Cloning and Expression of a \textit{Helicobacter bilis} Immunoreactive Protein

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Received 2 November 2001/Returned for modification 18 December 2001/Accepted 16 January 2002

In an effort to identify immunoreactive \textit{Helicobacter bilis} antigens with potential for serodiagnosis, sera from mice experimentally infected with \textit{H. bilis} were used to screen an \textit{H. bilis} genomic DNA expression library. Among 17 immunoreactive clones, several contained sequences that encoded a predicted 167-kDa protein (P167). Five overlapping P167 peptides (P167A to P167E) of approximately 40 kDa each were generated and tested. Immune sera reacted with fragments P167C and P167D at dilutions of 1:1,600 and 1:6,400, respectively, and reacted with an \textit{H. bilis} membrane extract at a dilution of 1:800 in an enzyme-linked immunosorbent assay. Sera from mice experimentally infected with \textit{H. hepaticus} did not react with P167C and P167D. Sera from mice naturally infected with \textit{H. bilis} but not sera from mice naturally infected with \textit{H. hepaticus} reacted with P167C and P167D. Hyperimmune sera against P167C peptide reacted with recombinant P167C and with a 120-kDa band in \textit{H. bilis} lysates but did not react with a protein of the same size on immunoblots prepared from \textit{H. hepaticus}, \textit{H. muridarum}, or unrelated \textit{Borrelia burgdorferi} and \textit{Campylobacter jejuni} whole-cell lysates. Nevertheless, the P167A, P167B, P167C, and P167D primers, but not the P167E primers, amplified DNA from \textit{H. hepaticus}, and all five primer sets amplified DNA from \textit{H. muridarum}. These results suggest that P167 is an immunodominant, \textit{H. bilis}-specific antigen that may have potential for use in serodiagnosis.

\textit{Helicobacter bilis} (11) is a member of an expanding and genetically diverse group of enterohemoplastic, commensal, and opportunistic \textit{Helicobacter} species that infect laboratory mice. In addition to \textit{H. bilis}, \textit{Helicobacter} species isolated from mice include \textit{H. hepaticus} (8), \textit{H. rodentium} (27), \textit{H. muridarum} (19), \textit{H. typhlonicus} (13), and others yet to be named (30). \textit{Flexispira rappini}, first described by Bryner et al. (2), has also been shown to be a \textit{Helicobacter} species (22, 24, 32), but recent studies suggest that it represents a mixture of \textit{Helicobacter} species (3). \textit{H. bilis} infections have been found to be widespread among research mouse colonies (11, 23). Infections are often subclinical but can produce liver and enteric diseases in some genotypes of mice, particularly mice with immune deficiencies. \textit{H. bilis} was isolated initially from aged inbred mice with chronic hepatitis and hepatomas in 1995 (11) and then subsequently from SCID mice that had enteritis and that were coinfected with \textit{H. rodentium} (28). Experimental inoculation of \textit{H. bilis} induces enteritis and hepatitis in SCID mice (12, 29) and enteric disease in athymic rats (16). Genomic alterations of mice can have both intentional and unpredicted immune perturbations that enhance the pathogenicity of these opportunistic pathogens. In addition to infecting mice, \textit{H. bilis} has been isolated from dogs, gerbils, rats, and cats (14), and its DNA has been amplified from bile and gall bladders of humans with cholecystitis (7).

For these reasons, there is a need for serodiagnostic assays that are both specific and sensitive. Currently available serologic assays for detecting \textit{Helicobacter} infection in mouse populations have relied on either bacterial lysates (9, 10, 33) or various types of membrane antigen preparations (14, 21, 34). Both are antigenically complex, with cross-reactive antigens causing a lack of specificity (34). In addition, these antigen preparations generally detect only low titers of serum reactivity in naturally infected mice and are not useful for detecting early stages of infection (9, 10, 21). Both fecal culturing and PCR have been shown to detect infection several weeks before positive membrane antigen seroconversion in sequentially sampled, experimentally \textit{H. bilis}-infected mice (17). Recently, an \textit{H. hepaticus} recombinant immunogenic protein (MAP18) was cloned and expressed (20). It proved to be \textit{H. hepaticus} specific but less sensitive than a membrane antigen extract (20). No recombinant proteins of \textit{H. bilis} have been characterized. In this report, we describe a novel recombinant \textit{H. bilis} gene product that is immunodominant and specific for \textit{H. bilis} and that has potential for further characterization as a serodiagnostic antigen.

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MATERIALS AND METHODS

Mice. Virus antibody- and \textit{Helicobacter}-free C3H/HeN (C3H) and C3H/Smn. C57Hsd-*cd (C3H-2cd) mice were purchased at 3 to 5 weeks of age from the National Cancer Institute Animal Production Program, Frederick Cancer Research Center, Frederick, Md. (C3H) or Harlan Sprague-Dawley, Indianapolis, Ind. (C3H-2cd). Upon arrival, fecal pellets from all mice were tested for \textit{Helicobacter} by culturing (see below) and PCR (17, 26). Mice were maintained in a pathogen-free room with restricted access on a 12-h light–12-h dark cycle. They were fed irradiated Pico Lab Mouse Diet 20 (PMI Nutrition International, Inc., Brentwood, Mo.). Mice were killed with CO\textsubscript{2} narcosis. The University of California laboratory animal care program is fully AAALAC accredited, and this study was reviewed and approved by the institutional animal care and use committee. All procedures and treatment of mice were in compliance with the Public Health Service Guide for the Care and Use of Laboratory Animals.
Bacterial culturing and isolation. *Helicobacter bilis* (ATCC 51630), *H. hepaticus* (ATCC 51448), and *B. burgdorferi* (ATCC 49282) were obtained from the American Type Culture Collection and cloned by threefold limiting dilution as described previously (27). The species identity of each clonal population was verified by PCR (17). To determine *H. bilis* infection in mice, freshly pooled fecal pellets were processed and cultured in brucella broth as described previously (17). Isolates were verified as *H. bilis* by PCR with *H. bilis*-specific 16S ribosomal DNA primers (23). For DNA extraction and PCR amplification from feces, samples were processed as described previously (26). Assays included negative controls from uninfected mice, and *H. bilis* genomic DNA served as a positive control. *Borrelia burgdorferi* sensu stricto cN40 was cultured in modified Barbour-Stoenner-Kelly medium at 33°C as described previously (6). Campylobacter jejuni was a gift from S. Jang, University of California, Davis.

Immune sera and antisera. *H. bilis* was grown under microaerobic conditions in brucella broth for 3 days at 37°C as described previously (17). Bacteria were adjusted to 10^8 CFU per ml, and 0.1 ml was inoculated intraperitoneally into 3- to 5-week-old C57BL-6 mice as described previously (17). Infection was established (4 to 8 weeks after inoculation) and confirmed by fecal PCR, the mice were killed and livers were collected. Liver tissue containing host-adapted *H. bilis* was homogenized in 10 ml of brucella broth, and then 0.25 ml of the homogenate was inoculated by gavage into C3H mice. Infection status was monitored weekly by fecal PCR and culturing. At 6 months after infection, blood was collected and sera were harvested from infection-positive mice.

Sera were also obtained and tested from naturally infected C3H mice. The mice were 12 to 14 weeks old when blood was collected. Identification of the infecting *Helicobacter* species was performed by fecal PCR with *Helicobacter* genus-specific primers (1) followed by restriction enzyme digestion of PCR amplicons to differentiate *H. hepaticus* from *H. bilis* (23). Briefly, a fresh fecal pellet was collected and suspended in 1.0 ml of phosphate-buffered saline (PBS). The fecal suspension was centrifuged at 700 x g for 5 min, and 60 μl of the suspension was combined with 140 μl of PBS. Purified DNA from the fecal suspension was obtained by using the Qiagen DNeasy tissue kit protocol for DNA isolation for a minimum of six wells containing uninfected normal mouse serum, was plated in 96-well plates as described previously (6). Duplicate samples of each mouse serum, including uninfected normal mouse serum as a control, were diluted 1:200 for probing. The secondary antibody was alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G secondary antibody diluted 1:4,500 (Sigma).

ELISA. For the ELISA, 100 μl of 1 μg of genomic DNA was added to each well. The optical densities at 405 nm were read on a Kinetic microplate reader (Molecular Devices, Sunnyvale, Calif.). The mean absorbance for duplicate experimental samples, as well as the mean absorbance and standard deviation for a minimum of six wells containing uninfected normal mouse serum, was calculated. Antibodies were considered present when the absorbance exceeded 3 standard deviations of the mean titer of the control (uninfected) mouse serum.

Amplification of P167A to P167E DNA fragments from genomic DNAs by PCR. Genomic DNAs were purified from *H. bilis*, *H. hepaticus*, *B. burgdorferi*, and *C. jejuni* as previously described (5). Each genomic DNA was used as a template separately, and each set of primers for P167A to P167E

| Primer         | Nucleotides corresponding to the P167 gene | Sequence                                                                 |
|---------------|--------------------------------------------|--------------------------------------------------------------------------|
| P167A forward | 58–81                                      | TATGCTGGCGATATTCAGGCGAT                                                 |
| P167A reverse | 110–121                                    | ATAGACAGTTATCCAGGTCTACC                                                 |
| P167B forward | 856–876                                    | TTATGATGCGTCCAGTACCAT                                                  |
| P167B reverse | 942–1092                                   | TGGTAGTCATCCACACTCAT                                                   |
| P167C forward | 1645–1668                                  | GCTATGGTGATAGTTAAGCAGTGT                                                 |
| P167C reverse | 2776–2799                                  | TGAAGCTACTGTTTCTTCTACTAC                                               |
| P167D forward | 2560–2583                                  | AGCTACACTTTACCAACACAGGGGAT                                              |
| P167D reverse | 3655–3675                                  | ATCTGTTACTCCAGGTGTTTGC                                               |
| P167E forward | 3409–3432                                  | CGTCTAGCAAGAGTAGCTAGCATT                                               |
| P167E reverse | 4609–4632                                  | ATTTGGTGAGGTGTTGTTTGC                                                  |

Helicobacter* bilis* genomic DNA expression library. Genomic DNA was isolated from *H. bilis*, and 200 μg of DNA was shipped to Stratagene, La Jolla, Calif., to construct a λZAP II *H. bilis* genomic DNA expression library. The λ ZAP II phage contains βluescript that can be excised and cloned directly with ExAssist helper phage (Stratagene). The library was screened with immune sera from *H. bilis*-infected mice. Immune sera were preabsorbed with *Escherichia coli* phage lysates to remove background reactivity. Immunoreactive clones were obtained by routine procedures as described previously (4). DNA sequencing was performed at the W. M. Keck Foundation Biotechnology Resource Laboratory at the Yale University School of Medicine. DNA sequence analysis was performed using the MacVector program (Kodak, New Haven, Conn.).

Expression and purification of recombinant proteins. The primers for the P167A DNA fragment corresponded to nucleotides 58 to 81 and 1102 to 1123 of the P167 gene. The primers for P167B were from 856 to 876 and from 942 to 1092. The primers for P167C were from 1645 to 1668 and from 2776 to 2799. The primers for P167D were from 2560 to 2583 and from 3655 to 3675. Finally, the primers for P167E were from 3409 to 3432 and from 4609 to 4632 of the P167 gene. These primer sets are summarized in Table 1. The synthetic DNA sequences were cloned in frame with the glutathione S-transferase gene into pMX, a pGEX-2T vector (Pharmacia, Piscataway, N.J.) with a modified polylinker (25). The PCR-amplified DNA sequences of the recombinant DNA were confirmed by sequence analysis with the original insert. Recombinant proteins were purifed on glutathione columns and freed of their glutathione S-transferase fusion partners by thrombin cleavage as described previously (4).

Immunoblotting. Four micrograms of membrane extract lysate or recombinant protein was resolved in sodium dodecyl sulfate–12% polyacrylamide gels by electrophoresis and transferred to nitrocellulose membranes. For dot blots, a Bio-Dot microfiltration apparatus (Bio-Rad) was used to transfer proteins to nitrocellulose membranes, and the protocol from the manufacture was modified as described below. A sheet of Bio-Rad Trans-Blot transfer medium nitrocellulose paper (9 by 12 cm) was soaked for 10 min in Tris-buffered saline (TBS) and then blotted with Whatman paper to dry. One hundred microliters of TBS was applied to each well to rewet the membrane, and then a vacuum was applied to the apparatus to remove the TBS. Proteins were diluted in TBS at 10 μg/ml, and 100 μl was added to each well. The TBS was replaced with 100 μl of nitrocellulose by gravity filtration. Once all the TBS had filtered through the nitrocellulose, the unit was disassembled and the nitrocellulose membranes were processed as immunoblots. Membranes were probed with immune sera (or uninfected normal mouse sera) diluted 1:100 and then labeled with alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G secondary antibody diluted 1:4,500 (Sigma).

ELISA. For the ELISA, 100 μl of 1 μg of membrane extract lysate or recombinant protein/ml in carbonate coating buffer (0.2 g of Na₂CO₃, 1.59 g of NaHCO₃, and 2.93 g of NaHCO₃ in 1 liter of distilled H₂O [pH 9.6]) was plated in 96-well plates as described previously (6). Duplicate samples of each mouse serum, including uninfected normal mouse serum as a control, were diluted 1:200 for probing. The secondary antibody was alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G diluted 1:5,000 (Jackson ImmunoResearch Laboratories Inc., West Grove, Pa.). The enzyme substrate was p-nitrophenyl phosphate (Sigma). The optical densities at 405 nm were read on a Kinetic microplate reader (Molecular Devices, Sunnyvale, Calif.). The mean absorbance for duplicate experimental samples, as well as the mean absorbance and standard deviation for a minimum of six wells containing uninfected normal mouse serum, was calculated. Antibodies were considered present when the absorbance exceeded 3 standard deviations of the mean titer of the control (uninfected) mouse serum.

Amplification of P167A to P167E DNA fragments from genomic DNAs by PCR. Genomic DNAs were purified from *H. bilis*, *H. hepaticus*, *B. burgdorferi*, and *C. jejuni* as previously described (5). Each genomic DNA was used as a template separately, and each set of primers for P167A to P167E
DNA was denatured at 94°C for 1 min, annealed at 55°C for 1 min, and extended at 72°C for 1 min. This process was repeated for 30 cycles. Amplicons were analyzed by gel electrophoresis.

Nucleotide sequence accession numbers. The sequences of P167 and P158 have been submitted to the GenBank nucleotide sequence database under accession numbers AF288477 and AF349728, respectively.

RESULTS

Immune sera obtained from mice that were experimentally infected with H. bilis at 6 months after gavage inoculation reacted to H. bilis membrane antigen extract in an ELISA at a dilution of 1:800. Immunoblots with the membrane antigen extract revealed reactivity of 6-month immune sera against 5 major bands with approximate molecular masses of 15, 30, 40, 50, and 60 kDa and 18 minor bands ranging from 10 to 100 kDa (Fig. 1). These results confirmed that sera from experimentally infected mice were reactive against a number of H. bilis antigens.

The H. bilis genomic DNA expression library was then probed with 6-month immune sera. Seventeen immunoreactive clones were obtained and sequenced. One clone contained a 4,633-bp open reading frame, and the predicted molecular mass of the gene product (P167) was 167 kDa. The open reading frame contained typical bacterial −10 (TTGTA) and −35 (TATAAA) potential promoters and a ribosome binding site (AAAAGAG) in the 5'-flanking region, a stop codon, and a translation terminator (a hairpin structure) in the 3'-flanking region. Another clone contained a gene encoding a product (P158) with a predicted molecular mass of 158 kDa. This gene had a fragment of 2,877 bp from bp 1490 to 4366 corresponding to bp 1756 to 4633 bp in the P167 gene. The P158 gene is 4,366 bp long and is 267 bp shorter than the P167 gene at the 5' end. Its first 1,100 bp share very little, if any, homology with the P167 gene. The middle section, from bp 1101 to 1489, shares 52% identity with its counterpart in the P167 gene, and its 3' section is 100% identical to that of the P167 gene. Among the 17 different immunoreactive clones, 13 were determined to contain either P167 or P158: 5 clones contained the complete sequence of P167; 6 clones contained partial sequences of P167; and 2 clones contained the complete sequence of P158. The remaining four clones reacted with antiserum to P167C. Therefore, they were not sequenced. These results suggest that one or both of these proteins (P167 and P158) are dominant immunoreactive antigens during H. bilis infection. A BLAST search did not reveal any significant homologue to P167, and its biological function is unknown.

Because the majority of clones contained the P167 gene, we focused on expressing the gene product. However, because of the potential difficulty in expressing a full-length protein of this size, we created five overlapping peptides (Fig. 2). The five fragments were designated P167A, P167B, P167C, P167D, and P167E. The predicted molecular mass of P167A was 39 kDa, and its DNA sequence extended from bp 58 to 1123, corresponding to the full-length gene. P167B was 40 kDa, and its DNA extended from bp 856 to 1962. P167C was 41 kDa, and its DNA extended from bp 1645 to 2799. P167D was 41 kDa, and its DNA extended from bp 2560 to 3675. P167E was 45 kDa, and its DNA extended from bp 3409 to 4632. Template DNA from the original reactive clone was used to amplify the gene fragments.

Immune sera from experimentally H. bilis-infected mice reacted on immunoblots against two of the five peptide fragments, P167C and P167D (Fig. 1, lanes 2 and 4). Immune sera reacted against recombinant P167C and P167D at dilutions of 1:800.
in protein band with a molecular mass of approximately 120 kDa reacted strongly with recombinant P167C and weakly with a control for both Western blotting and ELISA.

P167C antiserum, generated by hyperimmunization of mice, reacted strongly with recombinant P167C and weakly with a protein band with a molecular mass of 120 kDa in H. bilis whole-cell lysate immunoblots; the latter band apparently represented native P167 (data not shown). A protein band of this size was not seen on immunoblots prepared from the H. bilis membrane extract. These findings suggested that P167 may be preferentially expressed in vivo or is a secretory product or both. To test for the specificity of P167 for H. bilis, we next examined the immunoblot reactivity of P167C antiserum against whole-cell lysates of H. hepaticus and H. muridarum as well as two unrelated spirochetal species, B. burgdorferi and C. jejuni. The genus Helicobacter is closely related to the genus Campylobacter and was at one time included in the genus Campylobacter (15, 18). P167C antiserum did not react with bands of sizes similar to those of P167 from H. hepaticus, H. muridarum, B. burgdorferi, or C. jejuni whole-cell lysates. P167C antiserum, however, reacted weakly with an approximately 60-kDa band on immunoblots prepared from whole-cell lysates of all Helicobacter species as well as Campylobacter and Borrelia. Because these bands were not the same size as those of P167 and because similarly sized bands reacted with all species of bacteria, this result was interpreted as cross-reactivity of hyperimmune serum to a protein derived from E. coli from which P167C (used for hyperimmunization) was purified. When we probed P167C and P167D peptides with immune sera from mice experimentally infected with H. hepaticus, these sera showed no cross-reactivity with P167C or P167D (Fig. 1, lanes 3 and 5). These data suggest that at least the antigenic epitope(s) of P167 is H. bilis specific.

There are at least two explanations for why P167C antiserum did not react with equivalently sized proteins of H. hepaticus and H. muridarum. One is that these two species do not possess the P167 gene; another is that they possess a P167 gene homologue, but the gene products do not have similar antigenic epitopes. To further explore these possibilities, we performed PCR with all five sets of P167 fragment primers and H. hepaticus and H. muridarum genomic DNAs as templates. All five fragments were amplified from H. muridarum genomic DNA, and the amplicons were similar in size to those of all five H. bilis homologues. No product was amplified from C. jejuni or B. burgdorferi genomic DNA targets with any of the five primer sets. The density of amplicons from H. hepaticus and H. muridarum was much lower than that seen with H. bilis, suggesting a lower homology of P167 counterparts in H. hepaticus and H. muridarum. Sequence comparison has yet to be performed.

Finally, we examined whether naturally infected mice had antibodies to recombinant P167. Sera were collected from nine mice that had PCR-verified natural infections with H. bilis and from four mice that had PCR-verified natural infections with H. hepaticus. The duration of infection in these mice was unknown. These sera were used to probe dot immunoblots of the H. bilis membrane extract, P167C, and P167D (Fig. 4). Four of the nine sera from H. bilis-infected mice reacted with all three antigens, and one of the nine sera from H. bilis infected mice reacted with only the membrane extract. One serum from an H. bilis infected mouse had antibody to P167D only, and another had antibodies to both P167C and P167D. Two mice infected with H. bilis did not possess antibody to the H. bilis membrane extract, P167C, or P167D. None of the four sera from H. hepaticus-infected mice reacted with P167C or P167D, and one of the four sera from H. hepaticus-infected mice reacted with the H. bilis membrane extract. In summary, among seven of nine H. bilis-infected mouse sera that reacted with any one of the antigens, six reacted with P167D, five reacted with P167C, and five reacted with the membrane extract. Further studies with more serum samples from naturally infected mice are warranted, but these results show potential for these recombinant antigens to serve as diagnostic reagents.

DISCUSSION

Sera from mice infected with H. bilis react with a number of proteins that could serve as antigens for serodiagnosis, if cloned and expressed in recombinant form. Membrane antigens are logically favored targets, in that it is presumed that they are most apt to interface with the host during infection and thus elicit an antibody response. In one study, nine native proteins from H. bilis outer membrane protein (OMP) preparations were found to be immunoreactive. The proteins ranged from 20 to 80 kDa (14). Our current study revealed 5 immunoreactive native proteins with molecular masses of approximately 15, 30, 40, 50, and 60 kDa and 18 minor bands ranging from 10 to 100 kDa. We did not rule out the possibility that...
some of the smaller reactive bands might have been proteolytic degradation products. The difference in the results was due to different methods of antigen preparation. In the earlier study, OMP was generated by initially sonicating cells, centrifuging the samples to remove cell debris, and then adding 0.6% N-lauroyl sarcosine to the supernatants. OMP preparations consisted of detergent-solubilized proteins. It was therefore possible that the OMP preparation excluded some membrane proteins that could have been associated with cell debris and included cytosol proteins solubilized by detergent. In contrast, if we incubated intact cells with 1% n-octyl-β-D glucopyranoside to release membrane proteins and then removed cell debris, therefore, our preparation included as many membrane proteins as possible without including cytosol proteins. Whatever the method, these studies indicate that H. bilis elicits antibody responses during infection to a number of antigens, but the specificity and sensitivity of these individual antigens are unknown.

Membrane extracts are generally used as antigens for Helicobacter serodiagnostic purposes in ELISAs, but results have not been particularly sensitive or specific. For example, sera from mice experimentally infected with H. hepaticus cross-reacted with H. bilis and H. rodentium membrane extracts, making the specificities only 34 and 35%, respectively (34). Sera from mice that were naturally exposed to H. bilis cross-reacted with an H. hepaticus membrane antigen in an ELISA, and PCR and culturing had to be performed to determine if mice were coinfected (34).

The current study revealed a large, immunoreactive protein (P167) that appeared to elicit an antibody response in mice infected with H. bilis. A comparison of membrane extracts with whole-cell lysates as antigens on immunoblots revealed that antiserum to P167C reacted weakly with the lysates but not with the membrane extracts. This result suggested that P167 may not be associated with the membrane and may be a secretory product that stimulates host immunity. Alternatively, P167 may be preferentially expressed in vivo but not in vitro. Thus, despite the logic of focusing on membrane proteins as potential antigens for serodiagnosis, other antigens may be superior in terms of antigenicity, species specificity, and possibly sensitivity. Comparison of serum reactivity titers for membrane extracts and recombinant P167C and P167D peptides revealed higher titers for the recombinant peptides, with the added advantage of species specificity. Testing a limited number of serum samples from naturally infected mice suggested that membrane antigen extracts were less sensitive (and less specific) for detecting antibody reactivity to H. bilis than recombinant P167C and P167D. Further studies are needed to determine if P167C and P167D can detect early antibody responses during infection.

P167C hyperimmune serum did not recognize a protein with a size similar to that of P167 from H. hepaticus or H. muridarum whole-cell lysates, and H. hepaticus immune serum did not react with P167C or P167D recombinant proteins in immunoblotting. The fact that we were able to amplify all five fragments of P167 from H. muridarum genomic DNA and four out of five fragments from H. hepaticus indicates that H. bilis, H. hepaticus, and H. muridarum all share homologues of this protein that do not share antigenic epitopes. Furthermore, we found at least three clones that contained an operon that encoded a predicted 158-kDa gene product that shared regions of homology with P167. This result may indicate that recombination occurs at a variable middle section of the gene to create new genes. What drives the recombination is not clear, but this scenario may suggest that these proteins have important functions in the biology of these bacteria.

ACKNOWLEDGMENTS

We thank Sara Barrett Mulinyawe for technical support. This work was supported by NIH grant RR14034 from the Comparative Medicine Program, National Center for Research Resources, National Institutes of Health.

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