Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients


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Edited by Todd R. Klaenhammer, North Carolina State University, Raleigh, NC, and approved September 8, 2008 (received for review May 22, 2008)

A decrease in the abundance and biodiversity of intestinal bacteria within the dominant phylum Firmicutes has been observed repeatedly in Crohn disease (CD) patients. In this study, we determined the composition of the mucosa-associated microbiota of CD patients at the time of surgical resection and 6 months later using FISH analysis. We found that a reduction of a major member of Firmicutes, Faecalibacterium prausnitzii, is associated with a higher risk of postoperative recurrence of ileal CD. A lower proportion of F. prausnitzii on resected ileal Crohn mucosa also was associated with endoscopic recurrence at 6 months. To evaluate the immunomodulatory properties of F. prausnitzii we analyzed the anti-inflammatory effects of F. prausnitzii in both vitro (cellular models) and in vivo (2,4,6-trinitrobenzenesulphonic acid (TNBS)-induced) colitis in mice. In Caco-2 cells transfected with a reporter gene for NF-κB activity, F. prausnitzii had no effect on IL-1β-induced NF-κB activity, whereas the supernatant abolished it. In vitro peripheral blood mononuclear cell stimulation by F. prausnitzii led to significantly lower IL-12 and IFN-γ production levels and higher secretion of IL-10. Oral administration of either live F. prausnitzii or its supernatant markedly reduced the severity of TNBS colitis and tended to correct the dysbiosis associated with TNBS colitis, as demonstrated by real-time quantitative PCR (qPCR) analysis. F. prausnitzii exhibits anti-inflammatory effects on cellular and TNBS colitis models, partly due to secreted metabolites able to block NF-κB activation and IL-8 production. These results suggest that counterbalancing dysbiosis using F. prausnitzii as a probiotic is a promising strategy in CD treatment.

Inflammatory bowel disease (IBD) is a group of disorders characterized by a chronic and relapsing inflammation of the gastrointestinal tract frequent in Western countries (1). The two most common forms of IBD are Crohn disease (CD) and ulcerative colitis. The pathogenesis involves an inappropriate and ongoing activation of the mucosal immune system driven by the presence of the intestinal microbiota in a genetically predisposed patient. In CD, in particular, the intestinal microbiota is strongly suspected to play a role in initiating and triggering the immune system, leading to characteristic inflammation (2). Diversion of the fecal stream prevents postoperative recurrence of ileal CD (3). Two antagonistic strategies were proposed to define the role of microorganisms in IBD: “the candidate microorganism strategy” (4) and the “global description strategy” (5–7). We previously described the global specificities of the fecal microbiota in CD (6, 8). Using a metagenomic approach, we revealed a global decrease in the biodiversity of the fecal microbiota in CD (8). This was essentially due to a markedly reduced diversity of Firmicutes, and in particular of the Clostridium leptum phylogenetic group. Moreover, analysis by FISH combined with flow cytometry showed a significant quantitative reduction in the C. leptum group in CD patients compared with healthy subjects (6, 8). Using different molecular methods, other studies reported that Faecalibacterium prausnitzii was particularly depleted in IBD patients’ ileocolonic mucosa-associated microbiota (MAM) (9, 10).

In this study, we first analyzed the composition of the ileal MAM of CD patients at the time of surgical resection for active disease and 6 months later by FISH. We observed that the proportion of Firmicutes, and in particular of F. prausnitzii, which is the major bacterium of the C. leptum group, was low in patients that exhibited endoscopic recurrence 6 months after surgery. We hypothesized that counterbalancing dysbiosis using this deficient commensal bacterium as a probiotic in CD could be beneficial. Then, we evaluated the potential role of F. prausnitzii on intestinal inflammation using cellular and animal models.

Results

A Lower Proportion of F. prausnitzii on Resected Ileal Crohn Mucosa Is Associated with Endoscopic Recurrence. Previously, we demonstrated that administration of Lactobacillus johnsonii LA1 is ineffective for prophylaxis of postoperative endoscopic recurrence in CD (11). We did not observe any significant difference in the composition of the MAM between L. johnsonii LA1 and placebo groups at the time of surgery or 6 months after in surgical and biopsy samples from 21 human volunteers (data not shown). Focusing on endoscopic recurrence at 6 months (Rutgeerts score ≥2 for 13 patients, LA1 n = 6 and placebo n = 7), we observed: (i) a significantly lower proportion of F. prausnitzii at the time of surgery, consistently associated with endoscopic relapse; and (ii) a lower proportion of Firmicutes (i.e., C. coccoides and F. prausnitzii) 6 months after surgery in CD patients with endoscopic recurrence compared with CD patients who were still in remission [Fig. 1, supporting information (SI) Table S1, and SI Materials and Methods].


The authors declare no conflict of interest.

This article is a PNAS Direct Submission. See Commentary on page 16413.

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4This article contains supporting information online at www.pnas.org/cgi/content/full/0804812105/DCSupplemental.

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Values are expressed as the meanSEM in pg/ml for cytokines and in percentages for IL-10/IL-12 ratio (D). Different asterisks (*) indicate significant differences (*, P < 0.05; **, P < 0.01; ***, P < 0.003).

Methods. Based on these observations, we hypothesized that remission might be associated with the presence of F. prausnitzii, and probably with its anti-inflammatory effects.

F. prausnitzii Exerts Anti-inflammatory Effects in Peripheral Blood Mononuclear Cells (PBMCs). Because the cytokine profile released by PBMCs has been previously shown to correlate the in vitro and in vivo immunomodulation potential of different bacteria (29), we analyzed the capacities of F. prausnitzii to induce cytokine production in PBMCs (SI Materials and Methods). In addition, the immunostimulation by four other strains having well-documented in vitro and in vivo anti-inflammatory properties (Lactobacillus salivarius Ls33) (12), or lacking any anti-inflammatory activities (L. acidophilus NCFM, Lactococcus lactis MG1363, and Escherichia coli TG1) were also tested (Fig. 2). Interestingly, F. prausnitzii and L. salivarius Ls33 were the weakest inducers of proinflammatory/Th1 cytokines (e.g., IL-12 and IFN-γ) and the highest inducers of anti-inflammatory IL-10 compared with the other tested strains. The ratio of IL-10 to IL-12, frequently used to distinguish between strains exhibiting a proinflammatory (low ratio) vs. an anti-inflammatory (high ratio) profile, reveals that F. prausnitzii exhibit the highest anti-inflammatory profile (Fig. 2D).

F. prausnitzii Supernatant Reduces IL-8 Secretion by Caco-2 Cells. We examined the effects of UV-killed F. prausnitzii, bacterial fractions, supernatants, or sterile medium on IL-8 secretion in Caco-2 cells, with or without IL-1β stimulation (SI Materials and Methods and Fig. 3A). Either UV-killed F. prausnitzii, membrane extracts, or cytoplasmic extracts, but not DNA or sterile medium, induce low levels of IL-8 secretion. UV-killed F. prausnitzii, bacterial fractions, and sterile medium did not inhibit IL-8 secretion induced by IL-1β, compared with negative control. In contrast, supernatant from F. prausnitzii cultures significantly reduces IL-8 secretion induced by IL-1β.

F. prausnitzii Supernatant Abolishes NF-κB Activation in Caco-2 Reporter Cell Lines. To further explore the immunomodulatory properties of F. prausnitzii, we tested the effects of this bacterium and its supernatant on NF-κB activation using a Caco-2 reporter cell line (SI Materials and Methods). In Caco-2 cells, F. prausnitzii stimulates an NF-κB-dependent secreted alkaline-phosphatase (SEAP) production (Fig. 3B), whereas the supernatant decreases the basal NF-κB activity. After stimulation with IL-1β, NF-κB activity increases significantly. No significant effect on IL-1β stimulation was observed with UV-killed bacteria, whereas the F. prausnitzii supernatant strongly inhibits NF-κB activation by IL-1β in Caco-2 cells. Because F. prausnitzii produces high amounts of butyrate, which has well known anti-inflammatory effects (13), we evaluated the effect of butyrate in Caco-2 reporter cell lines at the concentration present in the F. prausnitzii supernatant (i.e., 40 mM). Butyrate did not reproduce the supernatant inhibitory effect after IL-1β stimulation, but in contrast strongly increased NF-κB activation (data not shown).
Effects of *F. prausnitzii* indicate significant differences (proteins. The values are expressed as the mean reporter gene with or without stimulation with IL-1. *F. prausnitzii*, its component, or its supernatant. Cells were stimulated or not with IL-1. Using two different Sokol et al. effects of intragastric administration of *F. prausnitzii* supernatant. Did Not Display In Vitro Antibacterial Effect. *F. prausnitzii* Supernatant Did Not Display In Vitro Antibacterial Effect. We investigated antibacterial properties of *F. prausnitzii* supernatant. Using two different *in vitro* techniques (critical dilutions of Mayr-Harting et al., ref. 14; and bacteriocin activity assay, ref. 15), we did not reveal any antibacterial effect on several anaerobic and aerobic bacterial species (see description in *Materials and Methods*).

**Severity of TNBS-Induced Colitis in Mice Is Reduced by *F. prausnitzii* and Its Supernatant.** We further explored the relevance of these observations *in vivo* by testing the ability of *F. prausnitzii*, its DNA and membrane fractions, and its supernatant to prevent acute colitis induced by TNBS in BALB/c mice (*SI Materials and Methods* and Fig. 4). A severe colitis was observed in the colitis control group. No improvement in colitis severity was observed with either UV-killed *F. prausnitzii*, its DNA, or its membrane (data not shown). In contrast, daily intragastric administration of living *F. prausnitzii* (5 × 10⁸ cfu) or its supernatant resulted in a marked attenuation of colitis with reduced weight loss, partial colon length normalization, and improvement of Wallace and Ameho scores. No significant protective effect was observed in mice receiving sterile culture medium.

**F. prausnitzii** and Its Supernatant Induce an Increased Secretion of IL-10 and a Decreased Secretion of TNF-α and IL-12 in TNBS-Induced Colitis in Mice. Forty-eight hours after TNBS instillation, colonic proinflammatory TNF-α and IL-12 cytokines and anti-inflammatory IL-10 were quantified by ELISA (*SI Materials and Methods* and Fig. 5). Compared with the no-colitis control group, TNF-α and IL-12 levels were increased, whereas IL-10 levels were barely modified. In the two TNBS groups treated with either *F. prausnitzii* or its supernatant, the secretion of TNF-α and IL-12 was significantly lower than in the colitis control group. Interestingly, IL-10 secretion was induced in the colon of mice treated with *F. prausnitzii* or its supernatant.

**F. prausnitzii** and Its Supernatant Tend to Counterbalance the TNBS-Induced Dysbiosis in Colitic Mice. To validate our initial hypothesis, we determined the composition of the fecal microbiota of mice from the colitis (TNBS-treated) and no-colitis control, the *F. prausnitzii*-treated, and the supernatant-treated groups by real-time qPCR (*SI Materials and Methods*, Table S2, and Fig. 6). A higher
concentration of total bacteria, bacteria from the *C. leptum* group, the *Bacteroides* group, and bacteria from the *C. coccoides* group was measured in the fecal microbiota of the colitis control mice compared with the no-colitis control mice. No difference was observed concerning the *Lactobacillus* group and *E. coli* species. *F. prausnitzii* was the only species diminished in colitis control mice. Treatment with either *F. prausnitzii* or its supernatant tended to counterbalance the dysbiosis observed in colitis control mice for all bacteria: *C. leptum*, *C. coccoides*, *Bacteroides*, and *F. prausnitzii* (Fig. 6). Strikingly, *F. prausnitzii* occurrence was nearly normalized in the *F. prausnitzii*-treated group.

**F. prausnitzii and Its Supernatant Can Act by a Gut-Independent Route.** To investigate whether *F. prausnitzii* or its supernatant could be active by a gut-independent pathway, we tested i.p. injection in a TNBS-induced colitis model. The survival rates 20 days after colitis induction were 100% and 10% in the no-colitis and colitis control groups, respectively (Fig. 7). In a third control group treated with dexamethasone, the survival rate was 40% at 20 days. In all groups treated with *F. prausnitzii* or the supernatant, the mortality rate was dramatically decreased. The group treated by live *F. prausnitzii* had a significantly better survival curve than the colitis control group (*P* = 0.039), with a survival rate of 50% at 20 days, close to the rate observed in the dexamethasone group. The mortality rate in the group treated with the culture supernatant was reduced to 0 (versus positive control, *P* < 10^{-4}; vs. dexamethasone group, *P* = 0.004). Interestingly, this strong anti-inflammatory effect was not due to butyrate presence, as the group of mice treated with butyrate (at the same concentration as supernatant) had a mortality rate similar to that in the colitis control group.

**Discussion**

In this study, we showed that a low proportion of *F. prausnitzii* on resected ileal Crohn mucosa is associated with endoscopic recur-
rence at 6 months. Moreover, this bacterium, deficient in CD patients’ microbiota, has anti-inflammatory effects in vitro as well as in vivo. On Caco-2 cells, secreted metabolites blocked NF-κB activation and IL-8 secretion. Moreover, on PBMCs, F. prausnitzii induced very low levels of IL-12 and IFN-γ, whereas high levels of IL-10 were obtained compared with other bacterial strains tested. Considering the IL10/IL12 ratio measured on PBMCs, F. prausnitzii displays the most anti-inflammatory properties. These in vitro effects were confirmed in vivo on TNBS-induced colitis.

It is now well established that microbial components of the resident microbiota can regulate gut inflammation (16). Moreover, some bacterial strains, like Bacteroides thetaitauraeomicron, have been particularly studied and their participation in the gut immune homeostasis elucidated (17). In parallel, the specificities of the gut microbiota composition in IBD patients have been assessed using molecular methods. Nevertheless, this recent knowledge has not yet been used to find new anti-inflammatory bacteria within the normal gut microbiota, to date.

An original and rational process led us to select F. prausnitzii as a candidate anti-inflammatory bacterium. Comparing any gut microbiota of healthy subjects and of CD patients, we observed that the C. leptum group within the dominant Firmicutes phylum was defective in CD patients’ microbiota (6, 8). This deficiency was quantitative and qualitative, as the biodiversity of the C. leptum was restricted. F. prausnitzii is one of the major members of the C. leptum group (18, 19) and one of the most prevalent bacteria within the human gut. In the present study, we showed that a low proportion of F. prausnitzii in the ileal MAM of CD patients at the time of a surgical resection for active disease was a risk factor for endoscopic recurrence 6 months later. Taken together, all these results suggested that F. prausnitzii could be crucial to the gut homeostasis and could have anti-inflammatory properties. F. prausnitzii and its components were then tested on Caco-2 cells stimulated with IL-1β. After stimulation by IL-1β, IL-8 secretion was not modified by F. prausnitzii and reduced by its supernatant. To better understand the mechanisms involved in this effect, we tested the bacterium components on Caco-2 cells stably expressing a reporter gene for NF-κB. We observed that F. prausnitzii increased the IL-1β-induced NF-κB activity, whereas the supernatant abolished it. It is noticeable, that F. prausnitzii DNA did not exhibit any inhibitory effect on either IL-8 secretion or NF-κB activation. Moreover, we showed that this blocking effect on NF-κB activation was not due to butyrate.

Besides, it is questionable whether the anti-inflammatory effect of the supernatant is shared by an F. prausnitzii-secreted metabolite(s) or due to an F. prausnitzii-induced change in product(s) of the culture medium. The fact that living F. prausnitzii given without supernatant exerts an anti-inflammatory effect in the in vivo experiments favors the hypothesis of secreted metabolite(s)-induced effect. Identification of the active molecule(s) involved in this protective effect is needed to specify this point.

Our results favor a secreted metabolite, which could be involved in inflammatory pathway control. Several targets can be evoked from the lumen to immune cells in the lamina propria, and we first speculated on an antimicrobial peptide (bacteriocin) able to modify the gut barrier. However, our in vitro tests did not reveal any antibacterial effect on several anaerobic and aerobic bacteria species. Antibacterial mode of action also could be mediated by stimulation of defensin secretion. In fact, it has been shown that a defect in HD-5 and HD-6 α-defensin secretion by epithelial cells (specialized epithelial cells from ileum) plays a crucial role in ileal CD and could be linked to NOD2 frameshift mutation (20, 21). The clinical part of our study focused on ileal CD patients requiring surgical resection. Thus, the dysbiosis in F. prausnitzii appears attractive regarding the α-defensin deficiency concept. As some probiotics have been shown to induce defensin secretion (22), a step further will be to investigate the ability of F. prausnitzii to induce such a secretion. Beside a gut barrier effect, our results suggest that a diffusible molecule (modulin) could block NF-κB activation. More precisely, our data showed that F. prausnitzii supernatant acts as a regulator of the NF-κB induction after stimulation by IL-1β. Nevertheless, the mechanism is probably much more complex, because NF-κB activation has been shown to play a crucial role in gut homeostasis (23). Indeed, F. prausnitzii itself without its supernatant exerts NF-κB induction effect on Caco-2 cells (Fig. 3B).

To evaluate the potential immunomodulatory capacity of F. prausnitzii, we measured the cytokine response of human PBMCs stimulated by the bacterium. F. prausnitzii had an anti-inflammatory profile because it induced very low secretion levels of IL-12 and IFN-γ and high secretion levels of IL-10. Moreover, F. prausnitzii has a higher IL10/IL12 ratio than the probiotic L. salivarius Ls33 strain known to have anti-inflammatory effects.

To confirm in vivo the anti-inflammatory effects observed in vitro, we tested F. prausnitzii in a murine TNBS-induced colitis model. F. prausnitzii and its supernatant had a protective effect in this colitis model, on macroscopic and histologic criteria as well as on colonic cytokine secretion profile. Mice treated with either F. prausnitzii or its supernatant had a reduced IL-12 and an elevated IL-10 colonic concentration compared with the colitis control group. Interestingly, this effect on cytokine profile is close to the one observed in vitro on PBMCs.

To go back to our initial hypothesis, we analyzed the composition of the mouse fecal microbiota by culture-independent methods. We observed that the dysbiosis associated with TNBS-induced colitis was partially counterbalanced by F. prausnitzii or its supernatant administration.

Several authors reported that probiotics could act by systemic routes of administration (24–26). To investigate whether F. prausnitzii can also be active by a gut-independent pathway, we tested it i.p. on a TNBS-induced colitis model. Systemic delivery of F. prausnitzii and, above all, of its supernatant significantly improved the survival curve related to TNBS-induced colitis. Here again, this effect was not related to butyrate presence.

In summary, a rational process led us to select F. prausnitzii as an anti-inflammatory bacterial candidate. Analysis revealed that F. prausnitzii exhibited anti-inflammatory effects, partly due to secreted metabolites blocking NF-κB activation and IL-8 secretion. Moreover, in vivo effects were associated with a decrease in proinflammatory colonic cytokine synthesis and with the induction of anti-inflammatory cytokine secretion. Counterbalancing dysbio-

sis using the commensal bacterium F. prausnitzii as a candidate probiotic agent appears to be a promising strategy in CD treatment. Nevertheless, clinical studies should establish diagnostic tools to define the best population of patients for this probiotic species, and especially whether the clinical benefit is more pronounced in patients with low levels of endogenous Firmicutes.

Materials and Methods

Patients. The present study was part of a double-blind controlled trial that compared the efficiency of the probiotic L. johnsonii LAL1 strain and a placebo to decrease endoscopic recurrence after curative surgery for CD (11). Although the whole human trial included 98 patients, the present study, which assessed the MAM, included only a subset of these patients.

Tissues, Histological Examination, FISH, and Quantification of Bacteria. Surgical samples and mucosal biopsy samples were processed directly in the operating room as previously described (27).

Bacterial Culture. F. prausnitzii A2–165 (DSM 17677), isolated from human fecal stool, was grown at 37°C in L1YBH medium [Brain–heart infusion medium supplemented with 0.5% yeast extract (Difco) and 5 mg/liter hemin] supple-
mented with cellobiose (1 mg/ml; Sigma–Aldrich), maltose (1 mg/ml; Sigma), and cysteine (0.5 mg/ml; Sigma) in an anaerobic chamber.

**Butyrate Quantification.** Butyrate was quantified in supernatant and culture medium using a gas-liquid chromatograph (Autosystem XL; Perkin–Elmer) as described (28).

**Antibacterial Assay.** Antibacterial effect of *P. prausnitzii* supernatant was investigated in vitro using two different techniques: the critical dilutions of May–Hartling (14) and the bacteriocin activity assay as described (15). This antibacterial effect exerted on six bacterial species: three aerobic bacteria (*E. coli* Nissle 1917, *E. coli* DH10B, and *Listeria monocytogenes* 11765), one facultative anaerobic bacterium (*Lactococcus subsp cremoris* MG1363), and two obligate anaerobic bacteria (*Clostridium perfringens* ATCC13124 and *Bifidobacterium infantis* DSM20088/ATCC15697). Antibiotic rifampicine and colistine were used as positive controls, whereas LYHBBH medium was used as negative control.

**PBMC Isolation and Stimulation.** PBMCs were isolated from the peripheral blood of healthy donors as described (29).

**TNBS-Induced Colitis.** After acclimatization, bacterial suspensions (10^7–10^10 cfu in 500 μl), bacterial supernatant, or control medium was administered to mice daily by intragastric gavage from day 5 before to day 1 after induction of colitis. For colitis induction, mice were anesthetized with isoflurane gas. TNBS (Sigma–Aldrich) was dissolved in 0.9% NaCl (50:50 vol/vol), and 50 μl solution (at a dose of 100 mg/kg body weight) was administered intrarectally (using a 3.5 French catheter; Solomon Scientific), and the mice then were kept in a vertical position for 30 seconds. No-colitis control mice received PBS intragastrically and 0.9% NaCl (50:50 vol/vol) intrarectally (30). Colitis control mice received PBS intragastrically and received TNBS intrarectally. Treated mice received bacteria (or bacterial components) intragastrically and TNBS intrarectally. Inflammation was monitored 48 h after TNBS administration. Mice were weighed before TNBS administration and at killing by cervical dislocation. The i.p. administration route experiments were performed using a protocol derived from the one previously described by Foligné et al. (24). The protective effect of a single i.p. injection of live microorganisms (10^7–10^10 cfu in 200 μl), supernatant (200 μl), dexamethasone (10 mg/kg), or butyrate (40 mM) was evaluated. Bacteria or components were injected 1 h before TNBS-colitis induction. The mortality rate was monitored day by day for 20 days.

**Inflammation Score Assessment of TNBS Colitis.** The colon was removed, dissected free of fat and mesentry, carefully opened, and cleaned with PBS. Colon length was measured. Colon damage and inflammation were assessed blindly according to the Wallace criteria (31).

**Histological Assessment of TNBS-Induced Colitis.** For histological assessment, a colon sample located in the most inflamed area was fixed in 4% paraformaldehyde and embedded in paraffin. Four-micrometer sections were stained with hematoxylin/eosin and examined blindly according to the Ameho criteria (32) (Fig. 5).

**ACKNOWLEDGMENTS.** We thank Alexandra Gruss, Sylvie Rabot, and Maarten van de Guchte for critical reading of this manuscript. We also thank Anne Lavergne-Slove for histological support. We thank the Groupe d’Etude Thérapeutique des Affections Inflammatoires du Tube Digestif members who participated in the sample collections for the clinical study. H.S. received a grant from Assistance Publique-Hôpitaux de Paris to achieve this work.

Supporting Information

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Materials and Methods

Bacterial Membranes and Cytosolic Fraction Preparation. Bacteria were grown in liquid medium. Cells were harvested by centrifugation at 5,000 × g for 10 min. The bacterial pellet from 20 ml culture was washed with and resuspended in 2 ml TE buffer (10 mM Tris-Cl, pH 8.0; 1 mM EDTA) and disrupted by ultrasonication on ice. Unbroken cells and debris were removed by centrifugation at 6,000 × g for 10 min. The supernatant was subjected to ultracentrifugation at 100,000 × g for 1 h. The cleared supernatant was retained as the cytosolic fraction and frozen at −80°C. The pellet, which is the total membrane fraction, was resuspended in 2 ml PBS and frozen at −80°C.

FISH Experiments: Tissue Samples. For each patient, we obtained samples from the resected ileum (2 × 2 cm) and biopsy samples of their neointestinal ileum 6 months later. Samples were processed directly in the operating room. Tissue samples were fixed in 4% formalin, incubated at 4°C for 48 h (72 h maximum), then processed and embedded into paraffin blocks according to routine procedures. Paraffin sections of 5 µm were placed on organosilane-coated slides and stored at room temperature. Biopsies were placed in Sarstedt 2.2-ml screw-cap tubes, immediately frozen in liquid nitrogen, and stored at −80°C until analysis.

Histological Examination. Sections were deparaffinized in xylene and rehydrated for 3 min in 100%, 96%, and 70% ethanol successively. They were then stained with hematoxylin and eosin for morphological assessment.

FISH. Before FISH, sections were deparaffinized, rehydrated, and postfixed in 4% paraformaldehyde for 5 min. Fixation was stopped after 5 min in PBS 3×, and slides were washed twice for 1 min in PBS 1×. Tissue sections were incubated 10 min at room temperature with Tris-EDTA buffer containing 10 mg/ml lysisoyze and then washed and hybridized using the hybridization solution (0.9 M NaCl, 20 mM Tris-HCl, pH 8, 0.01% SDS, and 30% formamide). Fixed tissue sections then were hybridized with the previous hybridization solution containing 4.5 ng/µl of 1 of the 5′-end Cy3-labeled 16S rRNA-targeted oligonucleotide probes. Hybridizations were performed at 35°C overnight in a microscope slide incubator, and stringent washings were carried out at 37°C (2 × 15 min) in a buffer containing 65 mM NaCl, 20 mM Tris-HCl, pH 8.0, 0.01 mM EDTA, and 0.01% SDS to remove nonspecific binding. The sections were mounted with Vectashield [mounting medium with 4′,6′ diamidino-2-phenylindole (DAPI); Vector Laboratories]. DNA was stained with DAPI to visualize all cells.

Quantification of Mucosa-Associated-Bacteria (MAB). Bacteria were visualized with an epifluorescence microscope Leica DMIRB using Cy3- and DAPI-specific filters at 100×, 400×, and 1000× magnification, and images were captured with a Leica DFC 300 FX camera and FW 4000 software (Leica Microsystems SAS). The entire mucosal surface and epithelium of each 4 cm² tissue section was examined for the presence of bacteria. Pure cultured bacteria belonging to each group were hybridized as positive and negative controls for the FISH procedure. The quantification of total MAB for each tissue sample was performed when bacteria were detected with both the Eub338 probe and DAPI staining and without hybridization with the nonEub338 nonsense probe. The total number of bacteria was determined for each tissue sample and then divided by the total mucosal surface (mucosa length ÷ thickness of paraffin sections). The proportion of bacteria hybridized with group-specific probes was expressed as a percent of total bacteria. Ten fields were examined for each sample, and the mean percentage was calculated.

PBMC Isolation and Stimulation. PBMCs were isolated from the peripheral blood of healthy donors as previously described (1). Briefly, after Ficoll gradient centrifugation (Amersham Pharmacia), mononuclear cells were collected, washed twice in RPMI medium 1640 (Life Technologies), and adjusted to 2 × 10⁶ cells/ml in RPMI 1640 supplemented with gentamicin (150 µg/ml), 2 mM l-glutamine and 10% decomplemented FCS (Gibco-BRL) and seeded in 24-well tissue culture plates (Corning). Twenty microliters of a thawed bacterial suspension at 10⁹ CFU/ml was added (bacteria:cell ratio of 10:1). PBS containing 20% glycerol was used as a negative (nonstimulated) control. After 24 h of stimulation at 37°C in an atmosphere of air with 5% CO₂, supernatants were collected, clarified by centrifugation, and stored at −20°C until cytokine analysis. Neither medium acidification nor bacterial proliferation was observed. Cytokines were measured by ELISA using BD PharMingen antibody pairs for IL-10, IL-12p70, and IFN-γ, according to the manufacturer’s recommendations.

Experiments on Caco-2 Cell Lines. The human colon cancer cell line Caco-2 from the European Collection of Cell Cultures (Wiltshire, United Kingdom) was cultured in 12- to 24-well culture plates in DMEM (Invitrogen) supplemented with 20% heat-inactivated FCS, 1% nonessential amino acids (Invitrogen) at 37°C in 10% CO₂/air atmosphere. Media were changed every day. Experiments were initiated on days 20–21 after seeding (previously shown as an early stage of differentiation, corresponding to the upper crypt–lower villus stage of differentiation). Twenty-four hours before bacterial challenge, the culture medium was changed for a medium without FCS. Bacteria were added at a multiplicity of infection (moi) of 100 in 0.5 ml DMEM. Cells were stimulated simultaneously with human recombinant IL-1β (15 µg/ml; Sigma–Aldrich) for 6 h. For some experiments, a challenge was made using bacterial supernatant at 40%, F. prausnitzii DNA, cytoplasm, or membrane in DMEM (bacterial supernatants were previously filtered through a 0.2-µm filter and added in DMEM). After incubation, cell supernatants were collected and frozen at −80°C.

IL-8 ELISA and Protein Concentration. Cells were washed twice with PBS and lysed in PBS containing 1% Triton X-100 and a protease inhibitor mixture (Roche Diagnostics GmbH). Protein concentrations were determined in cell lysates using bicinchoninic acid protein assay reagents (Pierce) according to the manufacturer’s instructions. The IL-8 level was determined in cell supernatants using ELISA kit DuoSet (R&D Systems) according to the manufacturer’s recommendations.

Construction of Stable NF-κB Reporting Cells. Stable transfectants of a Caco-2 cell line were obtained after transfection with the plasmid pNiFt2 (Invivogen). This plasmid combines five NF-κB sites with the SEAP reporter gene. Induction by NF-κB activates the promoter, resulting in the expression of the SEAP gene. Electroporation was performed using an amaxa nucleofector apparatus according to the manufacturer’s instructions (Amaxa GmbH). After electroporation, cells were resuspended in complete fresh medium and seeded in 24-well plates for 96 h until
experiments. To establish stable clones, zeocin (50 μg/ml; Invitrogen) was added to the medium from 2 days after transfection for 10 days, and the medium was changed every 2 days. Resistant clones were isolated using cloning glass cylinders and tested by IL-8 secretion. Selected clones were further cultured in the presence of 25 μg/ml zeocin.

**Analysis of NF-κB Activation.** SEAP-transfected Caco-2 cells were cultured using the same protocol as for nontransfected Caco-2 cells, with 25 μg/ml zeocin in 12-well culture plates. Experiments were initiated on days 20–21 after seeding. Twenty-four hours before bacterial challenge, the culture medium was changed for a medium without FCS. Bacteria were added at an moi of 100 in 0.5 ml DMEM. Cells were simultaneously stimulated with IL-1β (15 μg/ml) for 6 h. After incubation, cell supernatants were collected for determination of secreted alkaline phosphatase. SEAP in the supernatant was revealed using Quanti-Blue (Invitrogen). Absorbance at 655 nm was read after 3 h of revelation.

**Mice.** Seven- to eight-week-old conventional male BALB/c mice (Janvier) were used. All experiments were carried out in accordance with the institutional guidelines. Groups of 9 to 11 mice were used in each experiment. A total of 500 μl bacterial suspension, bacterial component solution, supernatant, or PBS was administered daily by intragastric gavage.

**Cytokine Assay.** Colon samples (segment of 0.5 cm located in the inflamed area) were mechanically homogenized in cold antiprotease buffer (Protease Inhibitor Mixture Tablets; Roche) using a miniBeadBeater (BioSpec Products), and after centrifugation for 30 min at 16,000 × g and 4°C, the supernatant was stored at −80°C. Total proteins were quantified in colon homogenates by a Bradford assay. Cytokines (TNF-α, IL-10, and IL-12) were quantified using ELISA kits (Ebioscience) according to manufacturer recommendations.

**DNA Extraction from Fecal Samples.** The distal colonic content from each mouse was collected after killing and aliquoted in Starstedt DNA extraction is described in the section on bacterial DNA extraction. DNA dissolved in 100 μl TE 1× buffer was stored at −20°C.

**Design of Oligonucleotide Primers and Probes.** The primers and probes for real-time quantitative PCR (qPCR) were designed using the Primer-Express software version 2.0 (Applied Biosystems). The selected primers and probe target sites (Table S2) were tested for specificity by submitting the sequences to the Probe Match program provided by Ribosomal Database Project II and on pure culture bacterial DNA (J.P.F., unpublished results). The primers were purchased from MWG-Biotech AG, and the TaqMan probes were synthesized by Applied Biosystems. The oligonucleotide probes used for FISH (2) were adapted to TaqMan probes as described by Furet et al. (unpublished data). The primers for this PCR were designed considering the RNA sequences (EMBL database) aligned with the program Clustal W. SYBR-Green qPCR was used to quantify subdominant species or group of species.

**Real-Time qPCR.** Real-time qPCR was performed by using an ABI 7000 Sequence Detection System apparatus with 7000 system software version 1.2.3 (Applied Biosystems). Amplification and detection were carried out in 96-well plates and with TaqMan universal PCR 2× master mix (Applied Biosystems) to quantify total bacteria and the bacterial-dominant group of microbiota, and with SYBR-Green PCR 2× master mix to the others. Each reaction was done in duplicate in a final volume of 25 μl with 0.2 μM final concentration of each primer, 0.25 μM final concentration of each probe, and 10 μl of appropriate dilutions of DNA samples. Amplifications were performed with the following temperature profiles: 1 cycle at 95°C for 10 min to denature DNA and activate AmpliTaq Gold polymerase, followed by 40 cycles of 95°C for 30 sec, 60°C for 1 min. For the SYBR-Green amplifications, the dissociation step was added to control the amplification specificity. We investigated the most important groups and species of the mouse fecal microbiota (Table S2).

**Statistical Analysis.** Statistical analysis for significant differences was performed according to the Student’s t-test for unpaired data or by the nonparametric Mann–Whitney. differences with P < 0.05 were considered significant. Animal experiments were performed at least three times, and results from a representative experiment are shown.

Fig. S1. Hematoxylin and eosin staining of representative cross-sections of murine (BALB/c) distal colon. (Magnification: ×20.) (a) Normal appearance of the colon from a negative control mouse. (b) Mild mucosal and submucosal inflammatory infiltrate (neutrophils) and edema. (c) Prominent inflammatory infiltrate (neutrophils) and edema, rare inflammatory cells invading the muscularis propriae without muscle necrosis. (d) Extensive coagulative necrosis bordered inferiorly by numerous neutrophils. Necrosis extends deeply into the muscularis propria.
Table S1. Composition of the ileal mucosa-associated microbiota (MAM) using fluorescent in situ hybridization (FISH) at the time of surgery and at 6 months after surgery. Assessment of the composition of the ileal MAM using FISH were performed using 6 group-specific probes (Eubacteria, Bacteroides, Enterics and relatives, Bifidobacterium genus, Clostridium coccoides and Faecalibacterium prausnitzii). The proportion of bacteria hybridized with group-specific probes was expressed as a percent of total bacteria. The Firmicutes proportion was obtained by adding C. coccoides and F. prausnitzii signals. MAM: Mucosa associated microbiota; * and § significant difference, $P_b = 0.02$

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<th></th>
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<th>C. coccoides</th>
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*Significant difference, $P = 0.02$

Assessment of the composition of the ileal MAM by using FISH was performed using six group-specific probes (Eubacteria, Bacteroides, Enterics, and relatives, Bifidobacterium genus, C. coccoides, and F. prausnitzii). The proportion of bacteria hybridized with group-specific probes was expressed as a percent of total bacteria. The Firmicutes proportion was obtained by adding C. coccoides and F. prausnitzii signals.
Table S2. Groups and species-specific 16S rRNA-targeted primers and probes used in this study. Probes are noted in bold.

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