Anaerobe 17 (2011) 137–141

Contents lists available at ScienceDirect

Anaerobe

journal homepage: www.elsevier.com/locate/anaerobe

Molecular Biology, Genetics and Biotechnology

The human commensal *Bacteroides fragilis* binds intestinal mucin

Julie Y. Huang [1], S. Melanie Lee, Sarkis K. Mazmanian*

Division of Biology, California Institute of Technology, 1200 E. California Bl., Mail Code 156-29, Pasadena, CA 91125, USA

ARTICLE INFO

Article history:
Received 28 October 2010
Received in revised form
14 May 2011
Accepted 16 May 2011
Available online 2 June 2011

Keywords:
Bacteroides fragilis
Gut commensal
Mucin
Adhesion
Microbiota

ABSTRACT

The mammalian gastrointestinal tract harbors a vast microbial ecosystem, known as the microbiota, which benefits host biology. *Bacteroides fragilis* is an important anaerobic gut commensal of humans that prevents and cures intestinal inflammation. We wished to elucidate aspects of gut colonization employed by *B. fragilis*. Fluorescence in situ hybridization was performed on colonic tissue sections from *B. fragilis* and *Escherichia coli* dual-colonized gnotobiotic mice. Epifluorescence imaging reveals that both *E. coli* and *B. fragilis* are found in the lumen of the colon, but only *B. fragilis* is found in the mucosal layer. This observation suggests that physical association with intestinal mucus could be a possible mechanism of gut colonization by *B. fragilis*. We investigated this potential interaction using an *in vitro* mucus binding assay and show here that *B. fragilis* binds to murine colonic mucus. We further demonstrate that *B. fragilis* specifically and quantitatively binds to highly purified mucins (the major constituent in intestinal mucus) using flow cytometry analysis of fluorescently labeled purified murine and porcine mucins. These results suggest that interactions between *B. fragilis* and intestinal mucin may play a critical role during host-bacterial symbiosis.

Following a sterile birth, the gastrointestinal (GI) tracts of humans and all mammals coordinately assemble a diverse multitude of microorganisms, collectively known as the microbiota. It has been acknowledged for decades that many of these microorganisms live symbiotically with their hosts, performing beneficial functions such as metabolizing complex carbohydrates and providing essential nutrients [1]. Recent studies have shown that the microbiota critically augments the development and function of the immune system (reviewed in [2] and [3]). Although the microbial diversity in the mammalian gut is vast (with an estimated 500–1000 species of microorganisms present in the human), organisms belonging to the genus *Bacteroides* represent one of the most abundant microbial taxa in both mice and humans [4]. *Bacteroides fragilis* is a ubiquitous Gram-negative anaerobic bacterium that inhabits the lower GI tract of most mammals [5]. Recent findings have revealed that this organism possesses the ability to direct the cellular and physical maturation of the host immune system and to protect its host from experimental colitis [6–8]. Therefore, the contributions of the microbiota to human health appear to be profound.

We wanted to understand how *B. fragilis* colonizes the mammalian gut. *B. fragilis* expresses at least eight distinct surface capsular polysaccharides (CPS), and previous studies have shown that CPS expression by the bacterium is required for successful intestinal colonization [9,10]. How these molecules mediate the initial interactions with the host, and whether they are involved in long-term persistence in the gut are currently unknown. Several mechanisms of gut colonization by symbiotic bacteria have been studied. Some organisms form biofilms, composed of a polymeric matrix secreted by the bacteria, which adhere to the epithelial layer. Others interact with components of the mucosal layer (reviewed in [11]). Mucus is a viscous stratum which separates epithelial cells from the lumen of the gut and acts as a crucial barrier against infection by pathogens. Various membrane-bound or secreted glycoproteins called mucins associate with one another to form the gel-like mucus. Interactions between gut bacteria and mucus have been hypothesized to be important for the assembly and stability of the microbiota [12]. Accordingly, we sought to determine whether or not *B. fragilis* binds intestinal mucus and purified mucin.

Initially, we visualized the spatial localization in the colon of 2 different commensal bacteria to determine potential differences in association with the mucus layer *in vivo*. Wild-type *B. fragilis* NCTC9343 was grown anaerobically in brain-heart infusion (BHI) supplemented with hemin (5 μg/ml) and vitamin K (0.5 μg/ml), and *Escherichia coli* BL21 was grown aerobically in BHI at 37 °C. Bacteria were grown to OD_{600} of 0.7–0.8, and 1×10^8 colony forming units (CFUs) were orally inoculated into germ-free Swiss Webster mice by gavage. Following 1 week of colonization, mice were sacrificed and colon tissue was fixed in Carnoy's solution and embedded in

* Corresponding author. Tel.: +1 626 395 2356.
E-mail address: sarkis@caltech.edu (S.K. Mazmanian).
[1] Present address: Dept. of Microbiology and Immunology, Fairchild Science Building D300, 299 Campus Drive, Stanford University, Stanford, CA 94305, USA.

1075-9964/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.anaerobe.2011.05.017

paraffin wax for sectioning. Fluorescence in situ hybridization was performed on tissue sections mounted on glass slides using a 6-carboxyfluorescein (6-FAM)-labeled oligonucleotide probe for *E. coli* (EnterbactB [AAGCCACGCCTCAAGGGCACAA]) and a Cy3-labeled oligonucleotide probe for *B. fragilis* (Bfra602 [GAGCCG-CAAACTTTCACAA]) (Integrated DNA Technologies, Inc.). Briefly, slides were deparaffinized, dried, and hybridized with both probes at 5 ng/μl concentration each for 2 h at 46 °C in hybridization buffer (0.9 M NaCl, 15% formamide, 20 mM Tris–HCl (pH 7.4), and 0.01% sodium dodecyl sulfate (SDS)). Slides were washed for 15 min at 48 °C in wash buffer (20 mM Tris–HCl (pH 7.4), 318 mM NaCl, and 0.01% SDS). For visualization of the colon epithelial cell nuclei, the slides were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI). The autofluorescence background allowed visualization of the tissue structures. The slides were examined with an Axioplan microscope (Zeiss, Oberkochen, Germany) using a 100× oil immersion objective. Epifluorescence images of a cross section through the colon of gnotobiotic mice that were dual-colonized with both *E. coli* and *B. fragilis* reveal that both bacteria are found in the lumen of the gut in high abundance (Fig. 1). Surprisingly however, only *B. fragilis* is found in the mucus layer that lies between the lumen and the gut epithelium tissue (Fig. 1). The spatial segregation of *B. fragilis* and *E. coli* across the colon mucus barrier suggests that *B. fragilis* may interact with mucus *in vivo* and this may be important for sustained colonization of commensal *B. fragilis*. Furthermore, these results reveal that not all bacteria are equally able to penetrate the mucus layer, suggesting dedicated mucus associating functions for *B. fragilis*.

To test the hypothesis that *B. fragilis* colonization of the distal gut is mediated by mucus binding, a standard mucus binding assay was used to determine if live bacteria are able to bind a crude, intestinal mucus preparation. Crude mucus was isolated from the colon and cecum of conventionally-colonized Swiss Webster mice as described in Cohen et al. [13]. Briefly, colonic and cecal mucus was scraped into HEPES-Hanks' Buffer (pH 7.4 with Calcium Chloride and Magnesium Chloride). Next, non-soluble material was removed by centrifuging once at 12,000 × g for 10 min at 4 °C, and then once at 26,500 × g for 15 min at 4 °C. The final concentration of the crude mucus solution was determined by the Bradford assay. The mucus was diluted with HEPES-Hanks' Buffer to 1 mg/ml. 0.2 ml of mucus was added into the wells of a 24-well tissue culture plate and incubated overnight at 4 °C. Controls included wells containing 0.2 ml of a 1 mg/ml solution of Bovine Serum Albumin (BSA, which served as a specificity control) or 0.2 ml of HEPES-Hanks' Buffer (which served as a negative control). The wells were washed with HEPES-Hanks' Buffer to remove non-immobilized proteins. The plate was UV-sterilized for 10 min and was then ready for use in the mucus binding assay. 1×10^8 CFUs of bacteria were added to both the immobilized mucus and the BSA control, and incubated at 37 °C for 1 h. Wells were washed with HEPES-Hanks' Buffer, treated with 0.05% trypsin for 10 min at room temperature to liberate bacteria. One milliliter of cold BHI was added to quench the trypsin activity. Samples were serially diluted and plated for CFUs. Fig. 2A shows that *B. fragilis* binds to crudely purified mucus *in vitro*, as determined by recovered CFUs. The BSA- and buffer-containing wells illustrate low background binding. A mutant strain of *B. fragilis* (CPM1), which only expresses one of the eight CPS [9], is able to bind mucus as effectively as wild-type *B. fragilis*, suggesting that CPS expression does not mediate mucus binding. Therefore, *B. fragilis* specifically binds intestinal mucus via a mechanism that appears not to involve expression of multiple surface polysaccharides.

Next, a mucus binding competition assay was performed to determine if the interaction between *B. fragilis* and mucus is saturable. We reasoned that as *B. fragilis* is pre-coated with higher concentrations of excess mucus, fewer putative receptors would be available to bind immobilized mucus in the well. Briefly, 1×10^8 CFUs of *B. fragilis* were incubated with excess mucus at 37 °C for 2 h under aerobic conditions with shaking. Bacteria were washed and added to

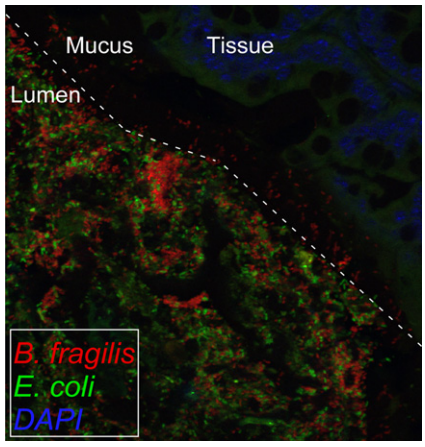

Fig. 1. Colon tissue section from a *B. fragilis* and *E. coli* dual-colonized Swiss Webster mouse. Epifluorescence image of bacteria visualized by FISH, and the epithelial cells counterstained with DAPI (blue) to visualize DNA. Both *E. coli* (green) and *B. fragilis* (red) are found in the lumen but only *B. fragilis* is found in the mucus layer. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

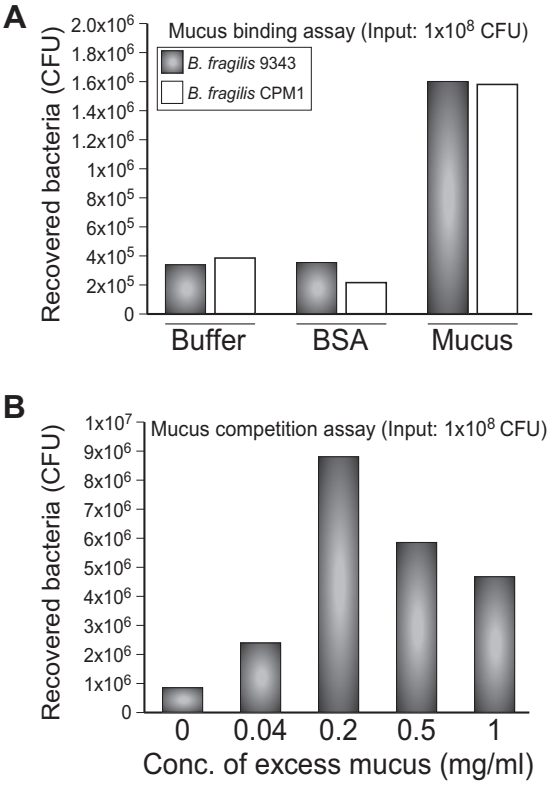

Fig. 2. *B. fragilis* binds intestinal mucus. (A) Number of *B. fragilis* (in CFUs) recovered after 1 h incubation in wells with an immobilized mucus layer, an immobilized BSA layer, or buffer only. Of the 1×10^8 CFUs incubated, 1.6×10^6 (1.6%) bound to immobilized mucus. The CPM1 mutant binds mucus similarly to wild-type bacteria. These data are representative of four independent trials. (B) Number of bacteria recovered from mucus binding assay after a 2 h pre-incubation with different concentrations of excess mucus. These data are representative of three independent trials.

wells of a 24-well tissue culture plate containing either immobilized mucus, BSA, or nothing (prepared as above). After 1 h, samples were treated with trypsin, serially diluted, and plated for CFUs. Unexpectedly, pre-incubation with excess mucus appeared to increase *B. fragilis* binding to mucus with a bi-phasing profile (Fig. 2B). Binding to immobilized mucus reached a peak when *B. fragilis* was pre-incubated with 0.2 mg/ml of excess mucus. Pre-incubation of bacteria with excess mucus at concentrations higher than 0.2 mg/ml resulted in a decrease in mucus binding, yet binding was still higher than without pre-incubation with mucus. Pre-incubation of bacteria with 0.4 mg/ml and 1 mg/ml of BSA did not affect binding, once again showing that the *B. fragilis*-mucus interaction is specific (data not shown). These results suggest that bacteria pre-incubated with mucus (and not BSA) have an increased ability to bind immobilized mucus until putative receptors are saturated at the highest mucus concentrations. Further experiments are required to determine if dedicated molecules on the bacterial surface mediate mucus binding.

In addition to mucin, intestinal mucus is known to contain host molecules, such as anti-microbial peptides, immunoglobulin A (IgA) antibodies, and lysozyme [13]. We wished to determine if mucus binding by *B. fragilis* was specific to mucin. As murine colonic mucin is not commercially available, we purified mucins from Swiss Webster mice based on the protocol by Shekels et al. [14] with a few modifications. Fig. 3 illustrates a schematic of this modified protocol and the analysis of mucin purity. We then tested the purified mucin and BSA for specific binding by *B. fragilis*. Purified mucin and the BSA control were labeled with Thermo Scientific DyLight Amine-Reactive Fluor 488, and unbound fluorophores were removed from the sample via dialysis against PBS. *B. fragilis* was pre-incubated with either unlabeled BSA or PBS and was subsequently incubated with labeled mucin or labeled BSA for 30 min at room temperature. The bacteria were washed after each incubation to remove non-adherent material. Percentage of mucin-binding bacteria in each sample was determined by flow cytometry. When *B. fragilis* was incubated with fluorescently labeled BSA, no binding was detected (Fig. 4A). However, when *B. fragilis* was incubated with labeled mucin, a significant number of *B. fragilis* was detected by flow cytometry. Pre-incubation with BSA did not diminish the percentage of *B. fragilis* adherent to mucin (Fig. 4A). Taken together, *B. fragilis* binds specifically to purified murine colonic mucin and not to BSA.

B. fragilis colonizes the intestines of most mammalian species studied to date [5]. In order to determine if mucin interactions extend beyond the murine host, we examined the ability of *B. fragilis* to bind porcine mucin. Starting with partially purified porcine gastric mucin purchased from Sigma Aldrich, we purified mucin to homogeneity using the same protocol as described above. Fig. 4B shows that a significant amount of mucin-binding bacteria were detected by flow cytometry, indicating that *B. fragilis* bound the fluorescently labeled purified porcine mucin. Both approaches we used in this study to demonstrate mucus binding resulted in only a small portion of

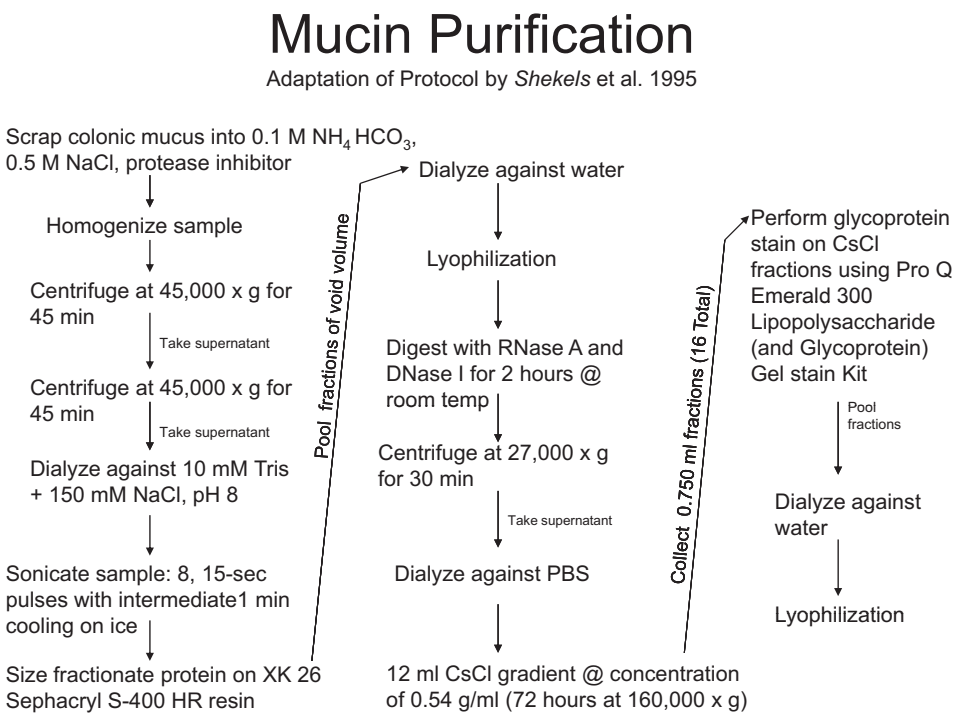

Fig. 3. Schematic of the mucin purification protocol. Briefly, crude mucus was scraped from the colon and cecum of conventionally-colonized 8-week-old male Swiss Webster mice into 0.1 M NH_4HCO_3, 0.5 M NaCl, and a cocktail of protease inhibitors on ice. The sample was then homogenized and centrifuged at 45,000 × g for 45 min at 4 °C. Non-soluble material was removed before centrifuging again at 45,000 × g for another 45 min at 4 °C. The supernatant was taken and dialyzed against 10 mM Tris pH 8.0 + 150 mM NaCl for about 24 h. Next, the sample was sonicated at eight 15-s pulses with intermediate 1 min cooling on ice on a Brason Sonicator at speed 3 to break up large aggregates and then centrifuged once more at 45,000 × g for 45 min. Next HPLC was employed whereby the supernatant was size fractionated on an XK 26/70 column containing Sephacryl S-400 resin (equilibrated in 10 mM Tris, pH 8.0). The void volume (which contained the large mucin glycoproteins) was collected and dialyzed against water for about 36 h and then lyophilized. The lyophilized glycoproteins were resuspended in a solution containing RNase A and DNase I and digested for 2 h at room temperature. After the digestion, the sample was centrifuged at 27,000 × g for 30 min at 4 °C and the supernatant was dialyzed against *p*hosphate *b*uffered saline (PBS) for 36 h. Cesium chloride was added to the dialyzed supernatant to a final concentration of 0.54 g/ml, and then centrifuged at 160,000 × g for 72 h. One milliliter fractions were collected and analyzed with the Pro Q Emerald Glycoprotein Staining Kit to determine which fractions contained the purified mucins. The mucin-containing fractions were pooled, dialyzed against water for 24 h, lyophilized, and then stored at −20 °C. Positive fractions from gel filtration chromatography were identified by absorbance readings at 280 nm. CsCl fractions and final product were assayed to contain mucin by glycoprotein staining (data not shown).

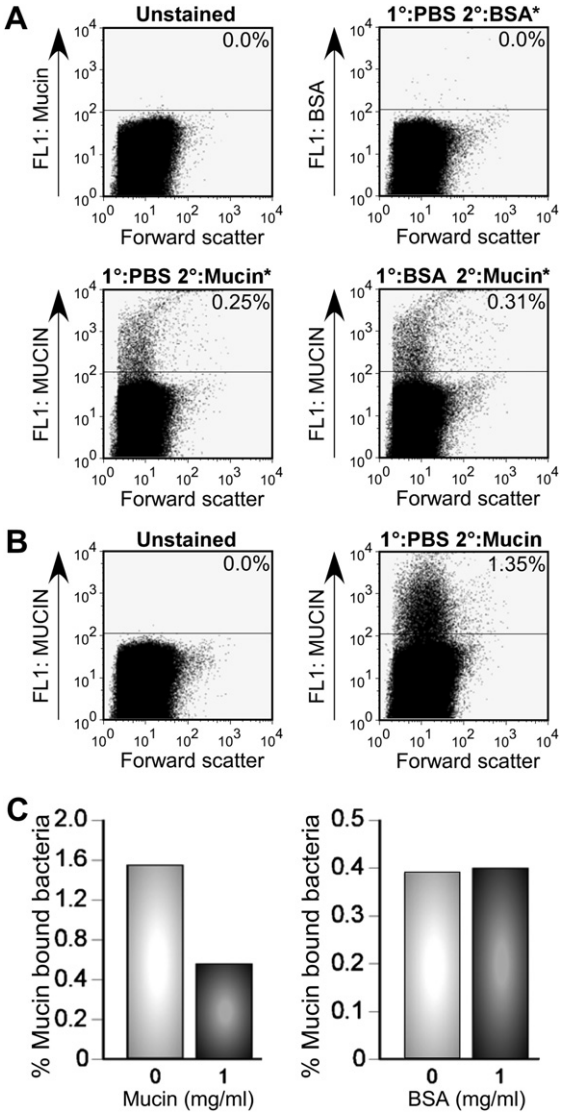

Fig. 4. *B. fragilis* binds soluble murine and porcine mucin. (A) Flow cytometry plots indicating percentage of *B. fragilis* bound to fluorescently labeled murine colonic mucin. Cells were either pre-incubated with BSA or not (1°), and secondary incubations were with fluorescently labeled BSA or mucin (denoted by asterisk). Percentages represent bacteria bound to fluorescently-labeled mucin relative to total number of bacteria analyzed per sample. These data are representative of two independent trials. (B) Percentage of *B. fragilis* bound to fluorescently labeled porcine mucin with no pre-incubation. These data are representative of two independent trials. (C) Percentage of *B. fragilis* bound to fluorescently labeled porcine gastric mucin following pre-incubation with unlabeled mucin (left) or unlabeled BSA (right). Porcine mucin was purchased from a commercial source and purified as described in Fig. 3 from the RNase/DNase digestion step. These data are representative of two independent trials.

bacterial binding (~1.6% for the immobilized plate assay and ~1.5% for the soluble mucin binding assay). This is consistent with the known ability of *B. fragilis* to be highly phase variable whereby only a portion of the bacterial population express a given surface molecule [15]. Fig. 4C shows that pre-incubation with 1.0 mg/ml of unlabeled mucin was able to compete with the fluorescently-labeled mucin, resulting in a lower percentage of bacteria binding to the fluorescently labeled mucin. Pre-incubation with BSA shows no inhibition (Fig. 4C), serving as a specificity control. Our results show that *B. fragilis* specifically binds porcine mucin in addition to murine mucin.

B. fragilis has emerged as a model symbiont for the study of host–microbial interactions with the immune system [3]. The mechanism by which *B. fragilis* maintains long-term colonization of the mammalian intestine remains unknown. Associations with mucus may involve bacterial binding, and/or nutrient utilization of mucin for bacterial growth. If binding to mucus is involved during the colonization process *in vivo*, we predict that *B. fragilis* would express defined and dedicated receptor(s) with specific affinity for mucin. Along these lines, the *B. fragilis* genome and other sequenced *Bacteroides* species express numerous homologs of the SusC/SusD proteins, which are known to bind starch and other carbohydrates that decorate the mucin glycoproteins [16]. Furthermore, SusC/SusD proteins of *B. fragilis* were recently shown to be phase variable [17]. This property is similar to the phase variability of capsular polysaccharides, whereby only a small fraction of bacteria express any one of the eight CPS of *B. fragilis* [9]. If mucus binding is also phase variable, this would explain why only a small percentage of bacteria invade the mucus layer (as shown in Fig. 1), and why only a small fraction of bacteria bind mucus and mucin *in vitro* (as shown in Figs. 2 and 4). A non-mutually exclusive function for mucus binding may be the use of host derived sugars as a carbon source. Several studies have shown that *B. fragilis* can degrade mucin and utilize it as a nutrient source for growth [18,19]. In fact, *B. fragilis* can utilize porcine mucin as a sole source for carbon and nitrogen [20], and structural analysis of the SusD homolog of *Bacteroides thetaiotaomicron* (also found in *B. fragilis*) suggests it binds sugars liberated from mucin glycoproteins [21]. Therefore, mucus binding may serve as a physical mechanism for sustained colonization, as a means to degrade and import nutrients into the bacterial cell for growth, or both. We have shown here that *B. fragilis* specifically binds intestinal mucin (although *B. fragilis* may also bind to other components in the mucus) and associates with the mucus layer *in vivo*. These findings, along with previous work, suggest that specific interactions between *B. fragilis* and mucus are relevant for *in vivo* colonization of animals. The identity of dedicated mucin-binding receptor(s), and a molecular mechanism during long-term association of the mammalian gut, await discovery.

Acknowledgments

We are grateful to Dr. William Clemons, Jr. (Caltech) and Justin Chartron (Caltech) for help with mucin purification. S.K.M. is a Searle Scholar. Work in the laboratory of the authors is supported by funding from the National Institutes of Health (DK078938, DK083633), Damon Runyon Cancer Research Foundation and the Crohn's and Colitis Foundation of America to S.K.M.

References

[1] Hooper LV, Gordon JI. Commensal host-bacterial relationships in the gut. Science 2001;292:1115–8.
[2] Macpherson AJ, Harris NL. Interactions between commensal intestinal bacteria and the immune system. Nat Rev Immunol 2004;4:478–85.
[3] Round JL, Mazmanian SK. The gut microbiota shapes intestinal immune responses during health and disease. Nat Rev Immunol 2009;9:313–23.
[4] Ley RE, Peterson DA, Gordon JI. Ecological and evolutionary forces shaping microbial diversity in the human intestine. Cell 2006;124:837–48.
[5] Ley RE, Hamady M, Lozupone C, Turnbaugh PJ, Ramey RR, Bircher JS, et al. Evolution of mammals and their gut microbes. Science 2008;320:1647–51.
[6] Mazmanian SK, Liu CH, Tzianabos AO, Kasper DL. An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. Cell 2005;122:107–18.
[7] Mazmanian SK, Round JL, Kasper DL. A microbial symbiosis factor prevents intestinal inflammatory disease. Nature 2008;453:620–5.
[8] Round JL, Mazmanian SK. Inducible Foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota. Proc Natl Acad Sci U S A 2010;107:12204–9.
[9] Liu CH, Lee SM, Vanlare JM, Kasper DL, Mazmanian SK. Regulation of surface architecture by symbiotic bacteria mediates host colonization. Proc Natl Acad Sci U S A 2008;105:3951–6.
[10] Coyne MJ, Chatzidaki-Livanis M, Paoletti LC, Comstock LE. Role of glycan synthesis in colonization of the mammalian gut by the bacterial symbiont *Bacteroides fragilis*. Proc Natl Acad Sci U S A 2008;105:13099–104.
[11] Macfarlane S, Dillon JF. Microbial biofilms in the human gastrointestinal tract. J Appl Microbiol 2007;102:1187–96.

[12] Sonnenburg JL, Angenent LT, Gordon JI. Getting a grip on things: how do communities of bacterial symbionts become established in our intestine? Nat Immunol 2004;5:569–73.
[13] Cohen PS, Laux DC. Bacterial adhesion to and penetration of intestinal mucus in vitro. Methods Enzymol 1995;253:309–14.
[14] Shekels LL, Lyftogt C, Kieliszewski M, Filie JD, Kozak CA, Ho SB. Mouse gastric mucin: cloning and chromosomal localization. Biochem J 1995;311:775–85.
[15] Kuwahara T, Yamashita A, Hirakawa H, Nakayama H, Toh H, Okada N, et al. Genomic analysis of *Bacteroides fragilis* reveals extensive DNA inversions regulating cell surface adaptation. Proc Natl Acad Sci U S A 2004;101: 14919–24.
[16] Xu J, Mahowald MA, Ley RE, Lozupone CA, Hamady M, Martens EC, et al. Evolution of symbiotic bacteria in the distal human intestine. PLoS Biol 2007; 5:e156.
[17] Nakayama-Imaohji H, Hirakawa H, Ichimura M, Wakimoto S, Kuhara S, Hayashi T, et al. Identification of the site-specific DNA invertase responsible for the phase variation of SusC/SusD family outer membrane proteins in *Bacteroides fragilis*. J Bacteriol 2009;191:6003–11.
[18] Roberton AM, Stanley RA. In vitro utilization of mucin by *Bacteroides fragilis*. Appl Environ Microbiol 1982;43:325–30.
[19] Tsai HH, Sunderland D, Gibson GR, Hart CA, Rhodes JM. A novel mucin sulphatase from human faeces: its identification, purification and characterization. Clin Sci (Lond) 1992;82:447–54.
[20] Macfarlane GT, Gibson GR. Formation of glycoprotein degrading enzymes by *Bacteroides fragilis*. FEMS Microbiol Lett 1991;61:289–93.
[21] Koropatkin N, Martens EC, Gordon JI, Smith TJ. Structure of a SusD homologue, BT1043, involved in mucin O-glycan utilization in a prominent human gut symbiont. Biochemistry 2009;48:1532–42.