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Structural and Functional Characterization of an Organic Hydroperoxide Resistance Protein from *Mycoplasma gallisepticum*▽

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As obligate parasites, *Mycoplasma* species are continuously exposed to oxidative damage due to host-generated peroxides and reactive oxygen species (ROS). In addition, the production of endogenous oxidants is believed to be a primary virulence mechanism of several Mollicute species, indicating that oxidative stress resistance is crucial to survival of these bacteria in the host milieu. Despite the abundance of oxidants at the site of infection, enzymes responsible for the detoxification of ROS have never been characterized in mycoplasmas. Here we characterize a homolog of the *ohr* (organic hydroperoxide resistance) family from *Mycoplasma gallisepticum* (encoding MGA1142). Unlike previously characterized *ohr* genes, the *mga1142* gene is not upregulated in response to oxidative stress but displays a novel pattern of expression. Both organic and inorganic peroxides can act as substrates for MGA1142, but they are degraded with various efficiencies. Furthermore, cumene hydroperoxide, an aromatic peroxide metabolized with high efficiency by other Ohr proteins, was shown to rapidly inactivate MGA1142, accounting for the sensitivity of *M. gallisepticum* cells to this compound. Comparative modeling of the MGA1142 quaternary structure revealed that the active site of this molecule has a relatively wide conformation. These data indicate that the natural substrate for MGA1142 differs from that for previously characterized Ohr proteins. Triton X-114 partitioning demonstrated that MGA1142 is located in both cytosol and membrane fractions, suggesting that in vivo this molecule plays a role in the detoxification of both endogenous and exogenous peroxides. A model describing how MGA1142 is likely to be oriented in the cell membrane is presented.

Mycoplasma gallisepticum is a highly contagious pathogen that is associated with avian mycoplasmosis, a severe and chronic respiratory disease complex of chickens. The bacterial cells colonize the mucosal surface of the host respiratory tract, causing inflammation and cilial shedding, which leaves the host susceptible to secondary infections (18). Vaccines and treatment therapies for controlling *M. gallisepticum* infection have been only partially successful, and avian mycoplasmosis continues to be a widespread problem, causing significant economic losses in the poultry industry worldwide (27).

The remarkable ability of *M. gallisepticum* to persist in the host is directly related to this organism's capacity to circumvent the host immune response. The best-studied mechanism is the phase switching of surface antigens of the pMGA family (25). However, like all pathogens, *M. gallisepticum* must also overcome nonspecific assaults from the host's innate immune response, such as the release of reactive oxygen species (ROS). These ROS include superoxide, hydroxy radicals, H_2O_2, and organic peroxides. The organic peroxides are particularly noxious compounds that are generated as reaction intermediates during the oxidative burst from inflammatory cells (3). Organic peroxides perpetuate oxidative damage by producing secondary free radicals (28) and are capable of modifying and inactivating various cellular macromolecules, including DNA, proteins, and lipids (3, 40).

In addition to the oxidative burst delivered by host immune cells, several *Mycoplasma* species (4, 24, 47, 48), including *M. gallisepticum*, are reported to release H_2O_2, superoxide anions and hydroxy radicals as a primary virulence mechanism, inflicting damage to host tissue. The abundance of ROS at the site of *Mycoplasma* infection further highlights the need for these organisms to possess a protective strategy for coping with oxidative challenge. The mechanisms of oxidative defense in mycoplasmas are not well understood but are predicted to play a significant role in virulence (5, 24, 47) and therefore warrant further investigation.

Pathogenic bacteria have evolved several strategies to detoxify oxidizing agents. Superoxide dismutase and catalase are involved in the neutralization of superoxide and hydrogen peroxide, respectively. Additional enzymes, such as alkyl hydroperoxide reductase (AhpR), a member of the peroxiredoxin family (34), and, more recently, the organic hydroperoxide resistance (Ohr) protein (28) and osmotically inducible protein C (OsmC) (23, 36), have been shown to be specifically involved in the detoxification of organic peroxides to the corresponding alcohols.

The OsmC superfamily consists of OsmC, Ohr, and a group of structurally related proteins with unknown functions (subfamily III) (3, 41). Ohr and OsmC are the best-studied members of the superfamily and are structurally and functionally homologous proteins, and each consists of a homodimer with two active sites on either side of the molecule (22, 23, 41). The

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active site consists of a hydrophobic pocket, designed to accommodate organic hydroperoxide (OHP) species, and contains two highly conserved redox-reactive cysteine residues which are directly involved in peroxide metabolism (22, 23, 28, 36). In the active form of the enzyme, one of the cysteine residues hydrogen bonds with an adjacent arginine (at approximately position 18 in Ohr and at approximately position 37 in OsmC), which reduces the pK_a of the thiol (-SH) group, deprotonating it to the more reactive thiolate ($-S^-$) form (22). During catalysis, the reactive cysteine becomes oxidized to a sulfenic acid intermediate (-SOH), which is then attacked by the sulfhydryl group of the second cysteine, creating a disulfide bridge (31). Regeneration of the dithiol form of the enzyme occurs via an unknown reducing agent, predicted to be dihydrolipoic acid (8, 26).

Mycoplasmas were previously thought to lack enzymes for detoxification of ROS, (5, 12, 47), and while genes for superoxide dismutase, catalase, and AhpC (the peroxidase subunit of AhpR) have not been discovered in any Mollicute genome, homologs of genes encoding members of the OsmC superfamily have been identified in members of the *Mycoplasma pneumoniae* phylogenetic cluster (3, 6, 41). In the *M. gallisepticum* genome there are two homologs encoding proteins belonging to this superfamily, *mga252*, which belongs to subgroup III (with an unknown function), and *mga1142* (GenBank accession number AE015450), which is annotated as a gene encoding an OsmC homolog, indicating a potential peroxidase function. Genes directly involved in peroxide metabolism have not been characterized previously in mycoplasma species but are predicted to play a significant role in virulence because of the propensity of these organisms to produce ROS (24, 47). Furthermore, data from a previous study identifying MGA1142 as a heparin-binding protein (17) indicates that this protein may play multiple roles in *M. gallisepticum* pathogenesis and may be of interest as a vaccine target. Here we examined the function of MGA1142 in *M. gallisepticum* in relation to its role in oxidative stress resistance. Below we present findings on the structure, pattern of expression, and peroxidase activity of this protein.

MATERIALS AND METHODS

Bacterial strains and culture. *M. gallisepticum* strain R_{low} (number of passages, <25) was cultured in Hayflick's medium (45) at 37°C overnight. Frozen stocks of log-phase cultures were maintained at −70°C. The solid medium used for the peroxide sensitivity assay was Hayflick's medium without the phenol red indicator and with 3% Noble agar added.

Phylogenetic analysis. OsmC and Ohr protein sequences were retrieved from the Swiss-Prot database and aligned using ClustalW (http://www.ch.embnet.org/software/ClustalW.html). Sequence gaps at the N and C termini were trimmed from the alignment for phylogenetic analysis. A tree was constructed using the PHYLIP suite of programs. Protdist with the Jones-Taylor-Thornton matrix was used to generate distances, and the tree was constructed using the neighbor-joining method. The data were subjected to 100 bootstrap replications.

Peroxide sensitivity assay. *M. gallisepticum* R_{low} was spread plated onto Hayflick's agar and allowed to dry. Sterile filter paper disks containing 10 μl of 0.5 M H_2O_2, *tert*-butyl hydroperoxide (tBOOH), or cumene hydroperoxide (CuOOH) were placed in the center of the plates, which were inverted and incubated at 37°C. After 2 days of incubation, the zones of inhibition were examined with a dissecting microscope and recorded.

RNA extraction and Northern blotting. An MGA1142 DNA probe was prepared by PCR digoxigenin (DIG) labeling using previously described primers (17). Exponential-phase cultures of *M. gallisepticum* R_{low} (6 ml) were subjected to oxidative stress (200 μM H_2O_2, tBOOH, or CuOOH), osmotic stress (0.5 to 2% NaCl), ethanol stress (2 to 4% ethanol), or heat shock (40 to 42°C) for 1 h. Cultures that were not exposed to any stress conditions were used as controls. Cells were harvested, and the RNA was extracted using a TRIzol Max bacterial RNA isolation kit (Invitrogen) according to the manufacturer's instructions. RNA pellets were resuspended in nuclease-free water, and the concentration was measured spectrophotometrically. For electrophoresis, RNA samples were diluted in denaturing buffer (1× 4-morpholinepropanesulfonic acid [MOPS], 50% formamide, 2% formaldehyde) and heated to 65°C for 15 min. After addition of tracking dye, 2 μg of each RNA sample was run in agarose gels under denaturing conditions (1% agarose, 0.5× MOPS, 2% formaldehyde) at 75 V for 2 h. The gel was stained to ensure that the sample loads were uniform. The RNA was transferred to Hybond N+ nylon (Amersham Biosciences, Piscataway, NJ) overnight via capillary transfer and UV cross-linked to the membrane. Membranes were prehybridised in DIG EasyHyb prehybridization solution (Roche, Basel, Switzerland) at 50°C for 2 h. Hybridization was carried out at 50°C overnight in the same solution containing 25 ng/ml preboiled MGA1142 probe. Membranes were washed and blocked prior to addition of anti-DIG antibody (1:10,000). Blots were developed with chloro-5-substituted adamantyl-1,2-dioxetane phosphate (CSPD) and detected on X-ray film.

Cloning and protein expression and purification. The *mga1142* gene was amplified by PCR as described previously (17) except that the forward primer used was 5′-CACCATGTTTAAAAAGGTTTATGAAACC-3′. The resulting PCR product was cloned into the pET100D-TOPO expression vector and transformed into *Escherichia coli* BL21 Star (Invitrogen, Carlsbad, CA). The MGA1142 protein was then expressed using isopropyl-β-D-thiogalactopyranoside (IPTG) induction according to the manufacturer's instructions and was purified under native conditions. *E. coli* cell pellets were resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole; pH 8.0) and treated with 1 mg/ml lysozyme for 30 min on ice. The cells were then sonicated with six 10-s pulses at 100% amplitude with a Dr Hielscher UP400s Ultraschallprozessor (Hielscher Ultrasonics, Stuttgart, Germany) interspersed with 10-s cooling periods. The cell debris was pelleted by centrifugation at 10,000 × *g* for 20 min, and Profinity IMAC Ni-charged resin (Bio-Rad Laboratories, Hercules, CA) was added to the cleared lysate at a ratio of 1:4. The lysate-resin mixture was incubated on a rotary shaker for 1 h at 4°C and loaded onto a glass column. The resin was washed twice with 2 volumes of wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole; pH 8.0) to remove unbound protein and eluted four times with 0.25 volume of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole; pH 8.0). Purified protein was analyzed by one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (17). The MGA1142 antiserum used for immunoblotting was generated in a previous study (17).

Enzymatic assays. Peroxidase activity was measured using the ferrous iron xylenol orange (FOX) assay as previously described (8). Purified MGA1142 protein was preincubated with 5 mM dithiothreitol (DTT) for 1 h on ice. The reaction mixtures contained peroxide and DTT at initial concentrations of 500 μM and 1 mM, respectively, and the reactions were initiated by addition of MGA1142. At various time points, 100-μl aliquots were collected from each sample and added to cuvettes containing 20 μl of 0.25 M H_2SO_4 to stop the reaction. When aliquots were collected for all the time points, the peroxide concentration was determined colorimetrically by adding 880 μl of freshly prepared FOX reagent to each cuvette. The optical density at 560 nm of each sample was determined after 20 min, when the color reaction had reached equilibrium. Peroxide removal by live cultures was determined as described above except that *M. gallisepticum* strain R and *Pseudomonas aeruginosa* cells grown overnight in broth culture and diluted to obtain an optical density at 600 nm of 0.35 were used.

TritonX-114 partitioning, 2D electrophoresis, and Western blotting. A 0.5-g pellet of *M. gallisepticum* cells was resuspended in 5 ml of 1% Triton buffer (1% Triton X-114, 10 mM Tris [pH 8.0], 150 mM sodium chloride, 1 mM EDTA) and incubated at 4°C overnight on a rotary shaker. The cell debris was pelleted by centrifugation at 10,000 × *g* for 15 min at 4°C. The supernatant was transferred to a fresh tube and incubated at 37°C for 10 min. Phase separation was induced by centrifugation at 8,000 × *g* for 10 min at room temperature. The aqueous phase was removed and placed in a fresh tube, and both phases were reextracted with 2% Triton X-114 at 4°C for 4 h. Phase separation was induced as described above, and both the aqueous and detergent phases were flooded with a 10 volumes of cold acetone. Proteins were precipitated overnight at −20°C and pelleted by centrifugation. The supernatants were removed, and the acetone was evaporated for 30 min. The aqueous phase pellet was resuspended in 2.5 ml of SSS buffer, which contained 8 M urea, 100 mM DTT, 4% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 0.8% ampholytes, and 40

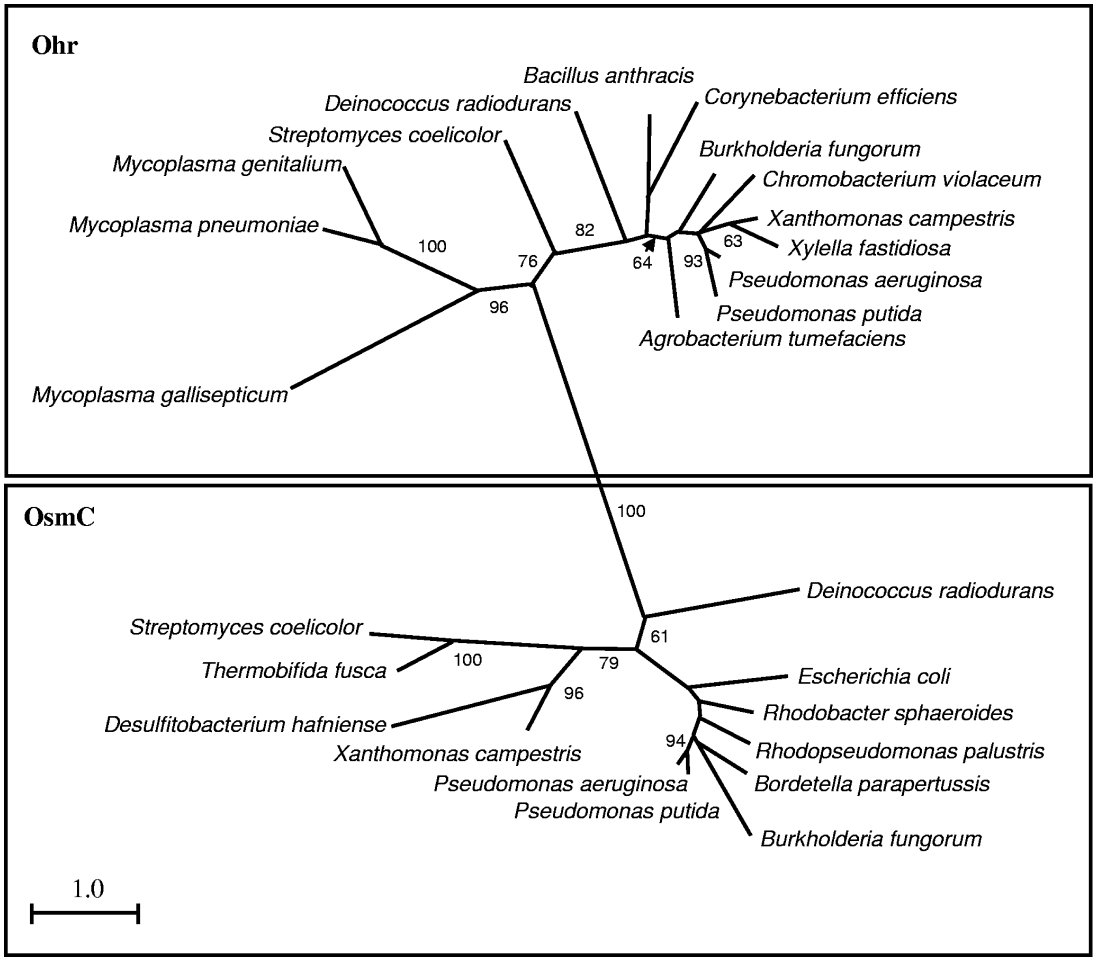

FIG. 1. Phylogenetic tree showing the relationship between the Ohr and OsmC protein subfamilies. MGA1142 is clearly a member of the Ohr subfamily and clusters with Ohr homologs from other members of the *M. pneumoniae* phylogenetic group. Bootstrap values greater than 50% are indicated at the nodes. Scale bar = 1.0 substitution per site.

mM Tris, and the detergent phase proteins were resuspended in 1.25 ml of MSS buffer, which contained 5 M urea, 65 mM DTT, 2% CHAPS, 0.8% ampholytes, 40 mM Tris, 2 M thiourea, and 2% sulfobetaine. Samples were sonicated at 10 W for 30 s and centrifuged, and the supernatants were collected for isoelectric focusing. Isoelectric focusing IPG ReadyStrips (11 cm; pH range, pH 3 to 10; Bio-Rad Laboratories) were rehydrated overnight with 200 μg of aqueous or detergent phase protein and then focused and separated in the second dimension as described previously (17). Western blot analyses of two-dimensional (2D) gels and whole-cell proteins from trypsin-treated *M. gallisepticum* were performed as described previously (11, 17).

Protein modeling. For modeling the three-dimensional structure of the MGA1142 homodimer, the Protinfo software (http://protinfo.compbio.washington.edu) was employed. The methods used to construct the MGA1142 structural model have been described in detail elsewhere (16, 38). Briefly, the X-ray structure of Ohr from *P. aeruginosa* (Protein Data Bank identifier 1n2f) was used as a template to construct the initial model using a minimum perturbation approach which retained as much information as possible from the template structure. Following this, a graph-theory clique-finding method, which surveys a range of potential conformations, was employed to construct the variable main and side chains. The optimal conformation was chosen using an all-atom scoring function.

Structure coordinates. The MGA1142 model represents residues 7 to 146 of the sequence deposited under GenBank accession number AAP56637. The coordinates for the MGA1142 structure are available at http://data.compbio.washington.edu/etc/downloads/ap_collaboration_ohr_mgal/.

RESULTS

Sequence analysis and phylogeny of MGA1142. The *mga1142* gene (GenBank accession number AE015450) is annotated as a gene encoding an OsmC homolog; however, preliminary BLASTX analyses returned matches to members of both the OsmC and Ohr subfamilies. To elucidate the phylogenetic position of the *mga1142* gene, we conducted a phylogenetic analysis of the corresponding protein sequence. Protein sequences of members of subgroup III were excluded due to their extremely low sequence homology with other members of the superfamily. The phylogenetic tree in Fig. 1 clearly demonstrates that MGA1142 belongs to the Ohr subfamily of proteins rather than the OsmC subfamily, indicating that MGA1142 likely plays a primary role in the degradation of organic peroxides. While Ohr homologs are also present in *Mycoplasma genitalium* (MG454) and *M. pneumoniae* (MPN668), no homologs of Ohr proteins were found in any other *Mycoplasma* genome sequences, indicating that within this genus Ohr proteins may be unique to the *M. pneumoniae* phylogenetic cluster. As indicated by the branch lengths, the

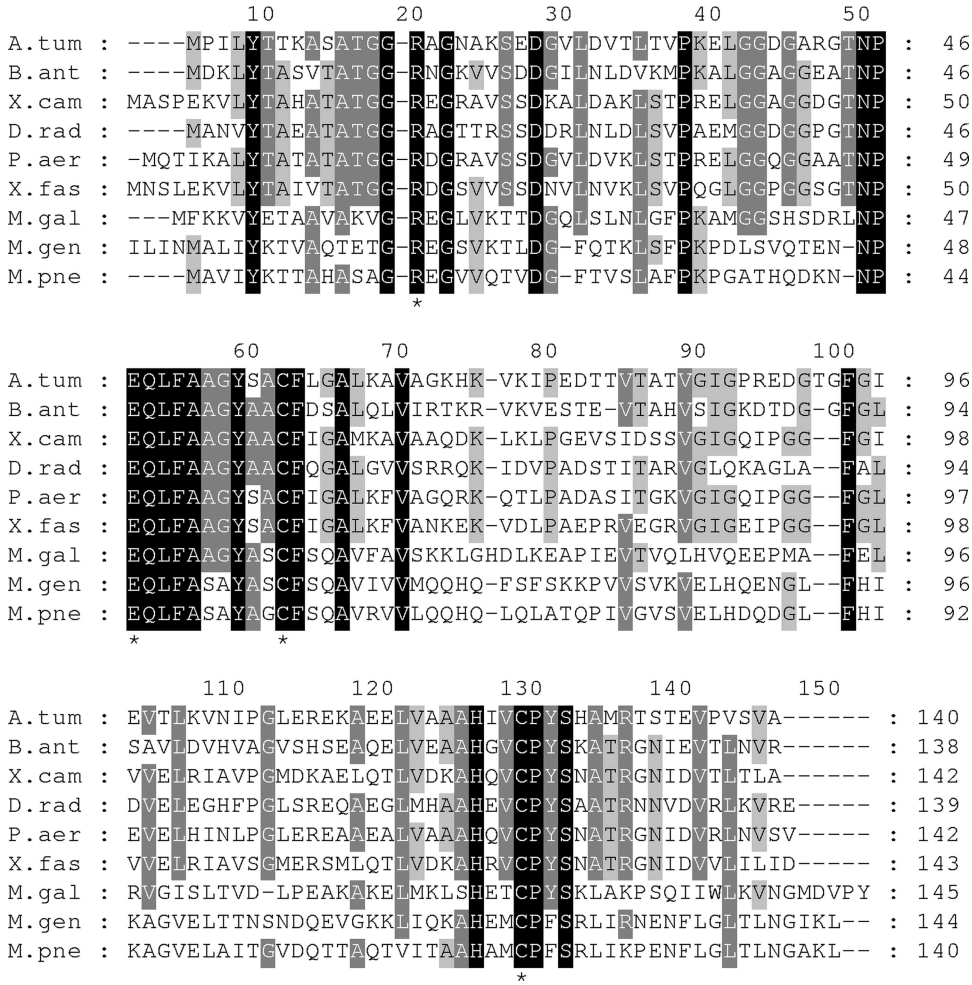

FIG. 2. Alignment of Ohr protein sequences from various bacterial species. Shading indicates areas where there is high conservation (black) and moderate conservation (gray and light gray). Asterisks indicate residues known to be directly involved in catalysis. A. tum, *Agrobacterium tumefaciens*; B. ant, *Bacillus anthracis*; X. cam, *Xanthomonas campestris*; D. rad, *Deinococcus radiodurans*; P. aer, *Pseudomonas aeruginosa*; X. fas, *Xylella fastidiosa*; M. gal, *Mycoplasma gallisepticum* strain R; M. gen, *Mycoplasma genitalium*; M. pne, *Mycoplasma pneumoniae*.

Mycoplasma Ohr sequences are not as highly conserved as other Ohr proteins. Sequences of members of the Ohr subgroup display 40 to 70% identity with each other (23), while the mycoplasma sequences display less than 32% identity with the other Ohr proteins (and only 15 to 20% identity with sequences of members of the OsmC subgroup). Despite this lower level of identity with other Ohr sequences, MGA1142 retains many of the functionally important residues, such as the catalytic cysteines (Cys58 and Cys122), the active site arginine (Arg16), and the glutamate residue (Glu48) (Fig. 2) which forms a salt bridge with the arginine when the enzyme is in the active form. One notable difference between the MGA1142 sequence and the sequences of other Ohr proteins is that the number of glycine residues varies considerably. Ohr proteins are typically quite glycine-rich, containing 11.5 to 13.5% glycine, while MGA1142 contains only 6.9% glycine. The sequences of the Ohr homologs from *M. genitalium* and *M. pneumoniae* also contain lower proportions of this amino acid, a feature most likely related to the fact that *Mycoplasma* genomes are AT rich.

Expression of *mga1142* under stress conditions. All Ohr homologs characterized in previous studies have been shown to be upregulated in the presence of organic peroxides but to be upregulated only weakly (28) or not at all (3) by hydrogen peroxide. In contrast, OsmC homologs are generally upregulated under osmotic or ethanol stress conditions (3). To determine whether the *mga1142* gene of *M. gallisepticum* is upregulated under oxidative stress conditions, as well as other stress conditions, we generated an *mga1142* probe for Northern analyses. As shown in Fig. 3A, *mga1142* was found to be constitutively expressed and was not upregulated under oxidative stress conditions regardless of the peroxide species tested. In contrast, expression of *mga1142* was upregulated approximately twofold in the presence of 2 to 4% ethanol (Fig. 3B) and was downregulated under osmotic stress conditions (0.5 to 2% NaCl) (Fig. 3C). Heat shock (40 to 42°C) did not appear to have a substantial effect on *mga1142* expression (data not shown).

Identification of the *mga1142* promoter region and N-terminal sequencing. A putative promoter for *mga1142* was identi-

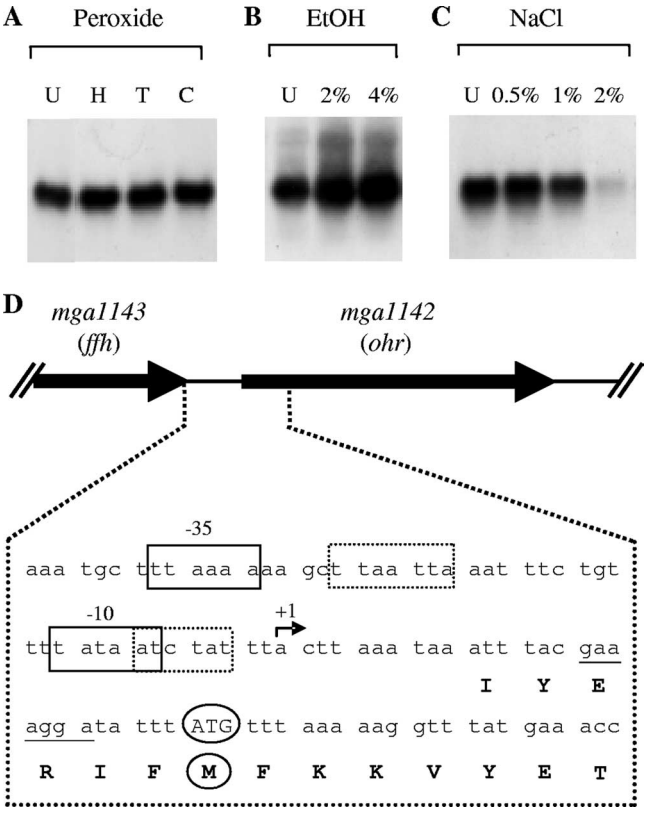

FIG. 3. (A to C) Northern analysis of *mga1142* expression under various stress conditions. As shown in panel A, the *mga1142* gene is expressed at high levels even when it is uninduced (lane U), and it is not upregulated in the presence of hydrogen peroxide (lane H), tBOOH (lane T), or CuOOH (lane C). In contrast, the expression levels increase approximately twofold in the presence of 2 to 4% ethanol (B) and decrease in the presence of 0.5 to 2% NaCl (C). (D) Schematic diagram of *mga1142*, showing the putative promoter region. The Pribnow box (−10) and −35 binding sites for σ^{70} are enclosed in solid boxes, and the likely transcriptional start site, as predicted by the Sequence Alignment Kernel program, is indicated by an arrow. Potential −10 and −35 binding sites for σ^{E} are enclosed in dotted boxes. The Shine-Dalgarno sequence is underlined. The translational start site, as determined by N-terminal sequencing, is circled and is located six residues downstream of the start predicted in the GenBank annotation.

fied (Fig. 3D) using the Sequence Alignment Kernel promoter predication program (14). The promoter region contains a Pribnow box (−10 site) which is an exact match to the consensus sequence (TATAAT) for the housekeeping sigma factor (σ^{70}). A putative −35 site (TTAAAA) was also identified upstream of the Pribnow box. In addition to these binding sites for σ^{70}, we also identified −10 and −35 sites matching the consensus binding sites for σ^{E} (Fig. 3D). A Shine-Dalgarno sequence was located downstream of the putative transcriptional start site and overlapped the proposed translational start codon identified in the *mga1142* GenBank entry (accession number AE0154500; proposed N terminus, IYERIFM). These data led us to the conclusion that the actual translational start codon resided at the methionine residue six amino acids downstream of the proposed start codon (Fig. 3D). This was subsequently confirmed via N-terminal sequencing of the MGA1142 protein excised from a 2D SDS-PAGE gel, and the amino acids MFKKVYET were identified at the N terminus.

Subcellular location of MGA1142. The subcellular location of Ohr proteins has not been examined yet but is of interest for determining whether the peroxide substrates for these enzymes are endogenous (by-products of bacterial metabolism), exogenous (host-generated oxidative challenge), or both. Furthermore, it has been speculated that homologs of both Ohr and OsmC may both be present in some organisms because they occupy disparate subcellular locations (23). While *M. gallisepticum* does not contain a member of the OsmC subfamily, it does contain both an Ohr homolog (encoded by *mga1142*) and a subgroup III homolog (encoded by *mga252*). A previous study of the heparin-binding properties of MGA1142 (17) indicated that this protein is surface accessible. Here, we used Triton X-114 partitioning and 2D Western blots to further elucidate the subcellular location of this protein. As shown in Fig. 4A, MGA1142 was prominent in both aqueous (cytoplasmic) and detergent (membrane) fractions. A TMPred analysis of the MGA1142 sequence also identified a putative transmembrane domain spanning amino acids 49 to 66. These data suggest that while significant quantities of MGA1142 are present in the cytoplasm, MGA1142 is also an integral membrane protein. To determine which portions of the MGA1142 molecule were surface exposed, we subjected *M. gallisepticum* cells to trypsin treatment and probed the whole-cell proteins with antiserum against MGA1142 and also the N- and C-terminal halves (amino acids 1 to 67 and 68 to 145, respectively) of MGA1142 (Fig. 4B). Partial degradation of both the N and C termini of MGA1142 was observed at the higher concentrations of trypsin but was not observed for the intracellular control protein (ribosomal protein L7/L12), indicating that portions of both the N and C termini of MGA1142 are exposed to the cell surface. Residual protein observed at the higher trypsin concentrations likely represented intracellular MGA1142 protein which was not degraded during trypsin treatment.

Disk inhibition assays. As Ohr proteins have been shown to preferentially degrade OHPs over H_2O_2, we decided to test the sensitivity of *M. gallisepticum* R_{low} to two different OHPs (tBOOH and CuOOH) and to H_2O_2 using a disk inhibition assay. As shown in Table 1, *M. gallisepticum* was highly resistant to tBOOH but displayed significant sensitivity to H_2O_2. Unexpectedly, and in contrast to other bacterial species containing Ohr, *M. gallisepticum* also displayed marked sensitivity to CuOOH, which is typically able to act as a substrate for Ohr. These data were further confirmed using the colorimetric FOX assay, which was used to detect peroxide degradation in cultures of both *M. gallisepticum* and *P. aeruginosa* (positive control). In *P. aeruginosa*, which contains multiple peroxidases, all peroxide species were rapidly degraded, while in *M. gallisepticum* only tBOOH was degraded with high efficiency, while H_2O_2 was degraded with low efficiency (after the additional effect of the medium was taken into account) and CuOOH did not appear to be degraded at all (data not shown).

Peroxidase activity. The ability of purified recombinant MGA1142 to degrade various peroxide species was also tested by the FOX assay. Like previously described Ohr proteins, MGA1142 did not show activity in the absence of a reducing

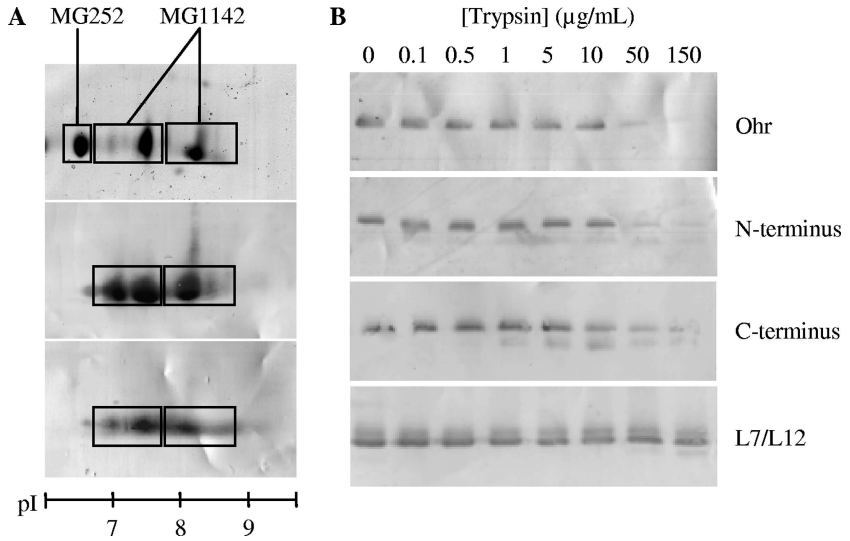

FIG. 4. (A) Protein spots corresponding to MGA1142 and MG252 from 2D SDS-PAGE and Western analysis of Triton X-114-partitioned proteins from *M. gallisepticum*. The top panel shows a section of a Coomassie blue-stained 2D gel which had been loaded with *M. gallisepticum* whole-cell proteins. The MGA1142 and MG252 proteins were identified by mass spectrometry (peptide matches are not shown). The MGA1142 protein was also subjected to N-terminal sequencing. Sections of Western blots of 2D SDS-PAGE-separated Triton X-114 proteins which were probed with MGA1142 antiserum are shown in the middle and bottom panels. Protein spots corresponding to MGA1142 are prominent in both the aqueous (middle panel) and detergent (bottom panel) phases. The bar indicates the isoelectric points (pI). (B) Western blots of whole-cell proteins from trypsin-treated *M. gallisepticum* cells reacted with antisera against MGA1142, the N-terminal half of MGA1142, the C-terminal half of MGA1142, and the intracellular L7/L12 ribosomal protein (negative control). The top three panels show partial degradation of MGA1142 with between 5 and 150 μg/ml of trypsin, indicating that portions of both the N and C termini of MGA1142 are surface exposed. The residual protein seen with 50 and 150 μg/ml of trypsin likely represents the intracellular portion of MGA1142. At the same trypsin concentrations, the intracellular L7/L12 protein (bottom panel) was not degraded.

agent (DTT) and preferentially metabolized organic hydroperoxide (tBOOH) over H_2O_2. At a concentration of 10 ng/μl MGA1142 was able to metabolize 300 μM tBOOH/min, while at a concentration of 100 ng/μl this enzyme was only able to degrade the same amount of H_2O_2 in 10 min. Thus, as previously reported, the activity of MGA1142 with tBOOH as a substrate is approximately 2 orders of magnitude greater than the activity with H_2O_2 (Fig. 5A and 5C). Unlike the results for previously described Ohr proteins, the degradation profile for CuOOH differed substantially from that for tBOOH (Fig. 5A and 5B). The specific activity of MGA1142 at a concentration of 10 ng/μl was the same for tBOOH and CuOOH during the first minute of catalysis (300 μM metabolized in 1 min); however, the kinetics of the reaction indicate that the capacity of this enzyme to degrade CuOOH decreased rapidly after this point. These data suggest that while CuOOH was able to act as a substrate for MGA1142, the enzyme was rapidly inactivated in the presence of this substrate. We hypothesized that either the structure or redox state of MGA1142 was altered during catalysis of CuOOH, inhibiting enzyme regeneration. A nonreducing SDS-PAGE analysis of peroxide-treated MGA1142 supported this hypothesis, with tBOOH- and CuOOH-treated MGA1142 displaying differential migration (Fig. 6). It has been demonstrated previously that purified Ohr migrates differently on SDS-PAGE gels depending on the redox state of the protein (8, 31).

TABLE 1. Sensitivity of *M. gallisepticum* to peroxide species

Peroxide species	Zone of inhibition (mm)
H_2O_2	8 ± 1.5
tBOOH	1.25 ± 0.5
CuOOH	10.5 ± 2.5

Structure of MGA1142. For comparison with existing Ohr X-ray diffraction structures, the quaternary structure of MGA1142 was constructed using computer modeling. Overall, the MGA1142 structure is similar to that of other members of the Ohr subfamily, with a positional root mean square deviation of 2.5 Å compared with the *P. aeruginosa* Ohr X-ray structure (calculated with DALI) (15). Two monomeric subunits intertwine to form a tightly packed homodimer (Fig. 7). The two large central helices were the helices identified by TMPred as putative transmembrane regions. The MGA1142 dimer, which was measured to be approximately 65 Å long, is slightly more elongated than other Ohr proteins (~60 Å) (Fig. 8A to C) and is more comparable to MPN625 belonging to subgroup III (~63 Å) (Fig. 8D).

Active sites are present on either side of the molecule and are formed by the two invariant cysteine residues residing within a hydrophobic pocket. In the MGA1142 model, the cysteines form a disulfide bridge, representing the inactive (oxidized) state of the enzyme. This conformation results from the modeling algorithm which predicts the disulfide form due to the spatial proximity of the two cysteine residues. As the model represents the oxidized form of the enzyme, the conserved arginine residue (Arg16) normally responsible for deprotonating the reactive cysteine (Cys58) is flipped outwards

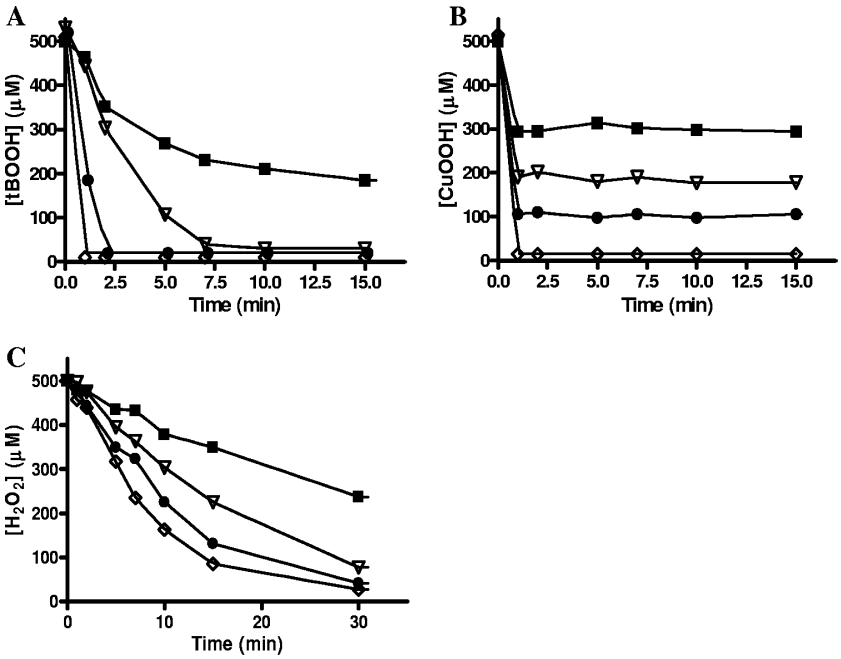

FIG. 5. Peroxidase activities of various concentrations of purified recombinant MGA1142 in the presence of different peroxide species and 0.5 mM DTT, as determined by the FOX assay. (A) tBOOH is rapidly degraded in an MGA1142 concentration-dependent (■, 5 ng/μl; ▽, 7.5 ng/μl; ●, 10 ng/μl; ◇, 20 ng/μl) and time-dependent manner. (B) CuOOH degradation profile is also dependent on the MGA1142 concentration (■, 5 ng/μl; ▽, 10 ng/μl; ●, 15 ng/μl; ◇, 20 ng/μl), but in each case catalysis ceases within 1 min of addition of CuOOH. (C) Degradation of H_2O_2 proceeds much more slowly and requires higher concentrations of MGA1142 (■, 50 ng/μl; ▽, 100 ng/μl; ●, 150 ng/μl; ◇, 200 ng/μl).

and in this respect the structure is comparable to the structure of Ohr from *Deinococcus radiodurans*, which was crystallized in the oxidized form (Fig. 8B). The conserved glutamate residue (Glu48), which in other Ohr proteins is known to form a stabilizing salt bridge with the arginine when the enzyme is reduced (22), is in the correct position to fulfil this function (Fig. 8A and C).

A major difference observed between the MGA1142 model and previously described Ohr structures is the size and shape of the active site pocket (Fig. 8A and C). Like the active sites of other Ohr proteins (22, 26, 31), the active site of MGA1142 is lined with numerous hydrophobic residues; however, in MGA1142 this region appears to be wider and more open. The relative size and shape of the active site of MGA1142 are most comparable to those of MPN625 (Fig. 8D), a protein from *M. pneumoniae* which is homologous to MGA252 and belongs to OsmC subgroup III. While the function of MPN625 has not been determined yet, this protein is predicted to possess peroxidase activity because of the overall structural similarity to other members of the OsmC superfamily and because of the position of the cysteine residues (6, 26, 41). The size of the

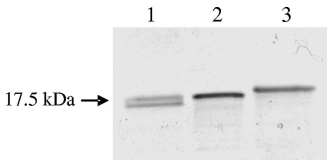

FIG. 6. One-dimensional SDS-PAGE gel showing the difference between the migration of MGA1142 treated with tBOOH and with CuOOH. Untreated MGA1142 formed a doublet due to the presence of multiple oxidation states (lane 1). Treatment of MGA1142 with 25 mM tBOOH (lane 2) or 25 mM CuOOH (lane 3) resulted in a single protein band in each case, but MGA1142 treated with CuOOH migrated slower than MGA1142 treated with tBOOH, indicating that there was a structural or redox shift.

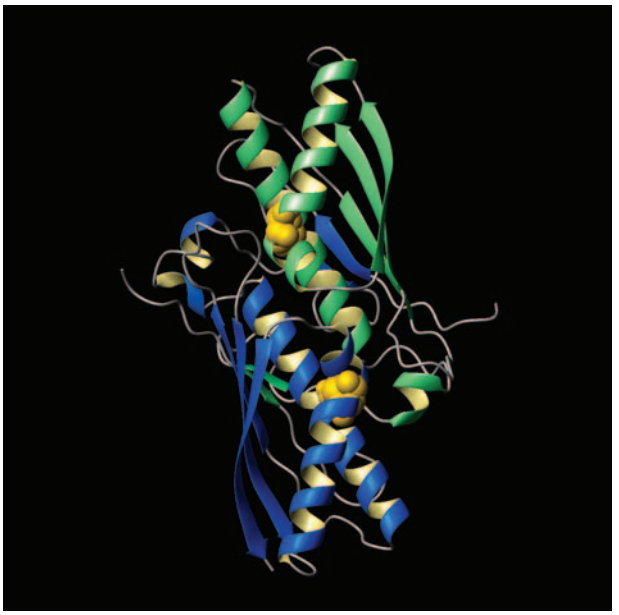

FIG. 7. Cartoon showing the structure of the MGA1142 homodimer produced by comparative modeling. The individual monomers represented by blue and green ribbons are tightly intertwined. Each monomer contains an intramolecular disulfide bridge formed by the active site cysteine residues (yellow spacefill), representing the inactive (oxidized) form of the enzyme.

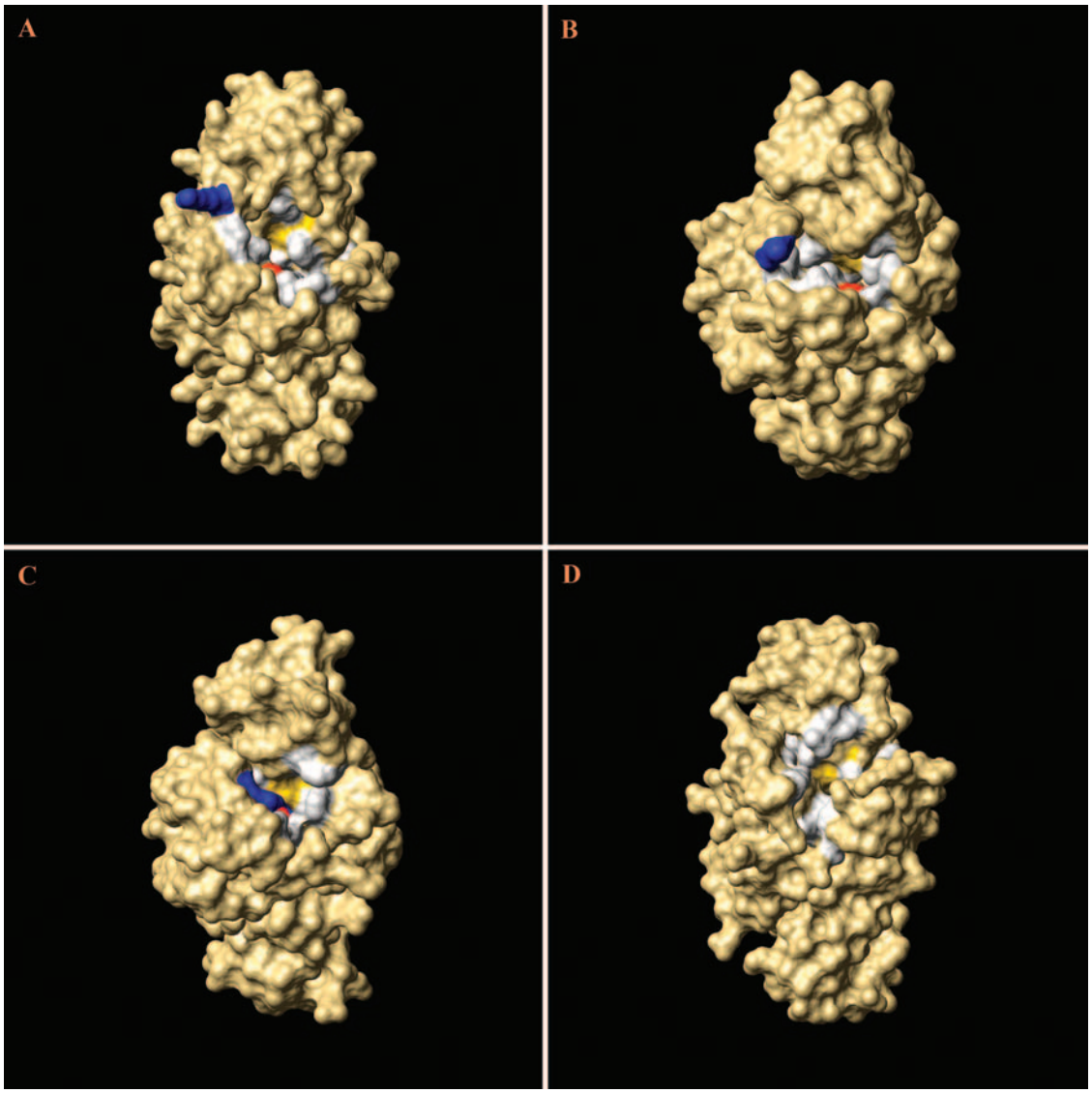

FIG. 8. Surface-rendered images of quaternary structures of Ohr proteins from *M. gallisepticum* (A), *D. radiodurans* (B), and *P. aeruginosa* (C) and a member of OsmC subfamily III from *M. pneumoniae* (MPN625) (D). Residues relevant to catalysis are indicated by different colors; arginine is blue, glutamic acid is red, cysteines are yellow, and hydrophobic regions are white. In panels A and B the conserved arginine residue is flipped outwards, representing the inactive form of the enzyme, while in panel C it is folded inwards toward the reactive cysteine and is stabilized by a hydrogen bond with the glutamic residue at the base of the catalytic cleft. Other notable differences between the four structures are the slightly more elongated shape of MGA1142 and MPN625 and the differences in the conformation of the hydrophobic pocket. Images were obtained using MOLMOL (20).

active site region in these molecules implies that they accommodate a larger substrate than other Ohr proteins.

DISCUSSION

In this study we characterized an Ohr protein of *M. gallisepticum* (MGA1142), the first peroxidase to be described in a *Mycoplasma* species. Our phylogenetic analyses indicate that the *mga1142* gene, previously annotated as encoding OsmC, actually encodes a member of the Ohr subgroup, a class of proteins that are found exclusively in microorganisms and are known to be particularly widespread among pathogens (3). It is notable that within the genus *Mycoplasma*, only members of the *M. pneumoniae* phylogenetic cluster contain Ohr homologs, highlighting the importance of the oxidative stress response in these ROS-producing organisms.

In contrast to other organisms possessing Ohr proteins, in *M. gallisepticum* the enzyme is not upregulated in the presence of peroxides but is expressed constitutively. It has been noted previously that in *D. radiodurans*, the basal levels of Ohr expression are also quite high; however, in this organism gene expression is still substantially induced by exposure to organic peroxides (3). In the vast majority of Ohr-containing bacteria, *ohr* gene expression is under the control of OhrR, which binds to the promoter region of *ohr* (OhrR box) and represses transcription (7, 32, 43). Under oxidative stress conditions, OhrR

becomes oxidized and is unable to bind to the OhrR box, resulting in derepression of *ohr* expression (13, 43). A search of the *M. gallisepticum* genome sequence failed to locate any identifiable OhrR homologs, and accordingly, no OhrR box was observed in the promoter region of *mga1142*. These data explain why *M. gallisepticum* is unable to upregulate *ohr* transcription in the presence of organic peroxides and instead exhibits constitutive expression. Quantitative regulation (as opposed to on/off regulation) of genes in *Mycoplasma* species is predicted based on their lack of typical sensor-regulator systems, as well as their obligate parasitism, meaning that they encounter relatively limited variation in environmental conditions (29). This appears to be the case for *mga1142* transcription, which is positively regulated (approximately twofold upregulation) under ethanol stress conditions and negatively regulated under osmotic stress conditions. This pattern of regulation is unique among the Ohr proteins studied thus far. Indeed, upregulation under ethanol stress conditions has been reported only for members of the OsmC subfamily, although OsmC is typically upregulated not only under ethanol stress conditions but also in response to osmotic shock (as opposed to the downregulation observed for MGA1142) and growth phase (3, 9, 29). OsmC expression is complex and is regulated by either NhaR, RcsB, or σ^S (during stationary phase) in *E. coli* (42) and by σ^B in *Bacillus subtilis*. In contrast, we found that MGA1142 was not upregulated during stationary phase (data not shown), and a search of the *M. gallisepticum* genome failed to reveal genes for σ^S, σ^B, NhaR, or RcsB. Therefore, while *M. gallisepticum* clearly possesses a mechanism for quantitative regulation of *mga1142*, it does not appear to use any of the typical Ohr or OsmC response regulators and therefore warrants further investigation. One possibility is that *mga1142* is regulated with an alternative sigma factor. While mycoplasmas are traditionally thought to lack all but the housekeeping sigma factor (σ^{70}), *M. gallisepticum*, along with some other members of the genus *Mycoplasma*, does contain a distant homolog of the alternative sigma factor encoded by *rpoE* (σ^E) (29), a known stress response sigma factor (35) which has been shown to be linked to virulence (21, 39, 44). In addition, partial matches to the consensus sequences for the σ^E binding site were identified within the *mga1142* promoter, raising the question of whether this gene may be regulated by σ^E as part of a global stress response.

Why *mga1142* should be upregulated in the presence of ethanol is uncertain; however, a previous study reported that in several mycoplasmas, including *M. gallisepticum*, oxygen uptake is increased in the presence of ethanol (1). While these organisms do not appear to derive energy from ethanol oxidation, it has been noted for some mycoplasmas that H_2O_2 production increases under these conditions (2). Thus, rather than responding directly to host-generated oxidative challenge, *M. gallisepticum* may instead upregulate its organic hydroperoxidase in response to stimulation of its own peroxide production pathway. As lipid peroxidation of cell membranes is one of the major toxic effects of H_2O_2 (22, 28), it follows that organisms that accumulate H_2O_2 also encounter high concentrations of the more noxious lipid peroxides, requiring a detoxification mechanism.

MGA1142 preferentially degrades linear organic peroxide (tBOOH) over H_2O_2 with a twofold order of magnitude of efficiency. This characteristic has been noted for all previously described Ohr proteins and is attributed to the highly hydrophobic nature of the Ohr active site (8, 22). Molecular modeling has demonstrated that the active site of MGA1142 is also rich in hydrophobic amino acids, accounting for the observed substrate preference. These data correlate with the results of the disk inhibition assay and the whole-cell FOX assay, which revealed that *M. gallisepticum* is more sensitive to H_2O_2 than to tBOOH. This finding is interesting given the known capacity of *M. gallisepticum* to produce H_2O_2 as a virulence mechanism (4, 47) and suggests that *M. gallisepticum* may lack specific enzymes for detoxifying inorganic peroxide. It has previously been suggested that mycoplasmas may rely heavily on cellular repair systems, such as methionine sulfoxide reductase (MsrA) (10) and thioredoxin (Trx) (5), to cope with oxidative challenge, and these systems may be among the mechanisms that *M. gallisepticum* uses to manage the effects of H_2O_2. Despite the presence of these repair enzymes in *M. gallisepticum*, the marked sensitivity to H_2O_2 of in vitro-grown cells indicates that this organism may rely on host cell catalase for detoxification (47) or that, as in the case *Mycoplasma mycoides* subsp. *mycoides* small colony, the proximity of the bacterium to host cells may result in specific targeting of the oxidative damage (33).

An unexpected result of our enzymatic assays was the degradation profile for CuOOH, an aromatic hydroperoxide, compared with that for tBOOH, a linear hydroperoxide. The Ohr proteins studied previously all confer resistance to both tBOOH and CuOOH (3, 28, 30, 40) and degrade them with equal efficiencies over time (8, 22). In contrast, MGA1142 appears to metabolize CuOOH with only slightly reduced efficiency compared with tBOOH metabolism over the first minute of catalysis but then becomes rapidly inactivated. Interestingly, a similar phenomenon has been observed for the type II peroxiredoxin from poplar, which was shown to be inactivated by CuOOH after approximately 20 s of catalysis (37). The distinct migration pattern on SDS-PAGE gels of MGA1142 treated with CuOOH, compared with MGA1142 treated with tBOOH, suggests that CuOOH alters either the structure or redox state of MGA1142. While the nature of the changes is not clear, it is proposed that they must inhibit enzyme turnover. Indeed, it has been shown previously that overoxidation of the reactive cysteine of Ohr from the mildly oxidized sulfenic acid form (-SOH) to the sulfonic acid form ($-SO_3H$) results in enzyme inactivation (31). Further clues that enzyme inactivation may result from overoxidation come from examining the active site pocket of MGA1142, which adopts a relatively open conformation. This conformation would be expected to leave the reactive cysteine residue (Cys58) more exposed to the solvent and therefore more prone to overoxidation. Together with the results of the disk inhibition and whole-cell FOX assays, which showed that *M. gallisepticum* has marked sensitivity to CuOOH but not to tBOOH, these data suggest that aromatic hydroperoxides are not the natural substrate for MGA1142. In this regard, it is notable that MGA1142 has a narrower substrate preference than previously characterized Ohr proteins and indicates that there has been evolutionary specialization in *M. gallisepticum*. As an obligate parasite, *M. gallisepticum* resides in a very restricted host environment and would be expected to encounter a specific subset of peroxide species. Based on the preference of MGA1142

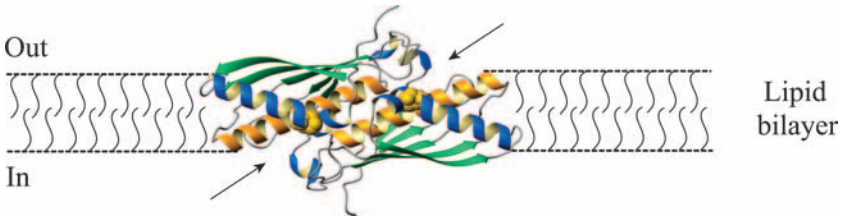

FIG. 9. Schematic diagram showing how MGA1142 may be oriented in the cell membrane. Portions of the N and C termini are exposed to the surface, while the two long central helices of each monomer (orange) span the membrane, as predicted by TMpred. In this orientation, the active site pockets (with catalytic cysteines indicated by yellow spacefill) can be accessed from either side of the membrane (arrows).

for linear hydroperoxide, along with the relative size of its hydrophobic pocket, it is surmised that the peroxide species degraded by MGA1142 are probably large lipid hydroperoxides. Indeed, the ability of at least one Ohr protein to protect against the effects of lipid hydroperoxide has already been demonstrated (19).

While the subcellular location of the OsmC family of proteins has been the subject of speculation (3, 23), this question has not been investigated experimentally. Members of the OsmC subgroup have been described previously as "putative envelope proteins" (42, 46) due to the presence of predicted transmembrane regions which also correlate to the large central helices of Ohr proteins. While *M. gallisepticum* lacks both a cell envelope and a cell wall, we provide evidence that in this organism Ohr is present in both the intracellular and membrane fractions, indicating that this protein is available to detoxify both endogenous peroxides (such as the products of metabolism and self-generated ROS) and exogenous peroxides (forming part of the host immune response to infection). Furthermore, the three-dimensional model of MGA1142, combined with the finding that this protein resides in the cell membrane with portions of both the N and C termini exposed to the surface, enabled us to establish a hypothetical model for how this protein is positioned within the membrane (Fig. 9). In this model, the dimer is rotated such that the predicted transmembrane helices from each monomer span the membrane, leaving the N terminus and portions of the C-terminal beta sheets exposed to both the inner and outer cell surfaces. In this orientation, the two active site pockets are open to the inner and outer areas of the cell membrane. Such an orientation would be ideal for scavenging peroxide species which come into contact with the membrane (from both internal and external sources), one of the major sites of oxidative damage (22, 28).

The identification of a peroxidase in *M. gallisepticum* may have important implications for understanding the pathogenesis of this organism. The apparent absence of toxins in *Mycoplasma* species has led researchers to speculate that the release of peroxides and other ROS is the primary virulence mechanism of these bacteria (47). In this regard, MGA1142 must be considered an excellent candidate for a vaccine target, as it likely represents one of the major strategies for protection against peroxides from both endogenous and exogenous sources. Indeed, the ability of this protein to bind heparin, established in a previous study (17), indicates that MGA1142 may play a multifactorial role in pathogenesis. Generation of an MGA1142 mutant is clearly desirable to determine if such a strain has attenuated virulence.

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