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 cytadhesin 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 Rlow+, 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 (ldp) has been identified as a virulence-associated determinant in M. gallisepticum using similar techniques (Hudson et al., 2006).

The aim of this study was to identify genes involved in colonization by and persistence of M. gallisepticum 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. gallisepticum 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; Knudtson & Minion, 1993), which contained the gentamicin resistance gene. Each signature tag consisted of a unique 40 bp oligonucleotide DNA sequence [(NK)30; 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 BamHI 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. gallisepticum 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 16 000 g at room temperature. The basic procedure for transformation of M. gallisepticum by electroporation was adapted from Hedredya et al. (1993) and Winner et al. (2003). The cells were resuspended in HEPES/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 electroporated 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 × 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 20 000 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 Knudtson & 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 IGstmGenmeF3 (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 consisted of 2–3 μl purified genomic DNA, 30 pmol IGstmGenmeF3 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 5 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 3.3.07 (Pearson & Lipman, 1988) to compare the DNA sequence to that of the M. gallisepticum strain Rlow genome (Papazisi et al., 2003).

**Southern blot analysis of transposon insertions in genome.** A total of 34 oligonucleotides complementary to each of the unique signature tags were synthesized (Geneworks, Australia), resuspended in TE buffer [0.01 M Tris/HCl (pH 8.0), 0.001 M EDTA] and 3 pmol applied by vacuum onto a Hybond-N+ membrane (Amersham Pharmacia Biotech) using the Bio-Dot SF Microfiltration Apparatus (Bio-Rad) following the manufacturer’s instructions. The DIG-labelled probes were hybridized to the membranes in DIG Easy Hyb buffer (Roche) for 16–18 h at 40 °C in a shaking water bath and then the membranes washed according to the manufacturer’s instructions, except that the second wash was conducted at 45 °C. Bound probes were detected using the DIG Luminescent Detection kit (Roche) and results recorded using Biomax film (Kodak).

**Calculation of numbers of viable cells of ST mutants for experimental infection.** The viable count for each cultured ST mutant, measured in colony forming 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 10⁷ 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; 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 (SPAFAS 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 (Gauzon et al., 2006a, b; Whithear et al., 1996).

### 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>ATCCCTACAACCTCAAGCT (18)</td>
</tr>
<tr>
<td>P4</td>
<td>Signature tag region PCR</td>
<td>ATCCCATTTAACAGACGC (18)</td>
</tr>
<tr>
<td>IGstmGenmeF3</td>
<td>DNA sequencing</td>
<td>GGACGTTATATGCTTGCTTT (26)</td>
</tr>
<tr>
<td>MG-F</td>
<td>M. gallisepticum 16S rRNA gene PCR</td>
<td>GTTGGCAATCCGTAAGGTT (20)</td>
</tr>
<tr>
<td>MG-R</td>
<td>M. gallisepticum 16S rRNA gene PCR</td>
<td>TTAGCAACGGTTTGTAGT (20)</td>
</tr>
<tr>
<td>EOGentSpeIRev</td>
<td>Gentamicin gene PCR</td>
<td>actagATCAGCAATGCTTTAT (26)</td>
</tr>
<tr>
<td>EPGentSpeIFor</td>
<td>Gentamicin gene PCR</td>
<td>actagCTGAGTTTATGGAAGGTT (26)</td>
</tr>
<tr>
<td>STM04-1</td>
<td>ST mutant PCR</td>
<td>CGGGGACACAGTAAGGCTAA (20)</td>
</tr>
<tr>
<td>STM06-1-Rev</td>
<td>ST mutant PCR</td>
<td>AATAGGTTAGGCTTTTC (20)</td>
</tr>
<tr>
<td>STM13-KE'-1-Rev</td>
<td>Wild-type Ap3AS PCR</td>
<td>CACAGGAACTTTGGAAG (17)</td>
</tr>
<tr>
<td>STM13-KF'</td>
<td>Wild-type Ap3AS PCR</td>
<td>TATAAACCCGTGACCG (16)</td>
</tr>
<tr>
<td>KB-STM33</td>
<td>ST mutant PCR</td>
<td>ACTACACGGTAGGAAAG (19)</td>
</tr>
</tbody>
</table>

*Lower case indicates nucleotide modifications to produce a restriction endonuclease cleavage site.*
Blood samples were collected from all birds before aerosol infection and before euthanization. The serum was tested using the rapid serum agglutination test (RSTA) to detect antibody responses against *M. gallisepticum*. The RSTA 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., 1997). 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 in MB.

Experiment 1 – preliminary ST mutant screening. The purpose of the preliminary screening experiment was to establish the strategy and methodology for subsequent *in vivo* screening experiments. Twelve 4-week-old chickens (SPAFAS Australia) were infected with a pool of ten ST mutants that included the two gapA mutants, 02-1 and 06-1 (Table 2). Three days after infection, four uninfected 4-week-old birds were placed in the isolator as in-contact controls to investigate the capacity for transmission of the ST mutants. Seventeen days after infection, six of the aerosol-infected birds were euthanized, post-mortem examinations conducted and samples from the respiratory tract collected for analysis. After 28 days, all the remaining birds (including the in-contact controls) were euthanized and samples collected at post-mortem.

Experiment 2 – initial ST mutant screening. Sixty 4-week-old chickens were allocated into three groups (A, B and C) of 20 birds each. Each group was infected by aerosol with 34 ST mutants, each with a unique sequence tag (a total of 102 ST mutants). As some mutants included in the different panels were subsequently found to be identical, all three groups were exposed to ST mutants 06 (as 06-1, 2 or 3; *gapA*-interrupted), 33 (as 33-1, 2 or 3; *crmA*-interrupted), 28 (as 28-1, 2 or 3) and 39 (as 39-1, 2 or 3) (*crmB*-interrupted), but ST mutant 04-1 (*malF*-interrupted) (Table 2) was only included in the pool used to infect group A. A further ten chickens were placed with the infected birds three days after aerosol exposure as in-contact controls. Sample collection and post-mortem examination were carried out as above with ten aerosol-infected birds examined at 14 days and the remaining 20 birds at 28 days after infection. In this study, only dot blot hybridization was used to identify the ST mutant(s) present in MB.

**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>Adherence protein A (GapA)</td>
</tr>
<tr>
<td>06-1/2/3</td>
<td>225057–225058 (54.6)</td>
<td>Cytadherence-related protein B (CrmB)</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 x 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 (Gausson 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.

**Haemagglutination (HA) and haemagglutination inhibition (HI) tests.** M. gallisepticum cells were cultured in MB and harvested at pH 6.8 as described above. The cell pellet was washed and resuspended in PBS to 1/50 of the original broth volume. The reciprocal of the highest dilution of mycoplasma cells showing complete agglutination was assigned a value of one HA unit (Beard, 1989; Noomhormadadi et al., 1997).

The HI test was performed using 4 HA units of M. gallisepticum cells in v-bottom microplates (Nunclon). The first well of each row was used as the antibody control and contained 25 μl rabbit anti-VlhA1.1 (M. gallisepticum strain S6) serum. To all remaining test wells, 25 μl PBS containing 0.5% FBS was added. To the second well of each row, 25 μl rabbit anti-VlhA1.1 was added and serial twofold dilutions were prepared by transferring 25 μl of the serum dilution into the wells in the next row. To all wells except the first row of the plate, a 25 μl volume of mycoplasma cell suspension containing 4 HA units was added. After one hour of incubation at room temperature, 25 μl of a 0.5% suspension of chicken erythrocytes in PBS containing 0.5% FBS was added to each well and the plate incubated for a further hour at room temperature. The reciprocal of the highest dilution of serum that completely inhibited haemagglutination was regarded as the HI titre.

**Colonial haemadsorption (HAd).** Colonies of ST mutants on MA plates were overlaid with 10 ml of a fresh 0.5% (v/v) suspension of chicken erythrocytes in PBS and incubated for 30 min at 37°C. The erythrocyte suspension was then carefully removed, followed by gentle washing with PBS, and HAd to all well-separated colonies assessed on each plate at low magnification using a binocular dissecting microscope. Colonies with ten or more adherent erythrocytes were defined as HAd positive (HAd⁺).

### 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 cytoadherence-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 (Papazizi, 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...
M. gallisepticum against A greater proportion of birds had antibody responses mutants Experiment 2 – initial screening using a pool of ST could not be recovered, including the trachea of one bird. The remaining six ST mutants following 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 2 – initial screening using a pool of ST mutants
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 2 – initial screening using a pool of ST mutants
A greater proportion of birds had antibody responses against M. gallisepticum detectable by RSA at 4 weeks than one at 2 weeks post-infection and all were positive by 4 weeks post-infection. Only one of the in-contact controls reacted in the RSA test, with a score of 1.

Mild air sac lesions (score of 0.5) were seen in 2/6 birds 2 weeks after infection and in 3/6 chickens at 4 weeks after infection, while one bird had severe lesions in the abdominal air sacs (score of 2.5). Only one of the in-contact controls had mild lesions (score of 0.5).

MA plates were examined for growth of M. gallisepticum in the presence and absence of gentamicin. There appeared to be no evidence of the loss of the transposon from ST mutants following in vivo passage and reisolation. M. gallisepticum was only isolated on MA plates inoculated with swabs of the air sacs of one bird and the tracheas of two birds at 2 weeks after inoculation, and of the tracheas of four chickens at 4 weeks after infection.

Four of the ten ST mutants used to infect the birds were recovered from the air sacs and tracheas of the birds, including the gapA mutant 02-1, which was recovered from the trachea of one bird. The remaining six ST mutants could not be recovered, including the gapA mutant 06-1.

Experiment 2 – initial screening using a pool of ST mutants
A greater proportion of birds had antibody responses against M. gallisepticum detectable by RSA at 4 weeks

Fig. 3. (a) SDS-PAGE and (b) Western blot analysis of selected ST mutants. Proteins of M. gallisepticum strain Ap3AS, strain ts-11 and five ST mutants were separated in a 12.5 % polyacrylamide gel, and transferred onto a nitrocellulose membrane. The membrane was then probed with rabbit anti-CrmA serum at a dilution of 1 : 20 000 and rabbit anti-GapA serum diluted 1 : 10 000. Lanes: 1 and 7, Ap3AS; 2, ST mutant 02-1; 3, ST mutant 06-1; 4, ST mutant 28-1; 5, ST mutant 33-1; 6, ST mutant 39-1; 8, ts-11.

<table>
<thead>
<tr>
<th>M</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
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<td>66.4</td>
<td>55.4</td>
<td>36.5</td>
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<td>21.5</td>
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<td>11.6</td>
<td>7.4</td>
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<td>3.5</td>
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<td>1.8</td>
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<td>0.8</td>
<td>0.6</td>
<td>0.4</td>
<td>0.3</td>
<td>0.2</td>
<td>0.1</td>
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</tbody>
</table>

(a) SDS-PAGE and (b) Western blot analysis of selected ST mutants. Proteins of M. gallisepticum strain Ap3AS, strain ts-11 and five ST mutants were separated in a 12.5 % polyacrylamide gel, and transferred onto a nitrocellulose membrane. The membrane was then probed with rabbit anti-CrmA serum at a dilution of 1 : 20 000 and rabbit anti-GapA serum diluted 1 : 10 000. Lanes: 1 and 7, Ap3AS; 2, ST mutant 02-1; 3, ST mutant 06-1; 4, ST mutant 28-1; 5, ST mutant 33-1; 6, ST mutant 39-1; 8, ts-11.
**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 *crmA* 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, 0.5)ab</td>
<td>0.25 (0, 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, 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, 2.5)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><em>malF</em></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><em>crmA</em></td>
<td>NT</td>
</tr>
<tr>
<td>33-1/2/3</td>
<td><em>crmA</em></td>
<td>NT</td>
</tr>
<tr>
<td>39-1/2/3</td>
<td><em>crmA</em></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 (Gaunson 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 (Gaunson 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;
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 M. pneumoniae 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 M. pneumoniae 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 M. pneumoniae 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 M. pneumoniae attachment bleb is similar to the attachment organelle of M. pneumoniae, but appears to protrude less from the cell (Abu-Zahr & Butler, 1978).
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; Jasco 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 & Tampé, 2002). Our studies are the first to demonstrate that MalF is required for 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 adherence. 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 cytadhesin 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 active malE gene. Arch Microbiol 189, 597–604.


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MalF is essential for persistence of M. gallisepticum in vivo


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