

Hydrogen Peroxide Production from Glycerol Metabolism Is Dispensable for Virulence of *Mycoplasma gallisepticum* in the Tracheas of Chickens

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Hydrogen peroxide (H_2O_2) is a by-product of glycerol metabolism in mycoplasmas and has been shown to cause cytotoxicity for cocultured eukaryotic cells. There appears to be selective pressure for mycoplasmas to retain the genes needed for glycerol metabolism. This has generated interest and speculation as to their function during infection. However, the actual effects of glycerol metabolism and H_2O_2 production on virulence *in vivo* have never been assessed in any *Mycoplasma* species. To this end, we determined that the wild-type (WT) R_{low} strain of the avian pathogen *Mycoplasma gallisepticum* is capable of producing H_2O_2 when grown in glycerol and is cytotoxic to eukaryotic cells in culture. Transposon mutants with mutations in the genes present in the glycerol transport and utilization pathway, namely, *glpO*, *glpK*, and *glpF*, were identified. All mutants assessed were incapable of producing H_2O_2 and were not cytotoxic when grown in glycerol. We also determined that vaccine strains ts-11 and 6/85 produce little to no H_2O_2 when grown in glycerol, while the naturally attenuated F strain does produce H_2O_2 . Chickens were infected with one of two *glpO* mutants, a *glpK* mutant, R_{low} , or growth medium, and tracheal mucosal thickness and lesion scores were assessed. Interestingly, all *glp* mutants were reproducibly virulent in the respiratory tracts of the chickens. Thus, there appears to be no link between glycerol metabolism/ H_2O_2 production/cytotoxicity and virulence for this *Mycoplasma* species in its natural host. However, it is possible that glycerol metabolism is required by *M. gallisepticum* in a niche that we have yet to study.

Pathogenic mycoplasmas have evolved a strategy for survival within specific niches in their hosts that involves mechanisms such as the loss of unnecessary genes via reductive evolution. Selective pressures exerted on these bacteria dictate that most intact genes should be functional and necessary for some aspect of the mycoplasma's life cycle. During their evolution, most mycoplasmas have lost the genes involved in the pentose phosphate pathway (PPP) and the tricarboxylic acid (TCA) cycle, leaving only glycolysis intact for the utilization of their primary carbon source, glucose (1). However, many mycoplasmas (including the avian pathogen *Mycoplasma gallisepticum*) carry genes that encode transporters and enzymes for the utilization of alternative carbon sources, such as fructose and glycerol (2). Glycerol utilization in *Mycoplasma* spp. has been a topic of much interest recently, because one of the primary by-products of glycerol metabolism is the cytotoxic reactive oxygen species hydrogen peroxide (H_2O_2).

Glycerol catabolism is associated primarily with the proteins encoded by the *glp* genes (for an overview, see Fig. 1). GlpF is responsible for the transportation of glycerol into the cell. GlpK then phosphorylates glycerol to glycerol-3-phosphate, and GlpO enzymatically converts glycerol-3-phosphate to dihydroxyacetone phosphate (DHAP), which is utilized during glycolysis, and H_2O_2 (by using water as an electron acceptor [1]). In addition to the Glp proteins, the UgpACE transport system can take up glycerol-3-phosphate directly into the cytoplasm of the bacterium, and the GlpQ protein in *Mycoplasma pneumoniae* can catabolize glycerophosphocholine into glycerol-3-phosphate as well (3). Grosshennig et al. (4) recently identified an additional glycerol transport protein in *M. pneumoniae* (GlpU) and characterized the roles of lipoproteins MPN133 and MPN284 in glycerol binding, leading to speculation that they act as a means of capturing glycerol and delivering it to the membrane transport proteins. While glycerol is a potential alternative carbon source, empirical evi-

dence from *M. pneumoniae* (5, 6) and metabolomic analysis of the *M. gallisepticum* genome (7) indicate that these pathogens can also utilize glycerol in conjunction with fatty acids to produce cardiolipin, a lipid used for membrane biosynthesis. Thus, there are multiple potential uses for glycerol in mycoplasmas to aid in their survival, but thus far, its actual utilization has been elucidated only for a few *Mycoplasma* species.

It has long been known that H_2O_2 is cytotoxic to cells grown in culture, and measuring cytotoxicity has been a common tool in determining the abilities of *Mycoplasma* spp. to grow in the presence of glycerol as a carbon source. Indeed, *in vitro* analyses of the growth and cytotoxicity of a *Mycoplasma mycoides* subsp. *mycoides* SC GlpO mutant (8) and of both GlpQ (3) and GlpD (9) (homologous to GlpO in other *Mycoplasma* spp.) mutants of *M. pneumoniae* have revealed that the loss of these enzymes results in abrogated H_2O_2 production and a concomitant loss of cytotoxicity toward cocultured eukaryotic cells. Molecular analysis of GlpO in *M. mycoides* subsp. *mycoides* SC demonstrated that the "FAD" domain (responsible for binding the flavin adenine dinucleotide cofactor) is essential for its enzymatic activity, H_2O_2 production, and cytotoxicity (8). Further insight into the mechanism by which H_2O_2 production may actually cause cytotoxicity toward host cells

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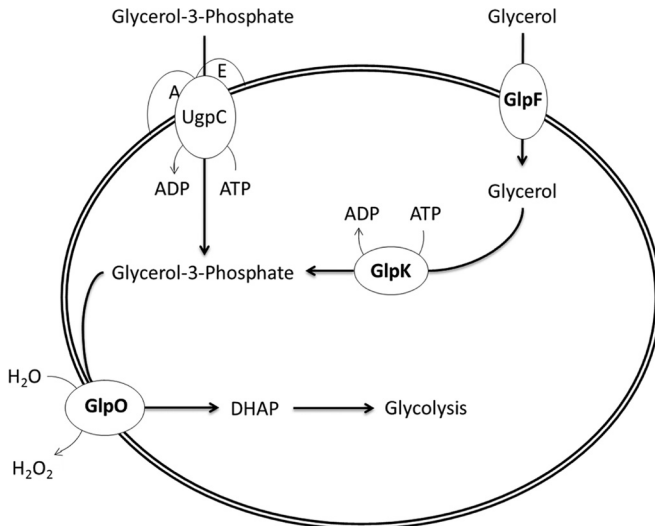


FIG 1 Overview of glycerol metabolism in *Mycoplasma gallisepticum*. Free glycerol is transported into the bacterial cell by the uptake facilitator GlpF. Glycerol is then phosphorylated by the glycerol kinase GlpK, producing glycerol-3-phosphate. Alternatively, if the UgpACE ABC transport system is functional in *M. gallisepticum*, it can take up and transport free glycerol-3-phosphate. Based on the work of others (6), we presume that the glycerol oxidase GlpO is a transmembrane enzyme that converts glycerol-3-phosphate to dihydroxyacetone phosphate (DHAP) and releases hydrogen peroxide as a by-product outside the bacterial cell.

was gained by immunogold labeling of virulent *M. mycoides* subsp. *mycoides* SC cells with anti-GlpO antibodies, which demonstrated that this protein is in fact membrane bound (10) and thus may allow the bacterium to produce H_2O_2 in the extracellular domain. Hence, H_2O_2 may be released into the growth medium or may also be directly transferred to attached host cells.

While we know that glycerol metabolism is active in many *Mycoplasma* spp. (1, 11), glycerol production (and H_2O_2 production) has never been demonstrated to be associated with the virulence of any mycoplasma *in vivo*. We sought to bridge this gap by directly assessing the correlation of glycerol metabolism and H_2O_2 production *in vitro* with the virulence of *M. gallisepticum* *in vivo*. Here we present the results of several genetic knockout experiments with the *glpF*, *glpK*, and *glpO* genes in *M. gallisepticum* and show that glycerol metabolism in this *Mycoplasma* species does result in H_2O_2 production and host cell cytotoxicity *in vitro*. However, these mutants remain fully virulent in the respiratory tract of their natural host (the chicken) during experimental infection. The implications of the discrepancy between the correlation of H_2O_2 production with *in vitro* cytotoxicity and the lack of correlation of H_2O_2 production with *in vivo* virulence are discussed.

MATERIALS AND METHODS

Culture conditions. *M. gallisepticum* strains were cultured at 37°C in Hayflick's complete medium (12) until mid-log-phase growth was achieved, as indicated by a color shift from red to orange. Mutants were grown in complete Hayflick's medium containing 50 μ g/ml of gentamicin.

***M. gallisepticum* R_{low} transformation and identification of isogenic mutants.** A library of 3,600 R_{low} transposon (Tn) mutants was generated via electroporation using plasmid pMT85, as described previously (13). The plasmid carries the mini-Tn4001-gent transposon, which encodes a gentamicin resistance gene. Pools of 30 mutants were grown, and then

genomic DNA was extracted. PCR screening of mutant pools for transposon insertions in the *glpO*, *glpF*, and *glpK* genes was performed in 96-well plates by using a gene-specific primer in conjunction with a 5' or 3' transposon-specific primer. PCR was run on positive pools in the reverse orientation (reverse gene primer with the opposite-end transposon primer). Pools with positive forward and reverse PCR products that exhibited the anticipated gene size for a transposon insertion were selected for a second round of screening. DNA was extracted from each individual pool member and was screened again as described above. The Tn insertion site was identified using Sanger sequencing, as described previously (14).

Hydrogen peroxide assay. *M. gallisepticum* cultures were grown to mid-log phase, and titers were determined by the optical density at 620 nm (OD_{620}). A total of 3×10^9 CFU was then harvested and was centrifuged at $10,000 \times g$ for 10 min at 4°C. Cells were then washed twice in 1 ml HEPES buffer (67.6 mM HEPES, 140 mM NaCl, 7 mM MgCl [pH 7.3]; Sigma-Aldrich, St. Louis, MO) each time. After the second wash, the cells were resuspended in 1 ml HEPES and were incubated for 1 h at 37°C. Glycerol was then added to each culture at a final concentration of 10 mM. After 20 min, Merckoquant peroxide test strips (Merck Millipore International) were used, according to the manufacturer's instructions, to determine the amount of peroxide generated by each culture.

Cytotoxicity assay. Twelve-well tissue culture plates were seeded with 2×10^5 MRC-5 human lung fibroblasts per well and were incubated at 37°C under 5% CO_2 for 24 h in Dulbecco's modified Eagle medium (DMEM) plus 10% fetal bovine serum until the cells reached confluence. *M. gallisepticum* cultures were started so as to coordinate mid-log-phase growth with the time of MRC-5 confluence. Each *M. gallisepticum* culture was centrifuged at $10,000 \times g$ for 10 min at 4°C and was then washed twice with 1 ml HEPES buffer. After the second wash, cells were resuspended in 1 ml DMEM plus 10% fetal bovine serum. The medium in the wells of the MRC-5 cells was removed, and 1,000 *M. gallisepticum* cells were inoculated into each well and were incubated at 37°C under 5% CO_2 for 1 h. Glycerol was then added to wells (as indicated in Fig. 4) at a final concentration of 10 mM, and the cells were incubated for an additional 24 h. Medium was then removed from the wells, and cell monolayers were stained with 0.1% crystal violet for 15 min. Cytopathic effects (CPE) were noted when the cells rounded up and lifted off from the bottoms of the plates.

Attachment assay. *M. gallisepticum* strains were radiolabeled in Hayflick's broth supplemented with [*methyl*- 3H]thymidine (10 μ Ci/ml), and the specific activity (calculated as cpm/CFU) of each culture was determined as measured by liquid scintillation (cpm) and optical density at 620 nm (CFU). MRC-5 lung fibroblasts seeded at 1×10^5 /well were incubated for 24 h prior to exposure to radiolabeled *M. gallisepticum* (at a multiplicity of infection [MOI] of 1,000 for 1 h at 37°C) in Hayflick's broth. Monolayers were washed twice with phosphate-buffered saline (PBS) and were lysed with 200 μ l of 50 mM NaOH, and activity was measured by liquid scintillation. The numbers of attached *M. gallisepticum* cells per well were calculated as the test activity (measured in cpm) divided by the specific activity of each strain (initial culture cpm/CFU).

Animals. Four-week-old female specific-pathogen-free White Leghorn chickens (Spafas, North Franklin, CT) were used in this study. Upon arrival, the chickens were divided into groups of 10, tagged, placed in HEPA-filtered isolators, and allowed to acclimate for 1 week prior to experimentation. Nonmedicated feed and water were provided *M. mycoides* throughout the experimental period. The chicken study procedures described here were in accordance with state and federal policies to ensure the humane use and care of research animals as approved by the University of Connecticut Institutional Animal Care and Use Committee (IACUC).

***In vivo* infection study.** Stocks of each *M. gallisepticum* culture were grown at 37°C with shaking at 130 rpm in fresh Hayflick's complete medium for 5 h prior to animal inoculation. *M. gallisepticum* concentrations were determined by the OD_{620} , and 10-fold serial dilutions were used to assess color-changing units (CCU) in order to ensure that the cells mea-

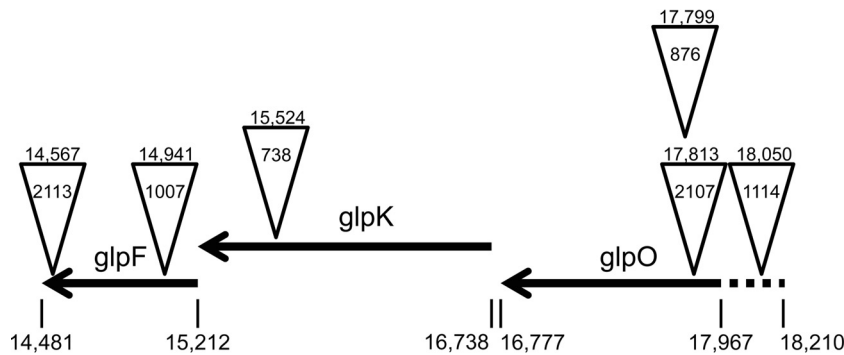


FIG 2 Map of the *glp* operon and Tn insertion sites for the different mutants tested. Arrows indicate open reading frames, with the genomic positions of the start and stop sites given below. Inverted triangles indicate Tn insertion sites. The mutant designation is given inside each triangle, and the insertion point is given above each triangle. The dashed line indicates the putative promoter region for the *glp* operon.

sured were viable. Chickens were challenged intratracheally as described previously (13), with 1×10^8 CFU in 200 μ l of each *M. gallisepticum* culture on days -2 and 0. Control chickens received 200 μ l Hayflick's complete medium on days -2 and 0. Chickens were euthanized and were immediately necropsied on day 14 postinfection (p.i.), and the trachea was collected from each bird. Gross and histopathological analyses of the tracheal lesions were conducted as described by Gates et al. (15).

Statistics. Statistical comparisons were conducted using analysis of variance (ANOVA), or the nonparametric ANOVA on ranks for tracheal lesion scores, with a Newman-Keuls *post hoc* test for multiple pairwise comparisons. Data were analyzed using GraphPad Prism, version 5 (GraphPad Software, San Diego, CA). Differences were considered significant if the *P* value was <0.05 .

RESULTS

Generation of *glp* mutants. In order to assess the role of the glycerol uptake and processing enzymes in the virulence of *M. gallisepticum*, we screened our previously described transposon library generated from the *M. gallisepticum* wild-type (WT) strain R_{low} (13) for isogenic mutants with alterations in the *glpO*, *glpK*, and *glpF* genes by using the haystack mutagenesis method. Insertion sites were mapped by Sanger sequencing using a unique outwardly directed primer. We identified two *glpO* mutants (mutants 2107 and 876), one *glpK* mutant (mutant 738), and two *glpF* mutants (mutants 1007 and 2113) (Fig. 2). An additional mutant was identified in the intergenic region upstream of the *glp* operon (mutant 1114).

H₂O₂ production by *M. gallisepticum* strains and *glp* mutants with glycerol as a carbon source. The amount of H₂O₂ can be measured directly in growth medium with Millipore semi-quantitative test strips. We used these strips to determine the amounts of H₂O₂ produced by multiple strains of *M. gallisepticum*. When R_{low} was grown in glycerol for 20 min, an average of 4.25 μ g/ml of H₂O₂ could be detected; however, this effect was abrogated in the absence of glycerol (Fig. 3). Mutant 1114 (with a Tn insertion in the intergenic region upstream of the *glp* operon) produced wild-type levels of H₂O₂ when grown in glycerol (3.5 μ g/ml), thus indicating that the mere presence of a Tn does not interfere with glycerol metabolism/H₂O₂ production. Inactivation of the *glpO*, *glpK*, or *glpF* gene by Tn insertion rendered each mutant unable to produce detectable levels of H₂O₂ when grown in glycerol. Additionally, we tested the abilities of attenuated vaccine strains and the high-passage-number R strain R_{high} (with a previously noted inactivating mutation in the *glpK* gene [16]) to

produce H₂O₂ in the presence of glycerol. Of the attenuated strains, only the F strain was capable of producing WT levels of H₂O₂ (4.0 μ g/ml; not significantly different from those produced by R_{low}) when grown in glycerol. ts-11 produced low levels of H₂O₂ in some experiments (1.25 μ g/ml), but its H₂O₂ production clustered statistically with that of R_{low} grown in the absence of glycerol and was significantly different from that of R_{low} grown in glycerol ($P < 0.05$).

Glycerol-dependent H₂O₂ production induces cytotoxicity in cocultured eukaryotic cells. To investigate the cytotoxic nature of H₂O₂ released from *M. gallisepticum* due to glycerol metabolism, MRC-5 human lung fibroblasts were infected in the presence and absence of glycerol. Uninfected cells served as a negative control. The WT strain R_{low} was able to induce cytopathic effects (CPE) in the presence of 10 mM glycerol 24 h after infection but induced no CPE without the addition of exogenous glycerol (Fig. 4). An intermediate phenotype was observed for the intergenic mutant 1114, with some wells exhibiting clearly demonstrable CPE and others less so. None of the *glp* mutants induced CPE in the presence of glycerol. Of note, Tn insertions in the *glp* genes did not affect the abilities of the mutants to bind to MRC-5 cells (Fig. 5); thus, their lack of cytotoxicity was not associated with an inability to attach to, and maintain intimate contact with, eukaryotic cells. We additionally tested the ability of strain R_{high} to induce

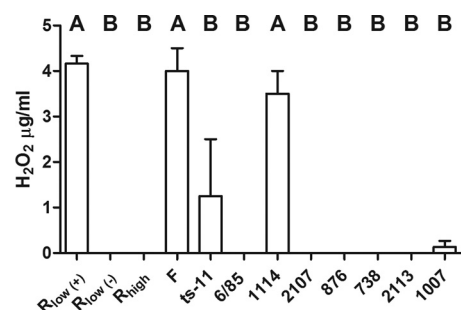


FIG 3 H₂O₂ production by *M. gallisepticum* strains and *glp* mutants grown in glycerol as the carbon source. The concentrations of H₂O₂ were measured in the growth media of *M. gallisepticum* strains grown in glycerol by using Merckoquant peroxide test strips. R_{low} was also grown without the addition of glycerol (-). Statistical significance was determined by ANOVA with a Newman-Keuls *post hoc* test for multiple pairwise comparisons. Different capital letters above the data indicate statistically significant differences ($P < 0.05$).

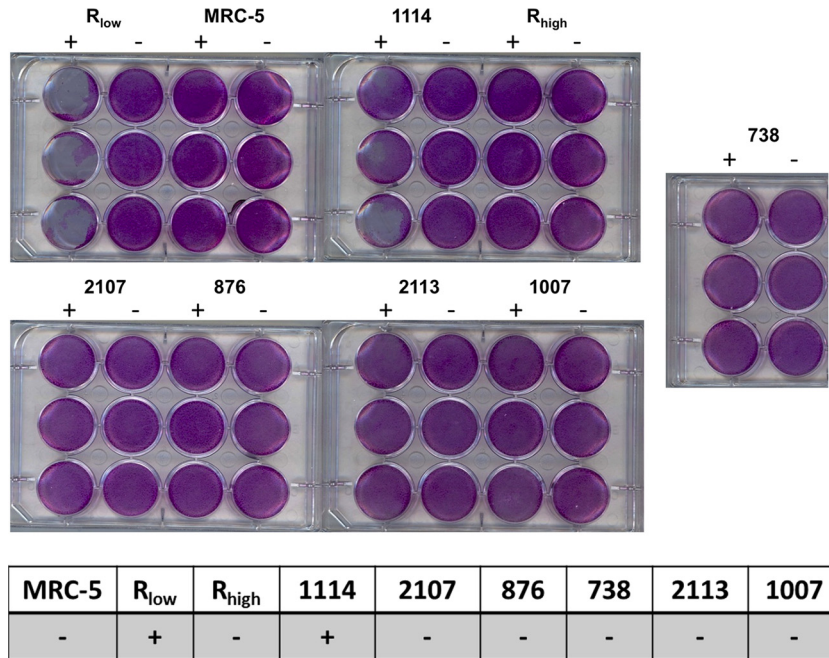


FIG 4 Cytotoxicities of *M. gallisepticum glp* mutants when cultured with MRC-5 human lung fibroblasts. (Top and center) Wells of confluent MRC-5 cells stained with crystal violet after inoculation with *M. gallisepticum* strains in the presence (+) or absence (-) of glycerol. Cytotoxicity was determined by the presence or absence of a cytopathic effect on MRC-5 cells. One thousand *M. gallisepticum* cells were inoculated into 12-well plates with MRC-5 cells grown to confluence. No cytopathic effects were observed for any strain when grown in the presence of glucose instead of glycerol. (Bottom) Presence (+) or absence (-) of cytotoxicity for MRC-5 cells cultured alone or with *M. gallisepticum* strains.

CPE in this system, but none was detected. When strains were grouped according to the findings of the H₂O₂ tests, cytotoxic strains clustered together with no significant difference among them, but they were significantly different from noncytotoxic strains (which clustered together as well). Thus, there appeared to be a correlation between H₂O₂ production/glycerol metabolism and cytotoxicity *in vitro*.

Glycerol metabolism and H₂O₂ production are not associated with *M. gallisepticum* virulence in the respiratory tracts of chickens. The relative virulence of the *glp* mutant strains was experimentally assessed in chickens as described previously (14). Groups of 10 female White Leghorn chickens were inoculated

intratracheally with either Hayflick’s medium as a negative control, R_{low} as a positive control, *glpO* mutant 2107 or 876, or *glpK* mutant 738. Two similar experiments had been conducted previously with essentially the same results. Tracheal lesions were scored for severity, and tracheal mucosal thickness was measured (Fig. 6). Chickens inoculated with medium alone differed significantly from those inoculated with R_{low}, both in terms of tracheal lesion scores and in terms of mucosal thickness (*P* < 0.05). All three *glp* mutants clustered statistically with R_{low} and were different from medium alone, again both in terms of lesion scores and in terms of mucosal thickness. Thus, H₂O₂ production appeared to be completely dispensable for *M. gallisepticum* virulence in the chicken respiratory tract, a primary natural niche for this pathogen. Furthermore, while glycerol metabolism and H₂O₂ production appeared to correlate with *in vitro* cytotoxicity, there appeared to be no link between glycerol metabolism/H₂O₂ production and the virulence of *M. gallisepticum*.

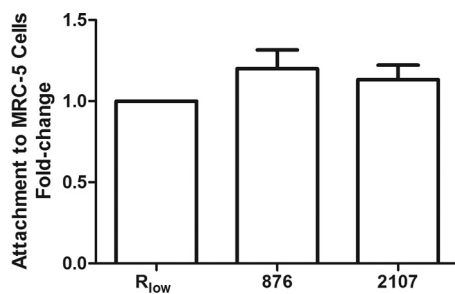


FIG 5 *glp* mutants 876 and 2107 maintain wild-type levels of attachment to eukaryotic cells. *M. gallisepticum* strains were radiolabeled, and their abilities to attach to MRC-5 cells were measured by liquid scintillation. Data represent the mean fold change (\pm standard error of the mean) in attachment to MRC-5 cells from R_{low} (whose value is set at 1), as measured over three separate experiments. No statistical difference was observed among the groups, as determined by one-way ANOVA with Dunnett’s multiple-comparison *post hoc* test (with R_{low} serving as the control group for comparison).

DISCUSSION

Much research has been dedicated recently to identifying and determining the functionality of glycerol metabolism genes and their products. Much of this work has focused on identifying H₂O₂ as a by-product of glycerol metabolism and demonstrating that H₂O₂-producing *Mycoplasma* spp. are cytotoxic when cocultured with eukaryotic cells. Genome sequencing has revealed that *M. gallisepticum* possesses the *glp* genes necessary for glycerol metabolism (2, 16), and earlier work showed that this pathogen can grow in the presence of glycerol (11). The studies presented here confirm these earlier reports and further demonstrate that H₂O₂ is produced during the metabolism of glycerol and that glycerol metabolism results in cytotoxicity toward cocultured eukaryotic cells.

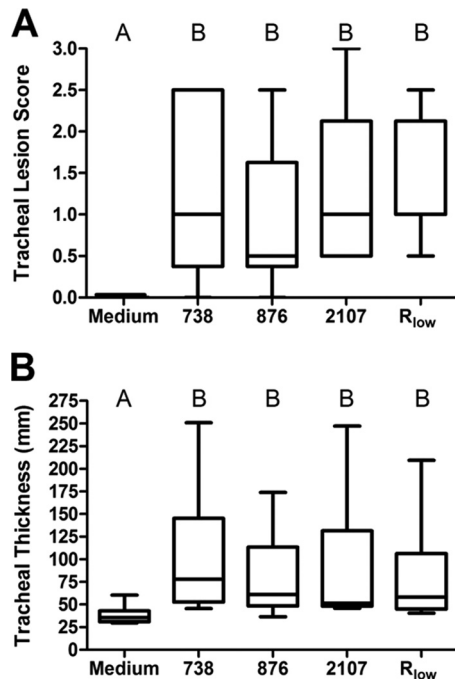


FIG 6 Tracheal lesion scores and measurements of thickness of histologic sections from chickens intratracheally infected with *M. gallisepticum* *glp* mutants. Chickens inoculated with *Mycoplasma* growth medium alone served as negative controls, and those inoculated with the WT strain R_{low} served as positive controls. Box plots represent the 25th and 75th percentiles, with the median score in between, and whiskers show the minimum and maximum for each group. Groups were compared by ANOVA (for thickness) or ANOVA on ranks (for lesion scores), with a Newman-Keuls *post hoc* multiple pairwise comparison using GraphPad Prism. Different capital letters above the data indicate statistically significant differences ($P < 0.05$).

However, this is the first report of the assessment of the role of glycerol metabolism in the virulence of any *Mycoplasma* species *in vivo*. To our surprise, this seemingly important enzymatic reaction had no bearing on the virulence of *M. gallisepticum* in the trachea of its natural host. We have found that lesions in tracheas (the primary site of infection) are a more consistent indicator of virulence than lesions in air sacs or lungs. Therefore, our measure of virulence is based on tracheal lesions. We utilized three different *glp* mutants and repeated the *in vivo* experiments, with similar results in replicate experiments. Thus, we have demonstrated that there is no link between glycerol metabolism/H₂O₂ production/cytotoxicity and virulence in the common host of *Mycoplasma gallisepticum*. These results indicate that glycerol metabolism, H₂O₂ production, and cytotoxicity do not correlate with virulence in all mycoplasmas.

There is direct evidence for the necessity of utilization of alternative carbon sources in the virulence of the closely related insect and plant pathogens in the genus *Spiroplasma*. As in previous studies on *Mycoplasma* spp., the presence of *glpO* has been associated with the virulence of *Spiroplasma culicicola* and *Spiroplasma taiwanense* in mosquitoes, and its loss has been associated with attenuation of the virulence of *Spiroplasma taiwanense* and *Spiroplasma sabaudiense* (17) in these insects. Perhaps more convincingly, direct evidence of the necessity of fructose as a carbon source and its link to virulence *in vivo* has been provided through infection studies of *fruR* Tn mutants of *Spiroplasma citri* in its

natural host, the periwinkle plant (18). The *fruR* mutants showed marked attenuation during infection, and virulence was restored upon complementation. Interestingly, *M. gallisepticum* encodes the genes necessary for fructose transport and has been shown previously to grow with fructose as a carbon source (11). Elucidation of the importance of alternative carbon sources (other than glycerol) for the growth and virulence of *Mycoplasma* spp. is warranted.

Given the selective pressure on *Mycoplasma* spp. to reduce unnecessary gene content, it is surprising that the *glp* genes are maintained yet are not involved in virulence in the tracheas of chickens. Multiple genomic studies have correlated the presence of genes involved in glycerol metabolism and/or H₂O₂ production with the relative virulence of field strains of different *Mycoplasma* spp. Indeed, there appears to be a direct correlation between H₂O₂ production during glycerol metabolism and the relative virulence of *M. mycoides* subsp. *mycoides* SC strains. Vilei and Frey (19) report that all the African field strains of *M. mycoides* subsp. *mycoides* SC contain the *gts* transport system (the glycerol transport system in this pathogen) and produce high levels of H₂O₂, while the relatively attenuated European strains are missing two of the *gts* genes and do not produce H₂O₂ in the presence of glycerol, an observation also made by Rice et al. (20). However, it is not currently known if other affected genes contribute to (or even are completely responsible for) attenuation in the European strains of *M. mycoides* subsp. *mycoides* SC.

Mycoplasma bovis and *Mycoplasma agalactiae* field strains appear to produce H₂O₂, but serial passage of one *M. bovis* strain (119B96) resulted in dramatically reduced H₂O₂ production and the loss of an associated protein (21). In a similar vein, we have shown that the high-passage-number attenuated R_{high} strain of *M. gallisepticum* has an inactivating mutation in the *glpK* gene (16), and we show in this work that this strain does not produce H₂O₂. Two other attenuated *M. gallisepticum* vaccine strains (ts-11 and 6/85) also produce little to no H₂O₂ in the presence of glycerol. These findings support the notion that *Mycoplasma* spp. experience selective pressure to maintain glycerol metabolism capabilities in the wild but often lose these genes when such pressures are removed under laboratory conditions. An interesting piece of this puzzle then arises from our findings with the naturally attenuated F strain of *M. gallisepticum*, which retained WT levels of H₂O₂ production in culture. The F strain has dramatically reduced virulence in chickens yet persists at WT levels in the respiratory tracts of chickens (22). The finding that this attenuated strain produces H₂O₂ supports the conclusion that H₂O₂ production does not affect *M. gallisepticum* virulence in chickens. However, it should be noted that the F strain is fully virulent in turkeys, providing a possible natural niche in which glycerol metabolism/H₂O₂ production may undergo the selective pressures for maintenance of this metabolic capability. Should this be the case, it is possible that glycerol metabolism is a species- or niche-specific virulence factor. This raises questions regarding the availability of glycerol in the tracheas of chickens, the relative resistance of chickens to H₂O₂ in their tracheas, the possibility that other tissue/organ systems in chickens (not assessed in this study) are negatively impacted by *M. gallisepticum* H₂O₂ production, and the activity of the *glp* genes of *M. gallisepticum* during infection. More work is needed to answer these questions and to test the hypothesis of species specificity as a driver of the maintenance of genes necessary for glycerol metabolism in *M. gallisepticum*.

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