A Mycoplasma gallisepticum Glycerol ABC Transporter Involved in Pathogenicity

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MalF has been shown to be required for virulence in the important avian pathogen Mycoplasma gallisepticum. To characterize the function of MalF, predicted to be part of a putative ABC transporter, we compared metabolite profiles of a mutant with a transposon inserted in malF (MalF-deficient ST mutant 04-1; ΔmalF) with those of wild-type bacteria using gas chromatography-mass spectrometry and liquid chromatography-mass spectrometry. Of the substrates likely to be transported by an ABC transport system, glycerol was detected at significantly lower abundance in the ΔmalF mutant, compared to the wild type. Stable isotope labeling using [U-13C]glycerol and reverse transcription-quantitative PCR analysis indicated that MalF was responsible for the import of glycerol into M. gallisepticum and that, in the absence of MalF, the transcription of gtsA, which encodes a second transporter, GtsA, was upregulated, potentially to increase the import of glycerol-3-phosphate into the cell to compensate for the loss of MalF. The loss of MalF appeared to have a global effect on glycerol metabolism, suggesting that it may also play a regulatory role, and cellular morphology was also affected, indicating that the change to glycerol metabolism may have a broader effect on cellular organization. Overall, this study suggests that the reduced virulence of the ΔmalF mutant is due to perturbed glycerol uptake and metabolism and that the operon including malF should be reannotated as golABC to reflect its function in glycerol transport.

Importance Many mycoplasmas are pathogenic and cause disease in humans and animals. M. gallisepticum causes chronic respiratory disease in chickens and infectious sinusitis in turkeys, resulting in economic losses in poultry industries throughout the world. Expanding our knowledge about the pathogenesis of mycoplasma infections requires better understanding of the specific gene functions of these bacteria. In this study, we have characterized the metabolic function of a protein involved in the pathogenicity of M. gallisepticum, as well as its effect on expression of selected genes, cell phenotype, and H2O2 production. This study is a key step forward in elucidating why this protein plays a key role in virulence in chickens. This study also emphasizes the importance of functional characterization of mycoplasma proteins, using tools such as metabolomics, since prediction of function based on homology to other bacterial proteins is not always accurate.

Keywords mycoplasma, metabolomics, glycerol metabolism

Mycoplasmas evolved from Gram-positive bacteria by reductive evolution, resulting in the loss of many metabolic pathways and a dependence on their hosts for...
many nutrients. Due to this dependence on their environmental niche, the genomes of many mycoplasmas encode numerous nutrient transport systems, but the definitive function of many of these transporters remains unknown (1–3). One of the reasons for this uncertainty is that the function of mycoplasma proteins is not always reliably predictable based on sequence similarity with proteins encoded by homologous genes in other bacteria. Recently, our laboratory demonstrated that the Mycoplasma gallisepticum OppD protein is part of an ABC transporter that appears to transport amino acids and related metabolites into the mycoplasma cell (4, 5), even though MalK, the most similar homologous protein in Escherichia coli, is part of an ABC transporter for maltose (6). The M. gallisepticum genome contains a gene homologous to malF, a gene encoding a component of the E. coli maltose transport system (7). However, in Mycoplasma pneumoniae this malF homologue has been predicted to encode part of a glycerol-3-phosphate ABC transporter system (8). Glycerol is not the preferred carbon source for many bacteria and, if glucose is present as a carbon source, the expression of genes involved in glycerol metabolism is generally reduced (9–11). However, in M. pneumoniae, the abundance of glycerol in the bacterium’s habitat and its role in modulating some regulatory proteins suggest that glycerol may be the preferred carbon source (12). The M. gallisepticum genome encodes three sets of proteins putatively involved in glycerol uptake and metabolism (Fig. 1).

The first of these sets are the Glp proteins. Glycerol is imported by the glycerol uptake facilitator (GlpF) and phosphorylated by glycerol kinase (GlpK) to glycerol-3-phosphate, which is converted to dihydroxyacetone phosphate (DHAP) by glycerol oxidase (GlpO), releasing the by-product, hydrogen peroxide, from the bacterial cell (13). The functions of the Glp proteins have been demonstrated previously in M. gallisepticum (14).

The second set of proteins involved in glycerol metabolism form a putative glycerol ABC transporter. This system is encoded by the gtsABC genes and the homologues of these genes encode an active glycerol transporter in Mycoplasma bovis strain PG50
and Mycoplasma mycoides subsp. mycoides SC strain Afade, but their function has not been confirmed in M. gallisepticum (15, 16).

The third set of proteins putatively involved in glycerol metabolism includes the MalF ABC transporter permease, as well as two other predicted proteins, all of which are encoded in the same predicted operon (see Fig. S1 in the supplemental material). The malF gene (also called ugpA) encodes a putative sn-glycerol-3-phosphate ABC transporter permease in M. pneumoniae, but its function has not been experimentally validated (8, 17). In some firmicutes, glycerol-3-phosphate can also be transported by the GlpT permease, but glpT is not present in the M. gallisepticum genome (18).

Glycerol metabolism has been implicated in the pathogenicity of several Mycoplasma species (16, 19–21). Recent studies in M. gallisepticum demonstrated the role of GlpO in H2O2 production from glycerol-3-phosphate. However, despite the correlation of this H2O2 production with in vitro cytotoxicity, loss of GlpO had no effect on the virulence of M. gallisepticum in vivo (14). A comparative genomic study of the virulent M. gallisepticum strain Rlow and the attenuated variant Rhigh identified an amino acid substitution in UgpC, the ATP binding protein of the Ugp ABC transporter, in Rhigh. The authors of that study suggested that this results in a functional change in UgpC that may affect glycerol metabolism and that this may be the reason for the attenuation of the Rhigh strain (17). In many bacteria, glycerol metabolism is primarily regulated by an antitermination regulatory protein, the expression of which is increased in response to low levels of intracellular glycerol or glycerol-3-phosphate (9, 11, 22, 23). However, in M. pneumoniae GlpQ, a glycerophosphocholine phosphodiesterase that is active in the cell under all conditions, modulates glycerol metabolism. The presence of glycerol or glycerol-3-phosphate inhibits the regulatory activity of GlpQ, resulting in increased activity of the glycerol kinase GlpK (8). It has been suggested that the Ugp ABC transporter system might also act in the regulatory process, but the nature of these regulatory functions is unclear (24).

We have previously shown that MalF, encoded by malF (MGA_0680), is essential for persistence and pathogenicity of M. gallisepticum strain Ap3AS in vivo (25). In the present study, we investigated the metabolic function of MalF using high performance liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS) to compare the metabolite profiles of a mutant with a transposon inserted in malF (MalF-deficient ST mutant 04-1; ΔmalF) and wild-type M. gallisepticum. Furthermore, uptake and metabolism of 13C-labeled glycerol was measured to confirm transporter function. To further investigate the regulation of glycerol metabolism, reverse transcription-quantitative PCR (RT-qPCR) studies were carried out on the mutant and the wild type. Since hydrogen peroxide is an important by-product of glycerol metabolism, levels of H2O2 were also assessed in the mutant and the wild type. Since glycerol can be utilized in lipid biosynthesis and thus may influence cell membrane function, the morphologies of the mutant and wild-type strains were also compared using electron microscopy.

RESULTS

Bioinformatic analysis of MalF. The MalF amino acid sequence was compared to other bacterial proteins using a BLASTP search of the UniProt database (https://www.uniprot.org), revealing 56% similarity with a putative ABC transporter permease protein of M. pneumoniae (MPN_135) belonging to the MalFG subfamily of the binding-protein-dependent transport system permease family. The Phyre2 web server was also used to predict MalF function (26), with 288 residues (88% of the malF sequence) modeled with 100.0% confidence to the crystal structure of the resting state of the maltose transporter of E. coli (see Fig. S2). Secondary structure and disorder prediction of MalF suggested the presence of six transmembrane helices, with both amino and carboxyl termini within the cytoplasm (see Fig. S3).

The presence or absence of glycerol in vitro does not affect the growth of the wild-type strain AP3AS or the ΔmalF mutant. To investigate the effect of glycerol on the growth of the M. gallisepticum wild-type strain AP3AS and the ΔmalF mutant, the concentrations of viable cells were measured in cultures incubated in the presence or absence of glycerol every 8 h for 24 h. The MalF mutant grew more slowly than did the
wild type, but the growth rate of neither strain was affected by the presence or absence of glycerol (Fig. 2).

Metabolite profiling revealed significant differences between the wild-type AP3AS strain and the ΔmalF mutant in a number of metabolites of glycerol and glucose metabolism. The total numbers of metabolites detected using GC-MS and LC-MS were 175 and 115, respectively. Statistical analyses (Holm-Bonferroni corrected t test, P < 0.05) found that the abundance of 105 metabolites (60%) detected by GC-MS and 38 (33%) detected by LC-MS differed significantly between the M. gallisepticum wild-type strain AP3AS and the ΔmalF mutant. Comparisons of the metabolites involved in glycerol metabolism and glycolysis found significantly higher abundances of maltose, sucrose, glucose-6-phosphate, fructose-1-phosphate, 2-phosphoglyceric acid, and 3-phosphoglyceric acid in the ΔmalF mutant. The levels of glycerol were significantly higher in the wild-type strain AP3AS than in the ΔmalF mutant in both GC-MS and LC-MS analyses (Fig. 3).

[U-13C]glycerol labeling suggests that MalF transports glycerol and that, in its absence, transport of glycerol-3-phosphate is significantly increased. The greater

![FIG 2 Growth of the M. gallisepticum wild-type strain AP3AS and the ΔmalF mutant in MB and MB-glycerol medium.](image-url)

![FIG 3 Fold change differences (log2) in the abundance of metabolites related to glycerol metabolism in the M. gallisepticum wild-type strain AP3AS and the ΔmalF mutant in two independent untargeted metabolite profiling experiments on GC-MS (a) and LC-MS (b) platforms. Only metabolites with statistically significant differences in abundance are shown (Holm-Bonferroni corrected t test; false discovery rate cutoff, 0.05).](image-url)
abundance of glycerol in the wild-type strain in the LC-MS steady-state analysis suggested that MalF may be involved in glycerol uptake, as previously suggested for *M. pneumoniae* (8). To validate this finding, both strains were labeled with 5 mM [U-13C] glycerol, and 13C-label enrichment in intracellular metabolites was determined by GC-MS. Interestingly, the intracellular abundance of labeled glycerol in AP3AS was much higher than in the ΔmalF mutant, resulting in GC-MS column overload in the wild type. However, there was significantly higher incorporation of label into glycerol-3-phosphate in the ΔmalF mutant compared to the wild type. These results suggest higher uptake of labeled glycerol-3-phosphate from the medium by the *malF* mutant. The downstream glycolytic pathway intermediates also incorporated more label in the ΔmalF mutant (Fig. 4).

RT-qPCR experiments suggest upregulation of *PtsG* and *GtsA* transporters in the absence of MalF. To investigate the expression of gene sets related to glycerol uptake and metabolism, both the *M. gallisepticum* wild-type AP3AS and the ΔmalF mutant strains were cultured to early (6 h) and mid-logarithmic (12 h) growth phase in the presence and the absence of glycerol, and total RNA was extracted from three replicates and reverse transcribed to cDNA. These assays detected significantly higher expression in the ΔmalF mutant compared to the wild type of *ptsG* after 6 h, *gtsA* after 12 h in mycoplasma broth (MB) medium, and *gtsA* after 12 h of incubation in MB-glycerol medium (Fig. 5). Interestingly, no significant difference in transcription was detected of the glycerol kinase gene *glpK* or the glycerol-3-phosphate oxidase gene *glpO*.

The ΔmalF mutant produces significantly less H₂O₂ than does wild-type *M. gallisepticum*. Semiquantitative QuantoFix peroxide test sticks (Macherey-Nagel) were used to determine the hydrogen peroxide concentrations in three replicate cultures of the *M. gallisepticum* wild-type AP3AS and the ΔmalF mutant. Significantly less hydrogen peroxide production was detected in the cultures of the ΔmalF mutant than in cultures of the wild type at the mid-logarithmic phase of growth in the presence or absence of glycerol. Interestingly, hydrogen peroxide production was significantly higher in the ΔmalF mutant in the presence of glycerol than in the absence of glycerol, although production was still significantly lower than in wild type grown under the same conditions (Fig. 6).

Electron microscopy demonstrated morphological differences between *M. gallisepticum* wild-type strain AP3AS and the ΔmalF mutant. Transmission electron microscopy showed that ΔmalF mutant cells, cultured in either the presence or the absence of glycerol, were significantly larger than those of the wild type (Fig. 7 and 8) under both conditions. When cultured in the absence of glycerol, the ΔmalF mutant cells had an average width of 467 nm and an average length of 626 nm compared to an average width of 433 nm and an average length of 520 nm for the wild type. In the presence of glycerol, the ΔmalF mutant cells had an average width of 870 nm and an average length of 1,024 nm compared to an average length of 462 nm and an average width of 389 nm for the wild type. The wild-type cells under both conditions, as well as the ΔmalF mutant cells cultured in the presence of glycerol, appeared to be more uniform in shape than those of the ΔmalF mutant cells cultured in the absence of glycerol, which also appeared to be more elongated. The intracellular structures also appeared less organized in the ΔmalF mutant than in the wild type in either the presence or absence of glycerol (Fig. 7).

**DISCUSSION**

In mycoplasmas ABC transporters play a major role in metabolism, importing nutrients that the mycoplasmas are unable to synthesize, and a number are multifunctional. They have also been shown to play critical roles in pathogenesis and survival *in vivo* (4, 27–29). The MalF transporter in *E. coli* is responsible for maltose uptake, but in *M. pneumoniae* the *malF* homologue has been predicted to be responsible for transporting sn-glycerol-3-phosphate (8). However, this has not been validated experimentally. In the study described here, the significantly higher abundance of maltose and sucrose, as well as glucose-6-
phosphate and fructose-1-phosphate, in the ΔmalF mutant, compared to the wild type, suggested that MalF is not a sugar transporter in *M. gallisepticum*.

Steady-state metabolite analyses using both GC-MS and LC-MS revealed a significantly lower abundance of glycerol in the ΔmalF mutant than in the wild type. The GC-MS analysis detected significantly higher abundances of 2-phosphoglyceric acid and 3-phosphoglyceric acid, compounds that are downstream intermediates of the glycolytic pathway, in the ΔmalF mutant compared to the wild type. The differing abundances of these metabolites suggested a significant change in glycerol metabolism in the absence of MalF. The [U-13C]glycerol labeling study resulted in overload of the GC-MS

**Fig 4** Metabolites labeled in the *M. gallisepticum* wild-type strain AP3AS and the ΔmalF mutant after culture in medium containing [U-13C]glycerol. The scale indicates the proportion of the metabolite containing the label. Values were normalized to the 13C/12C ratio in the medium. *, P < 0.05; **, P < 0.01; ***, P < 0.001 (Holm-Sidak corrected t test). A '?' indicates that the transporter is unknown (but is suggested to be GtsA based on the RT-qPCR results in this study).
column with labeled glycerol when labeled wild-type cells were analyzed, suggesting that glycerol uptake was much higher in this strain than in the \( \Delta \text{malF} \) mutant, which was not overloaded, suggesting that the substrate for the MalF transporter of \( M. \text{gallisepticum} \) was glycerol. However, somewhat surprisingly, there was significantly more label incorporation into glycerol-3-phosphate in the \( \Delta \text{malF} \) mutant, suggesting that labeled glycerol-3-phosphate was being transported into the mycoplasma cell at a higher rate in the \( \text{malF} \) mutant. There was no difference in transcription of \( \text{glpK} \) in the \( \Delta \text{malF} \) mutant, suggesting that intracellular conversion of glycerol to glycerol-3-phosphate was similar in the wild type and the mutant or possibly that regulation of this enzyme occurs at a posttranslational level. Uptake of labeled glycerol-3-phosphate

![Graph showing expression fold change of genes in Mycoplasma broth and Mycoplasma broth-glycerol](image)

**FIG 5** Comparative analysis of transcripts from genes involved in glycerol transport and metabolism in the \( M. \text{gallisepticum} \) wild-type strain AP3AS and the \( \Delta \text{malF} \) mutant cultured in MB (a) or MB-glycerol (b) medium for 6 or 12 h. *, \( P < 0.05 \); **, \( P < 0.01 \) (Holm-Sidak corrected t test).

**FIG 6** Hydrogen peroxide level scores for cultures of the \( M. \text{gallisepticum} \) wild-type strain AP3AS and the \( \Delta \text{malF} \) mutant (Mann-Whitney test; *, \( P < 0.05 \)).
would require extracellular conversion of glycerol to glycerol-3-phosphate by a currently unknown glycerol kinase on the mycoplasma cell surface. Many surface-displayed glycolytic enzymes have been described in mycoplasmas, suggesting that surface-displayed kinases could potentially be responsible for production of glycerol-3-phosphate from glycerol (30–32). The significantly higher expression of <i>ptsG</i>, annotated as the glucose-specific

![Figure 7](image1.png)

FIG 7 Transmission electron micrographs of the <i>M. gallisepticum</i> wild-type strain AP3AS in the presence (a) or absence (b) of glycerol and of <i>M. gallisepticum ΔmalF</i> mutant cells in the presence (c) or absence (d) of glycerol. Scale bar, 2 μm. Arrows indicate an electron-dense region, which is probably the terminal bleb, in some of the wild-type cells.

![Figure 8](image2.png)

FIG 8 Average cell size of the <i>M. gallisepticum</i> wild-type strain AP3AS and the <i>ΔmalF</i> mutant in the presence or absence of glycerol. ***, P < 0.001; ****, P < 0.0001 (one-way analysis of variance, followed by Tukey’s multiple-comparison test).
IIABC component of a phosphoenolpyruvate-dependent phosphotransferase system (PTS), in the ΔmalF mutant after 6 h of incubation, suggests that this PTS system may also be involved in glycerol transport. This system usually phosphorylates substrates as they are transported, which could also explain the higher abundance of glycerol-3-phosphate in ΔmalF mutant.

Our results indicated that MalF was unlikely to be transporting glycerol-3-phosphate into the cell, as has been proposed for the homologous transporter in *M. pneumoniae*, since this compound was significantly more abundant in the *malF* mutant. Rather, our results suggested that MalF was transporting glycerol and that, in the absence of this transporter, the decreased intracellular levels of glycerol or the absence of the MalF protein itself resulted in increased transcription of *ptsG* and *gtsA*. It also seems more likely that the GtsA transporter in *M. gallisepticum*, rather than transporting glycerol (as in *M. bovis*), is transporting glycerol-3-phosphate, resulting in the higher levels of glycerol-3-phosphate observed in the Δ*malF* mutant.

In *E. coli* the homologous ABC transporter, as well as importing maltose, plays a regulatory role (6, 7, 33–36). Neither the *glpQ* nor the *ugpQ* genes, which are thought to be key regulators of glycerol metabolism in firmicutes, are present in the *M. gallisepticum* genome. The higher incorporation of label from glycerol into downstream intermediates of glycolysis in the ΔmalF mutant suggested increased flux through this pathway in the absence of MalF, also suggesting that MalF may play a direct or indirect role in regulation of glycerol metabolism in *M. gallisepticum*. The ΔmalF mutant is less fit than the wild type both *in vitro* and *in vivo*, growing to lower cell densities *in vitro* (4) and failing to persist in chickens (25), suggesting that the loss of MalF has a fundamental effect on metabolism.

Significantly lower production of hydrogen peroxide was observed in the ΔmalF mutant than in the wild type, suggesting lower activity of GlpO, although the RT-qPCR results indicated that *glpO* was not differentially transcribed in the absence of MalF, which suggests possible posttranslational regulation of GlpO activity by MalF. Alternatively, MalF, or MalF in the presence of glycerol, could affect the enzymatic functionality of GlpO by modifying its structure or its location in the mycoplasma. Surprisingly, significantly higher production of hydrogen peroxide was detected in the ΔmalF mutant in the presence of glycerol, suggesting an increase in conversion of glycerol-3-phosphate to DHAP in the ΔmalF mutant when it was cultured in rich glycerol medium, which accords with the increased label incorporation into DHAP in the ΔmalF mutant in the glycerol labeling study. The increased flux through this pathway when glycerol is available as a carbon source, despite the absence of any change in expression of *glpO*, suggests that there may be posttranslational regulation of GlpO, mediated by the level of available glycerol. Future studies using various concentrations of labeled glycerol would be useful to confirm this. The activity of GlpO has been linked to virulence in several mycoplasmas (14, 19, 28, 37–40), and the reduction in hydrogen peroxide production by the *malF* mutant may also be linked to its inability to persist in chickens (25).

The morphological differences between the strains, as visualized using electron microscopy, suggest that the cellular structure was disturbed in the ΔmalF mutant, resulting in less uniform cells that were significantly larger than the AP3AS wild type. The presence or absence of glycerol in the growth medium also had a significant effect on cell morphology, but only when *malF* was interrupted, further suggesting that the role of MalF relates to glycerol metabolism. This may have affected the capacity of ΔmalF mutant cells to bind to host cells, contributing to their reduced ability to persist in the trachea of chickens (25). In *M. pneumoniae* glycerol-3-phosphate can be converted to 1-acyl-glycerol-3-phosphate by glycerol-3-phosphate acyltransferase and enter the lipid biosynthesis pathway (41, 42). Bioinformatic analysis demonstrated that the homologous pathway is also present in many *M. gallisepticum* strains such as R<sub>high</sub> (GenBank accession no. NC_017502), R<sub>low</sub> (GenBank no. NC_004829), and S6 (GenBank no. NC_023030). During attachment of mycoplasmas, the mycoplasma cytoplasmic
membrane is in direct contact with the cytoplasmic membrane of the host cell and thus plays a critical role in adherence. Increased flux of glycerol-3-phosphate into glycolysis in the ΔmalF mutant may have resulted in its reduced availability for lipid biosynthesis, and therefore altered cell membrane structures, resulting in a reduced capacity for colonization (25). An electron-dense polar terminal structure, the terminal bleb, which contributes to its flask-like morphology, has been described in M. gallisepticum, and this structure is thought to be involved in attachment, cell division, and gliding motility (43, 44). The terminal bleb has been characterized in the phylogenetically related human pathogen, M. pneumoniae, with several adhesion proteins clustered at the tip of the bleb shown to be essential for attachment (45). The absence of these electron-dense regions in the ΔmalF mutant may reflect changes in the structure of the terminal bleb, which could also reduce colonization of chickens by the ΔmalF mutant (25).

In conclusion, we have demonstrated that the MalF ABC transporter of M. gallisepticum does not import maltose or glycerol-3-phosphate. Rather, it appears to transport glycerol, and in the absence of MalF, glycerol metabolism is significantly perturbed, resulting in a loss of fitness that may explain the previously observed reduced pathogenicity of the ΔmalF mutant. Consequently, the operon including this gene should be reannotated as the GolABC transporter to reflect its function in glycerol transport. We also propose that the gtsA gene of M. gallisepticum encodes a glycerol-3-phosphate transporter, the activity of which is regulated by either intracellular glycerol concentrations or the GolB protein itself. This study provides functional characterization of GolB in M. gallisepticum and is a key step forward in understanding why this protein plays a key role in virulence in chickens. Since glycerol metabolism appears to be an important aspect of M. gallisepticum metabolism in general, the effect of other sugars, such as glucose, on glycerol metabolism needs further investigation.

MATERIALS AND METHODS

Bioinformatics and database searches for protein function. The UniProt web server was used to search for similarities between MalF and other proteins (https://www.uniprot.org). Sequences used for searching were the translated nucleotide sequences obtained from the M. gallisepticum Rlow genome sequence. The protein structure prediction tool Phyre2 was also used to assist in prediction of the function of MalF (26).

M. gallisepticum AP3AS strains and culture conditions. The M. gallisepticum wild-type strain Ap3AS was kindly provided by Anna Kanci Condello, Asia-Pacific Centre for Animal Health, Melbourne, Victoria, Australia. For routine culture M. gallisepticum Ap3AS was cultured at 37°C for 18 h in MB (0.75% Trypticase peptone, 0.25% phytone peptones, 0.05% proteose peptones, 0.5% yeast extract, 0.5% NaCl, 0.04% KCl, 0.035% MgSO4·7H2O, 0.005% Na2PO4, 0.2% DNA, 1% yeast autohydrolysate, 10% inactivated swine serum, 0.0048% phenol red solution and 5.56 mM glucose [pH adjusted to 8.1]) (46). When required, 5 mM glycerol was added to the MB medium. Previous work generated the ΔmalF mutant, which contains a Tn9001 transposon insertion at the 5’ end of the MGA_0680 gene (locus tag, MGA_RS00175; transposon insertion point in genome, positions 46569 to 46570; ratio of gene to inserter, 0.04% KCl, 0.035% MgSO4·7H2O, 0.005% Na2PO4, 0.2% DNA, 1% yeast autohydrolysate, 10% inactivated swine serum, 0.0048% phenol red solution and 5.56 mM glucose [pH adjusted to 8.1]) (46). When required, 5 mM glycerol was added to the MB medium. Previous work generated the ΔmalF mutant, which contains a Tn9001 transposon insertion at the 5’ end of the MGA_0680 gene (locus tag, MGA_RS00175; transposon insertion point in genome, positions 46569 to 46570; ratio of gene to inserter, 0.04% KCl, 0.035% MgSO4·7H2O, 0.005% Na2PO4, 0.2% DNA, 1% yeast autohydrolysate, 10% inactivated swine serum, 0.0048% phenol red solution and 5.56 mM glucose [pH adjusted to 8.1]) (46). When required, 5 mM glycerol was added to the MB medium. Previous work generated the ΔmalF mutant, which contains a Tn9001 transposon insertion at the 5’ end of the MGA_0680 gene (locus tag, MGA_RS00175; transposon insertion point in genome, positions 46569 to 46570; ratio of gene to inserter, 0.04% KCl, 0.035% MgSO4·7H2O, 0.005% Na2PO4, 0.2% DNA, 1% yeast autohydrolysate, 10% inactivated swine serum, 0.0048% phenol red solution and 5.56 mM glucose [pH adjusted to 8.1]) (46). When required, 5 mM glycerol was added to the MB medium. Previous work generated the ΔmalF mutant, which contains a Tn9001 transposon insertion at the 5’ end of the MGA_0680 gene (locus tag, MGA_RS00175; transposon insertion point in genome, positions 46569 to 46570; ratio of gene to inserter, 0.04% KCl, 0.035% MgSO4·7H2O, 0.005% Na2PO4, 0.2% DNA, 1% yeast autohydrolysate, 10% inactivated swine serum, 0.0048% phenol red solution and 5.56 mM glucose [pH adjusted to 8.1]) (46). When required, 5 mM glycerol was added to the MB medium. Previous work generated the ΔmalF mutant, which contains a Tn9001 transposon insertion at the 5’ end of the MGA_0680 gene (locus tag, MGA_RS00175; transposon insertion point in genome, positions 46569 to 46570; ratio of gene to inserter, 0.04% KCl, 0.035% MgSO4·7H2O, 0.005% Na2PO4, 0.2% DNA, 1% yeast autohydrolysate, 10% inactivated swine serum, 0.0048% phenol red solution and 5.56 mM glucose [pH adjusted to 8.1]) (46). When required, 5 mM glycerol was added to the MB medium. Previous work generated the ΔmalF mutant, which contains a Tn9001 transposon insertion at the 5’ end of the MGA_0680 gene (locus tag, MGA_RS00175; transposon insertion point in genome, positions 46569 to 46570; ratio of gene to inserter, 0.04% KCl, 0.035% MgSO4·7H2O, 0.005% Na2PO4, 0.2% DNA, 1% yeast autohydrolysate, 10% inactivated swine serum, 0.0048% phenol red solution and 5.56 mM glucose [pH adjusted to 8.1]) (46).
indicating growth of the bacteria, was used to determine the most probable number of mycoplasmas present in each of the inocula (49).

**Metabolic quenching of the cultures.** Six biological replicates of *M. gallisepticum* wild type and the ΔmalF mutant were cultured to mid-logarithmic phase in MB medium (based on growth curves determined previously (4)) and then rapidly quenched to approximately 0°C in an ethanol/dry ice bath to halt metabolic activity. The mycoplasma cells were harvested by centrifugation (20,000 × g, 20 min, 0°C) and washed twice in ice-cold phosphate-buffered saline (PBS; 17,000 × g, 5 min, 0°C). The pellet was then either immediately used for polar metabolite extraction or stored at −80°C for future extraction.

**Extraction of polar metabolites and GC-MS analysis.** To extract the polar metabolites, 250 μl of chloroform/methanol-water (CHCl3:CH3OH:H2O, 1:3:1 [vol/vol]), containing 1 nmol of [U-13C]sorbitol and 10 nmol of [U-13C]-N15-valine as internal standards, was added to cell pellets and the mixture vortexed vigorously. The samples were then incubated at 60°C for 15 min to lyse the cells and centrifuged (17,000 × g, 5 min, 0°C) to remove the cell debris. The supernatant was transferred to cooled microcentrifuge tubes containing 100 μl of ice-cold dH2O and then vortexed and centrifuged (17,000 × g, 5 min, 0°C) to achieve phase separation. The upper aqueous phase, containing the polar metabolites, was transferred into glass vials and completely dried in a rotational vacuum concentrator (RVC-2-33; John Morris Scientific) at 37°C, with 40 μl of 100% methanol added for the final drying stage. Metabolites were then derivatized in methoxyamine chloride (20 μl, 30 mg/ml in pyridine; Sigma) with continuous mixing (2 h, 37°C), followed by treatment with 20 μl of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS; Thermo Scientific) (1 h, 37°C, with continuous shaking) using a Gerstel MPS2 autosampler robot. A 1-μl volume of the derivatized sample was injected into an Agilent 7890A gas chromatograph (split/splitless inlet, 250°C) containing a VF-5ms column (30 m/0.25 m/0.25 μm/10 m [Eiguard precolumn]) coupled to an Agilent 5975C mass selective detector (Agilent Technologies) as described previously (4). Briefly, metabolites were separated by injecting a 10-μl volume of the samples into an autosampler at 4°C onto a SeQuant ZIC-pHILIC column (150 mm × 4.6 mm, 30 μm, Merck) at a column temperature of 40°C using solvent A (20 mM (NH4)2CO3, pH 9.0, Sigma-Aldrich) and solvent B (100% acetonitrile; Hyper grade for LC-MS Lichrosolv, Merck) at a flow rate of 300 μl/min. MS analysis was performed on an Agilent 6545 series quadruple time-of-flight mass spectrometer (QTOF-MS; Agilent Technologies). The metabolite ionization in negative mode was performed by directing the LC flow to electrospray ionization with a capillary voltage of 2,500 V, a drying gas (N2) pressure of 20 lb/in², a gas flow rate of 10.0 liters/min, a gas temperature in the capillary of 300°C, and fragmentor and skimmer cap voltages of 125 and 45 V, respectively. Data were collected in centroid mode with a mass range of 60 to 1,200 m/z and an acquisition rate of 1.5 spectra/s in all-ion fragmentor mode. Data matrices were generated using MassHunter quantitative analysis software (vB07.00; Agilent Technologies), and metabolites were identified by comparison of retention times and fragmented ion patterns of the representative chromatograms to those of the Metabolomics Australia in-house library using the MassHunter Quantitative analysis software (Agilent Technologies).

**Extraction of polar metabolites and LC-MS analysis.** The polar metabolites to be analyzed by LC-MS were extracted by adding 250 μl of chloroform/methanol-water (CHCl3:CH3OH:H2O, 1:3:1 [vol/vol]), containing 2 μM [U-13C]sorbitol, 10 nmol of [U-13C]-N15-valine, [U-13C]AMP, and [U-13C]UMP as internal standards, to the cell pellet. After vortexing for 10 s, samples were incubated at 4°C for 15 min to lyse the cells. Cell debris was removed by centrifugation (17,000 × g, 5 min, 4°C), and the supernatant, containing the polar metabolites, was analyzed by using an Agilent 1200 series HPLC system (Agilent Technologies) as described previously (4). Briefly, metabolites were separated by injection of a 10-μl volume of the samples into an autosampler at 4°C onto a ZIC-pHILIC column (150 mm × 4.6 mm, 4.6 μm, Merck) at a column temperature of 40°C using solvent A (20 mM (NH4)2CO3, pH 9.0, Sigma-Aldrich) and solvent B (100% acetonitrile; Hyper grade for LC-MS Lichrosolv, Merck) at a flow rate of 300 μl/min. MS analysis was performed on an Agilent 6465 series quadrupole time-of-flight mass spectrometer (QTOF-MS; Agilent Technologies). The metabolite ionization in negative mode was performed by directing the LC flow to electrospray ionization with a capillary voltage of 2,500 V, a drying gas (N2) pressure of 20 lb/in², a gas flow rate of 10.0 liters/min, a gas temperature in the capillary of 300°C, and fragmentor and skimmer cap voltages of 125 and 45 V, respectively. Data were collected in centroid mode with a mass range of 60 to 1,200 m/z and an acquisition rate of 1.5 spectra/s in all-ion fragmentor mode. Data matrices were generated using MassHunter quantitative analysis software (vB07.00; Agilent Technologies), and metabolites were identified by comparison of retention times and molecular masses with those of the in-house metabolites library. Data matrices were generated using MassHunter quantitative analysis software (vB07.00; Agilent Technologies), and metabolites were identified by comparison of retention times and molecular masses with those of the in-house Metabolomics Australia library (authentic standards).

**Stable isotope ([U-13C]glycerol) labeling study.** Four biological replicates of *M. gallisepticum* wild type and the ΔmalF mutant were cultured to late-logarithmic phase and then labeled for 12 h in MB medium containing 1 or 5 mM [U-13C]glycerol. Metabolic quenching and extraction of the polar metabolites were performed as described above for untargeted analysis on the GC-MS platform, with the exception that the Agilent 5975 mass selective detector (Agilent Technologies) was used. Metabolites were detected using Agilent MassHunter quantitative analysis software, and mass isotopomer distribution vectors were assigned to each fragment. The analyses were then corrected for naturally occurring background labeling (50, 51). The labeling level of each metabolite was recorded as a proportion of the total.

**Comparison of the metabolite profiles of *M. gallisepticum* AP3AS wild type and the ΔmalF mutant.** The data matrices were normalized to median, log transformed and analyzed using the multiple Holm-Bonferroni adjusted t test, with *P* < 0.05 considered significant. The GC data from one wild-type replicate were excluded because the quality of the chromatography was poor.

**Quantitative reverse transcription-PCR assay.** Three biological replicates of *M. gallisepticum* wild type and the ΔmalF mutant were cultured for 6 and 12 h (early and mid-logarithmic phase, respectively) in MB and MB-glycerol media, and harvested by centrifugation (500 × g, 20 min, 22°C), and RNA was extracted using an RNAeasy minikit (Qiagen) according to the manufacturer’s protocol. The purified nucleic acid was treated with RNase to eliminate any DNA contamination using the Turbo DNA-free kit (Invitrogen), followed by further clean-up using an RNAeasy minikit (Qiagen). The concentration and purity of the extracted RNA were determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific), and the RNA was then transcribed to cDNA. Briefly, 1 μg of RNA was added to a solution containing 50 μM random hexamers and 10 mM concentrations of each deoxynucleoside triphosphate (dNTP), and the reaction mixture was then incubated at 65°C for 5 min. The solution was then immediately incubated on ice, and 5 × first-strand buffer, 0.1 M dithiothreitol, 40 U of RNaseOUT recombinant RNase inhibitor, and 200 U of SuperScript III reverse transcriptase (Invitrogen) were added, followed by incubation at 55°C for 60 min. The enzyme was then inactivated by incubation at 70°C for 15 min. The cDNA was stored at −20°C.
**TABLE 1** Primers used in RT-qPCR

| Target gene    | Putative protein | Primer 1 | Sequence (5’–3’)
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>mnuA (MGA_0637)</td>
<td>Nuclease</td>
<td>mnuA-F</td>
<td>CAACCTTCTGATGCTGACGTTG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>mnuA-R</td>
<td>TTCAGAAGACTACCTAGTGCCGTC</td>
</tr>
<tr>
<td>glpK (MGA_0644)</td>
<td>Glycerol kinase</td>
<td>glpK-F</td>
<td>CTCGGTCTGATCTTCCGAAAG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>glpK-R</td>
<td>TCCACCAACACCTGTAATC</td>
</tr>
<tr>
<td>glpO (MGA_0646)</td>
<td>Glycerol-3-phosphate oxidase</td>
<td>glpO-F</td>
<td>CAGGAATATCGGCGCTTCA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>glpO-R</td>
<td>ACCAAATGAAGTTTCCGTTGG</td>
</tr>
<tr>
<td>gtsA (MGA_1076)</td>
<td>Glycerol ABC transporter</td>
<td>gtsA-F</td>
<td>TGACCCAGAACGATCGATG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>gtsA-R</td>
<td>TCGAACGATCGAATTGAGC</td>
</tr>
<tr>
<td>ptsG (MGA_0855)</td>
<td>PTS transporter</td>
<td>ptsG-F</td>
<td>GTGAAGACATCGAACAGAAG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ptsG-R</td>
<td>TCGAAGAAGTAAACGCCTGAC</td>
</tr>
</tbody>
</table>

The cDNA was used as the template for qPCR in reaction mixtures containing 2 mM MgCl₂, 200 μM concentrations of each dNTP, 400 nM concentrations of each primer, 8 μM SYTO 9 (Thermo Fisher Scientific), 0.04 U of GoTaq Flexi DNA polymerase (Promega), Colourless GoTaq Flexi buffer (Promega), and 3 μl of template in a 20-μl reaction. The PCR was incubated in a Rotor-Gene Q 2plex HRM instrument (Qiagen) with an initial incubation at 95°C for 3 min, followed by 60 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 60 s, with fluorescence acquisition at the end of each cycle. Every reaction was performed in triplicate and in three independent experiments. Each experiment included RT-negative reactions as a control for residual DNA and a qPCR targeting the mnuA gene, which has no role in glycerol metabolism, was used as a reference. The primers used in the qPCRs are listed in Table 1. The data were then processed using the Rotor-Gene Q series software (version 2.1.0 Build 9). The mnuA qPCR data were analyzed using the ΔΔCT method to achieve normalization. Statistical analysis was performed using a Holm-Sidak corrected t test, with P < 0.05 considered significant.

**Hydrogen peroxide assay.** To investigate the hydrogen peroxide levels produced by the *M. gallisepticum* wild type and the *ΔmalF* mutant, three replicate cultures of each of these strains were incubated to mid-logarithmic growth phase in MB or MB-glycerol media. The concentrations of hydrogen peroxide produced by each culture were measured as described previously (14). Briefly, 10⁷ color-changing units (CCU) of cells were harvested by centrifugation at 10,000 × g for 10 min at 4°C. The cells were washed twice in 1 ml of HEPES buffer (70 mM HEPES, 140 mM NaCl, 7 mM MgCl₂ [pH 7.4]) and then resuspended in 1 ml of HEPES buffer. The resuspended cells were then incubated for 1 h at 37°C, 5 mM glycerol was added to each suspension, and the cells were incubated for a further 20 min. The amount of hydrogen peroxide in each culture was determined using Quantofix peroxide test sticks (Macherey-Nagel) according to the manufacturer’s protocol. The results were analyzed using a Mann-Whitney test, with P < 0.05 considered significant.

**Transmission electron microscopy.** The *M. gallisepticum* wild type and the *ΔmalF* mutant were cultured to late-logarithmic phase. The cells were harvested by centrifugation (20,000 × g, 20 min) and washed twice in PBS (17,000 × g, 5 min). The pellets were then fixed in 2% glutaraldehyde overnight at 4°C and postfixed with 1% buffered osmium tetroxide and embedded in Spurr’s resin. Ultrathin sections were contrasted with uranyl acetate and Reynolds’s lead citrate and examined with a Philips CM10 transmission electron microscope at 60 kV. The image analysis was performed using ImageJ (52), and analyzed using an unpaired t test with Welch’s correction, with P < 0.05 considered significant.

**Statistical analysis.** The statistical analyses for all the experiments were performed using GraphPad Prism (v6.00 for Windows) and MetaboAnalyst software (53).

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1**, PDF file, 0.2 MB.

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**REFERENCES**


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