A *Mycoplasma gallisepticum* Glycerol ABC Transporter Involved in Pathogenicity

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ABSTRACT 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; $\Delta malF$) 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 $\Delta malF$ 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 $\Delta malF$ 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

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Mycoplasmas evolved from Gram-positive bacteria by reductive evolution, resulting in the loss of many metabolic pathways and a dependence on their hosts for

FIG 1 Overview of glucose, fructose, and glycerol uptake and metabolism by *M. gallisepticum* based on experimental data and genomic analyses. Question marks indicate that the transporter activity is not yet experimentally validated, but GtsA is predicted to transport glycerol, and MalF is the focus of the research described here. DHAP, dihydroxyacetone phosphate.

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 R_{low} and the attenuated variant R_{high} identified an amino acid substitution in UgpC, the ATP binding protein of the Ugp ABC transporter, in R_{high}. 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 R_{high} 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; $\Delta malF$) 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 $\Delta malF$ mutant. To investigate the effect of glycerol on the growth of the *M. gallisepticum* wild-type strain AP3AS and the $\Delta malF$ 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

FIG 2 Growth of the *M. gallisepticum* wild-type strain AP3AS and the $\Delta malF$ mutant in MB and MB-glycerol medium.

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 $\Delta malF$ 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 $\Delta malF$ 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 $\Delta malF$ mutant. The levels of glycerol were significantly higher in the wild-type strain AP3AS than in the $\Delta malF$ 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 3 Fold change differences ($\log_2$) in the abundance of metabolites related to glycerol metabolism in the *M. gallisepticum* wild-type strain AP3AS and the $\Delta malF$ 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).

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 $\Delta malF$ 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 $\Delta malF$ 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 $\Delta malF$ 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 $\Delta malF$ 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 $\Delta malF$ 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 $\Delta malF$ 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 $\Delta malF$ mutant. Significantly less hydrogen peroxide production was detected in the cultures of the $\Delta malF$ 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 $\Delta malF$ 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 $\Delta malF$ mutant. Transmission electron microscopy showed that $\Delta malF$ 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 $\Delta malF$ 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 $\Delta malF$ 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 $\Delta malF$ mutant cells cultured in the presence of glycerol, appeared to be more uniform in shape than those of the $\Delta malF$ mutant cells cultured in the absence of glycerol, which also appeared to be more elongated. The intracellular structures also appeared less organized in the $\Delta malF$ 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-

FIG 4 Metabolites labeled in the *M. gallisepticum* wild-type strain AP3AS and the $\Delta malF$ mutant after culture in medium containing $[\text{U-}^{13}\text{C}]$ glycerol. The scale indicates the proportion of the metabolite containing the label. Values were normalized to the $^{13}\text{C}/^{12}\text{C}$ 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).

phosphate and fructose-1-phosphate, in the $\Delta malF$ 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 $\Delta malF$ 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 $\Delta malF$ 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 $[\text{U-}^{13}\text{C}]$ glycerol labeling study resulted in overload of the GC-MS

FIG 5 Comparative analysis of transcripts from genes involved in glycerol transport and metabolism in the *M. gallisepticum* wild-type strain AP3AS and the $\Delta malF$ 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).

column with labeled glycerol when labeled wild-type cells were analyzed, suggesting that glycerol uptake was much higher in this strain than in the $\Delta malF$ mutant, which was not overloaded, suggesting that the substrate for the MalF transporter of *M. gallisepticum* was glycerol. However, somewhat surprisingly, there was significantly more label incorporation into glycerol-3-phosphate in the $\Delta malF$ mutant, suggesting that labeled glycerol-3-phosphate was being transported into the mycoplasma cell at a higher rate in the *malF* mutant. There was no difference in transcription of *glpK* in the *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

FIG 6 Hydrogen peroxide level scores for cultures of the *M. gallisepticum* wild-type strain AP3AS and the $\Delta malF$ mutant (Mann-Whitney test; *, $P < 0.05$).

FIG 7 Transmission electron micrographs of the *M. gallisepticum* wild-type strain AP3AS in the presence (a) or absence (b) of glycerol and of *M. gallisepticum* $\Delta malF$ mutant cells in the presence (c) or absence (d) of glycerol. Scale bar, 2 μ m. Arrows indicate an electron-dense region, which is probably the terminal bleb, in some of the wild-type cells.

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 *ptsG*, annotated as the glucose-specific

FIG 8 Average cell size of the *M. gallisepticum* wild-type strain AP3AS and the $\Delta malF$ 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 $\Delta malF$ 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 $\Delta malF$ 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 $\Delta malF$ 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 $\Delta malF$ 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 $\Delta malF$ 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 $\Delta malF$ 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 $\Delta malF$ mutant in the presence of glycerol, suggesting an increase in conversion of glycerol-3-phosphate to DHAP in the $\Delta malF$ mutant when it was cultured in rich glycerol medium, which accords with the increased label incorporation into DHAP in the $\Delta malF$ 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 $\Delta malF$ 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 $\Delta malF$ 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_{high} (GenBank accession no. [NC_017502](#)), R_{low} (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 $\Delta malF$ 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 $\Delta malF$ mutant may reflect changes in the structure of the terminal bleb, which could also reduce colonization of chickens by the $\Delta malF$ 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 $\Delta malF$ 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* R_{low} 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% $MgSO_4 \cdot 7H_2O$, 0.005% Na_2PO_4 , 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 $\Delta malF$ mutant, which contains a Tn4001 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 insertion point, 0.2%) (25). The $\Delta malF$ mutant was cultured as for the wild-type strain, with gentamicin added at 50 μ g/ml to maintain the selective pressure for carriage of the transposon. To confirm the presence and position of the transposon in the $\Delta malF$ mutant, PCR and sequencing analyses were performed as described previously (4). Briefly, the IGstmGenmeF3 primer (complementary to the transposon), with the sequence GGACTGTTATATGGCCTTTTGGATC, was paired with STM04-B-1 primer (complementary to MGA_0680), with the sequence CGGGACACAGTAAGGCTAA, and PCR was performed using Go Taq DNA polymerase (Promega) according to the manufacturer's instructions. The PCR was incubated in an iCycler thermocycler (Bio-Rad) with an initial denaturing step at 95°C for 3 min, followed by 35 cycles of denaturing (95°C, 30 s), annealing (52°C, 30 s), and extension (72°C, 60 s), with a final extension step at 72°C for 3 min. The PCR products were subjected to agarose gel electrophoresis and Sanger sequencing. The location of the transposon in the $\Delta malF$ mutant was assessed by comparing the nucleotide sequence to that of the genome of *M. gallisepticum* R_{low} (2).

Growth of AP3AS and $\Delta malF$ strains in vitro. To assess differences in growth between the wild type and the $\Delta malF$ mutant in MB media supplemented with 5 mM glycerol (MB-glycerol), growth curves were determined, with the number of viable mycoplasmas at each time point measured by titration, as described previously (47, 48). The strains were also cultured in standard MB medium for direct comparison. Briefly, the strains were recovered from -80°C by 1:10 dilution in fresh MB medium and incubated at 37°C for 18 h. These cultures were then diluted 1:10 in fresh medium (MB or MB-glycerol). At 0, 8, 16, and 24 h, samples were taken to determine the number of viable mycoplasmas by serial dilution in MB-glycerol broth in 96-well plates (Thermo Fisher Scientific). The color change in the medium,

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 $\Delta malF$ 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 $\times g$, 20 min, 0°C) and washed twice in ice-cold phosphate-buffered saline (PBS; 17,000 $\times g$, 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 μ l 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 $\times g$, 5 min, 0°C) to remove the cell debris. The supernatant was transferred to cooled microcentrifuge tubes containing 100 μ l of ice-cold dH₂O and then vortexed and centrifuged (17,000 $\times g$, 5 min, 0°C) to achieve phase separation. The upper aqueous phase, containing the polar metabolites, was transferred into glass vial inserts and completely dried in a rotational vacuum concentrator (RVC-2-33; John Morris Scientific) at 37°C, with 40 μ l of 100% methanol added for the final drying stage. Metabolites were then derivatized in methoxyamine chloride (20 μ l, 30 mg/ml in pyridine; Sigma) with continuous mixing (2 h, 37°C), followed by treatment with 20 μ l 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- μ l 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/250 μ m/0.25 μ m/10 m [Eziguard precolumn]) coupled to an Agilent 5975C mass selective detector. Metabolites were identified by comparison of the 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 μ l of chloroform-methanol-water (CHCl₃:CH₃OH:H₂O, 1:3:1 [vol/vol]), containing 2 μ M [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 $\times g$, 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 injecting a 10- μ l volume of the samples into an autosampler at 4°C onto a SeQuant ZIC-pHILIC column (150 mm $\times$ 4.6 mm, 5 μ m; Merck Millipore). The column temperature was maintained at 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 μ l/min. MS analysis was performed on an Agilent 6545 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 (vB.07.00; 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 $\Delta malF$ 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 Mass Hunter 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 $\Delta malF$ 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 $\Delta malF$ 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 $\times g$, 20 min, 22°C), and RNA was extracted using an RNeasy minikit (Qiagen) according to the manufacturer's protocol. The purified nucleic acid was treated with DNase to eliminate any DNA contamination using the Turbo DNA-free kit (Invitrogen), followed by further clean-up using an RNeasy 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 μ g of RNA was added to a solution containing 50 μ M 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 $\times$ 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	Sequence (5'–3')
<i>mnuA</i> (MGA_0637)	Nuclease	<i>mnuA</i> -F	CAACTTTCTGATGCTGACGTTG
		<i>mnuA</i> -R	TTCAAGAAGTACCTAGTGCCTC
<i>glpK</i> (MGA_0644)	Glycerol kinase	<i>glpK</i> -F	CTCGGTCGATTCTCCAGAAG
		<i>glpK</i> -R	TCACCAGCAACACCTGTAATC
<i>glpO</i> (MGA_0646)	Glycerol-3-phosphate oxidase	<i>glpO</i> -F	CAGGAATTATCGGCGCTTCA
		<i>glpO</i> -R	ACCCAATGAAGTTTCGTTTGC
<i>gtsA</i> (MGA_1076)	Glycerol ABC transporter	<i>gtsA</i> -F	TGACCAGAACGATGCGATG
		<i>gtsA</i> -R	TCAGGACGATCGAATTGAGC
<i>ptsG</i> (MGA_0855)	PTS transporter	<i>ptsG</i> -F	GTGTAAGCATCGAACCAGAAG
		<i>ptsG</i> -R	TCGAAGAAGTAAGCGTGACC

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 $\Delta\Delta CT$ 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 $\Delta malF$ 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 $\Delta malF$ 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 Reynold's lead citrate and examined with a Philips CM10 transmission electron microscope at 60 kV. The lengths and widths of 120 AP3AS wild-type and 120 $\Delta malF$ mutant cells cultured in the presence or absence of glycerol were measured across two sections 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

1. Kleven SH, Levisohn S. 1996. *Mycoplasma* infections of poultry, p 283–292. In Tully JG, Razin S (ed), *Molecular and diagnostic procedures in mycoplasmaology*, 2nd ed. Academic Press, San Diego, CA.
2. Papazisi L, Gorton TS, Kutish G, Markham PF, Browning GF, Nguyen DK, Swartzell S, Madan A, Mahairas G, Geary SJ. 2003. The complete genome sequence of the avian pathogen *Mycoplasma gallisepticum*

- strain R_{low}. Microbiology (Reading) 149:2307–2316. <https://doi.org/10.1099/mic.0.26427-0>.
3. Razin S. 1967. The cell membrane of mycoplasma. Ann N Y Acad Sci 143:115–129. <https://doi.org/10.1111/j.1749-6632.1967.tb27651.x>.
 4. Masukagami Y, Nijagal B, Tseng C-W, Dayalan S, Tivendale KA, Markham PF, Browning GF, Sansom FM. 2018. Metabolite profiling of *Mycoplasma gallisepticum* mutants, combined with bioinformatic analysis, can reveal the likely functions of virulence-associated genes. Vet Microbiol 223:160–167. <https://doi.org/10.1016/j.vetmic.2018.08.001>.
 5. Tseng C-W, Chiu C-J, Kanci A, Citti C, Rosengarten R, Browning GF, Markham PF. 2017. The *oppD* gene and putative peptidase genes may be required for virulence in *Mycoplasma gallisepticum*. Infect Immun 85:e00023-17. <https://doi.org/10.1128/IAI.00023-17>.
 6. Sharma S, Davis JA, Ayvaz T, Traxler B, Davidson AL. 2005. Functional reassembly of the *Escherichia coli* maltose transporter following purification of a MalF-MalG subassembly. J Bacteriol 187:2908–2911. <https://doi.org/10.1128/JB.187.8.2908-2911.2005>.
 7. Caldelari I, Palmer T, Sargent F. 2008. *Escherichia coli* *tat* mutant strains are able to transport maltose in the absence of an active *malE* gene. Arch Microbiol 189:597–604. <https://doi.org/10.1007/s00203-008-0356-8>.
 8. Schmidl SR, Otto A, Lluch-Senar M, Piñol J, Busse J, Becher D, Stülke J. 2011. A trigger enzyme in *Mycoplasma pneumoniae*: impact of the glycerophosphodiesterase GlpQ on virulence and gene expression. PLoS Pathog 7:e1002263. <https://doi.org/10.1371/journal.ppat.1002263>.
 9. Beijer L, Nilsson R-P, Holmberg C, Rutberg L. 1993. The *glpP* and *glpF* genes of the glycerol regulon in *Bacillus subtilis*. J Gen Microbiol 139:349–359. <https://doi.org/10.1099/00221287-139-2-349>.
 10. Darbon E, Servant P, Poncet S, Deutscher J. 2002. Antitermination by GlpP, catabolite repression via CcpA and inducer exclusion triggered by P~GlpK dephosphorylation control *Bacillus subtilis* *glpFK* expression. Mol Microbiol 43:1039–1052. <https://doi.org/10.1046/j.1365-2958.2002.02800.x>.
 11. Lin E. 1976. Glycerol dissimilation and its regulation in bacteria. Annu Rev Microbiol 30:535–578. <https://doi.org/10.1146/annurev.mi.30.100176.002535>.
 12. Halbedel S, Hames C, Stülke J. 2004. *In vivo* activity of enzymatic and regulatory components of the phosphoenolpyruvate: sugar phosphotransferase system in *Mycoplasma pneumoniae*. J Bacteriol 186:7936–7943. <https://doi.org/10.1128/JB.186.23.7936-7943.2004>.
 13. Snowden N, Wilson PB, Longson M, Pumphrey R. 1990. Antiphospholipid antibodies and *Mycoplasma pneumoniae* infection. Postgrad Med J 66:356–362. <https://doi.org/10.1136/pgmj.66.775.356>.
 14. Szczepanek S, Boccaccio M, Pflaum K, Liao X, Geary S. 2014. Hydrogen peroxide production from glycerol metabolism is dispensable for virulence of *Mycoplasma gallisepticum* in the tracheas of chickens. Infect Immun 82:4915–4920. <https://doi.org/10.1128/IAI.02208-14>.
 15. Djordjevic SP, Vilei EM, Frey J. 2003. Characterization of a chromosomal region of *Mycoplasma* sp. bovine group 7 strain PG50 encoding a glycerol transport locus (*gtsABC*). Microbiology (Reading) 149:195–204. <https://doi.org/10.1099/mic.0.25742-0>.
 16. Pilo P, Vilei EM, Peterhans E, Bonvin-Klotz L, Stoffel MH, Dobbelaere D, Frey J. 2005. A metabolic enzyme as a primary virulence factor of *Mycoplasma mycoides* subsp. *mycoides* small colony. J Bacteriol 187:6824–6831. <https://doi.org/10.1128/JB.187.19.6824-6831.2005>.
 17. Szczepanek S, Tulman E, Gorton T, Liao X, Lu Z, Zinski J, Aziz F, Frasca S, Kutish G, Geary S. 2010. Comparative genomic analyses of attenuated strains of *Mycoplasma gallisepticum*. Infect Immun 78:1760–1771. <https://doi.org/10.1128/IAI.01172-09>.
 18. Nilsson R-P, Beijer L, Rutberg B. 1994. The *glpT* and *glpQ* genes of the glycerol regulon in *Bacillus subtilis*. Microbiology 140:723–730. <https://doi.org/10.1099/00221287-140-4-723>.
 19. Blötz C, Stülke J. 2017. Glycerol metabolism and its implication in virulence in *Mycoplasma*. FEMS Microbiol Rev 41:640–652. <https://doi.org/10.1093/femsre/fux033>.
 20. Grosshennig S, Schmidl SR, Schmeisky G, Busse J, Stülke J. 2013. Implication of glycerol and phospholipid transporters in *Mycoplasma pneumoniae* growth and virulence. Infect Immun 81:896–904. <https://doi.org/10.1128/IAI.01212-12>.
 21. Hames C, Halbedel S, Hoppert M, Frey J, Stülke J. 2009. Glycerol metabolism is important for cytotoxicity of *Mycoplasma pneumoniae*. J Bacteriol 191:747–753. <https://doi.org/10.1128/JB.01103-08>.
 22. Deutscher J, Bauer B, Sauerwald H. 1993. Regulation of glycerol metabolism in *Enterococcus faecalis* by phosphoenolpyruvate-dependent phosphorylation of glycerol kinase catalyzed by enzyme I and HPr of the phosphotransferase system. J Bacteriol 175:3730–3733. <https://doi.org/10.1128/jb.175.12.3730-3733.1993>.
 23. Nikel PI, Kim J, de Lorenzo V. 2014. Metabolic and regulatory rearrangements underlying glycerol metabolism in *Pseudomonas putida* KT2440. Environ Microbiol 16:239–254. <https://doi.org/10.1111/1462-2920.12224>.
 24. Brzoska P, Rimmele M, Brzostek K, Boos W. 1994. The *pho* regulon-dependent Ugp uptake system for glycerol-3-phosphate in *Escherichia coli* is trans inhibited by Pi. J Bacteriol 176:15–20. <https://doi.org/10.1128/JB.176.1.15-20.1994>.
 25. Tseng C-W, Kanci A, Citti C, Rosengarten R, Chiu C-J, Chen Z-H, Geary SJ, Browning GF, Markham PFJM. 2013. MalF is essential for persistence of *Mycoplasma gallisepticum* *in vivo*. Microbiology (Reading) 159:1459–1470. <https://doi.org/10.1099/mic.0.067553-0>.
 26. Kelley LA, Mezulis S, Yates CM, Wass MN, Sternberg MJE. 2015. The Phyre² web portal for protein modeling, prediction, and analysis. Nat Protoc 10:845–858. <https://doi.org/10.1038/nprot.2015.053>.
 27. Fraser CM, Gocayne JD, White O, Adams MD, Clayton RA, Fleischmann RD, Bult CJ, Kerlavage AR, Sutton G, Kelley JM, Fritchman JL, Weidman JF, Small KV, Sandusky M, Fuhrmann J, Nguyen D, Utterback TR, Saudek DM, Phillips CA, Merrick JM, Tomb J-F, Dougherty BA, Bott KF, Hu P-C, Lucier TS, Peterson SN, Smith HO, Hutchison CA, Venter JC. 1995. The minimal gene complement of *Mycoplasma genitalium*. Science 270:397–404. <https://doi.org/10.1126/science.270.5235.397>.
 28. Szczepanek S, Frasca S, Schumacher V, Liao X, Padula M, Djordjevic S, Geary S. 2010. Identification of lipoprotein MslA as a neoteric virulence factor of *Mycoplasma gallisepticum*. Infect Immun 78:3475–3483. <https://doi.org/10.1128/IAI.00154-10>.
 29. Vilei EM, Frey J. 2001. Genetic and biochemical characterization of glycerol uptake in *Mycoplasma mycoides* subsp. *mycoides* SC: its impact on H₂O₂ production and virulence. Clin Diagn Lab Immunol 8:85–92. <https://doi.org/10.1128/CDLI.8.1.85-92.2001>.
 30. Chen H, Yu S, Shen X, Chen D, Qiu X, Song C, Ding C. 2011. The *Mycoplasma gallisepticum* α -enolase is cell surface-exposed and mediates adherence by binding to chicken plasminogen. Microb Pathog 51:285–290. <https://doi.org/10.1016/j.micpath.2011.03.012>.
 31. Grundel A, Pfeiffer M, Jacobs E, Dumke R. 2016. Network of surface-displayed glycolytic enzymes in *Mycoplasma pneumoniae* and their interactions with human plasminogen. Infect Immun 84:666–676. <https://doi.org/10.1128/IAI.01071-15>.
 32. Song Z, Li Y, Liu Y, Xin J, Zou X, Sun W. 2012. α -Enolase, an adhesion-related factor of *Mycoplasma bovis*. PLoS One 7:e38836. <https://doi.org/10.1371/journal.pone.0038836>.
 33. Boos W. 1998. Binding protein-dependent ABC transport system for glycerol 3-phosphate of *Escherichia coli*. Methods Enzymol 292:40–51. [https://doi.org/10.1016/s0076-6879\(98\)92006-7](https://doi.org/10.1016/s0076-6879(98)92006-7).
 34. Decker K, Plumbridge J, Boos W. 1998. Negative transcriptional regulation of a positive regulator: the expression of *malT*, encoding the transcriptional activator of the maltose regulon of *Escherichia coli*, is negatively controlled by Mlc. Mol Microbiol 27:381–390. <https://doi.org/10.1046/j.1365-2958.1998.00694.x>.
 35. Kühnau S, Reyes M, Sievertsen A, Shuman HA, Boos W. 1991. The activities of the *Escherichia coli* MalK protein in maltose transport, regulation, and inducer exclusion can be separated by mutations. J Bacteriol 173:2180–2186. <https://doi.org/10.1128/jb.173.7.2180-2186.1991>.
 36. Lippincott J, Traxler B. 1997. MalFGK complex assembly and transport and regulatory characteristics of MalK insertion mutants. J Bacteriol 179:1337–1343. <https://doi.org/10.1128/jb.179.4.1337-1343.1997>.
 37. Elkhali CK, Kean KM, Parsonage D, Maenpue S, Chaiyen P, Claiborne A, Karplus PA. 2015. Structure and proposed mechanism of L- α -glycerophosphate oxidase from *Mycoplasma pneumoniae*. FEBS J 282:3030–3042. <https://doi.org/10.1111/febs.13233>.
 38. Khan L, Miles R, Nicholas R. 2005. Hydrogen peroxide production by *Mycoplasma bovis* and *Mycoplasma agalactiae* and effect of *in vitro* passage on a *Mycoplasma bovis* strain producing high levels of H₂O₂. Vet Res Commun 29:181–188. <https://doi.org/10.1023/b:verc.0000047506.04096.06>.
 39. Maenpue S, Wathaisong P, Supon P, Sucharitakul J, Parsonage D, Karplus PA, Claiborne A, Chaiyen P. 2015. Kinetic mechanism of L- α -glycerophosphate oxidase from *Mycoplasma pneumoniae*. FEBS J 282:3043–3059. <https://doi.org/10.1111/febs.13247>.
 40. Wadher B, Henderson C, Miles R, Varsani H. 1990. A mutant of *Mycoplasma mycoides* subsp. *mycoides* lacking the H₂O₂-producing enzyme L- α -glycerophosphate oxidase. FEMS Microbiol Lett 72:127–130. [https://doi.org/10.1016/0378-1097\(90\)90358-W](https://doi.org/10.1016/0378-1097(90)90358-W).
 41. Dandekar T, Huynen M, Regula JT, Ueberle B, Zimmermann CU, Andrade MA, Doerks T, Sánchez-Pulido L, Snel B, Suyama M, Yuan YP, Herrmann R, Bork P. 2000. Re-annotating the *Mycoplasma pneumoniae*

- genome sequence: adding value, function and reading frames. *Nucleic Acids Res* 28:3278–3288. <https://doi.org/10.1093/nar/28.17.3278>.
42. Yus E, Maier T, Michalodimitrakis K, van Noort V, Yamada T, Chen W-H, Wodke JAH, Güell M, Martínez S, Bourgeois R, Kühner S, Rainieri E, Letunic I, Kalinina OV, Rode M, Herrmann R, Gutiérrez-Gallego R, Russell RB, Gavin A-C, Bork P, Serrano L. 2009. Impact of genome reduction on bacterial metabolism and its regulation. *Science* 326:1263–1268. <https://doi.org/10.1126/science.1177263>.
 43. Kirchhoff H. 1992. Motility, p 549–559. In Maniloff J, McElhaney RN, Finch LR (ed), *Mycoplasmas: molecular biology and pathogenesis*. American Society for Microbiology, Washington, DC.
 44. Shimizu T, Miyata M. 2002. Electron microscopic studies of three gliding mycoplasmas, *Mycoplasma mobile*, *M. pneumoniae*, and *M. gallisepticum*, by using the freeze-substitution technique. *Curr Microbiol* 44:431–434. <https://doi.org/10.1007/s00284-001-0014-8>.
 45. Krause DC. 1996. *Mycoplasma pneumoniae* cytoadherence: unraveling the tie that binds. *Mol Microbiol* 20:247–253. <https://doi.org/10.1111/j.1365-2958.1996.tb02613.x>.
 46. Markham P, Glew M, Brandon M, Walker I, Whithear K. 1992. Characterization of a major hemagglutinin protein from *Mycoplasma gallisepticum*. *Infect Immun* 60:3885–3891. <https://doi.org/10.1128/IAI.60.9.3885-3891.1992>.
 47. Stemke G, Robertson J. 1982. Comparison of two methods for enumeration of mycoplasmas. *J Clin Microbiol* 16:959–961. <https://doi.org/10.1128/JCM.16.5.959-961.1982>.
 48. Masukagami Y, Tivendale KA, Mardani K, Ben-Barak I, Markham PF, Browning GF. 2013. The *Mycoplasma gallisepticum* virulence factor lipo-protein MslA is a novel polynucleotide binding protein. *Infect Immun* 81:3220–3226. <https://doi.org/10.1128/IAI.00365-13>.
 49. Meynell GG, Meynell E, Press CU. 1970. *Theory and practice in experimental bacteriology*. Cambridge University Press, Cambridge, United Kingdom.
 50. Kowalski GM, De SD, Burch ML, Hamley S, Kloehn J, Selathurai A, Tull D, O'Callaghan S, McConville MJ, Bruce CR. 2015. Application of dynamic metabolomics to examine *in vivo* skeletal muscle glucose metabolism in the chronically high-fat fed mouse. *Biochem Biophys Res Commun* 462:27–32. <https://doi.org/10.1016/j.bbrc.2015.04.096>.
 51. Nanchen A, Fuhrer T, Sauer U. 2007. Determination of metabolic flux ratios from ¹³C-experiments and gas chromatography-mass spectrometry data, p 177–197. In Weckwerth W (ed), *Metabolomics: methods and protocols*. Humana Press, Totowa, NJ. https://doi.org/10.1007/978-1-59745-244-1_11.
 52. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez J-Y, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona A. 2012. Fiji: an open-source platform for biological-image analysis. *Nat Methods* 9:676–682. <https://doi.org/10.1038/nmeth.2019>.
 53. Chong J, Wishart DS, Xia J. 2019. Using MetaboAnalyst 4.0 for comprehensive and integrative metabolomics data analysis. *Curr Protoc Bioinformatics* 68:e86. <https://doi.org/10.1002/cpbi.86>.