RESEARCH ARTICLE

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 amylolytic 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 amylolytic 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*.**

Received 22 June 2015 **Accepted** 25 August 2015 **Published** 29 September 2015

Citation Ze X, Ben David Y, Laverde-Gomez JA, Dassa B, Sheridan PO, Duncan SH, Louis P, Henrissat B, Juge N, Koropatkin NM, Bayer EA, Flint HJ. 2015. Unique organization of extracellular amylases into amylosomes in the resistant starch-utilizing human colonic *Firmicutes* bacterium *Ruminococcus bromii*. mBio 6(5):e01058-15. doi:10.1128/mBio.01058-15.

Editor Julian Parkhill, Sanger Institute

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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

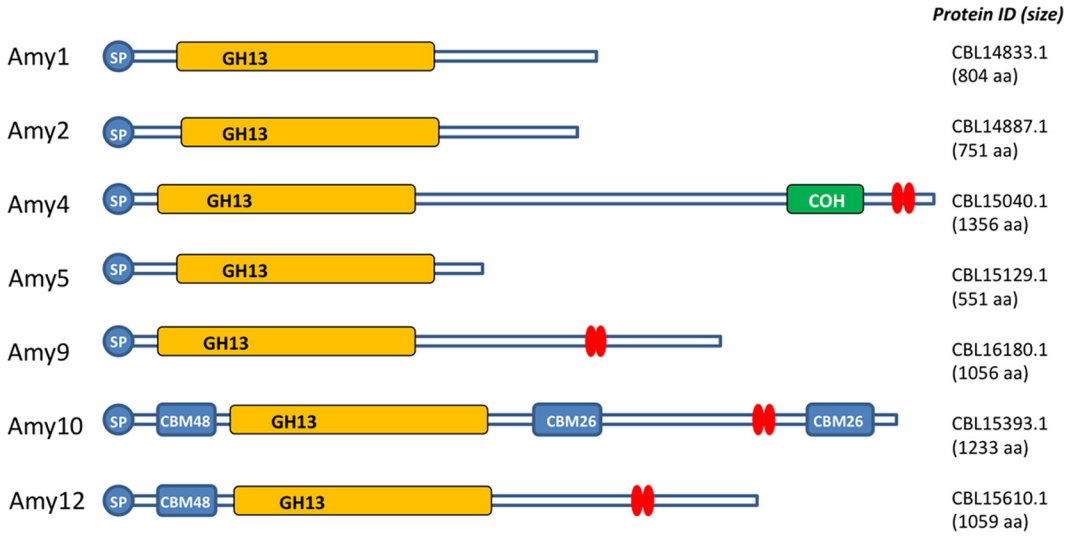


FIG 1 Major extracellular amylases of *R. bromii* L2-63. Modular organization is shown for seven predicted gene products that carry GH13 catalytic modules and signal peptides (SP). The cohesin (COH) of Amy4, the CBMs from family 26 or family 48, and the dockerins (red double ellipses) are shown schematically. aa, amino acids.

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 *Roseburia* 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 the ability of certain 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 nongrowing *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 β-glucosidase (GH3). N-terminal signal peptides (SP) 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 Amy 12) group with GH13 subfamily 14, which includes pullulanases (22), and one (Amy5) groups with *E. rectale* α-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).

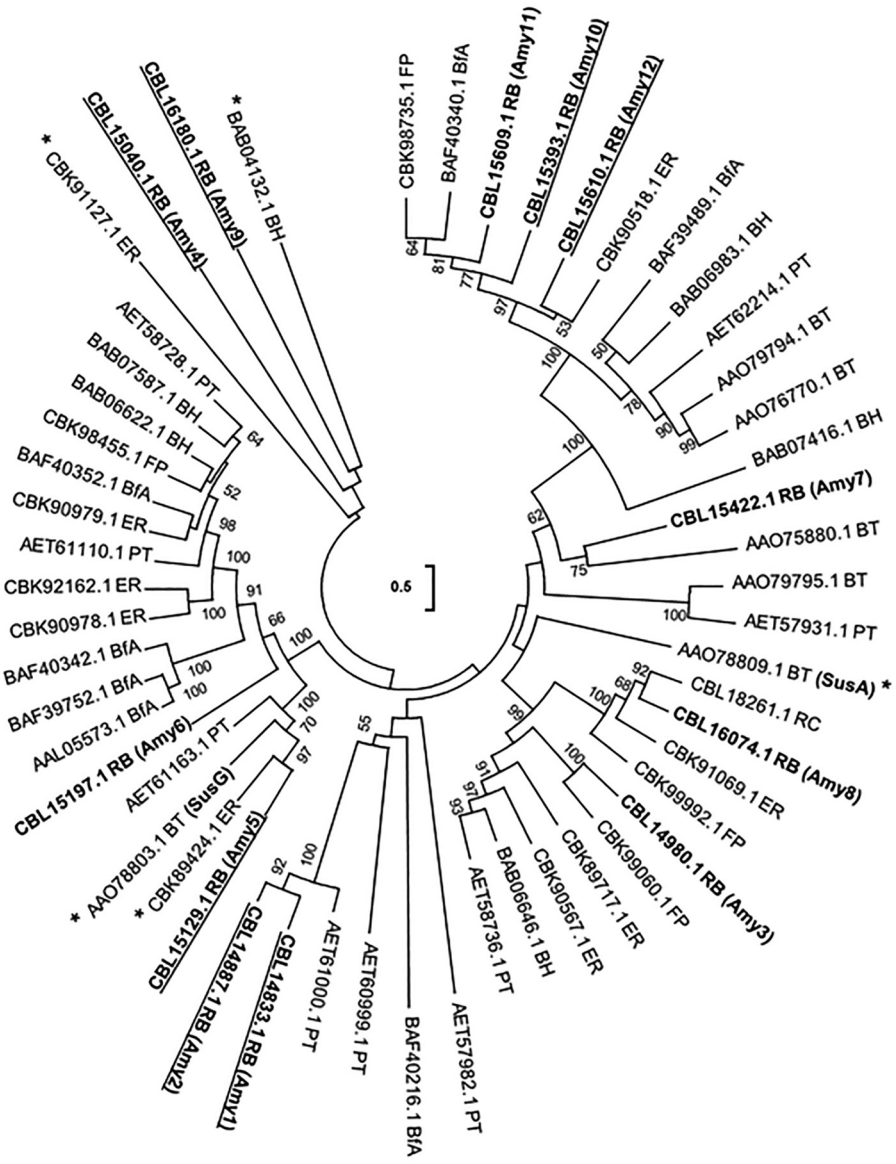


FIG 2 Phylogenetic tree comparing GH13 enzymes from *Ruminococcus bromii* and seven other bacterial genomes. *R. bromii* L2-63 (RB) GH13 sequences (labeled Amy1, Amy2, Amy3, etc.) are compared with those from other human gut species (the starch-utilizing strains *Eubacterium rectale* A1-86 [ER], *Bacteroides thetaiotaomicron* VPI-5482 [BT], and *Bifidobacterium adolescentis* ATCC15703 [BfA] and two other human colonic *Ruminococcaceae* species, *Ruminococcus champanellensis* 18P13 [RC] and *Faecalibacterium prausnitzii* L2-6 [FP]) and also with those from two non-gut species, *Bacillus halodurans* C-125 (BH) and *Paenibacillus terrae* HPL-003 (PT), that were known from the results of BLASTp queries of the NCBI database to possess proteins that were the closest matches to *R. bromii* Amy4. *R. bromii* sequences that are predicted to possess signal peptides are underlined. Sequences with functions concerned with glycogen or trehalose metabolism (including *R. bromii* L2-63 Amy 13, Amy14, and Amy15) that are predicted by KEGG GH annotation have been omitted from the tree. Sequences marked with an asterisk (*) encode enzymes that have been experimentally characterized. Bootstrap values, expressed as a percentage of 1,000 replications, are given at the branching nodes. This tree is unrooted and was constructed using the maximum-likelihood method. The scale bar (center of tree) refers to the number of amino acid differences per position.

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.

Dockerin 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. flavefaviens* 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

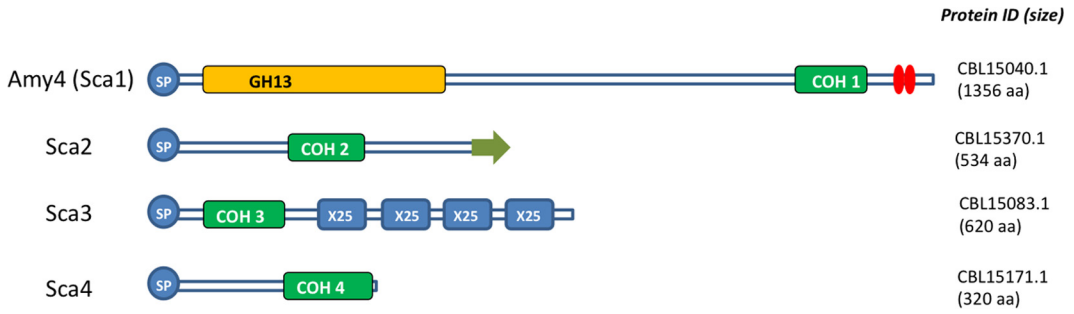


FIG 3 Schematic representation of cohesin-carrying proteins of *Ruminococcus bromii* L2-63. The four proteins are designated scaffoldins. Scaffoldin 1 (Sca1) contains a GH13 amylase module and is synonymous with the amylase Amy4. Sca2 carries a predicted C-terminal sortase signal (indicated by an arrow).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

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* 17 (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. 1), 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), which supports the growth of many other human colonic anaerobes. YCFA medium was therefore modified here to include additional filter-sterilized vitamins that enable good growth of *R. bromii* L2-63 (see Materials and Methods); we refer to this basal medium as RUM medium. As shown in Table 1, similar amylase activities were detected for mid-exponential-growth-phase cells grown in RUM medium with fructose or with soluble starch as the energy source, showing that amylase activity is expressed constitutively in this bacterium. Most (53% to 75%) of the amylase activity was detected in the cell pellet rather than in the culture supernatant, indicating that it was cell associated. *R. bromii* enzyme preparations from stationary-phase cultures were active against both raw and preboiled starches, but activity was greater against the boiled starches, especially in the case of potato starch (Table 2).

Proteomic analysis of prominent cell pellet-associated and supernatant proteins from starch-grown *R. bromii* cultures led to the detection of six proteins carrying GH13 modules (Table 3; see also Fig. S3 and S4 in the supplemental material). Five proteins carrying GH13 modules (identified as Amy1, Amy2, Amy4, Amy9, and Amy12) were detected among 23 prominent spots analyzed using two-dimensional (2D) gel separations of proteins from the culture supernatant. Amy4, Amy2, and Amy9, together with Amy10, were also identified in the cell pellet fraction from the 23 analyzed spots (Table 3). Thus, six of the seven predicted extracellular GH13 enzymes that carry signal peptides, and all four of dockerin-carrying enzymes Amy4, Amy9, Amy10, and Amy12, were detected among the major proteins expressed by *R. bromii* (Fig. 1).

Zymogram analysis of one-dimensional gel separations, involving pretreatment of the native protein fractions at 60°C followed by renaturation (11), was also used to compare the major amylases produced by *R. bromii* L2-63 grown on RUM medium with fructose or boiled RS3 starch as an energy source. As for total amylase assayable activity (Table 1), no difference between fructose- and starch-grown cultures was evident in activity profiles, further confirming that expression of these major amylases is constitutive (Fig. 4). Major activity bands were excised from the SDS gel and sequenced by liquid chromatography tandem mass spectrometry (LC MS/MS). Five bands were found to contain amylases; these corresponded to three dockerin-containing starch-degrading enzymes (two α-amylases, Amy4 [CBL15040] and Amy9 [CBL16180], and a pullulanase, Amy12 [CBL15610]) among the four discussed above. While the positions of Amy9 and

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

Growth substrate	Culture OD_{650} ± SD	Amylase activity (units/mg cell protein ± SD)[b]	
		Culture supernatant	Cell pellet
0.2% fructose	0.52 ± 0.03	0.022 ± 0.007	0.047 ± 0.025
0.2% potato starch	0.49 ± 0.01	0.027 ± 0.009	0.059 ± 0.039

[a] Data were determined in cultures growing exponentially on RUM medium and are presented as means and SDs of the results of three replicate experiments.
[b] 1 unit = 1 μM glucose/min.

TABLE 2 Activity of *R. bromii* L2-63 native amylases against different starches[a]

Type of starch	Total culture activity (%) under indicated condition	
	Boiled	Untreated
Potato starch (Sigma, S2004)	100	6.2
High-amylose corn starch (Sigma, S4180)	44.1	18.8
Corn starch (Sigma, S9679)	83.0	24.6
Novelose 330 (National Starch)	55.8	27.3

[a] Enzyme preparations from stationary-phase cultures grown for 48 h in RUM medium with boiled 0.2% Novelose 330 as the energy source were used. Data are shown as percentages of the activity obtained for boiled potato starch, based on the means of the results of triplicate assays (SD were less than 1% of the mean in all cases) combining data from supernatant, washings and residual pellet fractions.

Amy12 were consistent with their predicted molecular sizes, Amy4 sequences were detected in at least two activity bands of higher molecular mass (>250 kDa).

Dockerin-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 found in proteins with GH13 modules) and four cohesins (see Materials and Methods). The cohesin from Amy4 (Coh1) bound the dockerins of Amy9 and Amy10 as well as the dockerin of Amy4 itself (Table 4 and Fig. 5). The demonstration that Amy4-Coh1 recognizes its own C-terminal dockerin is intriguing, as this suggests the presence of a mechanism that could result in multimerization. Such cohesin-dockerin pairs that presumably result in multimerization are known in other microbial systems (24, 31, 32). Sca2-Coh2 bound strongly to two of the dockerins associated with GH13 modules (Amy4 and Amy9) and to three other dockerins, including one with a peptidase module and one containing leucine-rich-repeat (LRR)-type sequences (Table 4; see also Fig. S2 in the supplemental material). LRR sequences are also present in multiple dockerin-carrying gene products in *R. flavefaciens* (24) and 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 dockerins found in GH13 enzymes.

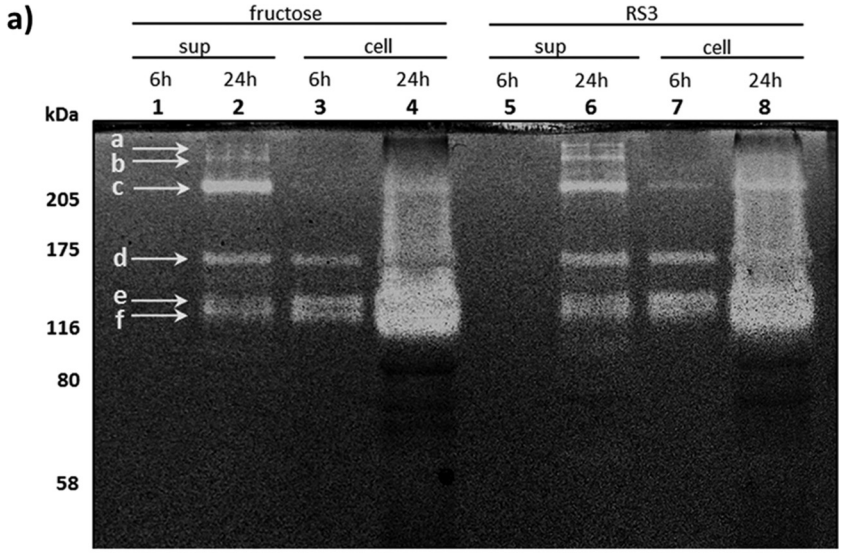
His6-tagged recombinant Amy4-Coh1 was also used to investigate interactions with native *R. bromii* proteins. A pulldown experiment using Coh1 detected the Amy9 and Amy12 proteins, two dockerin-containing membrane proteins of unknown function, and the Amy4 amylase itself (Fig. 6). This confirms that 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 dockerins and cohesins (Table 4).

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

R. bromii protein detected	Protein length (aa)	Cell pellet bit score (% coverage)	Culture supernatant bit score (% coverage)	Protein ID
Glycosidases				
Amy 4	1,356	759 (13)[b]	1,591 (25)	CBL15040.1
Amy 1	804		409 (11)	CBL14833.1
Amy 2	751	510	1,150	CBL14887.1
Amy 9	1,056	250 (4)	1,135 (21)	CBL16180.1
Pullulanases, type I				
Amy 10	1,233	1,190 (19)		CBL15393.1
Amy 12	1,059		552 (9)	CBL15610.1
Hypothetical	548		113	CBL14834.1
Pyruvate, phosphate dikinase	875	1,201 (28)	1,291 (29)	CBL14812.1
Hypothetical	630	389 (18)	449 (16)	CBL14592.1
Dockerin type I repeat	734		63	CBL15687.1
Chaperone (DnaK)	718	96 (4)	270 (10)	CBL15021.1
Archaea/vacuole-type H^+-ATPase subunit A	584	788 (25)		CBL15964.1
Chaperonin GroL	542	308 (11)		CBL15709.1
Aconitase	643	257 (9)	782 (27)	CBL15613.1
Putative uncharacterized	572		211 (11)	CBL14592.1
Hypothetical	1,495		1,247 (19)	CBL15066.1
Carbamoyl-phosphate synthase large subunit	1,350	187 (4)		CBL14589.1
Pyruvate-flavodoxin oxidoreductase	1,186	162 (3)		WP_021883784.1
Translocase subunit SecA	955	564 (14)		CBL15065.1
Isoleucyl-tRNA synthetase	921	337		CBL15771.1
Acetaldehyde dehydrogenase/alcohol dehydrogenase AdhE	873	640 (16)		CBL14797.1

[a] Cultures were grown on RUM medium containing 0.2% soluble starch for 48 h. Proteomic analysis and the preparation of cell pellet and culture supernatants are described in Materials and Methods. Data presented are based on analysis of the most prominent spots (molecular mass, >60 kDa) recovered from two SDS gel separations of cell pellet (23 spots) and supernatant (23 spots) preparations. aa, amino acid; ID, identifier.

[b] The scores and percent coverage values shown represent the top hits against an *R. bromii*-encoded protein for a given protein spot in the cell pellet or supernatant fractions. Several of these proteins were detected in multiple spots recovered from 2D gels, reflecting charge variants.



b)

Gene product	Predicted precursor mass (Da)	Detected in bands:-
Amy4	145,063	b, c, d
Amy12	116,603	e, f
Amy9	114,106	e

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.

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. champanellensis* is the only human intestinal bacterium so far reported to degrade microcrystalline cellulose (25, 36) and has

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

	Interaction				
Dockerin	Coh1 (Amy4)	Coh2 (Sca2)	Coh3 (Sca3)	Coh4 (Sca4)	Associated domain(s)
Amy9	+++++	+++	+++++	+++++	GH13
CBL16032.1	+++	++	+++++	+++++	Peptidase
CBL14720.1	+++++	+++	+++++	+++++	2× LRR
Amy4	++++	+++	+++++	+++++	GH13, Coh1
Amy10	+++++	+	+++++	+++++	GH13, CBM26, CBM48
CBL14834.1	+++++	+++	+++++	+++++	
CBL14836.1	−	−	−	+	Cysteine protease
CBL15647.1	−	−	−	−	
Amy12	−	−	++	+++++	GH13, CBM48
CBL15625.1	−	−	−	−	
CBL16049.1	−	−	−	−	

[a] 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.

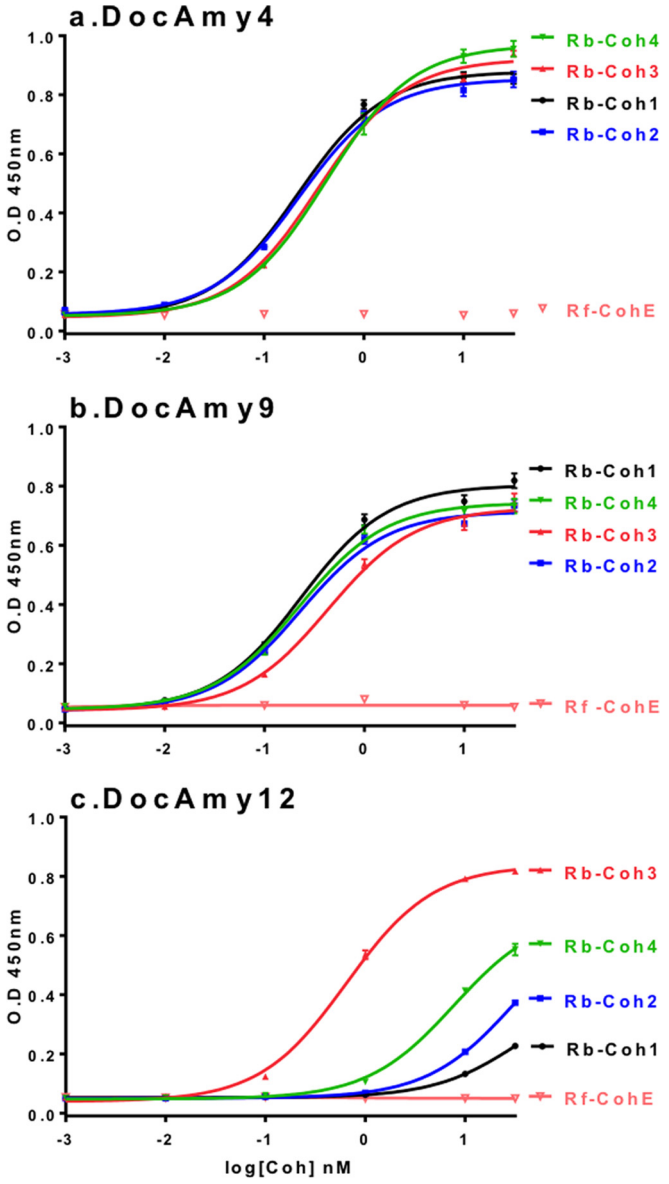


FIG 5 Interactions of recombinant dockerins and cohesins from *R. bromii* L2-63. Selected cohesin-dockerin interactions were examined by ELISA experiments. (a and b) Amy4 (a) and Amy9 (b) dockerins interact strongly with all four cohesins of *R. bromii*. (c) Strong interaction of the Amy12 dockerin with Coh3, moderate interaction with Coh4, and negligible interaction with Coh1 and Coh2. CohE from *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.

been detected mainly in methanogenic individuals (37). A related species from the human colon, "*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). *R. bromii*, on the other hand, is a remarkably specialized amylolytic 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 cellulolytic *Ruminococcus* species that have been shown to produce cellulosomes, notably, *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 *R. flavefaciens* (26, 39, 40) and in other *Clostridium*-related cellulolytic bacteria (27). A cellulosome complex has also been discovered recently in the human colonic species *R. champanellensis* (25). In contrast, the noncellulolytic human colonic species *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 noncellulolytic species. It was therefore of some surprise to detect 26 dockerin- and four cohesin-carrying proteins in the genome of the specialist starch-degrader *R. bromii* L2-63. We have shown here that some of these modules are involved in the organization of the major extracellular amylases of *R. bromii* into multiprotein complexes, or "amylosomes."

On the basis of the evidence presented here, the possible arrangements of the major extracellular *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 *R. flavefaciens* and *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 *B. thetaiotaomicron* and have also been observed in a pullulanase of *Bacillus acidopullulyticus* (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 *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 *R. bromii*, notably, the ATCC27255 type strain, which shows activity on RS as well as

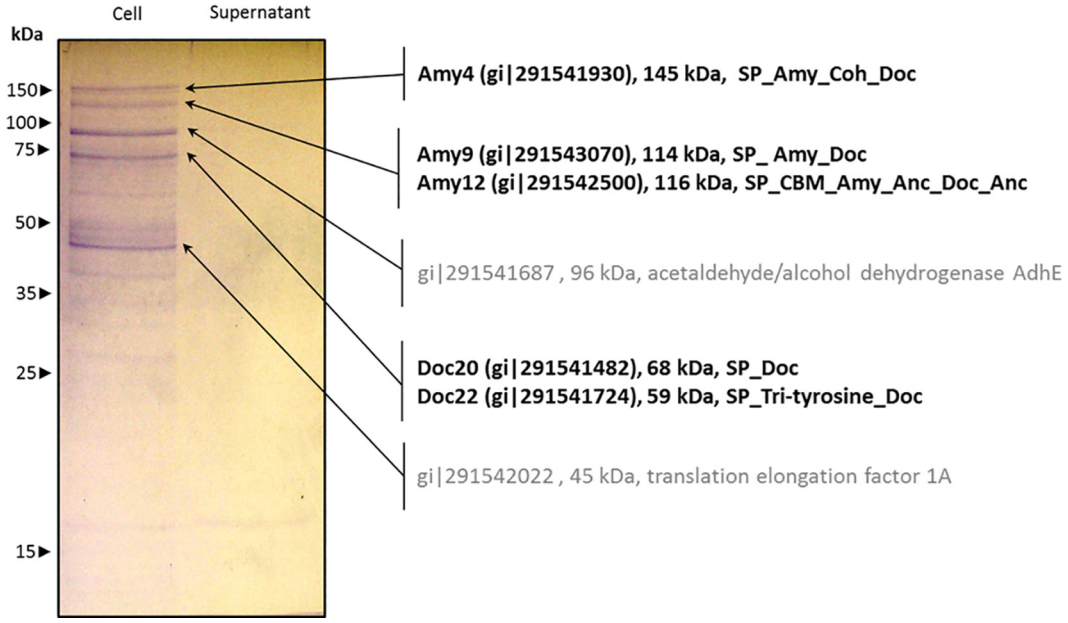


FIG 6 Identification of native *R. bromii* proteins that interact with overexpressed Amy4-Coh1. Cell-associated or cell-free supernatant proteins of *R. bromii* L2-63 grown with boiled Novelose RS3 starch as the energy source were incubated with His6-tagged Coh1 at 37°C as described in Materials and Methods. Proteins binding to Coh1 were recovered and separated by SDS-PAGE, followed by proteomic analysis of individual bands. Protein identifiers (ID), molecular-mass values, and conserved modules for identified proteins are shown on the right of the figure. Numbers on the left indicate sizes and positions of molecular mass markers. SP, signal peptide; Amy, amylase; Coh, cohesin; Doc, dockerin; Anc, cell wall surface anchor; CBM, carbohydrate-binding module; Tri-tyrosine, triple-tyrosine motif.

growth characteristics similar to those seen with *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 *R. bromii* L2-63.

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 *B. thetaiotaomicron* (8), *E. rectale* (13) and *Roseburia* spp. (11, 12) and provides further evidence

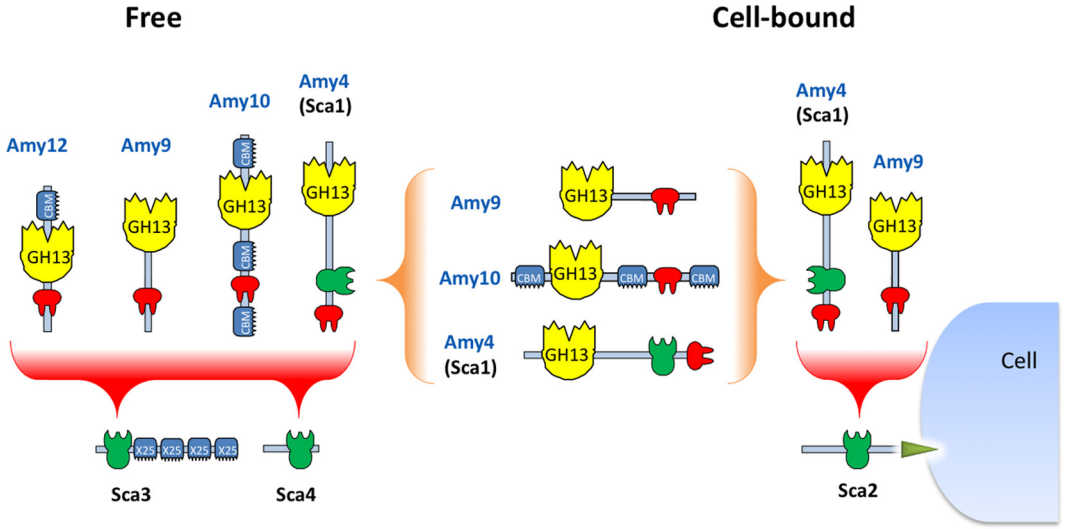


FIG 7 Potential dockerin-mediated interactions of *R. bromii* amylases with cohesin-carrying proteins and with each other. On the basis of observed interactions with recombinant cohesins and dockerins (Table 4 and Fig. 5), we can predict that the Amy4 and Amy9 enzymes are likely to bind to the cell surface via the Sca2 scaffoldin protein. Further complexes are likely to form between the Amy4, Amy9, and Amy10 proteins and between Amy4, Amy9, Amy10, and Amy12 and the Sca3 and Sca4 proteins. Binding of the enzymes to Sca3 would presumably confer starch-binding features to the resultant complex. Binding of dockerin-bearing amylases Amy9 and Amy10 to the cohesin of Amy4 would result in the formation of multienzyme complexes that can be part of a cell-bound or cell-free system. Amy4 also has the potential to self-aggregate through interactions between its own cohesin and dockerin.

that *R. bromii* is extremely specialized in its utilization of carbohydrate substrates.

In conclusion, we have presented evidence that four of the major extracellular starch-degrading enzymes in *R. bromii* are attached 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 cellulosomes 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 exceptional 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 project (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 dockerin 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 fecal 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), $NaHCO_3$ (0.4 g), resazurin (0.1 mg), biotin (1 μg), cobalamin (1 μg), *p*-aminobenzoic acid (3 μg), folic acid (5 μg), pyridoxamine (15 μg), K_2HPO_4 (0.045 g), KH_2PO_4 (0.045 g), NaCl (0.09 g), $(NH_4)_2SO_4$ (0.09 g), $MgSO_4 \cdot 7H_2O$ (0.009 g), and $CaCl_2$ (0.009 g) and the short-chain fatty acids (final concentrations) acetate (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 dispensed into Hungate tubes while the tubes were flushed with CO_2. After autoclaving, filter-sterilized solutions were added to give a final concentration of thiamine and riboflavin of 0.05 $\mu g \cdot ml^{-1}$ (each), pantothenate and nicotinamide of 1 $\mu g \cdot ml^{-1}$, pantethine of 50 $\mu g \cdot ml^{-1}$, and tetrahydrofolic acid of 0.1 $\mu g \cdot ml^{-1}$. Additional trace minerals $FeSO_4 \cdot 7H_2O$ (final concentration, 0.4 $\mu g \cdot ml^{-1}$), $ZnSO_4 \cdot 7H_2O$ (20 $ng \cdot ml^{-1}$), $MnCl_2 \cdot 4H_2O$ (6 $ng \cdot ml^{-1}$), H_3BO_3 (60 $ng \cdot ml^{-1}$), $CoCl_2 \cdot 6H_2O$ (40 $ng \cdot ml^{-1}$), $CuCl_2 \cdot 2H_2O$ (2 $ng \cdot ml^{-1}$), $NiCl_2 \cdot 6H_2O$ (4 $ng \cdot ml^{-1}$), $NaMoO_4 \cdot 2H_2O$ (6 $ng \cdot ml^{-1}$), and $NaSeO_3$ (15 $ng \cdot ml^{-1}$) and EDTA (1 $\mu g \cdot ml^{-1}$) 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 experiments. *R. bromii* L2-63 was grown anaerobically in 150 ml of RUM medium supplemented with 0.5% soluble potato starch (Sigma) at 37°C to an OD at 650 nm (OD_{650}) 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. Nonsolubilized 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 concentrations 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 Rabilloud buffer (7 M urea, 2 M thiourea, 4% CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}, 0.5% biolite Ampholite; pH 3 to 10) and 200 to 250 μg of protein was separated on two-dimensional gels using 17-cm-long immobilized 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 densest 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 inducibility 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 exponential phase (OD_{650} 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 $CaCl_2$ (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% fructose 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 supernatant was collected, dialyzed for 24 h with distilled H_2O (3 changes), frozen overnight at −20°C, and then lyophilized. The cell pellet was washed twice and resuspended in 50 mM sodium phosphate buffer (pH 6.5) before storage at −20°C. Cellular and supernatant protein samples 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 separating 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 washing 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 hydrolysis 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 GSRC-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 $CaCl_2$–0.05% Tween 20) at room temperature for 30 min. Afterward, chosen Xyn-Doc samples (3 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 $CaCl_2$–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 pulldown assay. The *R. bromii* L2-63 Coh1 module was cloned into pIVEX (5 Prime, Hamburg, Germany) and His6-tagged (N-terminal) versions of the product were overexpressed and purified *in vitro*. Coh1 was overexpressed using a cell-free RTS 100 *E. coli* HY overexpression system (5 Prime, Hamburg, Germany). His6-tagged proteins were bound to Talon Dynabeads (Invitrogen, Oslo, Norway) following the recommendations of the manufacturer. The beads with affinity-bound Coh1 modules were washed once, and then 500 μl of a lysate 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

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01058-15/-/DCSupplemental.

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.

ACKNOWLEDGMENTS

We acknowledge support from BBSRC grant no. BB/L009951/1, from the Scottish government Food, Land and People program, and from the Society for Applied Microbiology. E.A.B. is supported by a grant (no. 1349/13) from the Israel Science Foundation (ISF), Jerusalem, Israel, and by a grant from the United States-Israel Binational Science Foundation (BSF). E.A.B. is the incumbent of the Maynard I. and Elaine Wishner Chair of Bio-organic Chemistry.

Thanks are due to Fergus Nicol for proteomic analysis and to Auriane Bernard for enzyme assays on stationary-phase cultures. We also thank Julian Parkhill and Keith Turner (Wellcome Trust Sanger Institute, Cambridge, United Kingdom) for making the *R. bromii* L2-63 genome sequence available for analysis.

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