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Isolation and Characterization of "*Flexispira rappini*" from Laboratory Mice

DAVID B. SCHAUER,[1]†* NAFISA GHORI,[1] AND STANLEY FALKOW[1,2]

Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, California 94305-5402,[1] and Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, Montana 59840[2]

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A bacterium with an unusual ultrastructure and possessing a fusiform protoplasmic cylinder, spiral periplasmic fibers, and bipolar tufts of sheathed flagella was identified in the intestinal mucosae of laboratory mice. The organism was cultured under microaerophilic conditions and was found to rapidly hydrolyze urea. On the basis of 16S rRNA gene sequence analysis, the organism was shown to be "*Flexispira rappini*." "*F. rappini*" is closely related to members of the genus *Helicobacter* and has been reported to be associated with human gastroenteritis and ovine abortion. "*F. rappini*" has not previously been observed in the gastrointestinal tracts of mice.

"*Flexispira rappini*" is the provisional name of a microaerophilic, urease-producing bacterium with a fusiform protoplasmic cylinder, spiral periplasmic fibers, and bipolar tufts of sheathed flagella. "*F. rappini*" is the only member of its genus and is morphologically distinct from other bacterial organisms. "*F. rappini*" has been isolated from aborted sheep fetuses (9), the stools of humans with chronic diarrhea (20), and the stools of an asymptomatic human and a puppy without diarrhea, both of whom came into contact with an individual suffering from "*F. rappini*"-associated diarrhea (20). On the basis of 16S rRNA sequence analysis, "*F. rappini*" is most closely related to *Helicobacter muridarum* (4, 12), which is found in the intestinal mucosae of mice and rats (3, 6, 16). *H. muridarum* shares morphologic features with "*F. rappini*," including the presence of periplasmic fibers and bipolar tufts of sheathed flagella (12), but differs from "*F. rappini*" in that *H. muridarum* has a helical protoplasmic cylinder.

While *H. muridarum* is a component of the resident flora in the gastrointestinal tracts of laboratory mice and rats (3, 6, 16), the bacterium can invade from the gastrointestinal tract into deeper tissues in irradiated mice (17) and in mice treated with nitrogen mustard (8). An organism morphologically indistinguishable from *H. muridarum* has also been reported to be associated with gastritis in mice (18). "*F. rappini*" also has the potential to produce disease, since it has been associated with abortion in sheep (9), causes abortion when inoculated intraperitoneally into pregnant guinea pigs (2), and has been associated with gastroenteritis in humans (20).

We first observed an organism that was morphologically indistinguishable from "*F. rappini*" in the colons of mice that were infected with pure cultures of *Citrobacter freundii* (22). To confirm that the organism resembling "*F. rappini*" was a component of the resident flora in the murine gastrointestinal tract, we isolated the organism from normal, healthy mice. The nucleotide sequence of the 16S rRNA gene from the bacterium resembling "*F. rappini*" was determined to definitively identify the organism. Both "*F. rappini*" and *H. muridarum* are of interest because, as organisms closely related to the stomach-adapted *Helicobacter* species, including *Helicobacter pylori*, they provide insight into the evolution of gastric adaptation by bacteria. As resident flora in the lower bowels of easily manipulated laboratory animals, "*F. rappini*" and *H. muridarum* may also provide a model system for the identification of bacterial and host factors that permit gastric colonization.

MATERIALS AND METHODS

Animals. Twelve 3-week-old female CFW mice (Charles River Laboratories, Portage, Mich.) were euthanized. At necropsy, 1- to 2-cm sections of distal ileum, cecum, and colon were removed and were either placed into fixative for microscopy or incised longitudinally to expose the mucosal surface and inoculated onto solid bacterial culture medium.

Bacterial strains, media, and culture conditions. "*F. rappini*" was isolated and maintained on brucella agar (Difco Laboratories, Detroit, Mich.) supplemented with 5% fetal bovine serum (Biocell Laboratories, Rancho Dominquez, Calif.), 10 μg of trimethoprim (Sigma Chemical Co., St. Louis, Mo.) per ml, 5 μg of vancomycin (Sigma) per ml, and 2.5 μg of polymyxin B (Sigma) per ml. Moist plates were inoculated and incubated at 37°C in GasPak jars (BBL Microbiology Systems, Cockeysville, Md.) containing GasPak H_2 plus CO_2 generators (BBL) without catalyst to produce microaerophilic conditions and with palladium catalyst to produce anaerobic conditions. Urease and alkaline phosphatase production were detected with a RapID ANA II system (Innovative Diagnostic Systems, Inc., Atlanta, Ga.). For long-term storage of "*F. rappini*," plate-grown bacteria were suspended in brain heart infusion broth (Difco) with 20% glycerol and were stored at −80°C. The laboratory strain *Escherichia coli* DH5α (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) was grown in Lennox L (LB) broth and on LB agar (GIBCO Laboratories, Gaithersburg, Md.). For the selection of recombinant plasmids, 100 μg of ampicillin (Sigma) per ml was added to the medium.

Electron microscopy. (i) Transmission electron microscopy. Animal tissue was fixed in 2% gluteraldehyde buffered with 0.1 M sodium phosphate buffer (pH 6.3) for 2 h at 4°C. The tissue was then rinsed twice in phosphate buffer and was

* Corresponding author.
† Present address: Division of Toxicology, Massachusetts Institute of Technology, Cambridge, MA 02139.

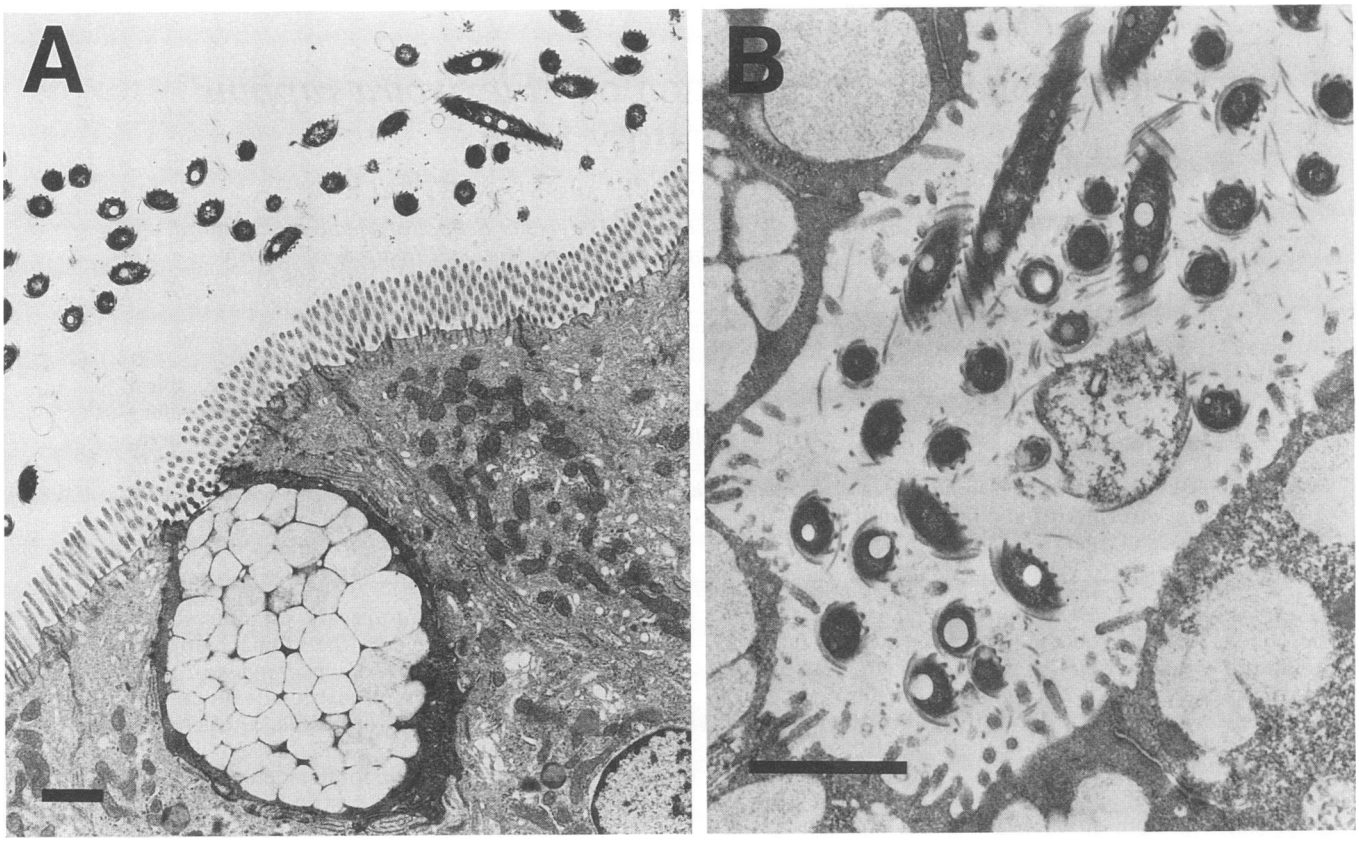

FIG. 1. Transmission electron micrographs of an organism resembling "*F. rappini*" at the apical surface of absorptive enterocytes and a goblet cell (A) and in an intestinal crypt in a mouse (B). Bars, 1 μm.

postfixed in 1% OsO_4 for 1 h at room temperature. After rinsing twice with distilled water, the tissue was stained en bloc with 2.5% aqueous uranyl acetate for 1 h in the dark. The tissue was again rinsed twice with distilled water, was then dehydrated through a graded series of ethanol solutions and then propylene oxide, and was embedded in a firm Spurr resin (Polysciences, Inc., Warrington, Pa.). Samples were sectioned, stained with uranyl acetate and lead citrate, and examined on a Philips 201c electron microscope.

(ii) Scanning electron microscopy. Animal tissue was fixed in buffered 2% gluteraldehyde as described above, rinsed twice with distilled water, and dehydrated through a graded series of ethanol solutions. Samples were dried in a critical-point apparatus, coated with gold-palladium, and examined on a JEOL scanning electron microscope.

(iii) Negative staining. Plate-grown bacteria were suspended in phosphate-buffered saline, applied to carbon-coated grids, and incubated for 2 min. Excess fluid was removed, and the specimen was rinsed with distilled water. After staining with 0.5% aqueous uranyl acetate for 1 min, the samples were examined on a Philips 201c electron microscope.

Polymerase chain reaction amplification of the 16S rRNA gene. Total bacterial DNA was prepared from plate-grown bacteria as described previously (15). The broad-range oligonucleotide primers 8FPL (5′-GCG GAT CCG CGG CCG CTG CAG AGT TTG ATC CTG GCT CAG-3′) and 1492RPL (5′-GGC TCG AGC GGC CGC CCG GGT TAC CTT GTT ACG ACT T-3′) complementary to the 16S rRNA genes of bacteria (*E. coli* 16S rRNA positions [underlined] 8 to 27 and 1510 to 1492) and containing restriction endonuclease sites (5, 14, 19) were used to amplify the "*F. rappini*" 16S rRNA gene from total bacterial DNA by polymerase chain reaction. Approximately 1 μg of DNA was used in a reaction volume of 100 μl containing 20 pmol of each primer and standard amounts of GeneAmp reagents (Perkin-Elmer Cetus Corp., Norwalk, Conn.). An overlay of sterile mineral oil was applied, and 25 cycles consisting of 1 min of denaturation at 94°C, 1 min of annealing at 55°C, and 1.5 min of extension at 72°C were used to amplify product in a programmable thermal controller (MJ Research Inc., Watertown, Mass.).

Cloning and nucleotide sequence determination. The polymerase chain reaction product was extracted with phenol-chloroform, ethanol precipitated, and isolated from a low-melting-point agarose gel (21). The DNA fragment had the expected size of 1.5 kb; it was digested with *Not*I (New England Biolabs, Inc., Beverly, Mass.) and was ligated to *Not*I-digested pBluescript KS- plasmid DNA (Stratagene, La Jolla, Calif.). *E. coli* DH5α was transformed with the ligation mixture by high-voltage electroporation with a Gene Pulser and Pulse Controller (Bio-Rad Laboratories, Richmond, Calif.). Plasmid DNA was isolated by alkaline lysis (21) from ampicillin-resistant transformants and was screened for the appropriate recombinant plasmid. The nucleotide sequence of the 16S rRNA gene from a plasmid with the correct insert was determined by using double-stranded DNA templates and oligonucleotide primers complementary to conserved bacterial 16S rRNA sequences (23)

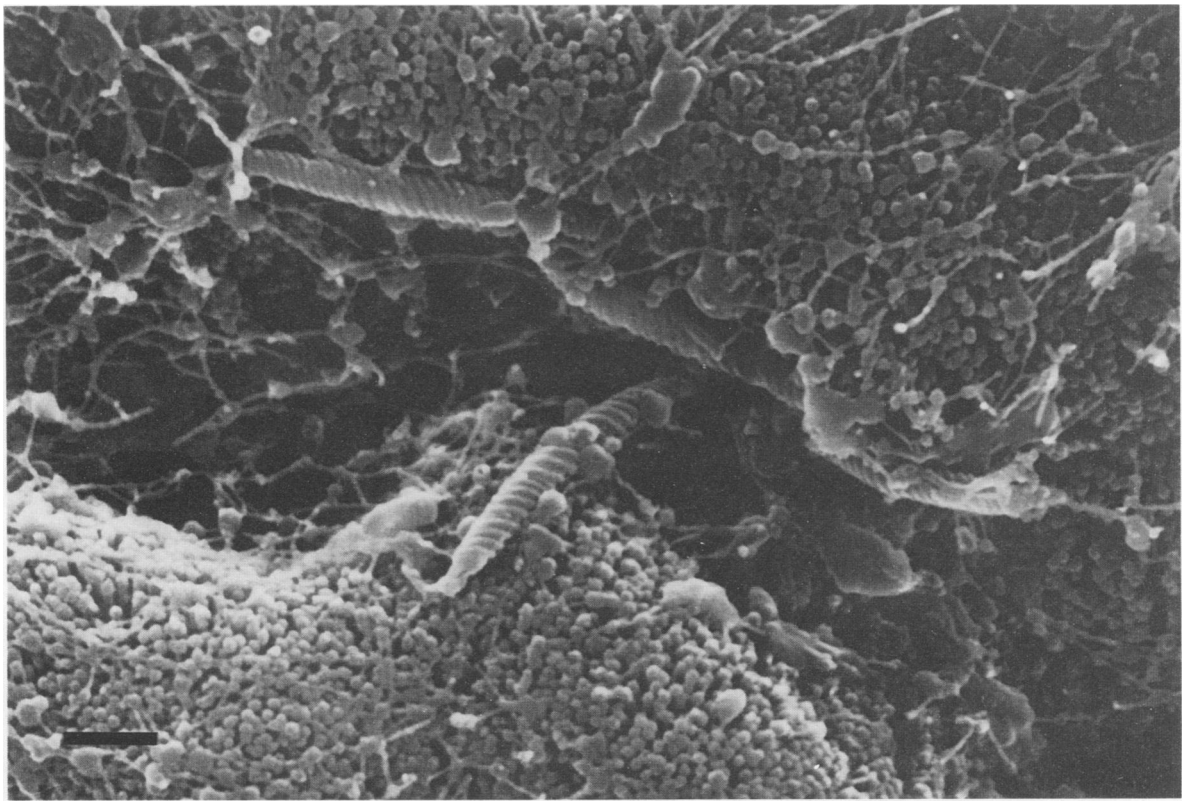

FIG. 2. Scanning electron micrograph of an organism resembling "*F. rappini*" among intestinal microvilli in a mouse. Bar, 1 μm.

by the dideoxy chain-termination method (Sequenase; U.S. Biochemical, Cleveland, Ohio).

Data analysis. Sequences were aligned manually with the *E. coli* 16S rRNA gene on the basis of conserved regions and secondary structures. Regions of umambiguous sequence were used to search against the GenBank data base (GenBank, Los Alamos, N.M.) by using the FastDB program of the IntelliGenetics Suite (release 5.4; IntelliGenetics, Inc., Mountain View, Calif.) and were aligned with the multiple sequence alignment program Genalign (release 5.4; IntelliGenetics).

Nucleotide sequence accession number. The partial 16S rRNA gene sequence of the mouse isolate of "*F. rappini*" has been submitted to GenBank, EMBL, and DDJB and has been assigned the accession number L12765.

RESULTS

Electron microscopy of animal tissue. We previously noted that disruption of the resident colonic flora of laboratory mice by transient colonization with the mouse pathogen *Citrobacter freundii* biotype 4280 led to the appearance of large numbers of an organism morphologically identical to "*F. rappini*" in the colonic mucosae of these mice (data not shown). To determine the normal niche for the organism resembling "*F. rappini*," we examined tissues from nine uninfected mice by electron microscopy. Few organisms were observed in the colonic mucosae of uninfected mice. Tissue from the distal ileum and cecum, however, was colonized by several morphologic types of organisms, including fusiform rods without periplasmic fibers, a segmented, filamentous organism (10), and the organism resembling "*F. rappini*." Both the luminal mucosal surface and the crypts, particularly in the terminal ileum, had large numbers of the organism resembling "*F. rappini*." Transmission electron microscopy revealed that the periphery of the organism was serrated (Fig. 1). A serrated appearance in transmission electron micrographs, previously noted by Erlandsen and Chase (6), is characteristic of both *H. muridarum* (16) and "*F. rappini*" (2). When the organisms were sectioned longitudinally, they appeared fusiform to slightly curved, but never coiled, which is in contrast to the coiled appearance of *H. muridarum* (16). By scanning electron microscopy, the periplasmic fibers appeared as regular, spiral ridges completely contained within an outer membrane (Fig. 2). The ultrastructure of the organisms was similar to that of *H. muridarum* (12).

Isolation and cultivation. The organism resembling "*F. rappini*" was isolated from all eight animals from which tissue was collected for culture. Occasional contaminants did grow on the brucella agar plates enriched with serum and containing antibiotics to inhibit the growth of other bacteria, but in general, overgrowth by more rapidly growing organisms did not occur. The organism grew as a thin, spreading film on the surface of the plate. Optimal growth occurred under microaerophilic conditions on plates that had not been predried as described previously (1, 16). The organism did not grow under anaerobic conditions, nor did it grow in the presence of ambient concentrations of O_2. The organism rapidly hydrolyzed urea, produced alkaline phosphatase, and was oxidase positive, but it was negative for catalase.

Morphology and ultrastructure. Bacteria from pure cultures were stained with uranyl acetate and were examined by electron microscopy (Fig. 3). Morphometric measurements

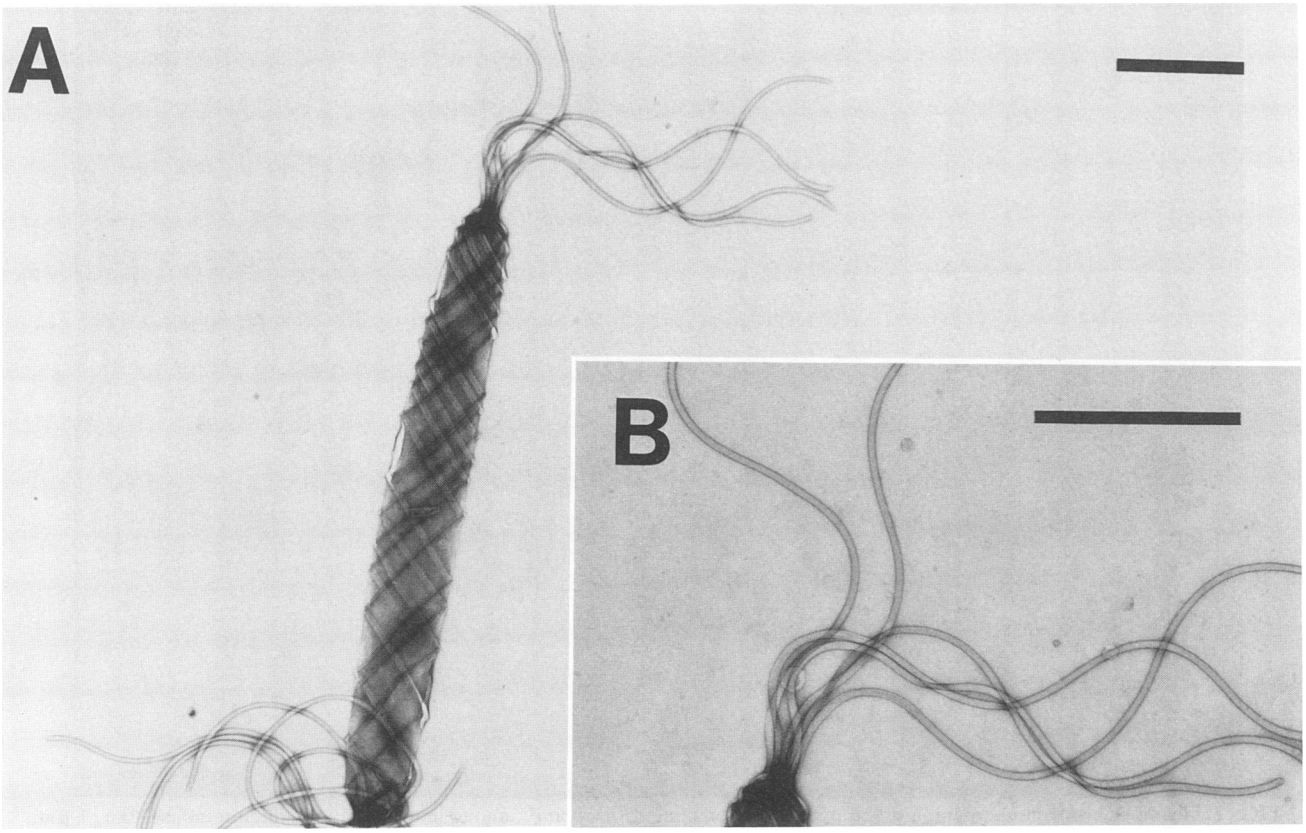

FIG. 3. Transmission electron micrographs of a bacterium taken from a pure culture of organisms resembling "*F. rappini*" and stained with uranyl acetate. The organism has spiral periplasmic fibers (A). Higher magnification of the organism shows 38-nm-wide sheathed flagella at the proximal end. (B). Bars, 1 μm.

were taken from independent micrographs of 20 representative bacteria. The organisms were 4 to 5 μm in length and approximately 0.5 μm in width. Organisms typically had between five and nine 38-nm-wide sheathed flagella originating from each pole, but some organisms with tufts of flagella at only a single pole were found. The periplasmic fibers coiling around the bacterial protoplasmic cylinder gave the surface of the organism a criss-cross appearance, which is characteristic of both "*F. rappini*" (1, 9) and *H. muridarum* (16, 18).

Cloning, nucleotide sequence determination, and alignment. Oligonucleotide primers complementary to phylogenetically conserved sequences near the 5′ and 3′ ends of the bacterial 16S rRNA genes (5, 19) were used to amplify almost the entire 16S rRNA gene from DNA isolated from a pure culture of the organism resembling "*F. rappini*." The nucleotide sequence of 967 bp of DNA, corresponding to *E. coli* 16S rRNA positions 492 to 1456, was determined unambiguously and was used to search for sequence homology within the GenBank nucleic acid sequence data base. The highest degree of nucleotide identity was found to exist between the query sequence and two "*F. rappini*" partial 16S rRNA sequences. The query sequence was found to have 99.3% identity with the 16S rRNA sequence from "*F. rappini*" NADC 1893 (4), which was isolated from an aborted sheep fetus (accession number M88137), and 98.0% identity with the 16S rRNA sequence from "*F. rappini*" ATCC 43879 (4), which was isolated from the stool of a human with gastroenteritis (accession number M88138). The query sequence was found to have 94.2% identity with the 16S rRNA sequence of *H. muridarum* (accession number M80205). An optimized alignment of the partial 16S rRNA gene sequence from the mouse isolate with the partial 16S rRNA sequences from two "*F. rappini*" isolates (4) and *H. muridarum* (12) is shown in Fig. 4.

DISCUSSION

We isolated an organism with an unusual ultrastructure from the gastrointestinal tracts of outbred CFW mice. The organism was morphologically indistinguishable from bacteria isolated from aborted sheep fetuses (9) and the stools of humans with chronic diarrhea (1); the organism has provisionally been named "*F. rappini*." The murine isolate grew under microaerophilic conditions, but not under strict anaerobic conditions or in air, as has been described for both *H. muridarum* (12, 16) and "*F. rappini*" (1). The original ovine isolate of "*F. rappini*" has been described as an anaerobic bacterium (9), but Archer et al. (1) could not grow this organism anaerobically. The murine isolate was negative for catalase, as were the human isolates of "*F. rappini*," while the sheep isolate of "*F. rappini*" is catalase positive (1). These differences may be due to strain variability. The murine isolate resembled *H. muridarum*, which can also be isolated from the intestines of normal mice (16, 18), but it could be distinguished from *H. muridarum* by the fact that *H. muridarum* has a helically coiled protoplasmic cylinder. We have examined a limited number of stocks and strains of

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Fr-mo    1  ACGAATAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTACTCGGAATCACTGGGCGTAAAGAGCGCGTAGGCGGGTG
FEXA   450  ..................................................N.................................................
FEXB   450  ..................................................N.................................................
Hmr    447  ................N.....................................................................T...C.......CT

Fr-mo  101  GTCAAGTCAGATGTGAAATCCTGTAGCTTAACTACAGAACTGCATTTGAAACTGACCATCTAGAGTATGGGAGAGGTAGGTGGAATTCTTGGTGTAGGGG
FEXA   550  ................................................................................................
FEXB   550  ................................................................................................
Hmr    547  AAT.................A..........T................ATTAG........G.....................................

Fr-mo  201  TAAAATCCGTAGAGATCAAGAGGAATACTCATTGCGAAGGCGACCTGCTGGAACATTACTGACGCTGATGCGCGAAAGCGTGGGGAGCAAACAGGATTAG
FEXA   650  .N..............................................................................................
FEXB   650  ..............................................................................K.................
Hmr    647  ..............................................A..................C....A.........................

Fr-mo  301  ATACCCTGGTAGTCCACGCCCTAAACGATGAATGATAGTTGTTGCCCTGCTTGTCAGGGCAGTAATGCAGCTAACGCATTAAGCATTCCGCCTGGGGAGT
FEXA   750  ................................C...............................................................
FEXB   750  ................................C...............................................................
Hmr    747  ................................C..........T......A.............................................

Fr-mo  401  ACGGTCGCAAGATTAAAACTCAAAGGAATAGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGATACGCGAAGAACCTTACCTAGGCT
FEXA   850  ................................................................................................
FEXB   850  ............................................NNNNNNNNNNN.............NNN.....................
Hmr    847  ..................................................NNN.............NNNN.....................

Fr-mo  501  TGACATTGATAGAATCCGCTAGAGATAGTGGAGTGCTGGCTTGCCAGAGCTTGAAAACAGGTGCTGCACGGCTGTCGTCAGCTCGTGTCGTGAGATGTTG
FEXA   950  ................................................................................................
FEXB   950  ................................................................................................
Hmr    947  ................TA........TG.......CACT.CTGTG.....................................................

Fr-mo  601  GGTTAAGTCCCGCAACGAGCGCAACCCTCGTCCTTAGTTGCTAGCAG TTCGGCTGAGCACTCTAAGGAGACTGCCTTCGTAAGGAGGAGGAAGGTGAGG
FEXA  1050  ..................................... C..........................................................
FEXB  1050  ................................N.... C..........................................................
Hmr   1047  ....N.....................................T..NA.....................................................

Fr-mo  700  ACGACGTCAAGTCATCATGGCCCTTACGCCTAGGGCTACACACGTGCTACAATGGGACATACAAAAAGATGCAATACCGCGAGGTGGAGCAAATCTCTAA
FEXA  1149  ................................................................................................N.
FEXB  1149  ................................................................................................
Hmr   1147  ..........................................................GTGC.....G..GA......T..T...................

Fr-mo  800  AATGTCTCTCAGTTCGGATTGTAGTCTGCAACTCGACTACATGAAGCTGGAATCGCTAGTAATCGTGAATCAGCCATGTCACGGTGAATACGTTCCCGGG
FEXA  1249  ................................................................................................
FEXB  1249  ................................................................................................
Hmr   1247  ..C.............................................................................................

Fr-mo  900  TCTTGTACTCACCGCCCGTCACACCATGGGAGTTGTATTCGCCTTAAGTCGGGATACTAAATTGGTTA  967
FEXA  1359  ..........................................................................  1426
FEXB  1359  ..........................................................................  1426
Hmr   1347  ..........................................................................  1414
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FIG. 4. Aligned partial 16S rRNA sequences from the mouse isolate of "*F. rappini*" (Fr-mo), "*F. rappini*" NADC 1893 (FEXA), "*F. rappini*" ATCC 43879 (FEXB), and *H. muridarum* (Hmr). Sequence identity with the mouse isolate of "*F. rappini*" is indicated by a dot, spaces indicate gaps, K indicates G or T, and N indicates A, C, T, or G. The GenBank accession number for the sequence for the mouse isolate of "*F. rappini*" is L12765, that for "*F. rappini*" NADC 1893 is M88137 (4), that for "*F. rappini*" ATCC 43879 is M88138 (4), and that for *H. muridarum* is M80205 (12).

laboratory mice from other vendors and have not detected the presence of organisms resembling "*F. rappini*" (data not shown). The frequency of colonization of laboratory mice and wild mice by "*F. rappini*" remains to be determined.

To identify the murine isolate resembling "*F. rappini*," the 16S rRNA gene was amplified and its nucleotide sequence was determined. It is possible that the portions of the amplified 16S rRNA gene for which the nucleotide sequence was not determined (corresponding to *E. coli* positions 28 to 491 and 1456 to 1492) contained additional differences from the published "*F. rappini*" 16S rRNA nucleotide sequences. The 967 bp of 16S rRNA for which the sequence was determined was 98.0 to 99.3% identical to that of "*F. rappini*." There were only 2 base mismatches, out of 967 nucleotides, with each of the "*F. rappini*" 16S sequences, or 99.8% homology. This level of sequence similarity suggests that the bacterial isolate from mice is "*F. rappini*" or is very closely related to that organism. While 16S rRNA identity

does not necessarily indicate species identity (7), it may be that under different conditions "*F. rappini*" can be an intestinal commensal organism of mice, can cause gastroenteritis in humans, and can induce abortion in sheep. Since the gastric species of the genus *Helicobacter* have evolved to be able to colonize as hostile an environment as the stomach, it is perhaps not surprising that the related organism "*F. rappini*," which is likely to be an intestinal *Helicobacter* species, can exploit such a wide range of niches.

We first observed the presence of an organism resembling "*F. rappini*" in the colonic mucosae of mice following transient colonization of the large bowel with *C. freundii* biotype 4280. Subsequently, the organism resembling "*F. rappini*" was identified in the ilea and ceca of uninfected mice. Both "*F. rappini*" and *H. muridarum* appear to be well suited for exploiting alterations in the gastrointestinal tract and can colonize sites other than the ileum and cecum, including the stomach (11) and colon (data not shown) of mice. In some instances, an organism resembling *H. muridarum* may be part of the resident flora in the mouse stomach, where it is associated with gastritis (18). Furthermore, bacteria resembling "*F. rappini*" have also been observed in the stomachs of dogs (13). Thus, it appears that both "*F. rappini*" and *H. muridarum* are intestinal *Helicobacter* species that are able to colonize the stomach, but the conditions that allow for gastric colonization have yet to be defined. We plan to determine whether drug-induced hypochlorhydria in mice intestinally colonized with "*F. rappini*" will lead to gastric colonization by these resident bacteria. Additionally, we hope to express putative virulence determinants from gastric-adapted *Helicobacter* species, including urease, in "*F. rappini*" and determine whether the recombinant bacteria are capable of gastric colonization in mice.

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