20% Lugol’s solution (Sigma) until clear zones of starch hydroly-
sis were visible.
Recombinant CBM-cohesin and xylanase-dockerin fusion products. Cloning of CBM-fused cohesins and xylanase-fused dockerins was performed as described by Ben David et al. (25). Procedures for expression in *E. coli* BL21(DE3) and purification of the recombinant proteins are described in the same reference.

**CBM-based microarray.** Microarray methods were as described in Ben David et al. (25). CBM-cohesin samples were diluted in Tris-buffered saline (TBS) (pH 7.4) to final concentrations of 9, 3, 1, 0.3, and 0.1 μM and printed onto cellulose-coated glass slides (type GSC-1; Advanced Microdevices Pvt. Ltd., Ambala Cantt, India) by the use of a Microbiology Grid 610 Microarrayer (Digilab, Inc., Marlborough, MA). The printed microarrays were blocked by incubating the slides in blocking buffer (1% bovine serum albumin–TBS–10 mM CaCl2–0.05% Tween 20) at room temperature for 30 min. Afterward, chosen Xyn-Doc samples (5 nM in blocking buffer) were incubated with the slide at room temperature for 30 min followed by 3 washing steps (5 min each) in washing buffer (TBS–10 mM CaCl2–0.05% Tween 20). Fluorescent staining was accomplished by adding Cy3-labeled anti-Xyn and Cy5-labeled anti-CBM (diluted 1:1,000) in blocking buffer for 30 min. The probed slides were again washed 3 times, air dried, and scanned for fluorescence signals using a Typhoon 9400 variable-mode imager (GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

**ELISA.** The ELISA procedure was performed as described earlier (25). The coating step was performed with 15 nM Xyn-Doc proteins. A concentration gradient (0.01 to 1,000 nM) of CBM-Coh was then applied to the coated Maxisorp 96-well plate (Greiner Bio-One, Belgium).

**Interactions of Coh1 with native *R. bromii* proteins.** The interaction of the cohesin module of Amy4 (Coh1) with proteins of *R. bromii* L2-63 was evaluated using a pull down assay. The *R. bromii* L2-63 Coh1 module was cloned into pIVEX (5 Prime, Hamburg, Germany) and His6-tagged *R. bromii* Coh1 was overexpressed using a cell-free RTS 100 expression system (5 Prime, Hamburg, Germany). His6-tagged Coh1 was overexpressed using a cell-free RTS 100 expression system (5 Prime, Hamburg, Germany). His6-tagged proteins of the cohesin module of Amy4 (Coh1) with proteins of *R. bromii* L2-63 cells in PBS was allowed to interact with them. After incubation for 20 min at room temperature (RT), the beads were washed 3 times and then the proteins were eluted as recommended by the manufacturer. The eluted proteins were separated on a 12% SDS-PAGE gel and subjected to Coomassie staining. The observed bands were excised from the gel and subjected to LC-MS/MS identification as described above.

**SUPPLEMENTAL MATERIAL**


Figure S1, PDF file, 0.1 MB.
Figure S2, PDF file, 0.1 MB.
Figure S3, PDF file, 0.2 MB.
Figure S4, PDF file, 0.1 MB.
Table S1, PDF file, 0.1 MB.
Table S2, PDF file, 0.02 MB.

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