



Reduction of Hydrogen Peroxide Accumulation and Toxicity by a Catalase from *Mycoplasma iowae*

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Abstract

Mycoplasma iowae is a well-established avian pathogen that can infect and damage many sites throughout the body. One potential mediator of cellular damage by mycoplasmas is the production of H₂O₂ via a glycerol catabolic pathway whose genes are widespread amongst many mycoplasma species. Previous sequencing of *M. iowae* serovar I strain 695 revealed the presence of not only genes for H₂O₂ production through glycerol catabolism but also the first documented mycoplasma gene for catalase, which degrades H₂O₂. To test the activity of *M. iowae* catalase in degrading H₂O₂, we studied catalase activity and H₂O₂ accumulation by both *M. iowae* serovar K strain DK-CPA, whose genome we sequenced, and strains of the H₂O₂-producing species *Mycoplasma gallisepticum* engineered to produce *M. iowae* catalase by transformation with the *M. iowae* putative catalase gene, *katE*. H₂O₂-mediated virulence by *M. iowae* serovar K and catalase-producing *M. gallisepticum* transformants were also analyzed using a *Caenorhabditis elegans* toxicity assay, which has never previously been used in conjunction with mycoplasmas. We found that *M. iowae katE* encodes an active catalase that, when expressed in *M. gallisepticum*, reduces both the amount of H₂O₂ produced and the amount of damage to *C. elegans* in the presence of glycerol. Therefore, the correlation between the presence of glycerol catabolism genes and the use of H₂O₂ as a virulence factor by mycoplasmas might not be absolute.

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Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All *Mycoplasma iowae* genome sequence files are available from the DDBJ/EMBL/GenBank database (accession number AWQU00000000).

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Introduction

Mycoplasma iowae is an economically important avian mycoplasma primarily associated with turkeys but also occasionally found in chickens [1]. Naturally occurring infections in turkeys commonly result in late embryo mortality, leading to a 2–5% decrease in hatchability, and leg abnormalities in offspring [2–4]. Experimental infections give rise to airsacculitis, stunting, poor feathering, and/or leg and joint problems [3,4]. *M. iowae* can be recovered from the respiratory tract, gastrointestinal tract, spleen, and kidneys of orally infected day-old poults, suggesting invasiveness [5]. Atypically among cultured mycoplasmas, *M. iowae* exhibits a predilection for the intestinal tract. Yolk sac inoculation of eight-day old turkey embryos results in intestinal binding and colonization [6]. Oral inoculation of day-old poults also results in intestinal colonization with no signs of disease, with mycoplasmas isolated more frequently from the intestinal walls than its contents, for at least 21 d after inoculation [5].

Phylogenetically, *M. iowae* is in the *Mycoplasma muris* cluster within the pneumoniae group, with *Mycoplasma penetrans* as its

best-characterized relative [7]. *M. iowae* has a terminal organelle with an internal cytoskeleton; this organelle attaches to host cells and is the leading cell pole during gliding motility [6,8]. *M. iowae* is one of two known avian mycoplasmas, along with *Mycoplasma lipofaciens*, that can obtain energy from catabolism of both glucose and arginine [9,10]. Despite considerable understanding of disease pathology resulting from *M. iowae* infection, as well as some knowledge about its cellular biology and biochemistry, the virulence factors of *M. iowae* are unidentified.

A draft genome sequence for *M. iowae* serovar I strain 695 [11] suggests some potential primary virulence factors, including two copies of genes for proteins related to an ADP-ribosylating and vacuolating toxin characterized in *Mycoplasma pneumoniae* [12] as well as genes for production of H₂O₂ through glycerol catabolism, a pathway that is widespread among mycoplasmas. In this pathway glycerol is imported into the cell via either the glycerol facilitator GlpF or an ABC transporter, phosphorylated by GlpK, and finally oxidized to dihydroxyacetone phosphate by GlpO (also known as GlpD in some species), using O₂ and resulting in production of H₂O₂ [13,14]. The H₂O₂ generated by

GlpO can cause cellular damage either directly or through altering host gene expression [15]. H₂O₂ has been described as a virulence factor for several pathogenic mycoplasmas, including *Mycoplasma mycoides* subsp. *mycoides*, *Mycoplasma pneumoniae*, and *Mycoplasma pulmonis*. Onset time of pneumonia caused by *M. pulmonis* infection is shortened in mice lacking catalase activity, implicating H₂O₂ [16]. A *glpD* mutant of *M. pneumoniae* produces less H₂O₂ and causes less cytotoxicity than wild-type cells [14]. An *M. mycoides* strain that lacks components of the glycerol ABC transporter produces less H₂O₂ and causes disease with delayed onset and decreased severity in experimentally infected cattle as compared with a strain that has all the transport [17–19]. Several enzymes have been implicated in reversing H₂O₂-mediated damage to mycoplasma cells [20,21], but direct degradation of H₂O₂ has not been described for these organisms.

It is unclear how well the existence of the glycerol catabolic pathway correlates with actual use of H₂O₂ as a virulence factor by mycoplasmas. The *M. iowae* serovar I genome sequence indicates the presence of *glpF*, *glpO*, and *glpK* [11], which could be taken to support a role for H₂O₂ in virulence of this organism. Thus far uniquely among mycoplasmas, it also contains a gene encoding a putative catalase, an enzyme that degrades H₂O₂, potentially interfering with H₂O₂-mediated virulence. In this report, we present the genome sequence of *M. iowae* serovar K strain DK-CPA, which also contains both the glycerol catabolism genes and the catalase gene, which we designate *katE*. The activity of the *katE* gene product as a catalase was tested by assaying for catalase activity in both *M. iowae* serovar K and *katE* transformants of *Mycoplasma gallisepticum*, a prolific H₂O₂ producer. All strains were also analyzed for the impact of *M. iowae* catalase on H₂O₂ production and resulting virulence via *Caenorhabditis elegans* toxicity assays modified for use with mycoplasmas. Our results, which constitute the first account of an active catalase in mycoplasmas, allow us to address the question of H₂O₂ as an *M. iowae* virulence factor.

Materials and Methods

Bacterial strains and growth conditions

Strains used in this study include *M. iowae* serovar K strain DK-CPA, *Mycoplasma genitalium* strain G37, *M. gallisepticum* strain R_{low}, and *M. gallisepticum* transformants 55A–C and 56 A and C. All strains were grown at 37°C in 175-cm² tissue culture flasks containing 50 mL SP-4 media [22] to mid-log phase (orange color). Transformants 55A–C and 56 A and C were grown in the presence of 4 µg mL⁻¹ tetracycline. *Escherichia coli* strain DH5α was used for cloning and was grown in Luria-Bertani broth in the presence of 100 µg mL⁻¹ ampicillin or 4 µg mL⁻¹ tetracycline.

Genome sequencing and analysis

Genomic DNA from *M. iowae* serovar K was sequenced at the Ohio State University Plant-Microbe Genomics Facility using the GS FLX system (454 Life Sciences). It was prepared with the GS FLX Titanium Rapid Library Preparation Kit (Roche) and sequenced using GS FLX Titanium Sequencing Kit XLR70. Shotgun sequencing data were assembled with the GS *De Novo* Assembler version 2.5.3 (Roche). Annotation was performed as previously described [23]. The draft genome project has been deposited at DDBJ/EMBL/GenBank under accession number AWQU00000000. The draft genomes of serovars K and I were compared using wgVISTA [24].

Catalase sequence analysis

The predicted amino acid sequence of *M. iowae* serovar K catalase was subjected to PSI-BLAST [25]. Matches reported as having e values of 0 were aligned by CLUSTALX 2.1 [26] using default parameters and the phylogram was inspected using NJplot [27]. Sequences that clustered separately from the group that included *M. iowae* catalase were removed and the remaining sequences were again subjected to alignment and tree generation. Secondary structure was predicted using SOPMA [28].

Detection of catalase activity

Cells from mid-log phase cultures of *M. iowae*, *M. gallisepticum*, and *M. gallisepticum* transformants were collected by centrifugation at 20,000×g for 20 min and washed three times with cold phosphate-buffered saline (PBS). Cells were smeared onto a clean microscope slide and one drop of 3% H₂O₂ was added. Catalase activity was indicated by the generation of bubbles.

To analyze H₂O₂ degradation, *M. iowae* and *M. genitalium* were grown in 24-well plate wells containing 1 mL of SP-4 media supplemented with 3% glycerol. Upon reaching mid-log phase, 1 mL of SP-4 containing 3% glycerol or mid-log phase *M. iowae* culture was added to *M. genitalium* culture and the amount of H₂O₂ remaining was measured over time using colorimetric test strips (EM Quant, range 0.5–25 mg L⁻¹). Statistical analysis of results was calculated using repeated measures ANOVA and unpaired Student's T-test. Results represent three biological replicates with one technical replicate each.

Construction of plasmids and transformation with *M. iowae katE*

To amplify *M. iowae katE* with a *SalI* cloning site with or without the addition of a C-terminal 6xHis tag, primers upstream (5'-ATCGGTTCGACAAATGCTGCAACAGCTGCAC-3') and downstream (5'-ATCGGTTCGACTAAACACAAAATTTGATT-TAATC-3' or 5'-ATCGGTTCGACTTAATGATGGT-GATGGTGGTGACCATATGCGTTTAAATGGCAAGGT-3', respectively) were synthesized (Fisher Scientific) and used for PCR with LongAmp Polymerase (NEB). Following addition of A overhangs using *Taq* polymerase (NEB), PCR products were ligated into linearized plasmid pCR2.1 (Invitrogen) and transformed into chemically competent *E. coli* cells. Sequencing at the Miami University Center for Bioinformatics and Functional Genomics confirmed 100% sequence identity to *M. iowae katE*. The resulting plasmids were named pOO54 and pOO53, respectively. These plasmids were digested with *SalI* and the resulting 1.8-kb DNA fragments were ligated together with pTF20 [29] that had been linearized using *SalI*. The resulting plasmids were named pOO56 and pOO55, respectively.

To produce *M. gallisepticum katE* transformants, electrocompetent *M. gallisepticum* cells were transformed with pOO55 and pOO56 as previously described [30]. Transformants were plated on SP-4 plates containing 4 µg mL⁻¹ tetracycline for selection. Transformants were subjected to three rounds of filter cloning and more than one transformant from each series (named 55A–C and 56 A and C) was analyzed to ensure that results observed were not due to polar effects from the chromosomal transposon insertion site.

H₂O₂ assay

Methods were adapted from Hames *et al.* [14]. Fifty-mL cultures of mycoplasmas were grown to mid-log phase. Cells were collected by centrifugation at 20,000×g for 20 min and washed three times with cold buffer containing 67.6 mM HEPES,

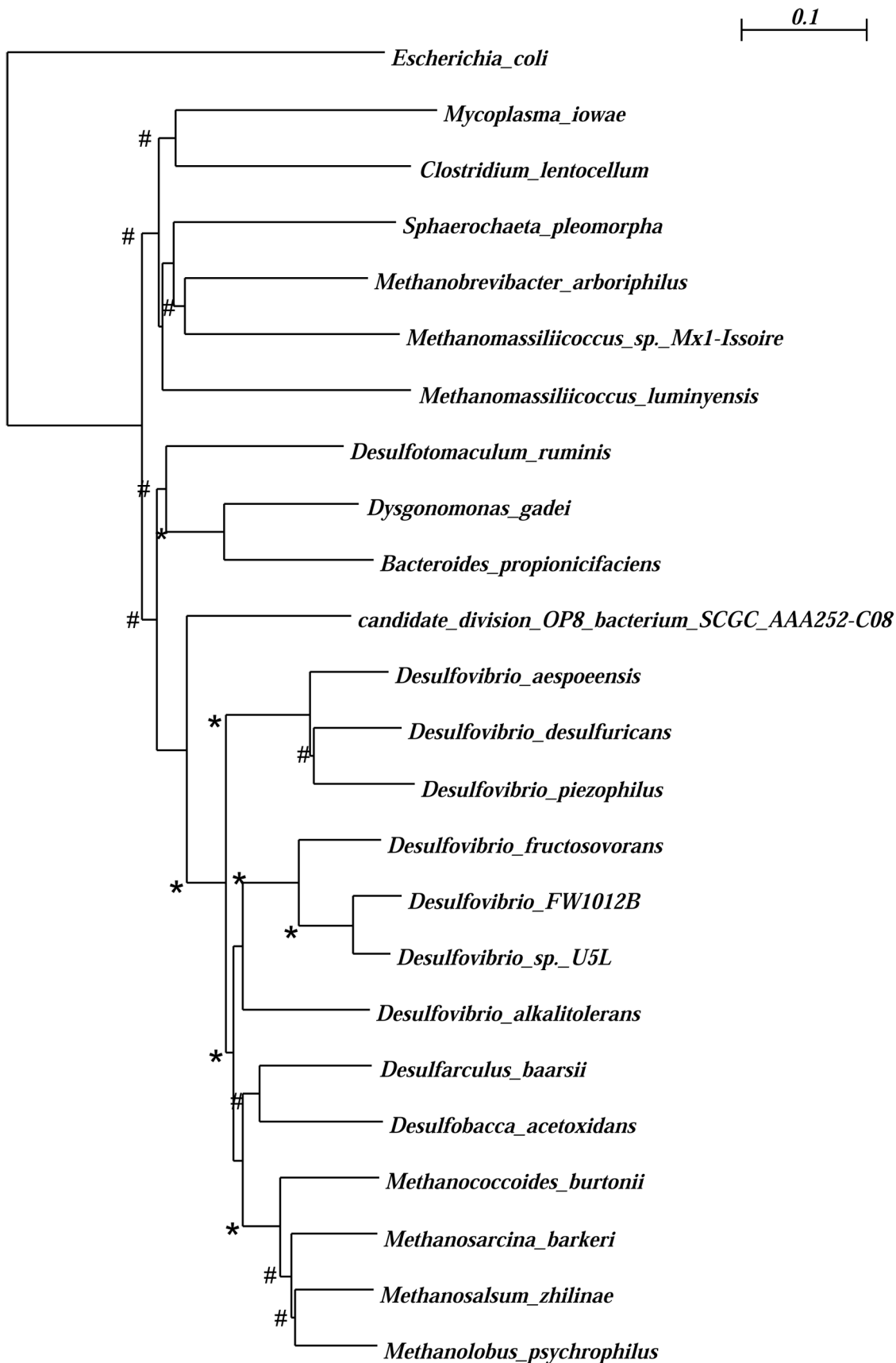


Figure 1. Phylogenetic tree of *M. iowae* serovar K catalase and close relatives, with *Escherichia coli* *katE* as an outgroup. All amino acid sequences included have an e value reported as 0 by PSI-BLAST with respect to *M. iowae* catalase. Scale bar, 0.05 substitutions per site. Bootstrap values out of 1000 trials are reported at nodes.
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140 mM NaCl, and 7 mM MgCl₂. Following resuspension to an OD₅₅₀ of 1.0, 1-mL aliquots were placed in microcentrifuge tubes and incubated at 37°C for 1 h. 100 μM glycerol was added to tubes and samples were incubated at 37°C for an additional 2 h. H₂O₂ levels were measured using colorimetric test strips. All analyses were performed in quadruplicate. Median and median absolute deviations values were calculated. Statistical significance of results was calculated using one-way ANOVA and Tukey's *post hoc* test. Results represent three biological replicates with at least two technical replicates each.

Preparation of cell extracts

Fifty-mL cultures of mycoplasma cells were collected by centrifugation at 20,000×g for 20 min and washed three times with cold PBS. The resulting cell pellet was resuspended in 1 mL cold PBS containing 1% sodium dodecyl sulfate and incubated at 37°C for 30 min. Protein concentration in cell lysates was determined using BCA assays (Pierce).

Western blot analysis

Five μg of cell lysates was separated on a 10% sodium dodecyl sulfate-polyacrylamide gel. Following electrophoresis, proteins were transferred to a nitrocellulose membrane (GE) overnight at 100 mAmp. Membrane was probed with anti-6XHis primary antibodies (Immunology Consultants Laboratory, Inc.) at a dilution of 1:1,000 followed by anti-rabbit immunoglobulin G-alkaline phosphatase secondary antibodies (Promega) at a dilution of 1:7,500. Bands were visualized with 5-bromo-4-chloro-3-indolyl phosphate and nitro-blue tetrazolium.

PCR analysis

Primers were designed to encompass *M. gallisepticum glpF*, *glpO*, and *glpK* to confirm absence of transposon insertion at this locus. Primers (5'-TCAAGTTCTGCTAGTAGCGG-3' and 5'-AATGTATCAGATCACGACC-3') were synthesized (Fisher Scientific) and used with *Taq* polymerase for PCR with chromosomal DNA. PCR products were subjected to agarose gel electrophoresis and transformants were compared to *M. gallisepticum* R_{low}.

Caenorhabditis elegans growth conditions

All assays were performed with *C. elegans* N2 (Bristol). Nematodes were cultivated using standard practices [31]. Briefly, worms were cultivated on Nematode Growth Media (NGM) plates seeded with *E. coli* strain OP50 as a food source at room temperature on the bench top.

C. elegans killing assays

Plates containing many large, gravid nematodes were bleached to obtain sterile eggs using standard procedures [31]. Eggs were hatched overnight in 10 mL of M9 buffer to obtain L1 larvae. Following incubation with 1 mM glycerol for 5–6 h, L1 larvae were washed with and resuspended in M9 buffer, and approximately 20–40 larvae were aliquotted into 24-well plate wells. The number of live worms per well (indicated by the presence of movement) was counted prior to adding any additional sample. Plates were incubated at room temperature on the bench top for 24 h and live worms were counted to measure survival. Worms were considered dead if they showed no movement in response to shaking of the plate.

To determine the susceptibility of worms to H₂O₂ using our assay conditions, L1 larvae were incubated with 1 mL of buffer containing 67.6 mM HEPES, 140 mM NaCl, and 7 mM MgCl₂

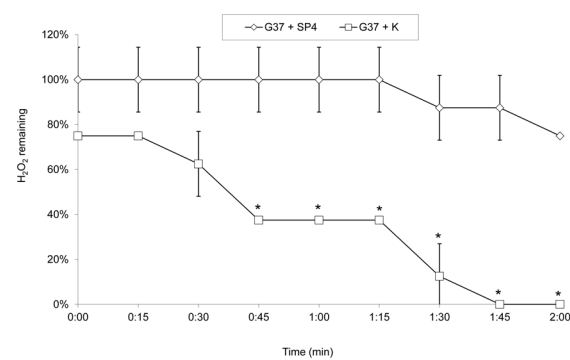


Figure 2. Degradation of H₂O₂ produced by an exogenous source of by *M. iowae*. H₂O₂ present in a mid-log phase culture of *M. genitalium* G37 was measured following the addition of an equal amount of SP-4 media (◇) or mid-log phase *M. iowae* serovar K culture (□). Error bars, SD. *, *p*<0.05 compared to control as determined by Student's T-test.

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and various concentrations of H₂O₂. Survival was measured at 24 h as described above. Statistical significance of results was calculated using one-way ANOVA. Results represent three biological replicates with at least two technical replicates each.

Mycoplasma samples were processed as described for hydrogen peroxide assays. Following initial 1-h incubation at 37°C, 1-mL samples were added to 24-well plate wells containing counted nematodes and glycerol. Worm survival was measured after 24 h incubation at room temperature. Conditions were optimized for maximum H₂O₂ production at room temperature by varying the OD₅₅₀ of *M. gallisepticum* R_{low} and concentrations of glycerol. The concentration of H₂O₂ under these optimized conditions was established at 2-h intervals for each strain using the colorimetric test strips. Final nematode killing assays were performed with mycoplasmas at an OD₅₅₀ of 0.75 in the presence or absence of 1 mM glycerol. Statistical significance of results was calculated using one-way ANOVA with Tukey's *post hoc* test and unpaired Student's T-test. Results represent three biological replicates with at least two technical replicates each.

Results

Analysis of *M. iowae* serovar K for catalase activity

We obtained a draft genome sequence of *M. iowae* serovar K, isolated from a turkey embryo [32]. The genomes of serovars K and I, which was isolated from a turkey air sac [32], are well conserved, with >99% nucleotide sequence conservation in most genes, and more substantive differences in genes encoding predicted lipoproteins and some hypothetical proteins. Differences in gene content are almost completely restricted to restriction systems, transposase fragments, and lipoprotein paralogs, plus the deletion in serovar K of two putative glycoconjugate synthesis genes.

glpF, *glpK*, and *glpO* were present, constituting a complete pathway for assimilation of free glycerol and H₂O₂ production. However, *glpU* and *glpQ*, which import and break down host glycerophospholipids to glycerol-3-phosphate, the substrate for GlpO [33,34], were absent. These findings suggest that *M. iowae* cannot use host cell phospholipids as a source of glycerol, leaving free glycerol, perhaps derived from host cells and/or other microflora, as the only source of substrate for GlpO. Although the genome had a typical complement of mycoplasma genes associated with antioxidant functions, including genes encoding

Table 1. Catalase activity and H₂O₂ production by *M. iowae* serovar K, *M. gallisepticum* R_{low}, and *M. gallisepticum* 55 and 56 series transformants containing *M. iowae* *katE*.

Strain	Catalase activity	H ₂ O ₂ production (mg L ⁻¹)	
		100 μM glucose	100 μM glycerol
<i>M. iowae</i> serovar K	+	0±0*	0±0*
<i>M. gallisepticum</i> R _{low}	–	1.6±0.1	3.8±0.3
55A	+	0±0*	0±0*
55B	+	0±0*	0±0*
55C	+	0±0*	0.1±0.1*
56A	+	0±0*	0±0*
56C	+	0.3±0.3*	0.6±0.4*

**P*<0.05 compared to *M. gallisepticum* R_{low} H₂O₂ production with respective carbohydrate.
doi:10.1371/journal.pone.0105188.t001

lipid hydroperoxide peroxidase, glutathione peroxidase, thioredoxins and thioredoxin reductases, flavodoxin and flavodoxin reductase, peroxiredoxin, and an OsmC-like protein possibly functioning in organic hydroperoxide reduction, two other genes stood out. One was a gene for superoxide dismutase, which among mycoplasmas has been found only in *Mycoplasma haemofelis* and *Mycoplasma haemocanis* [35,36]. The other was a gene for catalase, which we call *katE*. It is present in both serovar K and

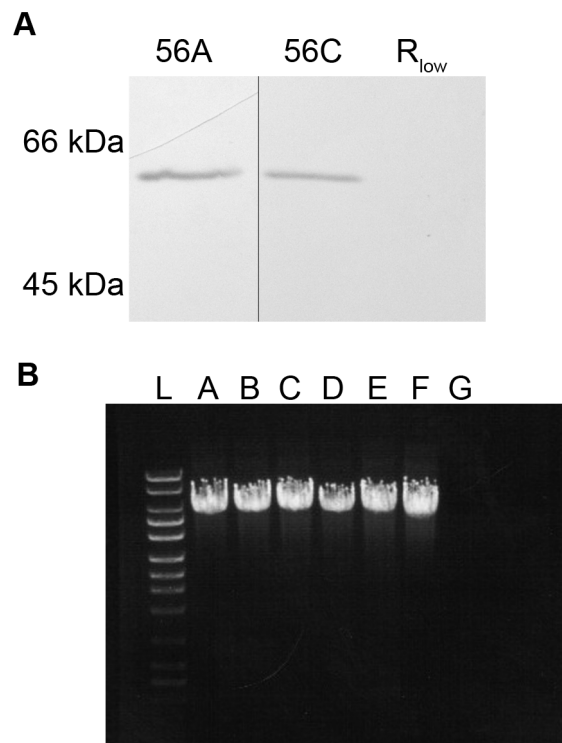


Figure 3. Production of catalase and integrity of glycerol metabolism operon in *M. gallisepticum* transformants. (a) Immunoblot of *M. gallisepticum* transformants 56A and 56C and *M. gallisepticum* R_{low} with anti-6X-His antibodies. (b) PCR products using primers for the putative *glpFOK* operon of *M. gallisepticum*. L, ladder with bands at 10, 8, 6, 5, 4, 3, 2.5, 2, 1.5, 1, 0.7, and 0.5 kb; A, *M. gallisepticum* R_{low}; B–D, *M. gallisepticum* transformants 55A–C; E–F, *M. gallisepticum* transformants 56A and C; G, *M. iowae* serovar K.
doi:10.1371/journal.pone.0105188.g003

the previously published serovar I [11], and the nucleotide sequence is nearly identical both in the coding region and the 434-bp non-coding region upstream. The predicted amino acid sequence for *M. iowae* serovar K catalase had 61% sequence identity to a homolog in the archaeon *Methanobrevibacter arboriphilus*, whose heme-dependent catalase activity has been characterized [37]. The predicted catalase protein contains all conserved amino acids of clade 3 heme-binding catalases as indicated on the Conserved Domain Database (CDD) [38], a group which it matches with an e-value of less than 10⁻¹⁰⁰. Its closest relatives are all found in anaerobic prokaryotes (Fig. 1), among which *Clostridium lentocellum* and *Desulfotomaculum ruminis* are the only members of the closely related *Firmicutes* lineage. The lack of a close relationship with catalases from most *Firmicutes* is more consistent with *M. iowae* having acquired *katE* by horizontal gene transfer from an anaerobe than having retained it during evolution from a common ancestor with Gram-positive bacteria. Sequence alignment reveals conservation of predicted secondary structural elements and active site residues (Fig. S1).

The presence of *katE* in *M. iowae* constitutes the first published account of a catalase gene in any mycoplasma species to date. Catalase activity was assessed in whole cells of *M. iowae* serovar K and *M. gallisepticum* strain R_{low}, which has no catalase gene. Upon the addition of 3% H₂O₂, *M. iowae* produced robust bubbling which was absent from *M. gallisepticum* (Table 1),

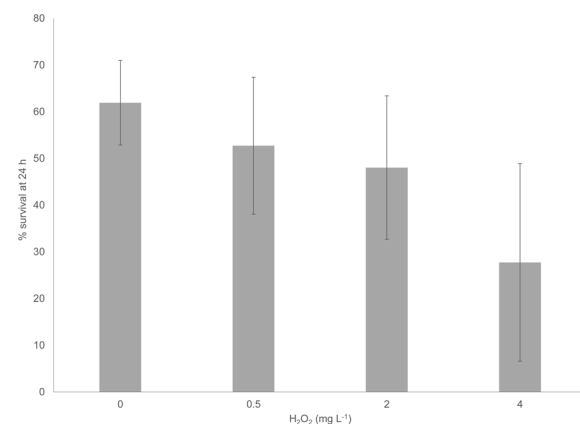


Figure 4. Survival of *C. elegans* upon exposure to increasing concentrations of H₂O₂. Error bars, SD.
doi:10.1371/journal.pone.0105188.g004

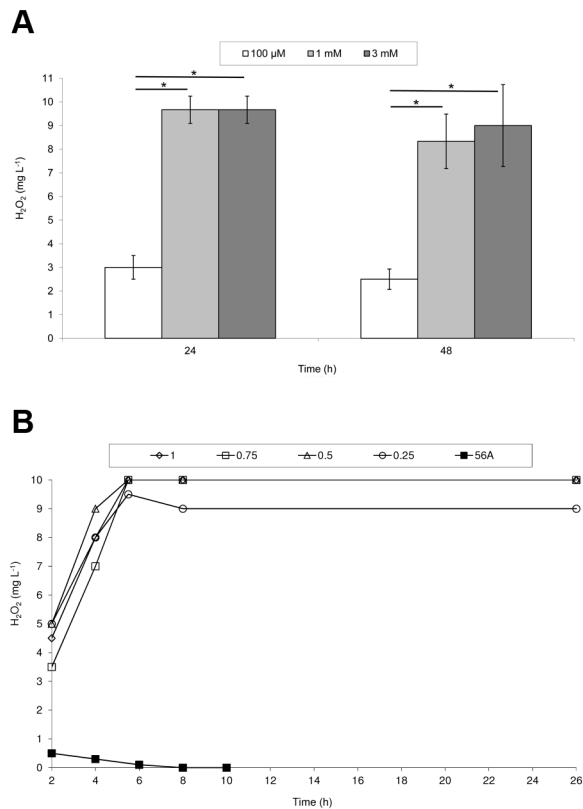


Figure 5. H₂O₂ accumulation by different amounts of *M. gallisepticum* with varying glycerol concentrations. (a) *M. gallisepticum* R_{low} at an OD₅₅₀ of 1.0 with 100 μM (white bars), 1 mM (light grey bars), or 3 mM (dark grey bars) glycerol. Experiments were performed in triplicate. *, $p < 0.05$ as determined by one-way ANOVA and Tukey's *post hoc* test. (b) *M. gallisepticum* R_{low} at an OD₅₅₀ of 1 (◇), 0.75 (□), 0.5 (Δ), or 0.25 (×) with 1 mM glycerol and *M. gallisepticum* catalase-producing transformant 56A at an OD₅₅₀ of 0.75 with 1 mM glycerol. Results are from one representative experiment. doi:10.1371/journal.pone.0105188.g005

consistent with the presence of catalase activity in only *M. iowae*. Furthermore, *M. iowae* was capable of breaking down H₂O₂ produced by *M. genitalium*, a species previously shown to be a robust H₂O₂-producer [39]. Cells of both species were grown to mid-log phase in media containing 3% glycerol, which stimulates H₂O₂ production [19,39]. Upon reaching mid-log phase, the cultures were combined and H₂O₂ concentration was monitored over time (Fig. 2). When an equal volume of *M. iowae* culture was added to the *M. genitalium* culture, the H₂O₂ concentration fell below detectable levels within 2 min. This decrease in the amount of H₂O₂ was not observed when warm SP-4 broth was added to *M. genitalium*. A repeated measures ANOVA revealed a significant difference between these two groups ($F(1,8) = 56.783$, $p < 0.05$), and significant differences were observed from 45 sec onward during the assay as determined by unpaired Student's T-test ($p < 0.05$). These data are consistent with catalase activity by *M. iowae*.

Expression of *M. iowae katE* in a hydrogen peroxide-producing mycoplasma

To test the role of *katE* in the catalase activity of *M. iowae*, *M. gallisepticum* was transformed with the gene either without or with a C-terminal 6X-His tag to create transformant series 55 and 56, respectively [30]. This species was chosen as the recipient due to its

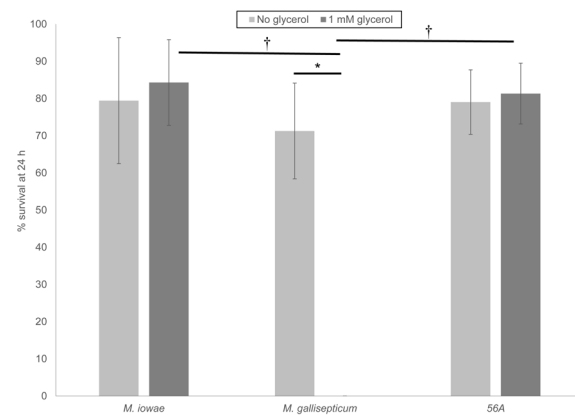


Figure 6. Toxicity of mycoplasmas to *C. elegans*. Toxicity was measured in the absence (light grey bars) and presence (dark grey bars) of 1 mM glycerol. Error bars, SD. *, $p < 0.05$ as determined by unpaired Student's T-test. † $p < 0.05$ as determined by one-way ANOVA and Tukey's *post hoc* test. doi:10.1371/journal.pone.0105188.g006

ability to produce H₂O₂ and lack of other known toxins [40,41]. We included the coding region of *katE* plus 239 nucleotides upstream of the start codon, anticipating that this region included a promoter, although no promoter sequences could be unambiguously identified by sequence inspection (not shown). Immunoblotting with anti-6X-His antibodies confirmed production by transformants of a protein migrating at approximately 60 kDa, the predicted size of *M. iowae* catalase, that was absent from untransformed *M. gallisepticum* (Fig. 3a). Addition of 3% H₂O₂ to all transformants resulted in bubbling, indicative of the presence of catalase activity (Table 1). Transformants were also analyzed for H₂O₂ production in the presence of 100 μM glucose or glycerol as previously described [14]. *M. iowae* produced no detectable H₂O₂ under any test conditions, including incubation with up to 3 mM glycerol (Table 1 and not shown). Although the parent strain produced robust H₂O₂ in the presence of 100 μM glycerol, all *katE* transformants displayed decreased H₂O₂ production, approaching or at undetectable levels. Individual transformants produced varying amounts of H₂O₂, but always less than the parent strain. There was a statistically significant difference between strains as determined by a one-way ANOVA ($F(7,21) = 73.102$, $p < 0.0001$ for 100 μM glucose and $F(7,22) = 96.076$, $p < 0.0001$ for 100 μM glycerol). A Tukey *post hoc* test revealed significant differences between *M. gallisepticum* and all catalase-containing strains, but no statistical differences between *M. iowae* and catalase-containing transformants following treatment with both carbohydrates, suggesting that *katE* is responsible for the catalase activity present in *M. iowae*. Because it was possible that introduction of the transposon carrying *katE* interfered with glycerol metabolism and therefore H₂O₂ production, PCR was performed on the *glpFOK* putative operon, which constitute the only genes known to be involved in glycerol metabolism in this organism. Wild-type *M. gallisepticum* and all transformants displayed a product of ~5.7 kb (Fig. 3b). These results are consistent with reduction in H₂O₂ accumulation in transformants being due solely to catalase activity provided by *M. iowae katE*.

H₂O₂-mediated toxicity in a *C. elegans* model

Multiple studies have demonstrated the susceptibility of *C. elegans* to H₂O₂ [42,43], but a *C. elegans* model for toxicity of H₂O₂ from mycoplasmas has never been reported. When *C.*

C. elegans L1 larvae were incubated with increasing concentrations of H₂O₂ at room temperature, a significant decrease in survival at 24 h was evident as indicated by a one-way ANOVA ($F(3,26) = 5.911$, $p = 0.003$) (Fig. 4). To maximize H₂O₂ output by *M. gallisepticum* after 24 h, thereby allowing for the most easily discernible results in subsequent assays, various concentrations of glycerol and *M. gallisepticum* cells were analyzed at room temperature to mimic conditions necessary for *C. elegans* toxicity assays (Fig. 5). H₂O₂ production by wild-type *M. gallisepticum* reached a distinct maximum level when the glycerol concentration was increased to 1 mM (Fig. 5a). There was a statistically significant difference between different glycerol concentrations at the same time point as determined by a one-way ANOVA ($F(7,6) = 124.842$, $p < 0.0001$ at 24 hr and $F(7,6) = 20.591$, $p < 0.0001$ at 48 hr). A Tukey post hoc test revealed a significant difference associated with treatment with 100 μ M glycerol but not with higher concentrations. No statistically significant differences were observed for any glycerol concentrations with an increase in incubation time from 24 to 48 hrs. OD₅₅₀ values ranging from 0.5 to 1.0 yielded the same level of H₂O₂ (Fig. 5b); therefore, we chose to use cells at an OD₅₅₀ of 0.75 in the *C. elegans* assays. When transformant 56A, representing the *katE* transformants, was analyzed for H₂O₂ production with cells at an OD₅₅₀ of 0.75 in the presence of 1 mM glycerol at room temperature, H₂O₂ was detectable, but present at more than an order of magnitude lower than wild-type *M. gallisepticum*, with H₂O₂ production peaking early and becoming undetectable after 10 h (Fig. 5b). Thus, the amount of H₂O₂ produced by both wild-type *M. gallisepticum* and transformant 56A under the conditions used for the *C. elegans* studies is markedly greater than those originally obtained with 100 μ M glycerol over 2 h of incubation at 37°C.

To compare H₂O₂ toxicity in the presence and absence of catalase-containing mycoplasmas, pre-counted *C. elegans* L1 larvae were incubated with 1 mL of *M. iowae*, *M. gallisepticum*, and *M. gallisepticum* transformant 56A with catalase activity at an OD₅₅₀ of 0.75 in the presence or absence of 1 mM glycerol at room temperature, and survival was measured at 24 h (Fig. 6). In the absence of glycerol, leaving H₂O₂ production absent or minimal (see Table 1), incubation with all strains resulted in 60–80% survival of *C. elegans*, which were statistically similar as determined by a one-way ANOVA ($F(2,21) = 0.962$, $p = 0.398$). Statistically significant differences were found between experiments with and without glycerol for *M. gallisepticum*, but not for *M. iowae* or transformant 56A as determined by Student's T-test. Wild-type *M. gallisepticum* allowed no survival of *C. elegans* at 24 h due to the high level of H₂O₂ production. Transformant 56A allowed increased survival of *C. elegans* as compared to wild-type *M. gallisepticum*, paralleling differences observed in H₂O₂ production under assay conditions (Fig. 5b). Catalase-producing *M. iowae* and transformant 56A were both statistically significantly different from untransformed *M. gallisepticum* in the presence of glycerol as determined by a one-way ANOVA ($F(2,21) = 292.531$, $p < 0.001$) and Tukey's *post hoc* test.

Discussion

Despite the presence in *M. iowae* of an entire set of genes sufficient for importing and using free glycerol as a metabolite, this organism produced no detectable H₂O₂ under any conditions tested. We speculated that the absence of H₂O₂ accumulation was due to the presence and activity of a catalase, likely intracellular, which provides *M. iowae* with the ability to break down exogenous H₂O₂ and might remove H₂O₂ generated through the glycerol catabolism pathway before it accumulates to detectable levels.

Consistent with this hypothesis, *M. gallisepticum* displayed catalase activity upon transformation with *M. iowae katE*. Significantly, catalase activity resulted in reduced or even undetectable H₂O₂ production by *M. gallisepticum* transformants, and a *C. elegans* toxicity assay revealed a correlation between reduced H₂O₂ production and decreased toxicity. A possible explanation for the absence of H₂O₂ produced by *M. iowae* in the presence of glycerol is that the GlpFKO pathway experiences low flux and produces only a small amount of H₂O₂. A second possibility is that *M. iowae* has a defect in its GlpFKO pathway resulting in the inability to utilize glycerol as a metabolite and therefore an absence of H₂O₂ production. Finally, the catalase activity may simply be so great as to prevent any accumulation of H₂O₂ generated by glycerol catabolism. Although the presence of these genes suggests that there is a use for these proteins in normal cellular function, further testing is needed to determine their expression, activity, and regulation. There might, for example, be conditions under which either catalase activity is reduced or glycerol catabolism is increased. Preliminary data suggest that a close relative of *M. iowae*, *M. penetrans*, produces minimal H₂O₂ amounts from glycerol catabolism (J. T. Newman and M. F. B., unpublished data), raising the possibility that this pathway is not very productive in this clade of mycoplasmas. Development of genetic tools for *M. iowae*, which have not been described, would be valuable for directly addressing the question of H₂O₂ production.

Our finding of reduced or no H₂O₂ production in the presence of catalase suggests a fundamental incompatibility between the presence of catalase and the use of H₂O₂ as a virulence factor by mycoplasmas, and provides insight into the correlation between the presence of glycerol catabolism genes and the use of H₂O₂ for virulence. If H₂O₂ is a widespread virulence factor among mycoplasmas, then this incompatibility is likely to explain the general absence of catalase from other mycoplasmas. The findings of this report support the idea that, although glycerol catabolism genes are widespread amongst mycoplasmas, their presence does not necessarily signal the use of H₂O₂ for virulence. In addition to its function in utilizing glycerol as an energy source, recent work has illustrated the importance of this pathway in other biochemical functions, such as the production of lipids [44].

C. elegans has previously been used to assay toxicity of several bacterial species via production of toxic molecules and infectious processes. The susceptibility of *C. elegans* to H₂O₂ has been well established and H₂O₂-mediated toxicity assays on solid media and in liquid have been used to study virulence caused via this mechanism [42,43], although this is the first report of a *C. elegans* assay addressing mycoplasma toxicity. There are several benefits of using this method as opposed to standard tissue culture models. First, *C. elegans* is a more complex model than tissue culture cells, possessing several of the same innate immune defense mechanisms as more complex organisms [45], although whether this feature is important in this particular study is not clear. Second, previous studies have established a time frame of 2–5 d until bacteria can adequately colonize the gut of *C. elegans* and therefore cause damage via an infectious process [43]. Any death observed in the first 48 h is therefore due to diffusible molecules like H₂O₂. Third, tissue culture models of virulence require infection for long periods of time, and they cannot distinguish damage caused by H₂O₂, which occurs rapidly, and damage caused by toxins, which occurs more slowly, without the use of mutants, which can be difficult at minimum to construct using currently available molecular techniques [14]. Although there are limitations associated with comparing results obtained from this method to those observed in the natural host, such as examining the impact of H₂O₂ produced

from exogenous or host-derived carbohydrates, it serves as a good system for examination of strains deficient in H₂O₂ production, such as the catalase-positive strains examined in this study.

One potential concern about the use of *C. elegans* as a reporter of H₂O₂-mediated toxicity is the question of whether binding of mycoplasmas to *C. elegans* impacts toxicity during the time frame required for these assays. Whether *M. iowae* binds to *C. elegans* under our experimental conditions, or under any conditions, is not known. Although *M. gallisepticum* can bind red blood cells at room temperature within 5 min [46], other pathogens with cell-associated toxins also bind externally to *C. elegans* within 2 d yet still require 4 d before any decrease in worm survival is observed [47]. Therefore, even though mycoplasmas might or might not bind to *C. elegans* during the 24-h incubation required for the toxicity assay employed in this study, one can be certain that any decrease in survival during this time frame is due to the production of diffusible molecules such as H₂O₂ and not cell-associated toxins.

Supporting Information

Figure S1 Alignment of catalase proteins closely related to that of *M. iowae*. The uppermost track plots alignment entropy, with hotter colors at more variable positions than colder ones. Beneath the entropy plot the sequence coordinates are given

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