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 Versalovic^{5,6}

Correspondence

James Versalovic

jxversal@texaschildrenshospital.org

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

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.

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INTRODUCTION

Helicobacter hepaticus is a Gram-negative, microaerobic bacterium that colonizes the murine large intestine and was associated with hepatic and intestinal inflammation in susceptible mice (Fox *et al.*, 1994). *H. hepaticus* was first isolated from A/JCr mice and naturally colonizes the lower intestinal tract (Avenaud *et al.*, 2003; Foltz *et al.*, 1998; Fox *et al.*, 1994, 1996). This bacterium was also isolated from multiple mutant mouse lines with inflammatory bowel disease. Chronic *H. hepaticus* infection is characterized by

the infiltration of reactive oxygen species (ROS)-generating neutrophils and macrophages in the mouse caecum or liver (Fox *et al.*, 1994, 1996). ROS are secreted during the course of *Helicobacter* infection and may exacerbate DNA damage in hepatocytes or intestinal epithelial cells, potentially contributing to the development of hepatitis and colitis in mice (Hagen *et al.*, 1994; Sipowicz *et al.*, 1997).

Bacteria have developed protective mechanisms such as the expression of catalases that convert hydrogen peroxide into molecular oxygen and water. *H. hepaticus* catalase may confer protection of enterohepatic helicobacters against hydrogen peroxide and other ROS that are derived from innate immune responses. In the related organism, *Helicobacter pylori*, the lack of catalase reduces the time of

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 *Helicobacter* 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 trypticase soy agar with 5% sheep blood (TSA with 5% sheep blood; Remel) in an anaerobic chamber (Forma Scientific) under anaerobic conditions (90% N₂, 5% H₂ and 5% CO₂) at 37 °C for 5 days. *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 *Nco*I and *Not*I respectively (forward, 5'-gccatggctATGTCAAAGAAATTTACGACAGCA-3'; reverse, 5'-cggccgcTTATAACCCCAAAAGCTCTGCCACTC-3'), Ex-*Taq* buffer with 2.5 mM MgCl₂, 10 mM dNTP and proofreading-capable Ex-*Taq* 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 (QIAquick Gel Extraction kit, QiaGen). Amplicons were analysed by dideoxy 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 *Nco*I and *Not*I 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 pEA-1. The plasmid pEA-1 was cloned in *E. coli* BL21 and transferred to the lysogenic expression host *E. coli* BL21(DE3) in

order to create the recombinant *E. coli* strain, EA-HCl. Prior to expression, the insert sequence was verified by dideoxy 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 Coomassie 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 (Slide-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 ($R^2=0.997$) 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 (PROFsec 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>; ScanProsite 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* (Loewen *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 deviations 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 C57/BL6 IL-10-deficient mice) were evaluated by immunoblotting with recombinant antigens. IL-10-deficient C57/BL6 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 $\sim 10^7$ 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 immunoblotting 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 μ m) for 60 min at 4 °C (60 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 SuperSignal West Pico Chemiluminescent Substrate kit (Pierce). Immunoblot images were processed by a cooled CCD camera in a Chemo-Imager 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 [²⁹⁶LNKNPENYFAEVEQ³⁰⁹ (14 amino acids)], peptide 2 [¹¹⁵YTNENGNWDIVGNNT¹²⁹ (15 amino acids)] and peptide 3 [¹⁴⁶QKRDPKTN¹⁵³ (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 (10000 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 Chemilmager system 5500 (Alpha Innotech).

In order to confirm specificities of peptide immunoneutralization experiments, peptide sequences were randomized [peptide 1r (PFYNELKEQNNVEA) and peptide 3r (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.cellbiol.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 pre-bled at the time of primary injection in order to obtain pre-immune sera. Sera were collected 10 weeks post-injection. Hepatic 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 organisms in hepatic tissue sections. Tissue sections (3–4 μ m) 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 enzymic 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-amino-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). *H.*

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 A_{240} . 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*

Fig. 1. *H. hepaticus*-infected mice demonstrate antibody responses to *H. hepaticus* and murine catalase. Immunoblot studies were performed to evaluate serological responses to *H. hepaticus* and murine catalases in *H. hepaticus*-infected IL-10-deficient C57/BL6 mice. (a, b) Immunoblot studies of anti-catalase immune responses with individual serum samples (1:1000 dilution) from two different *H. hepaticus*-infected C57/BL6 mice. Lane 1 contains recombinant murine catalase (60 kDa). Lane 2 contains recombinant *H. hepaticus* catalase (62 kDa). (c, d) SDS-PAGE (c) and immunoblot (d) studies of uninfected mice with pooled sera (1:1000 dilution) show lack of immunoreactivity to murine or *H. hepaticus* catalases. Lanes: 1, catalase-deficient *E. coli* UM 255; 2, protein A (50 kDa) added as a positive control to bind non-specific IgG; 3, murine catalase (60 kDa); 4, *H. hepaticus* catalase (62 kDa). Anti-mouse IgG-HRP conjugate (Rockland Immunochemicals) was used as a secondary antibody. M, molecular mass standards (Bio-Rad).

Fig. 2. Recombinant *H. hepaticus* catalase has functional enzymic activity. The decomposition of hydrogen peroxide (starting concentration 10 mM) was qualitatively assessed by recombinant *H. hepaticus* catalase (●). Catalase activity was demonstrated by the decline of A_{240} . Human erythrocyte catalase (▲), human erythrocyte catalase in the absence of hydrogen peroxide (◆) and hydrogen peroxide in the absence of catalase (■) were used as controls. The experiments were performed twice in duplicate. Error bars indicate standard deviations.

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 β -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 α -helices with β -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).

Sequence-structural correlations of *Helicobacter* catalases

The predicted secondary structure of *H. hepaticus* catalase indicated that α -helices represent approximately 28.3 % of the primary sequence, whereas β -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 α -helical with β -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

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

Name	Distal haem-ligand domain*	Proximal haem-ligand domain*
<i>B. pertussis</i>	RERIPERVHAKG	RLFSYGD
<i>C. jejuni</i>	RERIPERTVHAKG	RIFSYPD
<i>H. hepaticus</i>	RERIPERVVHAKG	RLFSYGD
<i>H. pylori</i>	RERIPERVVHAKG	RLFSYGD
<i>Homo sapiens</i>	RERIPERVVHAKG	RLFAYPD
<i>Mus musculus</i>	RERIPERVVHAKG	RLFAYPD
Consensus	RERIPERVVHAKG	RLFSYGD

*Underlined letters represent catalytically important amino acid residues.

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>).

of the three-dimensional structure of *H. pylori* catalase. The predicted surface motifs ²⁹⁶LNKNPENYFAEVEQ³⁰⁹, ¹¹⁵YTEEGNWDIVGNNT¹²⁹ and ¹⁴⁶QKRDPKTN¹⁵³ are highly conserved among *Helicobacter*, murine and human catalases (Fig. 3).

H. hepaticus-infected mice demonstrate cross-reactive immune responses to H. hepaticus and murine catalases

H. hepaticus-infected IL-10-deficient C57/BL6 mice generated cross-reactive immune responses to *H. hepaticus* and murine catalases (Fig. 1a, b). Immunoblot studies demonstrated antibody responses to *H. hepaticus* catalase and endogenous murine catalase only in individual serum samples or pooled sera derived from *H. hepaticus*-infected mice (Fig. 1a, b, and data not shown). Sera from eight different *H. hepaticus*-infected mice were tested individually,

and all mice demonstrated antibody responses to both *H. hepaticus* and murine catalases. Immune responses to bacterial or murine catalases were not observed in samples from uninfected control C57/BL6 mice (pooled $n=8$ mice including 5 males and 3 females) (Fig. 1c, d). The generation of cross-reactive antibody responses presumably results from the remarkable degree of sequence and structural conservation among mammalian and bacterial haem catalases (Fig. 3).

H. hepaticus catalase surface-predicted motifs represent functional epitopes recognized by murine antibodies

Peptides derived from surface-predicted motifs (Fig. 3) were designated peptide 1 (LNKNPENYFAEVEQ), peptide 2 (YTEEGNWDIVGNNT) 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).

Fig. 4. Surface-predicted peptides of *H. hepaticus* catalase comprise functional epitopes. Following removal of immune complexes after incubation of catalase peptides and mouse sera, supernatants were used to probe recombinant *H. hepaticus* catalase on a nitrocellulose membrane. Anti-mouse IgG HRP conjugate was used as a secondary antibody. Signals were visualized by chemiluminescence (see Methods). (a, b) Band signal intensities following immunoneutralization with catalase peptides 1 (a) and 3 (b) were assessed with ChemImager software by subtracting the background and measuring the relative band intensities. The signal intensities represent immune complex formation between sera and *H. hepaticus* catalase. The rectangular areas around each band were selected by the computer algorithm (areas ranged from 90 to 208 pixels² for peptide 1 and from 120 to 230 pixels² for peptide 3). The numerical values in each table represent relative band signal intensities due to peptide inhibition. The relative signal intensities indicate the percentage of the total signal measured for the positive control (Positive Ctrl with no peptide; $n=1.00$). The experiments were performed in duplicate (with replicates included), and standard deviations are indicated. The numbers above each table correspond to the lane numbers in the corresponding image.

***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.

Fig. 5. Random peptides do not neutralize murine anti-catalase responses. Following removal of immune complexes after incubation of random peptides and murine sera, supernatants were used to probe recombinant *H. hepaticus* catalase on a nitrocellulose membrane. Details as for Fig. 4. (a, b) Band signal intensities following immunoneutralization with random peptides 1r (a) and 3r (b). The signal intensities represent immune complex formation between sera and *H. hepaticus* catalase. The rectangular areas around each band were selected by the computer algorithm (areas ranged from 133 to 190 pixels² for peptide 1r and from 147 to 152 pixels² for peptide 3r). The numerical values in each table represent relative band signal intensities. The relative signal intensities indicate the percentage of the total signal measured for the positive control (Positive Ctrl with no peptide; $n=1.00$). The experiments were performed in duplicate (with replicates included), and the standard deviations are indicated. Differences in band signal intensities were not statistically significant ($P<0.05$). The numbers above each table correspond to the lane numbers in the corresponding image.

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 three 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

Fig. 6. Immunohistochemical demonstration of cross-reactivity between *H. hepaticus* and murine catalases. Anti-bovine catalase and anti-*H. hepaticus* catalase antibodies complexed with murine hepatocyte catalase in liver sections of *H. hepaticus*-infected A/JCr mice treated as indicated. (a) Section from mouse treated with polyclonal anti-bovine liver catalase antibodies (Abcam). These antibodies are known to react with murine catalase. (b) Section from mouse treated with rabbit IgG isotype control antibodies (Zymed, Invitrogen) as a negative control for non-specific staining. (c) Section from mouse treated with anti-*H. hepaticus* catalase antibodies (rabbit serum). (d) Section from mouse treated with pre-immune rabbit serum as a negative control. Bar, 100 μ m.

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 *Micrococcus 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 ⁴⁴⁴TFYTK⁴⁴⁸ 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*

Fig. 7. *H. hepaticus* catalase activity is present in the bacterial cytoplasm and periplasm. Cytoplasmic and periplasmic extracts were fractionated by native PAGE (10% polyacrylamide) for 3 h at 4 °C (25 mA) and stained with ferric chloride and potassium ferricyanide (Sigma). The gel was photographed with a cooled CCD camera (Chemi-Imager). Lanes: 1, recombinant *H. hepaticus* catalase (positive control); 2, catalase-deficient *E. coli* UM255 (negative control); 3, *H. hepaticus* periplasmic fraction; 4, *H. hepaticus* culture supernatant; 5, *H. hepaticus* cytoplasmic fraction.

catalase (⁷⁰TQYTK⁷⁴). This epitope is proximal to the ⁴⁴RERIPER⁵⁰ and ⁵¹VVHAKG⁵⁶ motifs by 19 and 13 amino acids, respectively.

Similar to KatA localization in *H. pylori* (Harris & Hazell, 2003), our data (Fig. 7) suggest that *H. hepaticus* KatA is present in the cytoplasm and periplasm. Its periplasmic localization is consistent with that of other catalases in Gram-negative bacteria including *Pseudomonas syringae* (Klotz & Hutcheson, 1992), *Pseudomonas aeruginosa* (Brown *et al.*, 1995), *Brucella abortus* (Sha *et al.*, 1994) and *Vibrio fischeri* (Visick & Ruby, 1998). The periplasmic location of catalases in *H. hepaticus* and other Gram-negative bacteria may facilitate antigenic exposure to the mammalian immune system and propensity to generate immune responses. Therefore, catalase may serve as an immunogenic target predisposing the host to recognize *H. hepaticus* and its own endogenous catalases (Klotz & Hutcheson, 1992; Klotz *et al.*, 1997; Miura *et al.*, 2000).

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'Elisio *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; Roozendaal *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.

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