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Phylogeny of the Defined Murine Microbiota: Altered Schaedler Flora

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The "altered Schaedler flora" (ASF) was developed for colonizing germfree rodents with a standardized microbiota. The purpose of this study was to identify each of the eight ASF strains by 16S rRNA sequence analysis. Three strains were previously identified as *Lactobacillus acidophilus* (strain ASF 360), *Lactobacillus salivarius* (strain ASF 361), and *Bacteroides distasonis* (strain ASF 519) based on phenotypic criteria. 16S rRNA analysis indicated that each of the strains differed from its presumptive identity. The 16S rRNA sequence of strain ASF 361 is essentially identical to the 16S rRNA sequences of the type strains of *Lactobacillus murinis* and *Lactobacillus animalis* (both isolated from mice), and all of these strains probably belong to a single species. Strain ASF 360 is a novel lactobacillus that clusters with *L. acidophilus* and *Lactobacillus lactis*. Strain ASF 519 falls into an unnamed genus containing [*Bacteroides*] *distasonis*, [*Bacteroides*] *merdae*, [*Bacteroides*] *forsythus*, and CDC group DF-3. This unnamed genus is in the *Cytophaga-Flavobacterium-Bacteroides* phylum and is most closely related to the genus *Porphyromonas*. The spiral-shaped strain, strain ASF 457, is in the *Flexistipes* phylum and exhibits sequence identity with rodent isolates of Robertson. The remaining four ASF strains, which are extremely oxygen-sensitive fusiform bacteria, group phylogenetically with the low-G+C-content gram-positive bacteria (*Firmicutes*, *Bacillus-Clostridium* group). ASF 356, ASF 492, and ASF 502 fall into *Clostridium* cluster XIV of Collins et al. Morphologically, ASF 492 resembles members of this cluster, *Roseburia cecicola*, and *Eubacterium plexicaudatum*. The 16S rRNA sequence of ASF 492 is identical to that of *E. plexicaudatum*. Since the type strain and other viable original isolates of *E. plexicaudatum* have been lost, strain ASF 492 is a candidate for a neotype strain. Strain ASF 500 branches deeply in the low-G+C-content gram-positive phylogenetic tree but is not closely related to any organisms whose 16S rRNA sequences are currently in the GenBank database. The 16S rRNA sequence information determined in the present study should allow rapid identification of ASF strains and should permit detailed analysis of the interactions of ASF organisms during development of intestinal disease in mice that are coinfected with a variety of pathogenic microorganisms.

The gastrointestinal tracts of mammals, including mice and rats, contain a diverse microecosystem. The ceca of normal mice contain numerous species, and the concentration of bacteria can be as great as 10^{11} bacteria/g of feces (41, 42). These microorganisms not only provide essential nutrients (e.g., vitamin K) for their hosts but also colonize mucosal niches, which in part helps protect the hosts against microbial pathogens (22, 25, 42, 49, 53, 55). For example, numerous studies have demonstrated the increased susceptibility of germfree mice to a variety of infectious agents compared to that of mice with the normal complement of microorganisms (17).

Gnotobiotic animals colonized with known microbiota have been used to great advantage as models for biomedical research (17). For certain studies, it is particularly desirable to colonize germfree mice with a defined microbiota.

In the mid-1960s, Russell W. Schaedler was the first researcher to colonize germfree mice with selected bacteria isolated from normal mice (40). He subsequently supplied animal breeders with this group of microorganisms (2) for use in colonizing their rodent colonies. These defined bacteria included aerobic bacteria that were easy to grow and some less-oxygen-sensitive anaerobic organisms. The so-called extremely oxygen-sensitive (EOS) fusiform bacteria, which make up the vast majority of the normal microbiota of rodents, were not included due to technical difficulties in isolating and cultivating EOS bacteria (26, 27). Of the defined microbiotas later used for gnotobiotic studies, the one known as the "Schaedler flora" was the most popular. This flora contained eight bacteria, which were designated *Escherichia coli* var. *mutabilis*, *Streptococcus faecalis*, *Lactobacillus acidophilus*, *Lactobacillus salivarius*, group N *Streptococcus*, *Bacteroides distasonis*, a *Clostridium* sp., and an EOS fusiform bacterium.

In 1978, the National Cancer Institute (NCI) decided to revise the Schaedler flora or "cocktail" consisting of eight bacteria in order to standardize the microbiota used to colonize axenic (germfree) rodents, including mice, at all NCI contractors, as well as mice used at NCI. Roger Orcutt, therefore, developed the new defined microbiota now known as the "altered Schaedler flora" (ASF), which consisted of four members of the original Schaedler flora (the two lactobacilli, *B. distasonis*, and the EOS fusiform bacterium), a spiral-shaped bacterium, and three new fusiform EOS bacteria (30).

Although very important, it is very difficult to monitor a gnotobiotic mouse colony with a defined microbiota. Not only is it necessary to demonstrate that the colony is free of any adventitious microorganisms, but it must also be demonstrated that the microorganisms of the specified microbiota are present. In the past, workers monitoring gnotobiotic animals relied on examining the morphology of the microorganisms and performing a limited evaluation of the biochemical traits and growth characteristics of the organisms.

The goals of this study were, therefore, to identify the bac-

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TABLE 1. PCR and sequencing primers

Primer	Position	Orientation	Specificity	Sequence
PCR primers				
F24	9-27	Forward	Universal	AGTTTGATYMTGGCTCAG
F25	1525-1541	Reverse	Universal	AAGGAGGTGWTCCARCC
Sequencing primers				
C75	7-27	Forward	Universal	GAGAGTTTGATYCTGGCTCAG
B34	344-358	Forward	Universal	ACGGGAGGCAGCAGY
F16	789-806	Forward	Universal	TAGATACCCYGGTAGTCC
F18	1099-1113	Forward	Most bacteria	GCAACGAGCGCAACC
F19	1099-1114	Forward	*Bacteroides*	ATAACGAGCGCAACCC
E94	1522-1541	Reverse	Universal	GAAGGAGGTGWTCCARCCGCA
F20	1226-1242	Reverse	Most bacteria	CCATTGTARCACGTGTG
F21	1226-1242	Reverse	β-*Proteobacteria*[a]	CCATTGTATGACGTGTG
F17	907-926	Reverse	Universal	CCGTCWATTCMTTTGAGTTT
F15	519-533	Reverse	Universal	TTACCGCGGCTGCTG
F22	344-358	Reverse	Universal	RCTGCTGCCTCCCGT

[a] Members of the β subclass of the class *Proteobacteria*.

teria in the ASF by 16S rRNA sequence analysis and to characterize the phylogenetic positions of these organisms relative to those of known bacteria. The long-term goal of our studies is to develop sensitive and specific molecular techniques for monitoring the microbiotas of gnotobiotic animals.

MATERIALS AND METHODS

Bacterial strains and cultivation. ASF bacteria, including four EOS fusiform anaerobes (Taconic stock culture strains ASF 356, ASF 492, ASF 500, and ASF 502), a spiral-shaped bacterium (Taconic strain ASF 457), two previously identified lactobacilli (Taconic strains ASF 360 and ASF 361), and a *Bacteroides* sp. (Taconic strain 519), were obtained from Taconic, Germantown, N.Y. The bacteria were cultured anaerobically on Schaedler agar (Difco Laboratories, Detroit, Mich.) supplemented with 5% sterile fetal calf serum (Summit Biotechnology, Ft. Collins, Colo.) in an anaerobic glove chamber containing a 10% CO_2–10% H_2–80% N_2 atmosphere (Coy Laboratory, Grass Lakes, Mich.). Anaerobiosis was monitored with a resazurin indicator. The media were prereduced by placing them inside the chamber 2 days prior to inoculation of bacteria. The temperature in the chamber was maintained at 33 to 35°C.

Extraction of DNA for sequence determination. Bacteria were harvested, washed twice with 1 ml of sterile phosphate-buffered saline, and then collected by centrifugation at 8,000 × g. The pellets were used for extraction of DNA templates that were used to amplify 16S rRNA by PCR. DNA was extracted from the cell pellets by using a commercial kit (High Pure PCR template preparation kit; Boehringer Mannheim) according to the manufacturer's instructions.

Amplification of 16S rRNA cistrons by PCR and purification of PCR products. The 16S rRNA cistrons were amplified with bacterial universal primers F24 and F25 (Table 1). PCR was performed in thin-walled tubes with a Perkin-Elmer model 9700 thermocycler. One microliter of the DNA template was added to a reaction mixture (final volume, 50 μl) containing 20 pmol of each primer, 40 nmol of deoxynucleoside triphosphates, and 1 U of *Taq* 2000 polymerase (Stratagene, La Jolla, Calif.) in buffer containing Taqstart antibody (Sigma Chemical Co.). In a hot-start protocol, samples were preheated at 95°C for 8 min, and this was followed by amplification in which the following conditions were used: denaturation at 95°C for 45 s, annealing at 60°C for 45 s, and elongation for 1.5 min with an additional 5 s for each cycle. A total of 30 cycles were performed, and then a final elongation step consisting of 72°C for 10 min was performed. The PCR amplification results were examined by electrophoresing preparations in a 1% agarose gel. The DNA was stained with ethidium bromide and visualized under short-wavelength UV light.

16S rRNA sequencing. Purified DNA obtained from the PCR was sequenced by using an ABI prism cycle sequencing kit (BigDye terminator cycle sequencing kit with AmpliTaq DNA polymerase FS; Perkin-Elmer). The primers in Table 1 were used for sequencing. Quarter dye chemistry was used with primers at a concentration of 80 μM and 1.5 μl of PCR product in a final volume of 20 μl. Cycle sequencing was performed by using a model ABI 9700 apparatus and 25 cycles consisting of denaturation at 96°C for 10 s, annealing, and extension at 60°C for 4 m. Sequencing reactions were performed with a model ABI 377 DNA sequencer.

16S rRNA data analysis. Sequence data were entered into RNA, a program set for data entry, editing, sequence alignment, secondary-structure comparison, similarity matrix generation, and dendrogram construction for 16S rRNA in Microsoft QuickBasic for use with PC computers, and sequences were aligned as previously described (31). Our database contains more than 1,000 sequences obtained in our laboratory and more than 500 sequences obtained from GenBank. Sequences were first checked by BLAST analysis versus all entries in the GenBank database (1). Neighboring sequences for the ASF organisms not already in our database were downloaded and added to our database. Dendrograms were constructed by the neighbor-joining method (37).

Nucleotide sequence accession numbers. The GenBank accession numbers for the strains examined in this study or used as reference strains are given in Table 2. The 16S rRNA sequences of ASF strains determined in this study have been deposited in the GenBank database under the following accession numbers: ASF 360, AF157050; ASF 361, AF157049; ASF 519, AF157056; ASF 457, AF157055; ASF 356, AF157052; ASF 492, AF157054; ASF 500, AF157051; and ASF 502, AF157053.

RESULTS

An essentially complete 16S rRNA sequence (length, 1,500 bases) was determined for each of the ASF strains. A neighbor-joining phylogenetic tree that included the closest neighbors of each ASF strain was constructed by using the sequences listed in Table 2 (Fig. 1). The strains previously presumptively identified as *L. acidophilus* (strain ASF 360), *L. salivarius* (strain ASF 361), and *B. distasonis* (strain ASF 519) based on phenotypic criteria were not members of these species but rather were members of neighboring species. The sequence of strain ASF 361 differed from the *L. salivarius* sequence but was essentially identical to the sequences of *Lactobacillus murinis* and *Lactobacillus animalis* (both isolated from mice). Strain ASF 360 is a novel lactobacillus that clusters with *L. acidophilus* and *Lactobacillus lactis*. Strain ASF 519 falls into an as yet unnamed genus along with [*Bacteroides*] *distasonis*, [*Bacteroides*] *merdae*, [*Bacteroides*] *forsythus*, and CDC group DF-3. The spiral-shaped strain, strain ASF 457, falls in the *Flexistipes* phylum and is most closely related to *Geovibrio ferrireducens* and an organism isolated from the stomach of a Colobus monkey (9). The remaining four ASF strains, which are EOS fusiform bacteria, grouped phylogenetically with the low-G+C-content gram-positive bacteria (*Firmicutes*, *Bacillus-Clostridium* group).

DISCUSSION

Germfree mice and rats that are monoassociated with a bacterium or a particular microbiota are commonly used in biomedical research. The ASF has been widely used since the 1980s as a group of defined bacteria for colonizing the gastrointestinal tracts of commercially available mice and rats used for biomedical research. In this report we provide an initial taxonomic description of these bacteria based on a 16S rRNA analysis.

TABLE 2. Strains examined in this study

Taxon	Strain	Identity	Sequence accession no.
Lactobacillus acidophilus	ASF 360	*Lactobacillus* sp.	AF157050
Lactobacillus salivarius	ASF 361	*L. murinus-L. animalis*	AF157049
Bacteroides distasonis	ASF 519	[*Bacteroides*] sp.	AF157056
Spiral-shaped organism	ASF 457	*Flexistipes* phylum	AF157055
Fusiform EOS bacteria	ASF 356	*Clostridium* sp.	AF157052
Fusiform EOS bacteria	ASF 492	*E. plexicaudatum*	AF157054
Fusiform EOS bacteria	ASF 502	*Clostridium* sp.	AF157053
Fusiform EOS bacteria	ASF 500	*Clostridium* sp.	AF157051
[*Bacteroides*] *forsythus*	ATCC 43037^T		L16495
[*Bacteroides*] *merdae*	ATCC 43184^T		X83954
[*Bacteroides*] *distasonis*	ATCC 8503^T		M86695
Acetitomaculum ruminis	ATCC 43876^T		M59083
Catonella morbi	ATCC 51271^T		X87151
CDC group DF-3	CDC F19047		U41355
Clostridium neopropionicum	DSM 3847^T		X76746
Clostridium piliforme	Uncultivable		L07416
Clostridium propionicum	ATCC 25522^T		X77841
Deferribacter thermophilus	MBA1		U756092
Eubacterium contortum	ATCC 25540^T		L34615
Eubacterium plexicaudatum[a]	ATCC 27514^T		AF157058
Flexistipes sinusarabic	DSM 4947		M59231
Flexistipes phylum Colobus	Lincoln Park 3		AF157057
Flexistipes phylum Rodent-1	HRI3liv		AF059188
Flexistipes phylum Rodent-2	UNSWRSp12		AF059190
Flexistipes phylum Rodent-3	UNSWMCS1		AF059189
Geovibrio ferrireducens	PAL-1		X95744
Johnsonella ignava	ATCC 51276^T		X87152
Lactobacillus acidophlus	ATCC 1968^T (= ATCC 4356^T)		M58802
Lactobacillus animalis	ATCC 35046^T		M58807
Lactobacillus delbrueckii subsp. *lactis*	ATCC 12315^T		M58823
Lactobacillus mali	ATCC 27053^T		M58824
Lactobacillus murinus	ATCC 35020^T		M58826
Lactobacillus salivarius	ATCC 11741^T		AF089108
Roseburia cecicola	ATCC 33874^T		L14676
Ruminococcus gnavus	ATCC 29149^T		X94967

[a] *E. plexicaudatum* type strain ATCC 27514 is not available because it was found to be nonviable.

Lactobacilli are common colonizers of the gastrointestinal mucosal and squamous epithelia of mice (20, 34, 38, 39, 41). Historically, most of the indigenous lactobacilli either have not been identified to the species level or have been identified as minor variants of human species by using a limited number of biochemical tests (20, 34, 39). Unfortunately, different vertebrate species often contain unique bacterial species that are distinct from phenotypically similar human-associated species. Thus, ASF 360 and ASF 361 were identified as minor phenotypic variants of *L. acidophilus* and *L. salivarius*, respectively. With the emerging use of molecular techniques, such as restriction endonuclease fingerprinting, DNA-DNA hybridization, plasmid content analysis, and 16S rRNA sequencing, classification and identification of lactobacilli have been greatly improved (6, 7, 16, 33, 36, 43, 47, 52, 58). 16S rRNA sequence analysis has clearly demonstrated that ASF 360 and ASF 361 are distinct from each other and distinct from *L. acidophilus* or *L. salivarius*. However, as shown in Fig. 1, the 16S rRNA sequence of ASF 361 appears to be essentially identical to the 16S rRNA sequences of the previously described species *L. murinus* and *L. animalis. L. murinus* strains have been isolated from the intestinal tracts of mice and rats (21). *L. animalis* strains have been isolated from the dental plaque and alimentary tracts of animals (8). The type strains of *L. murinus* and *L. animalis* appear to belong to a single species. While *L. animalis* strains isolated from mice may belong to the same species as *L. murinus* strains, strains isolated from other mammalian sources may belong to different species. Therefore, a thorough examination of *L. murinus* and *L. animalis* strains is necessary to resolve these taxonomic issues. The possibility that these species are identical was suggested previously (24). *L. murinus* was named 2 years before *L. animalis* was named, and therefore the name *L. murinus* has priority according to the rules of nomenclature.

Bacteroides spp. are microbes that are commonly found in the intestinal tracts of mammals. Many *Bacteroides* species, including *B. distasonis*, have been isolated from the ceca of conventional mice and characterized (39, 50, 51). These bacteria were included in the genus *Bacteroides* because they are nonmotile, gram-negative, strictly anaerobic, non-spore-forming rods which do not produce butyric acid (50, 51). However, after many of the early studies were performed, it was recognized that the genus *Bacteroides* contained species representing several genera. A majority of the species previously included in the genus *Bacteroides* have been placed in the genera *Porphyromonas*, *Prevotella*, and *Bacteroides* sensu stricto (44–46). [*B.*] *distasonis* is not a true member of the genus *Bacteroides* but rather falls in a novel genus closely related to the genus *Porphyromonas* (32). Strain ASF 519 is related to [*B.*] *distasonis* but is clearly a distinct species. [*B.*] *distasonis*, [*B.*] *merdae*, [*B.*] *forsythus*, CDC group DF-3 (54), and strain ASF 519 comprise a novel unnamed genus in the *Cytophaga-Flavobacterium-Bacteroides* phylum.

Strain ASF 457, a spiral-shaped obligately anaerobic bacterium, was described as a spirochete by Orcutt et al. (30). Bacteria with spiral-shaped morphology are commonly found in

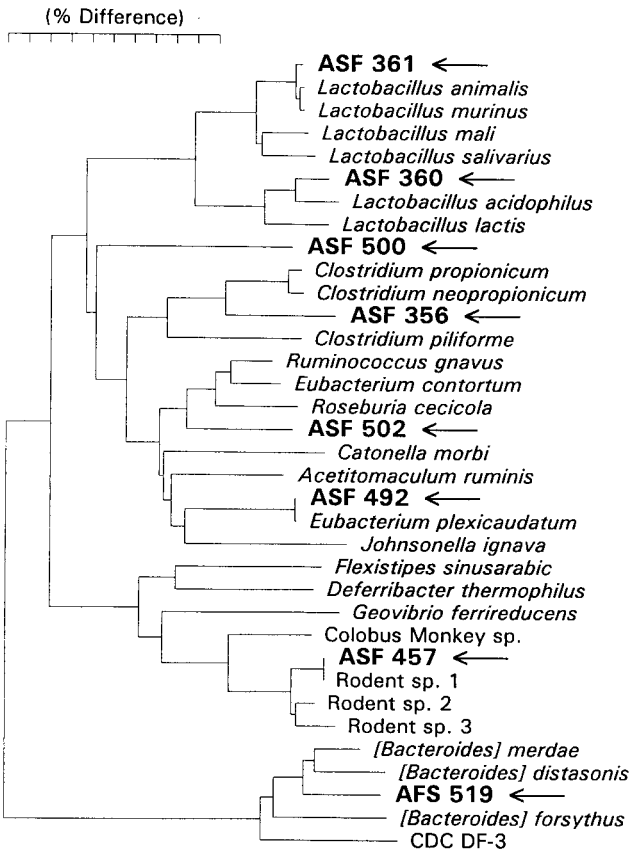

FIG. 1. 16S rRNA-based phylogenetic tree for ASF organisms and related species. The scale bar represents a 10% difference in nucleotide sequences, as determined by measuring the lengths of the horizontal lines connecting any two species. The Colobus monkey species isolate was obtained from Lincoln Park Zoo, Chicago, Ill., courtesy of Shelia Davis. The 16S rRNA sequence data for rodent species 1, 2, and 3 were obtained from reference 35.

large numbers mixed with tapered rods in the mucus layers of the ceca and colons of mice (18, 39). As determined by 16S rRNA analysis, this bacterium is related to *G. ferrireducens*, a dissimilatory, Fe(III)-reducing bacterium (3), *Deferribacter thermophilus* (19), and *Flexistipes sinusarabici* (10, 28) in the *Flexistipes* phylum (23). Within the level of sequencing error, the sequence of strain ASF 457 is identical to sequences of rodent isolates described by B. R. Robertson (35) and deposited in the GenBank database (accession no. AF059186 to AF05988). ASF 457 and the Robertson strains probably are isolates of the same species. Two other Robertson strains (accession no. AF059189 and AF05990) and a strain isolated from the stomach of a Colobus monkey (9) belong to related species. It appears that the *Flexistipes* phylum contains species that inhabit mammalian gastrointestinal tracts, as well as iron-reducing environmental isolates.

The majority of the members of the gastrointestinal microbiota of mice and rats are fusiform bacteria or tapered rods and are referred to in broad terms as EOS bacteria. These bacteria outnumber facultatively anaerobic bacteria by as much as 100 to 1 and aerobic bacteria by thousands to one (39). Although large numbers of the EOS fusiform bacteria or tapered rods are present (18, 20), only a few of these organisms have been cultivated, and fewer still have been named and extensively studied (48, 50, 57). Because it is difficult to identify these organisms at the species and genus levels, older taxonomic studies often grouped these bacteria on the basis of morphological criteria and growth characteristics (18) and in many cases considered them members of the genera *Eubacterium*, *Fusobacterium*, and *Clostridium* (20, 50, 57). The four EOS fusiform ASF strains belong in the low-G+C-content gram-positive bacterial group (*Firmicutes*, *Bacillus-Clostridium* group). Strain ASF 356 is most closely related to *Clostridium propionicum*. Strain ASF 492 possesses a subpolar tuft of flagella that is inserted subterminally, an unusual morphological characteristic shared by *Roseburia cecicola* (48) and *Eubacterium plexicaudatum* (57). The ASF 492 sequence clearly differentiates this organism from *R. cecicola*, but unfortunately, the type strain and other viable strains of *E. plexicaudatum* have been lost (56). The American Type Culture Collection still had vials of ATCC 27514^T that were never released because they were found to be nonviable. The complete 16S rRNA sequence of ATCC 27514^T (a nonviable strain kindly provided by the American Type Culture Collection) was determined, and this sequence was identical to the 16S rRNA sequence of strain ASF 492. Elsewhere, we will propose that ASF 492 should become the neotype strain for *E. plexicaudatum*. Our results demonstrate that 16S rRNA sequence analysis is an ideal tool for determining the molecular identities of archival or reference organisms which are no longer viable. Strain ASF 502 is most closely related to *Ruminococcus gnavus*. Strains ASF 356, ASF 492, and ASF 502 fall into *Clostridium* cluster XIV of Collins et al. (5). Strain ASF 500 branches deeply in the low-G+C-content gram-positive phylogenetic tree but is not closely related to any organism currently in the GenBank database.

Our findings again highlight the pitfalls of placing human and animal isolates with similar phenotypic characteristics in a single species. Taxonomic analysis of the family *Pasteurellaceae* (32), as well as many other organisms, has indicated that individual mammalian organisms have their own unique associated species. Molecular techniques, such as 16S rRNA sequencing, easily detect the existence of polyphyletic groups and can be used to prevent misclassification based phenotypic similarity.

Our findings also illustrate the taxonomic complexities of the normal flora of the mouse. Clearly, most mouse floras are much more diverse than the ASF in mice maintained under strict germfree conditions to prevent introduction of other bacterial species adept at colonizing the murine lower bowel. It is common for investigators to stipulate that mice have been maintained under specific-pathogen-free conditions. Unfortunately, this term is misinterpreted in most scientific publications and is commonly used to mask a lack of detailed information regarding the microbial pathogen status of the animals being studied. Because mice are housed in a "pathogen-free" environment and are periodically screened by viral serology and/or intestinal culture methods for known pathogenic bacteria and parasites, it is frequently assumed that infectious agents are not present and do not contribute to the pathogenesis of the disease being studied. Invariably, in these studies the intestinal flora is considered the normal flora. Indeed, a number of newly recognized murine enterohepatic helicobacters, which are fastidious microaerobes, were placed in this category; they were ignored because they are difficult to culture and because there were no previous data attributing any importance to the presence of large numbers of these spiral organisms in the crypts of the lower intestines of mice. It is now known that several of these helicobacters, most notably *Helicobacter hepaticus*, can cause serious gastrointestinal disease in a number of inbred and mutant mice (4, 11–13, 15). Also, it is important to recognize that certain members of the microflora of the intestine may be protective. This was clearly illustrated in young neonatal IL-10$^{-/-}$ mice susceptible to inflammatory

bowel disease at a young age. These mice were protected against the development of colitis by oral administration of *Lactobacillus* sp. (29). The authors hypothesized that the *Lactobacillus* sp. prevented bacterial adherence to gut mucosa and subsequent bacterial translocation.

This research provided an unambiguous molecular approach to identify the AFS organisms. Our information should allow workers who utilize ASF-colonized mice to more precisely monitor the microbiota of these gnotobiotic animals by using 16S rRNA-based probe or PCR techniques. The availability of the PCR probes should also result in more accurate quality control of the defined murine microbiota and prevent infections of mice with microbial pathogens (11, 14).

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