MalF is essential for persistence of *Mycoplasma gallisepticum* in vivo

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There is limited understanding of the molecular basis of virulence in the important avian pathogen *Mycoplasma gallisepticum*. To define genes that may be involved in colonization of chickens, a collection of mutants of the virulent Ap3AS strain of *M. gallisepticum* were generated by signature-tagged transposon mutagenesis. The collection included mutants with single insertions in the genes encoding the adhesin GapA and the cytadherence-related protein CrmA, and Western blotting confirmed that these mutants did not express these proteins. In two separate *in vivo* screenings, two GapA-deficient mutants (ST mutants 02-1 and 06-1) were occasionally recovered from birds, suggesting that GapA expression may not always be essential for persistence of strain Ap3AS. CrmA-deficient ST mutant 33-1 colonized birds poorly and had reduced virulence, indicating that CrmA was a significant virulence factor, but was not absolutely essential for colonization. ST mutant 04-1 contained a single transposon insertion in *malF*, a predicted ABC sugar transport permease, and could not be reisolated even when inoculated by itself into a group of birds, suggesting that expression of MalF was essential for persistence of *M. galliseptium* strain Ap3AS in infected birds.

**INTRODUCTION**

*Mycoplasma gallisepticum* is the most important mycoplasma pathogen of the poultry industry worldwide, causing infectious sinusitis in turkeys, and air sacculitis and tracheitis in chickens (Jordan, 1979; Yoder & Hodstad, 1964), resulting in significant economic losses. There is limited current understanding of the key virulence factors required for colonization of and survival in the host.

Attachment of the pathogen to a host cell is mediated by interactions between cytadhesins and host cell receptors (Razin et al., 1998). The adhesin GapA, encoded by the *gapA* gene, is considered to be the primary cytadherence of *M. gallisepticum* and has significant amino acid sequence similarity to the P1 cytadhesin of *Mycoplasma pneumoniae* (Goh et al., 1998). The cytadherence related protein A (crmA) gene is located downstream of and within the same operon as the *gapA* gene (Papazisi et al., 2000) and the product of this gene has significant sequence similarity to that of the ORF6 gene of *M. pneumoniae* (Mudahi-Orenstein et al., 2003; Papazisi et al., 2002). Both GapA and CrmA are present in the pathogenic *M. gallisepticum* strain Rlow, but are not expressed by the avirulent *M. gallisepticum* strain R₈, whilst GapA is absent in the majority of organisms in the *M. gallisepticum* vaccine strain ts-11 due to a frame shift caused by a 20 bp sequence duplication (Kanci et al., 2004), rather than a base substitution (Mudahi-Orenstein et al., 2003). Earlier studies have demonstrated that coexpression of GapA and CrmA is essential for effective cytadherence and virulence in *M. gallisepticum* strain R (Mudahi-Orenstein et al., 2003; Papazisi et al., 2002).
Signature-tagged (ST) mutagenesis is a powerful technique for identifying genes that are required by pathogens for growth in their host (Hensel et al., 1995). Individual mutants that are present in an input (pre-selection) pool, but missing from the pool recovered following inoculation of the host, are likely to contain mutations in genes responsible for colonization and persistence in the host, and that thus encode virulence determinants. The dihydrolipoamide dehydrogenase gene (lpd) has been identified as a virulence-associated determinant in _M. gallicepticum_ using similar techniques (Hudson et al., 2006).

The aim of this study was to identify genes involved in colonization by and persistence of _M. gallicepticum_ using ST mutagenesis, to assess whether mutations in cytadherence-related genes and a transport operon affected the capacity of this pathogen to persist in its natural host, the chicken, and to examine the pathogenicity of two of these mutants in experimentally inoculated chickens.

**METHODS**

**Bacterial strain and cultural conditions.** _M. gallicepticum_ Ap3AS was originally isolated from the air sacs of a broiler chicken in Australia and has been shown to be pathogenic (Soeripto et al., 1989). It was grown in modified Frey’s broth (MB) containing 10% swine serum (Higgins & Whithear, 1986) at 37°C until the late exponential phase of growth (pH approximately 6.8).

**Signature-tagged transposons.** A signature-tagged (ST) mutant library was prepared using the plasmid pISM 2062.2 carrying the transposon Tn4001mod (Byrne et al., 1989; Knudton & Minion, 1993), which contained the gentamicin resistance gene. Each signature tag consisted of a unique 40 bp oligonucleotide DNA sequence ([NK]20; N=A, C, G or T; K=G or T) flanked by two invariable arms of 20 bp that enabled the amplification and labelling of the unique regions by PCR using the primer pairs P2/P4 (Winner et al., 2003). The signature tag was cloned into the BamH I restriction endonuclease cleavage site of pISM 2062.2 (Fig. 1). A total of 34 distinct tags were available, enabling panels of 34 distinguishable mutants to be generated and simultaneously assessed in each group of infected animals.

**Signature-tagged mutagenesis**

**Transformation with signature tags.** Dilutions of _M. gallicepticum_ Ap3AS culture were grown in 1.5 ml MB overnight at 37°C. The dilutions showing an acid colour change were combined and the cells collected by centrifugation for 5 min at 16000 g at room temperature. The basic procedure for transformation of _M. gallicepticum_ by electroporation was adapted from Hedredya et al. (1993) and Winner et al. (2003). The cells were resuspended in HEPE/sucrose buffer and 100 μl aliquots of cells were placed on ice and 10 μg plasmid DNA was added. After electro-transformation, the cells were gently resuspended in 1 ml cold (4°C) MB and incubated at room temperature for 10 min and then at 37°C for up to 3 h until an acid colour change was seen. A 500 μl sample of the electrooporated culture was spread on a mycoplasma agar (MA) plate containing 160 μg gentamicin ml⁻¹ and the plate dried and incubated at 37°C for 7–10 days. Mycoplasma agar (MA) plates were prepared as for MB except that glucose and phenol red were omitted and the medium was solidified with 1% (w/v) noble agar (Difco).

**PCR and confirmation of ST mutants.** Colonies were cloned into 1 ml MB containing gentamicin (160 μg ml⁻¹). Following growth, cells in a 200 μl volume of the culture were collected by centrifugation and resuspended in 20 μl distilled water, heated at 100°C for 5 min and a 2 μl aliquot used as template for PCR. The oligonucleotide tag, the 16S rRNA gene and a region of the gentamicin resistance gene were amplified using the oligonucleotide primer pairs P2/P4, MG-F/MG-R (Boyle & Morrow, 1994) and EOGentSpeIRev/EPGentSpeIFor, respectively. Each PCR was conducted in a 20 μl reaction volume and contained 2 μl 10 x reaction buffer, 1 μM each primer, 200 μM each dNTP, 1.5 mM MgCl₂, 1.5 U Taq DNA polymerase (Promega) and 2 μl template DNA. PCRs to amplify the oligonucleotide tag were incubated through one cycle at 98°C for 2 min, then 35 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 40 s, and a final incubation at 72°C for 7 min; to amplify the 278 bp product from the gentamicin resistance gene, reactions were incubated at 95°C for 2 min, then through 28 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 15 s, and finally at 72°C for 5 min. PCR products were electrophoresed in a 2% agarose gel along with size standards (pUC18 digested with _HaeIII_).

**Determination of transposon insertion points.** Each ST transformant was grown in 40 ml MB supplemented with 160 μg gentamicin ml⁻¹ at 37°C until late exponential phase (~pH 6.8). The cells were harvested by centrifugation at 20000 g for 30 min, washed twice in chilled PBS, 25 μl of a 10% solution of SDS added to lyse the cells, and the solution then passed through a 26-gauge needle fitted to a 1 ml syringe to shear the genomic DNA. Genomic DNA

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**Fig. 1.** Basic structure of signature-tagged transposon (adapted and modified from Knudton & Minion, 1993). N=A, C, G or T; K=G or T. Gm, gentamicin resistance gene; Ap, ampicillin resistance gene; IS, insertion sequence; IR, inverted repeat sequence; ori, origin of replication.
extraction was performed with the HighPure PCR kit (Roche) according to the manufacturer’s protocol, but the initial lysozyme treatment was omitted and the DNA was eluted from the column using 50 µl 10 mM Tris/HCl buffer (pH 8.0). The amount of purified DNA was estimated by electrophoresis of a 1 µl sample in a 0.7% agarose gel together with molecular mass standards of known concentration (phage λ DNA digested with HindIII).

The procedure for genomic DNA sequencing was adapted and modified from Wada (2000). The primer I GstmGenmeF3 (Table 1), which binds to the modified Tn4001, was used for sequencing through the transposon–genomic DNA junction and into Ap3AS genomic DNA. The sequence was determined using ABI PRISM Big Dye 3.1 Terminator chemistry (Applied Biosystems). Each reaction contained genomic DNA. The sequence was determined using ABI PRISM Big Dye 3.1 Terminator chemistry (Applied Biosystems). Each reaction consisted of 2–3 µg purified genomic DNA, 30 pmol I GstmGenmeF3 primer, 4 µl Big Dye 3.1 enzyme mixture (Life Technologies), and water added to a final volume of 20 µl. Cycle sequencing was performed by incubation through one cycle at 95 °C for 3 min, followed by 60 cycles of 95 °C for 30 s, 55 °C for 30 s and 60 °C for 4 min. The sequencing products were then purified according to the manufacturer’s recommendations and analysed using an ABI 3100 Capillary Sequencer and relevant software.

The insertion site for each ST transposon was determined using BLAST (National Center for Biotechnology Information, NCBI) or FASTA version 3.3t07 (Pearson & Lipman, 1988) to compare the DNA sequence to that of the strain Rlow genome (Papazisi et al., 2003).

**Table 1. Selected oligonucleotides used in these studies**

<table>
<thead>
<tr>
<th>Name</th>
<th>Use</th>
<th>5’→3’ sequence (size; bp)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2</td>
<td>Signature tag region PCR</td>
<td>ATCCTACAACCTCAAGCT (18)</td>
</tr>
<tr>
<td>P4</td>
<td>Signature tag region PCR</td>
<td>ATCCCATTTAACAAGCC (18)</td>
</tr>
<tr>
<td>I GstmGenmeF3</td>
<td>DNA sequencing ST mutant PCR</td>
<td>GGACTGTTATATGGCCTTTTTGGATC (26)</td>
</tr>
<tr>
<td>MG-F</td>
<td>M. gallisepticum 16S rRNA gene PCR</td>
<td>GTTGCAAAATCCGGTAAAGTTGG (20)</td>
</tr>
<tr>
<td>MG-R</td>
<td>M. gallisepticum 16S rRNA gene PCR</td>
<td>TTAGCAACACGGTTTATAGAT (20)</td>
</tr>
<tr>
<td>EGoGentSpeIRev</td>
<td>Genticin gene PCR</td>
<td>actagtATCCGATTCCGATTTGATGG (26)</td>
</tr>
<tr>
<td>EPGentSpeIFor</td>
<td>Genticin gene PCR</td>
<td>actagtGTGATTTCACCGGAAAGG (26)</td>
</tr>
<tr>
<td>STM04-1</td>
<td>ST mutant PCR</td>
<td>CCGGGGACACGATGAGCTAAG (20)</td>
</tr>
<tr>
<td>STM06-1-Rev</td>
<td>ST mutant PCR</td>
<td>AACATGGAATTACGTTGG (20)</td>
</tr>
<tr>
<td>STM13-KF-C’</td>
<td>Wild-type Ap3AS PCR</td>
<td>TATAAACCTCGTTACCG (16)</td>
</tr>
<tr>
<td>KB-STM33</td>
<td>ST mutant PCR</td>
<td>ACTACACGTTAGTAC (19)</td>
</tr>
</tbody>
</table>

*Lower case indicates nucleotide modifications to produce a restriction endonuclease cleavage site.

**MalF is essential for persistence of M. gallisepticum in vivo**

**Calculation of numbers of viable cells of ST mutants for experimental infection.** The viable count for each cultured ST mutant, measured in colour changing units (CCU) ml⁻¹, was determined by limiting dilution titration as described previously (Whithear et al., 1983). The concentration was calculated using most probable number tables (Meynell & Meynell, 1970) and birds were exposed to an aerosol containing approximately 1 x 10⁶ CCU ml⁻¹ of each ST mutant.

**Expression of CrmA and GapA in selected ST mutants.** Whole-cell proteins of ST mutants 02-1, 06-1, 28-1, 33-1 and 39-1, which had Tn4001 insertions in or close to the cytadherence genes crmA and gapA (Fig. 2), were subjected to SDS-PAGE, Western blotting and immunostaining to detect the proteins expressed from these genes. Total cell proteins were separated in a 12.5% polyacrylamide gel along with molecular mass standards, electrophoretically transferred onto a nitrocellulose membrane (Hybond-C, GE Healthcare) and antigen-free sites blocked by overnight incubation in 5% skimmed milk (Devondale) in PBS containing 0.1% Tween 20 (PBS-T) at 4 °C. The following day the membrane was washed three times (10 min each) in PBS-T and then incubated in rabbit anti-CrmA (1:20 000 in PBS-T) or anti-GapA sera (1:5000 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 swine anti-rabbit horseradish peroxidase conjugate (DAKO) at a dilution of 1:2000 in PBS-T. After again washing as above, bound conjugate was detected using the enhanced chemiluminescence detection system (ECL Plus kit; GE Healthcare) following the manufacturer’s recommendations and results recorded on Biomax film (Kodak).

**Screening of ST mutants in infected birds.** The *in vivo* experiments were based on the original ST mutagenesis protocol (Hensel et al., 1995) using the chicken as the natural host with a number of modifications. Specific pathogen free White Leghorn chickens (SPAFAST Australia) were housed in a positive pressure fibreglass isolator and given feed and water *ad libitum*. A dilution series of each ST mutant was made in MB containing 160 µg gentamicin ml⁻¹ and incubated overnight at 37 °C. An inoculum containing a pool of ST mutants was prepared and used to infect chickens by aerosol as described previously (Gauin et al., 2006a, b; Whithear et al., 1996).
Blood samples were collected from all birds before aerosol infection and before euthanasia. The serum was tested using the rapid serum agglutination (RSA) test (Intervet, The Netherlands) to detect antibody responses against M. gallisepticum. The RSA test was scored using a scale from 0 to 4 (Whithear, 1993).

Chickens were euthanized by intravenous injection of sodium pentobarbitone and gross air sac lesions examined and scored for severity on a scale of 0 to 3 (Nunoya et al., 1987). Swabs were taken from the air sacs and trachea of each bird and used to inoculate MA plates containing 160 µg gentamicin ml⁻¹, as well as MA plates without gentamicin, and were then placed into 3 ml MB supplemented with 160 µg gentamicin ml⁻¹. MA plates were incubated at 37 °C and examined using a binocular dissecting microscope after 7–10 days. A previous study detected the loss of the Tn4001 transposon from some mutants during in vivo experiments 14 days after inoculation (Mudahi-Orenstein et al., 2003). These mutants regained the wild-type phenotype but were unable to survive the selection pressure of gentamicin. When there were tenfold or greater numbers of colonies on plates containing no gentamicin than on plates containing gentamicin, loss of the transposon was suspected. Cellular DNA was prepared from those broths showing a colour change and used as template in PCRs to amplify the unique tag region using the biotinylated P2 and P4 primers and the resultant amplicon used in dot blots to identify the ST mutant(s) present. Where the determination of the identity of the ST mutant was inconclusive, a PCR was performed using a primer specific for the ST mutant, together with the Tn4001 specific primer 1GsmGemmE3 (Table 1). The ST mutant-specific PCRs were incubated through one cycle at 95 °C for 2 min, followed by 35 cycles of 94 °C for 45 s, 52 °C for 45 s and 72 °C for 1 min, and a final incubation at 72 °C for 7 min.

**Figure 2.** Cytadherence operon and insertion site of transposons in cytadherence-related mutants. The transposon insertion sites in the genome of each ST mutant are indicated. The proportion of the gene 5′ to the insertion point was 68.3% in ST mutant 02-1, 54.6% in ST mutant 06-1, 67.6% in ST mutant 28-1, 79.2% in ST mutant 33-1 and 14.7% in ST mutant 39-1. Bar, 1 kb.

**Table 2.** Transposon insertion point within the ST mutants used in this study

<table>
<thead>
<tr>
<th>ST mutant ID</th>
<th>Insertion point in genome (% of gene to insertion point)</th>
<th>Function of disrupted gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>02-1</td>
<td>225517–225518 (68.3)</td>
<td>Adherence protein A (GapA)</td>
</tr>
<tr>
<td>04-1</td>
<td>46569–46570 (0.2)</td>
<td>ABC sugar transport permease (MalF)</td>
</tr>
<tr>
<td>06-1/2/3</td>
<td>225057–225058 (54.6)</td>
<td>Adherence protein A (GapA)</td>
</tr>
<tr>
<td>28-1/2/3</td>
<td>231845–231846 (67.6)</td>
<td>Cytadherence-related protein B (CrmB)</td>
</tr>
<tr>
<td>33-1/2/3</td>
<td>229137–229138 (79.2)</td>
<td>Cytadherence-related protein A (CrmA)</td>
</tr>
<tr>
<td>39-1/2/3</td>
<td>230367–230368 (14.7)</td>
<td>Cytadherence-related protein B (CrmB)</td>
</tr>
</tbody>
</table>
Four groups of 4-week-old specific pathogen free chickens were housed separately (20 birds per group) in positive pressure fiberglass isolators. The ST mutants (04-1 and 33-1) were cultured at 37 °C in MB supplemented with 160 μg gentamicin ml⁻¹ and wild-type Ap3AS in MB without gentamicin, and the concentrations of each culture adjusted to approximately 1 × 10⁷ CCU ml⁻¹. One group of birds was exposed to an aerosol of ST mutant 04-1, a second to ST mutant 33-1, whilst the third group was exposed to MB only and the fourth to wild-type Ap3AS.

All birds were euthanized at 14 days after infection and post-mortem examinations conducted, with samples collected as described above, except that sections of the trachea (upper, middle and lower) were taken from each bird. The tracheal sections were examined for histopathology and the mucosal thickness measured (Gauinson et al., 2006a, b; Nunoya et al., 1987).

To identify ST mutants reisolated in broth cultures, both dot blot hybridization and specific PCRs for the two mutants were used. Dot blot hybridization could not be used for cultures from the positive control group, as M. gallisepticum Ap3AS did not contain a signature tag, so a pair of PCR primers were used to confirm the presence of Ap3AS. The PCR used the primer pair STM13-KE-C', 1-Rev/STM13-KF-C and would yield a 400 bp product from Ap3AS, whilst no product would be generated from Ap3AS by the primer pair P2/P4 (Table 1). The PCR was incubated through one cycle at 95 °C for 2 min, followed by 35 cycles of 94 °C for 45 s, 52 °C for 45 s and 72 °C for 1 min, and a final incubation at 72 °C for 7 min.

Median tracheal lesion scores for each experimental group were compared using Mann–Whitney U tests (Minitab version 14.2 for Windows). Student’s t-tests and a one-way ANOVA were used to compare the mean tracheal mucosal thicknesses. A probability (P) ≤ 0.05 was regarded as significant.

**RESULTS**

**Identification of ST mutants of M. gallisepticum**

A total of 34 separate transposons, each containing a unique signature tag, were independently introduced into M. gallisepticum strain Ap3AS and transformants were selected for their resistance to gentamicin. From each unique tag transformation, three individual transformants were selected, identified by a number (signature tags 1–34) and allocated in the initial animal experiments to group A, B or C (see Methods and below). The insertion site of the transposon was determined in 91 ST mutants by direct genome sequencing. Reliable sequence data could not be obtained for 11 mutants, which were subsequently examined using Southern blotting to detect multiple insertions of the transposon. This group of 11 mutants was found by Southern blotting to contain five distinct mutants containing multiple insertions, with all mutants carrying the same sequence tag apparently identical. Several ST mutants were found with transposons inserted within the cytadherence-related genes, gapA, crmA and crmB. In ST mutants 02-1 and 06-1, the transposons were located within the gapA gene, but at different sites. Transposons were located within crmA in ST mutant 33-1 and within crmB in ST mutants 28-1 and 39-1, but at different locations (Table 2 and Fig. 2).

The transposon insertion site could not be determined in 3 ST mutants, as no significant match could be found with the existing M. gallisepticum genomic sequence in the database, including strains R, F, S6 and house finch isolate CA2006. This suggested that the insertion site might be unique to strain Ap3AS.

**Detection of CrmA and GapA expression in selected ST mutants by immunostaining**

Whole-cell proteins of the ST mutants with the transposon inserted in gapA (ST mutants 02-1 and 06-1), crmA (33-1) or crmB (28-1 and 39-1) were separated and immunostained using antisera to GapA and CrmA (Fig. 3). The results showed that CrmA was expressed in all of these mutants except for 33-1 (Fig. 3b, lane 5), whilst GapA was not detected in gapA ST mutants 02-1 and 06-1 (Fig. 3b, lanes 2 and 3) or the M. gallisepticum vaccine strain ts-11 (Fig. 3b, lane 8). The rabbit anti-CrmA serum was prepared by immunizing rabbits with SDS-PAGE gel extracts that predominantly contained CrmA, but also co-migrating proteins (Papazisi, personal communication). The resultant antisera recognized not only CrmA, but also another, more slowly migrating protein in the Western blot.

**Experiment 1 – preliminary ST mutant screening**

M. gallisepticum specific antibody was not detected in birds before infection. Of the chickens infected by aerosol with a pool of ten ST mutants, two thirds had RSA scores greater than that completely inhibited haemagglutination was regarded as the HI titre.
A greater proportion of birds had antibody responses against *M. gallisepticum* detectable by RSA at 4 weeks (84%) than at 2 weeks (60%) after infection, and no antibody response was detected in in-contact birds in any group. A greater number of birds had air sac lesions at 2 weeks (2, 2 and 4 in groups A, B and C, respectively) than at 4 weeks (0, 1 and 2 in groups A, B and C, respectively) after inoculation. More severe air sac lesions were observed at 2 weeks after inoculation (scores of 0.5 in group A, 1.0 to 2.0 in group B and 0.5 to 2.5 in group C), except that in group C one chicken had a lesion score of 3.0 at 4 weeks after inoculation. More air sac lesions were seen in group C.

The pattern of recovery of *M. gallisepticum* was similar to that seen in the preliminary experiment, with more isolations made from the tracheas than the air sacs. Generally, isolation rates were greater at 2 weeks than at 4 weeks after infection. No ST mutants were reisolated from the in-contact birds in group B, although five different ST mutants were reisolated from the in-contact birds in group A and two from those in group C (data not shown). The most commonly reisolated mutant from all groups (A, B and C) was ST mutant 28, which had the transposon inserted within *crmB*. A total of 16 ST mutants, marked with 12 different tags, including *malF* ST mutant 04-1, were not reisolated. When mycoplasmas were detected on solid media, they were usually also isolated by broth culture. No loss of the transposon was seen in any reisolated ST mutant.

**Experiment 3 – confirmatory screening**

A total of sixteen ST mutants that could not be detected in Experiment 2, including the *gapA* mutant 06-1 and the *crmA* mutant 33-1 were cultured, and allocated separately to one of two groups of inocula, which were each used to infect 20 birds. All infected birds were housed together and horizontal transmission between the two infected groups monitored by dot blot hybridization and by PCR where necessary.

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. No anti-*M. gallisepticum* antibody was detected in any bird prior to inoculation. At 2 weeks after inoculation, 15/19 chickens in group A were RSA positive, whilst 14/19 birds in group B were positive.

A total of 11 ST mutants, including *gapA* ST mutant 06-1, were reisolated from 16 chickens in group A and 10 were reisolated from 18 birds in group B.

Four ST mutants used to inoculate birds in group A were reisolated from group B, whilst four mutants used to inoculate birds in group B were reisolated from group A. Neither the MalF-deficient ST mutant 04-1 nor the CrmA-deficient ST mutant 33-1 were isolated from any bird in either group. No loss of the transposon was detected in any reisolated ST mutant.
Experiment 4 – virulence and infectivity of malF ST mutant 04-1 and crmA ST mutant 33-1

Air sac lesions were not seen in birds exposed to aerosols of the malF ST mutant 04-1 (group 2) or the crmA-deficient mutant 33-1 (group 3), or in the negative control birds (group 1), whilst mild to severe lesions (0.50 to 3.00) were seen in 11/18 birds infected with the virulent Ap3AS strain (group 4) (Table 3).

No anti-mycoplasma antibody was detected by the RSA test prior to infection or in the serum of the negative control birds (group 1) or in birds infected with the ST mutants (groups 2 and 3) 2 weeks after infection. However, strong reactions were detected in sera obtained from all of the positive control birds (group 4) 2 weeks after infection (Table 3).

The malF ST mutant 04-1 was not reisolated from any bird in group 2. However, crmA ST mutant 33-1 was recovered in MB from the tracheas of two birds (group 3) (Table 4) and its identity confirmed using the unique signature tag. M. gallisepticum strain AP3AS was reisolated from the air sacs of 9/18 birds and the tracheas of 17/18 birds in the positive control group (group 4).

The tracheal lesion scores of birds in the uninfected and the mutant infected groups differed significantly from those of birds in the positive control group (group 4) (P<0.0001), whilst no significant difference was seen between the negative control group (group 1) and the groups infected with the ST mutants in the lower trachea. However, a significant difference in lesion scores was seen between group 2 (infected with malF ST mutant 04-1) and the negative control group (group 1) or group 3 (infected with crmA ST mutant 33-1) in the upper trachea (Table 3).

The mean mucosal thicknesses of the upper, middle and lower trachea of the mutant infected groups (groups 2 and 3) were significantly less than that of the positive control group (group 4). Birds in the ST mutant infected groups (group 2 and 3) had greater mucosal thicknesses in the upper trachea than the negative control birds (group 1). No significant difference was observed between group 3 (infected with crmA ST mutant 33-1) and group 2 (infected with malF ST mutant 04-1) in any of the tracheal sections (Table 3).

HA, HI and colony HAd in mutants with interruptions in cytadhesin-related genes

The number of M. gallisepticum cells needed to produce one HA unit was determined (Table 5). The number of organisms required for strain ts-11 to produce one HA unit (3.9 × 10^6 CCU ml^-1) was twice the number for its parent strain 80083 (1.8 × 10^6 CCU ml^-1). The number of individual ST mutant cells required to produce one HA unit varied considerably compared to the parental AP3AS strain, which required 1.7 × 10^7 CCU ml^-1 to produce one HA unit. The gapA ST mutant 06-1 required 1.3 × 10^8 CCU ml^-1, whilst the crmB ST mutant 28-1 required 1.3 × 10^8 CCU ml^-1 to produce one HA unit.

The HI test was performed using a rabbit antiserum to VlhA1.1 of M. gallisepticum strain S6. The HI titre of this serum was 16 for strain ts-11 and 2 for strain 80083. The HI titre for strain Ap3AS was 8 and ranged from 4 to 256 for the different ST mutants.

More than 90 % of colonies of strains 80083 and Ap3AS were HAd^+, whilst approximately 70 % of the colonies of strain ts-11 were HAd^- (Table 5). For four of the five ST mutants, over 90 % of colonies exhibited HAd; the

### Table 3. Serology, air sac lesions, tracheal lesion scores and mucosal thicknesses in birds in the infectivity and virulence study

<table>
<thead>
<tr>
<th>Group</th>
<th>Inoculum</th>
<th>RSA assay</th>
<th>Air sac lesions</th>
<th>Median tracheal lesion score (range)</th>
<th>Mean (±sd) tracheal mucosal thickness (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Upper</td>
<td>Middle</td>
</tr>
<tr>
<td>1</td>
<td>Medium</td>
<td>0/20</td>
<td>0/20</td>
<td>0.25 (0.5)^a</td>
<td>0.25 (0.5)^a</td>
</tr>
<tr>
<td>2</td>
<td>ST mutant 04-1</td>
<td>0/20</td>
<td>0/20</td>
<td>0.5 (0.25, 1.5)^b</td>
<td>0.25 (0.5)^b</td>
</tr>
<tr>
<td>3</td>
<td>ST mutant 33-1</td>
<td>0/20</td>
<td>0/20</td>
<td>0.75 (0.25, 3)^b</td>
<td>0.25 (0.25, 1.5)^ab</td>
</tr>
<tr>
<td>4†</td>
<td>Ap3AS</td>
<td>18/18</td>
<td>11/18</td>
<td>1.5 (1, 3)^c</td>
<td>1.5 (0.5, 3)^c</td>
</tr>
</tbody>
</table>

*Values marked with the same superscript letter in the same column were not significantly different.
†Two birds died in group 4 before the conclusion of the experiment.

**Table 4. Frequency of reisolation of ST mutants from infected chickens**

<table>
<thead>
<tr>
<th>ST mutant</th>
<th>Gene interrupted</th>
<th>Experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>02-1</td>
<td>gapA</td>
<td>1</td>
</tr>
<tr>
<td>04-1</td>
<td>malF</td>
<td>NT</td>
</tr>
<tr>
<td>06-1/2/3</td>
<td>gapA</td>
<td>0</td>
</tr>
<tr>
<td>28-1/2/3</td>
<td>crmB</td>
<td>NT</td>
</tr>
<tr>
<td>33-1/2/3</td>
<td>crmA</td>
<td>NT</td>
</tr>
<tr>
<td>39-1/2/3</td>
<td>crmB</td>
<td>NT</td>
</tr>
</tbody>
</table>

NT, Not tested.
exception was the GapA-deficient mutant 02-1, with 64% of colonies being haemadsorptive.

**DISCUSSION**

In this study we used transposon mutagenesis to disrupt a number of genes in the avian pathogen *M. gallisepticum* strain Ap3AS. We modified the original ST mutagenesis method (Hensel et al., 1995) to avoid the need for the pre-screening process (Mei et al., 1997). The detection method used oligonucleotide primers end-labelled with DIG to identify tags by dot blotting, facilitating the use of non-radioactive hybridization (Fuller et al., 2000), reducing the level of background hybridization and eliminating the need to remove the invariable arms following PCR. We identified mutants with insertions in the genes encoding the primary adhesin and two cytadherence-related molecules of the organism. Among these ST mutants, two mutants had insertions in the gapA gene, one within the crmA gene and two within the crmB gene.

Previous studies have shown that 5-week-old chickens infected with wild-type Ap3AS by aerosol have peak systemic anti-mycoplasma RSA antibody concentrations 3 weeks after infection (Gausson et al., 2000). Gross air sac lesions were most severe at 2 weeks after infection, with lesions resolving over the following 3 weeks. Bacterial genome titres also changed over this period and peaked at 3 weeks after infection (Gausson et al., 2000). In the initial screening experiment, more chickens had antibody detectable by RSA at 4 weeks than at 2 weeks after infection, possibly due to low levels of infectious organisms in the inoculum. However, lesions were more common and more severe at 2 weeks after inoculation and this appeared to be the most appropriate time point to collect samples for analysis.

The genes identified as significant using ST mutagenesis can be separated into three classes: those with known biochemical function, those also found in other mycoplasma species or bacteria for which a function has yet to be determined, and those that are unique to *M. gallisepticum* based on searches of the existing database. Only half of the 102 ST mutants created in this study had interruptions in genes of known biochemical function, while the insertions in a further six were in intergenic regions. The gapA-crmA-crmB operon, which encodes cytadherence-related molecules, appeared to be a favoured site for insertion, as five ST mutants had the tagged transposon inserted within this operon. Nine mutants contained insertions in genes for hypothetical proteins and five transformants contained multiple insertions, as confirmed by Southern blotting.

As identification of ST transposon insertion sites and in vivo experiments were performed in parallel, the fact that most transformants in groups A, B and C in Experiment 2 were identical was not recognized initially, and thus the total number of ST mutants investigated in the initial screening experiment was reduced to thirty-nine. In many instances these mutants containing the same tags had the transposon inserted in identical positions, indicating that they were derived from the same parental mutant and that the three hours allowed after electroporation was excessive. Although unforeseen, this replicated the assessment of most mutants and thus increased confidence in the results. Those mutants not recovered in the second experiment, including the gapA mutant 06-1, were reassigned to new inoculum pools to confirm these findings. This additional screening decreased the number of mutants identified as incapable of colonization (Autret & Charbit, 2005; Darwin & Miller, 1999). In the confirmatory screening experiment, most ST mutants that had not been reisolated from infected birds in Experiment 2 were able to be recovered, leaving ST mutant 33-1, which had an insertion in crmA, as the only cytadherence operon mutant requiring further investigation. When chickens were exposed to aerosols of ST mutant 33-1 alone they did not develop disease, but the mutant could be isolated from the trachea of 2/20 exposed birds. These data suggested that ST mutant 33-1 was no longer virulent and that the presence of CrmA is important in pathogenicity, although not essential for colonization and persistence.

The attachment organelle possessed by some mycoplasma species is believed to be essential for attachment in these species. This organelle has been extensively investigated in *M. pneumoniae* (Balish et al., 2001; Balish & Krause, 2005).

### Table 5. HA, HI and HAd studies on selected ST mutants and *M. gallisepticum* strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Gene interrupted</th>
<th>1 HA unit (CCU ml⁻¹)</th>
<th>HI*</th>
<th>HAd (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ST mutant 02-1</td>
<td>gapA</td>
<td>1.1 × 10⁷</td>
<td>4</td>
<td>64</td>
</tr>
<tr>
<td>ST mutant 06-1</td>
<td>gapA</td>
<td>1.3 × 10⁴</td>
<td>32</td>
<td>&gt;90</td>
</tr>
<tr>
<td>ST mutant 28-1</td>
<td>crmA</td>
<td>1.3 × 10⁸</td>
<td>4</td>
<td>&gt;90</td>
</tr>
<tr>
<td>ST mutant 33-1</td>
<td>crmA</td>
<td>1.5 × 10⁶</td>
<td>256</td>
<td>&gt;90</td>
</tr>
<tr>
<td>ST mutant 06-1</td>
<td>crmA</td>
<td>1.8 × 10⁷</td>
<td>8</td>
<td>&gt;90</td>
</tr>
<tr>
<td>Ap3AS</td>
<td>–</td>
<td>1.7 × 10⁷</td>
<td>8</td>
<td>&gt;90</td>
</tr>
<tr>
<td>ts-11</td>
<td>gapA</td>
<td>3.9 × 10⁸</td>
<td>16</td>
<td>70–90</td>
</tr>
<tr>
<td>80083</td>
<td>–</td>
<td>1.8 × 10⁸</td>
<td>2</td>
<td>&gt;90</td>
</tr>
</tbody>
</table>

*Rabbit anti-VlhA1.1 antiserum used for HI test.*
Seto et al., 2001) and the cytadherence-related molecules, including P1, P30 and Proteins B and C from ORF6, which are localized in the attachment organelle, have been proven to be essential for cytadherence by *M. pneumoniae* (Balish & Krause, 2005; Baseman et al., 1987; Franzoso et al., 1993; Layh-Schmitt & Hermann, 1994; Layh-Schmitt et al., 1995, 2000; Morrison-Plummer et al., 1987). Therefore, the homologues of these molecules in *M. gallisepticum* are likely to be important for virulence (Goh et al., 1998; Hnatow et al., 1998; Mudahi-Orenstein et al., 2003; Papazisi et al., 2000, 2002). In *M. gallisepticum*, the attachment bleb is similar to the attachment organelle of *M. pneumoniae*, but appears to protrude less from the cell (Abu-Zahr & Butler, 1978). The homologue of P1, the adhesin that is required for close interaction between *M. pneumoniae* and host cells (Hu et al., 1977), in *M. gallisepticum* is GapA (or MGC1) (Goh & Keeler, 1996). GapA has been shown to play a crucial role in cytadherence in *M. gallisepticum* strain R (Goh et al., 1998; Mudahi-Orenstein et al., 2003; Papazisi et al., 2002). Proteins B and C in *M. pneumoniae* are cytadherence accessory proteins and are cleavage products of MPN142 (also known as ORF6), which is located immediately downstream of the P1 gene (Sperker et al., 1991). The crmA gene of *M. gallisepticum* is a homologue of MPN142 and, although the product of the gene is not cleaved, it has also been shown to be required for efficient colonization and pathogenicity (Papazisi et al., 2002). The crmB gene lies immediately downstream of crmA and, although its role has not been characterized (Papazisi et al., 2003), it is also thought likely to be involved in cytadherence. CrmB has low, but localized, sequence similarity to both GapA and P1 (Papazisi et al., 2000).

Both gapA and crmA are within the same operon (Fig. 2) (Goh et al., 1998; Papazisi et al., 2000) and previous studies by Papazisi et al. (2002) found that a high passage variant of the R strain (R<sub>high</sub>) that did not express GapA or CrmA was not cytadherent in vitro and did not induce disease in experimentally infected chickens. They further demonstrated that complementation of R<sub>high</sub> with either gapA or crmA did not restore cytadherence or virulence and that neither complemented mutant could be recovered from experimentally infected chickens. In contrast, complementation of R<sub>high</sub> with both gapA and crmA restored its cytadherence, its capacity to establish infection in the respiratory tract of chickens and its virulence (Mudahi-Orenstein et al., 2003; Papazisi et al., 2002). Previous studies have also shown that GapA is expressed at very low levels in the ts-11 vaccine strain and that variants of ts-11 that express GapA have enhanced capacity to establish infection and induce protective immunity (Mudahi-Orenstein et al., 2003; Shil et al., 2011). In our current study, Western blot analysis revealed that CrmA, but not GapA, was expressed in ST mutants 02-1 and 06-1, while ST mutant 33-1 did not express CrmA. The levels of expression of CrmA appeared to be lower in the GapA-deficient mutants 02-1 and 06-1, even though a similar number of organisms were used in the Western blot preparations. This suggested that CrmA expression may be affected by GapA, although the effect appeared not to be reciprocal, as the level of expression of GapA did not appear to be affected in the CrmA deficient mutant. It is possible that the mutants expressed carboxyl-terminal truncations of these proteins that could not be detected by the polyclonal antiserum, so further investigation is needed to confirm these observations. After two rounds of in vitro screening, mutants 33-1 (CrmA-deficient) and 06-1 (GapA-deficient) could not be reisolated, supporting previous work that has suggested a crucial role for CrmA and GapA in colonization. While gapA ST mutant 06-1 was not reisolated in the first two experiments, it was recovered from the trachea of one bird in the following experiment. In a previous study using ST mutants of gapA to infect birds, several reisolates of the GapA mutant had lost the transposon 14 days after infection, resulting in restoration of the parental phenotype (Mudahi-Orenstein et al., 2003); however, examination by PCR of reisolates of gapA ST mutant 06-1 for loss of the transposon confirmed that this was not the case in our studies. Thus our studies have shown that, while GapA is required for optimal levels of colonization of the respiratory tract and for induction of pathology in the trachea and air sacs of chickens, it does not appear to be absolutely required for colonization in *M. gallisepticum* strain Ap3AS. In our investigations of the infectivity and virulence of the crmA ST mutant 33-1, we exposed birds to aerosols of this mutant alone. The mutant was recovered from the tracheas of two inoculated birds and its identity confirmed. Thus, while it was clear that the ability of this mutant to colonize and cause disease had been dramatically reduced, it had not been completely abolished. These data suggest that factors other than GapA or CrmA mediate sufficient adhesion to facilitate the reduced survival and recovery observed in vivo. Insertion of the transposon into two different sites in the crmB gene did not appear to affect the capacity of *M. gallisepticum* to establish infection of the respiratory tract.

As binding to chicken red blood cells has been found previously to be influenced by some of the cytadherence-related proteins, colony HAd analyses were conducted on those mutants with insertions in the cytadherence-related loci. This showed that HAd of chicken red blood cells by the GapA-deficient ST mutant 02-1 was reduced by 30% compared to wild-type Ap3AS, whilst no difference was detected between wild-type Ap3AS and ST mutants 06-1 (GapA-deficient) or 33-1 (CrmA-deficient). In further analyses, the number of cells of GapA-deficient ST mutant 06-1 required for HA was 1000 times less than the number of wild-type organisms required and higher concentrations of anti-VlhA antibody were needed to inhibit HA. These results strongly suggested that perhaps another member of the VlhA family was expressed that was antigenically distinct from VlhA1.1 and not inhibited by the antiserum. The CrmA-deficient ST mutant 33-1 had slightly higher HA activity than the wild-type strain but this activity could...
be easily inhibited by very low concentrations of anti-VlhA1.1 antibody, suggesting that the tagged transposon not only interfered with CrmA expression, but also reduced VlhA expression in this ST mutant, or, alternatively, that VlhA was the sole agglutinin and HA was dramatically reduced by inhibiting its binding.

The mutant carrying an insertion in MalF could not be recovered from experimentally infected chickens, suggesting an essential role in persistence and pathogenicity. The function of MalF in *M. gallisepticum* is uncertain, although it appears likely that it is involved in carbohydrate transport into the cell. Its closest homologue in *Escherichia coli* has been extensively characterized as part of the maltose ABC transporter system (Austermuhle et al., 2004; Caldelari et al., 2008; Cui et al., 2010; Daus et al., 2009; Daus et al., 2007; Daus et al., 2006; Iacso et al., 2009; Mannering et al., 2001; Nikaido, 1994; Sharma et al., 2005). ABC transporters are multi-domain membrane proteins that bind ATP and utilize the energy from its hydrolysis to translocate solutes across cellular membranes (Noll et al., 2008; Pedersen, 2005; Young & Holland, 1999). ABC transporters consist of four domains, with a conserved core structure of two transmembrane domains and two nucleotide-binding domains (Garnory & Titball, 2004; Higgins, 1992; Jones & George, 2004; Schmitt & Tampe, 2002). Our studies are the first to demonstrate that MalF is required by a pathogenic mycoplasma for persistence in, and pathogenicity for, the respiratory tract. It should be noted that a number of mycoplasma proteins have been shown to play dual roles in addition to their apparent primary biochemical function. Many of these multifunctional proteins appear to be able to function as adhesins, so it is possible that the crucial role of MalF is not necessarily in carbohydrate transport, but potentially in other steps required for colonization, such as adhesion. Further investigation of the function of MalF will be required to determine why it is required for persistence in *vivo*.

Thus, in the work presented here, we used a modified ST mutagenesis technique to generate a series of mutants of *M. gallisepticum* and assess their capacity to establish infection of the respiratory tract of chickens. Our results suggest that expression of the cytdhesin GapA may not be essential for colonization of the respiratory tract in strain Ap3AS, although previous studies have shown that it is required for optimal colonization (Papazisi et al., 2002). We further showed that the loss of CrmA expression, while associated with greatly reduced capacity to establish infection and cause disease, did not completely abolish colonization. Insertions in the putative cytdhesin related gene *crmB* did not appear to affect the capacity of *M. gallisepticum* to colonize the respiratory tract of birds. We further showed that insertion of a transposon within the gene encoding MalF (MGA_0680) abolished the capacity of *M. gallisepticum* to persist or cause disease in inoculated birds, suggesting that this gene is essential for survival of strain Ap3AS in *vivo*.

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