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1 **The *oppD* gene and putative peptidase genes may be required for virulence in**

2 *Mycoplasma gallisepticum*

3

4

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17

18 Running title: Virulence genes in *Mycoplasma gallisepticum*

19

20 **Abstract**

21 Relatively few virulence genes have been identified in pathogenic mycoplasmas,  
22 so we used signature-tagged mutagenesis to identify mutants of the avian pathogen  
23 *Mycoplasma gallisepticum* with reduced capacity to persist *in vivo* and compared the  
24 virulence of selected mutants in experimentally infected chickens. Four mutants had  
25 insertions in one of the two incomplete *opp*ABCDF operons and a further three had  
26 insertions in distinct hypothetical genes, two containing peptidase motifs and one a  
27 member of a gene family. The three hypothetical gene mutants and the two with  
28 insertions in *oppD*<sub>1</sub> were used to infect chickens and all five were shown to have  
29 reduced capacity to induce respiratory tract lesions. One *oppD*<sub>1</sub> mutant and the  
30 MGA\_1102 and MGA\_1079 mutants had greatly reduced capacity to persist in the  
31 respiratory tract and to induce systemic antibody responses against *M. gallisepticum*.  
32 The other *oppD*<sub>1</sub> mutant and the MGA\_0588 mutant had less capacity than wild type  
33 to persist in the respiratory tract, but did elicit systemic antibody responses. Although  
34 *M. gallisepticum* carries two incomplete *opp* operons, one of which has been acquired  
35 by horizontal gene transfer, our results suggest that one of the copies of *oppD* may be  
36 required for full expression of virulence. We have also shown that three hypothetical  
37 genes, two of which encode putative peptidases, may be required for full expression  
38 of virulence in *M. gallisepticum*. None of these genes has previously been shown to  
39 influence virulence in pathogenic mycoplasmas.

40

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

43

44 **Introduction**

45 *Mycoplasma gallisepticum* is the most important mycoplasmal pathogen in

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

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

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

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

76

## 77 **Methods and Materials**

### 78 ***Bacterial strain and construction of mutants***

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

82 Plasmid pISM 2062.2, carrying the transposon Tn4001mod, was used to  
83 construct a signature-tagged (ST) library, as described previously (5, 12-15). The  
84 transposon contained a gentamicin resistance gene, allowing selective culture. The  
85 presence of the different tags in the ST mutants was confirmed by polymerase chain  
86 reaction (PCR) using the P2/P4 primer set (Table 1) (5). The insertion site of the  
87 transposon in each mutant was determined by genomic DNA sequencing using the  
88 IGstmGenmeF3 primer (Table 1) and comparing the sequence to that of the *M.*  
89 *gallisepticum* strain R<sub>low</sub> genome (16).

90

### 91 ***Screening of ST mutants in infected birds***

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

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

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

119

#### 120 ***Comparison of the persistence and virulence of selected mutants***

121 Several mutants were detected infrequently in the second screening experiment,  
122 suggesting that the genes that were interrupted in these mutants may have a significant  
123 influence on virulence. Two different *oppD*<sub>1</sub> ST mutants were recovered from birds at

124 distinct rates, suggesting that the different insertion sites may have differing impacts  
125 on persistence *in vivo* and potentially on pathogenicity. To assess this, and to assess  
126 the persistence and virulence of three mutants with insertions in hypothetical genes,  
127 the pathogenicities and rates of colonization of five distinct mutants were compared.  
128 Seven groups of 20 four-week-old SPF chickens were housed separately. Group 1 was  
129 exposed to an aerosol of mycoplasma broth only, Group 2 to MGA\_1102 ST mutant  
130 03-1, Group 3 to MGA\_0588 ST mutant 18-1, Group 4 to *oppD*<sub>1</sub> ST mutant 20-1,  
131 Group 5 to MGA\_1079 ST mutant 22-1, Group 6 to *oppD*<sub>1</sub> ST mutant 26-1 and the  
132 7th group to wild type Ap3AS. All birds were subjected to necropsies 14 days after  
133 infection, with samples collected as described above. Sections of the upper, middle  
134 and lower trachea were also taken from each bird.

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

149

150 ***SDS-PAGE electrophoresis and Western blot analysis of ST mutants***

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

157 Whole cell proteins of MGA\_0588ST mutant 18, *oppD*<sub>1</sub> mutant 20, *oppD*<sub>1</sub> mutant 26,  
158 and *M. gallisepticum* strain Ap3AS were subjected to sodium dodecyl sulphate  
159 polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting and  
160 immunostaining to detect differences in protein expressed in the mutants.

161 Total cell proteins of mutants with protein profiles that differed in Coomassie  
162 stained gels were separated in a 10% polyacrylamide gel along with molecular mass  
163 standards (PageRuler Prestained Protein Ladder, Thermo Scientific),  
164 electrophoretically transferred onto a PVDF membrane (Immobilon™-P Transfer  
165 Membrane, Millipore) and antigen-free sites blocked by overnight incubation in 5%  
166 skim milk (Devondale) in PBS containing 0.1% Tween 20 (PBS-T) at 4°C. The  
167 membrane was then washed three times (10 min each) in PBS-T and then incubated in  
168 pooled sera collected from birds infected with Ap3AS (1:2000 in PBS-T), at room  
169 temperature for 1 h with gentle rocking, again washed as above and then incubated for  
170 1 h at room temperature in anti-chicken horseradish peroxidase conjugate (DAKO) at  
171 a dilution of 1:2,000 in PBS-T. After again washing as above, bound conjugate was  
172 detected using enhanced chemiluminescence (CD/DAB Substrate Kit, Thermo  
173 Scientific) following the manufacturer's recommendations and results recorded  
174 digitally using a BioRad Molecular Imager (Bio-Rad) according to the manufacturer's  
175 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 *oppAB<sub>1</sub>C<sub>1</sub>D<sub>1</sub>F<sub>1</sub>* operon.  
184 Transposons were located within *oppB<sub>1a</sub>* in ST mutant 36-1 (and in 36-2 and 36-3)  
185 and within *oppC<sub>1</sub>* 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 *oppD<sub>1</sub>*. No mutants were obtained with insertions in the other copy of  
188 the *opp* operon in *M. gallisepticum* (*oppB<sub>2</sub>C<sub>2</sub>D<sub>2</sub>F<sub>2</sub>*). 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

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202 detectable air sac lesions.

#### 203 *Identification of recoverable ST mutants*

204 The recovery rates of ST mutants from birds at 14 and 28 days after inoculation  
205 are summarized in Tables 4 and 5. The *oppD*<sub>1</sub> ST mutant 20 (-1/-2/-3) was the  
206 mutant most frequently recovered from the air sacs, as well as from the tracheas, and  
207 was isolated at high frequency from all three groups. It was also recovered from the  
208 air sacs of one in-contact control bird in Group A 4 weeks after inoculation. In  
209 contrast, *oppD*<sub>1</sub> ST mutant 26 (-1/-2/-3) was only recovered from the trachea of one  
210 bird in Group A. Six and five isolations of *oppC*<sub>1</sub> ST mutant 24 (-1/-3) were made  
211 from the tracheas of birds in Groups A and C, respectively, whilst only one isolation  
212 of *oppB*<sub>1a</sub> ST mutant 36 (-3) was made, from the trachea of a bird in Group C.  
213 MGA\_0588 ST mutant 18 (-3) was only re-isolated from the air sacs of one bird in  
214 Group C, and from the trachea of one bird each in Groups A and C (-1/-3). Sixteen  
215 ST mutants, including ST mutants 03 (-1/-2/-3) (MGA\_1102 interrupted) and 22  
216 (-1/-2/-3) (MGA\_1079 interrupted), were unable to be recovered from any bird in any  
217 of the groups (Table 4A).

218

#### 219 *Confirmatory screening*

##### 220 *Pathological and serological assessments*

221 No anti-*M. gallisepticum* antibody was detected prior to inoculation in any bird  
222 (Table 4B). Severe air sac lesions (score of 2.5) were seen in one bird, and mild  
223 lesions (0.5 and 1.0) in another two chickens in Group A. One bird had mild air sac  
224 lesions (1.0) in Group B. At 2 weeks after inoculation, 15/19 chickens in Group A and  
225 14/19 birds in Group B had detectable antibody against *M. gallisepticum* (Table 4B).

##### 226 *Re-isolation of ST mutants*

227 Birds in Group A were infected with a pool containing MGA\_1102 ST mutant

228 03-1, and a pool that contained MGA\_1079 ST mutant 22-1 was used to inoculate the  
229 birds in Group B. A total of eleven ST mutants, including ST mutant 03-1, were  
230 re-isolated from sixteen chickens in Group A and ten, including ST mutants 22-1 and  
231 03-1, were re-isolated from eighteen birds in Group B (Table 4B).

232

### 233 ***Infectivity and virulence analysis of selected ST mutants***

#### 234 *Clinical signs and post mortem examination*

235 The prevalence and severity of lesions in chickens infected with individual  
236 mutants are shown in Table 6. Air sac lesions were not seen in the uninfected control  
237 birds (Group 1), or in birds exposed to aerosols of MGA\_1079 ST mutant 22-1  
238 (Group 5). Mild lesions (score of 0.25) were observed in one bird inoculated with  
239 MGA\_1102 ST mutant 03-1 (Group 2). Of the 20 birds infected with *oppD*<sub>1</sub> ST  
240 mutant 26-1 (Group 6), four had mild lesions (0.5 to 1.0), whilst lesions were only  
241 seen in the abdominal air sacs of five birds exposed to MGA\_0588 ST mutant 18-1  
242 (Group 3). Six of 20 birds had mild to severe lesions (0.5 to 2.5) in the group  
243 infected with *oppD*<sub>1</sub> ST mutant 20-1 (Group 4), while mild to severe lesions (0.5 to  
244 3.0) were seen in 11/18 birds infected with the virulent Ap3AS strain (Group 7).

#### 245 *Antibody responses and re-isolation of ST mutants*

246 No anti-*M. gallisepticum* antibody was detected at the time of infection in the  
247 serum of any birds (Table 6). Two weeks after exposure, antibody responses were  
248 not detected in any of the birds in Groups 5 (MGA\_1079 mutant 22-1 infected) or 6  
249 (*oppD*<sub>1</sub> mutant 26-1 infected), whilst a response was detectable in only one bird in  
250 Group 2 (MGA\_1102 mutant 03-1 infected). In contrast, strong antibody responses  
251 against *M. gallisepticum* were detectable in all the birds in Groups 3 (MGA\_0588  
252 mutant 18-1 infected) and 4 (*oppD*<sub>1</sub> mutant 20-1 infected). The uninfected control  
253 birds (Group 1) did not develop any antibody against *M. gallisepticum* over the course

10

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

256 *M. gallisepticum* was not isolated on MA plates inoculated with swabs of the air  
257 sacs of any birds in Groups 2 (MGA\_1102 mutant 03-1 infected) or 5 (MGA\_1079  
258 mutant 22-1 infected), but they were isolated from the trachea of two birds in Group 2  
259 (MGA\_1102 mutant 03-1 infected) and one bird in Group 5 (MGA\_1079 mutant 22-1  
260 infected). In Group 6 (*oppD*<sub>1</sub> mutant 26-1 infected), *M. gallisepticum* were isolated  
261 from the air sacs of one bird and the trachea of 6 birds. *M. gallisepticum* were also  
262 isolated from the trachea of 17/20 birds in Group 3 (MGA\_0588 mutant 18-1 infected)  
263 and of all the birds in Group 4 (*oppD*<sub>1</sub> ST mutant 20-1 infected), and also from swabs  
264 of the air sacs of 5/20 birds in Group 3 and 7/20 birds in Group 4. In the positive  
265 control group (Group 7), *M. gallisepticum* were isolated from the air sacs of 9/18  
266 birds and the trachea of 17/18 birds (Table 6).

267 *M. gallisepticum* was recovered in MB inoculated with swabs of the air sacs of  
268 one bird in Group 6 (*oppD*<sub>1</sub> mutant 26-1 infected), 7 birds in Group 3 (MGA\_0588  
269 mutant 18-1 infected) and 8 birds in Group 4 (*oppD*<sub>1</sub> mutant 20-1 infected).  
270 *M. gallisepticum* were recovered from the trachea of birds in all six of the groups  
271 exposed to ST mutants, with the number of colonized birds ranging from 3 in Group 5  
272 (MGA\_1079 mutant 22-1 infected) to 20 in Group 4 (*oppD*<sub>1</sub> mutant 20-1 infected).  
273 The identity of the recovered *M. gallisepticum* ST mutants was confirmed using the  
274 unique signature tags they carried (Table 6).

#### 275 *Tracheal lesions and mucosal thicknesses*

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

280 exception of those for the upper tracheas of the birds infected with MGA\_1102  
281 mutant 03-1. The upper tracheal lesion scores of the birds infected with MGA\_1102  
282 mutant 03-1 (Group 2) did not differ from those of birds in Groups 3, 5 and 6, but did  
283 differ significantly from those of birds in Group 4 (*oppD*<sub>1</sub> mutant 20-1 infected).

284 Mean tracheal mucosal thicknesses are shown in Table 7. The mean tracheal  
285 mucosal thicknesses of the wild type infected birds were significantly different from  
286 those of both the uninfected and the mutant infected birds ( $P < 0.0001$ ). The mean  
287 mucosal thicknesses in the middle and lower trachea did not differ significantly  
288 between the uninfected group and any of the mutant infected groups (Groups 2 to 6).  
289 However, they were significantly in the wild type infected group (Group 7). While  
290 there was no significant difference between the tracheal mucosal thickness in the  
291 upper trachea of the uninfected controls and Groups 3, 4, 5 and 6, the mean upper  
292 tracheal mucosal thickness in the group infected with MGA\_1102 mutant 03-1 was  
293 significantly greater than that of the uninfected birds and those infected with *oppD*<sub>1</sub>  
294 mutant 20-1. In summary, the wild type infected controls had significantly more  
295 severe tracheal lesions, as assessed by histological lesion score or mucosal thickness,  
296 than any of the groups infected with the ST mutants. Group 2 (MGA\_1102 mutant  
297 03-1 infected) was the most severely affected among the groups infected with the ST  
298 mutants.

299

### 300 ***SDS-PAGE electrophoresis***

301 Total cell proteins of the *M. gallisepticum* strains used in this study were  
302 separated by SDS-PAGE and stained with Coomassie brilliant blue (Figure 1).  
303 There were no detectable differences in the protein profiles of ST mutants 03-1  
304 (MGA\_1102 interrupted), 20-1 (*oppD*<sub>1</sub> interrupted), 22-1 (MGA\_1079 interrupted),  
305 24-1 (*oppC*<sub>1</sub> interrupted), 36-1 (*oppB*<sub>1a</sub> interrupted) and wild-type Ap3AS. However,

306 one protein band of about 40 kDa was absent in MGA\_0588 mutant 18-1, whilst  
307 *oppD*<sub>1</sub> 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 *oppD*<sub>1</sub> 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 *oppD*<sub>1</sub> mutant 26-1  
314 than in wild-type Ap3AS, while proteins of 20 kDa were less prominent in the *oppD*<sub>1</sub>  
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 (*oppB*<sub>1</sub>*C*<sub>1</sub>*D*<sub>1</sub>*F*<sub>1</sub>  
324 and *oppAB*<sub>2</sub>*C*<sub>2</sub>*D*<sub>2</sub>*F*<sub>2</sub>) in *M. gallisepticum* strain R (16). *OppB* and *OppC* are predicted  
325 to be transmembrane proteins, while *oppD* and *oppF* are predicted to encode  
326 ATP-binding proteins. The *oppB*<sub>1</sub> gene contains a frameshift mutation, *oppF*<sub>2</sub> 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 *oppA*, *oppB* and *oppF*, but two full length copies of  
331 *oppCD*.

332 One of these *opp* operons (MGA\_0237, MGA\_0235, MGA\_0234, MGA\_0232,  
333 and MGA\_0230, or *oppAB<sub>2</sub>C<sub>2</sub>D<sub>2</sub>F<sub>2</sub>*) is most similar to that of *M. synoviae* strain 53  
334 (GenBank accession number: AE017245), suggesting that the operon has been  
335 relatively recently acquired from *M. synoviae* or a closely related species within the  
336 Hominis phylogenic group (which includes *M. hyopneumoniae*, *M. synoviae* and  
337 *M. pulmonis*) (Figure 3). Phylogenetic analysis of these genes in a number of fully  
338 sequenced genomes suggested that this operon was duplicated in a member of the  
339 Hominis group, and that these duplicated operons then diverged, with one  
340 subsequently transferred horizontally to an ancestor of *M. gallisepticum* and  
341 *Ureaplasma urealyticum*. *Ureaplasma urealyticum* appears to have lost its original  
342 copy of the operon and retained only the horizontally transferred copy, whilst  
343 *M. gallisepticum* has retained most of both copies of the operon (Figure 3). Studies in  
344 *M. hominis* have shown that OppA is a cytoadhesin, and also that it binds oligopeptides,  
345 suggesting that this operon encodes a transporter responsible for import of  
346 oligopeptides (27-29). It is notable that duplication of the *oppABCDF* operon by  
347 horizontal gene transfer has also occurred in the ruminant pathogens *Mycoplasma*  
348 *agalactiae* and *Mycoplasma bovis*, although this horizontal gene transfer event was  
349 clearly independent of that in *M. gallisepticum* (30-32).

350 In the studies presented here, four mutants had transposon insertions in genes in  
351 the same *opp* operon (in *oppB<sub>1</sub>*, *oppC<sub>1</sub>*, and *oppD<sub>1</sub>*), suggesting that mutations in some  
352 of the original *opp* genes may be tolerated (except possibly for the only full length  
353 *oppF*, *oppF<sub>1</sub>*), but that the *oppB<sub>2</sub>C<sub>2</sub>D<sub>2</sub>* genes may be required for survival in  
354 *M. gallisepticum*. The transposon was inserted into the *oppD<sub>1</sub>* gene in mutants 20-1  
355 (-2/-3) and 26-1 (-2/-3), but in different positions (Table 2). Mutant 20-1 induced  
356 seroconversion in more birds than mutant 26-1, and also colonized a greater  
357 proportion of birds, even though they were both much less pathogenic than the wild

358 type strain. The insertion site in mutant 20-1 was close to the start of the gene, whilst  
359 in mutant 26-1 the first third of the gene may be able to be expressed. It is possible  
360 that the two different OppD proteins form heterodimers in the transport complex, and  
361 that, in the absence of expression of OppD<sub>1</sub> in mutant 20-1, OppD<sub>2</sub> could form  
362 functional homodimers. The partial copy of OppD<sub>1</sub> in mutant 26-1 may retain a  
363 capacity to form a heterodimer, but one that is less functional, resulting in less  
364 efficient transportation and thus the greater reduction in colonization and virulence  
365 seen in this mutant. Further work will be needed to establish whether the two copies  
366 of *oppD* in the genome can partially complement each other and whether they form  
367 heterodimers.

368 In ST mutant 36-1 (-2/-3), the transposon was inserted into the middle of the  
369 *oppB*<sub>1a</sub> gene (MGA\_0223). As there is a frame shift mutation within the *oppB*<sub>1</sub> gene,  
370 the two proteins predicted to be derived from it, OppB<sub>1a</sub> and OppB<sub>1b</sub> (MGA\_0223 and  
371 MGA\_0224), may not be fully functional, and thus *oppB*<sub>2</sub> may be the only fully  
372 functional copy in the genome. Although the *oppB*<sub>1a</sub> mutant 36-1 was isolated very  
373 infrequently in the initial screening experiment, it was only assessed as a member of a  
374 pool, so the impact of mutation of the *oppB*<sub>1a</sub> gene on infectivity and virulence  
375 requires further investigation. ST mutant 24-1 (-2/-3) had a transposon inserted into  
376 the *oppC*<sub>1</sub> gene (MGA\_0221) about two-thirds along the length of the gene and this  
377 mutant could be recovered frequently from birds in the initial screening, suggesting  
378 that either the essential region of OppC may be within the first two-thirds of the gene,  
379 or that *oppC*<sub>2</sub> can complement the loss of *oppC*<sub>1</sub>.

380 ST mutant 18-1, with an insertion in one of multiple paralogous genes for a  
381 hypothetical protein (MGA\_0588), was able to infect birds, but was significantly less  
382 virulent than wild type Ap3AS. This suggests that the paralog disrupted in this  
383 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 *oppD*<sub>1</sub> 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 *oppD*<sub>1</sub> 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 *oppD*<sub>1</sub> was expressed at sufficiently high levels to be detected  
408 in these assays, the presence of the product of the paralogous *oppD*<sub>2</sub> gene would be  
409 expected to obscure detection of changes in expression of *OppD*<sub>1</sub>. 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 *oppD*<sub>1</sub> 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 *oppD* 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 *oppD* 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

436 suggested that genes that appear to have been acquired by horizontal gene transfer  
437 from *M. synoviae*, MGA\_1102 and MGA\_1079, as well as the *oppD*<sub>2</sub> gene, which has  
438 been complemented by horizontal gene transfer from *M. synoviae*, may play a  
439 significant role in pathogenicity in this important pathogen, even though they are not  
440 essential for colonisation and persistence.

441

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447

448

#### 449 **References**

- 450 1. **Kleven SH.** 1981. Transmissibility of the F strain of *Mycoplasma*  
451 *gallisepticum* in leghorn chickens. *Avian Dis* **25**:1005-1018.
- 452 2. **Lin MY, Kleven SH.** 1982. Egg transmission of two strains of *Mycoplasma*  
453 *gallisepticum* in chickens. *Avian Dis* **26**:487-495.
- 454 3. **Hensel M, Shea JE, Gleeson C, Jones MD, Dalton E, Holden DW.** 1995.  
455 Simultaneous identification of bacterial virulence genes by negative selection.  
456 *Science* **269**:400-403.
- 457 4. **Struve C, Forestier C, Krogfelt KA.** 2003. Application of a novel  
458 multi-screening signature-tagged mutagenesis assay for identification of  
459 *Klebsiella pneumoniae* genes essential in colonization and infection.  
460 *Microbiology* **149**:167-176.
- 461 5. **Tseng CW, Kanci A, Citti C, Rosengarten R, Chiu CJ, Chen ZH, Geary SJ,**  
462 **Browning GF, Markham PF.** 2013. MalF is essential for persistence of  
463 *Mycoplasma gallisepticum* *in vivo*. *Microbiology* **159**:1459-1470.
- 464 6. **Hudson P, Gorton TS, Papazisi L, Cecchini K, Frasca S, Jr., Geary SJ.**  
465 2006. Identification of a virulence-associated determinant, dihydroloipoamide  
466 dehydrogenase (*lpd*), in *Mycoplasma gallisepticum* through *in vivo* screening  
467 of transposon mutants. *Infect Immun* **74**:931-939.
- 468 7. **Masukagami Y, Tivendale KA, Mardani K, Ben-Barak I, Markham PF,**

- 469        **Browning GF.** 2013. The *Mycoplasma gallisepticum* virulence factor  
470 lipoprotein MslA is a novel polynucleotide binding protein. *Infect Immun*  
471 **81**:3220-3226.
- 472 8.        **Szczepanek SM, Frasca S, Jr., Schumacher VL, Liao X, Padula M,**  
473 **Djordjevic SP, Geary SJ.** 2010. Identification of lipoprotein MslA as a  
474 neoteric virulence factor of *Mycoplasma gallisepticum*. *Infect Immun*  
475 **78**:3475-3483.
- 476 9.        **Soeripto, Whithear KG, Cottew GS, Harrigan KE.** 1989. Virulence and  
477 transmissibility of *Mycoplasma gallisepticum*. *Aust Vet J* **66**:65-72.
- 478 10.       **Higgins PA, Whithear KG.** 1986. Detection and differentiation of  
479 *Mycoplasma gallisepticum* and *M. synoviae* antibodies in chicken serum using  
480 enzyme-linked immunosorbent assay. *Avian Dis* **30**:160-168.
- 481 11.       **Gaunson JE, Philip CJ, Whithear KG, Browning GF.** 2000. Lymphocytic  
482 infiltration in the chicken trachea in response to *Mycoplasma gallisepticum*  
483 infection. *Microbiology* **146 ( Pt 5)**:1223-1229.
- 484 12.       **Byrne ME, Rouch DA, Skurray RA.** 1989. Nucleotide sequence analysis of  
485 IS256 from the *Staphylococcus aureus*  
486 gentamicin-tobramycin-kanamycin-resistance transposon Tn4001. *Gene*  
487 **81**:361-367.
- 488 13.       **Knudtson KL, Minion FC.** 1993. Construction of Tn4001lac derivatives to  
489 be used as promoter probe vectors in mycoplasmas. *Gene* **137**:217-222.
- 490 14.       **Hedreyda CT, Lee KK, Krause DC.** 1993. Transformation of *Mycoplasma*  
491 *pneumoniae* with Tn4001 by electroporation. *Plasmid* **30**:170-175.
- 492 15.       **Winner F, Markova I, Much P, Lugmair A, Siebert-Gulle K, Vogl G,**  
493 **Rosengarten R, Citti C.** 2003. Phenotypic switching in *Mycoplasma*  
494 *gallisepticum* hemadsorption is governed by a high-frequency, reversible point  
495 mutation. *Infect Immun* **71**:1265-1273.
- 496 16.       **Papazisi L, Gorton TS, Kutish G, Markham PF, Browning GF, Nguyen**  
497 **DK, Swartzell S, Madan A, Mahairas G, Geary SJ.** 2003. The complete  
498 genome sequence of the avian pathogen *Mycoplasma gallisepticum* strain  
499 R(low). *Microbiology* **149**:2307-2316.
- 500 17.       **Whithear KG, Harrigan KE, Kleven SH.** 1996. Standardized method of  
501 aerosol challenge for testing the efficacy of *Mycoplasma gallisepticum*  
502 vaccines. *Avian Dis* **40**:654-660.
- 503 18.       **Nunoya T, Tajima M, Yagihashi T, Sannai S.** 1987. Evaluation of respiratory  
504 lesions in chickens induced by *Mycoplasma gallisepticum*. *Nippon Juigaku*  
505 *Zasshi* **49**:621-629.
- 506 19.       **Whithear KG.** 1993. Avian Mycoplasmosis, p 1-12. *In* Corner LA, Bagust TJ

- 507 (ed), Australian Standard Diagnostic Techniques for Animal Diseases. CSIRO  
 508 for the standing committee on agriculture and resource management, East  
 509 Melbourne.
- 510 20. **Fowler J, Cohen L, Jarvis P.** 1998. Practical Statistics for Field Biology, 2nd  
 511 ed. John Wiley & Sons, Chichester, England.
- 512 21. **Pedersen PL.** 2005. Transport ATPases: structure, motors, mechanism and  
 513 medicine: a brief overview. *J Bioenerg Biomembr* **37**:349-357.
- 514 22. **Young J, Holland IB.** 1999. ABC transporters: bacterial exporters-revisited  
 515 five years on. *Biochim Biophys Acta* **1461**:177-200.
- 516 23. **Higgins CF.** 1992. ABC transporters: from microorganisms to man. *Annu Rev*  
 517 *Cell Biol* **8**:67-113.
- 518 24. **Jones PM, George AM.** 2004. The ABC transporter structure and mechanism:  
 519 perspectives on recent research. *Cell Mol Life Sci* **61**:682-699.
- 520 25. **Schmitt L, Tampe R.** 2002. Structure and mechanism of ABC transporters.  
 521 *Curr Opin Struct Biol* **12**:754-760.
- 522 26. **Garmory HS, Titball RW.** 2004. ATP-binding cassette transporters are targets  
 523 for the development of antibacterial vaccines and therapies. *Infect Immun*  
 524 **72**:6757-6763.
- 525 27. **Henrich B, Hopfe M, Kitzerow A, Hadding U.** 1999. The  
 526 adherence-associated lipoprotein P100, encoded by an opp operon structure,  
 527 functions as the oligopeptide-binding domain OppA of a putative oligopeptide  
 528 transport system in *Mycoplasma hominis*. *J Bacteriol* **181**:4873-4878.
- 529 28. **Fraser CM, Gocayne JD, White O, Adams MD, Clayton RA, Fleischmann**  
 530 **RD, Bult CJ, Kerlavage AR, Sutton G, Kelley JM, Fritchman RD,**  
 531 **Weidman JF, Small KV, Sandusky M, Fuhrmann J, Nguyen D, Utterback**  
 532 **TR, Saudek DM, Phillips CA, Merrick JM, Tomb JF, Dougherty BA, Bott**  
 533 **KF, Hu PC, Lucier TS, Peterson SN, Smith HO, Hutchison CA, 3rd,**  
 534 **Venter JC.** 1995. The minimal gene complement of *Mycoplasma genitalium*.  
 535 *Science* **270**:397-403.
- 536 29. **Himmelreich R, Hilbert H, Plagens H, Pirkl E, Li BC, Herrmann R.** 1996.  
 537 Complete sequence analysis of the genome of the bacterium *Mycoplasma*  
 538 *pneumoniae*. *Nucleic Acids Res* **24**:4420-4449.
- 539 30. **Sirand-Pugnet P, Lartigue C, Marena M, Jacob D, Barre A, Barbe V,**  
 540 **Schenowitz C, Mangenot S, Couloux A, Segurens B, de Daruvar A,**  
 541 **Blanchard A, Citti C.** 2007. Being pathogenic, plastic, and sexual while  
 542 living with a nearly minimal bacterial genome. *PLoS Genet* **3**:e75.
- 543 31. **Wise KS, Calcutt MJ, Foecking MF, Roske K, Madupu R, Methe BA.**  
 544 2011. Complete genome sequence of *Mycoplasma bovis* type strain PG45

- 545 (ATCC 25523). *Infect Immun* **79**:982-983.
- 546 32. **Qi J, Guo A, Cui P, Chen Y, Mustafa R, Ba X, Hu C, Bai Z, Chen X, Shi L,**  
547 **Chen H.** 2012. Comparative geno-plasticity analysis of *Mycoplasma bovis*  
548 HB0801 (Chinese isolate). *PLoS One* **7**:e38239.
- 549 33. **Jones MM, Johnson A, Koszelak-Rosenblum M, Kirkham C, Brauer AL,**  
550 **Malkowski MG, Murphy TF.** 2014. Role of the oligopeptide permease ABC  
551 transporter of *Moraxella catarrhalis* in nutrient acquisition and persistence in  
552 the respiratory tract. *Infect Immun* **82**:4758-4766.
- 553 34. **Moraes PM, Seyffert N, Silva WM, Castro TL, Silva RF, Lima DD, Hirata**  
554 **R, Jr., Silva A, Miyoshi A, Azevedo V.** 2014. Characterization of the Opp  
555 peptide transporter of *Corynebacterium pseudotuberculosis* and its role in  
556 virulence and pathogenicity. *Biomed Res Int* **2014**:489782.
- 557 35. **Felsenstein J.** 1989. PHYLIP -- Phylogeny Inference Package (Version 3.2).  
558 *Cladistics* **5**:164-166.
- 559 36. **Kumar S, Tamura K, Nei M.** 2004. MEGA3: Integrated software for  
560 Molecular Evolutionary Genetics Analysis and sequence alignment. *Brief*  
561 *Bioinform* **5**:150-163.
- 562 37. **Thompson JD, Higgins DG, Gibson TJ.** 1994. CLUSTAL W: improving the  
563 sensitivity of progressive multiple sequence alignment through sequence  
564 weighting, position-specific gap penalties and weight matrix choice. *Nucleic*  
565 *Acids Res* **22**:4673-4680.
- 566
- 567

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	ATCCATTCTAACCAAGC (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	CATCCCACCTGAAAACCTCGT (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 <sub>1</sub>
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 <sub>1</sub>
26-1/2/3	710587-719588 (34.2)	MGA_0220	Oligopeptide transporter ATP-binding protein OppD <sub>1</sub>
36-1/2/3	712897-712898 (45.4)	MGA_0223	Oligopeptide transporter permease (OppB <sub>1a</sub> )

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

<i>ST mutant</i>	<b>Transposon insertion site</b>	<b>Function</b>
<b><i>Cell envelope – Membranes, lipoproteins</i></b>		
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 <sub>low</sub>	Match to <i>vlhA</i> gene in draft Ap3AS sequence
<b><i>Cellular processes</i></b>		
01-1/-2/-3	MGA_1142	OsmC-like stress-induced protein
<b><i>Translation – Protein synthesis</i></b>		
19-1/-2/-3	MGA_0216	Elongation factor P (EF-P)
<b><i>Transport and binding proteins – ABC transport</i></b>		
20-1/-2/-3	MGA_0220	ATP-binding protein OppD <sub>1</sub>
24-1/-2/-3	MGA_0221	Permease protein OppC <sub>1</sub>
26-1/-2/-3	MGA_0220	ATP-binding protein OppD <sub>1</sub>
36-1/-2/-3	MGA_0223	Permease protein OppB <sub>1a</sub>
<b><u><i>Intergenic regions</i></u></b>		
10-1/-2/-3	MGA_0537 – MGA_0539	HsdM endonuclease – <i>hsdI</i> locus
16-1/-2/-3	MGA_0395 – MGA_0398	<i>vlhA</i> 3.09 – <i>malP</i>
23-1/-2/-3	MGA_0379 – MGA_0380	<i>vlhA</i> 4.04 – <i>vlhA</i> 4.05
27-1/-2/-3	MGA_0226 – MGA_0230	Conserved hypothetical protein – <i>oppF</i> (OppF)
31-1/-2/-3	MGA_0071 – MGA_0073	<i>vlhA</i> 1.05 – Putative transposase
38-1/-2/-3	MGA_0518 – MGA_0519	Unique hypothetical protein – conserved hypothetical protein
<b><i>Others</i></b>		
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 <i>M. gallisepticum</i> and <i>M.</i>
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
<b><i>Not determined</i></b>		
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) screens575 **A.**

Group	Antibody against <i>M. gallisepticum</i> *				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
<b>A</b> <sup>#</sup>	0/30	7/9	8/9	0/8	2/10	0/9	0/8	<i>oppD</i> <sub>1-20</sub> (2) <sup>§</sup>	<i>oppD</i> <sub>1-20</sub> (6), <i>oppC</i> <sub>1-24</sub> (6), <i>oppD</i> <sub>1-26</sub> (1) MGA_0588-18(1)
<b>B</b>	0/31	6/10	9/11	0/10	2/10	1/11	0/10	<i>oppD</i> <sub>1-20</sub> (1)	<i>oppD</i> <sub>1-20</sub> (6)
<b>C</b> <sup>#</sup>	0/29	5/10	9/10	0/7	4/10	2/10	0/7	<i>oppD</i> <sub>1-20</sub> (7)	<i>oppD</i> <sub>1-20</sub> (10), <i>oppC</i> <sub>2-24</sub> (5), <i>oppB</i> <sub>1a-36</sub> (1)

576

577 **B.**

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

578 \*Number positive/number examined.

579 <sup>#</sup>Two inoculated and two in-contact birds died in Group A and two in-contact birds died in Group C prior to necropsy.580 <sup>§</sup>including recovery from one bird in the in-contact group at 4 weeks after inoculation.581 <sup>†</sup>One bird died in Groups A and B before necropsy. Pool A contained mutant 03-1 and pool B contained mutant 22-1.

25

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) <sup>§</sup> , 20-1 (2) <sup>§</sup>	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 <sup>#</sup>	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 <sup>#</sup>	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 <sup>§</sup>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 <sup>#</sup>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).

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

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

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 $\pm$ SD* ( $\mu$ m)		
		Upper	Middle	Lower	Upper	Middle	Lower
1	Medium	0.25 (0, 0.5) <sup>a</sup>	0.25 (0, 0.5) <sup>a</sup>	0 (0, 0.25) <sup>a</sup>	50 $\pm$ 11 <sup>a</sup>	41 $\pm$ 8 <sup>a</sup>	37 $\pm$ 6 <sup>a</sup>
2	Mutant 03-1	1 (0.25, 3) <sup>b</sup>	0.375 (0, 1.5) <sup>a</sup>	0.25 (0, 1.5) <sup>a</sup>	89 $\pm$ 45 <sup>b</sup>	54 $\pm$ 12 <sup>a</sup>	46 $\pm$ 22 <sup>a</sup>
3	Mutant 18-1	0.25 (0, 10) <sup>ab</sup>	0.25 (0, 1.5) <sup>a</sup>	0.25 (0, 1.5) <sup>a</sup>	62 $\pm$ 15 <sup>ab</sup>	53 $\pm$ 15 <sup>a</sup>	49 $\pm$ 15 <sup>ab</sup>
4	Mutant 20-1	0.25 (0, 1.5) <sup>a</sup>	0.125 (0, 1.5) <sup>a</sup>	0.125 (0, 1.0) <sup>a</sup>	56 $\pm$ 22 <sup>a</sup>	49 $\pm$ 23 <sup>a</sup>	51 $\pm$ 19 <sup>a</sup>
5	Mutant 22-1	0.5 (0, 1) <sup>ab</sup>	0.25 (0, 1) <sup>a</sup>	0 (0, 0.25) <sup>a</sup>	64 $\pm$ 13 <sup>ab</sup>	49 $\pm$ 8 <sup>a</sup>	39 $\pm$ 8 <sup>a</sup>
6	Mutant 26-1	0.5 (0, 2) <sup>ab</sup>	0.25 (0, 1) <sup>a</sup>	0 (0, 2) <sup>a</sup>	64 $\pm$ 24 <sup>ab</sup>	49 $\pm$ 14 <sup>a</sup>	48 $\pm$ 22 <sup>a</sup>
7	Wild type Ap3AS	1.5 (1, 3) <sup>c</sup>	1.50 (0.5, 3) <sup>b</sup>	1.50 (0.25, 3) <sup>b</sup>	168 $\pm$ 70 <sup>c</sup>	153 $\pm$ 84 <sup>b</sup>	120 $\pm$ 56 <sup>b</sup>

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, *oppD*<sub>1</sub> mutant 20-1; lane 4, ST mutant 22-1 (MGA\_1079 interrupted); lane 5, *oppC*<sub>1</sub> mutant 24-1; lane 6, *oppD*<sub>1</sub>  
 606 mutant 26-1; lane 7, *oppB*<sub>1a</sub> mutant 36-1; Lane 8, wild-type strain Ap3AS. The positions of the broad range protein markers (Mark12™  
 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, *oppD*<sub>1</sub> ST mutant 20-1; Lanes 2,  
 613 4 and 8, Ap3AS; Lane 7, *oppD*<sub>1</sub> 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 *oppABCDF* operons of *Mollicutes*. Trees were inferred using DNAmI (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, *Mycoplasma*  
 621 *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*.

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Fig. 1

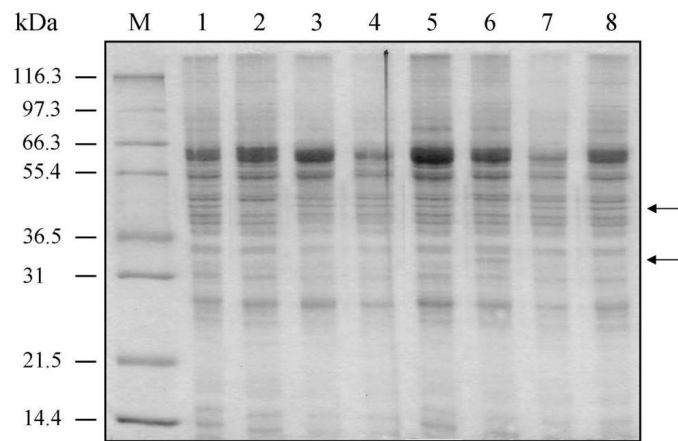
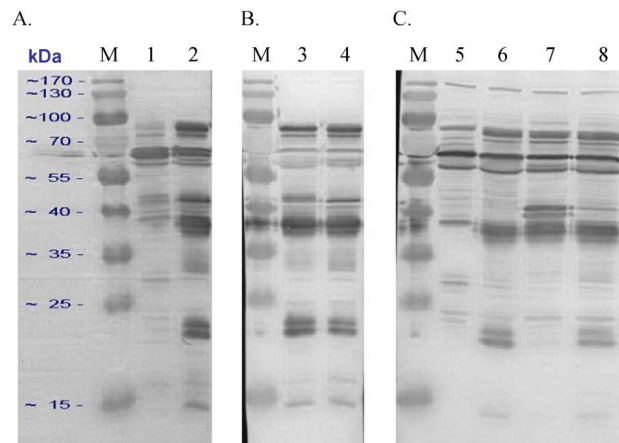




Fig. 2



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Fig. 3

