Viscosity Gradient Within the Mucus Layer Determines the Mucosal Barrier Function and the Spatial Organization of the Intestinal Microbiota

Alexander Swidsinski, MD, Beate C. Sydora, PhD,† Yvonne Doerffel, MD,‡ Vera Loening-Baucke, MD, Mario Vaneechoutte, MD,§ Maryla Lupicki,† Juergen Scholze, MD,‡ Herbert Lochs, MD, | and Levinus A. Dieleman, MD, PhD†*

Background: Migration is an important virulence factor for intestinal bacteria. However, the role of bacterial mobility in the penetration of viscous mucus and their spatial organization within the colon is relatively unknown.

Methods: Movements of fecal bacteria were assessed in gels of varying agarose concentrations and were compared with patterns of bacterial distribution observed in colons from conventional and *Enterobacter cloacae*-monoassociated mice. Bacteria were visualized using fluorescence in situ hybridization.

Results: Long curly bacteria moved best in moderate viscosity gels, short rods and cocci preferred a low viscous environment, whereas high viscosity immobilized all bacterial groups. The spatial distribution of bacteria in the murine colon was also shape- and not taxonomy-dependent, indicating the existence of vertical (surface to lumen) and longitudinal (proximal to distal colon) viscosity gradients within the mucus layer. Our results suggest that mucus viscosity is low in goblet cells, at the crypt basis and close to the intestinal lumen, whereas sites adjacent to the columnar epithelium have a high mucus viscosity. The mucus viscosity increased progressively toward the distal colon, separating bacteria selectively in the proximal colon and completely in the distal colon.

Conclusions: The site-specific regulation of mucus secretion and dehydration make the mucus layer firm and impenetrable for bacteria in regions close to the intestinal mucosa but loose and lubricating in regions adjacent to the luminal contents. Selective control of mucus secretion and dehydration may prove to be a key factor in the management of chronic diseases in which intestinal pathogens are involved.

(*Inflamm Bowel Dis 2007;13:963–970)*

Key Words: mucus barrier, spatial organization, intestinal microbiota, FISH, bacterial motility

Subcutaneous or intravenous injection of even small quantities of *Bacteroides, Enterobacteriaceae*, streptococci, or *Clostridium perfringens* is life-threatening. At the same time, these bacteria reside in every healthy colon and reach amounts of $\approx 10^{10}$ CFU/g of stool. Obviously, the untroubled coexistence with the so-called "normal intestinal microflora" is only possible as long as the mucosal barrier is intact. Many hypotheses address the mechanisms by which the mucosa is able to discriminate between pathogens and harmless commensal bacteria and to respond appropriately. Little is known about the local arrangement of this encounter and the exact composition of the microorganisms that contact the mucosa. Previous investigations have shown that intestinal bacteria are not evenly mixed, but are spatially organized.[1,2] The composition of bacteria differs in mucosa-adjacent and luminal regions and each segment of the mouse colon has characteristically assembled microbiota.[2] The reasons for the spatial organization of the intestinal microflora may be numerous: oxygen concentrations, innate and acquired immunity constituents (e.g., defensins, immunoglobulins, phosphotidylcholine, trefoil peptides), various nutrients, intraluminal pH and mucus viscosity differ depending on the intestinal region and the proximity to the colonic mucosa.[3] Each of these factors may interfere with bacterial growth. Complex bacterial communities can also self-regulate their composition. Many microorganisms co-aggregate and form biofilms displaying synergistic and antagonistic properties.[4,5] Detailed knowledge of host–microbial interactions with the mucosal surface may help us to understand the pathogenesis

Recieved for publication March 19, 2007; accepted March 20, 1007.

From the *Humboldt University, Charité Hospital, CCM, Laboratory for Molecular Genetics, Polymicrobial Infections and Bacterial Biofilms, Berlin, Germany, †Centre of Excellence for Gastrointestinal Inflammation and Immunity Research, University of Alberta, Edmonton, Alberta, Canada, ‡Outpatient Clinic, Humboldt University, Charité, CCM, Berlin, Germany, §Department Clinical Chemistry, Microbiology & Immunology, University Hospital Ghent, Belgium, | Department of Medicine, Section of Gastroenterology, Hepatology and Endocrinology, Humboldt University, Charité Hospital, CCM, Berlin, Germany.

*Reprints: Alexander Swidsinski, Humboldt University, Charité Hospital, CCM, Laboratory for Molecular Genetics, Polymicrobial Infections and Bacterial Biofilms, 10098 Berlin, Germany (e-mail: alexander.swidsinski@charite.de).

DOI 10.1002/ibd.20163

Published online 23 April 2007 in Wiley InterScience (www.interscience.wiley.com).

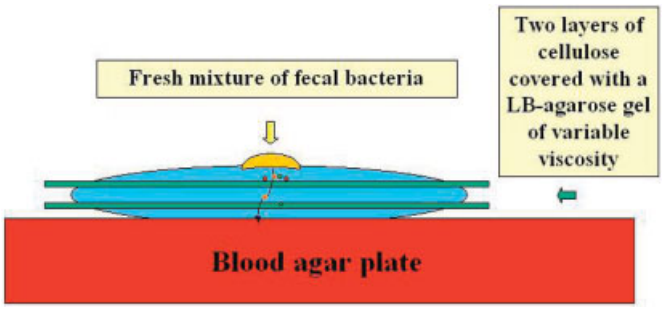


FIGURE 1. Diagrammatic representation of the culture medium, agarose, cellulose experimental setup to determine in vitro bacterial motility in different viscosities.

of inflammatory bowel disease (IBD) and to develop novel diagnostic and therapeutic approaches.

However, before bacteria contact the epithelial cells they must cross the viscous mucus layer, which continuously covers the colonic wall. Although the ability to move is a basic property of bacteria, the mechanisms of propulsion differ depending on bacterial morphology.[6,7] Coccoid bacteria float mostly by pili. Short rods swim using flagella or membranes. Long and curly shapes enable complex body movements such as gliding, crawling, and squeezing, which are crucial in semisolid environments.[6–8] Specific bacterial morphology brings advantages or shortcomings within a defined range of viscosity. Since bacterial metabolism and coaggregation are not shape-dependent, the finding of selected bacterial morphotypes within defined regions of intestine could indicate differences in local viscosity and allow mapping of viscosity gradients.

Therefore, our aims were to investigate the role of bacterial shape, mobility, and mucus viscosity in the spatial organization of murine colonic microbiota using fluorescence in situ hybridization (FISH).

MATERIALS AND METHODS

In Vitro Motility Assay of Fecal Bacteria Within Agarose Gels of Varying Concentrations

The ability of bacteria to move in viscous environments was investigated on gel layers fixed between 2 sheets of cellulose overlaying blood agar plates (Fig. 1). The gels were prepared with Luria Bertani base medium (LB Broth, Oxoid, Wesel, Germany) by adding different amounts of agarose (Agarose Standard EEO, Serva, Heidelberg, Germany) to adjust the semisolid viscosity. A diagram of the method is shown in Figure 1. Briefly, LB-agarose was autoclaved and chilled to 60°C. In a laminar flow box, 40 × 40 mm large sheets of autoclaved cellulose (wrapping paper, Schlecker, Ehingen, Germany) were dipped into LB-agarose, chilled for 5 seconds at room temperature, and laid on top of a blood agar plate, 2 sheets on top of each other. A set of plates was prepared for 0.2%, 0.3%, 0.4%, 0.6%, and 0.7% LB-agarose and the plates were oxygen prereduced overnight. A fresh fecal sample (50 mg) was suspended in 500 μL of LB medium. A drop of fecal suspension was pipetted onto the center of each LB-agarose set. The plates were incubated in anaerobe jars. The cellulose sheets with the enclosed gel layers were removed from the agar plates after 28 hours of anaerobic culture, cut to 3 × 20 mm size, fixed in Carnoy solution (6/3/1 vol. ethanol/glacial acetic acid/chloroform) for 3 hours, then embedded in paraffin.

The gel layer, which covered the surface of the upper cellulose sheet, and the gel layer between the 2 sheets were well preserved. The gel between the 2 cellulose sheets was used for the quantification of bacterial movement across the viscous environment.

Mice

Intestinal segments were derived from 5 euthanized germ free-raised 129/SvEv mice (between 12 and 20 weeks of age) that were monoassociated with *Enterobacter cloacae* after oral gavage 5–10 weeks before necropsy. These gnotobiotic mice were raised and maintained in sterile isolators at the Health Science Lab Animal Facility at the University of Alberta, Canada. In addition, intestines were obtained from 10 129/SvEv mice raised under SPF housing at the animal care unit of the Charité Hospital (Berlin, Germany). The intestinal segments were fixed in Carnoy's solution for 3–6 hours (6/3/1 vol. ethanol/glacial acetic acid/chloroform), embedded in paraffin using standard techniques, cut into 4-μm sections and placed on SuperFrost slides (R. Langenbrinck, Emmendingen, Germany) for pathologic examination and FISH studies. Portions of proximal and distal colon were obtained from each mouse and analyzed separately.

The patterns of spatial organization of intestinal bacteria within normal murine colon were compared to viscosity-dependent spatial arrangement of bacteria in vitro, as described above. Shape, taxonomy, and location of different bacterial groups with regard to each other and to the mucosal surface in murine colon were recorded and interpreted in terms of local viscosity, which would lead to similar segregation in vitro. To confirm the major impact of the mucus viscosity for spatial distribution of specific bacterial morphotypes in vivo, we also compared conventionally raised mice with gnotobiotic mice monoassociated with *Enterobacter cloacae,* in which other, potentially interfering intestinal bacteria were absent.

Fluorescence In Situ Hybridization

Oligonucleotide probes were synthesized with a Cy3- or Cy5-reactive fluorescent dye at the 5′ end (MWG Biotech, Ebersberg, Germany). Sixteen domain-, group-, and species-specific FISH probes and a bacterial universal probe (Eub 338) (Table 1) were applied to proximal and distal murine

TABLE 1. FISH Probes

Name[a]	Target	Reference
Eub338	Kingdom (*Eu)Bacteria*	9
Ebac	*Enterobacteriaceae* (incl. *Enterobacter cloacae*)	10
Erec	*Eubacterium rectale* group	11
Lach	subgroup of Erec (incl. *Lachnospira multipara*)	12
Ehal	subgroup of Erec (incl. *Eubacterium hallii*)	12
Clit135	*Clostridium lituseburense* group (incl. *Clostridium difficile*)	11
Lab158	*Lactobacillus* and *Enterococcus* group	13
Cor653	*Coriobacterium* group	14
Ecyl	*Eubacterium cylindroides*	12
Phasco	*Phascolarctobacterium faecium*	12
Veil	*Veillonella* group	12
Rbro	*Ruminococcus bromii*	12
Rfla	*Ruminococcus flavefaciens*	12
UroA	*Ruminococcus obeum*-like bacteria (subgroup of Erec)	15
Mib 661	Mice-specific *Bacteroides* groups	16
Bac303	*Bacteroides/Prevotella* group	17

[a]The numbers behind probes indicate the position within the *Escherichia coli* 16S rRNA-gene and are not repeated in the text.

colon sections in multicolor FISH. Formamide concentrations and hybridization temperatures were used as previously described to achieve optimal stringency.[9–17]

For in vitro experiments the Erec-Cy5 probe (*Eubacterium rectale* group) was chosen to visualize long rods (red fluorescence), the Bac-Cy3 probe (*Bacteroides* group) for small coccoid rods (yellow fluorescence), and the Eub-FITC (fluorescein isothiocyanate) probe for all bacteria (green fluorescence).

Microscopy was performed using a Nikon e600 fluorescence microscope. The images were photodocumented using a Nikon DXM1200F color camera and software (Nikon, Tokyo, Japan). Bacteria were identified according to previously described criteria.[18]

For each group-specific FISH probe, high-power ($\times$1000 magnification) images were made. Bacteria were counted within a 50-μm^2 area of the microscopic field representative of the region of interest. The conversion of the numbers within a microscopic field to concentrations of bacteria per mL was based on the calculation that a 10-μL sample with a cell concentration of 10^7 cells per mL has 40 cells per average microscopic field at a magnification of 1000; the details of conversion were previously described.[2] Additional light microscopic photos of successive sections stained with Alcian blue/PAS (Periodic Acid Schiff) were used for evaluation of mucus and leukocytes in tissue sections.

Statistics

Mean values and standard deviations (SDs) were calculated from the bacterial counts. Using Student's *t*-test and the chi-square test, a P-value < 0.05 was considered significant.

RESULTS

Bacterial Movement Across Gels of Different Agarose Concentrations

The numerical data illustrating the movement of single bacterial groups across LB agarose after 28 hours of anaerobic growth are shown in Figure 2.

The mobility of short coccoid rods, represented by bacteria that hybridized with the Bac-Cy3 probe (*Bacteroides* group), was highest in low-viscosity gels of 0.2% LB-agarose. The mobility of *Bacteroides* decreased with increasing viscosity and completely ceased at agarose concentrations of $\geq$0.5%.

Long curly rods represented by EREC-Cy5-positive organisms (*Eubacterium rectale* group) were immobile at agarose concentrations of 0.2%, started to move at 0.3%, and reached maximum velocity in gels with agarose concentrations between 0.4–0.5%. The velocity decreased progressively at higher agarose concentrations.

Spatial Segregation of Different Bacterial Groups In Vitro

Bacteria moved from the initial location and segregated in distinct, differently composed layers, which were spatially detached from each other and from the original mix. The segregation can be clearly seen in multicolor FISH analysis with simultaneous application of 3 differently labeled probes (Fig. 3A–D).

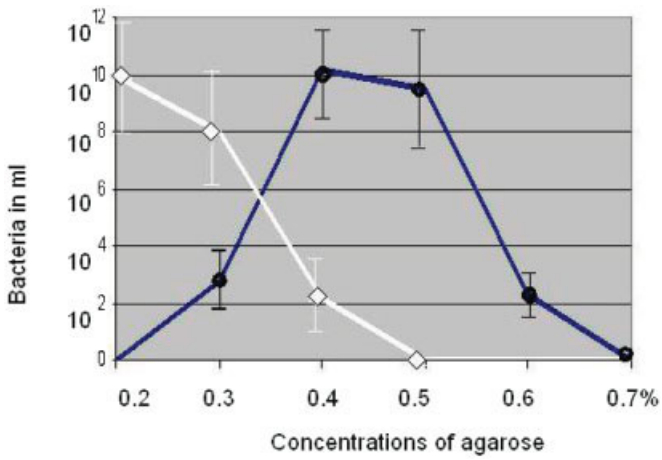


FIGURE 2. Motility of coccoid and bacillar bacterial types in different viscosity conditions as established by migration into agarose gels of different concentrations after 28 hours of anaerobic incubation. ◇: Bacteria that hybridize with the Bac probe (*Bacteroides*: coccoid bacteria). ●: Bacteria that hybridize with the EREC probe (*Eubacterium rectale* group: curly rod-shaped bacteria). Bars indicate standard deviation as obtained after 10 repeats.

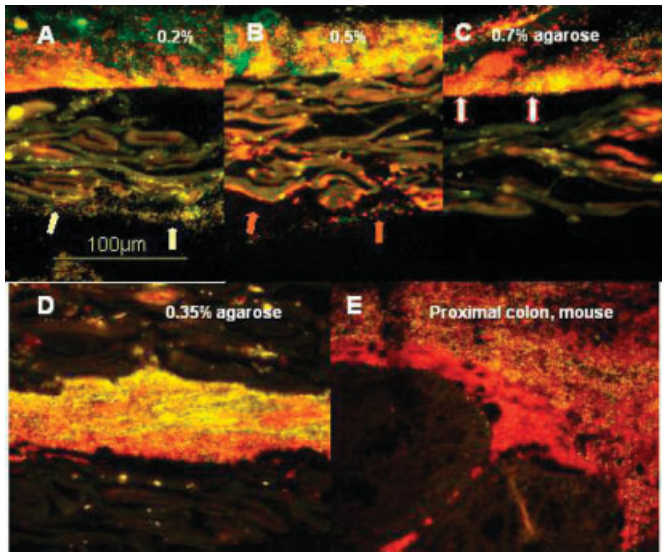


FIGURE 3. Multicolor analysis of bacterial movement and spatial distribution within agarose gels of different concentrations (A–D) and in the proximal colon of a mouse (E). Long rods of the *Eubacterium rectale* group are red (EREC-Cy5 probe), short coccoid bacteria of the *Bacteroides* group are yellow (Bac-Cy3 probe), and all other bacterial groups are green (Eub338-FITC probe). Bacteria are shown in real colors. The background fluorescence of cellulose fibers allows for a good orientation. At ×400 magnification the irregularly ordered cellulose fibers are clearly visible, with large spaces between that allow bacteria to move freely. Yellow arrows indicate a moving front of coccoid bacteria represented by *Bacteroides* (A). Red arrows indicate the moving front of long curly rods represented by *Eubacterium rectale* (B). White arrows demonstrate a lack of any bacterial movements and a gap free of bacteria (C).

At 0.2% agarose short coccoid rod-shaped bacteria that positively hybridized with the Bac-Cy3 probe (*Bacteroides,* yellow fluorescence, Fig. 3A) and coccoid bacteria that hybridized with the universal Eub 338-FITC probe (green fluorescence) built a distinct front below the membrane. They left an empty gap behind, since other bacterial groups with long rod morphology did not follow. Curly rods that positively hybridized with the EREC-Cy5 probe (*Eubacterium rectale* group, red fluorescence, arrows, Fig. 3B) moved at 0.5% agarose and nearly completely filled the gel with bacteria. Yellow-stained *Bacteroides* did not move at this concentration and remained at the original location. At 0.7% agarose no bacteria penetrated into the gel and the gel appeared as a gap devoid of any inclusions (Fig. 3C, white arrows).

At a 0.35% agarose concentration both short coccoid cells as well as long rods moved. However, curly bacteria, which positively hybridized with the EREC probe (red fluorescence), moved faster and built a dense front ahead of the *Bacteroides* group (yellow fluorescence), causing a separation of these bacterial groups into 2 distinct zones (Fig. 3D).

Spatial Distribution of Bacteria in the Murine Colon

Proximal Colon of Conventionally Raised Mice

A defined mucus layer was not apparent in Alcian-stained segments of the proximal colon. Instead, bacteria contacted the colonic wall, entered crypts, and were condensed to a bacterial biofilm flanking mucosa (up to 10^{11} bacteria/mL when hybridized with the Eub universal probe, data not shown). The width of this mucosa flanking bacterial film was 20–240 µm. However, the intimate contact with the mucosa was restricted to specific bacterial groups (Table 2) that hybridized with EREC (*Eubacterium rectale,* Fig. 4A,D), Phasco (*Phascolarctobacterium faecium,* Fig. 4B), Cor (*Coriobacterium*), and Lach (subgroup of EREC) probes. Only bacteria with the shape of long curly rods and filaments made direct contact with the colonic mucosa and entered crypts (Figs. 3E, 4A,B,D). In contrast, coccoid bacteria and short rods, which hybridized with Bac303 (*Bacteroides,* Figs. 3E, 4C), Ebac (*Enterobacteriaceae*), and Clit (*Clostridium lituseburense*) probes, had no contact with the mucosa, did not enter crypts, and were 20–60 µm away from the mucosa.

The Lab (*Lactobacillus*), Ehal, Ato, and Rbro probes hybridized equally often with bacterial groups of markedly different morphology. However, Lab-positive bacteria with coccoid morphology were observed exclusively in the lumen and did not contact the epithelial surface, whereas Lab-positive bacteria with a long rod morphology were mainly concentrated within the mucosa-adjacent bacterial layer (Table 2).

No bacteria could be observed in the epithelial cells or

TABLE 2. Concentrations of Bacterial Groups Identified Within Fecal Compartments and Within the Mucosa-adjacent Layer and Their Cellular Morphology

	Feces		Mucosa Adjacent Layer	
FISH Probe	Concentrations (range as cfu/mL)	Morphology	Concentrations (range as cfu/ml)	Morphology
Erec	10^9–10^{10}	Long rods	10^{10}–10^{12}	Long rods
Phasco	10^7–10^9	Long rods	10^8–10^9	Long rods
Ehal	10^5–10^8	Short and long rods	10^5–10^9	Long rods
Lab	10^7–10^9	Coccoid + long rods	10^5–10^8	Long rods
Cor	10^5–10^9	Long rods	10^5–10^8	Long rods
Ato	10^5–10^7	Short and long rods	10^5–10^8	Long rods
Rbro	10^5–10^8	Short and long rods	10^5–10^8	Long rods
Lach	10^6–10^8	Long rods	10^5–10^8	Long rods
Mib	10^9–10^{10}	Coccoid + short rods	Not found	—
Bac	10^8–10^9	Coccoid + short rods	Not found	—
Clit, Chis	10^7–10^9	Short rods	Not found	—
Ecyl	10^6–10^8	Coccoid/short rods	Not found	—
Ebac	10^5–10^7	Coccoid/short rods	Not found	
Veil	10^5–10^7	Coccoid	Not found	—
Ebac	10^5–10^8	Coccoid/short rods	Not found	—
UroA,B	10^5–10^6	Coccoid	Not found	—
Bif	10^5–10^8	Coccoid/short rods	Not found	—

in the submucosa. The spatial segregation of intestinal bacteria in mucosa flanking film composed of long curly rods and coccoid bacteria separated from the proximal colonic mucosa (Figs. 3E, 4C,D) was strikingly similar to a segregation observed in moderate viscosity gels, as seen in our in vitro mucus studies (Fig. 3D).

Distal Colon of Conventionally Raised Mice

In the distal colon of SPF mice all bacteria were separated from the colonic epithelium by a 50–240-μm thick mucus layer (Fig. 5A,B), which was completely free of bacteria. Occasionally, short rods of the *Bacteroides* group and long rods of the *Eubacterium rectale* group (Fig. 5A) could be seen partially segregated from each other on the border between the mucus layer and feces. However, the arrangement of single bacterial groups with regard to mucus and to each other was irregular, leading to a sandwich-like appearance with thin layers of *Bacteroides* located above, within, or below layers of the *Eubacterium rectale* group. Bacterial groups with different morphology were mixed to a homogeneous mass in the lumen of the colon. No mucosa-adherent bacteria were observed, and no bacteria were detected in crypts, within the epithelial cells or in the submucosa.

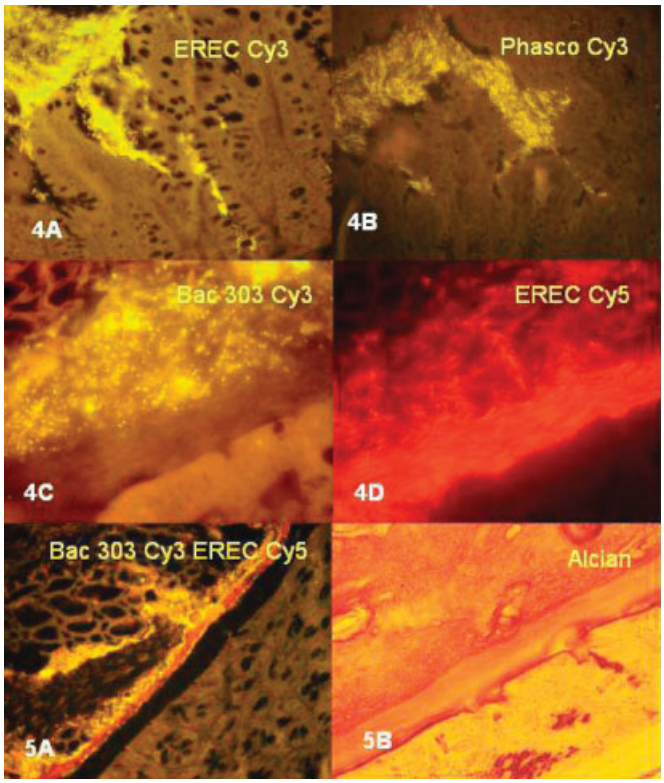
Proximal Colon of Mice Monoassociated with Enterobacter cloacae

Enterobacter cloacae appeared as coccoid rods and hybridized with the Ebac (*Enterobacteriaceae*) as well as with the Eub probe (universal for all bacteria). The Ebac and Eub signals were identical (data not shown), confirming the absence of other bacteria in the monoassociated mice.

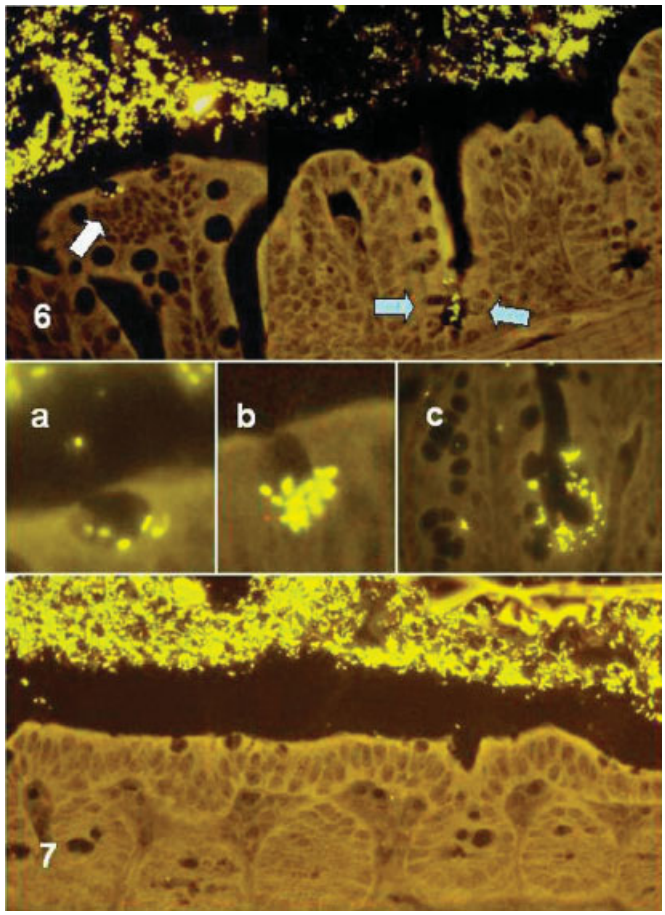
Enterobacter cloacae cells were separated from the columnar epithelium by a 10–80-μm thick mucus layer, which increased in width from the proximal to the distal colon. Despite this clear separation, single bacteria could be found sporadically below the mucus layer including the following locations: Individual goblet cells containing *E. cloacae* within open vacuoles (Fig. 6a,b) could be observed every 500 μm (the adjacent "closed" goblet cells were free of bacteria). Bacteria were also seen in one-third of the crypts. In longitudinally cut crypts containing bacteria, the mucus at the crypt necks was devoid of bacteria (Fig. 6, 6c). Single bacteria started to appear approximately halfway in the base of the crypts and their numbers increased toward the crypt base. Some bacteria were seen invading the cytoplasm of epithelial cells at the crypt base (Fig. 6, 6c). Similarly, the number of bacteria in occupied goblets cells was higher at the bottom of the crypts and decreased in an upward direction (Fig. 6, 6a,b).

Distal Colon of Mice Monoassociated with E. cloacae

Enterobacter cloacae bacteria were separated from the mucosa by a 60–250-μm thick mucus layer in the distal colon (Fig. 7). In contrast to the proximal colon, no bacteria were observed below the mucus layer: Crypts, vacuoles of goblet cells, and epithelial cell cytoplasm were all free of bacteria.



FIGURES 4, 5. Bacterial microbiota in relation to the mucosal surface within different colonic segments of conventional mice: proximal colon (4A–D, ×1000) and distal colon (5A,B, ×400). All bacteria contacting the mucosa and entering crypts in the proximal murine colon have the morphology of long curly rods, although they represent different bacterial taxonomic groups (4A,B). Bacteria with short coccoid rod morphology are separated from the colonic wall (*Bacteroides*, 4C). Bacteria with long rod morphology intimately contact the mucosa within the same microscopic field (*Eubacterium rectale* group, 4D). In the distal colon of conventional mice, all bacteria were separated from the colonic wall by a mucus layer, which was free of bacteria (gap in 5A and Alcian stain in 5B). Bacteria above the mucus segregate to irregular sandwich-like structures (multicolor fluorescence, *Bacteroides* is yellow, *Eubacterium rectale group* is red, 5A).



FIGURES 6, 7. Bacterial distribution within the colon of mice monoassociated with *Enterobacter cloacae*. FISH using the Ebac probe (yellow fluorescence) demonstrates *E. cloacae* bacteria with short coccoid rods morphology. Bacteria are clearly separated from the colonic wall throughout the colon by a mucus layer. Figure 6. Proximal colon (magnification ×400, with inserts a, b, and c: magnification ×1000). The mucus layer is thinner in the proximal colon than in the distal colon, and bacteria can be found in some of the emptying vacuoles of the goblet cells and within crypts. The white arrow indicates an emptying goblet cell filled with *E. cloacae*. Blue arrows indicate *E. cloacae* at the bottom of a longitudinally cut crypt. Bacteria within some of the crypts are infiltrating the epithelial cells of the crypt base (6c). Figure 7. Distal colon (magnification ×400). No bacteria can be found in crypts, goblet cells, or in cell cytoplasm of the distal colon.

DISCUSSION

In the healthy human colon the microflora is completely separated from the mucosa by a 40–240-μm thick mucus layer, which is free of bacteria.[19] In contrast, in the murine colon the mucus layer separates luminal bacteria from the mucosa only from the midcolon to the distal colon.[2] In the proximal murine colon, selective bacterial groups intimately contact the mucosa and enter the crypts and are concentrated to a 20–240-μm thick film flanking the mucosa. The composition of this bacterial film differs markedly from the remainder of the intestinal contents.[2] Only bacteria with a shape of long curly rods can be found here. Although no mucus layer is observed with Alcian stain, the number of mucus-secreting goblet cells in the proximal and distal colon is similar. Why can no mucus-filled gap be perceived between the bacterial film and the mucosa in the proximal colon, and what is the reason for the striking similarities in shape of bacteria contacting the mucosa? We suppose that some bacterial groups can penetrate mucus, move across, and completely fill the mucus layer, rendering the mucus layer invisible in Alcian stain. Do any known facts back this concept?

Mobility was recognized as a basic property of bacteria already in the 19th century.[6] It was shown that, depending on mechanisms of propulsion, each bacterial species has an optimal viscosity in which its velocity is the fastest. Bacterial

movement decreases outside of this "optimal" viscosity range. Mobility is shape-dependent: long rods with a spiral and curly form prefer a higher viscosity, whereas short coccoid bacteria favor a low viscosity and are trapped and immobilized by moderate viscosity.[7,8,20] The accumulation of long curly bacteria in the mucosa-adjacent regions by simultaneous separation of short rods and cocci could therefore indeed result from bacterial preference to a specific viscous environment. However, previous studies on bacterial motility are fragmentary. They are based on investigations of isolated aerobic strains. But colonic bacteria are mainly anaerobic and always occur in polymicrobial associations. Our in vitro mucus simulating studies confirm the general significance of shape for the velocity of unselected anaerobic and aerobic intestinal bacteria in a viscous environment. By testing segregation of bacteria moving from native fecal suspensions through gels of varying agarose concentrations we were also able to correlate in vitro mucus bacterial segregation with patterns of spatial- and segment-specific bacterial distribution within the colon of conventionally raised mice.

In gels of low viscosity, corresponding to agarose concentrations of 0.2%, only short rods, which hybridized with the Bac probe (*Bacteroides)*, moved, whereas long curly rods, which hybridized with the EREC probe (*Eubacterium rectale* group), were immobile. The mobility of short rods decreased with increasing viscosity and was completely lost in gels with 0.5% agarose. The long rods, belonging to the *E. rectale* group, started to move from concentrations of 0.3% agarose onwards, with maximal velocity at 0.4–0.5% and decreased at higher viscosity. Both morphotypes were mobile in a 0.35% agarose gel. However, while moving through these moderately viscous gels, bacteria segregated spatially in separate, independently moving fronts. After 28 hours of anaerobic culture a layer composed mainly of long curly rods (EREC probe) was followed by a layer of short coccoid rods (Bac probe), leading to an in vitro pattern of spatial distribution similar to the in vivo situation in the proximal murine colon. Gel concentrations higher than 0.7% agarose inhibited the movement of all bacterial groups. These gels remained bacteria-free as a continuous gap between the fecal mix and the membrane below, similar to the situation in the distal murine colon, where the mucosa is separated from feces by a continuous bacteria-free mucus layer.

To date there is no method to directly investigate the changes in mucus viscosity and its implications for the mucus barrier in vivo. However, since different bacterial morphotypes require a specific viscosity for their movements, the distribution of differently shaped bacteria can be used to map the areas of changing viscosity in vivo. The patterns of bacterial segregation within the murine colon indicate increasing viscosity of the mucus layer from the proximal colon (where curly shaped long rods enter mucus contacting mucosa, while short rods and coccoid bacteria are separated) to the distal colon (where penetration of the mucus by all bacterial groups stops). Similar cellular morphotypes within segregation zones hybridized with probes for different unrelated bacterial groups, indicating that bacterial morphology and not the biochemical properties or the taxonomical relationship is primarily responsible for the spatial distribution.

Another possible explanation for the segregation of bacteria in the mucosa-flanking bacterial film (composed exclusively of curly rods) and coccoid bacteria (staying at a marked distance from the mucosa) could be bacterial interference, which promotes or antagonizes selective bacterial groups. In monoassociated mice the bacterial interference can be excluded. *E. cloacae* is a short, coccoid rod, which belongs to *Enterobacteriaceae*, and ceases to move in a moderate viscous environment.[8] The spatial distribution of *E. cloacae* in monoassociated wildtype mice is in accordance with the assumption of a mucus layer that continuously envelops the entire colon but regionally differs in viscosity. In contrast to the observations in conventionally raised mice, where the proximal colon has no perceivable mucus layer using Alcian stain, we found in monoassociated mice that *E. cloacae* was separated from the epithelial surface along the entire length of the colon by a mucus layer. The thickness of the mucus layer increased from the proximal (10–80 μm) to the distal (60–250 μm) colon. Additionally, the distribution of *E. cloacae* in monoassociated mice revealed the existence of a second, vertical viscosity gradient within the colonic mucus layer. In the proximal colon, where the separating mucus layer was partially thin, *E. cloacae* could occasionally penetrate it and spread into areas of moderate viscosity located below. Here *E. cloacae* was found in isolated crypts and within vacuoles of some goblet cells. In all these cases of translocation the numbers of *E. cloacae* were highest at the bottom of the crypts (or even goblet cells) and decreased in an upward direction. No bacteria were found in the crypt necks or within mucus adjacent to the columnar epithelium. Obviously, viscosity is low at the sites of mucus production, i.e., within goblet cells and crypts, but increases after contact of the mucus with the columnar epithelium.

These facts have an interesting counterpart in previously performed studies on colonic electrolyte and water transport, which demonstrated that the undifferentiated epithelial cells at the base of crypts are primarily mucus-secreting cells, whereas differentiated cells of the columnar epithelium are mainly absorptive cells, removing water and electrolytes from the mucus.[21–23] The epithelial stem cells at the crypt base proliferate and replace surface cells within 4–8 days.[24] The dissemination of *E. cloacae* in crypt bases and goblet cells outline zones of lower viscosity and confirms independently that during the journey from the crypt base toward the surface epithelium crypt cells become increasingly differentiated and absorptive. The adsorptive cells of the crypt necks and of the epithelial cells of the columnar epithelium dehydrate the mucus layer. Dehydration

makes the mucus layer solid and impenetrable for bacteria and protects sites of mucus production and the mucosa from encounters with potential pathogens. The lower viscosity of the mucus at the crypt base promotes emptying of crypts and prevents obstruction, but as a drawback it may make these types of cells more vulnerable to invasion by potential pathogens. Indeed, invasion of epithelial cells by *E. cloacae* was observed exclusively at the crypt bottom, whereas no *E. cloacae*-containing cells were observed within the cytoplasm of the columnar epithelial cells in monoassociated mice. Interestingly, crypt abscesses, which are typical histomorphologic findings in human self-limiting colitis and IBD, are also more abundant toward crypt bases.

Our findings may have important therapeutic implications. Most of the current therapies of IBD concomitantly interfere with the viscosity gradient of the intestinal mucus barrier. Corticosteroids enhance intestinal mucus production in general (glucocorticosteroid effects) but also promote its dehydration (mineralocorticosteroid effects), increasing the thickness and viscosity of the mucus layer and decreasing its permeability for bacteria.[21,22] TNF-alpha antibodies are mainly regarded as anti-inflammatory, but TNF-alpha antibodies also reduce apoptosis of differentiated epithelial cells.[25] The resulting prevalence of differentiated resorptive epithelial cells over immature secretory cells stabilizes the mucus barrier while solidifying it at the mucosal surface, and thus may be partially responsible for the clinical efficiency of this therapy. Similarly, mesalazine not only suppresses mucosal biofilm propagation,[26] but also controls the differentiation of the epithelial cells via the prostaglandin cycle.[27] Antibiotics decrease the number of bacterial pathogens that could destroy the mucus barrier and make it penetrable to other bacterial groups. Probiotics stimulate intestinal mucus production and reshape its properties.

Our data stress that the regulation of the viscosity of the intestinal mucus barrier can determine the penetration of specific disease-inducing intestinal bacteria. These novel findings may have important implications for how resident intestinal bacteria can induce and perpetuate acute and chronic intestinal inflammation.

ACKNOWLEDGMENT

We thank the Broad Medical Research Program of The Eli and Edythe L. Broad Foundation and the Crohn's and Colitis Foundation of Canada for their financial support.

REFERENCES

1. Palestrant D, Holzknecht ZE, Collins BH, et al. Microbial biofilms in the gut: visualization by electron microscopy and by acridine orange staining. *Ultrastruct Pathol.* 2004;28:23–27.
2. Swidsinski A, Loening-Baucke V, Lochs H, et al. Spatial organization of bacterial flora in normal and inflamed intestine: a fluorescence in situ hybridization study in mice. *World J Gastroenterol.* 2005;11:1131–1140.
3. Rhodes JM. Colonic mucus and mucosal glycoproteins: the key to colitis and cancer? *Gut.* 1989;30:1660–1666.
4. Rickard AH, Gilbert P, High NJ, et al. Bacterial coaggregation: an integral process in the development of multi-species biofilms. *Trends Microbiol.* 2003;11:94–100.
5. Davey ME, O'Toole GA. Microbial biofilms: from ecology to molecular genetics. *Microbiol Mol Biol Rev.* 2000;64:847–867.
6. Pijper A. Shape and motility of bacteria. *J Pathol Bacteriol.* 1946;58:325–342.
7. Young KD. The selective value of bacterial shape. *Microbiol Mol Biol Rev.* 2006;70:660–703.
8. Greenberg EP, Canale-Parola E. Motility of flagellated bacteria in viscous environments. *J Bacteriol.* 1977;132:356–358.
9. Amann R, Krumholz L, Stahl DA. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. *J Bacteriol.* 1990;172:762–770.
10. Bohnert J, Hübner B, Botzenhart K. Rapid identification of *Enterobacteriaceae* using a novel 23S rRNA-targeted oligonucleotide probe. *Int J Hyg Environ Health.* 2000;203:77–82.
11. Franks AH, Harmsen HJ, Raangs GC, et al. Variations of bacterial populations in human feces measured by fluorescent in situ hybridization with group-specific 16S rRNA-targeted oligonucleotide probes. *Appl Environ Microbiol.* 1998;64:3336–3345.
12. Harmsen HJ, Raangs GC, He T, et al. Extensive set of 16S rRNA-based probes for detection of bacteria in human feces. *Appl Environ Microbiol.* 2002;68:2982–2990.
13. Harmsen HJ, Elfferich P, Schut F, et al. A 16S rRNA-targeted probe for detection of *Lactobacilli* and *Enterococci* in fecal samples by fluorescent in situ hybridization. *Microbiol Ecol Health Dis.* 1999;11:3–12.
14. Harmsen HJ, Wildeboer-Veloo AC, Grijpstra J, et al. Development of 16S rRNA-based probes for the *Coriobacterium* group and the *Atopobium* cluster and their application for enumeration of *Coriobacteriaceae* in human feces from volunteers of different age groups. *Appl Environ Microbiol.* 2000;66:4523–4527.
15. Zoetendal EG, Ben-Amor K, Harmsen HJ, et al. Quantification of uncultured *Ruminococcus obeum*-like bacteria in human fecal samples by fluorescent in situ hybridization and flow cytometry using 16S rRNA-targeted probes. *Appl Environ Microbiol.* 2002;68:4225–4232.
16. Salzman NH, de Jong H, Paterson Y, et al. Analysis of 16S libraries of mouse gastrointestinal microflora reveals a large new group of mouse intestinal bacteria. *Microbiology.* 2002;148:3651–3660.
17. Manz W, Amann R, Ludwig W, et al. Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum *Cytophaga-flavobacter-bacteroides* in the natural environment. *Microbiology.* 1996;142:1097–1106.
18. Swidsinski A. Standards for bacterial identification by fluorescence in situ hybridization within eukaryotic tissue using ribosomal rRNA-based probes. *Inflamm Bowel Dis.* 2006;12:824–826.
19. Swidsinski A, Loening-Baucke V, Theissig F, et al. Comparative study of the intestinal mucous barrier in normal and inflamed colon. *Gut.* 2006;56:343–350.
20. Schneider WR, Doetsch RN. Effect of viscosity on bacterial motility. *J Bacteriol.* 1974;117:696–701.
21. Kunzelmann K, Mall M. Electrolyte transport in the mammalian colon: mechanisms and implications for disease. *Physiol Rev.* 2002;82:245–289.
22. Turnamian SG, Binder HJ. Aldosterone and glucocorticoid receptor-specific agonists regulate ion transport in rat proximal colon. *Am J Physiol.* 1990;258:G492–498.
23. Geibel JP. Secretion and absorption by colonic crypts. *Annu Rev Physiol.* 2005;67:471–490.
24. Hermiston ML Gordon JI. Organization of the crypt-villus axis and evolution of its stem cell hierarchy during intestinal development. *Am J Physiol.* 1995;G268:813–822.
25. Zeissig S, Bojarski C, Buergel N, et al. Downregulation of epithelial apoptosis and barrier repair in active Crohn's disease by tumour necrosis factor alpha antibody treatment. *Gut.* 2004;53:1295–1302.
26. Swidsinski A, Loening-Baucke V, Bengmark S, et al. Azathioprine and mesalazine-induced effects on mucosal flora in patients with IBD colitis. *Inflamm Bowel Dis.* 2007;13:51–56.
27. Gaginella TS, Walsh RE. Sulfasalazine; multiplicity of action. *Dig Dis Sci.* 1992;37:801–812.