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Abundant and Diverse Fungal Microbiota in the Murine Intestine

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Enteric microbiota play a variety of roles in intestinal health and disease. While bacteria in the intestine have been broadly characterized, little is known about the abundance or diversity of enteric fungi. This study utilized a culture-independent method termed oligonucleotide fingerprinting of rRNA genes (OFRG) to describe the compositions of fungal and bacterial rRNA genes from small and large intestines (tissue and luminal contents) of restricted-flora and specific-pathogen-free mice. OFRG analysis identified rRNA genes from all four major fungal phyla: *Ascomycota*, *Basidiomycota*, *Chytridiomycota*, and *Zygomycota*. The largest assemblages of fungal rRNA sequences were related to the genera *Acremonium*, *Monilinia*, *Fusarium*, *Cryptococcus*/*Filobasidium*, *Scleroderma*, *Catenomyces*, *Spizellomyces*, *Neocallimastix*, *Powellomyces*, *Entophlyctis*, *Mortierella*, and *Smittium* and the order *Mucorales*. The majority of bacterial rRNA gene clones were affiliated with the taxa *Bacteroidetes*, *Firmicutes*, *Acinetobacter*, and *Lactobacillus*. Sequence-selective PCR analyses also detected several of these bacterial and fungal rRNA genes in the mouse chow. Fluorescence in situ hybridization analysis with a fungal small-subunit rRNA probe revealed morphologically diverse microorganisms resident in the mucus biofilm adjacent to the cecal and proximal colonic epithelium. Hybridizing organisms comprised about 2% of the DAPI (4′,6-diamidino-2-phenylindole, dihydrochloride)-positive organisms in the mucus biofilm, but their abundance in fecal material may be much lower. These data indicate that diverse fungal taxa are present in the intestinal microbial community. Their abundance suggests that they may play significant roles in enteric microbial functions.

Gut microflora play a variety of roles in health and disease. Some bacteria synthesize nutrients utilized by the host, while others transform dietary substances into carcinogens (8). Standard indigenous bacteria appear to be involved in both the development of a normal gut immune system and, in the case of inflammatory bowel disease, the induction of inappropriate inflammatory responses (8, 41, 50, 57). In addition, specific intestinal microbial populations in infants have been correlated with increased incidence of atopy (9, 30). To better understand the roles that specific organisms or consortia of organisms play in such functions, investigators will need a variety of experimental capabilities, not the least of which is the ability to identify the microorganisms inhabiting the gut.

Obtaining detailed descriptions of microbial community composition is a continuing challenge. Microorganisms have been traditionally identified through characterization of their morphological and physiological traits. However, this approach is limited by its reliance on culture techniques, which detect only a fraction of microorganisms (5, 33, 54). While the development of strategies to directly analyze rRNA molecules and genes from environmental samples has provided a means to examine microbial communities without the culture bias, most of these methods, including denaturing gradient gel electrophoresis (42), terminal restriction fragment length polymorphisms (38), ribosomal intergenic spacer analysis (11), and amplified ribosomal DNA restriction analysis (61), do not typically generate detailed descriptions of microbial communities. Nucleotide sequence analysis of rRNA gene clone libraries can be used to facilitate thorough depictions of microbial composition, yet this approach is usually impractical because of the high costs associated with examining large numbers of samples from complex communities such as those found in the gut.

In this study, we used an array-based method termed oligonucleotide fingerprinting of rRNA genes (OFRG) (10, 58, 59) to examine fungal and bacterial rRNA genes from small and large intestines (tissue and luminal contents) of restricted-flora (RF) and specific-pathogen-free (SPF) mice. OFRG provides a cost-effective means to extensively analyze microbial community composition. RF mice are descendants of a colony established by antibiotic treatment and inoculation with several *Clostridium* spp. (13, 17). RF mice possess three immunologic phenotypes: abnormal development of naive T-cell subsets and regulatory B-cell subsets and the induction of colitis in susceptible mouse strains (13, 16, 17, 62). To our knowledge, this report provides the first culture-independent analysis of fungal rRNA genes from mammalian intestine.

MATERIALS AND METHODS

Mice. SPF C57BL/6 mice bear normal aerobic and anaerobic enteric commensal microflora but are negative for a panel of pathogenic microorganisms based on serologic and microbiologic screening by the UCLA Division of Laboratory Animal Medicine. An RF colony was established 12 years ago in a separate facility maintained by the UCLA Department of Radiation Oncology by extensive antibiotic treatment and recolonization with six species of nonpathogenic *Clostridium* (13). RF C57BL/6 mice were established 6 years ago by cesarean

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section delivery of SPF fetal C57BL/6 mice and adoptive transfer to RF foster mothers. RF mice were housed in enclosed racks with filtered air and autoclaved bedding, food, and water. For both SPF and RF mice, animals of either gender were used at age 6 to 12 weeks. All animal procedures were performed in accordance with current UCLA institutional review board-approved protocols.

Intestinal sample collection. Mice were euthanatized by isofluorane inhalation, and the intestines were excised. For DNA extraction, 5- to 10-cm segments of small intestine or colon were collected, and luminal contents were moved to one end of the intestinal segment with a forceps. Two to 3 cm of the tissue containing the condensed luminal contents was placed in a lysis tube (screw-cap tubes with beads) containing 1 ml CLS-Y buffer from a FastDNA kit (Qbiogene, Carlsbad, CA) and immediately frozen at −70°C. For fluorescence in situ hybridization (FISH) samples, small intestine (including jejunum) was harvested and divided into three equally long segments (11 to 12 cm each). In the large intestine, the cecal appendix was excised, and the remaining large bowel was divided into two equal segments (7 to 8 cm each). These tissue samples were divided longitudinally, gently washed with RPMI 1640 (Gibco, Grand Island, NY) to remove fecal material and luminal debris, fixed at room temperature for 24 h in Carnoy's solution (15% glacial acetic acid, 85% ethanol), and processed for conventional paraffin embedding.

DNA extraction from intestinal samples. Samples in the FastDNA lysis tubes described above were thawed on ice and lysed by bead beating in a FastPrep instrument (Qbiogene) for 30 s at setting 5.0. DNA was purified using the FastDNA Kit as described by the manufacturer (Qbiogene). DNA was further purified and size fractionated by electrophoresis in 0.6% agarose gels. After staining with ethidium bromide, DNA larger than 3 kb was excised and recovered using the QIAquick gel extraction kit (QIAGEN, Valencia, CA).

DNA extraction from mouse chow. DNA was extracted from the mouse chows (200-mg crushed pellet) using the FastDNA kit and the CLS-Y buffer as described by the manufacturer (Qbiogene). DNA was further purified and size fractionated as described above. Details about the chows can be found in Table 4.

PCR amplification of bacterial and fungal small-subunit rRNA genes. Bacterial and fungal rRNA genes from small- and large-intestinal samples were obtained by PCR as previously described (60), using 35 amplification cycles. For each sample type, the PCR template was composed of pooled DNA from replicate samples from five mice.

OFRG analysis. The compositions of fungal and bacterial rRNA genes from mouse intestinal samples were obtained by OFRG analyses as previously described (60). Briefly, rRNA gene clone libraries were constructed. rRNA genes from the libraries were then PCR amplified, arrayed on nylon membranes, and hybridized with ^{33}P-labeled DNA probes 10 nucleotides in length. Hybridization signals were used to generate OFRG fingerprints, which were clustered with OFRG fingerprints from taxonomically classified rRNA gene sequences by using the unweighted-pair group method with arithmetic mean. Intestinal rRNA gene clones were categorized by their association with the taxonomically classified rRNA gene sequences and by nucleotide sequence analysis of representative clones distributed throughout the tree determined by the unweighted-pair group method with arithmetic mean.

rRNA gene analysis of mouse chow. DNAs extracted from the mouse chows were PCR amplified using two sets of universal primers, one targeting all bacterial small-subunit rRNA genes and the other targeting all fungal small-subunit rRNA genes, as described above. rRNA clone libraries were constructed from the resulting amplicons as described above. Nucleotide sequence analysis was performed on 19 randomly selected colonies each from the bacterial and fungal libraries as described below. Two of the bacterial and six of the fungal clones either did not contain an rRNA gene insert or they did not produce reliable nucleotide sequence data and were not analyzed further.

Chow DNA was also analyzed by taxon-selective PCR analyses. Sequence-selective PCR primers were developed for four of the largest assemblages of rRNA gene clones identified by the bacterial and fungal OFRG analyses. Primers were designed by locating DNA sequences that were conserved among the rRNA gene clones within each group by using Pretty (Accelrys, San Diego, CA) and which had few, if any, identical matches to rRNA gene sequences from unrelated taxonomic groups by using BLAST (NCBI) (3). The primer sequences were for *Firmicutes* (FirmF7639, GCGTGAGTGAAGAAGT; FirmR7641, CTACG CTCCCTTTACAC), *Filobasidium* (FiloF7632, AGCGTTTAGCTGTTG; FiloR7633, GCCAAGGTTATAAACTC), *Lactobacillus* (LactoF7627, GAGCT TGCCTAGATGA; LactoR7631, TATTAGCATCTGTTTCCA),and *Smittium* (SmitF7566, TGTTTTCATTAATCAAGAAC; SmitR7567, CAAGTCTTTAAA TTATAACATT). The sizes of the predicted PCR products were as follows: *Firmicutes*, 161 bp; *Filobasidium*, 109 bp; *Lactobacillus*, 96 bp; and *Smittium*, 116 bp. Amplification reactions were performed in a Bio-Rad iCycler MyiQ real-time detection system (Bio-Rad Laboratories Inc., Hercules, CA) with 25-μl mixtures containing the following reagents: 50 mM Tris (pH 8.3), 500 μg/ml bovine serum albumin, 2.5 mM $MgCl_2$, 250 μM of each deoxynucleoside triphosphate, 400 nM of each primer, 1 μl template DNA, and 1.25 U *Taq* DNA polymerase. The cycling parameters for the *Firmicutes* amplification reactions were as follows: 94°C for 5 min; 35 cycles of 94°C for 20 s, 56.8°C for 30 s, and 72°C for 30 s; and then 72°C for 2 min. The cycling parameters for the *Filobasidium* amplification reactions were as follows: 94°C for 5 min; 35 cycles of 94°C for 20 s, 58°C for 30 s, and 72°C for 30 s; and then 72°C for 2 min. The cycling parameters for the *Lactobacillus* amplification reactions were as follows: 94°C for 5 min; 35 cycles of 94°C for 20 s, 60°C for 30 s, and 72°C for 30 s; and then 72°C for 2 min. The cycling parameters for the *Smittium* amplification reactions were as follows: 94°C for 5 min; 35 cycles of 94°C for 20 s, 57.8°C for 30 s, and 72°C for 30 s; and then 72°C for 2 min. PCR products were resolved on 1% agarose gels, stained with ethidium bromide, and photographed under UV illumination. PCR products were excised from the gels, purified using the QIAquick gel extraction kit (QIAGEN), and cloned into pGEM-T (Promega). For each of the four sequence-selective groups, nucleotide sequence analysis was performed on two randomly selected colonies as described below.

Nucleotide sequence analysis of rRNA gene clones. Nucleotide sequences of partial bacterial and fungal rRNA gene sequences were determined using the ABI PRISM BigDye Terminators version 3.0 cycle sequencing kit and a 3100 genetic analyzer (ABI) and assembled using ContigExpress (Vector NTI, Invitrogen, Carlsbad, CA). Sequence identities were determined using BLAST (NCBI) (3) and AlignX (Vector NTI).

FISH fungal analysis. Paraffin-embedded intestinal specimens were sectioned at 4-μm thickness; baked on microscope slides for 60 min at 65°C; deparaffinized by sequential washes in xylene, ethanol, and water; and air dried. Sample regions were circumscribed with a liquid blocking pen, to which was applied 100 μl hybridization solution (0.9 M NaCl, 20 mM Tris, 10% formamide, 0.1% sodium dodecyl sulfate, pH 8.0) containing 2 ng/μl 5′-fluorescein isothiocyanate (FITC)-labeled PF2 fungal small-subunit rRNA antisense probe (CTCTGGCTTCAC CCTATTC) (GeneDetect, Bradenton, FL) (31). In some cases, a 5-FITC-labeled nonsense probe (CCTCCCATCCGGTAGAACA) or the universal eubacterial probe EUB 338 (GCTGCCTCCCGTAGGAGT) was used as a negative control. Selectivity of the PF2 probe was previously demonstrated by Kempf et al. (31). Selectivity was also assessed in this study by performing FISH analyses on a few bacterial and yeast strains with the PF2 and EUB338 probes: for EUB338, FISH signals were detected from *Escherichia coli* and *Staphylococcus aureus* but not from *Saccharomyces cerevisiae*; for PF2, FISH signals were detected from *S. cerevisiae* but not from *E. coli* and *S. aureus* (data not shown). Slides were denatured at 70°C for 5 min, hybridized for 3 h at 48°C, washed for 5 min at 48°C in washing solution (0.45 M NaCl, 20 mM Tris, 0.01% sodium dodecyl sulfate, pH 8), rinsed in water, and air dried. Slides were then stained for 5 min at room temperature with 0.1 μg/ml DAPI (4′,6-diamidino-2-phenylindole, dihydrochloride) (Molecular Probes, Eugene, OR) and coverslipped with mounting medium (Vectashield; Vector, Burlingame, CA). Slides were viewed using a Zeiss Axiophot fluorescence microscope equipped with single-, dual-, and triple-pass filters (DAPI/green/red) and a PlanApo 100× oil objective. The images were captured with CytoVision FISH software (Applied Imaging Corp., San Jose, CA), and single-plane images were captured and processed using Adobe Photoshop 7.0 (Adobe, San Jose, CA).

The relative abundance of PF2-positive microorganisms (compared to the total DAPI-positive microorganisms) in cecal samples was determined by FISH. For each slide, five or more high-magnification fields (×1,000) with moderate to high levels of DAPI-positive organisms were selected and scored for DAPI- and PF2-positive organisms (structures with coherent microbial morphologies). In areas with organisms at higher densities, enumeration was performed with the assistance of higher power (up to ×2,500) and a grid ocular. Fields were also examined at different focal depths, because the organisms were dispersed throughout the 4-μm sections. Three independent experiments were scored. Similar rates of DAPI-positive and PF2-positive microorganisms were observed among these experiments, so the results were pooled for quantitative analysis.

Nucleotide sequence accession numbers. Sequences represented by GenBank accession numbers AY987400 to AY987459 and AY987461 to AY987476 were generated by PCR amplification with primers targeting fungal small-subunit rRNA genes, and those represented by accession numbers AY994018 to AY994051 were generated with primers targeting bacterial small-subunit rRNA genes. Sequences are designated by a clone number followed by a suffix indicating their origin: -1, RF large intestine; -2, RF small intestine; -3, SPF large intestine; and -4, SPF small intestine.

TABLE 1. Taxonomic distribution of rRNA gene clones obtained by OFRG analysis of murine intestine with fungus-selective PCR primers

Taxon	No. of clones[a]					Nearest relative(s) (accession no.)[b]	% Identity to nearest relatives[b]
	Total	RF-C	RF-SI	SPF-C	SPF-SI		
Ascomycota	477	153	40	164	120		
Acremonium	57	7	3	39	8	*Acremonium alternatum* (AY083232)	99
Alternaria	28	13	5	1	9	*Alternaria alternata* (AF229504)	93–97
Monilinia	151	70	1	28	52	*Monilinia laxa* (Y14210)	96–99
Fusarium	63	4	5	50	4	*Gibberella pulicaris* (AF149875), *Fusarium oxysporum* (AF141951), *Fusarium equiseti* (AF141949), *Fusarium culmorum* (AF141948), *Fusarium cerealis* (AF141947), *Gibberella avenacea* (AF141946), *Cordyceps sinensis* (AB067700)	99
Unidentified	178	59	26	46	47		
Basidiomycota	154	46	11	61	36		
Cryptococcus/ *Filobasidium*	51	1	4	40	6	*Filobasidium globisporum* (AB075546), *Filobasidium elegans* (AB075545), *Cryptococcus magnus* (AB032643), *Cryptococcus ater* (AB032622), *Filobasidium floriforme* (D13460)	98–99
Scleroderma	45	19	0	11	15	*Scleroderma citrina* (AF026621)	96–97
Unidentified	58	26	7	10	15		
Zygomycota and *Chytridiomycota*	239	139	13	23	64		
Cluster 1	162	86	6	21	49	*Catenomyces* sp. (AY635830), *Spizellomyces punctatus* (AY546684), *Spizellomyces acuminatus* (M59759), *Neocallimastix* sp. (M59761), *Powellomyces* sp. (AF164245), *Neocallimastix frontalis* (X80341), *Entophlyctis helioformis* (AY635826), *Mortierella alpina* (AJ271630), *Smittium culisetae* (D29950)	94–99
Mucorales	54	36	7	1	10	*Amylomyces rouxii* (AY228093), *Rhizopus oryzae* (AF113440), *Backusella ctenidia* (AF157122)	98–99
Unidentified	23	17	0	1	5		
Mammalia	428	20	247	75	86	Several mammals, including *Mus musculus* (BK000964) and *Sus scrofa* (AY265350)	98–100
Unidentified	111	33	10	36	32		

[a] Determined by adding the number of clones in the major taxonomic groups identified by the OFRG analysis (Fig. 1).
[b] Determined by BLAST (NCBI) (3) analysis of representative clones from the major taxonomic groups identified by the OFRG analysis (Fig. 1). RF-C, colon samples from restricted-flora mice; RF-SI, small intestine samples from restricted-flora mice; SPF-C, colon samples from specific-pathogen-free mice; SPF-SI, small intestine samples from specific-pathogen-free mice.

RESULTS

Composition of intestinal fungal small-subunit rRNA genes. The compositions of fungal rRNA genes from small and large intestine (tissue and luminal contents) of RF and SPF mice were obtained by OFRG analysis (Table 1 and Fig. 1). Most of the rRNA gene clones were distributed among nine well-defined taxonomic clusters. Two hundred ninety-nine clones were associated with four *Ascomycota* taxa: *Acremonium*, *Alternaria*, *Monilinia*, and *Fusarium*. Ninety-six clones were associated with three *Basidiomycota* taxa: *Filobasidium*, *Cryptococcus*, and *Scleroderma*. Two hundred sixteen clones were associated with two major *Chytridiomycota* and *Zygomycota* assemblages: cluster 1 and *Mucorales*. Four hundred twenty-eight clones had high sequence identity to mammalian rRNA genes. Other rRNA gene sequences identified from small clusters or taxonomically mixed clusters were related to *Paecilomyces javanicus*, *Oxyporus* sp., *Armillaria borealis*, *Sordariomycete* sp., *Galactomyces citri-aurantii*, *Rhodosporidium toruloides*, *Plectosphaerella cucumerina*, *Sordaria fimicola*, and *Myrothecium verrucaria*. The taxonomic identities of the major clusters identified by the OFRG analysis were validated by nucleotide sequence analysis of representative rRNA gene clones. Accession numbers for all of these sequences are listed in "Nucleotide sequence accession numbers" in Materials and Methods. The percent sequence identities to their nearest relatives are shown in Table 1. For several fungal taxa, differences in the number of clones between SPF and RF mice were detected. However, it should be emphasized that OFRG is not a quantitative methodology, so validation of these apparent differences would require additional experimentation.

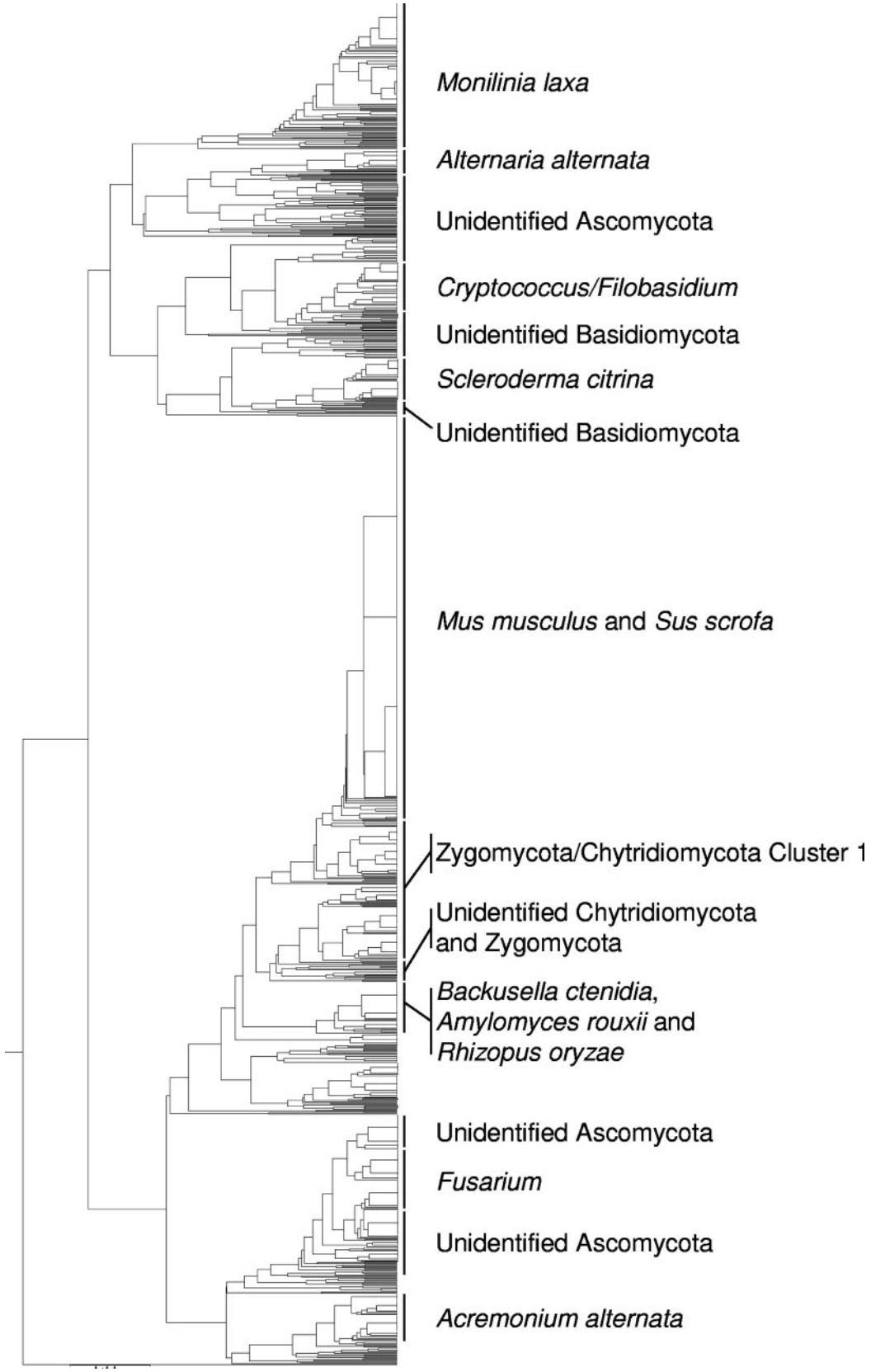

FIG. 1. Taxonomic depiction of fungal small-subunit rRNA genes identified by OFRG analysis of murine intestine. Major taxonomic groups are indicated. Taxonomic distribution of the rRNA gene clones by mouse and tissue type is listed in Table 1.

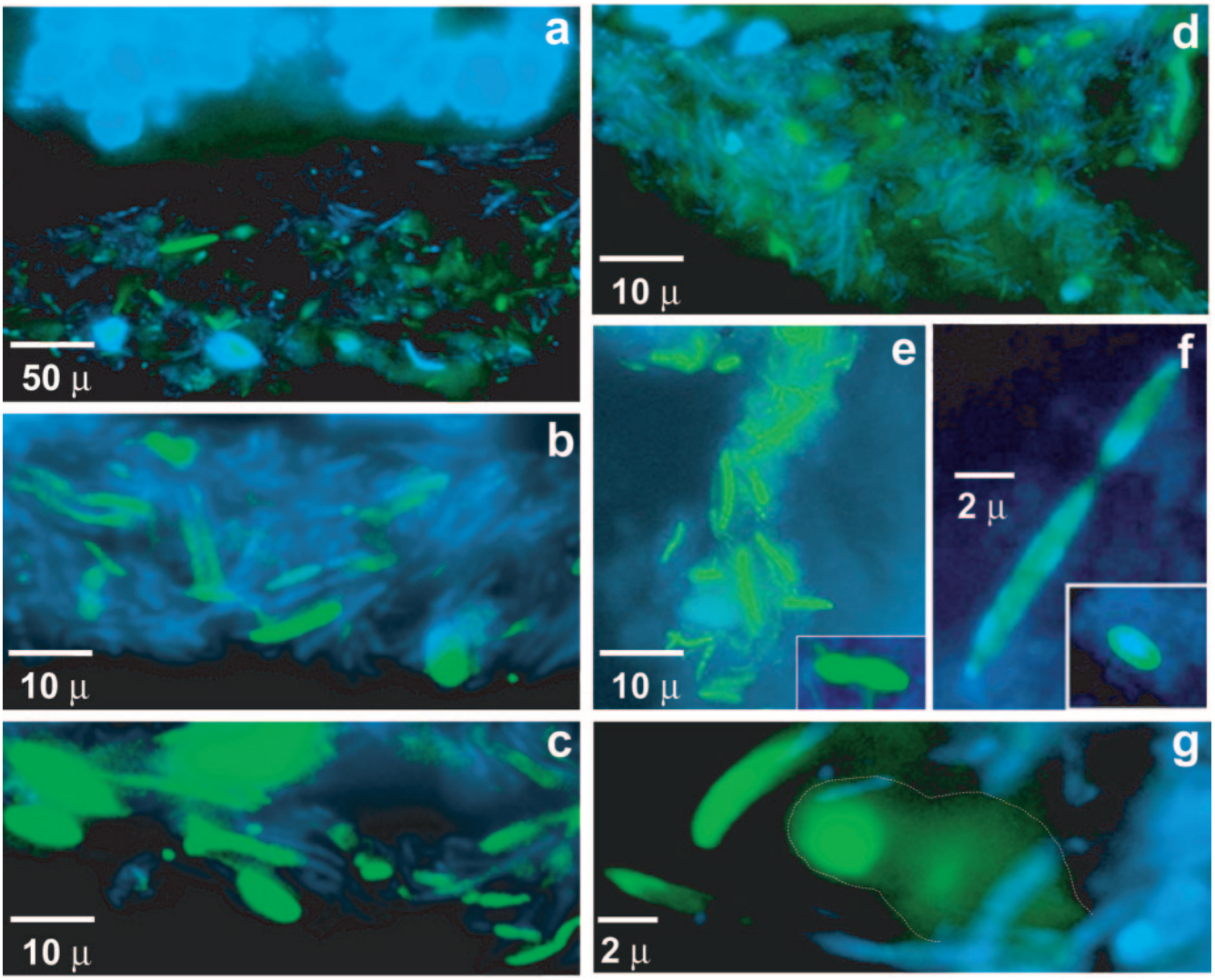

FIG. 2. Fungal FISH analysis of the mucus biofilm. Four-micrometer sections of SPF mouse cecum were hybridized with a fungal small-subunit rRNA probe (PF2, 5′-FITC) and counterstained with DAPI. Magnifications: (a) ×200; (b to e) ×1,000; (f and g) ×2,500. For panel g, a white line was added to the FISH image to highlight the outline of this structure.

FISH analysis of the murine intestine with a universal fungal small-subunit rRNA probe. The OFRG analysis provided evidence suggesting that the murine intestine harbors a diverse array of intestinal fungi. To corroborate these findings, specimens of mouse intestine were subjected to FISH analysis. DAPI staining of the cecum revealed the glandular microanatomy of the mucosa, a thin (0- to 30-μm) mucus layer relatively devoid of organisms, and a luminal biofilm containing organisms and sloughed senescent epithelial cells (Fig. 2a). The organisms in this biofilm were mainly DAPI positive and PF2 negative and were relatively small with rod-like or filamentous morphologies. Previous FISH studies using universal (EUB338) or species-selective small-subunit rRNA probes (55, 56) have shown that most of these microorganisms were of bacterial origin.

When the interlaced layer biofilm was examined by hybridization with a fungus-selective FISH probe (PF2), occasional but distinct PF2-positive microorganisms were observed (Fig. 2a). Morphotypes included filamentous and stout rods, small and large ovoid structures, and very large fusiform structures. These morphotypes were interspersed with each other and the more abundant PF2-negative population. Examples of their morphologies and spatial distribution in different cecal specimens are shown in Fig. 2b to g. These findings were consistent in more than five independent experiments. Only rare DAPI- or PF2-positive microorganisms were detected in cecal crypts or epithelial fields. In three independent experiments, the relative abundance of PF2-positive microorganisms (compared to DAPI-positive microorganisms) in the biofilm was enumerated. The frequency of PF2-positive per DAPI-positive microorganisms ranged from 0 to 10%, with a median of 2%.

Examination of other regions of the intestine revealed much lower levels of DAPI- or PF2-positive microorganisms (data not shown). In the proximal colon, the interlaced layer was colonized with only sparse clusters of DAPI-positive microor-

TABLE 2. Taxonomic distribution of rRNA gene clones obtained by OFRG analysis of murine intestine with bacterium-selective PCR primers

Taxon	No. of clones[a]					Nearest relative(s) (accession no.)[b]	% Identity to nearest relatives[b]
	Total	RF-C	RF-SI	SPF-C	SPF-SI		
Acinetobacter	313	24	5	145	139	*Acinetobacter* sp. (AJ244765), *Acinetobacter* sp. (AY588958), *Acinetobacter calcoaceticus* (AY800383)	99
Bacteroidetes	37	5	7	13	12	Uncultured gut bacterium (DQ014784)	99
Firmicutes	763	323	305	70	65	Uncultured gut bacteria (DQ014768, DQ015223, AF132269, AF371582, AF371836, AY916380, AY991721, AY991912, AY992417, AY992630, AY992840, AY993011)	99, 94, 91, 93, 98, 91, 96, 96, 95–96, 94, 95, 99, respectively
Lactobacillus	105	0	7	56	42	*Lactobacillus johnsonii* (AE017206)	99
Unidentified	200	28	54	53	65		

[a] Determined by adding the number of clones in the major taxonomic groups identified by the OFRG analysis (data not shown). RF-C, colon samples from restricted-flora mice; RF-SI, small intestine samples from restricted-flora mice; SPF-C, colon samples from specific-pathogen-free mice; SPF-SI, small intestine samples from specific-pathogen-free mice.
[b] Determined by BLAST (NCBI) (3) analysis of representative clones from the major taxonomic groups identified by the OFRG analysis (data not shown).

ganisms (~1% of cecal density). Occasional PF2-positive organisms of different morphologies were detected among this population, roughly proportional to the reduced DAPI-positive microbiota. As previously reported, the mucus layer was thicker in the middle and distal colon, with only rare DAPI-positive microorganisms (56); no PF2-positive microorganisms were detectable in this region. DAPI- and PF2-positive organisms were rare in the ileum and undetected in the jejunum.

The fecal compartments of these intestinal segments were also examined by FISH analyses. However, fecal material in the lumen of the intestinal specimens was generally lost during sample preparation. On the occasions where fecal material was present, fungi were detectable in the cecum, rare in the proximal colon, and undetected in the distal colon or expelled fecal pellets. As expected, fecal material itself was unapparent in the small intestine. Because of this sampling problem, fungal abundance in the fecal compartment is uncertain.

Composition of intestinal bacterial small-subunit rRNA genes. The compositions of bacterial rRNA genes from small and large intestines (tissue and luminal contents) of RF and SPF mice were obtained by OFRG analysis (Table 2). Most of the rRNA gene clones were distributed among four well-defined taxa: *Bacteroidetes*, *Firmicutes*, *Acinetobacter*, and *Lactobacillus*. The taxonomic identities of the major clusters identified by the OFRG analysis were validated by nucleotide sequence analysis of representative rRNA gene clones. Accession numbers for all of these sequences are listed in "Nucleotide sequence accession numbers" in Materials and Methods. The percent sequence identities to their nearest relatives are shown in Table 2. A striking predominance of *Firmicutes* clones was detected in RF mice, among both the small and large intestinal compartments. Conversely, a more balanced distribution of clones from the predominant bacterial taxa was observed in the SPF mice. As noted above, validation of this conclusion would require additional quantitative experimentation. However, this observation is concordant with the origin of RF mice, which were produced by conventionalization of germfree mice with a mixture of *Clostridium* species.

rRNA gene analysis of mouse chow. The mouse chows were examined by PCR analyses using primers targeting all bacterial small-subunit rRNA genes, all fungal small-subunit rRNA genes, and four of the largest assemblages of rRNA gene clones identified by the OFRG analyses of the murine intestine. Analysis of the SPF chow with universal bacterial primers identified four corn mitochondrial rRNA gene clones, six rice chloroplast rRNA gene clones, three wheat chloroplast rRNA gene clones, and four different bacterial small-subunit rRNA gene clones, none of which were identified in the intestinal analysis (Table 3). Analysis of the SPF chow with universal fungal primers identified one wheat small-subunit rRNA gene clone, one

TABLE 3. Taxonomic distribution of rRNA gene clones obtained by PCR amplification of DNA extracted from mouse chow[a] using bacterium and fungus-selective primers

Analysis	Taxon	No. of clones	Nearest relative (accession no.)[b]	% Identity to nearest relative[b]
Universal bacterial	*Oryza sativa* (chloroplast)	6	AE017114	98–100
	Pantoea agglomerans	1	AY849936	99
	Pantoea sp.	1	AF451269	99
	Pseudomonas sp.	1	AY131221	99
	Triticum aestivum (chloroplast)	3	AJ239003	99–100
	Uncultured bacterium	1	AF371481	99
	Zea mays (mitochondrion)	4	AY506529	98–100
Universal fungal	*Cladosporium cladosporioides*	10	AF548071	95–100
	Phaeosclera dematioides	1	Y11716	98
	Setomelanomma holmii	1	AY161121	99
	Triticum aestivum	1	AJ272181	99

[a] Chow fed SPF mice.
[b] Determined by BLAST (NCBI) (3).

TABLE 4. Detection of rRNA genes[a] by sequence-selective PCR amplification of DNA extracted from six different mouse chows

Taxon	PCR products from mouse chows[b]					
	RF	SPF	Mouse diet, no. 5015	Mouse diet 9F, no. 5020	Autoclavable mouse breeder diet, no. 5021	PicoLab mouse diet 20, no. 5058
Firmicutes	+	+[c]	+[c]	+[c]	+[c]	+[c]
Filobasidium	−/+	+	+	+	+	+
Lactobacillus	+	−	−/+	+	−	−/+
Smittium	−/+	−/+	−/+	+	+	+

[a] Four of the largest assemblages of rRNA gene clones identified by the OFRG analyses of murine intestine (Tables 1 and 2).
[b] The presence or absence of PCR products is indicated as follows: +, product was detected; −, product was not detected; −/+, detection of product was variable in replicate experiments. RF designates the type of Harlan Teklad (Madison, Wisconsin) diet fed to RF mice, autoclaved sterilizable rodent diet (W) (catalog no. 8656); SPF designates the diet fed to SPF mice, rodent diet (W) (catalog no. 8604). The four other diets are different types of PMI LabDiet chow.
[c] PCR products were produced but had high identity to corn mitochondrial rRNA genes.

Phaeosclera dematioides small-subunit rRNA gene clone, one *Setomelanomma holmii* small-subunit rRNA gene clone, and 10 *Cladosporium cladosporioides* small-subunit rRNA gene clones, none of which were identified in the intestinal analyses (Table 3). No PCR products were detected when DNA from the RF chow (which was autoclaved) was amplified with universal bacterial and fungal primers (data not shown). Sequence-selective PCR assays detected *Firmicutes*, *Filobasidium*, and *Smittium* rRNA genes in both the RF and SPF chows and *Lactobacillus* rRNA genes in the RF chow, although some of the amplification reactions did not consistently produce PCR products (Table 4). Analysis of four additional mouse chows detected *Filobasidium* and *Smittium* rRNA gene sequences in all four chows and *Lactobacillus* in three of the chows (Table 4). Analysis of the four additional chows with the *Firmicutes*-selective PCR assay produced corn mitochondrial rRNA genes (Table 4). Unless otherwise indicated, nucleotide sequence analysis of the PCR products reported in Table 4 confirmed their identities (data not shown).

DISCUSSION

Culture-independent analyses of microorganisms inhabiting humans and animals have uncovered a considerable diversity of previously undescribed organisms, the roles of which are just beginning to be understood. Such analyses have been performed for bacterial and viral components of the intestinal microbial community (12, 49, 54). However, to our knowledge, no culture-independent analyses of fungal rRNA genes have been performed on the mammalian intestine. The data presented in this report suggest that the murine intestine, like many other environments (26, 27), contains a diverse array of fungi. OFRG analysis of the murine intestine identified rRNA genes from all four major fungal phyla: *Ascomycota*, *Basidiomycota*, *Chytridiomycota*, and *Zygomycota*. FISH analysis corroborated these data by revealing fungus-like structures with diverse morphotypes. Our discussion addresses the OFRG analysis of intestinal microbiota, fungus-selective FISH analysis, interpretation of the intestinal fungal data, and the contribution of food as a source of luminal bacterial and fungal rRNA genes.

OFRG analysis of intestinal bacteria. Common enteric bacterial taxa identified in previous studies have included *Bacillus*, *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Enterobacter*, *Enterococcus*, *Eubacterium*, *Fusobacterium*, *Helicobacter*, *Lactobacillus*, *Peptostreptococcus*, *Ruminococcus*, and segmented filamentous bacteria (8, 49, 51, 54). In this study, most of the bacterial rRNA gene clones identified by OFRG analysis were affiliated with the taxa *Bacteroidetes*, *Firmicutes*, *Acinetobacter*, and *Lactobacillus*. Although *Acinetobacter* spp. were not described in these prior gut studies, members of this genus have been associated with a variety of environments and conditions, including human gastritis (48), nosocomial infections (15), and food (22, 34). As with other culture-independent analyses of bacteria from mice (35, 49), large numbers of bacterial clones identified in this study had relatively low sequence identities to previously described rRNA genes. This result provides additional evidence for the variable and diverse nature of gut bacteria.

OFRG analysis of intestinal fungi. OFRG of the mouse intestinal system revealed a diversity of rRNA genes from all major fungal taxa in both the small and large intestines. The *Ascomycota* sequences were most closely associated with the genera *Acremonium*, *Alternaria*, *Monilinia*, and *Fusarium*. *Acremonium* species can form endophytic associations with grass species (52) and cause infections in humans (20). *Alternaria alternata* is a ubiquitous environmental saprophyte which has been associated with allergic disorders and ocular infections (25, 63). *Monilinia laxa* has been described as a causal agent of brown rot of stone fruits (21). The form genus *Fusarium* includes a variety of saprophytes, plant pathogens, human pathogens, and mycotoxin producers (18, 45, 53). The *Basidiomycota* sequences were most closely associated with the genera *Filobasidium*, *Cryptococcus*, and *Scleroderma*. Members of the genus *Scleroderma* are found throughout the world and commonly form ectomycorrhizal associations with shrubs and trees (2). *Filobasidium* and *Cryptococcus* are yeasts found in soil and on plant tissues (7, 19). Most species appear to be nonpathogenic to humans and animals, with the notable exception of *Cryptococcus neoformans*.

The majority of the *Chytridiomycota* and *Zygomycota* sequences were assembled into two major clusters: cluster 1 and another comprised of members of the order *Mucorales*. Cluster 1 contains members of the genera *Catenomyces*, *Entophlyctis*, *Mortierella*, *Neocallimastix*, *Powellomyces*, *Smittium*, and *Spizellomyces*. *Catenomyces* and *Entophlyctis* have been identified as inhabitants of aquatic environments (24, 29, 32). *Mortierella alpina* is a common soil fungus that produces large amounts of arachidonic acid (14, 37). *Neocallimastix* spp. are anaerobic fungi commonly found in the guts of herbivores (28), and *Powellomyces* spp. have been identified in soil environments (39). *Smittium culisetae* is a member of the *Trichomycetes*, which are

obligate inhabitants of arthropod guts (36). Although *Trichomycetes* are thought to interact with their host in a commensalistic manner, pathogenic behavior of some species has also been described (36). *Spizellomyces* spp. are commonly found in soil and can live saprophytically or parasitize fungi and nematodes (40). Sequences belonging to the order *Mucorales* were related to the genera *Amylomyces*, *Backusella*, and *Rhizopus*. *Amylomyces rouxii* is involved in rice fermentation for the production of tape ketan, a traditional Indonesian food (6). *Rhizopus oryzae*, which is also called *Rhizopus arrhizus*, is a pathogen of plants and humans (4, 46).

FISH analysis of intestinal fungi. FISH analysis showed that fungi of diverse morphotypes inhabit a poorly organized biofilm of the cecum and, to a lesser extent, the proximal colon. Quantitative analysis indicated that fungi form ~2% of the cecal biofilm microbial population, but their levels were much lower in the biofilms of other intestinal segments. In fecal material, fungi were observed in the cecal region, but they were not detected in the small intestine and the distal colon. Several analytic issues should be noted. First, there are technical limitations of FISH analysis. For example, FITC-PF2 hybridization of cultured *S. cerevisiae* detects only 30 to 50% of organisms, reflecting factors such as rRNA levels and permeabilization efficiency (31). rRNA probes also may fail to detect certain fungal taxa. Also, fungal taxa may vary in their morphological integrity during intestinal passage or after histologic processing. If these are important components of the biofilm, then the relative fungal contribution to the microbial biofilm may be further underestimated. Second, the intestinal mucosal biofilm has received only limited study, and these studies have relied (as in this report) on FISH assessment of microanatomic sections preserved with Carnoy's solution (which preserves the extramucosal mucus layer during histologic processing). The advent of other recovery or preservation systems would validate and possibly refine biofilm scale and composition.

Role of intestinal fungi. The discovery of these fungal rRNA genes raises important functional questions. What role, if any, do fungi play in food metabolism for nutrient and energy bioavailability? Since anaerobe fungi contribute to the digestion of fibrous materials in ruminants (1, 23), they may play a similar role in nonruminant animals. Similarly, how might fungi contribute to microbial immune homeostasis? Several recent reports have implicated fungi in the development of allergic and immune responses. In mice, antibiotic perturbations of gastrointestinal communities coupled with *Candida albicans* supplementation led to an increase in levels of eosinophils, mast cells, interleukin-5, interleukin-13, gamma interferon, immunoglobulin E, and mucus-secreting cells in response to exposure to *Aspergillus fumigatus* (44). The authors of that study suggest that this response may be caused by the production of prostangladin-like oxylipins by the fungus. In addition, as fungi produce an assortment of immune-modulating compounds, it has also been suggested that these organisms could be associated with a range of immune alterations (43). These findings, coupled with the diverse array of rRNA genes identified in this study, suggest that fungi may play an important role in microbial immune homeostasis.

Microbial analysis of mouse chow. Analysis of the mouse chow suggested that some of the microbial rRNA genes identified in the intestinal analysis, and/or the organisms represented by these genes, were present in the chow. When assays targeting some of these specific rRNA sequences were used, most of them were detected in the chow. However, when assays targeting all bacteria and fungi were used, none of the rRNA genes identified by analysis of the intestine were detected in the chow. Given that the universal bacterial primers anneal to corn mitochondrial, rice chloroplast, and wheat chloroplast rRNA genes with either one or no mismatches, it is not surprising that these plant-derived sequences predominated this analysis. Taken together, these results suggest that some of the intestinal rRNA genes were present in the chow but that they were a minor constituent compared to the plant components.

Interpreting the data from this study is complicated by the detection of some of the same microbial rRNA genes in both the intestine and chow. The rRNA genes identified in the intestinal samples may have come from intestinal inhabitants, or they may have originated from the chow in the form of living organisms, dead organisms, or their DNA. Although distinguishing among these possibilities is not a simple task, it may be an important one. For example, in a study examining the probiotic bacterium VSL-3, the viable bacterium and its DNA attenuated colitis, while the heat-killed bacterium did not (47). At this point, however, it is unclear how such studies could be performed on complex communities of intestinal microorganisms, many of which are yet to be described.

Detection of some of the same microbial rRNA genes in both the intestine and chow could also influence the design of future experimentation as well as the interpretation of data from past and future investigations. Experimental design considerations derived from these results should include chow type selection. For example, when comparing germfree and conventionally raised animals fed different chows, chow type should be considered a potential variable. Concerning data analysis, when interpreting results from rRNA gene analysis of intestine or feces, it may be important to consider the origin of the sequences.

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