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Running title: Virulence genes in *Mycoplasma gallisepticum*

Abstract

Relatively few virulence genes have been identified in pathogenic mycoplasmas, so we used signature-tagged mutagenesis to identify mutants of the avian pathogen *Mycoplasma gallisepticum* with reduced capacity to persist *in vivo* and compared the virulence of selected mutants in experimentally infected chickens. Four mutants had insertions in one of the two incomplete *opp*ABCDF operons and a further three had insertions in distinct hypothetical genes, two containing peptidase motifs and one a member of a gene family. The three hypothetical gene mutants and the two with insertions in *opp*D_1 were used to infect chickens and all five were shown to have reduced capacity to induce respiratory tract lesions. One *opp*D_1 mutant and the MGA_1102 and MGA_1079 mutants had greatly reduced capacity to persist in the respiratory tract and to induce systemic antibody responses against *M. gallisepticum*. The other *opp*D_1 mutant and the MGA_0588 mutant had less capacity than wild type to persist in the respiratory tract, but did elicit systemic antibody responses. Although *M. gallisepticum* carries two incomplete *opp* operons, one of which has been acquired by horizontal gene transfer, our results suggest that one of the copies of *opp*D may be required for full expression of virulence. We have also shown that three hypothetical genes, two of which encode putative peptidases, may be required for full expression of virulence in *M. gallisepticum.* None of these genes has previously been shown to influence virulence in pathogenic mycoplasmas.

Abbreviations: MB, mycoplasma broth; MA, mycoplasma agar; ST, signature-tagged; RSA, rapid serum agglutination

Introduction

Mycoplasma gallisepticum is the most important mycoplasmal pathogen in

poultry (1, 2) and a particularly useful model of mycoplasmal pathogenesis, in part as a typical representative of the Pneumoniae phyogenic group, which includes the important human pathogen *M. pneumoniae*. There have been relatively few studies attempting to identify virulence genes in mycoplasmas, in part because of the limited availability of tools for such studies. Signature-tagged mutagenesis (STM) is a useful technique for identifying genes likely to be involved in virulence, as it enables rapid comparison of populations of mutants used to inoculate animals with those recovered from these animals during the course of infection, allowing identification of mutants with reduced capacity to persist in experimentally infected animals (3, 4). However the examination of only pools of mutants may result in a failure to distinguish between a mutation in a gene that is essential *in vivo* and a mutation in a gene that reduces the capacity of the mutant to compete with other mutants *in vivo*. In addition, it is not possible to fully assess the virulence of individual mutants within populations. Therefore STM needs to be complemented with direct comparisons of isolated mutants for their capacity to infect, persist, and cause disease.

In a previous study we showed that several signature-tagged (ST) mutants of *M. gallisepticum* that were recovered infrequently from chickens inoculated with pools of mutants were significantly attenuated when assessed for their virulence in isolation (5). These mutants contained insertions in genes encoding the cytadhesin GapA and its accessory protein CrmA. Three other virulence-associated determinants in *M. gallisepticum*, *lpd*, *mal*F, and *msl*A, which has recently been shown to encode a novel oligonucleotide binding protein, have also been identified previously using transposon mutagenesis (5-8). Two of these (*mal*F and *msl*A) are members of ABC transporter operons.

The aims of the study reported here were to characterize further ST mutants that were recovered infrequently from chickens infected with a pool of mutants and to then

examine and compare the virulence of these mutants and their capacity to colonize the respiratory tract of experimentally infected chickens, with the aim of identifying additional genes that play a significant role in the pathogenesis of respiratory mycoplasmosis.

Methods and Materials

Bacterial strain and construction of mutants

The virulent *M. gallisepticum* strain Ap3AS was originally isolated from the air sacs of a broiler chicken with airsacculitis (9). It was cultured in modified Frey's broth (MB) containing 10% swine serum (10), as described previously (11).

Plasmid pISM 2062.2, carrying the transposon Tn*4001*mod, was used to construct a signature-tagged (ST) library, as described previously (5, 12-15). The transposon contained a gentamicin resistance gene, allowing selective culture. The presence of the different tags in the ST mutants was confirmed by polymerase chain reaction (PCR) using the P2/P4 primer set (Table 1) (5). The insertion site of the transposon in each mutant was determined by genomic DNA sequencing using the IGstmGenmeF3 primer (Table 1) and comparing the sequence to that of the *M. gallisepticum* strain R_{low} genome (16).

Screening of ST mutants in infected birds

The experimental design has been described previously (5). Briefly, in the initial screening, 102 ST mutants labeled with 34 different signature tags were cultured, combined into three pools, each containing 34 different mutants with a distinct tags (Tables 2 and 3), and each pool used to infect 20 specific pathogen free (SPF) chickens by aerosol (17), with an additional 10 uninfected chickens included with each group to assess transmissibility of the mutants. Necropsies were conducted on 10

inoculated birds 14 days after infection and on the remaining 20 birds 28 days after infection. Gross air sac lesions were assessed for severity as described previously (18). Swabs of the trachea and air sacs of each bird were assessed by culture in mycoplasma broth and on mycoplasma agar, as described previously (5), and the signature tags present amplified by PCR using the P2/P4 primer pair if the broth culture showed signs of growth of *M. gallisepticum*. The tags present were identified by dot blotting, as described previously (5). Blood samples were collected from all birds before inoculation and before euthanasia and the serum assessed for antibody against *M. gallisepticum* using commercial agglutination antigen (Intervet International) in the rapid serum agglutination (RSA) test (19).

Dilutions of the ST mutants that were not re-isolated in the initial screening experiment were subjected to confirmatory screening to further reduce the number of candidate ST mutants for definitive testing. The mutants were separated into two pools, with the mutants in each pool distinguishable from each other by their distinct tags. Each pool contained a similar concentration of each mutant and each pool was used to infect 20 birds, as described above. In-contact controls were not included, but all 40 birds were placed into the same positive pressure fibreglass isolator so each group could be used as an in-contact group for the other. Necropsies and sample collection were performed as described above at 14 days after inoculation. Dot blot hybridization and ST mutant specific PCRs were performed to identify the ST mutants recovered.

Comparison of the persistence and virulence of selected mutants

Several mutants were detected infrequently in the second screening experiment, suggesting that the genes that were interrupted in these mutants may have a significant influence on virulence. Two different *opp*D_1 ST mutants were recovered from birds at

distinct rates, suggesting that the different insertion sites may have differing impacts on persistence *in vivo* and potentially on pathogenicity. To assess this, and to assess the persistence and virulence of three mutants with insertions in hypothetical genes, the pathogenicities and rates of colonization of five distinct mutants were compared. Seven groups of 20 four-week-old SPF chickens were housed separately. Group 1 was exposed to an aerosol of mycoplasma broth only, Group 2 to MGA_1102 ST mutant 03-1, Group 3 to MGA_0588 ST mutant 18-1, Group 4 to *opp*D_1 ST mutant 20-1, Group 5 to MGA_1079 ST mutant 22-1, Group 6 to *opp*D_1 ST mutant 26-1 and the 7th group to wild type Ap3AS. All birds were subjected to necropsies 14 days after infection, with samples collected as described above. Sections of the upper, middle and lower trachea were also taken from each bird.

Dot blot hybridization and specific PCRs were used to identify ST mutants reisolated in broth cultures. Because strain Ap3AS did not have a specific sequence tag, dot blot hybridization could not be used for cultures from the positive control group. A pair of PCR primers, STM13-KE-C'-1-Rev and STM13-KF-C, which yielded a 400 bp product, were used to confirm the presence of Ap3AS (Table 1) (5). Sections of the trachea were processed and stained for histological examination, scored for mucosal lesions and the mucosal thickness measured (18). Statistical analyses were conducted using Kruskall-Wallis tests to compare median tracheal lesion scores, with the Dunn post-hoc test used to assess the significance of differences between pairs of groups, and an analysis of variance and Tukey's test to evaluate the mean tracheal mucosal thicknesses. Differences in the proportions of birds with lesions, of birds with serum antibody against *M. gallisepticum*, and of birds positive by culture for *M. gallisepticum* were assessed using Fisher's exact test (20). A probability less than or equal to 0.05 was regarded as significant.

SDS-PAGE electrophoresis and Western blot analysis of ST mutants

The cells in a 1 ml sample of a mid-log phase culture of each of the ST mutants, as well as the Ap3AS strain, were collected by centrifugation at 16,000 *g* for 5 min, then resuspended in 1 x SDS-PAGE lysis buffer and incubated at 100°C for 5 min before rapid cooling on ice. Total cell proteins were separated in a 12.5% polyacrylamide gel together with molecular mass standards (Marker 12 Wide Range Protein Standard, Novex) and then stained with Coomassie brilliant blue.

Whole cell proteins of MGA_0588ST mutant 18, *opp*D_1 mutant 20, *opp*D_1 mutant 26, and *M. gallisepticum* strain Ap3AS were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting and immunostaining to detect differences in protein expressed in the mutants.

Total cell proteins of mutants with protein profiles that differed in Coomassie stained gels were separated in a 10% polyacrylamide gel along with molecular mass standards (PageRuler Prestained Protein Ladder, Thermo Scientific), electrophoretically transferred onto a PVDF membrane (Immobilon™-P Transfer Membrane, Millipore) and antigen-free sites blocked by overnight incubation in 5% skim milk (Devondale) in PBS containing 0.1% Tween 20 (PBS-T) at 4°C. The membrane was then washed three times (10 min each) in PBS-T and then incubated in pooled sera collected from birds infected with Ap3AS (1:2000 in PBS-T), at room temperature for 1 h with gentle rocking, again washed as above and then incubated for 1 h at room temperature in anti-chicken horseradish peroxidase conjugate (DAKO) at a dilution of 1:2,000 in PBS-T. After again washing as above, bound conjugate was detected using enhanced chemiluminescence (CD/DAB Substrate Kit, Thermo Scientific) following the manufacturer's recommendations and results recorded digitally using a BioRad Molecular Imager (Bio-Rad) according to the manufacturer's instructions .

176

177 **Results**

178 ***Identification of insertion sites in ST mutants***

179 The insertion points of the transposon in ST mutants examined in detail in this
180 study are shown in Table 2. The insertion points in the other ST mutants in the pools
181 are listed in Table 3. In most cases the insertion site for a specific tagged transposon
182 was the same in each of the three initial mutant pools. There were four ST mutants
183 that had a tagged transposon inserted within the *opp*$AB_1C_1D_1F_1$ operon.
184 Transposons were located within *opp*B_{1a} in ST mutant 36-1 (and in 36-2 and 36-3)
185 and within *opp*C_1 in ST mutant 24-1 (and in 24-2 and 24-3). In ST mutant 20-1 (and
186 20-2 and 20-3) and 26-1 (and 26-2 and 26-3) the transposons were inserted at
187 different sites in *opp*D_1. No mutants were obtained with insertions in the other copy of
188 the *opp* operon in *M. gallisepticum* (*opp*$B_2C_2D_2F_2$). Three ST mutants, 03-1 (and 03-2
189 and 03-3), 18-1 (and 18-2 and 18-3) and 22-1 (and 22-2 and 22-3), had the transposon
190 inserted within genes encoding distinct hypothetical proteins.

191

192 ***Initial screening***

193 *Pathological and serological findings*

194 No anti-*M. gallisepticum* antibodies was detected in the serum of any bird at the
195 time of inoculation. More sera were anti-*M. gallisepticum* antibody-positive at 4
196 weeks than at 2 weeks after infection, but no antibody response was detected in
197 uninoculated in-contact birds in any group (Table 4). Fewer birds had air sac lesions
198 at 4 weeks than at 2 weeks after inoculation and more severe air sac lesions were
199 generally observed at 2 weeks after infection (scores of 0.5 in Group A, 1.0 to 2.0 in
200 Group B and 0.5 to 2.5 in Group C). The severity of the lesions did not correlate
201 closely with the serology results, with some birds having high RSA scores but no

detectable air sac lesions.

Identification of recoverable ST mutants

The recovery rates of ST mutants from birds at 14 and 28 days after inoculation are summarized in Tables 4 and 5. The *opp*D_1 ST mutant 20 (-1/-2/-3) was the mutant most frequently recovered from the air sacs, as well as from the tracheas, and was isolated at high frequency from all three groups. It was also recovered from the air sacs of one in-contact control bird in Group A 4 weeks after inoculation. In contrast, *opp*D_1 ST mutant 26 (-1/-2/-3) was only recovered from the trachea of one bird in Group A. Six and five isolations of *opp*C_1 ST mutant 24 (-1/-3) were made from the tracheas of birds in Groups A and C, respectively, whilst only one isolation of *opp*B_{1a} ST mutant 36 (-3) was made, from the trachea of a bird in Group C. MGA_0588 ST mutant 18 (-3) was only re-isolated from the air sacs of one bird in Group C, and from the trachea of one bird each in Groups A and C (-1/-3). Sixteen ST mutants, including ST mutants 03 (-1/-2/-3) (MGA_1102 interrupted) and 22 (-1/-2/-3) (MGA_1079 interrupted), were unable to be recovered from any bird in any of the groups (Table 4A).

Confirmatory screening

Pathological and serological assessments

No anti-*M. gallisepticum* antibody was detected prior to inoculation in any bird (Table 4B). Severe air sac lesions (score of 2.5) were seen in one bird, and mild lesions (0.5 and 1.0) in another two chickens in Group A. One bird had mild air sac lesions (1.0) in Group B. At 2 weeks after inoculation, 15/19 chickens in Group A and 14/19 birds in Group B had detectable antibody against *M. gallisepticum* (Table 4B).

Re-isolation of ST mutants

Birds in Group A were infected with a pool containing MGA_1102 ST mutant

03-1, and a pool that contained MGA_1079 ST mutant 22-1 was used to inoculate the birds in Group B. A total of eleven ST mutants, including ST mutant 03-1, were re-isolated from sixteen chickens in Group A and ten, including ST mutants 22-1 and 03-1, were re-isolated from eighteen birds in Group B (Table 4B).

Infectivity and virulence analysis of selected ST mutants

Clinical signs and post mortem examination

The prevalence and severity of lesions in chickens infected with individual mutants are shown in Table 6. Air sac lesions were not seen in the uninfected control birds (Group 1), or in birds exposed to aerosols of MGA_1079 ST mutant 22-1 (Group 5). Mild lesions (score of 0.25) were observed in one bird inoculated with MGA_1102 ST mutant 03-1 (Group 2). Of the 20 birds infected with *opp*D_1 ST mutant 26-1 (Group 6), four had mild lesions (0.5 to 1.0), whilst lesions were only seen in the abdominal air sacs of five birds exposed to MGA_0588 ST mutant 18-1 (Group 3). Six of 20 birds had mild to severe lesions (0.5 to 2.5) in the group infected with *opp*D_1 ST mutant 20-1 (Group 4), while mild to severe lesions (0.5 to 3.0) were seen in 11/18 birds infected with the virulent Ap3AS strain (Group 7).

Antibody responses and re-isolation of ST mutants

No anti-*M. gallisepticum* antibody was detected at the time of infection in the serum of any birds (Table 6). Two weeks after exposure, antibody responses were not detected in any of the birds in Groups 5 (MGA_1079 mutant 22-1 infected) or 6 (*opp*D_1 mutant 26-1 infected), whilst a response was detectable in only one bird in Group 2 (MGA_1102 mutant 03-1 infected). In contrast, strong antibody responses against *M. gallisepticum* were detectable in all the birds in Groups 3 (MGA_0588 mutant 18-1 infected) and 4 (*opp*D_1 mutant 20-1 infected). The uninfected control birds (Group 1) did not develop any antibody against *M. gallisepticum* over the course

of the experiment, while all birds infected with the wild type Ap3AS strain (Group 7) had strong antibody responses against *M. gallisepticum.*

M. gallisepticum was not isolated on MA plates inoculated with swabs of the air sacs of any birds in Groups 2 (MGA_1102 mutant 03-1 infected) or 5 (MGA_1079 mutant 22-1 infected), but they were isolated from the trachea of two birds in Group 2 (MGA_1102 mutant 03-1 infected) and one bird in Group 5 (MGA_1079 mutant 22-1 infected). In Group 6 (*opp*D_1 mutant 26-1 infected), *M. gallisepticum* were isolated from the air sacs of one bird and the trachea of 6 birds. *M. gallisepticum* were also isolated from the trachea of 17/20 birds in Group 3 (MGA_0588 mutant 18-1 infected) and of all the birds in Group 4 (*opp*D_1 ST mutant 20-1 infected), and also from swabs of the air sacs of 5/20 birds in Group 3 and 7/20 birds in Group 4. In the positive control group (Group 7), *M. gallisepticum* were isolated from the air sacs of 9/18 birds and the trachea of 17/18 birds (Table 6).

M. gallisepticum was recovered in MB inoculated with swabs of the air sacs of one bird in Group 6 (*opp*D_1 mutant 26-1 infected), 7 birds in Group 3 (MGA_0588 mutant 18-1 infected) and 8 birds in Group 4 (*opp*D_1 mutant 20-1 infected). *M. gallisepticum* were recovered from the trachea of birds in all six of the groups exposed to ST mutants, with the number of colonized birds ranging from 3 in Group 5 (MGA_1079 mutant 22-1 infected) to 20 in Group 4 (*opp*D_1 mutant 20-1 infected). The identity of the recovered *M. gallisepticum* ST mutants was confirmed using the unique signature tags they carried (Table 6).

Tracheal lesions and mucosal thicknesses

Median tracheal lesion scores are shown in Table 7. The scores of birds in all the mutant infected groups differed significantly from those of birds in the wild type infected group (Group 7) ($P < 0.0001$). There was no significant difference between the lesions scores for uninfected group (Group 1) and the mutant infected groups, with

exception of those for the upper tracheas of the birds infected with MGA_1102 mutant 03-1. The upper tracheal lesion scores of the birds infected with MGA_1102 mutant 03-1 (Group 2) did not differ from those of birds in Groups 3, 5 and 6, but did differ significantly from those of birds in Group 4 (*opp*D_1 mutant 20-1 infected).

Mean tracheal mucosal thicknesses are shown in Table 7. The mean tracheal mucosal thicknesses of the wild type infected birds were significantly different from those of both the uninfected and the mutant infected birds ($P < 0.0001$). The mean mucosal thicknesses in the middle and lower trachea did not differ significantly between the uninfected group and any of the mutant infected groups (Groups 2 to 6). However, they were significantly in the wild type infected group (Group 7). While there was no significant difference between the tracheal mucosal thickness in the upper trachea of the uninfected controls and Groups 3, 4, 5 and 6, the mean upper tracheal mucosal thickness in the group infected with MGA_1102 mutant 03-1 was significantly greater than that of the uninfected birds and those infected with *opp*D1 mutant 20-1. In summary, the wild type infected controls had significantly more severe tracheal lesions, as assessed by histological lesion score or mucosal thickness, than any of the groups infected with the ST mutants. Group 2 (MGA_1102 mutant 03-1 infected) was the most severely affected among the groups infected with the ST mutants.

SDS-PAGE electrophoresis

Total cell proteins of the *M. gallisepticum* strains used in this study were separated by SDS-PAGE and stained with Coomassie brilliant blue (Figure 1). There were no detectable differences in the protein profiles of ST mutants 03-1 (MGA_1102 interrupted), 20-1 (*opp*D_1 interrupted), 22-1 (MGA_1079 interrupted), 24-1 (*opp*C_1 interrupted), 36-1 (*opp*B_{1a} interrupted) and wild-type Ap3AS. However,

306 one protein band of about 40 kDa was absent in MGA_0588 mutant 18-1, whilst
307 *opp*D_1 mutant 26-1 contained an additional band of about 33 kDa compared to the
308 other mutants and wild-type Ap3AS.

309 Two protein bands, of 38 and 30 kDa, were more prominently detected in
310 MGA_0588 mutant 18-1 than in wild-type Ap3AS by sera from birds infected with
311 the parent strain Ap3AS (Figure 2, Figure S1). One protein of 43 kDa was not
312 detected in *opp*D_1 mutant 20-1 by sera from birds infected with Ap3AS and two
313 proteins of 40 kDa and 43 kDa were more prominently detected in *opp*D_1 mutant 26-1
314 than in wild-type Ap3AS, while proteins of 20 kDa were less prominent in the *opp*D_1
315 mutant 26-1 than in the Ap3AS strain (Figure 2, Figure S1).

316

317 **Discussion**

318 ABC transporters are a family of multi-domain membrane proteins that use
319 energy from hydrolysis of ATP to translocate solutes across cellular membranes (21,
320 22). The four domains within ABC transporters form a conserved core structure
321 composed of two transmembrane domains (TMDs) and two nucleotide-binding
322 domains (NBDs) (23-26).

323 There are two adjacent copies of the *opp* ABC transporter operon (*opp*$B_1C_1D_1F_1$
324 and *opp*$AB_2C_2D_2F_2$) in *M. gallisepticum* strain R (16). OppB and OppC are predicted
325 to be transmembrane proteins, while *opp*D and *opp*F are predicted to encode
326 ATP-binding proteins. The *opp*B_1 gene contains a frameshift mutation, *opp*F_2 appears
327 to have been truncated, and there is only one gene encoding OppA, the
328 substrate-binding lipoprotein (27), in *M. gallisepticum*. Thus it appears that *M.*
329 *gallisepticum* may contain two complementary partial copies of the *opp* operon, with
330 only a single full-length copy of *opp*A, *opp*B and *opp*F, but two full length copies of
331 *opp*CD.

One of these *opp* operons (MGA_0237, MGA_0235, MGA_0234, MGA_0232, and MGA_0230, or *opp*$AB_2C_2D_2F_2$) is most similar to that of *M. synoviae* strain 53 (GenBank accession number: AE017245), suggesting that the operon has been relatively recently acquired from *M. synoviae* or a closely related species within the Hominis phylogenic group (which includes *M. hyopneumoniae*, *M. synoviae* and *M. pulmonis*) (Figure 3). Phylogenetic analysis of these genes in a number of fully sequenced genomes suggested that this operon was duplicated in a member of the Hominis group, and that these duplicated operons then diverged, with one subsequently transferred horizontally to an ancestor of *M. gallisepticum* and *Ureaplasma urealyticum*. *Ureaplasma urealyticum* appears to have lost its original copy of the operon and retained only the horizontally transferred copy, whilst *M. gallisepticum* has retained most of both copies of the operon (Figure 3). Studies in *M. hominis* have shown that OppA is a cytadhesin, and also that it binds oligopeptides, suggesting that this operon encodes a transporter responsible for import of oligopeptides (27-29). It is notable that duplication of the *opp*ABCDF operon by horizontal gene transfer has also occurred in the ruminant pathogens *Mycoplasma agalactiae* and *Mycoplasma bovis*, although this horizontal gene transfer event was clearly independent of that in *M. gallisepticum* (30-32).

In the studies presented here, four mutants had transposon insertions in genes in the same *opp* operon (in *opp*B_1, *opp*C_1, and *opp*D_1), suggesting that mutations in some of the original *opp* genes may be tolerated (except possibly for the only full length *opp*F, *opp*F_1), but that the *opp*$B_2C_2D_2$ genes may be required for survival in *M. gallisepticum*. The transposon was inserted into the *opp*D_1 gene in mutants 20-1 (-2/-3) and 26-1 (-2/-3), but in different positions (Table 2). Mutant 20-1 induced seroconversion in more birds than mutant 26-1, and also colonized a greater proportion of birds, even though they were both much less pathogenic than the wild

type strain. The insertion site in mutant 20-1 was close to the start of the gene, whilst in mutant 26-1 the first third of the gene may be able to be expressed. It is possible that the two different OppD proteins form heterodimers in the transport complex, and that, in the absence of expression of $OppD_1$ in mutant 20-1, $OppD_2$ could form functional homodimers. The partial copy of $OppD_1$ in mutant 26-1 may retain a capacity to form a heterodimer, but one that is less functional, resulting in less efficient transportation and thus the greater reduction in colonization and virulence seen in this mutant. Further work will be needed to establish whether the two copies of *opp*D in the genome can partially complement each other and whether they form heterodimers.

In ST mutant 36-1 (-2/-3), the transposon was inserted into the middle of the *opp*B_{1a} gene (MGA_0223). As there is a frame shift mutation within the *opp*B_1 gene, the two proteins predicted to be derived from it, $OppB_{1a}$ and $OppB_{1b}$ (MGA_0223 and MGA_0224), may not be fully functional, and thus *opp*B_2 may be the only fully functional copy in the genome. Although the *opp*B_{1a} mutant 36-1 was isolated very infrequently in the initial screening experiment, it was only assessed as a member of a pool, so the impact of mutation of the *opp*B_{1a} gene on infectivity and virulence requires further investigation. ST mutant 24-1 (-2/-3) had a transposon inserted into the *opp*C_1 gene (MGA_0221) about two-thirds along the length of the gene and this mutant could be recovered frequently from birds in the initial screening, suggesting that either the essential region of OppC may be within the first two-thirds of the gene, or that *opp*C_2 can complement the loss of *opp*C_1.

ST mutant 18-1, with an insertion in one of multiple paralogous genes for a hypothetical protein (MGA_0588), was able to infect birds, but was significantly less virulent than wild type Ap3AS. This suggests that the paralog disrupted in this mutant may have a significant influence on virulence, although not on colonisation.

384 Mutant 22-1 (MGA_1079 interrupted) was relatively apathogenic and had a reduced
385 capacity to colonize. ST mutant 03-1 (-2/-3) (MGA_1102 interrupted) was not
386 recovered from any bird in the initial screen, but was able to be re-isolated in the
387 confirmatory screen, albeit at a relatively low frequency, and was also able to spread,
388 as it was recovered from birds in both groups. Although only one bird infected with
389 this mutant developed detectable serum antibodies, it was the most virulent of all the
390 mutants assessed in isolation, suggesting that mutants need to be assessed individually
391 in hosts to examine the severity and extent of the lesions they induce if their virulence
392 is to be fully assessed, and that capacity to induce seroconversion is not always a
393 reliable indicator of virulence.

394 The *opp*D_1 mutant 20-1, which caused mild air sac lesions and minor damage in
395 the trachea, induced a detectable anti-mycoplasma antibody response. The site of
396 insertion of the transposon would allow expression of only the N-terminal 7.9% of the
397 protein. In contrast, the less virulent *opp*D_1 mutant 26-1, could express the N-terminal
398 34% of the protein, and did not induce detectable anti-mycoplasma antibody.
399 Western blotting detected differences between this mutant and wild-type Ap3AS strain,
400 suggesting that the transposon insertion did result in changes in protein expression.

401 However, the protein profiles of the mutants did not provide clear evidence of the
402 loss of expression of the proteins encoded by the genes that had been interrupted in
403 each mutant, although some differences in the protein profiles were detected. The lack
404 of a clear correlation between the site of insertion of the transposon and the changes in
405 protein profiles may be attributable to the prevalence of post-translational cleavage in
406 mycoplasmas, which complicates direct attribution of a protein band to a specific gene.
407 In addition, assuming *opp*D_1 was expressed at sufficiently high levels to be detected
408 in these assays, the presence of the product of the paralogous *opp*D_2 gene would be
409 expected to obscure detection of changes in expression of OppD_1. In other bacteria

410 signals imported through the Opp system can have diverse effects on expression of
411 other genes, so the changes detected in these mutants could reflect similar effects in *M.*
412 *gallisepticum*. We would predict that the two *opp*D_1 mutants might have differing
413 patterns of protein expression as a result of the differing extents of their effects on the
414 function of the Opp transport system.

415 As it is possible that the mutants described here could have other mutations in
416 addition to the transposon insertion, complementation would be required to
417 definitively establish the role of the genes interrupted by the transposon in virulence
418 in *M. gallisepticum.* Unfortunately, stable extrachromosomal expression of introduced
419 genes, which would be needed to perform experimental infection studies, is difficult
420 to achieve with our current tools for genetic manipulation of *M. gallisepticum*. In
421 addition, the *opp*D insertions may have polar effects on expression of other genes in
422 the operon. However, the attenuation of virulence, albeit to different levels, by two
423 independent insertions in the *opp*D gene strongly suggests that this gene in particular
424 does play a role in virulence. Furthermore, recent studies have shown that this operon
425 can play a role in virulence in both Gram negative and Gram positive bacteria (33,
426 34).

427 Notably, genes MGA_1102 and MGA_1079, which, based on the studies
428 described here, may be required for full pathogenicity in *M. gallisepticum*, appear to
429 have been transferred horizontally from *M. synoviae*, as all their most closely related
430 homologs are found in mycoplasmas in the Hominis phylogenic group. The protein
431 encoded by MGA_1102 has a zinc peptidase-like motif at its carboxyl end, while the
432 protein encoded by MGA_1079 has a trypsin-like peptidase motif at its carboxyl end.

433 Thus, in the work presented here, we used STM to screen pools of mutants of *M.*
434 *gallisepticum* and identified a number of genes that may have a role in establishing
435 infection in the natural host of this pathogen, and on pathogenicity. Our results

suggested that genes that appear to have been acquired by horizontal gene transfer from *M. synoviae*, MGA_1102 and MGA_1079, as well as the *opp*D_2 gene, which has been complemented by horizontal gene transfer from *M. synoviae*, may play a significant role in pathogenicity in this important pathogen, even though they are not essential for colonisation and persistence.

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References

1. **Kleven SH.** 1981. Transmissibility of the F strain of *Mycoplasma gallisepticum* in leghorn chickens. Avian Dis **25:**1005-1018.
2. **Lin MY, Kleven SH.** 1982. Egg transmission of two strains of *Mycoplasma gallisepticum* in chickens. Avian Dis **26:**487-495.
3. **Hensel M, Shea JE, Gleeson C, Jones MD, Dalton E, Holden DW.** 1995. Simultaneous identification of bacterial virulence genes by negative selection. Science **269:**400-403.
4. **Struve C, Forestier C, Krogfelt KA.** 2003. Application of a novel multi-screening signature-tagged mutagenesis assay for identification of *Klebsiella pneumoniae* genes essential in colonization and infection. Microbiology **149:**167-176.
5. **Tseng CW, Kanci A, Citti C, Rosengarten R, Chiu CJ, Chen ZH, Geary SJ, Browning GF, Markham PF.** 2013. MalF is essential for persistence of *Mycoplasma gallisepticum in vivo*. Microbiology **159:**1459-1470.
6. **Hudson P, Gorton TS, Papazisi L, Cecchini K, Frasca S, Jr., Geary SJ.** 2006. Identification of a virulence-associated determinant, dihydrolipoamide dehydrogenase (*lpd*), in *Mycoplasma gallisepticum* through *in vivo* screening of transposon mutants. Infect Immun **74:**931-939.
7. **Masukagami Y, Tivendale KA, Mardani K, Ben-Barak I, Markham PF,**

Browning GF. 2013. The *Mycoplasma gallisepticum* virulence factor lipoprotein MslA is a novel polynucleotide binding protein. Infect Immun **81:**3220-3226.

8. **Szczepanek SM, Frasca S, Jr., Schumacher VL, Liao X, Padula M, Djordjevic SP, Geary SJ.** 2010. Identification of lipoprotein MslA as a neoteric virulence factor of *Mycoplasma gallisepticum*. Infect Immun **78:**3475-3483.

9. **Soeripto, Whithear KG, Cottew GS, Harrigan KE.** 1989. Virulence and transmissibility of *Mycoplasma gallisepticum*. Aust Vet J **66:**65-72.

10. **Higgins PA, Whithear KG.** 1986. Detection and differentiation of *Mycoplasma gallisepticum* and *M. synoviae* antibodies in chicken serum using enzyme-linked immunosorbent assay. Avian Dis **30:**160-168.

11. **Gaunson JE, Philip CJ, Whithear KG, Browning GF.** 2000. Lymphocytic infiltration in the chicken trachea in response to *Mycoplasma gallisepticum* infection. Microbiology **146 (Pt 5):**1223-1229.

12. **Byrne ME, Rouch DA, Skurray RA.** 1989. Nucleotide sequence analysis of IS256 from the *Staphylococcus aureus* gentamicin-tobramycin-kanamycin-resistance transposon Tn4001. Gene **81:**361-367.

13. **Knudtson KL, Minion FC.** 1993. Construction of Tn*4001lac* derivatives to be used as promoter probe vectors in mycoplasmas. Gene **137:**217-222.

14. **Hedreyda CT, Lee KK, Krause DC.** 1993. Transformation of *Mycoplasma pneumoniae* with Tn*4001* by electroporation. Plasmid **30:**170-175.

15. **Winner F, Markova I, Much P, Lugmair A, Siebert-Gulle K, Vogl G, Rosengarten R, Citti C.** 2003. Phenotypic switching in *Mycoplasma gallisepticum* hemadsorption is governed by a high-frequency, reversible point mutation. Infect Immun **71:**1265-1273.

16. **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 **149:**2307-2316.

17. **Whithear KG, Harrigan KE, Kleven SH.** 1996. Standardized method of aerosol challenge for testing the efficacy of *Mycoplasma gallisepticum* vaccines. Avian Dis **40:**654-660.

18. **Nunoya T, Tajima M, Yagihashi T, Sannai S.** 1987. Evaluation of respiratory lesions in chickens induced by *Mycoplasma gallisepticum*. Nippon Juigaku Zasshi **49:**621-629.

19. **Whithear KG.** 1993. Avian Mycoplasmosis, p 1-12. *In* Corner LA, Bagust TJ

(ed), Australian Standard Diagnostic Techniques for Animal Diseases. CSIRO for the standing committee on agriculture and resource management, East Melbourne.

20. **Fowler J, Cohen L, Jarvis P.** 1998. Pratical Statistics for Field Biology, 2nd ed. John Wiley & Sons, Chichester, England.

21. **Pedersen PL.** 2005. Transport ATPases: structure, motors, mechanism and medicine: a brief overview. J Bioenerg Biomembr **37:**349-357.

22. **Young J, Holland IB.** 1999. ABC transporters: bacterial exporters-revisited five years on. Biochim Biophys Acta **1461:**177-200.

23. **Higgins CF.** 1992. ABC transporters: from microorganisms to man. Annu Rev Cell Biol **8:**67-113.

24. **Jones PM, George AM.** 2004. The ABC transporter structure and mechanism: perspectives on recent research. Cell Mol Life Sci **61:**682-699.

25. **Schmitt L, Tampe R.** 2002. Structure and mechanism of ABC transporters. Curr Opin Struct Biol **12:**754-760.

26. **Garmory HS, Titball RW.** 2004. ATP-binding cassette transporters are targets for the development of antibacterial vaccines and therapies. Infect Immun **72:**6757-6763.

27. **Henrich B, Hopfe M, Kitzerow A, Hadding U.** 1999. The adherence-associated lipoprotein P100, encoded by an opp operon structure, functions as the oligopeptide-binding domain OppA of a putative oligopeptide transport system in *Mycoplasma hominis*. J Bacteriol **181:**4873-4878.

28. **Fraser CM, Gocayne JD, White O, Adams MD, Clayton RA, Fleischmann RD, Bult CJ, Kerlavage AR, Sutton G, Kelley JM, Fritchman RD, Weidman JF, Small KV, Sandusky M, Fuhrmann J, Nguyen D, Utterback TR, Saudek DM, Phillips CA, Merrick JM, Tomb JF, Dougherty BA, Bott KF, Hu PC, Lucier TS, Peterson SN, Smith HO, Hutchison CA, 3rd, Venter JC.** 1995. The minimal gene complement of *Mycoplasma genitalium*. Science **270:**397-403.

29. **Himmelreich R, Hilbert H, Plagens H, Pirkl E, Li BC, Herrmann R.** 1996. Complete sequence analysis of the genome of the bacterium *Mycoplasma pneumoniae*. Nucleic Acids Res **24:**4420-4449.

30. **Sirand-Pugnet P, Lartigue C, Marenda M, Jacob D, Barre A, Barbe V, Schenowitz C, Mangenot S, Couloux A, Segurens B, de Daruvar A, Blanchard A, Citti C.** 2007. Being pathogenic, plastic, and sexual while living with a nearly minimal bacterial genome. PLoS Genet **3:**e75.

31. **Wise KS, Calcutt MJ, Foecking MF, Roske K, Madupu R, Methe BA.** 2011. Complete genome sequence of *Mycoplasma bovis* type strain PG45

(ATCC 25523). Infect Immun **79:**982-983.

32. **Qi J, Guo A, Cui P, Chen Y, Mustafa R, Ba X, Hu C, Bai Z, Chen X, Shi L, Chen H.** 2012. Comparative geno-plasticity analysis of *Mycoplasma bovis* HB0801 (Chinese isolate). PLoS One **7:**e38239.

33. **Jones MM, Johnson A, Koszelak-Rosenblum M, Kirkham C, Brauer AL, Malkowski MG, Murphy TF.** 2014. Role of the oligopeptide permease ABC transporter of *Moraxella catarrhalis* in nutrient acquisition and persistence in the respiratory tract. Infect Immun **82:**4758-4766.

34. **Moraes PM, Seyffert N, Silva WM, Castro TL, Silva RF, Lima DD, Hirata R, Jr., Silva A, Miyoshi A, Azevedo V.** 2014. Characterization of the Opp peptide transporter of *Corynebacterium pseudotuberculosis* and its role in virulence and pathogenicity. Biomed Res Int **2014:**489782.

35. **Felsenstein J.** 1989. PHYLIP -- Phylogeny Inference Package (Version 3.2). Cladistics **5:**164-166.

36. **Kumar S, Tamura K, Nei M.** 2004. MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. Brief Bioinform **5:**150-163.

37. **Thompson JD, Higgins DG, Gibson TJ.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res **22:**4673-4680.

568 **Table 1.** Oligonucleotides used in this study

Name	Use	5 -3' sequence (size in bp)
P2	Signature tag region PCR	ATCCTACAACCTCAAGCT (18)
P4	Signature tag region PCR	ATCCCATTCTAACCAAGC (18)
STM13-KE-C'-1-Rev	Wild-type Ap3AS PCR	CCACAGAGAACTTGAAG (17)
STM13-KF-C'	Wild type Ap3AS PCR	TATAAACCTGGTACGG (16)
IGstmGenmeF3	DNA sequencing and ST mutant PCR	GGACTGTTATATGGCCTTTTTGGATC (26)
STM03-B-Rev	ST mutant PCR	ACAGCTTGACGTTTTCCA (18)
STM18-Rev	ST mutant PCR	AGCAAAATTTCCACCCAAGA (20)
STM20-Rev	ST mutant PCR	GAGACATCAATCGCGGTTTT (20)
JZ-STM22	ST mutant PCR	CTCAATTGAAGAATTATATGATG (23)
STM26-Rev	ST mutant PCR	CATCCCACCTGAAAACTCGT (20)

569

570 **Table 2.** Transposon insertion sites in ST mutants used in this study (inocula A, B and C)

ST mutant	Insertion site in genome (% of gene to insertion point)	Gene Name	Function of disrupted gene
03-1/2/3	380999-381000 (16.0)	MGA_1102	Hypothetical membrane protein, zinc peptidase-like motif
18-1/2/3	980912-980913 (25.1)	MGA_0588	Hypothetical membrane protein, multiple paralogs
20-1/2/3	710237-710238 (7.9)	MGA_0220	Oligopeptide transporter ATP-binding protein $OppD_1$
22-1/2/3	365386-365387 (22.3)	MGA_1079	Hypothetical lipoprotein, trypsin-like peptidase motif
24-1/2/3	712236-712237 (67.8)	MGA_0221	Oligopeptide transporter permease $OppC_1$
26-1/2/3	710587-719588 (34.2)	MGA_0220	Oligopeptide transporter ATP-binding protein $OppD_1$
36-1/2/3	712897-712898 (45.4)	MGA_0223	Oligopeptide transporter permease ($OppB_{1a}$)

571 **Table 3** Classification of transposon insertion sites in ST mutants in this study

ST mutant	**Transposon insertion site**	**Function**
Cell envelope – Membranes, lipoproteins		
11-1/-2/-3	MGA_0964	VlhA 4.02 (Hemagglutinin)
17-1/-2	MGA_0379	VlhA 3.02 (Hemagglutinin)
13-1/-2/-3	No match in strain R_{low}	Match to *vlh*A gene in draft Ap3AS sequence
Cellular processes		
01-1/-2/-3	MGA_1142	OsmC-like stress-induced protein
Translation – Protein synthesis		
19-1/-2/-3	MGA_0216	Elongation factor P (EF-P)
Transport and binding proteins – ABC transport		
20-1/-2/-3	MGA_0220	ATP-binding protein $OppD_1$
24-1/-2/-3	MGA_0221	Permease protein $OppC_1$
26-1/-2/-3	MGA_0220	ATP-binding protein $OppD_1$
36-1/-2/-3	MGA_0223	Permease protein $OppB_{1a}$
Intergenic regions		
10-1/-2/-3	MGA_0537 – MGA_0539	HsdM endonuclease – *hsd1* locus
16-1/-2/-3	MGA_0395 – MGA_0398	*vlh*A 3.09 – *mal*P
23-1/-2/-3	MGA_0379 – MGA_0380	*vlh*A 4.04 – *vlh*A 4.05
27-1/-2/-3	MGA_0226 – MGA_0230	Conserved hypothetical protein – *opp*F (OppF)
31-1/-2/-3	MGA_0071 – MGA_0073	*vlh*A 1.05 – Putative transposase
38-1/-2/-3	MGA_0518 – MGA_0519	Unique hypothetical protein – conserved hypothetical protein
Others		
02-2	MGA_0549	Unique hypothetical protein
02-3	MGA_0145	Putative transposase
03-1/-2/-3	MGA_1102	Hypothetical membrane protein, zinc peptidase motif
04-3	MGA_0073	Putative transposase
07-1/-2/-3	MGA_0554	Hypothetical lipoprotein
09-1/-2	MGA_0662	Unique hypothetical membrane protein
12-1/-2/-3	16S rRNA	16S rRNA
14-1/-2/-3	MGA_0549	Hypothetical membrane protein unique to *M. gallisepticum* and *M.*
18-1/-2/-3	MGA_0588	Hypothetical membrane protein, multiple paralogs
21-1/-2/-3	MGA_0554	Hypothetical lipoprotein
22-1/-2/-3	MGA_1079	Hypothetical lipoprotein
32-1/-2/-3	MGA_0817b	Unique hypothetical protein
35-1/-2/-3	MGA_0758	Putative hemoxygenase
Not determined		
09-3	Multiple insertions	
15-1/-2/-3	Multiple insertions	
17-3	Multiple insertions	
25-1/-2/-3	Multiple insertions	
34-1/-2/-3	Multiple insertions	

572 Mutants 02-1, 04-1, 06-1/2/3, 28-1/2/3, 33-1/2/3, and 39-1/2/3 have been described previously (5).

573

574 **Table 4.** Serology, air sac lesions and mutants detected in each group in the initial (A) and confirmatory (B) screens

575 **A.**

Group	Antibody against *M. gallisepticum**				Air sac lesions*			Mutant-tag detected (number of times detected)	
	Week 0	Week 2	Week 4	Control	Week 2	Week 4	Control	Air sacs	Trachea
A[#]	0/30	7/9	8/9	0/8	2/10	0/9	0/8	*opp*D_1-20 (2)[§]	*opp*D_1-20 (6), *opp*C_1-24 (6), *opp*D_1-26 (1) MGA_0588-18(1)
B	0/31	6/10	9/11	0/10	2/10	1/11	0/10	*opp*D_1-20 (1)	*opp*D_1-20 (6)
C[#]	0/29	5/10	9/10	0/7	4/10	2/10	0/7	*opp*D_1-20 (7)	*opp*D_1-20 (10), *opp*C_2-24 (5), *opp*B_{1a}-36 (1)

576

577 **B.**

Group	Antibody against *M. gallisepticum**		Air sac lesions*	Mutant-tag detected (number of times detected)	
	Week 0	Week 2	Week 2	Air sacs	Trachea
A[†]	0/20	15/19	3/19	MGA_1102-03 (1)	MGA_1102-03 (2)
B[†]	0/20	14/19	1/19		MGA_1102-03 (1), MGA_1079-22 (2)

578 *Number positive/number examined.

579 [#]Two inoculated and two in-contact birds died in Group A and two in-contact birds died in Group C prior to necropsy.

580 [§]including recovery from one bird in the in-contact group at 4 weeks after inoculation.

581 [†]One bird died in Groups A and B before necropsy. Pool A contained mutant 03-1 and pool B contained mutant 22-1.

582 **Table 5** Mutants detected in each experimental group in the initial screening and confirmatory screening

Group	Signature tags detected (number of times detected)	
	Air sacs	Trachea
Initial - A*	16-1 (1)[§], 20-1 (2)[§]	07-1 (3), 11-1 (1), 12-1 (4), 14-1 (1), 18-1 (1), 19-1 (2), 20-1 (6), 21-1 (1), 24-1 (6), 26-1 (1), 27-1 (2), 31-1 (1), 32-1 (1), 35-1 (1), 38-1 (1)
Initial - B	12-2 (1), 20-2 (1)	07-2 (1), 11-2 (1), 12-2 (3), 14-2 (1), 16-2 (1), 20-2 (6), 27-2 (1)
Initial - C*	11-2 (1), 12-2 (1), 16-2 (3), 18-2 (1), 20-2 (7), 27-2 (1)	01-3 (1), 02-3 (8), 11-3 (1), 12-3 (5), 13-3 (1), 14-3 (1), 16-3 (3), 18-3 (1), 20-3 (10), 24-3 (5), 27-3 (6), 36-3 (1), 38-3 (2)
Confirmatory - A[#]	03-1 (1), 09-1 (1), 10-1 (2), 15-1 (1), 23-1 (2)	02-2 (4), 03-1 (2), 04-3 (1), 09-1 (3), 10-1 (10), 15-1 (1), 17-2 (12), 23-1 (2), 25-1 (1), 34-1 (1)
Confirmatory - B[#]	09-3 (2), 17-3 (1), 23-1 (2)	02-2 (1), 03-1 (1), 09-3 (14), 10-1 (5), 15-1 (2), 17-3 (10), 23-1 (15), 25-1(3), 34-1 (2)

583 *One inoculated and two in-contact birds died in Group A and two in-contact birds died in Group C prior to necropsy.

584 [§] including recovery from one bird in the in-contact group at 4 weeks after inoculation.

585 The following tags were not detected in the initial screen: 02-2, 03, 04-3, 09, 10, 15, 17, 22, 23, 25 and 34.

586 [#]One bird each died in Groups A and B before necropsy.

587 Birds in Group A were inoculated with a pool containing ST mutants 02-2, 03-1, 04-1, 06-1, 09-1, 10-1, 15-1 and 17-2; birds in Group B were inoculated with

588 a pool containing ST mutants 04-3, 09-3, 17-3, 22-1, 23-1, 25-1, 33-1 and 34-1.

589 Mutants 02-1, 04-1, 06-1/2/3, 28-1/2/3, 33-1/2/3, and 39-1/2/3 have been described previously (5).

590

591 **Table 6.** Serology, air sac lesion scores and re-isolation rates in the infectivity and virulence study

Group	Inoculum	Antibody against *M. gallisepticum*[*]	Air sac lesions[*] (range of scores)	Positive by agar culture		Positive by broth culture[†]	
				Air sacs	Trachea	Air sacs	Trachea
1	Medium	0/20[a]	0/20[a] (0 – 0)	0/20[a]	0/20[a]	0/20[a]	0/20[a]
2	Mutant 03-1	1/20[a]	1/20[a,b] (0–0.25)	0/20[a]	2/20[a,b]	0/20[a]	5/20[b,c]
3	Mutant 18-1	20/20[b]	5/20[b] (0.5–3.5)	5/20[b,c]	17/20[c]	7/20[b]	19/20[d]
4	Mutant 20-1	20/20[b]	6/20[b] (0.5–4.0)	7/20[c]	20/20[c]	8/20[b]	20/20[d]
5	Mutant 22-1	0/19[a]	0/19[a] (0–0)	0/19[a]	1/19[a,b]	0/19[a]	3/19[a,b]
6	Mutant 26-1	0/20[a]	4/20[a,b] (0.5–1.0)	1/20[a,b]	6/20[b]	1/20[a]	10/20[c]
7	Wild type Ap3AS	18/18[b]	11/18[c] (0.5–8.5)	9/18[c]	18/18[c]	10/18[b]	15/18[d]

592 [*] Number positive/number examined. One bird died in Group 5 and two birds died in Group 7 before necropsy.

593 [†]The only signature tag detected in each group was that carried by the mutant used to infect that group. No signature tags were detected in cultures from
594 groups 1 and 7.

595 Data in the same column marked with the same superscript letter were not significantly different.

596

597 **Table 7** Tracheal lesion scores and mucosal thicknesses in birds in the infectivity and virulence study

Group	Inoculum	Median tracheal lesion score (minimum, maximum)			Mean tracheal mucosal thickness ± SD* (µm)		
		Upper	Middle	Lower	Upper	Middle	Lower
1	Medium	0.25 (0, 0.5)[a]	0.25 (0, 0.5)[a]	0 (0, 0.25)[a]	50 ± 11[a]	41 ± 8[a]	37 ± 6[a]
2	Mutant 03-1	1 (0.25, 3)[b]	0.375 (0, 1.5)[a]	0.25 (0, 1.5)[a]	89 ± 45[b]	54 ± 12[a]	46 ± 22[a]
3	Mutant 18-1	0.25 (0, 10)[a,b]	0.25 (0, 1.5)[a]	0.25 (0, 1.5)[a]	62 ± 15[a,b]	53 ± 15[a]	49 ± 15[ab]
4	Mutant 20-1	0.25 (0, 1.5)[a]	0.125 (0, 1.5)[a]	0.125 (0, 1.0)[a]	56 ± 22[a]	49 ± 23[a]	51 ± 19[a]
5	Mutant 22-1	0.5 (0, 1)[a,b]	0.25 (0, 1)[a]	0 (0, 0.25)[a]	64 ± 13[a,b]	49 ± 8[a]	39 ± 8[a]
6	Mutant 26-1	0.5 (0, 2)[a,b]	0.25 (0, 1)[a]	0 (0, 2)[a]	64 ± 24[a,b]	49 ± 14[a]	48 ± 22[a]
7	Wild type Ap3AS	1.5 (1, 3)[c]	1.50 (0.5, 3)[b]	1.50 (0.25, 3)[b]	168 ± 70[c]	153 ± 84[b]	120 ± 56[b]

598 * SD : standard deviation

599 Data in the same column marked with the same superscript letter were not significantly different.

600 **FIGURE LEGENDS**
601
602 **Figure 1** Protein profiles of ST mutants. Total cell proteins were separated in a 12.5% polyacrylamide gel together with molecular
603 mass standards and then stained with Coomassie brilliant blue. The position of the main differences in the protein profiles (in lanes 2
604 and 6) are indicated by arrows on the left. Lane 1, ST mutant 03-1 (MGA_1102 interrupted); lane 2, ST mutant 18-1 (MGA_0588
605 interrupted); lane 3, *opp*D_1 mutant 20-1; lane 4, ST mutant 22-1 (MGA_1079 interrupted); lane 5, *opp*C_1 mutant 24-1; lane 6, *opp*D_1
606 mutant 26-1; lane 7, *opp*B_{1a} mutant 36-1; Lane 8, wild-type strain Ap3AS. The positions of the broad range protein markers (Mark12^{TM}
607 Wide Range Protein Standard, Novex) are indicated on the left.
608
609
610 **Figure 2.** Western blot analysis of selected ST mutants. Whole cell proteins of ST mutants, and the parent strain Ap3AS were separated
611 in 12.5% polyacrylamide gels, then transferred onto a PVDF membrane. The membranes were then probed with pooled sera from birds
612 infected with Ap3AS diluted 1:2,000 (C). Lanes 1 and 5, MGA_0588 ST mutant 18-1; Lanes 3 and 6, *opp*D_1 ST mutant 20-1; Lanes 2,
613 4 and 8, Ap3AS; Lane 7, *opp*D_1 ST mutant 26-1. The positions of the broad range protein markers (PageRuler Prestained Protein Ladder,
614 Thermo Scientific) are indicated on the left.
615
616
617 **Figure 3.** Phylogenetic analysis of *opp*ABCDF operons of *Mollicutes*. Trees were inferred using DNAml (35) and then drawn using
618 MEGA version 3.1 (36). Clustal W (37) aligned sequence data were analyzed using global rearrangement and randomized input orders to
619 find the most likely tree. The optimal transition/transversion ratio was found to be 0.8 when analyzing the *oppB* genes, so this ratio was
620 used for analysis of all other genes. A single mutation rate was assumed. MG, *Mycoplasma genitalium*; MGA,
621 *Mycoplasma gallisepticum*; MMOB, *Mycoplasma mobile* 163K; mph, *Mycoplasma hyopneumoniae*; MPN, *Mycoplasma pneumoniae*;
622 MS, *Mycoplasma synoviae*; MSC, *Mycoplasma mycoides* subspecies *mycoides* small colony type; MYPE, *Mycoplasma penetrans*;
623 MYPU, *Mycoplasma pulmonis*; UU, *Ureaplasma urealyticum/parvum*.

624

Fig. 1

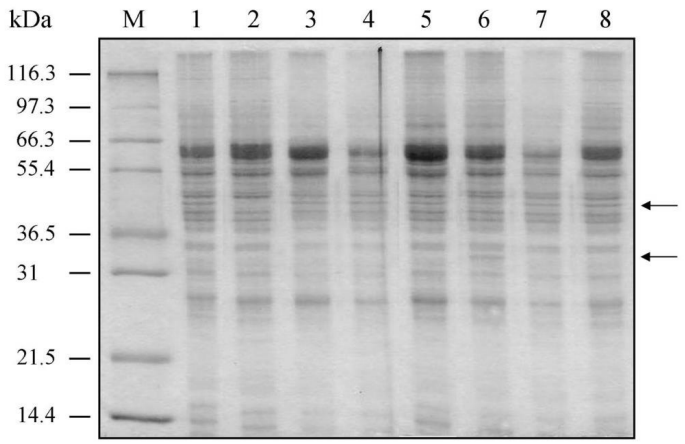

Fig. 2

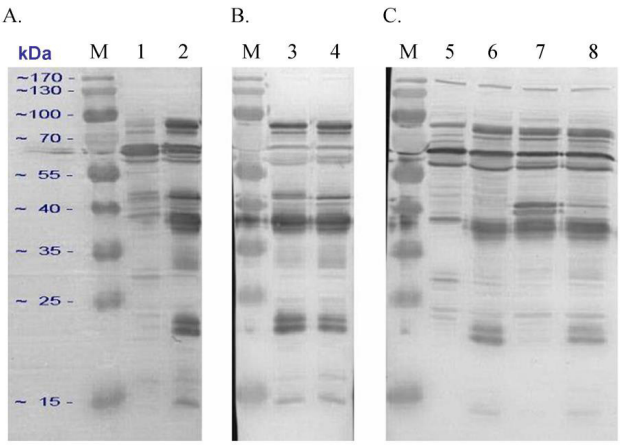

Fig. 3