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Analysis of 16S libraries of mouse gastrointestinal microflora reveals a large new group of mouse intestinal bacteria

Nita H. Salzman,[1]† Hendrik de Jong,[1] Yvonne Paterson,[1] Hermie J. M. Harmsen,[2] Gjalt W. Welling[2] and Nicolaas A. Bos[3]

Author for correspondence: Nita H. Salzman. Tel: +1 414 456 4244. Fax: +1 414 456 6535. e-mail: nsalzman@mcw.edu

[1] Department of Microbiology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA

[2] Department of Medical Microbiology, University of Groningen, PO Box 30001, 9700 RB Groningen, The Netherlands

[3] Department of Cell Biology, Histology and Immunology Section, University of Groningen, A. Deusinglaan 1, 9713 AV Groningen, The Netherlands

Total genomic DNA from samples of intact mouse small intestine, large intestine, caecum and faeces was used as template for PCR amplification of 16S rRNA gene sequences with conserved bacterial primers. Phylogenetic analysis of the amplification products revealed 40 unique 16S rDNA sequences. Of these sequences, 25% (10/40) corresponded to described intestinal organisms of the mouse, including *Lactobacillus* spp., *Helicobacter* spp., segmented filamentous bacteria and members of the altered Schaedler flora (ASF360, ASF361, ASF502 and ASF519); 75% (30/40) represented novel sequences. A large number (11/40) of the novel sequences revealed a new operational taxonomic unit (OTU) belonging to the *Cytophaga–Flavobacter–Bacteroides* phylum, which the authors named 'mouse intestinal bacteria'. 16S rRNA probes were developed for this new OTU. Upon analysis of the novel sequences, eight were found to cluster within the *Eubacterium rectale–Clostridium coccoides* group and three clustered within the *Bacteroides* group. One of the novel sequences was distantly related to *Verrucomicrobium spinosum* and one was distantly related to *Bacillus mycoides.* Oligonucleotide probes specific for the 16S rRNA of these novel clones were generated. Using a combination of four previously described and four newly designed probes, approximately 80% of bacteria recovered from the murine large intestine and 71% of bacteria recovered from the murine caecum could be identified by fluorescence *in situ* hybridization (FISH).

Keywords: commensal bacteria, intestinal colonization, *Bacteroides*

INTRODUCTION

The indigenous intestinal microflora of mammals is involved in host nutrition, mucosal defence and host immunity. The presence of commensal bacteria in the intestinal tract provides the first line of defence against pathogenic bacteria, by the establishment of colonization resistance (van der Waaij, 1989). The use of probiotics as dietary supplements is being explored for the prevention of intestinal infection (Goldin & Gorback, 1992) by the modulation or re-establishment of the normal commensal bacteria. More recently, abnormal host immune responses to bacteria colonizing the intestine have been implicated in the pathologic process of inflammatory bowel disease (Kuhn *et al.*, 1993; Sadlack *et al.*, 1993). Standard selective culture techniques have been applied in studies of the gastrointestinal microflora of mice (Dubos *et al.*, 1965; Schaedler *et al.*, 1965), humans and many other mammalian species. Since the pioneering studies of Schaedler *et al.* (1965) and Dubos *et al.* (1965), only a limited number of studies have explored the gastrointestinal population of mice by using traditional culturing methods. From these original studies, it was shown that the cultivable microflora of the mouse intestine includes facultative anaerobes, including *Lactobacillus* spp., *Enterococcus* spp. and *Enterobacillus*

†**Present address:** Medical College of Wisconsin, Department of Pediatrics, 8701 Watertown Plank Rd, Milwaukee, WI 53226, USA.

Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; FISH, fluorescence *in situ* hybridization; MIB, 'mouse intestinal bacteria'; OTU, operational taxonomic unit; SFB, segmented filamentous bacteria.

The GenBank accession numbers for the clone sequences reported in this paper can be found in Table 1; the accession number for isolate MIB-CB3 is AJ418059.

spp., and obligate anaerobes, including *Bacteroides* spp. and *Clostridium* spp. (Dubos *et al*., 1965; Schaedler *et al*., 1965). Morphologically, the majority of the bacteria of the mouse gastrointestinal tract are fusiform. Only a few representatives of these organisms have been cultivated, but on the basis of their morphological and growth characteristics those that have been cultivated are generally considered to belong to the genera *Eubacterium*, *Fusobacterium* and *Clostridium* (Dewhirst *et al*., 1999). Segmented filamentous bacteria (SFB), belonging to the *Clostridium* group on the basis of 16S rRNA gene analysis (Snel *et al*., 1994), are a clear example of a population of organisms that cannot be cultured using current methods. However, these bacteria have been shown to account for a significant percentage of the mouse gut microflora, especially in young mice (Talham *et al*., 1999). The more recent application of molecular approaches to studying human intestinal bacterial populations suggests that the majority of intestinal bacteria cannot be identified by the culturing techniques currently available (Franks *et al*., 1998; Langendijk *et al*., 1995; Suau *et al*., 1999). It has been suggested that >90% of the organisms that make up the gastrointestinal tract ecosystem have not yet been identified (Savage, 1977); this may be largely due to our inability to create adequate culture environments in which to grow many of these organisms.

Molecular methodologies relying on 16S rRNA gene sequences are now commonly used for the identification and classification of bacterial species within mixed microbial populations (Amann *et al*., 1991; Amann *et al*., 1995; Suau *et al*., 1999; Ward *et al*., 1990). Oligonucleotide probes that are specific for the 16S rRNA of distinct bacterial species have been developed for use in fluorescence *in situ* hybridization (FISH) studies (Amann *et al*., 1990, 1995; Giovannoni *et al*., 1988; Harmsen *et al*., 1999). Probes specific for bacteria found in the human intestine have also been generated and characterized (Franks *et al*., 1998; Langendijk *et al*., 1995; McCartney *et al*., 1996; Yamamoto *et al*., 1992).

Although the population studies detailed above concentrated on the human intestinal microflora, many studies on the interaction between the intestinal microflora and the mucosal immune system are being done in mouse models (Cebra *et al*., 1999). The intestinal microflora of specific pathogen-free mice is still ill-defined. Many inbred strains of mice started out as germ-free strains that were colonized with a defined population of bacteria, the altered Schaedler flora (Dewhirst *et al*., 1999) – the 16S rRNA gene sequences of the eight bacterial strains of the altered Schaedler flora were published by Dewhirst *et al*. (1999). Under specific pathogen-free conditions, no monitoring of the population is performed, except for the exclusion of certain defined pathogenic organisms. A number of new bacterial strains have recently been identified in the murine intestinal tract. For example, a new species of *Bacteroides*, *Bacteroides acidifaciens*, isolated from the mouse caecum was described recently (Miyamoto & Itoh, 2000). Also, a new unidentified Gram-positive rod (HCDA-1), involved in the metabolism of bile acids, was isolated from the rat intestinal microflora (Eyssen *et al*., 1999).

Because the intestinal microflora of mice is still ill-defined, we were interested in evaluating the utility of existing probes used to study the human intestinal microflora to identify murine intestinal bacteria. We were also interested in the development of new 16S rRNA probes for the detection of murine intestinal bacterial species that did not hybridize to the existing probes. In this work, we examined the bacteria present in the murine small intestine, caecum, large intestine and faeces directly, and identified the bacterial species within these regions by their 16S rRNA gene sequences. Using the 16S rRNA gene sequences derived from these bacteria, we developed oligonucleotide probes and found them to be useful for the identification and quantification of bacterial populations in the mouse intestinal tract. These newly designed probes can be applied to study the role of intestinal bacteria in diverse mouse disease models.

METHODS

Experimental animals. FvB mice were purchased from Taconic Laboratories. C57BL/6 mice were obtained from Harlan (Harlan Netherlands). All mice were housed in a specific pathogen-free facility (SPF) and fed a Purina 5015 rodent lab diet (FvB mice; Purina Mills) or an RMHB rodent diet (C57BL/6 mice; Hope Farms). Faecal samples were obtained from the C57BL/6 mice, while small intestine, large intestine and caecal samples were obtained from the FvB mice. Intestinal samples were obtained from adult FvB and C57BL/6 mice, which were between 5 and 6 weeks of age. The mice studied were age-matched litter-mates.

Bacterial cultures and isolation of intestinal bacterial DNA. Mice were killed using CO_2, and total DNA was isolated from the large intestine, small intestine, caecum and faeces of the mice. Each specimen was homogenized in TE buffer. Lysozyme (5 mg ml^{-1}) was added to the homogenate and the mixture was incubated at 37 °C for 1 h. Proteinase K (2 mg ml^{-1}) was added to the homogenate, which was incubated for a further 1 h at 56 °C. SDS (1%, w/v) was then added to the homogenate, which was incubated at 37 °C for 30 min. The DNA was extracted from the homogenate with equal volumes of phenol/chloroform/isoamyl alcohol. The sample was centrifuged and the DNA (in the aqueous layer) was precipitated overnight with 3 M sodium acetate/ethanol. The sample containing the precipitate was centrifuged, the supernatant removed, and the resulting DNA pellet resuspended in TE buffer. A species belonging to the 'mouse intestinal bacteria' (MIB) group was isolated from the caecum sample by plating dilution series of the untreated homogenate onto pre-reduced *Brucella* blood agar plates supplemented with 5% sheep blood (Summanen *et al*., 1993); the plates were incubated anaerobically for 48 h at 37 °C. After 48 h, random colonies were picked from the agar, and these were simultaneously subcultured and hybridized with the MIB661 probe. One MIB661-positive isolate was obtained. A partial DNA sequence was obtained from this isolate by direct sequencing of its 16S rDNA, after PCR amplification of the 16S rRNA gene.

PCR amplification of 16S rRNA gene sequences, cloning and sequencing. 16S rRNA genes were specifically amplified by

PCR using the conserved 16S-rRNA-specific primer pairs 8FE (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-GGMTACCTTGTTACGACTT-3′), and 519FB (5′-ATTG-GATCCCAGCMGCCGGGGTAA-3′) and 1392RS (5′-TGA-GTCGACACGGGCGGTGTGTRC-3′). The PCR conditions used were 30 cycles at 96 °C for 1 min, 48 °C for 2 min and 72 °C for 2 min, followed by a final elongation at 72 °C for 5 min. PCR products were purified by using preparative low-melting-point-agarose gel electrophoresis to separate the products. The band corresponding to 1500 bp was excised from the gel. The excised piece of gel was heat-sealed in a plastic pouch (Kapak) and frozen at −20 °C; it was then squeezed to remove the DNA. The DNA was precipitated from the recovered liquid by using 3 M sodium acetate/ethanol. The sample containing the PCR product was centrifuged, the supernatant discarded, and the resulting pellet was dissolved in TE buffer. The PCR product was then introduced into pPUC18 by using the TA Cloning Kit (Invitrogen). pPUC18 containing the PCR product was used to transform *Escherichia coli* DH5α; plasmids of the resulting clones were isolated for sequencing or slot-blot hybridization using the Wizard Mini-Prep Kit (Promega) and extraction with phenol/chloroform. The clone sequences were obtained by using vector-specific primers and an ALFwin automated sequencing device (Amersham Pharmacia).

Analysis of the 16S rRNA gene sequences and the design of new probes. The clone sequences were compared with sequences of reference organisms from the Ribosomal Database Project (release 8.0) (Maidak *et al*., 2001), which contains about 16000 sequences, including sequences of the altered Schaedler flora (Dewhirst *et al*., 1999). The clone sequences and the 16S rDNA sequences of their nearest relatives were aligned; the ARB package (Ludwig *et al*., 1998) was used to produce the multiple-sequence alignment. A phylogenetic tree, generated by using the neighbour-joining method with Jukes–Cantor 2-correction parameter, was produced to illustrate the clustering of the different clone groups with their nearest relatives. The distance matrix (generated from the multiple-sequence alignment) used in the neighbour-joining analysis included stretches of sequence corresponding to *E. coli* positions 41–1464 and only used positions with more than 50 % invariability, as implemented in the ARB software. Hence, the phylogenetic analysis was based on 1192 nt. The topology of the tree was analysed by bootstrap analysis (1000 replications). New probes were designed from the sequences of the clones. Existing probes were tested to identify the target groups of bacteria that were grouped on the basis of the obtained sequences, by screening for group-specific target sequences using the ARB software (Ludwig *et al*., 1998). Specificity of the probes was tested by slot-blot hybridization of clones with known sequences using increasing stringency, i.e. increasing the temperature and the formamide concentration, as described previously (Franks *et al*., 1998).

Slot blots and hybridization with 16s rRNA oligonucleotide probes. A series of slot blots were generated for each set of clones obtained from the different intestinal sites. ^{32}P-labelled 16S rRNA oligonucleotides were used to probe the slot blots, as described previously (Franks *et al*., 1998). Hybridizations were done overnight at 50 °C in 30 % formamide. The samples were then washed at room temperature for 1 h, followed by washing at 37 °C in 2× SSC and 0·1 % SDS for 1 h.

Sample preparation and enumeration of intestinal bacteria. The complete contents of the caeca and the large intestines of five 5-week-old FvB mice were collected and frozen at −20 °C. After thawing the samples on ice, PBS (137 mM NaCl, 2·7 mM KCl, 5·4 mM Na_2HPO_4, 1·8 mM KH_2PO_4, pH 7·4) was added to them to give 1 g sample (10 ml PBS)$^{-1}$. For each sample, the suspension was homogenized by using a Polytron homogenizer for 5 s and centrifuged at 35 ***g*** for 15 min. The supernatant was mixed with 3 vols of freshly prepared 4 % paraformaldehyde in PBS. The bacteria were fixed overnight at 4 °C. An aliquot (2 ml) of the fixed bacteria was centrifuged at 12000 ***g*** for 10 min and washed twice with PBS. The pellet was taken up in 400 µl of 50 % ethanol in PBS, which was kept at −20 °C. This procedure typically yielded 1×10^9 bacteria (ml ethanol-fixed stock)$^{-1}$ (as counted with a Petroff–Hausser counting chamber; Hausser Scientific).

FISH. From the ethanol-fixed stock, 6×10^6 cells were added to 300 µl of 50 °C hybridization buffer [0·9 M NaCl, 20 mM Tris/HCl (pH 7·2), 0·1 % SDS] containing 10 ng oligonucleotide probe µl^{-1} (oligonucleotide probes with FITC incorporated into their 5′ and 3′ ends were synthesized by Operon Technologies). The bacteria/hybridization buffer/probe mixture was incubated overnight at 50 °C, to allow hybridization. The mixture was then centrifuged at 12000 ***g*** for 5 min. The resulting pellet was washed once with a 50 °C washing solution (hybridization buffer without SDS) and once with PBS (pH 8·5); it was then taken up in 500 µl PBS (pH 8·5).

Fluorescence microscopy. To 200 µl of the hybridized bacteria, 10 µl of 4′,6-diamidino-2-phenylindole (DAPI; 15 µg ml^{-1}) was added. The bacteria were suspended in 3 ml PBS and then collected on a 0·2 µm filter (Millipore). The filter was mounted onto a slide using Vectashield fluorescence mounting medium (Vector Laboratories). Slides were viewed under oil immersion, using a Nikon microphot FXA epifluorescence microscope equipped with DAPI and FITC filter cubes and an MTI 3CCD camera. DAPI and FITC images were captured and analysed using COUNT-PRO PLUS software (Media Cybernetics). Percentages of bacterial populations of which the ribosomes hybridized to specific 16S fluorescently labelled oligonucleotide probes were counted in a total of 1000 DAPI-positive bacteria.

RESULTS

Cloning of 16S rRNA genes and sequence analysis

In total, 10 independent libraries of cloned 16S rRNA gene sequences were produced – one from a faecal sample, one from a caecal sample, three from large intestine samples and five from small intestine samples. This resulted in 152 clones, 70 of which were used for 16S rRNA gene sequence analysis. Four additional clones were sequenced, but one was found to contain an eukaryotic sequence and the other three were found to contain chimeric sequences. In total, 40 unique 16S rRNA gene sequences were found; these sequences were defined as unique if they showed >2 % nucleotide differences with the sequence of their nearest relatives, or if they came from different isolation sites. These unique sequences, referred to here as 'sequences' rather than 'clones', were submitted to the EMBL database, and their accession numbers can be found in Table 1. A phylogenetic tree was constructed using 24 of the full-length sequences along with the sequences of their closest relatives (Fig. 1). We also performed partial sequencing on 46 clones, which confirmed our phylo-

Table 1. Summary of sequenced clones used for phylogenetic analysis

Group/species*	Unique sequences	Identity with closest relative (%)	Closest relative	Origin	Sequence length (nt)	No. of clones	Accession no.
Eubacterium rectale–Clostridium coccoides ($n=20$)	C13-5	98·5	*Clostridium* sp. ASF502	Caecum	1493	1	AJ308396
	L7-1	97·3	*Clostridium clostridiiformes*	Large intestine	886	4	AJ400247
	L7-6	94·7	*Ruminococcus schinkii*	Large intestine	886	1	AJ400250
	L10-2	90·6	*Clostridium celerecrescens*	Large intestine	1502	1	AJ400265
	L10-3	98·3	*Clostridium* sp. ASF502	Large intestine	1494	1	AJ400255
	L10-8	93·2	'*Clostridium fusiformis*'	Large intestine	1497	5	AJ400260
	L11-5	93·2	*Clostridium* sp. ASF502	Large intestine	1495	6	AJ400261
	L11-10	91·8	*Clostridium polysaccharolyticum*	Large intestine	547	1	AJ400272
SFB ($n=5$)	S25-6	99·5	SFB	Small intestine	1495	5	AJ308387
Bacillus mycoides ($n=4$)	S37-11	91·9	*Bacillus mycoides*	Small intestine	882	4	AJ308388
Helicobacter ($n=1$)	L10-17	99·8	*Helicobacter* sp.	Large intestine	509	1	AJ308389
Lactobacillus sp. ($n=8$)	F15	99·1	*Lactobacillus acidophilus* subsp. *johnsonii*	Faeces	790	1	AJ400238
	L10-37	99·9	*Lactobacillus* sp. ASF360	Large intestine	1528	1	AJ308390
	S25-1	99·3	*Lactobacillus acidophilus* subsp. *johnsonii*	Small intestine	583	1	AJ308391
	S25-5	99·7	*Lactobacillus reuteri*	Small intestine	1537	2	AJ308392
	S25-9	99·3	*Lactobacillus murinus*	Small intestine	1529	3	AJ308393
Verrucomicrobium ($n=4$)	L10-6	83·2	Candidatus *Xiphinematobacter*	Large intestine	1472	4	AJ400275
Bacteroides sp. ($n=3$)	L7-8	99·8	*Bacteroides acidofaciens*	Large intestine	878	1	AJ400252
	L7-16	97·8	*Bacteroides* sp. ASF519	Large intestine	858	1	AJ400245
	L7-17	96·9	*Bacteroides vulgatus*	Large intestine	857	1	AJ400246
MIB ($n=16$)	F1	82·9	*Bacteroides distasonis*	Faeces	1494	1	AJ400234
	F2	83·8	*Porphyromonas* sp.	Faeces	1494	1	AJ400235
	F3	83·7	*Bacteroides forsythus*	Faeces	1493	1	AJ400236
	F5	81·0	*Prevotella* sp.	Faeces	1501	1	AJ400266
	F8	82·1	*Prevotella* sp.	Faeces	1500	2	AJ400267
	F18	83·6	*Bacteroides distasonis*	Faeces	1491	1	AJ400254
	L7-2	86·0	*Bacteroides forsythus*	Large intestine	884	1	AJ400249
	L7-10	90·0	*Bacteroides distasonis*	Large intestine	955	1	AJ400243
	L11-6	84·3	*Porphyromonas* sp.	Large intestine	1496	1	AJ400264
	S30-4	84·3	*Bacteroides distasonis*	Small intestine	887	1	AJ400241
	S30-5	84·7	*Bacteroides distasonis*	Small intestine	893	5	AJ400242
Miscellaneous ($n=9$)	F13	86·7	*Clostridium methylpentosum*	Faeces	1488	1	AJ400237
	F16	95·7	*TM7* phylum sp.	Faeces	1489	1	AJ400239
	L7-7	90·1	*Clostridium* sp.	Large intestine	881	1	AJ400251
	L10-4	92·0	*Clostridium celerecrescens*	Large intestine	1495	1	AJ400256
	L10-14	90·0	*Acetivibrio cellulosolvens*	Large intestine	690	1	AJ418058
	L10-21	87·8	*Ruminococcus gnavus*	Large intestine	1462	1	AJ308386
	L11-2	92·6	*Eubacterium desmolans*	Large intestine	1491	1	AJ400270
	S26-5	91·8	*Desulfovibrio* sp.	Small intestine	1518	1	AJ308394
	S26-9	87·5	*Ralstonia* sp.	Small intestine	1505	1	AJ308395
Total	**40**					**70**	

* n, Total no. of clones.

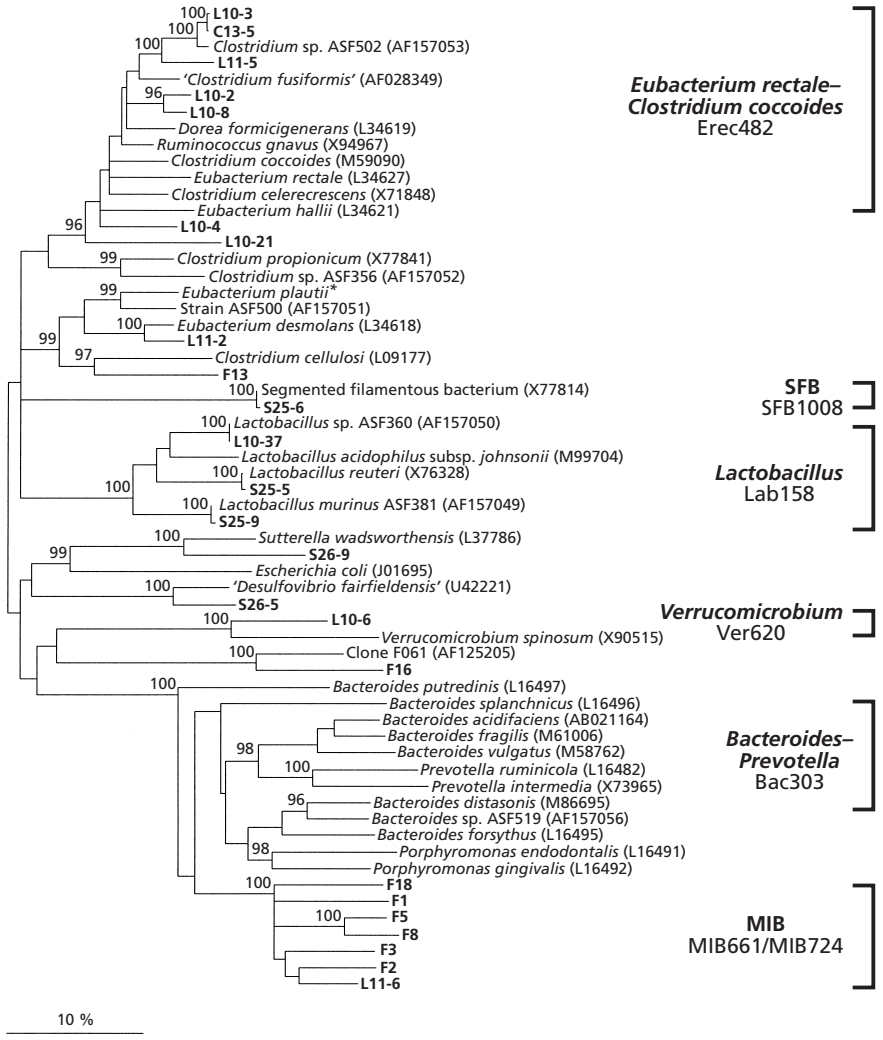

Fig. 1. Phylogenetic tree showing the relationships of 24 unique sequences from our libraries with published sequences. The published sequences are those most closely related to the unique sequences on the basis of multiple-sequence alignments of 16S rRNA gene sequences, which included *E. coli* positions 41–1446 (almost-complete sequences). The groupings, shown on the right of the figure, were made according to sequence similarity data and hybridization of the unique sequences with the group-specific probes. The group names, as well as the probes with which the groups could be detected, are shown. Bootstrap values, based on 1000 replications, at the nodes of the tree display the significance of these nodes; only values displaying >95 % confidence are given. Accession numbers for each of the published sequences are given, except for *Eubacterium plautii*, which has no available GenBank accession number. The 16S rRNA gene sequence of this species is, however, available from the ARB database under ARB_6D1EE797. ASF strains are representatives of the altered Schaedler flora. Bar, 10 % sequence divergence.

Table 2. List of oligonucleotides used in this study and their target organisms

Probe	OPD*-code	Probe sequence (5′→3′)	Targeted organisms	Reference
Bact338	S-D-Bact-0338-a-A-18	GCTGCCTCCCGTAGGAGT	Bacteria	Amann *et al*. (1995)
non-Bact338	S-D-Bact-0338-a-S-18	ACTCCTACGGGAGGCAGC	Negative control	Amann *et al*. (1995)
Erec482	S-*-Erec-0482-1-A-19	GCTTCTTAGTCARGTACCG	*Eubacterium rectale–Clostridium coccoides*	Franks *et al*. (1998)
SFB1008	S-S-SFB-1008-a-A-22	GCGAGCTTCCCTCATTACAAGG	SFB	Snel *et al*. (1995)
Lab158	S-G-Lab-0158-a-A-20	GGTATTAGCAYCTGTTTCCA	*Lactobacillus–Enterococcus*	Harmsen *et al*. (1999)
Bmy843	S-*-Bmy-0843-a-A-18	CTTCAGCACTCAGGTTCG	*Bacillus* spp.	This study
Ver620	S-*-Ver-0620-a-A-18	ATGTGCCGTCCGCGGGTT	*Verrucomicrobium* spp.	This study
Bac303	S-*-Bac-0303-a-A-17	CAATGTGGGGGACCTT	*Bacteroides* spp.	Manz *et al*. (1996)
MIB661	S-S-Bacm-0661-a-A-18	GCATTCCGCATACTTCTC	MIB	This study
MIB724	S-S-Bacm-0724-a-A-18	CCGGTATGCTGCCTTCGC	MIB	This study

* OPD, Oligonucleotide Probe Database (Alm *et al*., 1996).

genetic analysis (data not shown). Sequences in the tree were divided into different groups based on the presence of the target sequences of existing or newly designed 16S-rRNA-targeted probes (Fig. 1; see below).

Several of our sequences were identical to published 16S rRNA gene sequences, including those of members of the altered Schaedler flora (ASF; Fig. 1), *Lactobacillus* spp., *Bacteroides* spp., SFB and one *Helicobacter* sp.

(MIT 97-6810; in this study, sequence L10-17) that was recently found in the microflora of the mouse intestinal tract (Fox *et al.*, 1999). The rest of these sequences revealed novel sequences of, as yet, unidentified bacterial species.

Along with three known *Bacteroides* spp., a large group of our sequences ($n=16$) belonged to a separate, so far unrecognized branch of the *Bacteroides* group of bacteria. This new operational taxonomic unit (OTU) is designated here as MIB. Unique sequences in this OTU were obtained from samples from the small intestine ($n=2$), the large intestine ($n=3$) and the faeces ($n=6$) of two different mouse strains (FvB and C57BL/6) that were housed in two independent laboratories. To confirm the presence of these species within the mouse intestinal microflora, we isolated a bacterial species from the mouse caecum by plating serial dilutions of a caecal sample onto *Brucella* blood agar medium supplemented with 5% sheep blood. Random colonies from the plates were screened with a probe specific for the MIB OTU (MIB661; see below). Analysis of the 16S rRNA gene sequence of this isolate (MIB-CB3), obtained by direct sequencing of the PCR-amplified 16S rRNA gene (accession no. AJ418059), confirmed that this isolate belonged to the MIB OTU.

Eight of our library sequences clustered within the *Eubacterium rectale–Clostridium coccoides* group. Two sequences, L10-4 and L10-21, were more distantly related to the *E. rectale–C. coccoides* group; these sequences had two and four mismatches, respectively, with the Erec482 probe. One sequence (L10-6), which occurred four times, was related (although distantly) to *Verrucomicrobium spinosum* and was grouped within the '*Verrucomicrobium*' group (Suau *et al.*, 1999; Wilson & Blitchington, 1996). Another sequence, also occurring four times, was distantly related to *Bacillus mycoides* and was referred to as belonging to the '*Bacillus mycoides*' group. Nine sequences represented by only one clone did not cluster together and were designated as miscellaneous (Table 1). Four of these sequences (F13, L10-14, L10-21 and L11-2) belonged to the heterogeneous *Clostridium leptum* group, which includes the fusiform bacteria *Clostridium* sp. ASF356 and *Eubacterium* sp. ASF500. This number of clones was surprisingly low, since fusiform bacteria are morphologically the most dominant group of organisms present in mouse faecal samples.

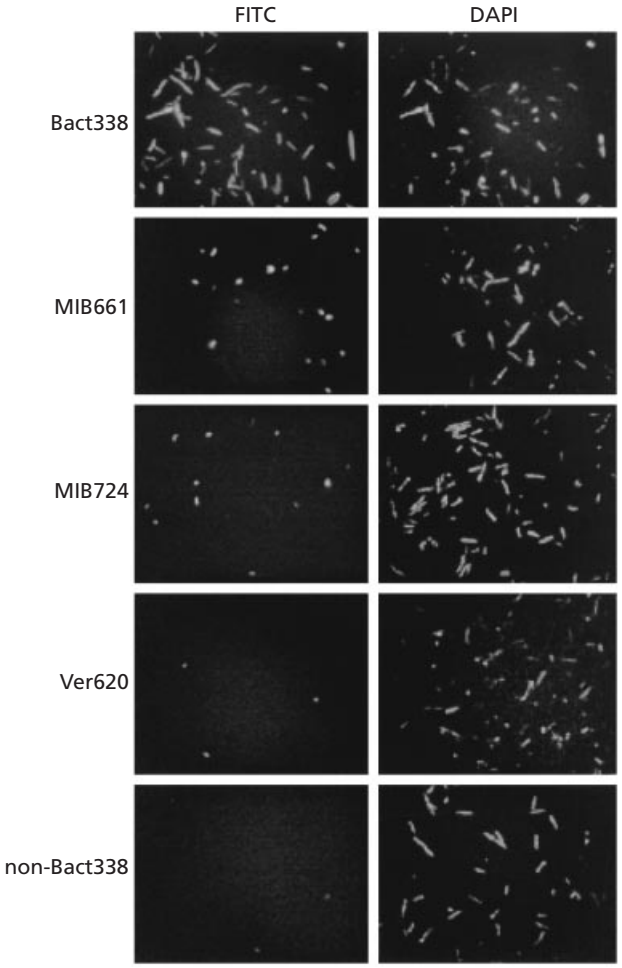

Fig. 2. *In situ* hybridization of mouse large intestinal samples with the newly designed 16S rRNA probes. FISH was performed on bacteria from the large intestine of 5-week-old FvB mice by using probes Bact338, MIB661, MIB724, Ver620 and non-Bact338 (Table 2). The bacteria were counterstained with DAPI.

Design of new probes and testing of existing probes

To obtain probes that could be used for the quantitative analysis of mouse intestinal and faecal bacterial populations we tested existing probes to see whether they could be used to identify the different groups of bacteria within these environments. For the *E. rectale–C. coccoides* group (Franks *et al.*, 1998), the *Bacteroides* group (Manz *et al.*, 1996), the SFB (Snel *et al.*, 1995) and the *Lactobacillus* group (Harmsen *et al.*, 1999), the published probes were appropriate for identifying the different target groups (Table 2). The Erec482 probe could target all of the new clones that belonged to the *E. rectale–C. coccoides* group. All of the sequences belonging to the new MIB OTU had one or more mismatches with Bac303. Therefore, we designed two new oligonucleotide probes, MIB661 and MIB724, which could identify members of this new OTU (Fig. 2). MIB661 revealed all new members of the MIB OTU, except for clone F1, which had one mismatch with the probe. MIB724 could target most (9/11) members of the new OTU, but it had one mismatch with two sequences, S30-4 and S30-5. Furthermore, probes were designed that could bind to the '*Verrucomicrobium*' group (Ver620) and the '*Bacillus mycoides*' group (Bmy843) of bacteria (Table 2; Fig. 2). The specificity of the newly designed probes was tested using slot-blot hybridization, with the target clones as reference. Hybridizations were found to be optimal at 50 °C in a buffer containing 30% formamide. Increasing the formamide concentration to

Table 3. Dot blot of plasmid clone library with 16S rRNA probes

Location	Total no. of clones	No. of clones positive for probe						
		Bact338	Erec482	Ver620	MIB661/724*	Bac303	SFB1008	Lab158
Large intestine	61	56	38†	1	3*	1*	2	6†
Caecum	15	15	6‡	0	3‡	0	0	4
Small intestine	39	35	3	0	0	0	16	14

* For the clones derived from the large intestine, the Bac303-positive clone cross-reacted with the MIB probes. This clone is counted within the Bac303 group. Two of the MIB subclones hybridized only with MIB724, and one hybridized with both probes. For the clones derived from the caecum, all three subclones hybridized to both MIB661 and MIB724.

† Three of the Erec482-positive clones also cross-reacted with the Lab158 probe; these are counted in the Erec482 group.

‡ One clone in the Erec482-positive group cross-reacted with the MIB probes. This clone is counted within the Erec482 group. All of the caecal MIB clones hybridized with both MIB661 and MIB724.

50 % or the hybridization temperature to 65 °C resulted in the loss of stable hybridization. Decreasing the formamide concentration did not significantly decrease the specificity of hybridization, unless it was accompanied by a decrease in the hybridization temperature.

Slot-blot analysis of the clone libraries generated from different sites in the mouse gastrointestinal tract

Slot blots were made from 115 clones from the small intestines, the large intestines and the caeca of mice. These clones included 34 clones that had been identified by sequencing, which served as positive controls, and 81 unidentified clones. All of the clones contained almost-complete bacterial 16S rRNA gene sequences, which had been amplified from samples taken from the mouse gastrointestinal tract. Successive hybridizations with the established and the newly generated oligonucleotide probes identified 90 % of the clones (Table 3). By using this approach, we avoided redundantly sequencing clones with previously identified 16S rRNA gene sequences. As expected, we found that clones belonging to the MIB group reacted with either one or both of the MIB probes. One clone reacted with both of the MIB probes and with probe Bac303. We did not find any other clones that reacted with Bac303 in our dot-blot analysis.

Five clones from the large intestine and four from the small intestine were negative in the dot-blot assay for Bact338. Sequence analysis of these clones revealed that they either contained fragments of cloning vector and, rarely, fragments of mouse genomic DNA or had sequences similar to previously identified bacteria, including some *Lactobacillus*, *Verrucomicrobium*, *Clostridium* and *Eubacterium* species, and SFB. It is unclear as to why these latter clones were negative for the Bact338 probe in the dot-blot assay.

Sequence analysis of three of the clones that hybridized with Bact338 but not with any of the other specific probes confirmed that these clones lacked the target sequences for the probes used. The three clones were found to represent a *Helicobacter* species (L10-17) or additional, unrecognized species (L10-4 and L10-21).

Quantification of the bacterial species found within the murine caecum and large intestine by *in situ* hybridization

For a more accurate determination of the size of the bacterial population within the mouse intestinal tract, we isolated intestinal bacteria and quantified populations by *in situ* hybridization and fluorescence microscopy, using both the established and the newly designed fluorescently labelled 16S-rRNA-targeted oligonucleotide probes. Quantification of the bacterial populations was performed on specimens from the caeca and large intestines of 5-week-old FvB mice. The Bact338 probe was able to account for approximately 95 % of all of the DAPI-stained bacteria in the large intestine and caecum. Since MIB661 and MIB724 targeted strongly overlapping sequences and revealed almost similar counts in the initial counts, only MIB661 was used for quantification of the tested samples. In the large intestine samples, $27 \pm 7{\cdot}1$ % of the bacteria were labelled with Erec482, 0 % were labelled with SFB1008, $10 \pm 3{\cdot}6$ % were labelled with Lab158, 1 % were labelled Ver620, $11 \pm 2{\cdot}2$ % were labelled with Bac303 and $31 \pm 4{\cdot}6$ % were labelled with MIB661; these values accounted for 80 % of all of the bacteria present within these samples. In the caecal samples, $33 \pm 7{\cdot}1$ % of the bacteria were labelled with Erec482, 1 % were labelled with SFB1008, $11 \pm 2{\cdot}5$ % were labelled with Lab158, $5 \pm 0{\cdot}5$ % were labelled with Bac303, $19 \pm 4{\cdot}0$ % were labelled with MIB661 and 2 % were labelled with Ver620; these values accounted for 71 % of the bacteria present within the caecal samples.

DISCUSSION

By cloning and sequencing the 16S rRNA gene sequences derived from the mouse intestinal tract, we have shown that the majority of identified sequences were derived

from previously unrecognized bacteria. This confirms that our current knowledge of the complex microflora of this area is limited. In this study, we have provided novel information about the microflora of the mouse intestinal tract and have designed new probes to be used in the analysis of this population.

Of the 16S rRNA gene clones analysed, 57% contained unique sequences. Thirty of these sequences were represented only once, whereas 10 sequences were present in multiple clones. About 25% of our 16S rRNA gene sequences differed only slightly (<2% nucleotide differences) from those of known bacterial species. This percentage includes non-cultivable members of the gut microflora, such as SFB, which dominate the small intestine of the mouse, especially around weaning; the numbers of SFB present in the small intestine decline after weaning, although they remain present in low numbers within the caecum (Snel *et al.*, 1998). We identified five clones from the small intestine that belonged to the SFB; however, our *in situ* hybridization studies revealed that only 1% of the caecal bacteria belonged to this group. Our method for the isolation of intestinal bacteria may favour the retrieval of lumenal bacteria over that of attached bacteria, which may result in an underestimation of numbers for this bacterial group.

Four members of the altered Schaedler flora (ASF360 and ASF361, *Lactobacillus* sp.; ASF502, *C. coccoides*; ASF519, *Bacteroides* sp.) were identified in our clone libraries. Surprisingly, no clones were found in our libraries that corresponded to the fusiform bacteria of the altered Schaedler flora (ASF356, ASF492 and ASF500), even though fusiform bacteria are morphologically very dominant in faecal samples. These fusiform bacteria may be represented by the sequences belonging to the *E. rectale*–*C. coccoides* group or by the four sequences belonging to the *C. leptum* group. No new probe was developed to target these sequences belonging to the *C. leptum* group, since only four clones were found and they did not cluster in a clear manner. However, in clone libraries generated from human faecal samples, this group of bacteria is strongly represented (Suau *et al.*, 1999; Wilson & Blitchington, 1996). In future studies on the mouse gut microflora, it will be important to test new probes designed based on the sequences from the human faecal bacteria libraries. The fusiform bacteria are of particular interest, as a Gram-positive rod that belongs to this group was isolated from the rat intestine and has been found to be important in bile-acid metabolism (Eyssen *et al.*, 1999).

Of the unique sequences in our clone libraries, eight belonged to the *E. rectale*–*C. coccoides* group; these sequences could all be targeted with the existing Erec428 probe (Franks *et al.*, 1998). The majority of these eight clones were derived from the large intestine, where bacteria of this group are commonly found.

One clone (L10-17) corresponded to a *Helicobacter* sp. (MIT 97-6810) that was recently isolated from the caecum of IL-10-deficient mice and was shown to be associated with the occurrence of colitis in these mice (Fox *et al.*, 1999). As we have been able to isolate the 16S rRNA gene from the same *Helicobacter* sp. from immunocompetent mice, we have shown that this species can belong to the resident murine gut microflora without inducing pathology. Therefore, under conditions of a compromised immune system, this bacterium may opportunistically challenge the host.

We identified several *Bacteroides* spp., related or identical to known species, within our clone libraries that could be targeted with the existing Bac303 probe. A very surprising and important finding of this study was that the majority of these new sequences ($n=11$) formed a novel OTU within the *Cytophaga–Flavobacter–Bacteroide*s phylum; we designated this OTU MIB. The phylogenetic position of this OTU was within the highly diverse *Bacteroides* group, although members of the OTU clustered tightly as a new lineage. This clustering was similar to that of the genera *Prevotella* and *Porphyromonas*, which form separate, distinct lineages within the *Bacteroides* group. The presence of members of the MIB OTU within the mouse intestinal tract was confirmed by isolation of a bacterial species belonging to this OTU from the mouse caecum. Analysis of all known *Bacteroides* sequences in the database showed no overlap between species containing the Bac303 sequence and our sequences that contained the new MIB-probe sequences. In the dot-blot assay, we found one clone that reacted with both of the MIB-probes and with the Bac303 probe; this result could represent some false hybridization by a clone that only had one mismatch with Bac303.

Clones of members of the MIB group were isolated from all parts of the mouse gastrointestinal tract, including the small and large intestine and the faeces. The new MIB probes, MIB661 and MIB724, identified a large population of small, rounded, rod-like bacteria that have not been detected previously with existing probes (Fig. 2). This suggests the usefulness of the MIB probes in quantification studies on intestinal populations.

Additional unique sequences from the libraries, while less abundant, were distantly related to the bacteria *V. spinosum* and *Bacillus mycoides*. Members of the '*Verrucomicrobium*' group of bacteria have also been identified in low numbers in human-derived 16S rRNA gene libraries (Suau *et al.*, 1999; Wilson & Blitchington, 1996) and in clone libraries of environmental samples, such as soil and water, but members of this group have not yet been identified in cultured bacteria. A new probe, Ver620, targeting the '*Verrucomicrobium*' group of bacteria has been designed; this probe detected low numbers (1–2%) of small, round bacteria in both the caeca and the large intestines of the mice studied (Fig. 2). This probe may be more useful in defining bacterial populations within the small intestine, as it was identified from clones derived from that segment of the gastrointestinal tract. The *Bacillus mycoides*-related species identified in this study has not previously been

described and was only represented by one sequence in our libraries, although this sequence did occur four times. The newly designed probe Bmy843, which was targeted at this species, did not detect *Bacillus mycoides*-related bacteria in the caeca or the large intestines of the mice studied here.

The application of 16S rRNA gene sequence analysis to studying the bacterial populations within the mouse gastrointestinal tract has uncovered large populations of previously unidentified bacterial species that contribute significantly to the composition of the intestinal commensal microflora. Using a combination of four pre-existing probes and our four newly designed probes we were able to identify the majority of bacteria within the murine caecum and large intestine. By using the newly designed and existing probes in FISH studies, we have established baseline information on the composition of the gut microflora from unmanipulated strains of inbred laboratory mice kept under specific pathogen-free conditions. Ultimately, similar approaches to the ones used here should allow a rapid assessment of commensal bacterial populations in a variety of mouse model systems looking at responses to diet, stress and disease.

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