**Helicobacter hepaticus** catalase shares surface-predicted epitopes with mammalian catalases

Essam J. Alyamani,1,6 Petra Brandt,2 Jeremy A. Pena,6 Angela M. Major,6 James G. Fox,3 Sebastian Suerbaum4 and James Versalovic5,6

1Department of Pharmaceutical Sciences, Northeastern University, Boston, MA 01225, USA  
2MWG Biotech AG, Ebersberg, Germany  
3Massachusetts Institute of Technology, Cambridge, MA 02139, USA  
4Institute of Medical Microbiology and Hospital Epidemiology, Hannover Medical School, Hannover, Germany  
5Departments of Pathology, Molecular Virology & Microbiology, and Molecular and Human Genetics, Baylor College of Medicine, Houston, TX 77030, USA  
6Department of Pathology, Texas Children’s Hospital, Houston, TX 77030, USA

**INTRODUCTION**

*Helicobacter hepaticus* colonizes the murine intestine and has been associated with hepatic inflammation and neoplasia in susceptible mouse strains. In this study, the catalase of an enterohepatic *Helicobacter* was characterized for the first time. *H. hepaticus* catalase is a highly conserved enzyme that may be important for bacterial survival in the mammalian intestine. Recombinant *H. hepaticus* catalase was expressed in *Escherichia coli* in order to verify its enzymic activity in vitro. *H. hepaticus* catalase comprises 478 amino acids with a highly conserved haem-ligand domain. Three conserved motifs (R-F-Y-D, RERIPER and VVHAKG) in the haem-ligand domain and three surface-predicted motifs were identified in *H. hepaticus* catalase and are shared among bacterial and mammalian catalases. *H. hepaticus* catalase is present in the cytoplasmic and periplasmic compartments. Mice infected with *H. hepaticus* demonstrated immune responses to murine and *H. hepaticus* catalase, suggesting that *Helicobacter* catalase contains conserved structural motifs and may contribute to autoimmune responses. Antibodies to *H. hepaticus* catalase recognized murine hepatocyte catalase in hepatic tissue from infected mice. Antibodies from sera of *H. hepaticus*-infected mice reacted with peptides comprising two conserved surface-predicted motifs in *H. hepaticus* catalase. Catalases are highly conserved enzymes in bacteria and mammals that may contribute to autoimmune responses in animals infected with catalase-producing pathogens.

**Abbreviations:** HRP, horseradish peroxidase; ROS, reactive oxygen species.
survival in macrophage phagosomes (Basu et al., 2004). Due to the biological importance of catalase in bacteria–host interactions, this enzyme is highly conserved in many different species. *H. pylori* catalase (KatA) is essential for persistent colonization in the SS1 mouse model (Harris et al., 2002).

Monofunctional catalases are highly conserved among bacteria that colonize mammalian hosts and are frequently expressed by commensal and pathogenic organisms. The genomic sequence of *H. hepaticus* (Suerbaum et al., 2003) includes the katA gene (HH 0043) encoding catalase (http://www.mwg-biotech.com/html/i_information/i_helicobacter.shtml). In this study, an enterohepatic *Heliobacter* catalase was characterized by enzymic studies and sequence analyses for the first time. *H. hepaticus* and other catalase sequences were compared in order to define conserved motifs that may be important for immune recognition. Antibody responses to intact *H. hepaticus* catalase and synthetic peptides derived from surface-predicted sequences were studied as potential epitopes with sera from *H. hepaticus*-infected mice. Immune responses to *H. hepaticus* and murine catalase were evaluated in mice infected with *H. hepaticus* in order to study the possible role of bacterial catalases in the generation of autoimmune responses.

**METHODS**

**Bacteriological culture.** *H. hepaticus* (ATCC 51448) was cultured on tryptase soy agar with 5% sheep blood (TSA with 5% sheep blood; Remel) in an anaerobic chamber (Forma Scientific) under 90% N2, 5% H2 and 5% CO2 at 37 °C. *E. coli* EA-HCl was cultured on trypticase soy agar with 5% sheep blood (TSA with 5% sheep blood; Remel) in an anaerobic chamber (Forma Scientific) under 90% N2, 5% H2 and 5% CO2 at 37 °C. *Escherichia coli* BL21, BL21(DE3) (Novagen) and catalase-deficient UM255 (kindly provided by Daniel Hassett, University of Cincinnati College of Medicine) strains were cultured in Luria-Bertani (LB) broth at 37 °C.

**Cloning of *H. hepaticus* katA.** Genomic DNA was extracted according to the manufacturer’s instructions (MoBio kit). *H. hepaticus* catalase-specific primers were designed on the basis of the published katA sequence (Suerbaum et al., 2003). The PCR reaction consisted of 100 ng DNA template and 10 pmol *H. hepaticus* catalase-specific primers containing terminal restriction sites. Lower case denotes pET-vector restriction sites Ncol and Ncol respectively (forward, 5′-ggccgtctaggtgttaaccgctacagca-3′; reverse, 5′-gggctttataaccgccacgctcactc-3′). Ex-Tag buffer with 2.5 mM MgCl2, 10 mM dNTP and proofreading-capable Ex-Tag DNA polymerase (1.5 U) (Fisher Scientific), Following an initial denaturation (95 °C, 5 min), the PCR consisted of 35 cycles of denaturation (95 °C, 45 s), annealing (65 °C, 60 s and extension (72 °C, 90 s). The PCR product (1437 bp) was purified by agarose gel electrophoresis and excised from the gel prior to DNA sequencing (Qiagen). Amplified genes were sequenced by dyeoxy sequencing and capillary electrophoresis using a model 310 Genetic Analyser (Applied Biosystems) in the Baylor College of Medicine Core DNA Sequencing facility.

The katA amplicon was cloned in multiple cloning sites flanked by restriction sites Ncol and Ncol downstream of the N-terminal hexahistidyl tag (His-tag) and S-tag (327–344 bp and 249–293 bp respectively) in the inducible expression vector pET30a (Novagen) in order to create the recombinant E. coli strain, EA-HCl. Prior to expression, the insert sequence was verified by dyeoxy DNA sequencing in the Baylor College of Medicine Core Sequencing facility. DNA sequences were aligned with known catalase sequences including that of *H. pylori* in order to verify the identity of cloned DNA.

**Expression and purification of *H. hepaticus* and murine catalases.** Expression of recombinant *H. hepaticus* catalase was induced by addition of 1 mM IPTG for 4 h at 30 °C in 55.5 mM glucose in LB. Induction was followed by lysis with the BugBuster extraction reagent according to the manufacturer’s instructions (Novagen). Subsequently, *E. coli* EA-HCl lysates were resolved by 10% SDS-PAGE followed by Coomasie blue and silver staining (Invitrogen). Hexahistidyl-tagged *H. hepaticus* catalase was bound to Ni-NTA cations in a His-bind resin (Novagen) and eluted with 1 M imidazole. Protein purity was assessed by SDS-PAGE. The protein was dialysed against PBS in a cassette Dialyser (Sild-A-lyser, Pierce) and concentrated by column-facilitated centrifugation (Ultrafree 0.5 μm filter unit, Millipore). The murine catalase cDNA was cloned in pQE-60 (Qiagen) to create the plasmid containing recombinant murine catalase and was kindly provided by Dr Ken Tsutsui of Okayama University School of Medicine (Wang et al., 2001). The plasmid containing murine catalase was transformed into *E. coli* JM109 and induced overnight by addition of 1 mM IPTG at 30 °C in 55.5 mM glucose in LB medium. Murine catalase was purified under denaturing conditions by addition of 8 M urea. The hexahistidyl-tagged murine catalase was bound to Ni-NTA cations in a His-bind resin (Novagen) and eluted with 1 M imidazole. Murine catalase was dialysed against PBS to remove the denaturing agent and was concentrated as described above. Relative protein purity was assessed by SDS-PAGE. Molecular mass standards (Bio-Rad) were included in SDS-PAGE gels.

**Assessment of *H. hepaticus* catalase activity.** In a qualitative assay, the decomposition of hydrogen peroxide by catalase was explored by adding 10 mM hydrogen peroxide to recombinant *H. hepaticus* catalase. The catalytic conversion of hydrogen peroxide was measured by observing the reduction in absorbance at 240 nm per unit time. In quantitative catalase assays, the changes in the slope of time-dependent reduction in hydrogen peroxide absorbance at 240 nm (PBS, pH 7.2) were used as a measure of catalase activity and were calculated according to the method of Beers & Sizer (1952). Reaction mixtures contained 10 mM hydrogen peroxide (Sigma-Aldrich) and the activities were recorded at 30 s intervals (2 min duration) at 240 nm in a SmartSpec 3000 spectrophotometer (Bio-Rad). One unit of catalase activity decomposes 1 μmol hydrogen peroxide at 240 nm min⁻¹ at a substrate concentration of 10 mM. Enzymic activities were measured as U per mg total protein. Recombinant *H. hepaticus* catalase and total bacterial protein concentrations were quantified by the bicinchoninic acid (BCA) assay (Pierce) (Smith et al., 1985). Unknown protein concentrations were automatically calculated using the BSA standards (R² = 0.997) by a SmartSpec 3000 spectrophotometer at an absorbance wavelength of 562 nm.

**Sequence–structural correlations of *H. hepaticus* catalase with other prokaryotic and mammalian catalases.** BLASTN (GenBank version 2.2.6) queries and pairwise DNA and amino acid sequence alignments confirmed the identity of recombinant *H. hepaticus* katA. Amino acid sequences of different catalases were compared by using the MegAlign program in Lasergene version 5.06 (DNAstar) and MultiAlign (Corpet, 1988) with hierarchical clustering. *H. hepaticus* catalase secondary structure was predicted (PROSec version 2000.6, PSIPRED version 2.3). Further comparisons were performed by using conserved domain databases (CD, NCBI; http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi; ScanFroosite database) (Hoffman & Wright, 1985). Using the PROSITE motif and conserved domain (CD) databases, primary sequence conservation
within the haem--ligand domain in H. hepaticus catalase and other catalases was demonstrated. Three-dimensional structures of human erythrocyte (Putnam et al., 2000) and H. pylori (Loevelen et al., 2004) catalases were visualized and compared with the primary sequence of H. hepaticus catalase in order to generate predicted structures (Cn3D version 4.0, NCBI, Swiss-PdbViewer version 3.7, Swiss-model). The three-dimensional structure of H. hepaticus catalase was predicted on the basis of protein comparative modelling with the program Swiss-model (data not shown). The three-dimensional model of H. hepaticus catalase was constructed by submitting the primary amino acid sequences (target) to the Swiss-model databases (http://swissmodel.expasy.org/SWISS-MODEL.html). The H. pylori catalase was used as a template (3D H. pylori KatA) for alignment with the target protein (GueX & Peitsch, 1997; Peitsch et al., 1995; Schwede et al., 2003). The accuracy of the three-dimensional model of H. hepaticus catalase has been evaluated by the WhatCheck program in the Swiss-model suite. The program generates Z-scores for each criterion used in the evaluation. The Z-scores are defined as the standard deviation from the mean of the expected value (Hooft et al., 1996).

Serological studies of immune responses to H. hepaticus and murine catalases in H. hepaticus-infected mice. In order to study cross-reactive immune responses to H. hepaticus and endogenous murine catalases, two groups of mice (H. hepaticus-infected and uninfected C57BL6 IL-10-deficient mice) were evaluated by immuneblotting with recombinant antigens. IL-10-deficient C57BL6 mice were housed in the animal care facility in the Division of Comparative Medicine, Massachusetts Institute of Technology under specific pathogen-free conditions in micro-isolator cages. Six- to 13-week-old mice were age- and sex-matched. Animals received three doses of ~10^8 c.f.u. H. hepaticus per dose by orogastric gavage. H. hepaticus doses were administered three times (once per day, every other day). Control animals received sterile Brucella broth for H. hepaticus. Ten weeks post-infection, animals were killed by CO2 asphyxiation.

Sera from H. hepaticus-infected mice and uninfected controls were prepared by centrifugation. Serum dilutions (1: 1000 dilution of pooled and eight individual sera samples) were tested by immuneblotting for reactivity against recombinant H. hepaticus and murine catalases. Individual and pooled samples of murine sera were obtained from H. hepaticus-infected mice (n=8 mice: 5 males and 3 females). Pooled specimens from uninfected control mice included samples of equal volume from each of 5 males and 3 females. Purified recombinant H. hepaticus catalase was electrophoresed by SDS-PAGE (4–20 % gradient, Bio-Rad) for 90 min at 120 V prior to transfer to a nitrocellulose membrane (0.45 μm) for 90 min at 120 V prior to transfer to a nitrocellulose membrane (0.45 μm) for 90 min at 120 V prior to transfer to a nitrocellulose membrane (0.45 μm) for 90 min at 120 V prior to transfer to a nitrocellulose membrane (0.45 μm) for 90 min at 120 V prior to transfer to a nitrocellulose membrane (0.45 μm) for 90 min at 120 V. After incubation with SuperBlock reagent (Pierce), the membrane was probed with mouse sera (1:1000 dilution). Anti-mouse IgG conjugated with horseradish peroxidase (HRP) (Rockland Immunochemicals) was used as a secondary antibody. Chemiluminescent signal detection was performed with a cooled CCD camera in a Chemi-Imager system 5500 (Alpha Innotech). Immunoblot images were processed with a cooled CCD camera in a ChemiImager system 5500 (Alpha Innotech).

Immunoneutralization by peptides derived from H. hepaticus catalase. In order to explore candidate epitopes derived from H. hepaticus catalase, three peptides were synthesized (Bio-Synthesis) including peptide 1 (126LNKPNPENFAEVEQ^169 (14 amino acids)), peptide 2 (115YTNEGWDVIGNNT^129 (15 amino acids)) and peptide 3 (QKRDPKTN^113 (8 amino acids)). Peptides derived from H. hepaticus catalase were added in different quantities directly to sera from infected mice (1:1000 dilution in TBST). Controls contained all components except the peptides of interest. All tubes were incubated at room temperature for 16 h on a rotator (20 r.p.m.). The tubes were centrifuged for 15 min at 4 °C in a microfuge (10 000 r.p.m.) to pellet immune complexes. After centrifugation, supernatants were used to probe recombinant H. hepaticus catalase that was separated in a 4–20 % gradient by SDS-PAGE and transferred to a nitrocellulose membrane (Immunetics). Anti-mouse IgG HRP conjugate was used as the secondary antibody. Detection of the bands was performed with SuperSignal West Pico Chemiluminescent Substrate kit (Pierce). Immunoblot images were processed with a cooled CCD camera in a ChemiImager system 5500 (Alpha Innotech).

In order to confirm specificities of peptide immunoneutralization experiments, peptide sequences were randomized [peptide 1 (PFYNELKEQNNVEA) and peptide 3 (KNKTRQPD)]. Amino acid sequence composition remained the same. Peptide sequence randomization was performed by the web-based computer program DNA Protein Sequence Randomizer (http://www.cellbio.com/cgi-bin/randomizer/sequence_randomizer.html).

Immunohistochemical studies of antigenicity of murine catalase in vivo. Rabbit polyclonal anti-bovine liver catalase antibodies were obtained from Abcam Inc.; these antibodies react with mouse catalase as verified by routine quality-control procedures (Abcam). Rabbit sera containing polyclonal anti-H. hepaticus catalase antibodies were produced commercially by Bio-Synthesis Laboratories. Briefly, 2 mg purified recombinant H. hepaticus catalase was injected into rabbits. The animals were bled at the time of primary injection in order to obtain pre-immune sera. Sera were collected 10 weeks post-injection. Heparinated sections from uninfected and infected A/JCr mice were formalin-fixed and paraffin-embedded by standard histological procedures. Infections due to H. hepaticus were demonstrated by silver staining of organs in hepatic tissue sections. Tissue sections (3–4 μm) were deparaffinized in three changes of xylene for 5 min, gradually hydrated in descending fractions of ethanol, and rinsed in water. For antigen retrieval, all sections were treated by boiling specimens in citrate buffer for 20 min followed by rinsing samples in de-ionized water. In order to prevent endogenous enzymatic activity from confounding signal detection, sections were blocked with 3 % hydrogen peroxide in methanol for 15 min and rinsed in de-ionized water. Sections were incubated with an avidin and biotin blocking reagent (Vector Laboratories) consecutively for 15 min and rinsed with de-ionized water prior to blocking in 20 % goat serum for 20 min. Subsequently, tissue sections were incubated with primary anti-catalase antibodies or sera for 1 h at room temperature prior to rinsing with TBST. A 1:50 dilution (sera with anti-H. hepaticus catalase antibodies or pre-immune control sera) or 1:1500 dilution (anti-bovine liver catalase or rabbit IgG isotype control) of primary antibody was used for these studies. Concentrated goat biotinylated anti-rabbit IgG (BioGenex) in 2 % mouse serum was added and used as a secondary antibody according to the manufacturer’s instructions prior to rinsing with TBST. Sections were incubated with concentrated peroxidase-conjugated streptavidin (Biogenex) for 20 min at room temperature. Following a rinse in TBST, sections were incubated in 3-aminio-9-ethylcarbazole (peroxidase substrate) before a final rinse in TBST. The sections were rinsed in water, counterstained with haematoxylin, and mounted for microscopic evaluation with an Axioskop 40 microscope (Zeiss). Negative controls included rabbit IgG isotype controls (Zymed, Invitrogen) for studies with anti-bovine liver catalase antibodies (Abcam) and pre-immune rabbit sera for experiments with anti-H. hepaticus sera.

Cellular localization of H. hepaticus catalase. To investigate whether H. hepaticus catalase is localized in the periplasm or cytoplasm, the cellular compartments of H. hepaticus were isolated. Catalase activity from each compartment was evaluated by anti-H. hepaticus catalase antibodies and in situ catalase assays. The isolation of catalase from each compartment was based on osmotic shock of the outer membrane and lysozyme-mediated digestion of the cell wall (PeriPreps Periplasting Kit, Epicentre Biotechnologies).

Microbiology Research

E. J. Alyamani and others

1008

Downloaded from www.microbiologyresearch.org by IP: 54.70.40.11 On: Mon, 05 Nov 2018 08:02:00
hepaticus was grown to confluence as described earlier, and the cells were digested with lysozyme in the presence of sucrose/EDTA. Subsequently, the cells were subjected to osmotic shock in cold distilled water followed by centrifugation at 13,000 g for 2 min at room temperature. Supernatants (periplasmic fractions) were transferred to sterile tubes and stored at −80 °C until needed. The residual cell pellets were lysed with lysozyme in order to obtain soluble cytoplasmic proteins and were stored at −80 °C until required. In order to verify catalase activity in periplasmic or cytoplasmic extracts in situ (Woodbury et al., 1971), native PAGE was used to fractionate proteins from the cytoplasm or periplasm (10% native PAGE for 3 h at 4 °C; 25 mA). Catalase in the gel was exposed to hydrogen peroxide (0.003 %, v/v) followed by staining with ferric chloride (2%, w/v) and potassium ferricyanide (2%, w/v) (Sigma-Aldrich). Gels were washed and photographed with a cooled CCD camera. Appropriate markers were used to validate the periplasmic extraction process. Alkaline phosphatase is an enzyme that is active exclusively in the periplasmic compartment in many prokaryotes (Hoffman & Wright, 1985; Inouye et al., 1982). Therefore, its activity was utilized as a marker to evaluate the quality of the periplasmic extractions. Similarly, malate dehydrogenase is a citric acid cycle enzyme that functions entirely in the bacterial cytoplasm, and has been used frequently as a cytoplasmic marker in order to exclude the possibility of cytoplasmic contamination in periplasmic extracts (Harris & Hazell, 2003; Pitson et al., 1999).

RESULTS

Expression and enzymic studies of H. hepaticus KatA

H. hepaticus KatA was fractionated by SDS-PAGE and yielded a predominant protein of the expected size (62 kDa) with hexahistidyl and S-epitope tags (additional 7 kDa) (Fig. 1c). Recombinant murine catalase (60 kDa) was tagged with hexahistidyl tag and lacked an S-epitope tag (Fig. 1c). Immunoblot-based confirmation of H. hepaticus KatA and murine CatA was performed with antibodies to the S-epitope tag or His-tag. Recombinant H. hepaticus catalase activity was demonstrated using in vitro assays, and results were consistent with studies of recombinant H. pylori KatA (Bai et al., 2003; Hazell et al., 1991). In a qualitative assay (Fig. 2), the catalytic conversion of hydrogen peroxide by recombinant H. hepaticus catalase immediately resulted in reduction of A240. Recombinant H. hepaticus catalase activity yielded a mean value of 6498 U (mg total protein)−1 by quantitative studies at 22 °C and pH 7.2 (data not shown). The effects of different pH values (3.5, 5.5, 7.4 and 9.0) on H. hepaticus...
catalase activity were explored. *H. hepaticus* catalase activity was reduced at pH 3.5 ($P<0.01$), but remained similar at pH values of 5.5 and 9.0 when compared to activity at physiological pH 7.4 (data not shown).

**Primary sequence analysis of *H. hepaticus* catalase: definition of conserved motifs**

*H. hepaticus* catalase comprises 478 amino acids and multiple conserved domains shared with diverse eukaryotic and prokaryotic catalases. *H. hepaticus* catalase was 62% identical when compared to *H. pylori* catalase by amino acid sequence. Pairwise sequence alignments of diverse catalases with *H. hepaticus* catalase yielded different levels of amino acid sequence identity when compared to *Bordetella pertussis* (65%), *H. pylori* (two strains, 62%), *Campylobacter jejuni* (60%), *Mus musculus* (50%) and *Homo sapiens* (50%).

Using the PROSITE motif and conserved domain (CD) databases, primary sequence conservation within the haem-ligand domain in *H. hepaticus* catalase and other catalases was demonstrated (Table 1). The 44RERIPER50 motif in the distal portion of the prosthetic haem-ligand domain is entirely conserved in *H. hepaticus*, *H. pylori*, *C. jejuni*, *B. pertussis*, *Mus musculus* and *Homo sapiens*. A second conserved motif, 51VVHAKG56, was identified in the haem-ligand domain (Table 1). These motifs contribute to a beta-barrel domain in catalase (Fita & Rossmann, 1985; Murthy et al., 1981; Zamocky & Koller, 1999). The R-F-Y-D motif in the proximal haem-ligand domain is also entirely conserved among bacteria and mammals (Table 1). According to secondary structural analysis, the distal haem-ligand domain connects z-helices with beta-strands. The distal haem-ligand motif likely faces the core of the tetramer and lies within the inter-domain connection known as the wrapping domain (Fita & Rossmann, 1985; Murthy et al., 1981; Zamocky & Koller, 1999).

**Table 1. Delineation of conserved motifs in the haem-ligand domains of catalases**

<table>
<thead>
<tr>
<th>Name</th>
<th>Distal haem-ligand domain*</th>
<th>Proximal haem-ligand domain*</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. pertussis</em></td>
<td>RERIPERVVHAKG</td>
<td>RLFSYGD</td>
</tr>
<tr>
<td><em>C. jejuni</em></td>
<td>RERIPERTVHAKG</td>
<td>RIFSYPD</td>
</tr>
<tr>
<td><em>H. hepaticus</em></td>
<td>RERIPERVVHAKG</td>
<td>RLFSYGD</td>
</tr>
<tr>
<td><em>H. pylori</em></td>
<td>RERIPERVVHAKG</td>
<td>RLFSYGD</td>
</tr>
<tr>
<td><em>Homo sapiens</em></td>
<td>RERIPERVVHAKG</td>
<td>RLFAYPD</td>
</tr>
<tr>
<td><em>Mus musculus</em></td>
<td>RERIPERVVHAKG</td>
<td>RLFAYPD</td>
</tr>
<tr>
<td>Consensus</td>
<td>RERIPERVVHAKG</td>
<td>RLFAYPD</td>
</tr>
</tbody>
</table>

*Underlined letters represent catalytically important amino acid residues.

**Sequence–structural correlations of *Helicobacter* catalases**

The predicted secondary structure of *H. hepaticus* catalase indicated that z-helices represent approximately 28.3% of the primary sequence, whereas beta-strands constitute 16.3% of the protein. Coiled or loop regions represent the majority (55.7%) of *H. hepaticus* catalase by primary sequence. The three-dimensional structure of the haem-ligand domain of human erythrocyte catalase was compared with *H. hepaticus* catalase and yielded structural predictions for this region in *H. hepaticus* catalase (Putnam et al., 2000). The distal haem-ligand domain connects z-helical with beta-strand regions and lies adjacent to each haem group in the centre of the tetrameric enzyme (data not shown). This domain includes the R-F-Y-D, 44RERIPER50 and 51VVHAKG56 motifs that were defined in this study (Table 1).

*H. pylori* catalase was crystallized (Loewen et al., 2004) and was localized in the periplasm and cytoplasm (Harris & Hazell, 2003). The proposed three-dimensional structure of *H. hepaticus* catalase was predicted on the basis of the known structure of *H. pylori* catalase (data not shown). Three conserved amino acid sequences (Fig. 3) were identified by comparing *H. hepaticus*, *H. pylori*, *Mus musculus* and *Homo sapiens* catalases and mapped onto the surface regions.
of the three-dimensional structure of H. pylori catalase. The predicted surface motifs of H. pylori catalase (PDB ID # 1QWL). The general colour scheme is as follows: positively charged amino acid residues in blue, negatively charged residues in red and neutral residues in grey. Motifs 1–3 are indicated in yellow. The image was generated by Cn3D alignment viewer version 4.0 (http://130.14.29.110/Structure/CN3D/cn3d.shtml/) and was based on structural alignments of conserved domains with the primary amino acid sequence of H. hepaticus catalase (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml).

Fig. 3. Surface-predicted amino acid sequence motifs of H. hepaticus catalase (KatA) predicted from the three-dimensional structure of H. pylori catalase (PDB ID # 1QWL). The general colour scheme is as follows: positively charged amino acid residues in blue, negatively charged residues in red and neutral residues in grey. Motifs 1–3 are indicated in yellow. The image was generated by Cn3D alignment viewer version 4.0 (http://130.14.29.110/Structure/CN3D/cn3d.shtml/) and was based on structural alignments of conserved domains with the primary amino acid sequence of H. hepaticus catalase (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml).

H. pylori catalase epitopes

Peptides derived from surface-predicted motifs (Fig. 3) were designated peptide 1 (LNKNPENYFAEVEQ), peptide 2 (YTNGWNWIVGNNTP) and peptide 3 (QKRDPKTN). These peptides were synthesized and tested for their relative abilities to neutralize antibody binding to H. hepaticus catalase. Peptides 1 and 3 demonstrated effective immunoneutralization (Fig. 4). Peptide 2 lacked evidence of any neutralizing capability. In order to further investigate specific effects of catalase-derived peptides, peptide sequences 1 and 3 were randomized and tested similarly for their abilities to neutralize anti-catalase binding. Randomized peptides 1r and 3r lacked evidence of effective immunoneutralization (Fig. 5). These results indicate that surface-predicted peptides 1 and 3 form functional epitopes capable of binding anti-H. hepaticus catalase antibodies.

Immunohistochemical demonstration of cross-reactivity between H. hepaticus and murine hepatocyte catalases in vivo

Murine catalase was expressed in the mouse liver as demonstrated by immunoreactivity with polyclonal antibodies to bovine liver catalase (Fig. 6a). The anti-bovine liver catalase antibodies are known to recognize mouse catalase. By contrast, the rabbit IgG isotype controls were negative (Fig. 6b). Hepatic tissues from infected and uninfected mice were reactive with anti-bovine catalase antibodies (especially prominent with hepatic sections of H. hepaticus-infected mice), presumably due to the high degree of conservation between bovine and murine catalases. In order to demonstrate that sera with anti-H. hepaticus catalase antibodies could recognize murine catalase in the liver, rabbit sera were diluted and tested with murine liver sections from infected and uninfected mice. Livers of H. hepaticus-infected mice were treated with pooled sera from rabbits challenged with H. hepaticus catalase. The murine liver sections demonstrated evidence of cross-reactivity between H. hepaticus and murine hepatocyte catalase (Fig. 6c). The pattern of immunoreactivity was consistent with endogenous hepatocyte catalase and distinct from reactivity to extracellular bacteria. The degree of immunostaining in infected animals correlated with chronic hepatic inflammation in infected animals. The pre-immune rabbit sera served as a control and did not react with murine liver sections (Fig. 6d).
**H. hepaticus** catalase is present in the bacterial cytoplasm and periplasm

*H. hepaticus* catalase activity was present in the periplasmic and cytoplasmic compartments. Purified recombinant *H. hepaticus* catalase and catalase-deficient *E. coli* UM255 were used as positive and negative controls, respectively. Bands consistent with the sizes of native bacterial catalase were observed by immunoblotting studies using anti-*H. hepaticus* catalase antibodies (data not shown). Furthermore, catalase activity was detected in both periplasmic and cytoplasmic fractions by *in situ* catalase gel-based assays (Fig. 7). Periplasmic and cytoplasmic protein extractions yielded control enzymic activities including alkaline phosphatase (periplasm) and malate dehydrogenase (cytoplasm), respectively.

### DISCUSSION

An enterohepatic Helicobacter monofunctional catalase was cloned and characterized for the first time. Three conserved motifs were identified in the haem-ligand domain of *H. hepaticus* catalase and are shared among bacterial and mammalian catalases. The proximal R-F-Y-D and distal 44RERIPER50 motifs within the haem-ligand domain are entirely conserved among evolutionarily distant catalases and represent signature sequences for haem catalases in diverse genomes. The 51VVHAKG56 motif is a conserved motif in the distal haem-ligand domain and contains functionally important histidine and alanine residues. Three surface-predicted motifs were identified in *H. hepaticus* catalase and are also shared among bacterial and mammalian catalases. Two of these surface-predicted motifs formed functional epitopes recognized by murine antibodies from animals infected with *H. hepaticus*. Murine hepatocytes express catalase, and antibodies to *H. hepaticus* catalase reacted with murine hepatocyte catalase in infected mice, suggesting that immunological cross-reactivity between...
bacterial and host catalases may contribute to hepatopathology. The generation of autoantibodies to murine catalase in H. hepaticus-infected mice highlights the potential contributions of highly conserved haem catalases to post-infectious immunopathology.

The first prokaryotic catalase was isolated from Microoccus luteus by Herbert and Pinsent in 1948 (Herbert, 1948). Investigators (Mayfield & Duvall, 1996; von Ossowski et al., 1993) have classified catalases into haem (true catalases) and non-haem manganese catalases (pseudocatalases). Bacteria such as Lactobacillus plantarum differentially express haem and non-haem manganese catalases (Kono & Fridovich, 1983). H. hepaticus catalase belongs to the haem catalase group based on the presence of the conserved haem-ligand domain with newly described catalase motifs within this domain. A β-barrel-containing region includes the distal haem-ligand domain (Fita & Rossmann, 1985; Murthy et al., 1981) that contains the highly conserved motifs defined in this study. The presence of highly conserved motifs within the haem-ligand domain highlights preserved enzymic features of haem catalases among diverse bacteria and mammals.

Conserved epitopes in mammalian catalases were found capable of inducing autoreactive antibodies (Miura et al., 2000). Different catalase epitopes have been identified which are capable of cross-reacting with catalases of different species. An epitope from bovine 444TFYLK448 and rat 445TFYTK449 catalases yielded cross-reactive autoantibody responses in rats (Miura et al., 2000). Interestingly, the epitope 445TFYTK449 from rat catalase is 100% conserved in mouse catalase (445TFYTK449) and conserved in H. hepaticus catalase.
Helicobacter catalases may represent immunodominant bacterial antigens recognized by mammalian hosts. H. pylori catalase was used successfully as a vaccine in BALB/c mouse models (Radcliff et al., 1997). Interestingly, immunization of mice with H. pylori catalase delivered by an attenuated Salmonella typhimurium strain offered protection against H. pylori infection (Chen et al., 2003). In addition, S. typhimurium catalase provided protection against H. pylori infection in mouse models. These results indicate that heterologous bacterial catalases may offer broad-spectrum immune protection due to conserved structural domains. The limitation of such a broad-spectrum vaccination approach may be the possible generation of autoimmune responses in the host.

Cross-reactive anti-catalase immune responses in mice infected with H. hepaticus may be due to the presence of conserved epitopes between mammalian and bacterial catalases. Mice infected with H. hepaticus by orogastric gavage generated antibody responses to H. hepaticus and mouse catalases, secondary to documented intestinal colonization of H. hepaticus (Pena et al., 2005). Molecular mimicry between H. hepaticus and mammalian antigens may stimulate autoimmune responses in mouse models (Ward et al., 1996). Sera obtained from H. hepaticus-infected mice demonstrated detectable antibodies that cross-reacted with multiple proteins overlapping in size with murine catalase (Ward et al., 1994a, b). These antigens were not characterized in further detail but may include catalase as a potential autoantigen. Other pathogenic Helicobacter species such as H. pylori strains display Lewis blood group antigens similar to antigens expressed on the mucosal surface of the human stomach and contributing to gastric autoimmunity (Bergman et al., 2006; D’Elios et al., 2004; Hynes et al., 2005). Furthermore, H. pylori urease was considered to be a trigger that activates autoimmune responses and immunopathogenesis in the context of H. pylori infection (Yamanishi et al., 2006).

Of relevance to autoimmunity, two new catalase-derived surface-predicted motifs have been defined as functional epitopes and are highly conserved among bacterial and mammalian catalases. H. hepaticus is known to colonize the gastrointestinal and hepatobiliary tracts in laboratory mice and produces cytolethal distending toxin (Ge et al., 2005; Pratt et al., 2006). Cytolethal distending toxin is a cytotoxin with nuclease activity (Dassanayake et al., 2005) that may contribute to the exposure of the adaptive immune system to endogenous antigens following infection with H. hepaticus. H. hepaticus-infected mice produced antibodies to endogenous murine catalase as a result of intestinal colonization or invasive hepatic infection. Immunohistochemical data showed that murine hepatocyte catalase was immunoreactive with antibodies to H. hepaticus catalase, and immunoreactivity was correlated with chronic inflammation in infected animals. Antibodies to H. hepaticus catalase reacted directly with murine catalase in hepatocytes, indicating that murine and Helicobacter catalases are structurally conserved in vivo. Antibodies and autoreactive T cells targeting H. hepaticus catalase may recognize endogenous catalase and contribute to immunopathology.

Anti-catalase autoimmune responses may be relevant to human disease. Human catalase was reported to be a target autoantigen for anti-neutrophil cytoplasmic autoantibodies (ANCA) in patients with hepatobiliary diseases, inflammatory bowel disease and primary sclerosing cholangitis (PSC) (Orth et al., 1998; Roozendael et al., 1998). Our preliminary data suggest that patients with PSC have anti-Helicobacter
and anti-human catalase responses (data not shown). The high degree of conservation among bacterial and mammalian catalases raises intriguing questions regarding the immunopathogenic roles of shared epitopes in conserved microbial enzymes. Bacteria colonizing mucosal surfaces may contribute to autoimmune responses in genetically predisposed individuals by expressing highly conserved, surface-exposed proteins in sufficient quantities to stimulate adaptive immunity.

ACKNOWLEDGEMENTS

The authors acknowledge Ken Tsutsui (Okayama University School of Medicine in Japan) for providing the cloned murine catalase gene, Daniel Hassett (University of Cincinnati College of Medicine) for sending E. coli UM255, Yanhong Huang for technical support and Tiffany Morgan for administrative assistance. The authors also acknowledge Milton J. Finegold for assisting with immunohistochemical experiments, and Arlin Rogers for his assistance and helpful insights. This work was supported by National Institutes of Health (NIH) grant KO8DK02705 award (J.V.), the Crohn’s and Colitis Foundation of America (CCFA)-sponsored Senior Research Award, and the Moran Foundation (J.V.). J.V. was also supported by the US Public Health Service Grant K08DK02705 award (J.V.), the Crohn’s and Colitis Foundation of America (CCFA)-sponsored Senior Research Award, and the Moran Foundation (J.V.). J.V. was also supported by the US Public Health Service Grant DK56338, which funded the Texas Gulf Coast Digestive Diseases Center (renamed as the Texas Medical Center Digestive Diseases Center). The Helicobacter hepaticus genomic sequencing project was supported by a grant from the Bundesministerium fuer Bildung und Forschung PathoGenoMik network (S.S.). The authors also acknowledge NIH grants RO1CA67529 and RO1AI50952 (J.G.F.) and grant SFB621/B8 from the Deutsche Forschungsgemeinschaft (S.S.).

REFERENCES


Edited by: N. J. High