Failure of Surface Ring Mutant Strains of *Helicobacter mustelae* To Persistently Infect the Ferret Stomach

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Helicobacter mustelae, the gastric pathogen of ferrets, produces an array of surface ring structures which have not been described for any other member of the genus *Helicobacter*, including *H. pylori*. The unique ring structures are composed of a protein named Hsr. To investigate whether the Hsr rings are important for colonization of the ferret stomach, ferrets specific pathogen free for *H. mustelae* were inoculated with an Hsr-deficient mutant strain or the wild-type *H. mustelae* strain. Quantitative cultures from antral biopsy specimens obtained at 3, 6, and 9 weeks postinoculation demonstrated no significant difference in the levels of bacteria in the ferrets that received the Hsr-negative strain and the ferrets infected with the parent strain. However, when the ferrets were biopsied at 12 and 15 weeks and necropsied at 18 weeks after infection, the levels of bacteria of the Hsr-negative strain in the stomach antrum were significantly reduced. This decline contrasted the robust antral colonization by the wild-type strain. The Hsr-negative strain did not efficiently colonize the gastric body of the study ferrets. Histological examination at 18 weeks postinoculation revealed minimal gastric inflammation in the animals that received the mutant *H. mustelae* strain, a finding consistent with its waning infection status, whereas lesions characteristic of helicobacter infection were present in ferrets infected with the wild-type strain. Scant colonization by the Hsr-negative *H. mustelae* strain at the end of the 18-week study, despite initial successful colonization, indicates an inability of the mutant to persist, perhaps due to a specific host response.

Helicobacter mustelae in ferrets shares a number of features with *H. pylori* in humans. Both helicobacters are causally associated in their host species with subclinical mild gastritis, chronic gastritis, gastroduodenal ulceration, and gastric cancer (6, 10, 11, 15, 28). Urease activity, motility by means of flagella, and ability to adhere to the gastric mucosa typify both bacteria (20). Gastric infection in ferrets with *H. mustelae* and in humans with *H. pylori* can be lifelong, and a humoral, nonprotective immune response to each microorganism is elicited (5, 17). To elucidate the pathogenesis of *H. pylori* in humans, experiments have been designed to investigate aspects of these common characteristics in ferrets that are specific pathogen free (SPF) for and dosed with *H. mustelae*. Flagellar motility as a colonization factor has been studied with isogenic flagellar mutants of *H. mustelae* (1); likewise, experiments with urease-negative isogenic strains of *H. mustelae* have demonstrated the necessity of urease activity for colonization of the ferret stomach (2). Ferrets have also been used to study the epidemiology of gastric helicobacter infections. For example, fecal shedding of *H. mustelae* is dependent in part on gastric pH (9), and ferrets in which *H. mustelae* has been eradicated by antimicrobial therapy have been shown to be susceptible to both natural and experimental reinfections (4).

One proposed virulence determinant of *H. mustelae* is miss-

ing from *H. pylori* and has not been described for other *Helicobacter* species to date. The unique attribute is the laterally extensive array of rings that cover the cell surface of *H. mustelae* (20, 21). The uniformly distributed rings are 8.5 nm in diameter, project 6 nm from the outer membrane of the cell, and are composed of a 150-kDa monomer protein referred to as Hsr (for *Helicobacter* surface ring). It is estimated that the Hsr protein comprises 25% of the total envelope protein of *H. mustelae* (21). Immunochemical analysis with an affinity-purified antibody against Hsr has shown that this protein is expressed by all *H. mustelae* strains that have been tested, although the affinity was highest for the strain against which the antibody was raised (21). The suggestion of antigenic variability in Hsr proteins among *H. mustelae* strains was supported recently in a study that investigated recombination between the *hsr* gene and its flanking sequences (7). This process may occur because the *hsr* gene is flanked by an extensive array of sequences that are identical to regions within the 5' two-thirds of the *hsr* coding sequence (7), providing a mechanism for antigenic variation.

The exposed location of Hsr on the *H. mustelae* cell surface, as well as the probability of antigenic diversity through differential *hsr* recombination among strains of *H. mustelae*, raises questions about possible functions for the ring structures. It is also intriguing that such a prominent and abundant feature found on a ferret gastric pathogen is absent from its counterpart in humans, *H. pylori*. Despite superficial similarities, such as molecular structure and noncovalent binding to the outer membrane, the Hsr surface rings do not form a classical surface layer (S layer), found in many prokaryotes, because no

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highly ordered or paracrystalline symmetry is achieved in their arrangement (21). Nevertheless, comparable roles have been suggested for the Hsr surface rings and S layers (25). Roles ascribed to and demonstrated for S layers include involvement in protection, cell adhesion, surface recognition, and immunity (24). However, the Hsr surface rings of *H. mustelae* have been shown not to function as an adhesin (7).

In the work reported here, the importance of the ring structures for *H. mustelae* colonization was examined by inoculating SPF ferrets with an isogenic Hsr-negative strain or the Hsr-positive parent strain. Histopathological changes concomitant with infection by the strains of *H. mustelae* were also compared. A preliminary study was performed to test two isogenic Hsr-negative mutant strains, and the ferrets were necropsied at 12 weeks postinfection. Based on results from this preliminary study showing no difference in colonization behavior between the two Hsr-negative strains, an expanded study (more animals and a longer period of observation) with only one Hsr-negative *H. mustelae* strain was undertaken.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. *H. mustelae* strain 4298 was recovered from a naturally infected ferret obtained from the same commercial ferret colony from which the type strain of *H. mustelae*, ATCC 43772, was originally isolated. *Escherichia coli* strain ER2206 (7) and plasmid pUC18 (29) were used for cloning experiments that followed standard protocols (23). Plasmid pHM002 was described previously (20). The chloramphenicol acetyltransferase gene (*cat*) was derived from plasmid pRY109 (30).

Culturing of *H. mustelae* for mutant construction was performed with chocolate blood agar (Oxoid Columbia agar base plus 5% [vol/vol] defibrinated horse blood) plates incubated at 37°C in a microaerobic atmosphere. *E. coli* was grown on Luria-Bertani agar with antibiotic supplementation as required. Antibiotics were added to the *E. coli* growth media at the following levels: ampicillin, 100 µg/ml; kanamycin, 50 µg/ml; and chloramphenicol, 10 µg/ml. *H. mustelae* transformants were selected on chocolate blood agar plates with chloramphenicol incorporated at 10 µg/ml.

Insertional inactivation of the *hsr* gene. A construct (pHM219) was made to allow *cat* gene insertion into the nonvariable β-barrel junction region of *hsr*, according to the organization of the Hsr molecule defined elsewhere (7). For construction of pHM219, a 1.8-kb fragment of the *hsr* gene and downstream flanking sequence (Fig. 1) was cloned into pUC18 to generate intermediate pHM217. The *hsr* fragment was derived from a region encompassing the *DraI* site at coordinate 3034 of the *hsr* gene (21) through a vector-derived *SphI* site from the polylinker in pHM002, which comprises the *hsr* gene and flanking sequences cloned in pUC18 (21). A cleavage site which incorporates the recognition sequence for restriction enzyme *BglII* was introduced into the insert in pHM217 by inverse PCR (14) with primers NTF011 and NTF012. These primers are complementary to coordinates 3661 to 3678 (NTF011) and 3642 to 3660 (NTF012) of the *hsr* gene. The *cat* gene of pRY109 was cloned into the novel *BglII* site of the *hsr* fragment in pHM217 to generate pHM219.

Construction of Hsr-deficient *H. mustelae*. Plasmid pHM219, containing the mutated allele, was introduced into *H. mustelae* strain 4298 by natural transformation, and chloramphenicol-resistant transformants were selected. One clone was designated *H. mustelae* strain 4298-219. The clone was verified for a single *cat* gene insertion in a syngenic orientation at the expected genomic site by PCR with primers flanking the insertion site. This PCR resulted in products corresponding in size to the wild-type chromosomal fragment plus the size of the *cat* gene (data not shown). Porwollik et al. previously showed by transcript analysis that this *cat* marker did not lead to polar effects in *H. pylori* because the *cat* transcript runs off the end of the *cat* gene into downstream sequences (22). In addition, there are no coding sequences (whose expression might be affected) within 2,880 bp of the 3' end of the *hsr* gene, since Forester et al. previously showed that this region is occupied by flanking repeat sequences (7).

The transformant was assayed for the lack of expression of Hsr by Western blotting of whole-cell lysates separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (21). No immunoreactive peptide of any size was detected by Western blotting, although truncated proteins were detect-

FIG. 1. Insertional inactivation of the *hsr* gene. (Top) Organization of the Hsr protein (7), including respective numbers of amino acid residues. (Middle) Site of insertion of a chloramphenicol acetyltransferase gene (*cat*) in mutagenic construct pHM219. (Bottom) Nucleotide coordinates of the *hsr* gene (21) relevant for the construction of pHM219. The diamond marker indicates a restriction site introduced by PCR. The lines in the middle and bottom diagrams represent cloned *H. mustelae* insert DNA only; vector sequences are not shown, except for a small part of a multiple cloning site (broken lines) containing a vector-derived *SphI* recognition site.

able in *E. coli* (data not shown), presumably reflecting more severe proteolysis for *H. mustelae*.

***H. mustelae* inocula for dosing of ferrets.** *H. mustelae* strains 4298 and 4298-219 were grown on 5% sheep blood agar with cefoperazone, vancomycin, and amphotericin B (Remel, Lenexa, Kans.) under microaerobic conditions at 37°C. Both strains were positive for catalase, oxidase, and urease; sensitive to nalidixic acid; and resistant to cephalothin. Morphology consistent with *H. mustelae* was found to be present in the two strains by Gram staining and phase-contrast microscopy. Prior to dosing, the organisms were suspended in brucella broth (Difco Laboratories, Detroit, Mich.) with 20% glycerol added; the approximate concentration of organisms in an aliquot of broth was determined by ascertaining the optical density.

Animals. To achieve pathogen-free status with regard to *H. mustelae*, pregnant jills purchased from a commercial ferret supplier (Marshall Farms, North Rose, N.Y.) were placed on an oral triple-antibiotic therapy regimen which was used previously to eradicate *H. mustelae* (4). Jills were given antibiotics until their kits were 2 weeks old. After the kits were weaned, their dams were confirmed to be *H. mustelae* free by culturing and histological examination of endoscopic gastric biopsy specimens. When the kits were 3 months old, they were likewise biopsied to determine by culturing and histological examination that they were not colonized by *H. mustelae*.

For the duration of the study, the ferrets were housed by group in plastic cages (MediCage Lock Solutions, Kenilworth, N.J.) in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Ferrets dosed with the isogenic Hsr-negative *H. mustelae* strain were kept in a cubicle separate from that housing the ferrets that were sham dosed or inoculated with the wild-type *H. mustelae* strain. Food (Lab Diet 5L14 [PMI Feeds, Inc., St. Louis, Mo.] and Feline Growth [Hill's Pet Nutrition, Inc., Topeka, Kans.]) and water were available ad libitum. Prior to necropsy, the ferrets were anesthetized with intramuscular xylazine-ketamine (see below) and then given an overdose of carbon dioxide.

Study design. Eighteen SPF kits were entered into the study when they were 4 months old. Under sedation with intramuscular acepromazine (0.25 mg/kg of body weight) and ketamine (25 mg/kg of body weight), four of these ferrets (males) received 3 ml of sterile brucella broth-glycerol via an orogastric feeding tube, seven ferrets (four males and three females) received 3 ml of broth containing $\sim 1.0 \times 10^8$ CFU of *H. mustelae* wild-type strain 4298/ml, and seven ferrets (five males and two females) received 3 ml of broth containing $\sim 1.0 \times 10^8$ CFU of *H. mustelae* isogenic strain 4298-219/ml.

At 3, 6, 9, 12, and 15 weeks postinfection, endoscopic gastric biopsy specimens were obtained from anesthetized (intramuscular xylazine at 4 mg/kg and ket-

FIG. 2. Quantitative culture data for antrum and body samples. Average log CFU per gram is shown on the y axis; sample time points in weeks postinfection are shown on the x axis. The closed and open circles represent average quantitative culture results obtained from wild-type ($n = 7$) and Hsr-negative ($n = 7$) *H. mustelae*-infected ferrets, respectively. Error bars represent confidence intervals.

amine at 40 mg/kg) ferrets. Two 2-mm punch biopsy specimens each from the pyloric antrum and the gastric body were placed in 500 μ l of brucella broth-glycerol for quantitative culturing. The ferrets were euthanized and necropsied at 18 weeks postinoculation, when three samples each were taken from the antrum and the body for quantitative culturing.

Quantitative culturing. Quantitative culturing was performed by weighing each tissue sample and then homogenizing the tissue. Hundredfold serial dilutions of the homogenate (10^0 , 10^{-2} , and 10^{-4}) were cultured on sheep blood agar-cefoperazone-vancomycin-amphotericin B plates under microaerobic conditions at 37°C. The growth of *H. mustelae* strains was monitored every 3 to 5 days for 2 weeks. The phenotypic characteristics of *H. mustelae* were tested for all strains isolated. Isolates recovered from selected ferrets in each group were assessed for the presence or absence of Hsr proteins by SDS-PAGE (16). The culture results were examined by a repeated-measures analysis of variance (SuperANOVA), as samples were obtained repeatedly from each animal over the course of the experiment.

Histopathological changes. Stomach tissue samples were collected at baseline via endoscopy and at necropsy. After fixation in 10% neutral buffered formalin and routine processing, 5- μ m sections were stained with hematoxylin-eosin and with the Warthin-Starry silver stain.

RESULTS

***H. mustelae* quantitative cultures.** The sham-infected animals were not included in the repeated-measures analysis of variance, as they had no positive cultures. Overall, the average log CFU per gram of tissue recovered from the ferrets infected with wild-type *H. mustelae* versus Hsr-negative *H. mustelae* differed significantly ($P = 0.0001$). When the two-way interaction of *H. mustelae* strain (wild type versus Hsr negative) with time point of the study was examined, the patterns of colonization differed significantly (the P value was 0.0239, as determined with the Greenhouse-Geisser correction factor) between the two organisms. The graphs in Fig. 2 illustrate the colonization behaviors over time for the *H. mustelae* strains, as measured in the two stomach areas. From Fig. 2 it is evident that the patterns differed as the study progressed, in both the antrum and the body, between the ferrets dosed with the wild-type strain and those dosed with the mutant strain. One important difference, for example, was the dramatic increase in

the wild-type log CFU per gram obtained from antral biopsy specimens at 18 weeks postinfection and the simultaneous dramatic decrease in the mutant log CFU per gram. At time points at which there was a significant difference in quantitative culture results between the wild-type- and the mutant-infected animals, the confidence intervals for one group did not overlap the mean for the other group. Antral cultures were not significantly different between the two groups at the 3-, 6-, or 9-week time points. From 12 to 18 weeks postinoculation, however, antral cultures from the ferrets that received Hsr-negative *H. mustelae* showed significantly reduced levels of bacteria. Cultures from the gastric body showed significantly lower levels of bacteria in mutant-infected animals than in wild-type-infected animals at all time points. SDS-PAGE of isolates obtained at the 18-week study conclusion confirmed the absence of Hsr in the *H. mustelae* mutant strain (Fig. 3).

Histopathological changes. Microscopically, there were minimal or no inflammatory cells throughout the antral mucosa at the 18-week time point (Fig. 4A) in the ferrets that were sham infected with sterile broth. Organisms consistent with *H. mustelae* were not shown to be present by Warthin-Starry staining. At the conclusion of the study, the ferrets that received wild-type *H. mustelae* 4298 had moderate to diffuse multifocal leu-

FIG. 3. SDS-polyacrylamide gel showing Coomassie blue-stained total protein from *H. mustelae* isolates recovered from ferrets at the 18-week time point. Lane 1, isolate from a ferret infected with wild-type strain 4298; lanes 2 to 6, isolates from ferrets infected with the Hsr-negative mutant strain; and lane S, molecular mass markers. k, kilodaltons.

FIG. 4. Sections of gastric antrum samples from ferrets. (A) Gastric antrum from sham-infected ferret at 18-week time point with minimal inflammatory cells. (B) Gastric antrum from ferret infected with wild-type *H. mustelae* at 18-week time point showing moderate infiltration of mononuclear cells in the lamina propria. (C) *H. mustelae* in gastric pits of wild-type-infected ferret (18 weeks). (D) Gastric antrum from ferret infected with Hsr-negative mutant strain of *H. mustelae* at 12-week time point of a preliminary study showing a diffuse inflammatory infiltrate. (E) Gastric antrum from ferret infected with Hsr-negative mutant strain at 18-week time point showing decreased inflammation. (A, B, D, and E) Hematoxylin-eosin staining; bars, 100 μ m. (C) Warthin-Starry stain; bar, 10 μ m.

kocytic infiltrates in the antral submucosa (Fig. 4B). The inflammatory cell type was primarily mononuclear. *H. mustelae* organisms were visible in the gastric pits of ferrets infected with this wild-type strain (Fig. 4C). Ferrets from the preliminary study that underwent gavage with an isogenic Hsr-negative mutant strain of *H. mustelae* and were necropsied at 12 weeks postinfection had pathological changes like those seen in ferrets infected with the wild-type strain at the same time point (Fig. 4D). Inflammation was evident in these ferrets at 12 weeks despite the diminished levels of Hsr-negative *H. mustelae* seen in quantitative cultures; these levels were comparable to those seen at 12 weeks (and thereafter) in the expanded study. In addition, organisms consistent with *H. mustelae* were rare to nonexistent in antral sections of these mutant-infected ferrets at 12 weeks. When the stomachs of isogenic mutant-infected ferrets were evaluated histologically at 18 weeks postinfection, evidence of antral inflammation was minimal (Fig. 4E), and silver-stained bacteria were absent.

Stomach body sections examined from all of the ferrets lacked appreciable evidence of inflammation. At 18 weeks postinfection, bacteria were also absent from the body mucosa of the ferrets, other than the wild-type-infected ferrets, in which scattered *H. mustelae* cells were visible.

DISCUSSION

The Hsr surface rings of *H. mustelae* were not critical for early events of colonization, but their absence did impact the long-term survival of *H. mustelae* in the ferret stomach. At 3, 6, and 9 weeks postinoculation, quantitative antral cultures of the Hsr-negative mutant were not significantly different from antral cultures of wild-type strain 4298, whereas from 12 weeks on, the levels of the Hsr-negative strain were reduced substantially. Quantitative culture data from the ferrets inoculated with the wild-type strain were of the same magnitude as those reported for wild-type *H. mustelae* in an earlier study that examined the colonization features of isogenic flagellar mutants of *H. mustelae* (1). *H. mustelae* is known to colonize the stomach antrum before it colonizes the body; this pattern was observed in ferrets infected with wild-type strain 4298 in the present study. In contrast, the *H. mustelae* mutant strain was recovered at low levels from gastric body tissue samples throughout the 18-week study.

In a preliminary study in which ferrets were necropsied at 12 instead of 18 weeks postinfection, antral gastritis was evident in ferrets infected with the Hsr-negative strain 12 weeks after dosing; this gastritis was similar to the gastritis observed in ferrets infected with the parent strain, except that many fewer *H. mustelae* organisms were visible in the gastric pits for the former. Hence, the inflammatory effects of colonization by Hsr-negative *H. mustelae* were comparable to those of colonization by wild-type *H. mustelae*. By 18 weeks postinfection, however, when the mutant-dosed ferrets had experienced reduced helicobacter colonization for several weeks, inflammatory infiltrates had subsided. Previous studies found gastritis in ferrets to be reduced but persistent following the eradication of *H. mustelae* with antimicrobial agents (4, 17). In any case, it is difficult to compare these earlier findings with those of the present study due to differences in strains of *H. mustelae* used and study designs as well as subjective assessments of his-

topathological inflammation used by various investigators. It is conceivable that the elimination of established long-term *H. mustelae* colonization by antibiotic therapy affects manifestations of inflammation in unique ways.

The mechanism whereby the Hsr-negative *H. mustelae* strain was depleted from ferret stomachs is unknown. A direct effect on bacterial viability would be expected to preclude even the transient infection that occurred. Instead, the 9-week duration between initial colonization and statistically significant reduction in the levels of Hsr-negative mutant *H. mustelae* suggests that some relationship between host and bacteria is responsible, most likely involving the host immune system. Such dynamic host-bacterium interactions have been explored with several pathogenic species that have classical lattice S layers. For example, isogenic mutants of *Campylobacter fetus* that lacked the S-layer protein were unable to colonize in an ovine abortion model (13). This failure to colonize is unlike the delayed loss of the Hsr-negative *H. mustelae* strain in the study ferrets. A second *Campylobacter* species, *C. rectus*, also has S-layer proteins, and these proteins appear to be important in the development of periodontal disease (26). One effect of the S layer of *C. rectus* is to downregulate proinflammatory cytokines; however, so far this effect has been assayed only in the short term with an in vitro epithelial cell model (26).

Antigenic or phase variation is another method that is potentially used by bacteria to modulate the host immune response, and it also involves superficial structures such as S layers. In *C. fetus*, differential S-layer protein expression has been well characterized and demonstrated to occur in vivo (12). Variable expression of the Hsr proteins of *H. mustelae*, which was reported recently (7), could also have a role in evasion of host immunity over time. The Hsr-negative mutant that was absent from the 12- to 18-week gastric samples may have had invariant antigenic epitopes that rendered the bacteria ineffectual against host immune attack. Whether antigenic variation in the surface ring structures of *H. mustelae* takes place during the course of an actual infection remains to be demonstrated. It was shown recently that ferrets naturally infected with *H. mustelae* develop antibody to Hsr (8). Interestingly, phase variation in *H. pylori* is associated with the blood group-related Lewis antigens, particularly Lewis x and Lewis y, found in the lipopolysaccharide layer of the bacteria (3, 27). Although *H. mustelae* does express blood group-like antigen A, it lacks Lewis x and Lewis y (18, 19). These two gastric helicobacters may have evolved different strategies, Hsr proteins in *H. mustelae* versus Lewis antigens in *H. pylori*, to help achieve long-term survival by avoiding the bactericidal effects of the host immune system.

This study confirms that the unique Hsr proteins are integral to the pathogenesis of *H. mustelae* because these surface rings are required for persistent infection. Additional in vivo experiments are warranted to further characterize these surface structures in response to the host environment. It would be instructive to determine, for example, whether infection with an *H. mustelae* Hsr-negative mutant strain and the resultant host immune response would afford any protection to subsequent challenge with Hsr-positive *H. mustelae* wild-type strains (4). By studying fundamental differences in the biology of *H. mustelae* and that of *H. pylori*, an understanding of both of these gastric pathogens can be achieved.

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