Cloning and Expression of a Helicobacter bilis Immunoreactive Protein

Sunlian Feng, Emir Hodzic, Lon V. Kendall, Amy Smith, Kimberly Freet, and Stephen W. Barthold*

Center for Comparative Medicine, Schools of Medicine and Veterinary Medicine, University of California at Davis, Davis, California

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

In an effort to identify immunoreactive Helicobacter bilis antigens with potential for serodiagnosis, sera from mice experimentally infected with H. bilis were used to screen an 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 H. bilis membrane extract at a dilution of 1:800 in an enzyme-linked immunosorbent assay. Sera from mice experimentally infected with H. hepaticus did not react with P167C and P167D. Sera from mice naturally infected with H. bilis but not sera from mice naturally infected with H. hepaticus reacted with P167C and P167D. Hyperimmune sera against P167C peptide reacted with recombinant P167C and with a 120-kDa band in H. bilis lysates but did not react with a protein of the same size on immunoblots prepared from H. hepaticus, H. muridarum, or unrelated Borrelia burgdorferi and Campylobacter jejuni whole-cell lysates. Nevertheless, the P167A, P167B, P167C, and P167D primers, but not the P167E primers, amplified DNA from H. hepaticus, and all five primer sets amplified DNA from H. muridarum. These results suggest that P167 is an immunodominant, H. bilis-specific antigen that may have potential for use in serodiagnosis.

Helicobacter bilis (11) is a member of an expanding and genetically diverse group of enterohepatic, commensal, and opportunistic Helicobacter species that infect laboratory mice. In addition to H. bilis, Helicobacter species isolated from mice include H. hepaticus (8), H. rodentium (27), H. muridarum (19), H. typhlonicus (13), and others yet to be named (30). Flexispira rappini, first described by Bryner et al. (2), has also been shown to be a Helicobacter species (22, 24, 32), but recent studies suggest that it represents a mixture of Helicobacter species (3). H. bilis infections have been found to be widespread among research mouse colonies (11, 23). Infections are often subclinical but can produce liver and enteral diseases in some genotypes of mice, particularly mice with immune deficiencies. 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 H. rodentium (28). Experimental inoculation of H. bilis induces enteritis and hepatitis in SCID mice (12, 29) and enteral 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, 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 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 H. bilis-infected mice (17). Recently, an H. hepaticus recombinant immunogenic protein (MAP18) was cloned and expressed (20). It proved to be H. hepaticus specific but less sensitive than a membrane antigen extract (20). No recombinant proteins of H. bilis have been characterized. In this report, we describe a novel recombinant H. bilis gene product that is immunodominant and specific for H. bilis and that has potential for further characterization as a serodiagnostic antigen.

MATERIALS AND METHODS

Mice. Virus antibody- and Helicobacter-free C3H/HecN (C3H) and C3H/Snu. C57Hud-secl (C3H-secl) 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-secl). Upon arrival, fecal pellets from all mice were tested for 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 CO2 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.
TABLE 1. Primer sets for five P167 fragments (P167A to P167E)

<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotides corresponding to the P167 gene</th>
<th>Sequence</th>
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</thead>
<tbody>
<tr>
<td>P167A forward</td>
<td>58–81</td>
<td>TATGCTGGGATATTCAAGGCAGGAT</td>
</tr>
<tr>
<td>P167A reverse</td>
<td>1102–1123</td>
<td>ATAGCAGTATGACCACGTACTCC</td>
</tr>
<tr>
<td>P167B forward</td>
<td>856–876</td>
<td>TGGCATGTGCGATGAGCCAC</td>
</tr>
<tr>
<td>P167B reverse</td>
<td>942–1092</td>
<td>TGGTACCTGCTACACATCTCAT</td>
</tr>
<tr>
<td>P167C forward</td>
<td>1645–1668</td>
<td>CGTATTGGGATAGTAGTTAAGCATGTC</td>
</tr>
<tr>
<td>P167C reverse</td>
<td>2776–2799</td>
<td>TGACGCTTACGTTTCTCTACATC</td>
</tr>
<tr>
<td>P167D forward</td>
<td>2560–2583</td>
<td>AGCTCACATCTACACACAAAGGGGAGGT</td>
</tr>
<tr>
<td>P167D reverse</td>
<td>3655–3675</td>
<td>ATCTGTTACCCATTGTTGGTGC</td>
</tr>
<tr>
<td>P167E forward</td>
<td>4409–4462</td>
<td>CGTCTAGCAAGAGTAGCTAGCATT</td>
</tr>
<tr>
<td>P167E reverse</td>
<td>4609–4632</td>
<td>ATTTGTGTTAGGGTGTGTTTGTTTG</td>
</tr>
</tbody>
</table>

Bacterial cultivation and isolation. *H. bilis* (ATCC 51630), *H. hepaticus* (ATCC 51448), and *H. muridarum* (ATCC 49282) were obtained from the American Type Culture Collection and cloned by threefold limiting dilution as described previously (17). 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 fees, 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* strains were cultured in modified Barbour-Stoenner-Kelly medium at 33°C as described previously (6). Campylobacter jejuni and *Campylobacter jejuni* 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. Jiang, University of California, Davis.

Immune sera and antiserum. *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). Once 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 C57BL/6 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 C57BL/6 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 × g for 5 min, and 80 μl of the suspension was combined with 140 μl of PBS. Purified DNA from the fecal supernatant was obtained by using the Qiangen DNeasy easy tissue kit protocol for total DNA isolation (23). Following PCR amplification with Helicobacter genus-specific primers, amplicons were digested separately with restriction enzymes *Mbo* I and *Hha* I and analyzed by gel electrophoresis.

Hyperimmune antiserum to recombinant proteins was generated in C57BL/6 mice by subcutaneous injection of 20 μg of recombinant protein in 0.1 ml of Freund’s complete adjuvant, followed by two boosts of 10 μg of protein each in incomplete Freund’s adjuvant at 2-week intervals. Sera were collected and tested by an enzyme-linked immunosorbent assay (ELISA), and the antibody reactivity of antiserum was verified at a serum dilution of 1:100,000.

Native bacterial antigens. To prepare whole-cell lysates, broth cultures of *H. bilis*, *H. hepaticus*, *H. muridarum*, *B. burgdorferi*, and *C. jejuni* were pelleted by centrifugation, washed with cold PBS, and then sonicated to lyse cells. Laemml* sample buffer (Bio-Rad Laboratories, Hercules, Calif.) was added to the lysates, and the mixtures were stored at –20°C. The membrane antigen extract of *H. bilis* was prepared as described previously (21). Briefly, bacteria were incubated for 2 days at 37°C in brucella broth with 5% fetal calf serum on a shaker. Bacterial cells were pelleted by centrifugation, washed with PBS, resuspended in PBS with 1% n-octyl-β-D-glucopyranoside (Sigma Diagnostics, Inc., St. Louis, Mo.) to release membrane proteins, and then centrifuged to remove insoluble proteins. The supernatants were dialyzed to remove detergent.

*H. bilis* genomic DNA expression library. Genomic DNA was isolated from *H. bilis*, and 200 μg of DNA was sheared to Stratagene, La Jolla, Calif., to construct a λZAP II *H. bilis* genomic DNA expression library. The λ ZAP II phage contains pLuetscript 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. Immune-reactive 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. The DNA sequence was analyzed by 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 1942 to 1962. 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 sequences are summarized in Table 1. DNA from the original reactive clone was used as a template. Amplified P167A to P167E DNA fragments were cloned in frame with the gluthathione S-transferase gene into pMX, a pGEX-2T vector (Pharmacia, Piscaway, N.J.) with a modified polylinker (25). The PCR-amplified DNA sequences of the recombinant DNA were confirmed by sequence comparison with the original insert. Recombinant proteins were purified 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 manufacturer 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 absorbed by the 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 NaN₃, 1.5 g of Na₂CO₃, 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*, *H. muridarum*, *B. burgdorferi*, and *C. jejuni* as described previously (5). Each genomic DNA was used as a template separately, and each set of primers for P167A to P167E was

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(described above) was used for each reaction. 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

![FIG. 1](http://cvi.asm.org/)

**FIG. 1.** Immunoblots of an *H. bilis* membrane extract and recombinant proteins probed with *H. bilis* or *H. hepaticus* immune sera. Lane 1, *H. bilis* membrane extract probed with *H. bilis* immune serum. Lane 2, P167C probed with *H. bilis* immune serum. The asterisk indicates P167C, and the lower bands are probably degraded products of P167C. Lane 3, P167C probed with *H. hepaticus* immune serum. Lane 4, P167D probed with *H. bilis* immune serum. The asterisk indicates P167D, and the lower bands are probably degraded products of P167D. Lane 5, P167D probed with *H. hepaticus* immune serum.

![FIG. 2](http://cvi.asm.org/)

**FIG. 2.** Relative sizes, interrelationships, and locations of peptides P167A to P167E in relation to the entire P167 molecule.
1:1,600 and 1:6,400, respectively, in an ELISA. Both reacted at higher titers than the membrane extract (1:800) when tested simultaneously. Sera from *Helicobacter*-free mice did not react with either P167C or P167D and were used as negative controls 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 approximately 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 *H. bilis* (Fig. 3). In contrast, the P167A, P167B, P167C, and P167D primers amplified DNA from *H. hepaticus*, but the P167E primers did not. The amplification products were equivalent in size to their corresponding *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 un-

![FIG. 4. Dot blot with sera from mice naturally infected with *H. bilis* or *H. hepaticus*. The top row contains *H. bilis* membrane antigen extract; the middle row contains recombinant P167C antigen; and the bottom row contains recombinant P167D antigen. Lanes 1 to 9 contain sera from nine mice naturally infected with *H. bilis*; lanes 10 to 13 contain sera from four mice naturally infected with *H. hepaticus*.](http://cvi.asm.org/)
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, 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 titters 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 opernon 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.

**REFERENCES**


