

Intestinal Fungal and Bacterial Composition of the Murine Intestine

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ABSTRACT

Fungi are a diverse group of microorganisms involved in a variety of processes. In the mammalian intestine, the extent of fungal diversity and their roles in physiological and disease processes remains unclear. This study utilized a culture-independent method termed oligonucleotide fingerprinting of ribosomal RNA genes (OFRG) to describe the intestinal fungal and bacterial composition 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*. Most of the bacterial rRNA gene clones were affiliated with three genera: *Acinetobacter*, *Clostridium*, and *Lactobacillus*. This report provides the first culture-independent, rRNA gene-based analysis of the fungal composition of a mammalian gut, to our knowledge. Most of these fungi have not been previously associated with mammalian intestine.

INTRODUCTION

Gut microflora play a variety of roles in health and disease. 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 (IBD), the induction of inappropriate inflammatory responses. Obtaining thorough descriptions of microbial community composition has been and continues to be a challenge. Culture techniques detect only a fraction of microorganisms. A variety of strategies to directly analyze rRNA molecules has provided a means to examine microbial communities without the culture bias, but generate non-comprehensive and non-quantitative descriptions of microbial composition. rRNA-targeted fluorescent in situ hybridization (FISH) provides a quantitative means to examine microbial composition, but only detect pre-selected taxa. Finally, nucleotide sequence analysis of rRNA clone libraries permit thorough depictions of microbial composition, but this approach remains restricted to centers for very high-throughput sequencing and analysis.

FIGURE 1

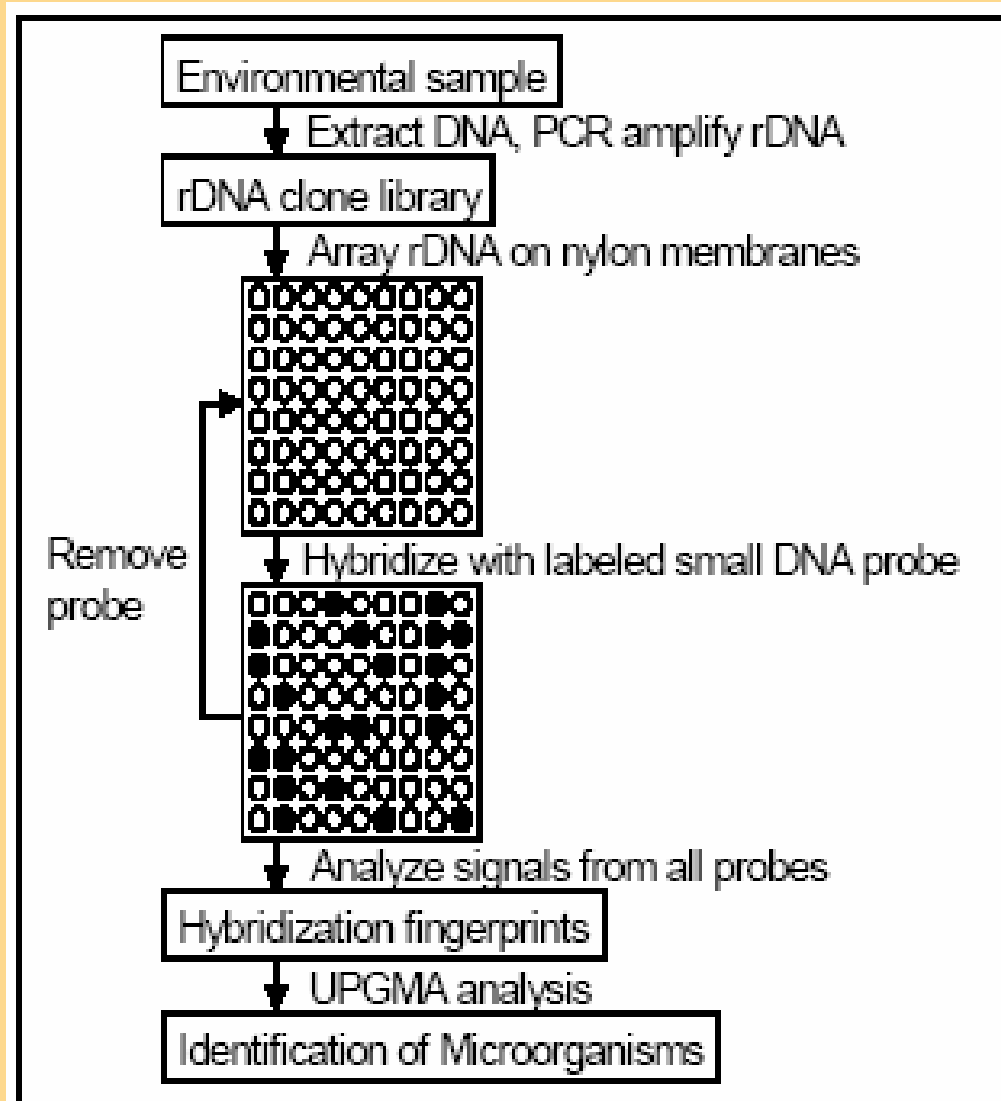


FIGURE 1

To provide a cost-effective method for extensive analysis of microbial community composition, a method termed oligonucleotide fingerprinting of ribosomal RNA genes (OFRG) was developed (Appl. Environ. Microbiol. 68:3243, 2002; Appl. Environ. Microbiol. 69:1573, 2003). Clone libraries are constructed using PCR primers designed to selectively amplify rDNA from a specific taxonomic group such as bacteria or fungi. Cloned rRNA gene fragments are arrayed on nylon membranes and then subjected to a series of hybridization experiments, each using a single DNA oligonucleotide probe. For every hybridization experiment, the signal intensities are transformed into three discrete values 0, 1, and N, where 0 and 1 respectively specify negative and positive hybridization events and N designates an uncertain assignment. This process creates a hybridization fingerprint for each clone, which is a vector of values resulting from its hybridizations with all probes. The clones are identified by clustering their hybridization fingerprints with those from taxonomically identified organisms and by nucleotide sequence analyses of representative clones within a cluster.

OFRG offers several useful features which result from the manner in which it classifies gene sequences. Unlike most array-based approaches, which have at least one probe for each target sequence, OFRG uses a small set of probes to coordinately distinguish a much larger set of sequences. Probe sets are designed from training data, which are assemblages of all known rRNA genes within a desired group. OFRG analyses can be designed to identify a taxonomically narrow group of organisms such as all *Candida* species, or, it can be designed to identify a larger group of organisms such as all known fungi. In addition, unlike other array-based methods, OFRG can also identify rRNA gene sequences that have not been previously described. This is accomplished through nucleotide sequence analysis of representative clones within clusters that do not associate with known sequences.

This poster describes the use of OFRG to identify fungi and bacteria inhabiting small intestine and colon from restricted flora (RF) and specific pathogen free (SPF) mice. To our knowledge, this report provides the first culture-independent, rRNA gene-based analysis of fungal composition of a mammalian intestine.

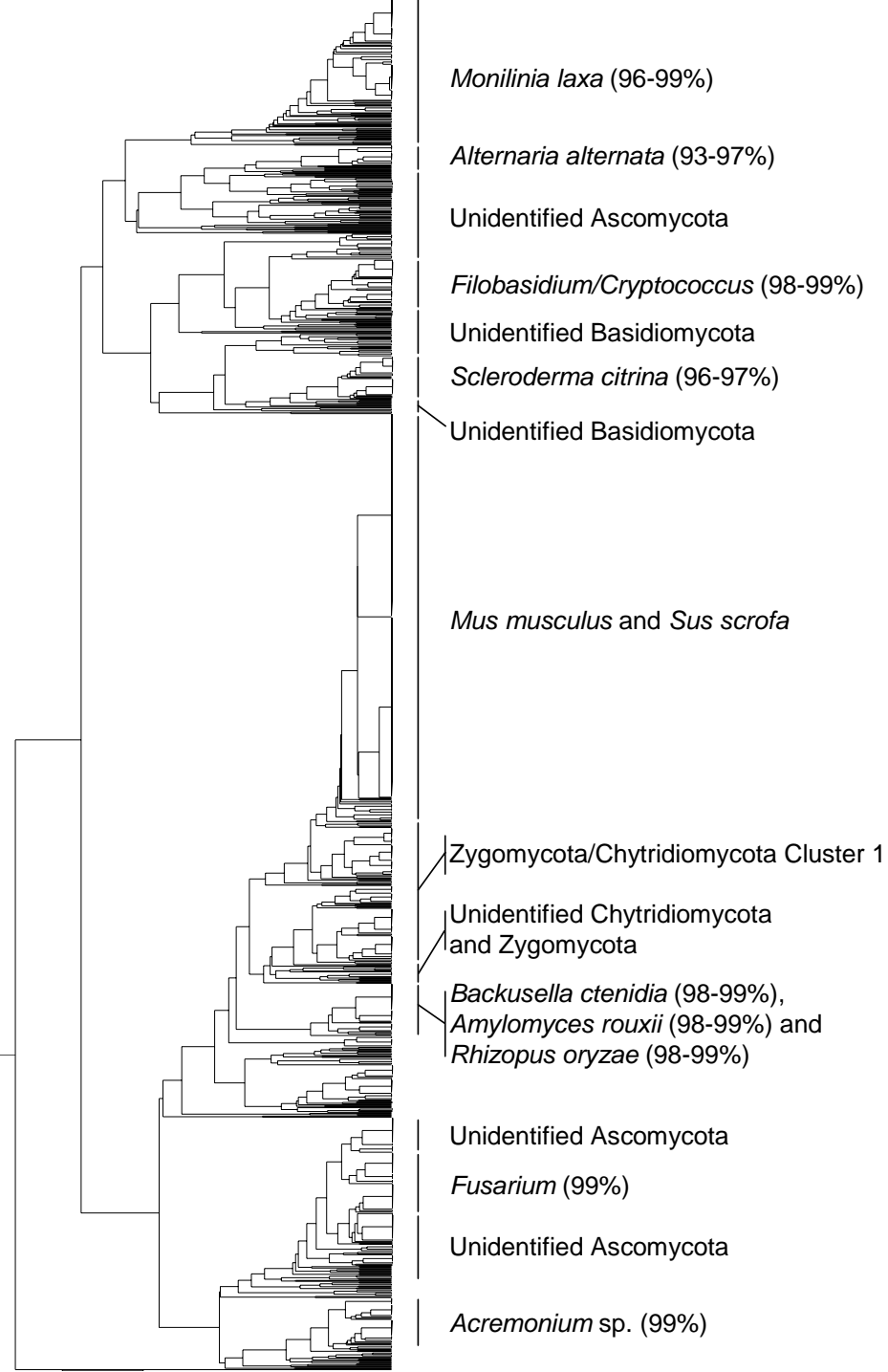


FIGURE 2

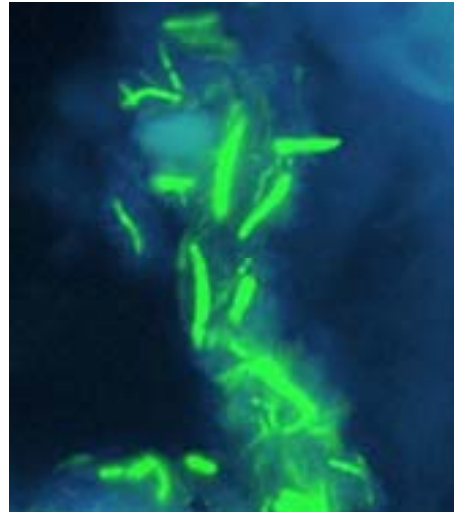
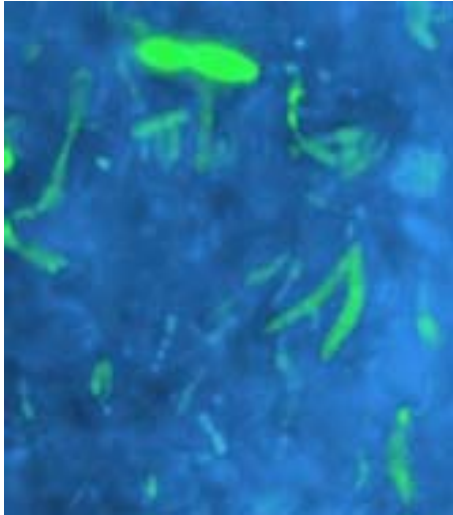
TABLE 1. Taxonomic distribution of rRNA gene clones obtained through PCR amplification of small and large intestine samples from RF and SPF mice using fungi-selective primers.

Taxon	No. of clones ^a					Nearest relatives (accession) ^b	% Identity to nearest relatives ^b
	Total	RF-C	RF-SI	SPF-C	SPF-SI		
<i>Ascomycota</i>	477	153	40	164	120		
<i>Acremonium</i>	57	7	3	39	8	<i>Acremonium alternatum</i> (AY083232)	99
<i>Alternaria</i>	28	13	5	1	9	<i>Alternaria alternata</i> (AF229504)	93-97
<i>Monilinia</i>	151	70	1	28	52	<i>Monilinia laxa</i> (Y14210)	96-99
<i>Fusarium</i>	63	4	5	50	4	<i>Gibberella pulicaris</i> (AF149875), <i>Fusarium oxysporum</i> (AF141951), <i>Fusarium equiseti</i> (AF141949), <i>Fusarium culmorum</i> (AF141948), <i>Fusarium cerealis</i> (AF141947), <i>Gibberella avenacea</i> (AF141946), <i>Cordyceps sinensis</i> (AB067700)	99
Unidentified	178	59	26	46	47		
<i>Basidiomycota</i>	154	46	11	61	36		
<i>Cryptococcus/Filobasidium</i>	51	1	4	40	6	<i>Filobasidium globisporum</i> (AB075546), <i>Filobasidium elegans</i> (AB075545),	98-99

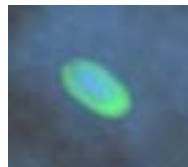
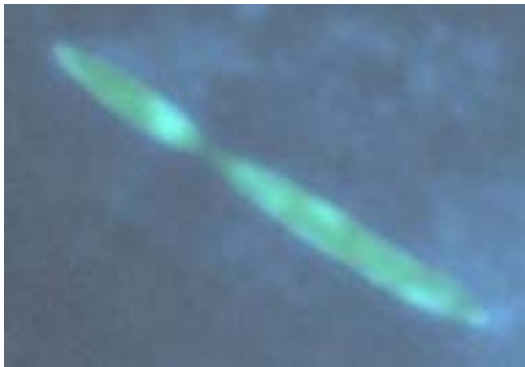
Fungal intestinal composition

Fungal composition of small and large intestine from RF and SPF mice were obtained by OFRG analysis (**FIGURE 2 and TABLE 1**). Most of the rRNA gene clones were distributed among nine well-defined taxonomic clusters. Two hundred and 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 and 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*, *Rhodosporeidium toruloides*, *Plectosphaerella cucumerina*, *Sordaria fimicola*, and *Myrothecium verrucaria*; accession numbers for these sequences are listed in the “Nucleotide sequence data” section of the Materials and Methods. The taxonomic identities of the major clusters identified by the OFRG analysis were validated by nucleotide sequence analysis of representative rRNA gene clones. The percent sequence identities to their nearest relatives are shown in Table 1.

Fungal FISH

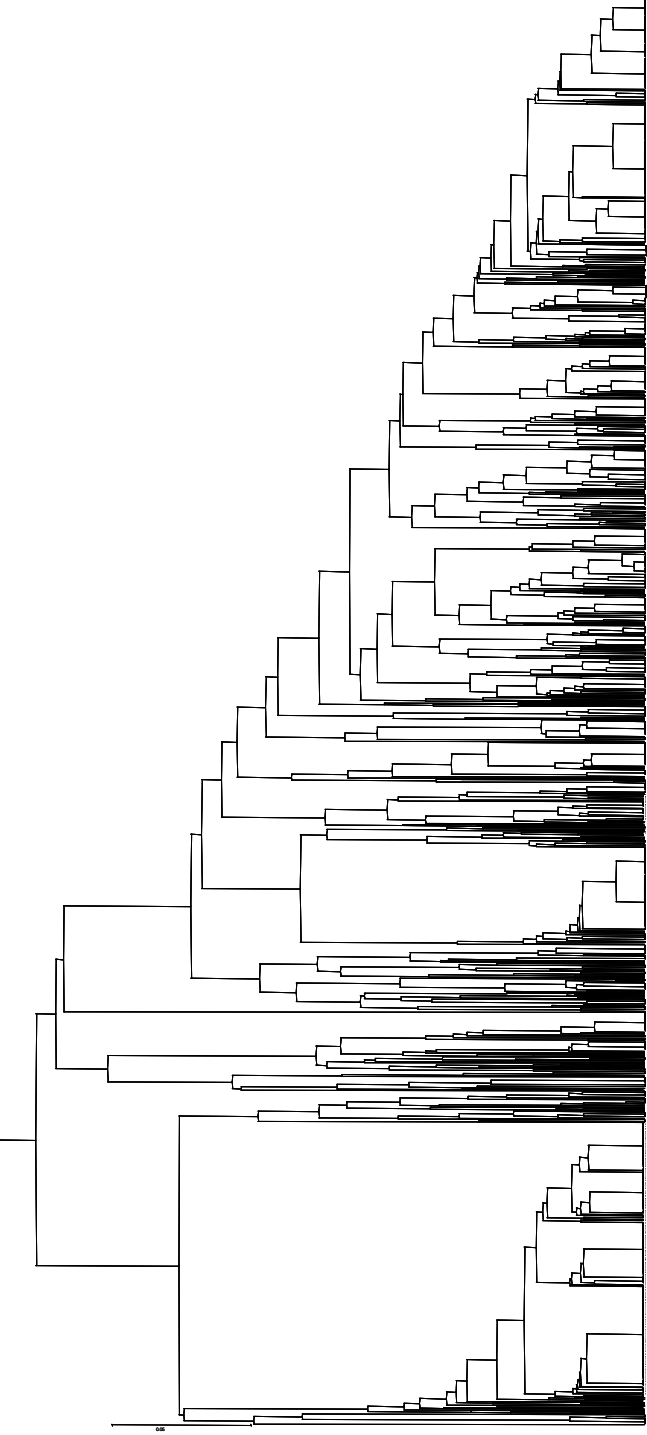


1000x



2500 x

Mouse intestinal specimens were hybridized with a FITC-conjugated universal fungal rRNA probe (PF2), and examined by immunofluorescence. Microorganisms were exclusively detected in the extra-mucosal mucus layer, and were anatomically localized to the ileum, cecum, and proximal intestine.



Clostridium

CFB

Lactobacillus

Acinetobacter

FIGURE 3

TABLE 2. Taxonomic distribution of rRNA gene clones obtained through PCR amplification of small and large intestine samples from RF and SPF mice using bacteria-selective primers.

Taxon	No. of clones ^a					Nearest relatives (accession) ^b	% Identity to nearest relatives ^b
	Total	RF-C	RF-SI	SPF-C	SPF-SI		
<i>Bacteria</i>							
<i>Acinetobacter</i>	313	24	5	145	139	<i>Acinetobacter</i> sp (AJ303013), <i>Acinetobacter</i> sp. (AJ244765), <i>Acinetobacter johnsonii</i> (AB099655)	99
<i>Clostridium</i>	763	323	305	70	65	Uncultured gut bacterium (AJ409005, AY305312, AY442823, AJ408985, AJ408992, AJ576354), <i>Ruminococcus</i> sp. (AB125231), <i>Clostridium boltei</i> (AJ508452), <i>Clostridium amygdalinum</i> (AY353957), Butyrate-producing gut bacterium (AY305310, AJ270478, AY305318), <i>Clostridium disporicum</i> (Y18176)	88, 85, 87, 87, 85, 89, 86, 87, 92, 87, 89, 86, 98, respectively
<i>Lactobacillus</i>	105	0	7	56	42	<i>Lactobacillus johnsonii</i> (AB017206)	99
Unidentified	237	33	61	66	77		

^aDetermined by adding the number of clones in the major taxonomic groups identified by the OFRG analysis (data not shown).

^bDetermined by BLAST (NCBI) analysis of representative clones from 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.

Bacterial composition of small and large intestine from RF and SPF mice were obtained by OFRG analysis (**FIGURE 3, TABLE 2**). Most of the rRNA gene clones were distributed among three well-defined taxonomic clusters: *Acinetobacter*, *Clostridium*, and *Lactobacillus*. Other rRNA gene sequences identified from small clusters or taxonomically mixed clusters were related to *Enterococcus* sp., and *Cytophaga* sp; accession numbers for these sequences are listed in the “Nucleotide sequence data” section of the Materials and Methods. The taxonomic identities of the major clusters identified by the OFRG analysis were validated by nucleotide sequence analysis of representative rRNA gene clones. The percent sequence identities to their nearest relatives are shown in Table 2.

SUMMARY

This report describes the fungal composition of small and large intestines from isogenic mice containing normal and restricted microflora. OFRG analysis identified rRNA genes from all four major fungal phyla: *Ascomycota*, *Basidiomycota*, *Chytridiomycota* and *Zygomycota*. The discovery of these fungi raises many more questions than it answers. What role, if any, do these fungi play in immune-microbe homeostasis? Are these organisms beneficial or detrimental? Do diverse communities of fungi inhabit the gut of most animals? Do fungi colonize other tissues, cells or organs? Although these questions and others remain unanswered, this study establishes that the mammalian gut contains a diverse array of fungi that are likely to contribute to intestinal homeostasis and disease susceptibility.