AUSTRALIAN POULTRY CRC

FINAL REPORT

Program 2B

Project No: UM 03-11

Development of New Generation Mycoplasma Based Vaccines

PROJECT LEADER: Dr Philip F Markham

UNSW Collaborator Dr Wendy Muir

DATE OF COMPLETION: 30th July 2008

ISBN 978 1 921890 22 2

Development of New Generation Mycoplasma Based Vaccines

Project No. 03-11

Researcher Contact Details

Dr Philip F Markham

Veterinary Preclinical Centre
The University of Melbourne
Parkville Victoria 3010

Phone: 03 8344 7422
Fax: 03 8344 7374
Email: pmarkham@unimelb.edu.au
Website:

In submitting this report, the researcher has agreed to the Australian Poultry CRC publishing this material in its edited form.

Australian Poultry CRC Contact Details

PO Box U242
University of New England
ARMIDALE NSW 2351

Phone: 02 6773 3767
Fax: 02 6773 3050
Email: info@poultrycrc.com.au
Website: http://www.poultrycrc.com.au

Published in July 2009

Executive Summary

This project had four major goals, each were met with varying degrees of success, several approaches complemented others producing an outcome that in some cases was unexpected but mostly very productive.

The first goal was to produce an alternative method for the diagnosis of the vaccine strain produced a qPCR test and ELISA to measure chicken IgG and IgA from serum and tracheal washes. Though no cytokines were detectable nor did the ELISA out perform the RSA test or qPCR prove reliable a High Resolution Melt curve test based on DNA sequences of *M. gallisepticum* ts-11 vaccine strain was developed and shown to be sensitive and specific fulfilling the initial goals. The test can be used in a farm situation or on isolates from the field and with form the basis of another HRM curve based PCR at the APCAH facility in Werribee.

The genetic manipulation of *M. gallisepticum* ts-11 vaccine strain was attempted using a plasmid carrying the MG origin of replication and the tetracycline resistance gene. The oriC vector was developed for homologous recombination, the first developed worldwide for MG. There were difficulties in producing homologous recombinants without an antibiotic selection marker in the gene knockout with several methodologies attempted. More recent publications suggest alternative methods to remove the antibiotic marker following genomic insertion, these could be used in the future.

The development of *M. gallisepticum* ts-11 vaccine strain as a vaccine vector would enable expression of foreign genes and produce a bi-valent vaccine. Attempts to express the HagA haemagglutinin of *Avibacterium paragallinarum* were unsuccessful using the oriC vector system and as such the chicken interferon gene (chIFN-γ) was cloned in the pISM plasmid carrying the Tn4001 transposon and expressed in *M. gallisepticum* ts-11 vaccine strain. The level of expression of chIFN-γ was low and produced a detectable immune response in chickens suggesting that the vaccine is capable of expressing foreign genes that can interact or manipulate the immune system of the host.

Microarray analysis was conducted on VlhA the most abundant cell surface antigen of *M. gallisepticum* strains. The VlhA peptide belongs to a gene family that is predicted to be involved in immune evasion. The expressed VlhA gene was identified using microarray analysis, the gene chip produced has the potential to fingerprint other *M. gallisepticum* strains to aid in epidemiological studies.

A series of Signature Tagged mutants were tested for attenuation, from these experiments a single clone STM26 was chosen for further investigations in a protection experiment. The mutation in STM26 was located in the *oppD* gene of the oligopeptide transport operon. The efficacy experiment was successful with the ST mutant 26 providing protection against wild type challenge whilst proving safe when used alone in chickens.

The potential to use this specific attenuation in other bacteria producing disease in chickens was assessed using the colibacillosis causing strain *E. coli* E956. Both *oppD* and *dppD* knockouts were produced and assessed in a chick experiment assessing safety and efficacy. The *oppD* knockout was found to be both safe and efficacious when compared to the parental strain.

This attenuation was the subject of a patent application and forms the basis for a platform technology to produce attenuated vaccine strains.

Contents

Introduction

Alternatives to serology for determining an effective immune response to *Mycoplasma gallisepticum* ts-11 vaccine.

Mycoplasma gallisepticum (MG) is an important pathogen of poultry worldwide causing chronic respiratory disease in chickens and turkeys, reduction in egg production and considerable economic losses to the poultry industry (Ley, 2008). Diagnosis of MG infection can be made by various methods but the gold standard test for confirmation of diagnosis is isolation and identification of the organism (Ley, 2008). Serology has been traditionally used to detect infection of flocks with MG. Infection with wild-type MG usually produces a serological response that is detectable using tests such as rapid serum agglutination (RSA), haemagglutination inhibition and ELISA (Whithear, 1996). With the introduction of live attenuated vaccine strains, such as ts-11, producers have required an indicator that their flocks have been effectively vaccinated and hence protected against any future challenge with wild-type MG. Not surprisingly, they turned to serology as a means of assessing a successful vaccine 'take'. However, strain ts-11 is highly attenuated and does not elicit a strong serological response. Not infrequently there is barely detectable or even no MG-specific serum antibody. An ELISA based on autologous pMGA (a major membrane antigen) from ts-11 was somewhat more sensitive than the RSA test but it still failed to detect antibodies in all vaccinated birds (Noormohammadi *et al.*, 2002a).

However, it is well known that there is no correlation between level of serum antibody and protection (Noormohammadi *et al.*, 2002b). It has also been shown that ts-11 vaccinated chickens showing no detectable antibody are resistant to challenge with virulent MG. By contrast, studies have shown the generation of MG-specific IgA and IgG immunoglobulins in respiratory secretions following challenge, which was correlated to reduced MG colonisation of the trachea, highlighting the likely significance of local immunity in protection from MG (Avakian & Ley, 1993).

The objective of this project is to discover and evaluate alternatives to serology which correlate with protective immunity, ideally using a simple sampling technique such as a swab from trachea or choanal cleft. Two approaches will be examined. The first is to use polymerase chain reaction (PCR) to quantify vaccinal organisms in the respiratory tract at critical times after vaccination. This will be correlated with ability to resist subsequent challenge. The aim will be to develop a test which establishes an acceptable threshold of vaccinal replication that will confidently predict a successful vaccine 'take'. The second approach is more direct and will attempt to detect and measure a key component of the mucosal immune response that correlates with immunity, for example immunoglobulins and/or cytokines present in respiratory secretions or tears.

There is no information about the kinetics of growth of ts-11 in the chicken although one study failed to detect MG organisms in tracheal washings by PCR at 3 weeks after vaccination of chickens with ts-11 (Gaunson *et al.*, 2000). Despite this, the vaccinated chickens were able to resist aerosol challenge with virulent MG and at 2 weeks after challenge had significantly less tracheal lesions and MG in tracheal washings than unvaccinated controls challenged in the same way. This suggests that the replication of ts-11 necessary to stimulate a protective immune response occurs in the first 2 weeks after vaccination although it is also possible that the organism may be replicating in sites other than the trachea. It would therefore seem appropriate to focus studies on PCR detection of organisms between 1 and 2 weeks after vaccination and mucosal immune responses between 3 and 4 weeks after vaccination.

Objectives

To develop alternatives to serology for determining an effective immune response to *Mycoplasma gallisepticum* ts-11 vaccine.

Methodology

Quantification *of M. gallisepticum* ts-11

qPCR detection of *M. gallisepticum* in the respiratory tract by PCR and correlation with protection.

Optimisation of the PCR will initially be done with known numbers of ts-11 and other MG strains grown *in vitro* using 16S and ts-11-specific primer sets. The optimised tests will then be evaluated *in vivo* using swabs collected from the choanal cleft and upper trachea of chickens vaccinated 2 weeks previously with ts-11.

A time course study to measure replication of ts-11 will be done on samples collected by swabbing of the choanal clefts and tracheas at 1, 2 and 3 weeks after vaccination and processed for both viable counts and mycoplasmal genomic DNA titres. At 4 weeks after vaccination washings collected from nasal sinuses and tracheas will be processed for viable counts, mycoplasmal genomic DNA titres and measurement of immunoglobulins and cytokines (part 2). Remaining birds will be challenged by aerosol with virulent MG and 2 weeks later will be euthanised and samples collected and tested as described above. In addition, post mortem examinations will be performed including histological examinations of tracheal mucosae. Results will be statistically analysed including correlations between mycoplasmal DNA genomic titres post vaccination and resistance to challenge. The experiment will be repeated using commercial broiler breeders.

Quantification of immunoglobulins and cytokines in respiratory tract and to correlation with protection.

Part 2 will use the same *in vivo* experiments as described in Part 1. Both total and MG-specific IgM, IgG and IgA immunoglobulin titres will be determined by ELISA. Initial establishment and standardisation of the ELISA technique will be undertaken to ensure accurate replication of results, and quantification of antibody concentration. The immunoglobulin response, as determined by ELISA, in nasal sinuses, tracheal washings and tears (if possible) will be determined and aligned with protection (from part 1).

Those cytokines that are identified as being pivotal in the generation of an immune response to MG, as determined by real time PCR in the strategy above, will be investigated in samples collected from immunised birds. Assuming a local immune response is central to protection it is expected that Th2 type cytokines as IL-6 and TGF-beta, will be assessed. However, cytokines more typical of a cell mediated Th-1 type response, as IFN-gamma and IL-2 may also be measured. The correlation between cytokine levels in respiratory samples and tears will be investigated, possibly providing an *in-vivo* sample site for identification of a protective immune response following immunisation with ts-11.

Part 3. Evaluate test procedures in field vaccinated birds

The most promising procedures developed in Parts 1 and 2 will be further evaluated in field vaccinated birds, including challenge studies.

Results

Development of a quantitative TaqMan PCR assay

A quantitative TaqMan PCR was developed using the translational elongation factor gene (Ef-tu, *tuf*) as the target, the forward and reverse primer sequences were CACGTTGACTGTCCCGGTC and ACCACCGTCCATTTGAGCA respectively and the FAM labeled probe sequence was CGCCGACTACGTTAAAAACATGATTACAGGTG. The PCR was conducted using a Stratagene MX3000p thermocycler with Platinum Quantitative PCR Supermix UDG (Invitrogen, California USA) with the conditions of 50°C for 2 min, 95°C for 5 min followed by 40 cycles of 94°C for 30s, 60°C for 60s and 72°C for 30s and analysis performed using the Stratagene supplied software.

Standard curves were constructed using 10-fold dilution series of *M. gallisepticum* ts-11 vaccine strain DNA starting 10^7 diluted to 10^3 genome copies. The reactions were performed in triplicate and a standard curve generated and number of genome copies for each sample interpolated from the standard curve generated (example standard curve shown in Fig. 1). Initial studies compared recovery of Mycoplasma DNA by qPCR from swabs collected in phosphate buffered saline (PBS) and extracted using either the HighPure DNA extraction kit (Roche, GMBH) or QIAX bead method (Sykes *et al.*, 2001). Due to the observed efficiency and reliability of the HighPure DNA extraction method (Table 1) we subsequently employed this method for extraction of all further samples.

Table 1. MG ts-11 3 weeks post VAXSAFE vaccinated, comparison of extraction methods

Bird ID*	Roche	QIAX	Site sampled
982	2.84E+05	9.78E+04	choanal cleft
	1.80E+04	2.13E+02	occular
	2.17E+03	1.13E+03	tracheal
996	1.07E+04	2.30E+02	choanal cleft
	1.91E+04	No Ct	occular
	1.45E+05	2.79E+02	tracheal

*Bird selected based on rapid serum agglutination score: Bird 982 and 996 with an RSA score of 3 and 1 respectively

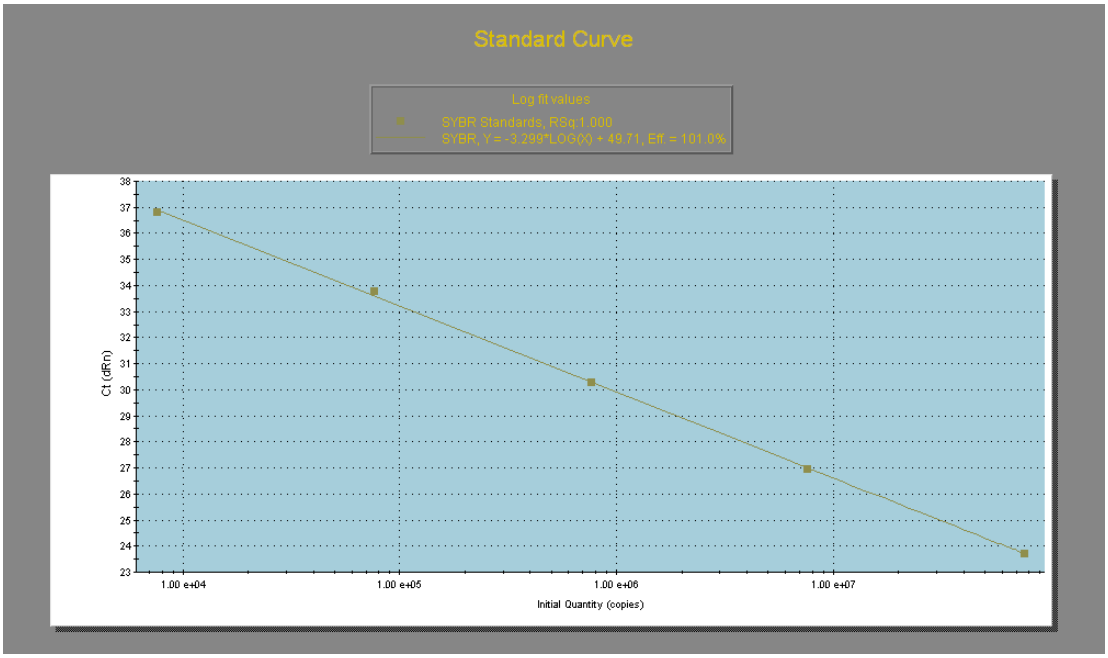


Figure 1. Detection of MG ts-11 genome determined by quantitative PCR amplification of the *tuf* gene. A $\log_{10}$ dilution series of MG ts-11 genome copies was made and the PCR cycle (cycle threshold value) at which it was reproducibly detectable is shown. The efficiency of the PCR was typically around 100% with a standard curve R^2 value of 1.000.

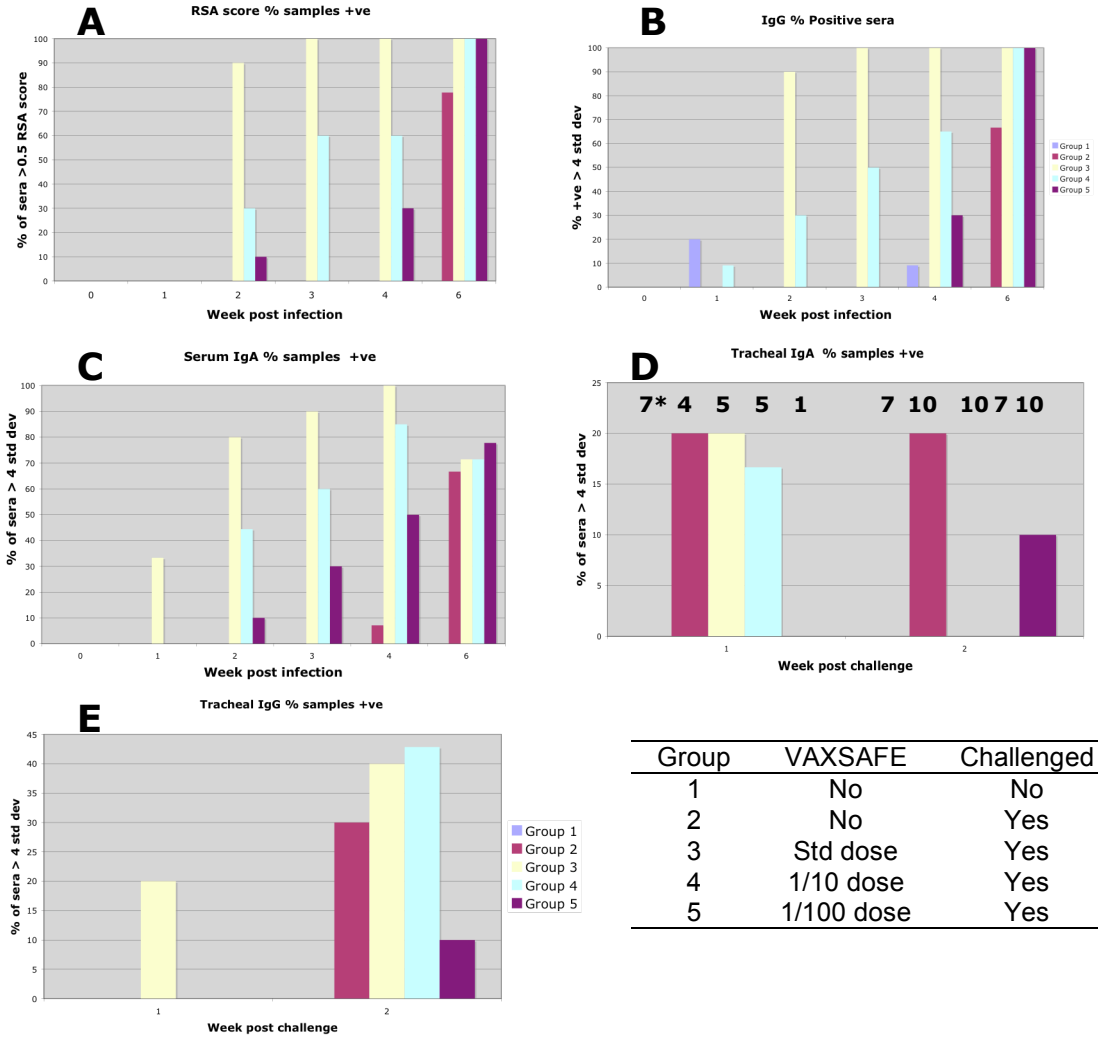
Experimental ts-11 vaccination of chickens: dose reduction experiment

An animal experiment was conducted following the protocol outlined in Appendix 1. Serum samples were taken from birds and tested at the University of Sydney in ELISA for antibody production to the monoclonal antibody purified VlhA antigen of *M. gallisepticum* ts-11. Serum and tracheal washings were tested in ELISA for IgG and IgA production. Serum was also tested by the Rapid Serum Agglutination (RSA) test, a standard serology test typically employed in the farm situation to determine immuno-reactivity to *M. gallisepticum*. Cytokine production of serum samples was processed at the Animal Health Laboratories, Geelong.

Tracheal swab samples were taken for qPCR analysis and processed using the Roche DNA purification kit.

ELISA results

The graphs depicted in Figure 2 show the production of antibody assessed by RSA and ELISA over the period of the experiment. The unvaccinated and non-challenged sera or tracheal washing optical density was converted to a percentage of a standard serum and used to calculate the mean and standard deviation for each ELISA type. Samples greater that the mean plus 4 standard deviations of each ELISA were considered positive. It can be seen that by day 14 in 1x VAXSAFE vaccinated birds up to 90% would be considered reactors with an RSA score of 0.5 or greater with only 2 birds having an RSA score of 0.5. This follows the results of the IgG antibody response with all birds considered positive by the third week post vaccination. The birds vaccinated with either a tenth or one-hundredth dose of VAXSAFE showed a steady rise in the percentage of positive birds in either the RSA test or IgG ELISA. Several sera from the unvaccinated group gave non-specific reaction in the IgG ELISA and a concern in regard to specificity. Comparison of the percentage of birds positive in the IgA ELISA with either the RSA test or IgG ELISA showed the IgA ELISA was overall less sensitive and transient in nature, with IgA levels peaking in Group 3 VAXSAFE vaccinated birds in week four post vaccination. Interestingly, 33% of birds from Group 3 were considered positive one week post-vaccination in the IgA ELISA as compared to none in the RSA test or 10% by IgG ELISA. The tracheal washings ELISA examining the IgG and IgA levels. The IgA response was seen in a small percentage of birds one-week post challenge and declined by the second week whilst the IgG response was seen in 20% of birds in Group 3 one-week post challenge increasing to 40% by the second week post challenge and also observed in other groups.



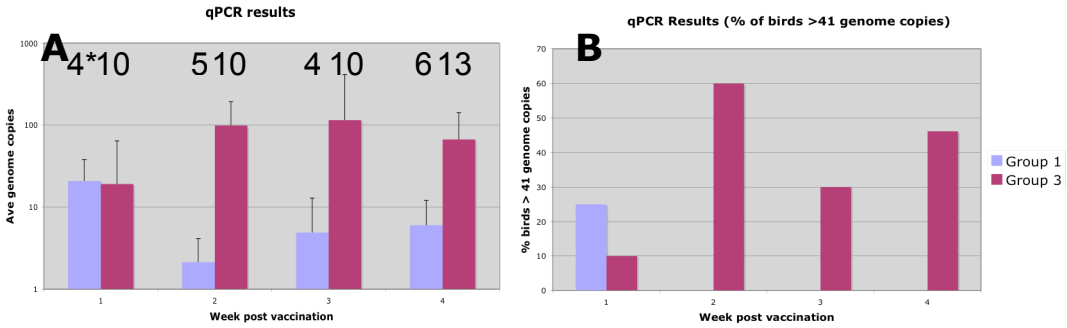
Group	VAXSAFE	Challenged
1	No	No
2	No	Yes
3	Std dose	Yes
4	1/10 dose	Yes
5	1/100 dose	Yes

*Number of birds sampled

Figure 2. ELISA results for experimental groups. (A) The percentage of birds with an RSA score greater than 0.5 for each group in the accompanying table. (B) The percentage of birds in each group with an IgG level greater than the average plus 4 std dev is shown at weekly time intervals. (C) The percentage of birds in each group with a serum IgA level greater than the average plus 4 std dev. is shown at weekly time intervals. (D) The percentage of birds in each group with a mucosal IgA level greater than the average plus 4 std dev. is shown at one and two weeks after challenge. (E) The percentage of birds in each group with a mucosal IgG level greater than the average plus 4 std dev. is shown at one and two weeks after challenge.

qPCR Detection of *M. gallisepticum* genome

Quantitative PCR was used to detect the number of VAXSAFE genome copies from swabs taken of the trachea of birds. The graphs depicted in Figure 3 show the qPCR results from tracheal swabs taken over the first 4 weeks of vaccinated and unvaccinated birds. The average genome copies in Figure 3A show an increase in genomic copies by the second and third week of vaccination and decreasing in the fourth week though the standard deviation is relatively high for each time point. This is somewhat mirrored in the graph Figure 3 B where the percentage of birds with a genomic copy value greater than the ave +3 std dev increases by week 2 and is lower in weeks 3 and 4.



*Number of birds sampled

Figure 3. Results from tracheal qPCR analysis. Panel A shows the average number of genome copies with one standard deviation from unvaccinated (Group 1) and VAXSAFE vaccinated (Group 3) at each week following vaccination. Panel B shows the percentage of birds with a genome copy number greater than the ave plus 3 std dev at each week following vaccination.

Detection of Cytokine Response in Chickens

Tracheal washings of birds from the experiment were taken and transported to CSIRO Animal Health Laboratories in Geelong and tested for the presence of specific cytokines. The level and/or type of cytokines present were unable to be detected using the assays developed and this line of enquiry was not pursued further.

Development of High Resolution Melt Curve Test to Detect *M. gallisepticum* ts-11

HRM Methodology

Due to the lack of consistency with recovery of *M. gallisepticum* from samples and subsequent qPCR an alternative method was developed using the newly developed High Resolution Melt technology. A pair of oligonucleotide primers were designed ts-11-F (5'-GTTTGGAGTTGGTGTATAGTTAG -3') and ts-11-R (5'-TCTTCTTCGAAAACAAAAGG -3'), based on a region preceding the trinucleotide repeat of a member of the *vlhA* gene family and amplicons of 145-352 bp were generated from cultures of 10 different *M. gallisepticum* strains including the ts-11, F and 6/85 vaccine strains (Ghorashi *et al.*, 2009). Amplification of DNA was performed in 25 µl reaction volume on an I-Cycler thermal cycler (Bio-Rad). The reaction mixture contained 3 µl extracted genomic DNA, 25 µM of each primer, 1.5 mM $MgCL_2$, 1250 µM of each dNTP, 5 µM SYTO® 9 green fluorescent nucleic acid stain (Invitrogen), 1× GoTaq® Green Flexi Reaction Buffer (Promega) and 1 U of Go Taq DNA polymerase (Promega). PCR conditions were one cycle of 94 ºC for 60 s, 35 cycles of 94 ºC for 10 s, 50 ºC for 10 s and 72 ºC for 10 s, and a final cycle of 72 ºC for one min. In each set of reactions, MG ts-11 genomic DNA and distilled H_2O were included as positive and negative controls, respectively. The PCR products together with standard molecular weight marker, were analysed by electrophoresis in 1.7% agarose gels stained with GelRed™ (Biotium, Hayward, CA, USA), and visualised by UV transillumination. HRM curve analysis was performed in a Rotor-Gene™ 6000 thermal cycler (Corbett Life Science Pty Ltd). In order to determine the optimal melting condition for differentiation of MG strains, the PCR products were subjected to three different ramping rates of 0.1°C, 0.2°C and 0.3°C/s between 72°C and 82°C. All specimens were tested in triplicate and their melting profiles analysed using Rotor Gene 1.7.27 software and the HRM algorithm provided.

Differentiation of *M. gallisepticum* strains by HRM

High resolution melting (HRM) curve analysis of the resultant amplicons could differentiate all *M. gallisepticum* strains (Ghorashi *et al.*, 2009). Analysis of the nucleotide sequence of the amplicons from each strain revealed that each melting curve profile related to a unique DNA

sequence. The HRM curve profiles (for ts-11) remained consistent after at least five passages under laboratory condition. PCR HRM curve analysis of 33 DNA extracts derived from respiratory swabs, or mycoplasma cultures grown from respiratory swabs, of ts-11 vaccinated commercial or specific pathogen free chickens identified all these specimens, according to their sequences, as ts-11.

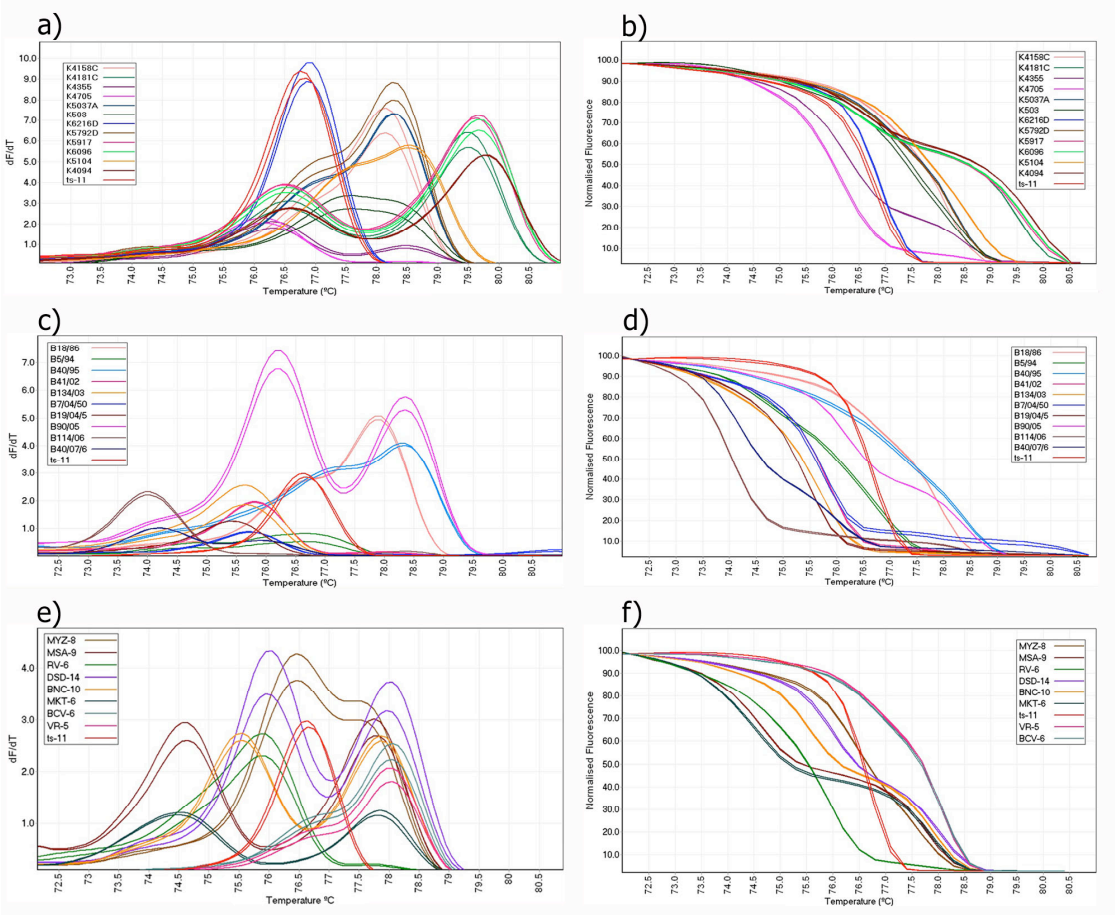


Figure. 4 (a, c and e) Conventional melting-curve and (b, d and f) normalised HRM curve analysis of PCR products of the *vlhA* gene promoter region from ts-11 and *M. gallisepticum* isolates from USA (a and b), Europe (c and d) and Israel (e and f).

Visual examination of the conventional and normalised melt curves of the *M. gallisepticum* isolates from USA (Fig. 4a) revealed that *M. gallisepticum* isolate K6216D had a single peak at 76.8 °C highly similar to the ts-11 and was genotyped as ts-11 with 95 GCP. The nucleotide sequence of the amplicon (226 bp) was also identical to that of ts-11. *M. gallisepticum* isolates K5917 and K6096 had two similar conventional melt curve peaks at 76.5 and 79.7 °C and similar normalisation curves at GCP of 99. The sequence of the PCR amplicons (323 bp) was also identical. The *M. gallisepticum* isolates K5037A and K5792D also generated similar normalised and conventional melt curves with one peak at 78.3 °C and a shoulder peak at a lower temperature. These two isolates produced a major DNA band (319 bp) with identical nucleotide sequences. The *M. gallisepticum* isolate K4181C and K4094 generated slightly different pattern to those of K5917 and K6096 with two peaks at 76.6 and 79.5°C, and 76.6 and 79.8°C respectively. The nucleotide sequence analysis of these two isolates revealed 99.7% identity to each other and 93.7-100% identity to K5917/K6096. The rest of *M. gallisepticum* isolates from USA (K4158C, K4355, K4705, K503 and K5104) each had curve shapes that were distinct to the other USA and Australian *M. gallisepticum* strains/isolates used in this study.

Visual examination of conventional and normalised melt curves of *M. gallisepticum* isolates from Europe revealed that MG isolate B40/95 and F strain generated amplicons with similar melt curve with one peak at 78.4 °C and a shoulder peak at lower temperature. Nucleotide sequences of amplicons revealed 99.7% sequence identity (one nucleotide substitution in 319 bp). This similarity was also reflected in similar normalised melt curves with GCP of 88. All other MG isolates from Europe did not have similarity to each other (Fig. 4b) or to any other reference strains/isolates characterised (Ghorashi *et al.*, 2009).
Examination of the Israeli *M. gallisepticum* isolates revealed that the isolate MSA-9 and MKT-6 generated highly similar conventional melt curves with two peaks at 74.6 and 77.8°C (Fig. 4e). The normalized melt curves for the amplicons of these isolates also were highly similar and had GCP of 92 (Fig. 4f). The nucleotide sequence of the respective amplicons (355 bp) was found to be identical. Similarly the *M. gallisepticum* isolates BCV-6 and VR-5 generated amplicons with similar conventional melt curves with one peak at 78.0°C and a shoulder peak at a lower temperature. Nucleotide sequences (319 bp) of amplicons were identical to each other and to the MG strain K1453 (examined earlier in this study). This similarity was also reflected in similar normalised melt curves with GCP of 90. A full review of this work can be found as Appendix 2.

Discussion of Results

Antibody Response and qPCR Results

An ELISA was developed using as an antigen the VlhA peptide. The VlhA peptide was affinity purified using monoclonal antibody from the vaccine strain *M. gallisepticum* ts-11 strain. The ELISA assay detected both chicken IgG and IgA from sera and tracheal washings. Chicken sera reactivity in the RSA test and the IgG and IgA ELISA were compared at weekly intervals of the *in vivo* experiment. The IgG ELISA showed it was sensitive and relatively specific in detecting serum IgG response to vaccination as it was in detecting serum IgA levels. The RSA test was at least as sensitive as the IgG serum ELISA with both tests detecting 100% of neat vaccinated birds by week 2. The IgA serum ELISA showed a delayed response of one week compared to the IgG serum ELISA or RSA test. Tracheal washings showed variable IgG and IgA ELISA responses with a lower number of positive reactors compared to serum ELISA results. A qPCR assay was developed that used a complementary probe (Taqman assay) format, the qPCR was shown to be sensitive for the detection of *M. gallisepticum in vitro*. Attempts to detect *M. gallisepticum* ts-11 vaccine genome from swabs of several anatomical sites showed inconsistent results. When birds were tested at weekly intervals in the experiment not all birds produced a qPCR signal with only 60% of birds positive by qPCR two weeks after vaccination. The effect of site sampling and processing of swabs and extraction procedures need to be further assessed.

Cytokine Detection

No specific cytokines were detected in samples sent for analysis; as such this line of investigation was not pursued further.

HRM curve analysis

Following the success of high resolution melt curve analysis in tests developed at APCAH Laboratories in Werribee, a HRM test was developed for detection of the *M. gallisepticum* vaccine. The test was shown to be reliable and robust and able to successfully detect the vaccine from *M. gallisepticum* vaccine culture, reisolates from the field and tracheal swabs.

Implications

The poor antibody response typical of field birds vaccinated with the *M. gallisepticum* ts-11 vaccine was not evident in SPF birds vaccinated with undiluted vaccine in the ELISA and RSA tests in this study. However, birds vaccinated with dilutions of the vaccine showed decreased antibody response as is typical of a dose dependant vaccine. The ELISA results suggest there is no inherent improvement in detection compared to the RSA test though testing of birds from the field may show differences not readily seen under laboratory conditions. The increasing use of the ts-11 vaccine and of its introduction in other countries

will create an opportunity for the ts-11 ELISA test to be included in central laboratory testing, it may also complement other established ELISA set-ups and reporting formats.
Manufacturers of MG ELISA test should be approached with the intent of including the antigen as a standard for MG serological survey and vaccine "take".
The results from qPCR show that it is an imperative to be consistent in the sampling from chickens for determining genome copies of the vaccine. This may be better suited to reporting a "yes/no" result for vaccine take as has been recently reported (Collett *et al.*, 2005). What appears to be a more user friendly and is becoming more widely accepted is the use of HRM curve analysis to determine the presence of particular strains or organism variants. The HRM curve analysis could be instituted with a positive/negative result and problems of inconsistent sample amount overcome by pooling of swabs. The test is relatively fast to complete and could be supported by isolation of the organism in the first instance, and once established and supported by the industry, a stand alone diagnostic.

Recommendations

To support the use of HRM for differential diagnosis of vaccine take and/or presence of wildtype *M. gallisepticum.* The test would be conducted at APCAH laboratory facility at the University of Melbourne Werribee where they have the machinery and expertise to perform the test, the facility is also supported by the Australian Poultry CRC.

Introduction

Production of Genetically Distinguishable *M. gallisepticum* vaccine

In this project we will investigate better methods for detecting and assessing vaccine take. While some flocks do not produce detectable immune responses to the ts-11 vaccine, many do. As this immune response is indistinguishable from a low level response to infection with virulent *M. gallisepticum* it is not possible to determine whether virulent *M. gallisepticum* has been eradicated from flocks. It has been established that ts-11 can, if used for extended periods, displace more virulent strains from flocks.

It would be advantageous to poultry producers if use of the current mycoplasma vaccines, ts-11 and MS-H, could be distinguished from infection of stock with the wild-type pathogen, as this would then enable the vaccine to be incorporated into eradication campaigns. This would not only assist poultry producers, but would also enhance the market of the vaccine in countries where eradication or control programmes are already in place.

Work in our laboratory has also established a protocol for successful disruption of a gene in the chromosome of *M. gallisepticum* by homologous recombination (Markham *et al.*, 2003). The target gene for interruption by homologous recombination was that encoding p47, the basis of a commercially available serological assay, which is currently in use internationally for detection of *M. gallisepticum* infection. We have thus been able to establish that the gene for p47 is dispensable *in vitro*. We have further shown that the strain carrying this disruption can colonise tracheal organ cultures as effectively as the parental strain.
Current work is introducing this disruption into the ts-11 vaccine strain. Once this has been achieved it will be necessary to assess whether p47- ts-11 is as effective as a vaccine as the original. This work will form the basis of this project. The p47 gene appears to be present in most mycoplasmas, so should the deletion of this gene from *M. gallisepticum* result in a promising vaccine candidate.

Objectives

Assessment of the immunogenicity of ts-11 strain of *M. gallisepticum* lacking p47 gene
Assessment of safety of ts-11 strain of *M. gallisepticum* lacking p47 gene
Assessment of ability to discriminate vaccinated and wild type infected chickens when using $p47^-$ ts 11 as a vaccine

Methodology

Genetic manipulation *M. gallisepticum* vaccine

A plasmid carrying the *M. gallisepticum* origin of replication and the tetracycline resistance gene were used to construct the plasmid (pOriC) and used in transformation studies in *M. gallisepticum* strain S6. To produce a *M. gallisepticum* vaccine that does not possess an antibiotic resistance marker the OriC plasmid was made that contained the *M. gallisepticum* p47 gene interrupted with the poly-Histidine tag (pOric/p47/HIS). This construct was then used to generate a tagged *M. gallisepticum* vaccine strain through homologous recombination with the genomic located p47 gene. Recombinant clones could then be detected using a monoclonal antibody specific for the poly-histidine epitope.

This approach was unsuccessful and studies were undertaken to verify transcription of the poly-HIS tag and further methods developed to enrich for recombinant clones.

A further two methods for detection were devised. The first method used the same construct to transform MG ts-11 vaccine strain and select tetracycline resistant clones, plate several of the antibiotic resistant clones, allow colonies allowed to form and use a labelled oligonucleotide specific for the tag sequence to probe the colonies in Southern blot.

The second method relied on the expression of a monoclonal antibody epitope from the *M. gallisepticum* VlhA gene sequence that is absent in the vaccine strain. This research group had previously produced a monoclonal antibody mMAb66 to the VlhA peptide, which can then be used to detect or "immuno-pan" for *M. gallisepticum* vaccine recombinants expressing the epitope.

Results

Production of an oriC vector

This work resulted in the development of the first oriC plasmid for *M. gallisepticum* and subsequent manuscript being published in the journal Microbiology (Lee *et al.*, 2008).

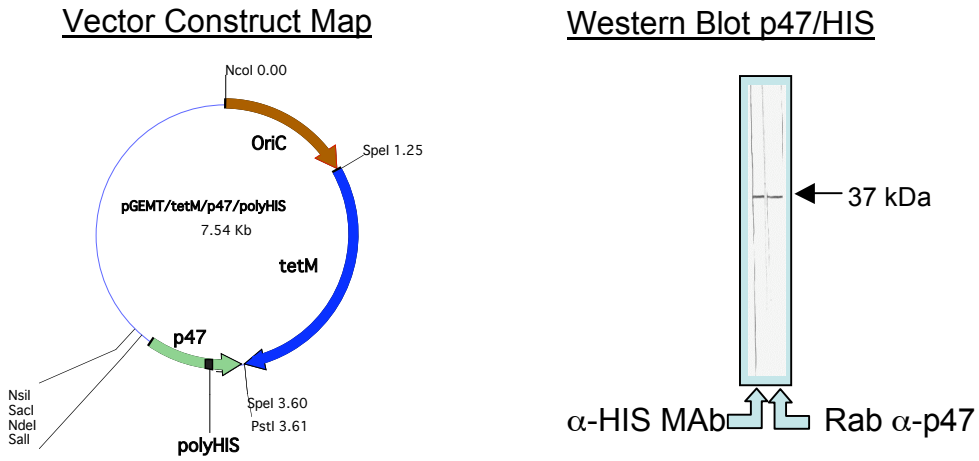


Figure 5. Vector map of oriC/p47/HIS construct and reactivity of *E. coli* expressed gene construct HIS peptide and p47 using a mouse monoclonal antibody to poly-HIS or rabbit anti-p47 specific antibody.

Homologous Recombination Trials

A trial experiment using the *M. gallisepticum* S6 strain transformed with the pOriC/p47/HIS was successful and the strain shown to contain the construct. It was expected that the interrupted gene would express the poly-HIS epitope and be detected by commercially purchased anti-HIS monoclonal antibody. The construct's epitope was readily detected by the anti-HIS MAb in the *E. coli* host (Fig. 5) prior to introduction into *M. gallisepticum.*

Following several attempts the epitope was unable to be detected in the *M. gallisepticum* host. To ascertain whether the tagged gene was transcribed a quantitative PCR was developed to detect native *p47* and tagged *p47* messenger RNA. Total RNA was extracted from an MG clone containing the construct and the level of each mRNA species determined. It was found that tagged mRNA was transcribed at the same or at slightly higher levels than the native p47 gene. This suggests that factors other than mRNA transcription are responsible for the failure of P47 expression, one reason may be the appending of P47 with 6x histidine residues, which is basic in nature, does not allow for efficient translation or transport through the cell membrane.

Table 2. qPCR analysis of p47 mRNA: copy number P47-polyHIS (oriC plasmid) and genomic p47

	Copy Number from standard curve*	
	P47/HIS region	P47 genomic region
MG S6 oriC/p47/HIC	1.8E+07	2.1E+07

* Copies/5µl

We selected 50 clones and using PCR determined the presence of the tetracycline resistance gene and the P47/polyHIS construct. We then chose one clone and passaged it in media without tetracycline. A PCR reaction was developed that used one oligonucleotide primer to the HIS DNA sequence and another to a region of the p47 gene located in the genome, this PCR configuration would only produce an amplicon if homologous recombination had occurred. An amplicon of the correct size was present in culture grown without tetracycline and detectable up to 1/100 dilution (Fig. 6).

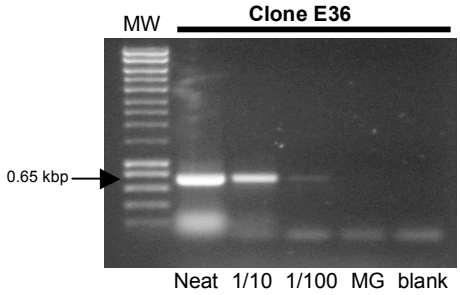


Figure 6. Homologous recombination of P47/HIS construct with *M. gallisepticum* genome as determined by specific PCR. Dilutions of *M. gallisepticum* clone E36 were subjected to PCR together with controls including *M. gallisepticum* genomic DNA and water as template. The amplicons were separated in a 1% agarose gel and stained with ethidium bromide. MW: molecular weight standards

As homologous recombination occurred in cultures grown in the absence of tetracycline a preliminary screening exercise was conducted to identify homologous transformants using qPCR.
A total of 1342 clones were selected, grown and subjected to qPCR. The initial PCR was conducted to detect the tetracycline gene and if negative was then screened by qPCR for the presence of the HIS sequence. Of the 1342 clones screened 84 were negative for the tetracycline PCR and when tested for the presence of the HIS PCR were also negative suggesting recombination frequency was at least <1E-03/clone. This screening process was discontinued due to the low frequency of homologous recombinants and the ability to screen larger numbers of clones using agar plates.

Since the expressed poly-HIS tag was to be used to "immuno-pan" by anti-HIS antibody to select for *M. gallisepticum* expressing the HIS tag a further two methods for detection were devised.

The first method was to use the same construct to transform MG ts-11 vaccine strain and select tetracycline resistant clones, plate several of the antibiotic resistant clones on non-selective media, allow colonies to form and use a labelled oligonucleotide specific for the tag sequence to probe the colonies in Southern blot. Two Southern blot methods were assessed to detect the HIS DNA tag. One method labelled a PCR product using the Roche PCR DIG detection system. The DIG labelled PCR product contained the p47-HIS gene region from the vector construct. Initial studies were conducted of Southern blotted endonuclease restriction digests of pOric/p47/HIS DNA with the DIG-labelled probe and the method optimised using RFLP Southern blots. The method was not successfully transferred to screening lifts as background staining occurred with *M. gallisepticum* genomic DNA, most likely due to the small amount of p47 gene present in the DIG-PCR probe. As an alternative an oligonucleotide complimentary to the HIS tag was 3' labelled with poly-thymine-digoxigenin or 5' labelled with α-^{32}P ATP and used to probe endonuclease restriction digests of pOric/p47/HIS DNA. The sensitivity was similar with both methods, the Roche DIG system was chosen for further study as it was non-radioactive. Conditions were optimised according to the manufacturer's instructions and controls included of transformed or untransformed ts-11 colonies grown with or without tetracycline respectively. Initial problems with non-specific binding of the probe were overcome by addition of poly-adenine to the hybridisation reaction though no recombinant p47-HIS *M. gallisepticum* colonies were detected using this method.

Expression of Monoclonal-antibody Epitopes

The second method relies on the expression of an epitope recognised by a monoclonal antibody from the *M. gallisepticum* VlhA gene sequence, the epitope is absent in the VlhA profile expressed by the vaccine strain. It could be expected that *M. gallisepticum* vaccine strain would express this homologous DNA sequence that is present and expressed in high levels in *M. gallisepticum* strain S6. The location of the MAb66 epitope was narrowed down to a 500 bp region of the VlhA1.1 gene following cloning, subcloning and expression of the different VlhA gene regions. This region was subsequently amplified by PCR and cloned into the pOriC/p47 construct and tested for expression in *E. coli* before being introduced into the vaccine strain (Fig. 7). The monoclonal antibody epitope was recognised in *E. coli* expressed *M. gallisepticum* clones and can then be selected by immuno-panning and/or Western blotting of colony lifts.

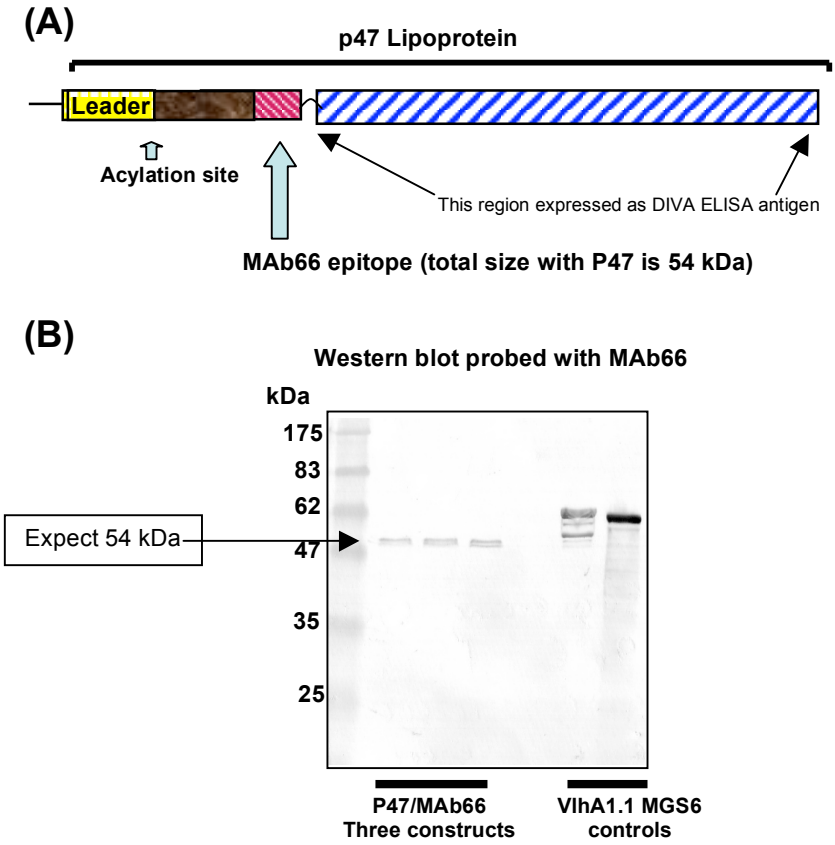


Figure 7. Schematic diagram of p47 construct containing the MAb66 and Western blot of construct expressed in *E. coli*.
(A) Schematic diagram of p47/MAb66 construct showing the P47 peptide interrupted by the MAb66 epitope, the construct contains the necessary leader sequence and acylation signal sequences for expression on the cell surface. The region to be used an antigen in a DIVA ELISA is shown.
(B) Western blot of *M. gallisepticum* strain S6 whole cells and *E. coli* strains carrying the oriC/P47/Mab66 construct probed with MAb66. Controls are: VlhA1.1 peptide affinity purified from *M. gallisepticum* strain S6, MGS6 whole cells.

The *M. gallisepticum* vaccine strain was transformed with the oriC/p47/MAb66 construct and a number of tetracycline resistant transformants selected. The recombinants were passaged in non-selective media and plated onto agar. Colony lifts were conducted a number of times but was unsuccessful as no MAb66 expressing clones were detected.
A promoterless construct containing the p47 gene and phosphatase enzyme (phoA) was also produced and attempts made to introduce this construct into the genome failed.

Discussion of Results

Gene Constructs Produced

Several gene constructs were prepared, each of these were used in homologous recombination experiments to knockout the p47 gene and create a marked serologically distinguishable vaccine. In order to achieve this aim we initially had to produce a vector containing the origin of replication of *M. gallisepticum*, this was successfully produced and is the first oriC vector developed for *M. gallisepticum.* This oriC vector construct was fused with the tetracycline resistance gene and then several different versions of the p47 gene were added. A version of the p47 genes construct contained a polyHIS epitope at the carboxyl end of the gene and another containing the MAb66 epitope. Attempts to identify recombinants by immuno-staining colony lifts with specific monoclonal antibodies to the HIS or MAb66 epitope were

unsuccessful. Interestingly recombinants could be detected by PCR in cultures grown without antibiotics and attempts were made to identify one of these clones, as such over 1346 clones were screened by qPCR without success. Screening for recombinants by Southern blotting of *M. gallisepticum* colonies was attempted but a number of technical issues with probe specificity and background staining inhibited our ability to identify recombinant clones,
It is most likely the frequency of recombination was extremely low and that without having a selective marker for enrichment and elimination of untransformed cells we were unable to recover transformants.

Implications

The ability to produce recombinants without selective markers such as antibiotics that inhibit cell growth or cause cell death is a major impediment to this approach. The inclusion of an antibiotic resistance gene in the vaccine would be unacceptable to the regulatory authorities and an alternative method should be sought to either remove the selective marker or produce a more efficient method for producing recombinants.

Recommendations

As the major draw back to the above approach is the removal of the antibiotic selective marker then alternative methods could be used to specifically remove the marker. An oriC plasmid carrying the FLP system from Yeast has been used in *E. coli* to remove genes flanked by specific inverted DNA repeats. This could be used to excise the antibiotic resistance marker once a p47 knockout is produced. Another alternative is to produce an oriC vector containing the Xer1 gene from *M. agalactiae,* Xer1 is responsible for recombination in the Vpma system and could possibly be used to remove the antibiotic selective marker when introduced into a transformed *M. gallisepticum* clone.

Introduction

Development of *M. gallisepticum* ts-11 strain as a Vaccine Vector

The key aim of this strategy is to capitalise on the characteristics of *M. gallisepticum* ts-11 to use it to develop and commercialise vaccines that over enhanced, single dose protection against respiratory pathogens such as Infectious Bronchitis Virus. A number of factors limit effective vaccination in poultry including the cost of repeated handling for administration of vaccines, effective delivery of vaccine antigens to the mucosal immune system, and the requirement for repeated vaccination to achieve adequate life-long protection.
One approach to addressing these problems is to use a live vector to deliver antigens to the chicken. The optimal vector is an attenuated vaccine strain of a bacterium or virus which is normally administered to poultry, as these will have been assessed for their safety, the birds are generally known to have not been previously exposed to wild-type strains (otherwise the vaccine is unlikely to be effective), and the attenuated strain has generally been chosen for its efficacy in inducing a protective mucosal immune response. The requirements a live vaccine vector can be expected to need to be efficacious are a capacity to deliver multiple antigens, a capacity to persist for sufficient time on the mucosal surface to induce a protective immune response against the antigen, and a capacity to induce both humoral and cell mediated immunity.
It has been established that the ts-11 vaccine strain can successfully control the respiratory disease caused by virulent *M. gallisepticum*, and, if used for extended periods, can displace more virulent strains from flocks. Furthermore it colonises the respiratory tract of birds for extensive periods, resulting in life-long immunity after a single administration, even though levels of circulating antibody remain relatively low. These characteristics suggest that it may be an appropriate vector for administration of other vaccine antigens to the respiratory mucosa of poultry. Furthermore studies in our laboratory have established that the immune response to *M. gallisepticum* recruits large numbers of CD8+TCRab T cells to the respiratory

mucosa, suggesting that mycoplasma vectors may have the potential to induce the cytotoxic immunity necessary for enhanced antiviral immunity. Furthermore studies by other workers have demonstrated that mycoplasma lipoproteins, which have a unique structure among bacterial lipoproteins, have a potent capacity to activate macrophages through toll-like receptor 2, and hence enhance immune responses.

Previous work in the Department of Veterinary Science at the University of Melbourne has resulted in the development of a series of constructs for expression of foreign genes in *M. gallisepticum*. These constructs, upon introduction into *M. gallisepticum*, express foreign proteins cytoplasmically, on the cell surface, and as a lipoprotein. Further work has placed a series of different promoters in front of these constructs, and current work is identifying the best constructs for expression. Preliminary work has developed constructs that should express portions of either the S1 or N proteins of infectious bronchitis virus, but thus far, while expression of foreign bacterial proteins has been achieved, expression of eukaryotic and viral genes has not.
Current work is examining the expression of a protective antigen of *Avibacterium paragallinarum*, the cause of infectious coryza, in the ts-11 strain.

Objectives

The aim of this project was to examine the safety and efficacy of a ts-11 vaccine expressing HagA of *Avibacterium paragallinarum*. HagA was to be expressed cytoplasmically, on the cell surface or as a lipoprotein in different ts-11 transformants.
A series of bird experiments were planned for *M. gallisepticum* ts-11 strains expressing HagA but these experiments were postponed and as there was doubt as to the involvement of HagA in haemagglutination and difficulty in obtaining a clone that expressed the peptide. In place of this approach the chicken interferon gene was expressed in the *M. gallisepticum ts-11* vaccine strain and assessed as an immune adjuvant.

Methodology

Expression of HagA

The HagA gene was codon optimised for expression in *M. gallisepticum* and commercially synthesised to contain appropriate endonuclease restriction sites, a schematic of the gene construct is shown in Figure 8.

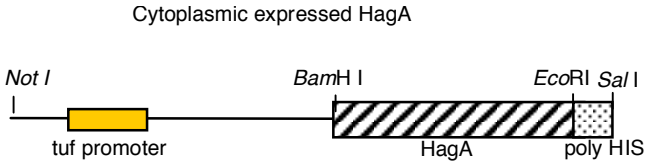


Figure 8. Schematic representation of cytoplasmically expressed HagA.

The HagA construct will be cloned in the oriC vector and introduced into *M. gallisepticum* by electroporation and expression of HagA determined by specific ant-HagA antibody and anti-polyHIS antibody.

Expression of chIFN-γ

The chicken interferon gamma (chIFN-γ) gene tagged with the FLAG epitope was cloned into the pISM2062.2 plasmid containing the Tn4001 transposon. The chIFN-γ gene was placed under the control of the tuf promoter and was preceded by phoA and a *M. gallisepticum* cleavage motif. The methodology to produce this construct can be found in Appendix 3

(Muneta *et al.*, 2008). A bird experiment was conducted to assess the immunogenicity of chickens vaccinated with ts-11 expressing gamma interferon.

Results

Cloning and Expression of HagA

The HagA gene was codon optimised for expression in *M. gallisepticum* and cloned into the oriC vector carrying the tetracycline resistance gene. The oriC/hagA/poyHIS construct was introduced into *M. gallisepticum* and recombinants tested for HagA expression in Western blot. No immunoreactive band was seen following probing of recombinants in Western blot with either anti-HagA or anti-polyHIS reagents. The HagA/polyHIS construct was then cloned into the pGEX expression vector (pGEX4T-1) and recombinants selected. An immunoreactive band of the predicted size was observed when probed with chicken anti-HagA and anti-polyHIS antibodies. Further investigations into the reasons why HagA was not expressed in *M. gallisepticum* were not pursued.

Cloning and Expression of chIFN-γ

The transposon Tn4001 based vector pISM2062.2 was used for construction of chIFN-γ expression vector. The primers used for the construction of the plasmid are shown in Table.1 (Appendix 3). The *M. gallisepticum* tuf promotor, signal sequence, and mouse alkaline phosphatase gene (phoA) fused with cleavage signal (QASETQ) for *M. gallisepticum* VlhA1.1 was amplified by PCR and cloned into pGEM-t vector (Promega) by TA cloning and transformed *E. coli* DH5α pGEMt-tufp-sig-phoA-QASETQ). The mature part of chIFN-γ cDNA fused with FLAG sequence to the 3' end of it was amplified from a plasmid containing chicken IFN-γ cDNA as a template (kindly provided from Dr. Andrew Bean) and cloned into pGEMt-tufp-sig-phoA-QASETQ using *Spe* I and *Nsi* I site, and transformed DH5α (pGEMt-tufp-sig-phoA-QASETQ-IFNγ-FLAG). The entire sequence containing tuf promotor, signal sequence, phoA, secretion signal, chIFN-γ, and FLAG was amplified and cloned into the *Bam*HI site of pISM 2062.2 and transformed *E. coli* XL10-Gold (Stratagene).

Transformation and the Characterisation of *M. gallisepticum* clone expressing chIFN-γ

The resulting plasmid (pISM-tufp-sig-phoA-QASETQ-IFNγ-FLAG) was used for the transformation of *M. gallisepticum*. The DNA sequence of the construct was confirmed by cycle sequencing using the ABI PRISM Big Dye Terminator reagent (Applied Biosystems) following manufacturer's instruction. Colonies of putative gentamycin-resistant transformants were selected randomly from agar plates and inoculated into 1 ml fresh *M. gallisepticum* broth containing gentamycin. After colour change, the supernatants were collected and screened for chIFN-γ secretion using chicken IFN-γ sandwich ELISA. A positive clone for chIFN-γ secretion (ts-11 C3) was further sub-cultured three times and stored. The cell pellets of ts-11 C3 were used as template for PCR to determine the presence of gene construct. The level of chIFN-γ was assessed by ELISA using chIFN-γ recombinant protein as a control (Fig. 8). The alkaline phosphatase expression and activity of transformants was also determined using specific anti-PhoA monoclonal antibody (Sigma) and the substrate BCIP/NBT (Sigma), respectively.

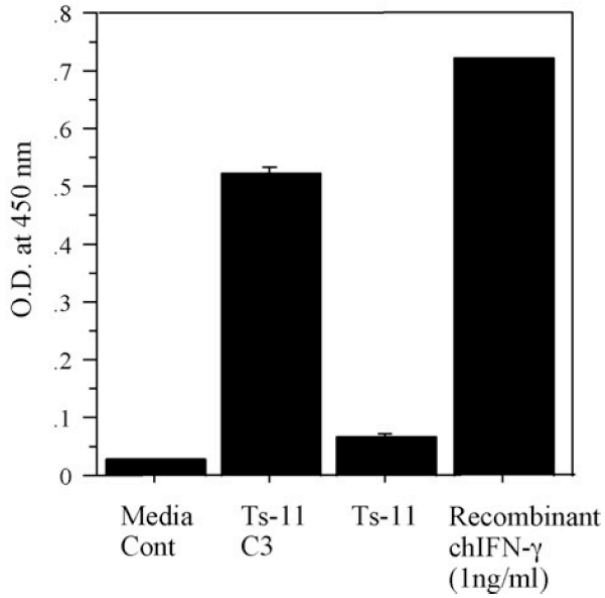


Figure 8. IFN-γ secretion into the supernatants of cultures of *M. gallisepticum* ts-11 transformants.
The supernatants (diluted twofold) of cultures of 27 gentamicin-resistant *M. gallisepticum* transformants were screened in duplicate using the chIFN-γ ELISA. A positive clone (ts-11C3) was obtained. Recombinant chIFN-γ and MG broth only were used as a positive and a negative control, respectively. ELISA ODs at 450nm are shown

Experimental Infection of Chickens with *M. gallisepticum* Ts-11 C3

To assess the immunogenicity of ts-11 C3 in chickens, 30 two-week-old white leghorn specific-pathogen-free chickens (SPAFAS Pty Ltd, Woodend, Victoria, Australia) were divided into three treatment groups (Groups 1-3). Group 1, which was immunized by MB broth only, Group 2, which was immunized by ts-11 (5.0×10^7 CCU/bird), and Group 3, which was immunized by ts-11 C3 (7.5×10^7 CCU/bird). All immunisation was done by eye drop method.
Blood was collected from each bird every week and fresh serum was examined for systemic antibody response against MG using the rapid serum agglutination test (RSA) shown in Table 3.

Table 3. RSA score in chickens vaccinated with ts-11 and ts-11C3

Group	Number of chickens	Mean RSA score (range)		
		0 week	1 week	2 week*
1	10	0 (0,0)	0 (0,0)	0 (0,0)[b]
2	10	0 (0,0)	0 (0,0)	1.4 (1,2)[a]
3	10	0 (0,0)	0 (0,0)	0 (0,0)[b]

Values with different superscript letters are significantly different ($P<0.01$).
*RSA score of 5 birds from each group.

Serum IFN-γ concentration was estimated by chicken IFN-γ ELISA, daily weight gain was calculated using the following formula. (The weight of postmortem day – The weight of first day of experiment)/duration of the experiment and re-isolation of MG from choanal swab samples was done by culture and confirmed by PCR (all shown in Table 4).

Table 4. Serum IFN-γ, daily weight gain and re-isolation of *M. gallisepticum*

Groups	Serum IFN-g	Daily weight gain	Re-isolation of MG		
			Colony on agar	Color change broth	Broth PCR +ve
1	0/10	13.25±1.139	0/10	0/10	0
2	0/10	12.72±0.769	10/10 (1-<100)	10/10	9
3	0/10	12.51±1.021	1/10 (1)[a]	0/10	0/10

Values in parenthesis indicate the range in the number of *M. gallisepticum* colonies. Group 1: media; Group 2: ts-11; Group 3: ts-11C3. [a] This colony was unable to be sub-cultured. Results from birds 1 and 2 weeks after vaccination.

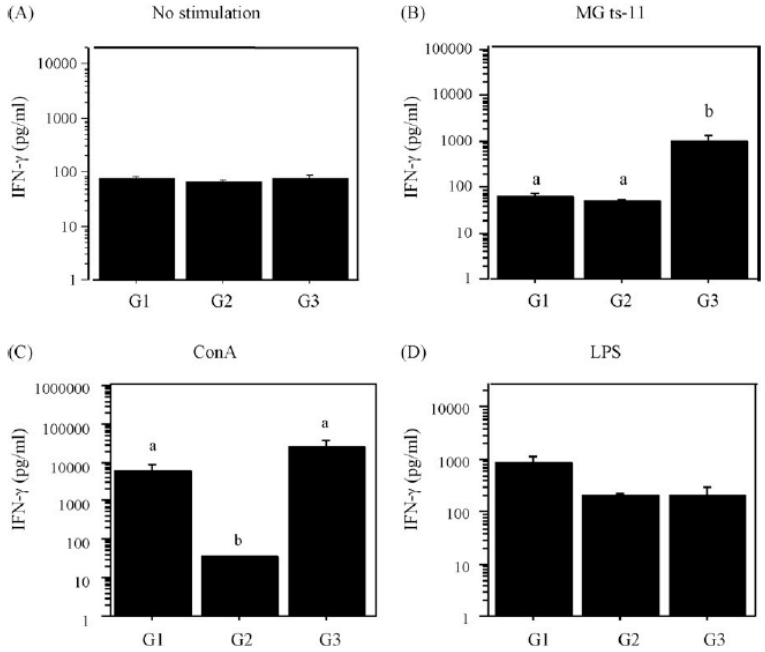


Figure 9. IFN-γ production by stimulated spleen cell cultures. Fifteen chickens (5 from each group) were killed at 2weeks after immunisation, spleens were collected and the spleen cells were isolated. A total of 5 x 10^7 cells were left unstimulated (A) or stimulated with either MG ts-11 heat-killed antigen (B), 10 mg/ml ConA (C), and 10 mg/ml LPS (D) and cultured at 39°C. After 5 days the IFN-γ concentration in the supernatant was measured by chIFN-γ ELISA. a vs b Columns labelled with different lower case letter are significantly different (P<0.05).

Spleens were collected from each bird 2 weeks after immunisation. Spleen was mashed using sterile metal mesh and filtered through sterile gauze. The cell suspension was washed two times by minimum essential medium. 5x10^7 cell/ml of cells were cultured in DMEM containing 10 % FCS and stimulated with either 10 μg/ml Concanavalin A (Sigma), 10 μg/ml Lipopolysaccaride (LPS) from *E. coli* O55B5 (Sigma) or heat-killed ts-11 whole antigen (cell: ts-11 = 1:1). Then, cells were incubated in 5% CO_2 at 39°C for 5 days and IFN-γ concentration in the supernatant was measured using chicken IFN-γ ELISA (Fig. 9).

Upper and lower tracheal samples were fixed in 10% neutral buffered formalin, embedded in paraffin and 2 μm sections were prepared and stained with haematoxylin and eosin. The mean mucosal thickness of the trachea of each bird was determined by measuring the thickness at six random points in sections from anterior and posterior ends of the trachea.

The number of heterophils infiltrating the trachea epithelium was estimated by microscopy using x1000 magnification. The number of heterophils in six randomly selected tracheal epithelium were counted and the mean numbers were calculated.

Table 5. Tracheal mucosal thickness and the number of heterophils in the tracheal epithelium

Gp	Tracheal mucosal thickness (μm±S.E.)		No. of heterophils/ high powered field (mean±S.E.)	
	Upper trachea	Lower trachea	Upper trachea	Lower trachea
1	50.1±3.6	33.3±1.8	0.60±0.22[a]	0.25±0.15[a]
2	53.1±3.2	32.3±1.8	0.40±0.14[a]	0.32±0.13[a]
3	52.5±1.8	33.4±2.3	8.77±2.18[b]	6.00±1.32[b]

The data from birds killed at 1 and 2 weeks after vaccination were pooled for comparison. Values with a different superscript are significantly different (P<0.05)

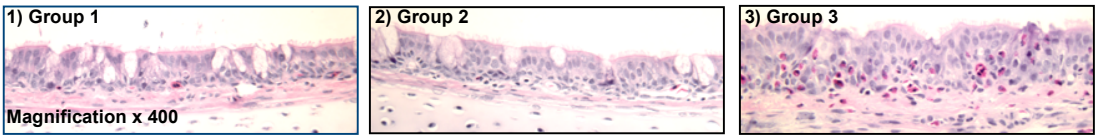


Figure 10. Heterophil infiltration into the tracheal Hematoxylin and eosin stained sections of trachea from immunized chickens were prepared and examined as described above. (1) Group 1; (2) Group 2; (3) Group 3. Significant heterophil infiltration was observed in the tracheal epithelium of birds in Group 3 (400 x magnification).

Discussion of Results

Expression of HagA

The HagA protein of *Avibacterium paragallinarum* was codon optimised and cloned into an oriC vector for expression in the *M. gallisepticum* strain ts-11. The vector was introduced into *M. gallisepticum* cells but HagA expression was not detected, though the protein was immunoreactive in Western blot when expressed in the pGEX system. As there was some doubt as to the importance of HagA involvement in cell attachment another line of investigation was undertaken in foreign gene expression in *M. gallisepticum*.

Expression of chIFN-γ in *M. gallisepticum* ts-11

In this study, a strain of MG ts-11 expressing chIFN-γ (ts-11 C3) was generated using a transposon expression vector. This is the first report of successful expression of a cytokine in any mycoplasma species. The ts-11 C3 strain secreted chIFN-γ into the culture supernatant, but still retained its temperature-sensitive phenotype. PCR analysis showed that the 20 bp insertion in the *gapA* gene found in ts-11 was also preserved in ts-11 C3, indicating that this clone possessed similar genotypic and phenotypic properties to the ts-11 vaccine. ChIFN-γ expression in ts-11 C3 was unable to be detected by Western blotting (data not shown), and was only detected in the supernatants of ts-11 C3 cultures by ELISA (Fig. 8) and at a concentration of approximately 1 ng/ml. This level of expression is considered very low for a foreign gene, but is enough to modulate the immune response *in vivo*, as most cytokines work at ng and/or pg levels.

Indeed, when ts-11 C3 was inoculated into chickens by eye drop, the host immune response was altered compared with immunisation with the parent ts-11 strain. However, no IFN-γ was detected in the serum and no daily body weight change was observed in ts-11 C3 vaccinated chickens (Table 4), suggesting there were no adverse side effects of expression of IFN-γ at this level by ts-11 C3 under these conditions.

Interestingly, ts-11 C3 immunised chickens did not produce detectable RSA antibodies, even at 2 weeks after vaccination (Table 3), although all ts-11 immunised chickens had a moderate RSA score by this time. Strain ts-11 usually induces sero-conversion detectable by the RSA test at 2 weeks after immunisation. Moreover, ts-11 C3 immunization of chickens induced significantly higher MG ts-11 specific IFN-γ responses in spleen cells (Fig. 9B). The suppression of T cell responses has been reported in other mycoplasmas (Cole & Wells, 1990; Teh *et al.*, 1988), and may be related to the effects of co-infection with MG on disease caused by viral respiratory pathogens (Roussan *et al.*, 2008). Indeed, IFN-γ production after ConA stimulation, a non-specific T cell mitogen, was suppressed in ts-11 immunized birds 2 weeks after immunisation. However, the amount of IFN-γ produced by spleen cells from ts-11 C3 immunized chickens after ConA stimulation was similar to that seen in unvaccinated chickens (Fig. 9C). These results, when considered together with those seen in spleen cells stimulated with ts-11, indicate that IFN-γ production by ts-11 C3 stimulates a host cellular immune response that overcomes the immunosuppressive effects of ts-11. Chickens immunised with ts-11 C3 also had significant heterophil infiltration of the tracheal epithelium (Fig. 10 and Table 5). Heterophils are the avian equivalent of mammalian neutrophils. IFN-γ stimulates IL-8 production by human bronchial epithelial cells (HBECs) and enhances ICAM-1 expression on HBECs (Striz *et al.*, 2000), which is critical for neutrophil migration and adherence. IFN- γ also enhances neutrophil transmigration across the human airway epithelium through its effect on IL-8 production (Kidney & Proud, 2000). Previous studies with recombinant chIFN-γ have shown that it can activate and enhance phagocytosis of *Salmonella enteritidis* by heterophils as well as cytokine gene expression in heterophils (Kogut *et al.*, 2001; Kogut *et al.*, 2005). These results suggest that IFN-γ production by ts-11 C3 stimulates the mucosal cellular immune response and recruits heterophils into the tracheal epithelium. The increased concentration of heterophils may account for the difficulty experienced in isolating ts-11 C3 from the mucosa after immunisation even though ts-11 was readily re-isolated from the mucosa of all birds after immunization (Table 4) as ts-11 C3 cells might be expected to be phagocytosed and killed by the heterophils. In conclusion, a strain of MG ts-11 expressing chIFN-γ was successfully created and was examined for its immunogenicity in chickens.

Implications

The strain ts-11 C3 induced greater cellular immunity and less humoral immunity compared to the parent ts-11 strain, and was also able to induce greater mucosal heterophil infiltration. These results indicate that IFN-γ produced by ts-11 C3 can alter the host's immune response and Th1/Th2 balance, and can also induce greater mucosal cellular immune responses. These results also suggest that ts-11 C3 is a promising vaccine vector, capable of delivering protective antigens from other chicken respiratory pathogens such as avian influenza virus as well as generating non-specific protection against mucosal bacterial pathogens of chickens such as *E. coli* and *S. enteritidis*. Since these studies were undertaken we have established the ts-11 vaccine is composed of two temperature sensitive strains, one that is predominant and contains a non-functional attachment peptide (GapA) and another that is less abundant and contains a functional GapA peptide. This last strain is most likely the protective vaccine component as it is able to attach and persist and is the strain recovered from vaccinated birds. The ts-11 C3 belonged to the first class of strains and its ability to persist was most likely hampered by the lack of a functional GapA peptide and exacerbated by the cellular immune response induced.

Recommendations

To explore the use of *M. gallisepticum* ts-11 GapA +ve strain as a carrier for foreign genes. It was shown that expression of antigens such as cytokines even at very low levels produce an immune effect in the chicken and partnered with other foreign antigens could be used to produce multivalent vaccines against bacterial and/or viral antigens.

Introduction

Detection of Virulence Genes in *M. gallisepticum*

Little is known of how genes of an organism are controlled on a genome level during infection. The technology to achieve this has only recently become available and relies upon knowledge of the DNA sequence of the genome. The completion of the genome sequence of *M. gallisepticum* provides an opportunity to investigate gene regulation in mycoplasma vaccines and wild type organisms (Papazisi *et al.*, 2003). Although current vaccines are able to control mycoplasmosis there is not yet a complete understanding of why the live vaccine is protective. Knowledge of the features responsible for protection using DNA microarray technology will enable improvement in efficacy and functionality of vaccines and will be able to be applied to development of vaccines against other pathogens.

Microarray Analysis

Nucleic acid hybridisation is the basis for DNA microarray technology. The technique allows the simultaneous detection of tens to hundreds of thousands of unique hybrids in one experiment. Utilising this technology, we are able to detect differentially expressed genes under varying conditions of growth *in vitro* or *in vivo*. With the completion of the genome sequence of *Mycoplasma gallisepticum,* it is now possible to determine what genes are important to the organism in establishing infection within the host.

Signature Tagged Mutagenesis

Several approaches can be undertaken in determining what genes are involved in pathogenesis and survival within the host. One method is Signature Tagged Mutagenesis (STM). STM is able to establish which genes are involved in the initial stages of infection of the host and persistence within the host. The method relies on the random insertion of a signature tagged transposon into one of the 742 putative coding sequences of *M. gallisepticum*. This procedure is suited to finding those genes that are necessary for infection such as proteins involved in attachment of the organism to the host, but fails to address gene families or regulatory proteins involved in pathogenesis. Previous animal experiments screened (under an Egg Industry Research and Development Corporation grant UM54A) a total of 39 ST mutants containing 34 different signature tags, and found that 2 ST mutants were not recoverable in the post-selection pools. Several other mutants were detected infrequently, suggesting that the genes carrying the tagged transposon in these mutants might also be important *in vivo*. The main aim of the study described was to examine the pathogenicity of individual ST mutants by infecting groups of birds with single mutants and then identifying a mutant that may provide protection from challenge with wild-type strain.

In contrast, microarray technology employs a holistic approach, whereby every gene is interrogated as to its expression level in response to conditions that mimic the host or are found within the host. This allows identification of genes involved in pathogenesis and survival within the host. It is expected that results from the STM studies may complement or support our microarray-based approach in identifying genes involved in pathogenicity.

Objectives

To identify novel genes for attenuation of *Mycoplasma gallisepticum*
To improve the efficacy and functionality of mycoplasma based vaccines
To develop techniques for application to other avian pathogens

Methodology

Two different approaches were used to attain our objectives, the first will investigate the use of microarray to analyse gene expression and whilst the other will investigate several attenuated mutants produced through Sequence Tagged Mutagenesis.

Microarray Analysis of *vlhA* genes

For microarray analysis the *M. gallisepticum* R strain was cultured until late log growth phase. Mycoplasma cells were pelleted by centrifugation at 16,000 xg in a bench-top microfuge for 5 min at room temperature and resuspended in RNase free water. Total RNA was extracted using the RNeasy Kit (Qiagen) following the manufacturer's instructions. The amount of RNA was quantitated using a BioPhotometer spectrophotometer (Eppendorf) and RNA integrity assessed by agarose gel electrophoresis. For agarose gel electrophoresis all solutions were produced using 0.1% diethyl pyrocarbonate treated water. The RNA gel contained 0.35 g of agarose, 1 μl of 10 mg/ml ethidium bromide, 20 mM of guanidinium isothiocyanate in 35 ml of 0.5× TBE (1 x TBE is 0.89M TRIS-borate 0.89M boric acid, l mM EDTA). RNA samples were mixed 10:1 with dye loading buffer (0.4% w/v bromophenol blue, 50 mM EDTA, 50% glycerol, 2% w/v SDS) and loaded into agarose gels and electrophoresed for 3 hours at 4 V/cm. The gel was examined using an ultraviolet transilluminator.

The relative transcription levels of several mycoplasma genes were determined using quantitative PCR (qPCR). Quantitative PCR assays were developed for *trkG*, *uvrD, vlhA4.01, vlhA2.01*, *vlhA5.04* and *tufB.* The oligonucleotide primers for each gene assay (Table 6) were designed using Primer3 software (http://frodo.wi.mit.edu/). The qPCR assays were developed using genomic DNA of MG R strain as template and detection of amplicon by SYBR green (Invitrogen). The optimal ratio of oligonucleotides for each assay was ascertained and assay efficiency determined using 10 fold dilutions of a DNA template.

For reverse transcriptase (RT) qPCR, total RNA was extracted from *M. gallisepticum* R strain using RNeasy Kit (Qiagen) as described above. To remove contaminating genomic DNA an aliquot containing 2 μg of total RNA was mixed with 2 μl 10 x DNase I reaction buffer, 2 μl DNase I (Invitrogen) and RNase free water added to total 20 μl. The mixture was incubated at room temperature for 15 min after which 2 μl of 25 mM EDTA (Invitrogen) was added and heated to 65°C for exactly 10 min and placed on ice. Two identical RT reactions were prepared as follows in 1.5 ml microfuge tubes (one tube did not contain the RT enzyme to control for DNA contamination in subsequent qPCR assays), each tube contained: 1 ug of DNase treated RNA, 2 μl (100 ng) of random hexamers (Invitrogen), 1 μl of 10 mM each of dNTPs (BioRad) and heated to 65° C for 5 min and then placed on ice after which 4 μl first strand reaction buffer (Invitrogen), 2 μl 0.1 M dithiothreitol (Invitrogen) were added and incubated at 25° C for 2 min. One tube received 1 μl SuperScript II (Invitrogen) whilst the no-RT control tube 1 μl of water was added, both tubes were incubated at 25° C for 10 min followed by 42° C for a further 50 min with a final RT inactivation step of 70° C for 15 min. The reactions were then diluted 1:30 in sterile water for use as template in qPCR.

The qPCR assays for *trkG*, *uvrD, vlhA4.01, vlhA2.01*, *vlhA5.04, vlhA3.03* and *tufB* genes were similarly prepared, with 10 μl of 2 x Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen), 0.02 μl ROX reference dye (Invitrogen), 0.12 μl of 25 mM each primer, 5 μl DNA template and water added to 20 μl. Each assay was tested in triplicate and included no RT DNA control as template in duplicate. The reactions were run using a Stratagene MX3000P (Stratagene) real-time thermocycler using the following conditions: 55° C for 2 min, 95° C for 5 min followed by 40 cycles of 95° C for 30 sec, 60° C for 60 sec.

Table 6. Oligonucleotides used in qPCR

Oligonucleotide Name	Oligonucleotide Sequence 5'-3'
JVvlhA_3.03For	GTTACAACTGATCCTACTAATAAAAAGACG
JWvlhA_3.03Rev	TAAATTCTAAATATCCAAAATTTGGAGCAC
LKtrkGFor	GCCCTTGAATTTTTGGAAGATTG
LLtrkGRev	GATTACGTGCTTAACCTGTGGT
LMUvrDFor	TGCTTACACTAGAGCCAAACACA
LNUvrDRev	CAATCCCTTCACCATAAAGTTGG
LOvlhA_4.01For	CAATTAGTGGTTCTGCTCCTACTCC
LPvlhA_4.01Rev	GGCATCGTGTTTTTGCTCTG
LQvlhA_2.01For	CGGGACATTTGCTAAGACTGG
LRvlhA_2.01Rev	ATTCAGCTTGTGCTGCTGGT
LSvlhA_5.04For	CGGTTGAAGGGGTTGTACTTG
LTvlhA_5.04Rev	TGGTTGAGGTGCCATGTTTG

A total of 45 oligonucleotide probes were designed for use in the DNA microarray to detect transcriptional level of each member of the *vlhA* multigene family (Table 9). A further seven oligonucleotide probes were used as controls, these were designed to non-*vlhA* genes with high and low expression levels as predicted from transcription patterns of *M. pneumoniae* Oligonucleotides were around 60 nucleotides in length with a with a 5' 10 mer polyT spacer that included a 5' amine group. In instances where a single oligonucleotide could not distinguish between *vlhA* genes a second oligonucleotide primer was designed. To aid in the design of the oligonulceotide probes the software programs OligoWiz (http://www.cbs.dtu.dk /services/OligoWiz2/) and OligoPicker (http://pga.mgh.harvard.edu/oligopicker/) were used. The software programs *in silico* (http://insilico.ehu.es/) and FASTA were used to assess cross hybridisation of the probes with the template.
Oligonucleotides were resuspended in water and printed in a 50% DMSO solution with a DNA concentration range of 100-250 ng/µl on Corning CMT-GAPS aminosilane slides (Corning). The DNA was UV cross-linked at 70 mj and baked at 80ºC for 2-4 hours.

Table 7. Oligonucleotides used for DNA microarray

Gene	Oligonucleotide sequence	Cross Binding
vlhA 1.01pic	GTTTGAACATGCTGAGGCTGCTAGAAATAATGGTTCTATCGATTTAGTAG	
vlhA 1.02wiz	CATTAGTACCAGTTGAAGGTATGAGCCCCACTAAGGATGAAGTAACT	
vlhA 1.03wiz	CTTATGAGAACAGATGTAGAAAAGGTTAATACTGAATTGATGGGTGCTG	
vlhA 1.04wiz	AAAATCTCTTTTAAGAGCAAATTTCTCTTCAGAAAACAGCCAAACACAACC	
vlhA 1.05wiz	AAGAATTGCTAGTTTGCTAGGTCTAGGTTCAATTATGAGTTTAGGGGTG	
vlhA 1.06wiz	AAACTAAAACAAACGCTGCAGCTAAAGTTACAGAACTTGAATCTCTTG	
vlhA 1.07pic	TACAAATATCACGAATGGCACTACTGGAAACACTGGTATGCAAACAGCTA	
vlhA 1.08pic	GTAAAACAATTGTCTACAGGTAATTATTCTACTATTACATTAAGAACCTT	*vlhA* 4.01
vlhA 1.08wiz	ATTAGTGGTGAGATTCCAACATCTAAGATGATTACAGATGGAACTGCTAATAT	*vlhA* 5.08
vlhA 2.01wiz	TTAAGTGTTGGCTCGATTTTAGGCTTAATACTGTCTAGTTGTACATCACTG	
vlhA 2.02wiz	CAAGTTGGTATGTATAACGAATACAAACAGATTCAAGCCGGACTTAA	
vlhA 3.01wiz	GGTGTAACTGGAATGCAAAGTGGACAAACTACTATTCCCAACTGGAATTT	
vlhA 3.02pic	TGGTTTTAGTAATGATTTAGATCATAAAATTGCAGGTAATGCAGGAAATC	
vlhA 3.03wiz	GTAAAACAATTGTTTACAGGTAATTTAACTACTACATTAAGAACCTTTGA	
vlhA 3.04wiz	TTAAAGAAAATTTAGTCACATTGTATAACGCCGGCAAAATGCTTACT	
vlhA 3.05wiz	TGGTCCTGGTAGAAGACAGCTTGTTAACAACAGTAATAGAATTCAGAGT	
vlhA 3.06wiz	AAACAAACATTGAATCCAGTGAATGGTAATCTTCCAGTTGTAGCT	*vlhA* 4.08
vlhA 3.07wiz	CAGTTAGTGGAATTGTTCCTGCTGCTAATACAATCACAGAAGAGATT	
vlhA 3.08pic	AAAGAGTAGTTTGAAAACCACAAGAAGGTCGGTCAAGTACCTATGTGCCA	
vlhA 3.09wiz	CAAATCCTCCTAGTGGTGGTAACATGAATGGTGGAGATACTAATC	*vlhA* 3.04
vlhA 4.01wiz	ACTGTAAGCCAAAAATCAGCTACTGTAAGTTCATTAGGATTAAGTAATCCTCAA	
vlhA 4.02wiz	AGCTTCTCTAACATCACTTCAAGATCTAGTTTCTAAAGGCCTTGCAAT	
vlhA 4.03pic	ATTGTTTGAAAACCAAAAATGGGTAGTGGCAGTACTTATGAGCCATTAGA	
vlhA 4.04wiz	GATTTATTAGCAACACAAAGCAGTAACCTTGCAAAATATGCAGATTATACA	
vlhA 4.05wiz	ACTAAACAAATTAACTTTGTACAAACTCCAGCTTCTGGTCCAAGTGA	*vlhA* 5.09
vlhA 4.06wiz	CAGCTGACAATATGAATAATTCTGTTACGTTTACAGTCCAAGGAAAGAAT	
vlhA 4.07pic	TACTGGAATAACAGAAAAAACAAACGAGCAAAGATCTCCAGTAGTTAGTA	*vlhA* 1.08, 5.08
vlhA 4.08wiz	AAACAAACATTGAATCCAGTGAATGGTAATCTTCCAGTTGTAGCT	*vlhA* 3.06
vlhA 4.09wiz	ATTTGATACAAGTGATACGACTAACAATGTTGTATCAGGCAATATGGC	
vlhA 4.10wiz	ATTTGATACAAGTGATACGACTAACAATGTTGTATCAGGCAATATGGC	
vlhA 4.11pic	TTTACTTTAAGTGCTACTCCACAAACTGCTGCTAACCCTAGTTCTACTAG	
vlhA 4.12pic	TTAAATGATGAAATGGCAAAAGCAGTTGATTTTAAGACATCATCTTCAGC	

Name	Sequences	Cross Binding
vlhA 5.01wiz	CAATCATCTCTAGACACTTAAGAAATAGTAGTGGTACAACTCCCCAAGACC	
vlhA 5.02pic	TGCAACTGGTCCAACGAATATTCAAAATTCTCCTAATGTAGATAATGATA	
vlhA 5.03wiz	ACTACTGGTGCTCAAGCTGCTATGCAAACTGCTAATAAAACCATT	
vlhA 5.04wiz	CTAATAGAACCTTAGAAACGGTTGAAGGGGTTGTACTTGATACTCAAGC	
vlhA 5.05pic	TCAACATTTATAGCTGAATATAGTGGCACAGGTCTAACATTAGATAATCA	
vlhA 5.06wiz	AAAACTGGTGCAACTCTAAATGAGGTTAATGAGGCAAAAACTACATT	
vlhA 5.07wiz	TTAAAGCTAGAACAGATTTCGATGCAGTTAATGGTCCATTAGTGG	
vlhA 5.08wiz	GCTAATATCGAAAAAGTAATAAATAGCGAAACTCTTATGTCGCAAAAAGAGAAC	*vlhA* 1.08
vlhA 5.09pic	TGGTATGAATGGTGGAAATACCCCAACACCTAATCCAAATCCTCATAGTG	*vlhA* 3.03, 4.10, 5.06/11
vlhA 5.09wiz	TTAGTGTTGATGTTAATGGTCCGCAAAATAATAATCGCCCTAATT	*vlhA* 4.05
vlhA 5.10wiz	AAGCAGAAGAGGTAACTAGCGCAAATAATGGCATTAAAAATGCTA	
vlhA 5.11wiz	ACTAAAACGCAAATACTAGATTCTTTAACGGCAGAACAATTTAGCTCAATTAGT	
vlhA 5.12wiz	AATTAACATCTGGTAGTAGTGAAACTAGTATGCAAACACAACCTCAACCAG	
eno	TCTAAGTACAGGTCAGATCAAGACTGGTTCAATGTCACGATCTGAAAGAA	
lon	ACACCCAATATGGAGGTGATCTTTTACCAATCGAAGTAAGTA	
trkG	AAGATTTAGTATCATGGACGTGTTCTTTGAATACTCTTCAGGTT	
tufB	AAAGAAGGAACTGAAATGGTTATGCCTGGTGATAACACAGAAATTA	
dnaK	AATACAATTAACGAAATCCTTGGTTCTAAAGAAGCAGAAGC	
uvrD	AGCCAAACACAATCTTTGATTATGTTCAAACCAAGATTATG	
cysS	TTTTGCCAACGCAACTAAAGTTATCTGAGACTTAATCAAAGTT	

The target cDNA used to interrogate the microarray was produced using the SuperScript Indirect cDNA Labeling System (Invitrogen) following the manufacturer's instructions. Briefly, 5 µg of total RNA from *M. gallisepticum* R strain and 5 µg of random hexamers (Invitrogen) were combined, RNAse free water added to give a final volume of 15.5 µl and the mixture heated to 70˚C for 10 min. The mixture was then cooled on ice and first strand cDNA synthesis conducted following the manufacturer's instructions with the synthesis buffer containing aminoacyl-dUTP, dTTP, dATP, dCTP and dGTP at a ratio of 2:3:5:5:5 respectively together with 150 U of Superscript II. Synthesis was carried out at 42˚C for 2 h after which the RNA template was removed by basic hydrolysis. The cDNA was labeled whilst bound to a Qiaquick PCR purification kit (Qiagen) column. The cDNA remained bound to the column by following the manufacturer's instructions except the elution step was not completed. The Cy5 fluorescent dye (GE Healthcare) was reconstituted in 0.1 M sodium carbonate pH 9.0 and added to the column. Coupling of the dye to aa-dUTP proceeded in the dark at room temperature for 1 h after which the mixture was eluted from the column with water. Uncoupled Cy5 fluorescent dye (GE Healthcare) was removed using the Qiaquick PCR purification kit (Qiagen) and the eluted labeled template dried under vacuum and reconstituted to an appropriate volume with water for addition to the microarray slide. Microarray slides were prehybridised in a prewarmed solution containing 10 mg/ml BSA, 25% formamide, 5× SSC (20 SSC is 3.0 M Sodium Chloride, 0.3 M Sodium Citrate, pH 7.0), 0.1% SDS in a Coplin jar for 45 min at 42˚C, then rinsed twice in distilled water and dried using compressed air. The vacuum dried Cy5 dye labeled cDNA was resuspended in 10 µl of water and 12 µl of 2× hybridization buffer (20% formamide, 5× SSC, 0.2% SDS) and 2 µl of Salmon sperm DNA (10 mg/ml) added. The labelled cDNA mixture was heated for 2 min at

100˚C and aliquots carefully placed between a Lifter Slip (Grale Scientific) and the microarray features on the slide. The slide was hybridised overnight at 42˚C in a hybridisation chamber and washed in 1× SSC, 0.2% SDS with agitation for 5 min at room temperature. The slide was further washed in 0.1× SSC, 0.2% SDS for 5 min with shaking and then washed twice more in 0.1× SSC for 2 min each after which the slide was dried using compressed air and scanned using a GenePix 4000B (Molecular Devices Corporation).

Experimental Infection of Chickens using Signature Tagged mutants

Two ST mutant experiments were conducted, one experiment investigated the infectivity of 6 different ST mutants while the final experiment investigated the efficacy of one particular ST mutant STM26.

Two ST mutants, 04-1 and 33-1, which had not been detected after the initial or confirmatory screening experiments, and five that were detected infrequently (ST mutants 03, 18, 20, 22 and 26) were cultured at 37°C in MB supplemented with gentamicin at 160 μg/ml until late logarithmic phase. Wild-type Ap3AS was cultured at 37°C in MB that did not contain gentamicin. The concentration of each strain was adjusted to approximately 1×10^7 CCU/ml. Eight groups of four-week-old SPF chickens were housed separately (20 birds per group) in positive pressure fibreglass isolators. Each of the six groups was inoculated with a different ST mutant by aerosol exposure. Negative control birds were exposed to MB and positive control birds to wild-type Ap3AS.

The birds were euthanased at 14 days after infection and *post mortem* examinations conducted. Sera and swabs were collected from each bird and for anti-mycoplasma antibody detection and mycoplasma isolation were conducted as described in Section 3.2.6.3, with the exception of swabs taken from the Ap3AS infected group, which were inoculated onto MA plates and then placed in MB without gentamicin. DNA was isolated from each broth culture showing a colour change and used as template in PCRs to amplify the unique tag region using the P2/P4 primer pair. The PCR products were used as probes in dot blot hybridisations to detect the presence of specific tags. PCRs were also performed using the IGstmGenmeF3 primer and a primer specific for each ST mutant as an additional tool to identify the ST mutants in inoculated birds. The upper, middle and lower sections of the trachea were taken from each bird, examined histopathologically and the mucosal thickness measured.

Dot blot hybridisation and PCR amplification were used to detect each ST mutant in the broth cultures. Dot blot hybridisation could not be used for cultures from the positive control group, as wild-type Ap3AS did not contain a signature tag. Briefly, the tag regions in the DNA extracted from the cultures showing a colour change were amplified using the DIG-labelled primer set. Oligonucleotides corresponding to the seven signature tags that identified the mutants used in the experiment were spotted onto nylon membrane and subsequently used in hybridisations. The DIG Luminescent Detection Kit (Roche) was used to detect hybridisation following the manufacturer's instructions.

PCR primers specific for each ST mutant were designed using the sequencing data for each mutant. The primer pair (STM13-KE-C'-1-Rev and STM13-KF-C') used to confirm the re-isolation of wild-type strain Ap3AS targeted the region identified when sequencing ST mutants 13-1, 13-2 and 13-3.

PCR reactions were conducted using 2 μl of extracted DNA as template in a 20 μl reaction containing 2 μl of 10 x reaction buffer, 1 μM of each primer, 200 μM of each dNTP and 1.5 U of *Taq* DNA polymerase (Promega). PCRs were incubated 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, with a final incubation at 72°C for 7 min.

Samples of upper, middle and lower trachea were collected and immersed in 10% neutral buffered formalin (10% formalin, 4 g NaH_2PO_4 and 6.5 g Na_2HPO_4 per litre) for at least 24 h for fixation. Tissues were then processed into paraffin wax, followed by vacuum embedding. Sections 2 μm thick were cut and collected onto glass slides. Following dewaxing and rehydration, the sections were stained with haematoxylin and eosin, and examined by light microscopy for lesions and for measurement of mucosal thickness.

Histological lesions in upper, middle and lower sections of trachea were scored for severity on a scale of 0 to 3:
0 = no significant changes;
0.5 = very small aggregates of lymphocytes (less than 2 foci) or very slight, diffuse lymphocytic infiltration;
1 = small aggregates of lymphocytes (more than 2 foci) or minor thickening of the mucosa caused by diffuse infiltration of lymphocytes;
2 = moderate thickening of the mucosa due to heterophil and lymphocyte infiltration, as well as oedema accompanied by degeneration of epithelia with or without luminal exudation;
3 = considerable thickening caused by infiltration of heterophils and lymphocytes and oedema with squamous metaplasia or epithelial degeneration and luminal exudation.

The mucosal thickness of the trachea of each bird was determined by measuring the thickness at 6 points on each section from the upper, middle and lower trachea from each bird. The mean thickness in micrometres was then calculated for each of the three regions.
Median tracheal histological lesion scores for each experimental group were compared using Mann-Whitney U tests (Minitab version 14.2 for Windows). Student's t-test and a one-way analysis of variance (ANOVA) were used to compare the mean tracheal mucosal thicknesses. A probability (P value) ≤ 0.05 was regarded as significant.
Results from the above chicken experiment showed chickens infected with ST mutant 26-1 did not produce a detectable systemic anti-mycoplasma antibody response or airsacculitis at 14 days after inoculation and had only minor tracheal lesions. The aim was to investigate the protective immunity elicited by ST mutant 26-1. Chickens were administered ST mutant 26-1 as a vaccine then challenged with a virulent *M. gallisepticum* strain Ap3AS. Birds vaccinated with ST mutant 26-1 were then compared to unvaccinated as well as unchallenged birds to assess weight gain, anti-mycoplasma antibody titres, gross air sac lesions and histological tracheal lesions. From these results it was possible to assess the protective immunity induced by ST mutant 26-1.

Results

Microarray Analysis of *vlhA* genes

The microarray slide was spotted with oligonucleotides to detect each of the VlhA gene members (Fig. 11 and control genes).

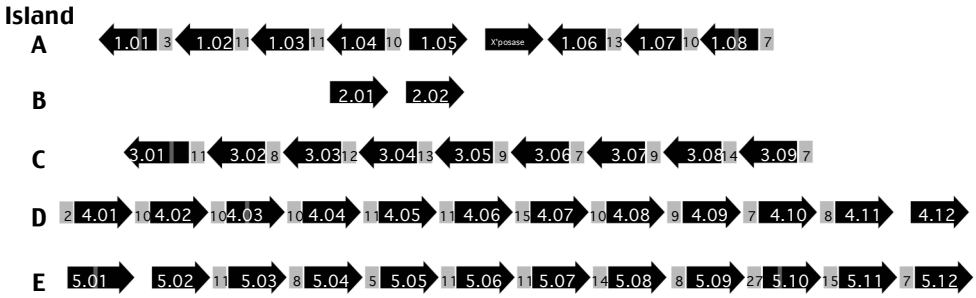


Figure 11. Schematic diagram of VlhA gene islands found in *M. gallisepticum* R strain genome. The light grey boxes represent GAA repeats (the number of repeats are indicated) involved in the regulation of VlhA gene control whilst the large black arrows are VlhA genes with text indicating the gene ID number.

Transcription levels for each of the reference genes was assessed using qPCR and their expression levels calculated against highly expressed Tuf gene that was arbitrarily set at 100% (Table 8).

Table 8. qPCR Ct values and fluorescent levels for reference genes

Gene ID	Ct value	std dev	% efficiency of PCR	Fl value
trkG	22.14	0.12	96.3	404.5
UvrD	23.39	0.07	94	157
MG_4.01	18.64	0.23	90.5	825.5
MG_2.01	19.02	0.03	98.4	1113.5
MG_5.04	25.19	0.02	90.7	77
tuf	16.79	0.06	106	1730
MG_3.03	16.51	0.08	70	5334

Duplicate slides were hybridised with the fluorescent labelled cDNA and the average level for each VlhA and reference gene determined. The fluorescence levels for each of the reference genes were then used to create a standard curve against % expression level of the Tuf gene. The fluorescence values obtained for each VlhA was then interpreted from the graph (Fig. 10) to calculate the % expression levels for each, R^2=0.928.

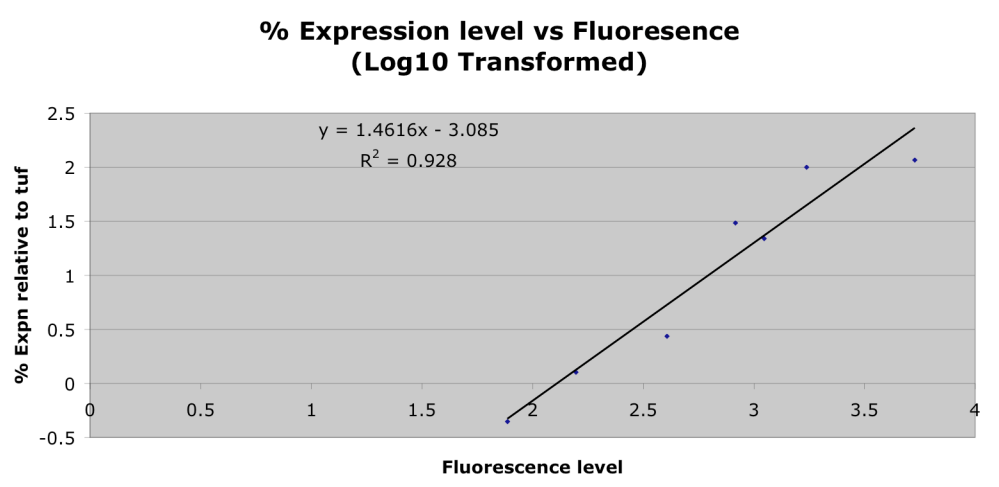


Figure 12. Expression level of reference genes relative to fluorescence. The % expression level of each reference gene (relative to tuf) were plotted against its fluorescence level (log10 transformed) and a line of best fit calculated.

The relative expression level for reach VlhA gene was calculated from the graph in Figure 12 and the expression level graphically presented in Figure 13.

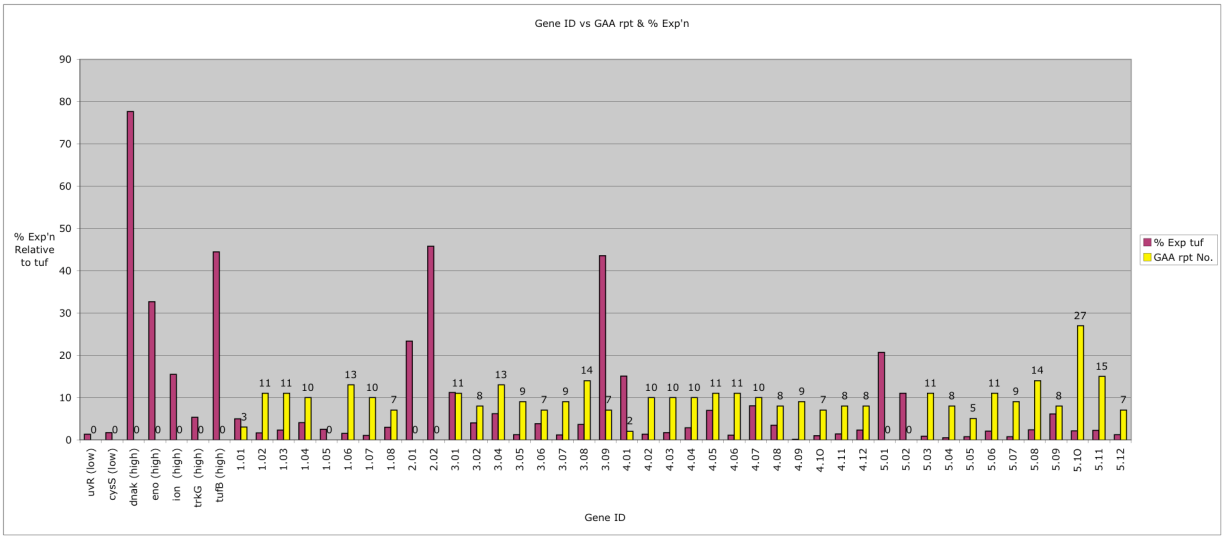


Figure 13. Percentage expression level of VlhA and reference genes. The expression level of individual VlhA genes was calculated from the graph in Figure 10 and together with the number of GAA repeats preceding the VlhA gene is shown in the graph. The most highly expressed VlhA gene *vlhA*3.03 was omitted from the graph as its expression level was greater than tuf (Table 8).

Expression Level of *vlhA*3.03 Highest

The results show that *vlhA*3.03 was the most highly expressed member of the VlhA gene family with higher expression levels than the highly transcribed tuf gene. The regulatory region preceding each of the VlhA genes possesses a trinucleotide repeat containing the nucleotides GAA. Previous studies have predicted that for expression VlhA the number of repeats should be twelve. This is the case with VlhA3.03 in *M. gallisepticum* R strain as this gene is preceded by 12 repeats suggesting this is critical for gene expression.

Analysis of STM Experimental Infection of Chickens

Results for in vivo experiments using 6 different ST mutants show that air sac lesions were not seen in birds exposed to aerosols of ST mutants 04-1 (Group 2), 33-1 (Group 3) or 22-1 (Group 8), or in the negative control birds (Group 1). Mild lesions (score of 0.25) were observed in one bird inoculated with ST mutant 03-1 (Group 4). Of the 20 birds infected with ST mutant 26-1 (Group 5), four had mild lesions (0.50 to 1.00), whilst lesions were only seen in the abdominal air sacs of four birds exposed to ST mutant 18-1 (Group 6). Six of 20 birds had mild to severe lesions (0.50 to 2.50) in the group infected with ST mutant 20-1 (Group 7), while mild to severe lesions (0.50 to 3.00) were seen in 11/18 birds infected with the virulent Ap3AS strain (Group 9). The results are summarised in Table 9 and shown graphically in Figure 14.

Table 9. RSA results, air sac lesion scores and re-isolation rate of *M. gallisepticum* from birds in the virulence and infectivity study.

Group	Inoculum	RSA*	Air sac	MA plate#		MB^	
		Week 2	lesions*	Air sacs	Trachea	Air sacs	Trachea
1	Medium	0/20	0/20	0/20	0/20	0/20	0/20
2	ST mutant 04-1	0/20	0/20	0/20	0/20	0/20	0/20
3	ST mutant 33-1	0/20	0/20	0/20	0/20	0/20	2/20
4	ST mutant 03-1	1/20	1/20	0/20	2/20	0/20	5/20
5	ST mutant 26-1	0/20	4/20	1/20	6/20	1/20	10/20
6	ST mutant 18-1	20/20	5/20	5/20	17/20	7/20	19/20
7	ST mutant 20-1	20/20	6/20	7/20	20/20	8/20	20/20
8	ST mutant 22-1	0/19	0/19	0/19	1/19	0/19	3/19
9	Ap3AS	18/18	11/18	9/18	18/18	10/18	15/18

* Results are indicated as number of positive responses/number examined. Complete RSA and air sac lesion score data are shown in Appendix E. One bird died in Group 8 and two birds died in Group 9 before *post mortem*

Results are indicated as number of positive samples/number collected.

^ Results are indicated as number of colour-change samples/number collected. Signature tags were only detected as the ones carried by ST mutants that infected birds in Groups 2 to 8.

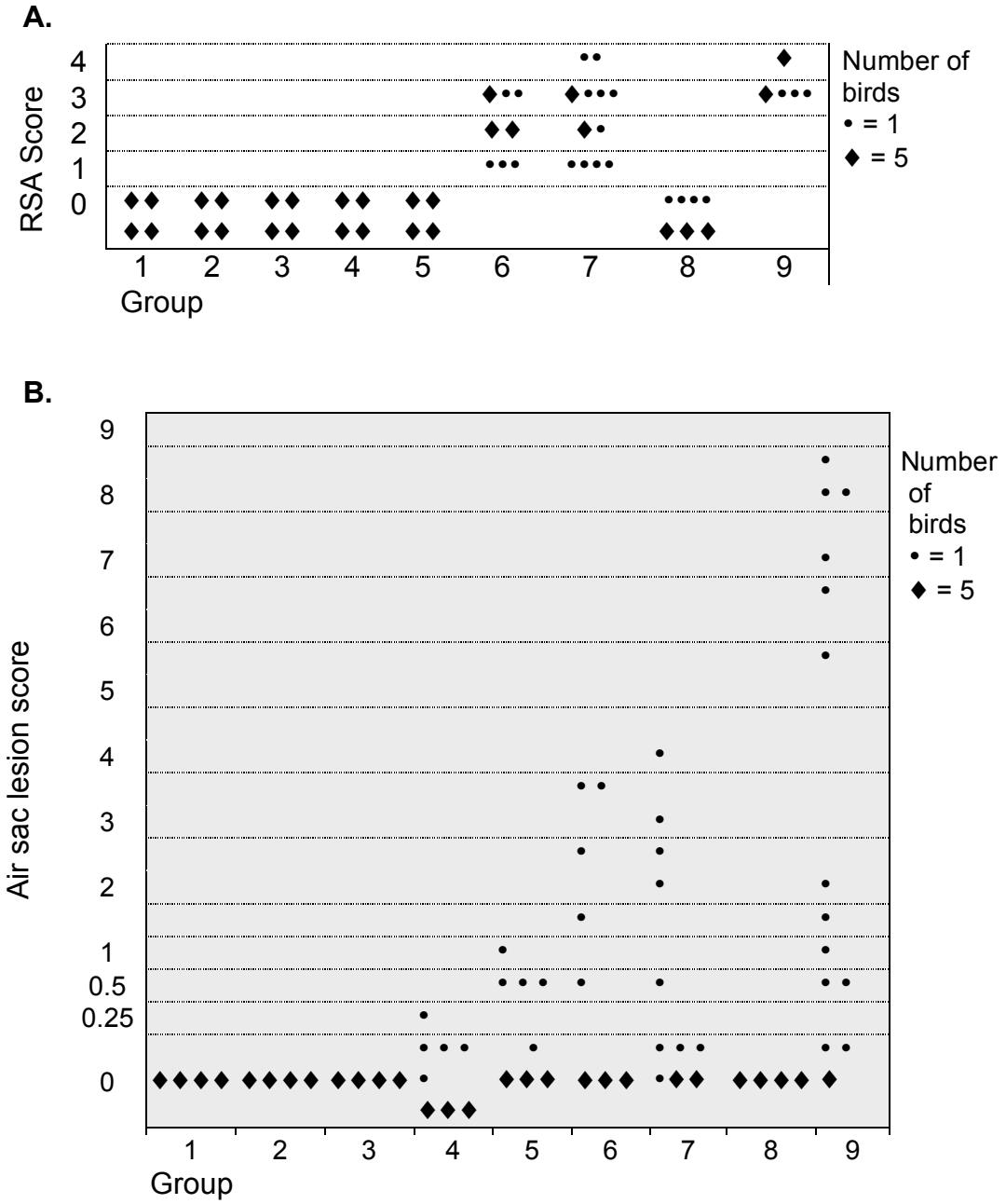


Figure 14. Distribution of RSA and air sac lesion scores in birds in the virulence and infectivity study. A. RSA score at 2 weeks after infection. B. Air sac lesion score. Group 1, negative control; Group 2, ST mutant 04-1 infected; Group 3, ST mutant 33-1 infected; Group 4, ST mutant 03-1 infected; Group 5, ST mutant 26-1 infected; Group 6, ST mutant 18-1 infected; Group 7, ST mutant 20-1 infected; Group 8, ST mutant 22-1 infected; Group 9, positive control (wild-type Ap3AS infected).

Serology Results

No anti-mycoplasma antibody was detected at the time of infection in the serum of any experimental birds using the RSA test. Two weeks after infection, antibody responses were not detected in any of the birds in Groups 2, 3, 5 and 8, whilst a response was detectable in only one bird in Group 4. In contrast, strong RSA reactions against *M. gallisepticum* were detectable in all the birds in Groups 6 and 7. The negative control birds (Group 1) did not show reactivity against *M. gallisepticum* in the RSA test, while all the positive control birds (Group 9) had strong RSA reactions against MG (Table 9, Figure 14).

Reisolation of ST mutants

M. gallisepticum was not isolated on MA plates inoculated with swabs of the air sacs or trachea of any bird in Group 2 (ST mutant 04-1 infected) or Group 3 (ST mutant 33-1 infected) (Table 9). *M. gallisepticum* was not isolated on agar plates inoculated with swabs of the air sacs of any birds in Groups 4 (ST mutant 03-1 infected) or 8 (ST mutant 22-1 infected), but they were isolated from the trachea of two birds in Group 4 and one bird in Group 8. In Group 5 (ST mutant 26-1 infected), *M. gallisepticum* were isolated from the air sacs of one bird and the tracheas of 6 birds. *M. gallisepticum* were also isolated from the tracheas of 17/20 birds in Group 6 (ST mutant 18-1 infected) and all the birds in Group 7 (ST mutant 20-1 infected), and also from swabs of the air sacs of 5/20 birds in Group 6 and 7/20 birds in Group 7. In the positive control group (Group 9), *M. gallisepticum* were isolated from the air sacs of 9/18 birds and the tracheas of 17/18 birds.

M. gallisepticum was more frequently isolated from swabs incubated in broth than on agar plates. *M. gallisepticum* was not isolated in broth inoculated with swabs of the air sacs or tracheas of any bird exposed to ST mutant 04-1 (Group 2) (Table 9). In the other mutant-infected groups, *M. gallisepticum* was recovered in broth inoculated with swabs of the air sacs of one bird in Group 5 (ST mutant 26-1), 7 birds in Group 6 (ST mutant 18-1) and 8 birds in Group 7 (ST mutant 20-1). *M. gallisepticum* were recovered from the tracheas of birds in 6 of the groups exposed to ST mutants, with the number of infected birds ranging from 2 in Group 3 (ST mutant 33-1 infected) to 20 in Group 7 (ST mutant 20-1 infected). The identity of the recovered *M. gallisepticum* ST mutants was confirmed using the unique signature tags they carried (Table 9).

Severity of ST mutant Infection

Median tracheal lesion scores are shown in Table 10. The scores of birds in all the mutant infected groups differed significantly from those of birds in the positive control group (Group 9) ($P < 0.0001$). There was no significant difference between the negative control group (Group 1) and Groups 5 (ST mutant 26-1 infected) or 7 (ST mutant 20-1 infected). The lower tracheal lesion scores of the birds infected with ST mutant 18-1 (Group 6) did not differ from those of birds in Groups 2, 3, 4, 5 and 7, but did differ significantly from those of birds in Group 8 (ST mutant 22-1 infected) and in the negative control group (Group 1), while only lesion scores in the middle trachea of the birds in the positive control group differed from those of birds in any of the other groups.

Table 10. Tracheal lesion scores and mucosal thicknesses in birds in the virulence and infectivity study.

Grp	STM	Median tracheal lesion score (min, max)			Mean tracheal thickness ± SD* (μm)		
		Upper	Middle	Lower	Upper	Middle	Lower
1	Broth	0.25 (0, 0.5)[a]	0.25 (0, 0.5)[a]	0 (0, 0.25)[a]	50 ± 11 [a]	41 ± 8 [a]	37 ± 6 [a]
2	04-1	0.5 (0.25, 1.5)[b]	0.25 (0, 0.5)[b,c]	0.25 (0, 0.25)[a,b]	67 ± 11 [b]	53 ± 12 [a,b]	40 ± 9 [a,b,c]
3	33-1	0.75 (0.25, 3)[b]	0.25 (0, 2.5)[a,b]	0 (0, 0.25)[a,b]	73 ± 32 [b,c]	47 ± 13 [b]	38 ± 8 [b]
4	03-1	1 (0.25, 3)[b]	0.375 (0, 1.5)[c]	0.25 (0, 1.5)[c]	89 ± 45 [d]	54 ± 12 [b]	46 ± 22 [c]
5	26-1	0.5 (0, 2)[a,b]	0.25 (0, 1)[a,b]	0 (0, 2)[a,c]	64 ± 24 [b,e]	49 ± 14 [b]	48 ± 22 [b,c]
6	18-1	0.25 (0, 10)[a]	0.25 (0, 1.5)[a,b,c]	0.25 (0, 1.5)[b,c]	62 ± 15 [b,e]	53 ± 15 [b]	49 ± 15 [c,d]
7	20-1	0.25 (0, 1.5)[a]	0.125 (0, 1.5)[a]	0.125 (0, 1.0)[a,c]	56 ± 22 [a,c,e]	49 ± 23 [a,b]	51 ± 19 [c,d]
8	22-1	0.5 (0, 1)[a,b]	0.25 (0, 1)[a,b]	0 (0, 0.25)[a]	64 ± 13 [b,e]	49 ± 8 [b]	39 ± 8 [a,b]
9	Ap3	1.5 (1, 3)[c]	1.50 (0.5, 3)[d]	1.50 (0.25, 3)[d]	168 ± 70 [f]	153 ± 84 [c]	120 ± 56 [e]

* SD : standard deviation

Data in the same column with the same superscript are not significantly different.

The mean mucosal thicknesses in the middle trachea did not differ significantly between any of the mutant infected groups (Groups 2 to 8). However, they were all significantly greater than that of the negative control group (Group 1), except for those in Groups 2 and 7, and they were significantly less than that of the positive control group (Group 9). There was no

significant difference between the negative controls and Group 7 (ST mutant 20-1 infected) in the mucosal thicknesses of the upper and middle tracheas, but there was a difference in the lower trachea (P = 0.004).
In summary, the positive controls had significantly more severe tracheal lesions, as assessed by histological lesion score or mucosal thickness, than any of the groups infected with the ST mutants. Group 4 (ST mutant 03-1 infected) was the most severely affected among the groups infected with the ST mutants..
Total cell proteins of the *M. gallisepticum* strains used in this study were separated by SDS-PAGE and stained with Coomassie brilliant blue (Figure 15). There were no detectable differences in the protein profiles of ST mutants 03-1, 04-1, 20-1, 22-1 and wild-type Ap3AS. However, one protein band of about 45 kDa was absent in ST mutant 18-1, whilst ST mutant 26-1 contained an additional band of about 33 kDa compared to the other mutants and wild-type Ap3AS.

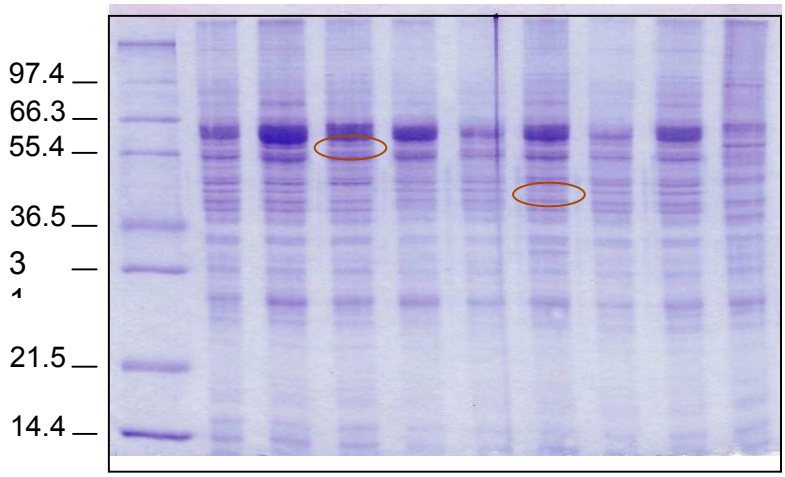


Figure 15. Protein profiles of ST mutants. Total cell proteins were separated in a 12.5% polyacrylamide gel together with molecular mass standards and then stained with Coomassie brilliant blue. The main differences in the protein profiles are circled. Lane 1, ST mutant 03-1; Lane 2, ST mutant 04-1; Lane 3, ST mutant 18-1; Lane 4, ST mutant 20-1; Lane 5, ST mutant 22-1; Lane 6, ST mutant 26-1; Lane 7, ST mutant 33-1; Lane 8, wild-type strain Ap3AS; Lane 9, ts-11 vaccine strain. The positions of the broad range protein markers (Mark12^{TM} Wide Range Protein Standard, Novex) are indicated on the left.

Protection Studies in Chickens Using STM26

For the protection experiment using STM26 gross air sac lesions were only seen in birds in the positive control group (Group 2). In this group, 5 birds had air sac lesions, with total air sac lesion scores ranging from 0.5 to 2.5. The results are summarised in Table 11 and shown graphically in Figure 16.

Table 11. Results of RSA test, air sac lesion scores and re-isolation of *M. gallisepticum* of all groups in the immune protection study.

Grp	Vaccine	Challge AP3	RSA[#] Day14	RSA[#] Day28	Air sac lesions	MA plate[^] Air sacs	MA plate[^] Trachea	MB[&] Air sacs STM26	MB[&] Air sacs Ap3	MB[&] Trachea STM26	MB[&] Trachea Ap3
1	Broth	No	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20
2	Broth	Yes [*]	0/20	20/20	5/20	1/20	19/20	0/20	2/20	0/20	0/20
3	STM26	No	0/20	16/20	0/20	1/20	2/20	1/20	0/20	2/20	0/20
4	STM26	Yes	0/20	20/20	0/20	0/20	7/20	0/20	0/20	0/20	9/20

[*] wild-type Ap3AS

[#] Complete RSA and air sac lesion score data are shown in Appendix G.

[^] Number of samples showing *M. gallisepticum* colonies growing on the MA plate / total number of samples collected.

[&] PCRs were conducted to determine the presence of ST mutant 26-1 and strain Ap3AS

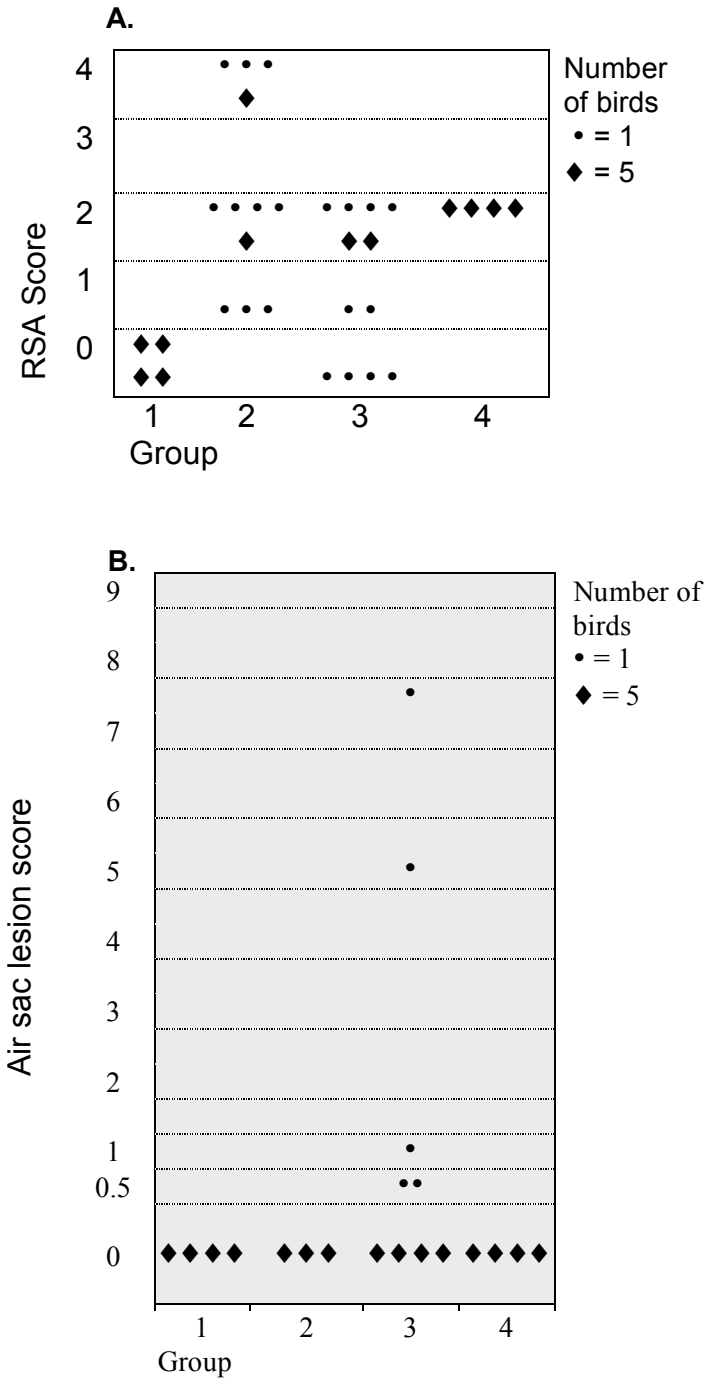


Figure 16. Distribution of RSA and air sac lesion scores of birds. A. RSA score at day 28. B. Air sac lesion score. Sum score of 6 different air sacs. Group 1, negative control; Group 2, positive control (wild-type Ap3AS infected); Group 3, vaccination control (ST mutant 26-1 vaccinated); Group 4, vaccinated (ST mutant 26-1) and challenged (wild-type Ap3AS infected).

Sera collected from birds at the time of vaccination and challenge contained no detectable anti-mycoplasma antibody. None of the chickens in the negative control group (Group 1) had any detectable serum antibody against *M. gallisepticum* at the time of *post mortem*. In contrast, a large proportion of birds in the other 3 groups had detectable antibodies against

M. gallisepticum at *post mortem*. Every bird in the positive control group (Group 2) had produced a moderate to strong RSA antibody response against *M. gallisepticum*. Anti-mycoplasma antibodies were detected in 16/20 serum samples from Group 3, with the RSA scores ranging from low to moderate. Moderate anti-mycoplasma antibody titres were detected in all birds in Group 4 (vaccinated with ST mutant 26-1 and infected with the wild-type strain). The results are summarised in Table 11.
M. gallisepticum were more frequently isolated from tracheal swabs than swabs of the air sacs in Groups 2, 3 and 4, and were not isolated from the air sacs or tracheas of any bird in Group 1 (negative controls). *M. gallisepticum* colonies were grown from swabs of the air sacs in one bird each in Groups 2 (challenged only) and 3 (vaccinated only), whilst re-isolation was achieved from the trachea of 19 and 2 birds, respectively. In Group 4, which had been given ST mutant 26-1 as a vaccine and then infected with strain Ap3AS, Mg was recovered on MA plates from the air sacs of one bird and from the tracheas of 7 birds (Table 11).
As in previous experiments, re-isolation rates were higher in broth than on agar plates. PCR amplification confirmed that the only mycoplasmas re-isolated from Groups 2 and 3 were the strain that had been administered to that group. Following PCR amplification using strain specific primer pairs, the only mycoplasmas re-isolated from Group 4 were wild-type Ap3AS. The results are shown in Table 11.
There was no significant difference in weight gain between any of the groups over the 14 days following vaccination. At week 4 (14 days after challenge), the positive control birds (Group 2) had lower mean weight gains than the birds in Groups 1 (negative controls) or 3 (vaccination controls) (P = 0.006 and 0.027, respectively), but not than the birds in Group 4 (vaccinated and challenged) (P = 0.332) (Table 12).
In the period between challenge and *post mortem* (day 14 to day 28), the mean weight gain of the chickens in Group 2 (challenged only) was significantly lower than that of the birds in each of the other groups. The mean weight gain of birds in Group 3 (vaccinated, not challenged) was not greater than that of the negative controls, but was significantly greater than that of the birds in Group 4 (vaccinated and challenged) (P = 0.016). However, the mean weight gain of the birds in Group 4 did not differ significantly from that of the birds in Group 1 (P = 0.580). The results are shown in Table 12

Table 12. Weight gain of chickens in all experimental groups in the immune protection study

Grp	Vaccine	Challenge Wildtype	Day 0 weight (g)	Day 0–14 gain (%)	Day 0–28 gain (%)	Day 14–28 gain (%)
1	Medium	No	382 ± 41	53 ± 6 [a]	106 ± 12 [a]	34 ± 7 [a,c,d]
2	Medium	Yes	422 ± 33	52 ± 7 [a]	94 ± 13 [b]	28 ± 4 [b]
3	STM26	No	387 ± 43	48 ± 10 [a]	104 ± 15 [a]	38 ± 6 [c]
4	STM26	Yes	415 ± 58	50 ± 9 [a]	100 ± 13 [a,b]	33 ± 6 [d]

Results are expressed as mean ± standard deviation.
Values with the same superscript in the same column are not significantly different.
Complete data are shown in Appendix H.
wild-type Ap3AS

The median tracheal lesion scores are shown in Table 13. The lesions were severe in the birds in Group 2 (challenged only). The median tracheal lesion scores of birds in Group 2 (positive controls) differed significantly from those in the other three groups (P < 0.0001). The lesion scores of the birds in the other groups did not differ significantly from each other. The mean mucosal thicknesses of the upper, middle and lower trachea of the birds in Group 2 (challenged only) were significantly greater than those of the birds in the other three groups (P < 0.0001), but there was no significant difference between those of the birds in these other three groups (Table 13). However, in middle and lower tracheas, mean mucosal thickness was less in the birds in Group 4 than in the birds in Groups 1 (P = 0.028) or 3 (P = 0.036).

Table 13. Tracheal lesion scores and mucosal thicknesses of birds in the immune protection study

Grp	Vaccine	Challenge WtAp3A*	Median tracheal lesions (min, max)			Tracheal mucosal thickness±SD# (μm)		
			Upper	Middle	Lower	Upper	Middle	Lower
1	Medium	No	0(0,0.5)[a]	0(0,0)[a]	0(0,0)[a]	41±6[a]	32±5[a]	28±4[a]
2	Medium	Yes	2.5(0.5,3)[b]	2.5(0.5,3)[b]	2(1,3)[b]	233±101[b]	206±107[b]	173±98[b]
3	STM26	No	0(0,0.25)[a]	0(0,0.5)[a]	0(0,0.25)[a]	43±11[a]	34±8[a]	28±6[a]
4	STM26	Yes	0(0,1)[a]	0(0,0.25)[a]	0(0,0)[a]	44±14[a]	28±5[c]	25±3[c]

*wild-type strain Ap3AS
#SD: standard deviation
Data in the same column with the same superscripts are not significantly different.

Discussion of Results

Microarray analysis

Microarray analysis is a valuable method to determine expression levels of gene and regulation of those genes under different environmental conditions. We developed a small array containing the probes for the VlhA gene family. The VlhA peptides play an important role in *M. gallisepticum* attachment and most likely immune evasion. The mechanism responsible for controlling the VlhA gene expression is poorly understood though it is thought that the number of GAA repeats preceding each gene is critical for expression. This work has shown that 12 GAA repeats is necessary for VlhA expression and provides a tool to for analysis of VlhA expression under various conditions. The costs for generating a chip for expression of the genome of *M. gallisepticum* was prohibitive in the current grant and due to IP issues the use of a chip from another institution was thought unwise and as such the efforts of strategy 4 were directed to the development of ST mutants.

Signature-tagged mutagenesis

Signature-tagged mutagenesis (STM) is a valuable technique for genetic analysis of factors involved in infection and virulence, and most studies conducted using STM are designed to investigate the interaction between pathogens and their hosts. However, the use of pools of mutants may result in a failure to distinguish between a mutation in a gene that is essential *in vivo* and a mutation in a gene that reduces the capacity of the mutant to compete with other mutants *in vivo*.

Identification of Attenuating Mutation

A third round of *in vivo* screening was conducted by exposing birds to individual ST mutants instead of a pool, and this further reduced the number of mutants that were identified as unable to infect. ST mutant 04-1, which had not been detectable in either of the two previous screening experiments, was not able to be re-isolated from any of the chickens exposed to it, suggesting that the expression of the gene that had been interrupted (MGA_0680, encoding the ABC sugar transport permease MalF) is essential for survival of Mg strain Ap3AS *in vivo*. This gene would thus appear to be worthy of further investigation as a target for development of treatments to prevent *M. gallisepticum* infection.

In ST mutant 33-1, the insertion of the tagged transposon within the *crm*A gene (MGA_0939) interfered with CrmA expression. Previous studies have concluded that both GapA and CrmA are essential for *in vivo* colonisation by *M. gallisepticum* the tracheas of two inoculated birds and the identity of the mutant that was recovered was confirmed by both dot blot hybridisation and PCR amplification using a primer pair specific for the junction between the tagged transposon and the genome of strain Ap3AS. While it was clear that the ability of this mutant to colonise and cause disease had been dramatically reduced, it had not been

completely abolished. This finding suggests that other factors may be able to partially compensate for the role of CrmA in cytadherence.
SDS-PAGE revealed one missing band in ST mutant 18-1, in which the tagged transposon was located within an open reading frame encoding a unique hypothetical protein. The insertion site of the transposon would allow the amino terminal 70% of the gene to be translated. This mutant was able to induce an antibody response, cause mild air sac lesions, and colonise both the air sacs and tracheas of birds (results not shown). Thus ST mutant 18-1 was still able to infect, but was significantly less virulent than wild-type Ap3AS. This suggests that the gene disrupted in ST mutant 18-1 may have a significant influence on virulence, although not in colonisation. A similar situation was seen in the birds infected with ST mutant 20-1. The transposon carrying Tag 20 inserted into a site close to the start of gene MGA_0220, which encodes the ATP-binding protein OppD (or DppD), and thus was likely to block translation of this gene. In spite of the probable lack of this molecule, ST mutant 20-1 could still colonise birds and cause mild lesions, indicating that this protein may play a role in virulence, but not in colonisation.
In previous screening experiments, ST mutants 18-1 and 20-1 were detected at low frequency in the output pools. However, when they were the only strain present they could be recovered from the respiratory system of chickens at a high frequency. A key problem in STM studies is that insufficient signal may be generated from some mutants by PCR amplification from the output pools. It is possible that transposon integration might significantly influence the replication *in vivo* of those mutants that were either undetectable or detected at low frequency compared to other ST mutants in the same pre-selection pool, thus resulting in recovery of fewer cells, low levels of signal production and a failure to detect them by hybridisation.
In ST mutant 26-1, the tagged transposon was also inserted into the gene (MGA_0220) encoding the ATP-binding protein OppD. SDS-PAGE revealed the presence of an additional protein band in this mutant, possibly as a consequence of transposon mutagenesis. The identity and role of this additional protein is yet to be determined and requires further investigation. The birds inoculated with this mutant had only minor respiratory lesions, and the mutant was recovered from the air sacs and tracheas at a reasonable frequency. These results suggest that ST mutant 26-1 had lost most of its virulence but retained a capacity for *in vivo* colonisation. Similarly, ST mutant 22-1 was relatively apathogenic, and had a reduced capacity to colonise. Neither ST mutant 26-1 nor 22-1 induced detectable anti-mycoplasma antibody in infected birds. However, ST mutant 20-1, which caused mild air sac lesions and only minor damage in tracheas, induced a detectable anti-mycoplasma antibody response. These three ST mutants, 26-1, 20-1 and 22-1, may warrant further investigation as potential vaccine candidates.
As the age of chickens and the dose of vaccine play a key role in successful vaccination with *M. gallisepticum* ts-11. A number of studies have indicated that vaccination of chickens with *M. gallisepticum* ts-11 at less than 3 weeks of age generates poor levels of protection against disease caused by virulent *M. gallisepticum* and the recommended dose for eye drop vaccination is more than $1 \times 10^{7.7}$ CCU. In the study described ST mutant 26-1 was assessed as a vaccine because in studies of its pathogenicity it only induced mild air sac lesions and caused very little tracheal pathology in infected birds. The dose, method of administration and the age of chickens used to conduct this experiment were adapted from the recommended procedures for vaccination with ts-11.
As in the study reported in the previous study, no anti-mycoplasma serum antibody could be detected 14 days after vaccination with ST mutant 26-1. However, a relatively consistent antibody response, as assessed by RSA, was detected at 4 weeks after inoculation. This indicates that ST mutant 26-1 is able to stimulate production of measurable antibody in birds 4 weeks after infection at a similar level to that seen in birds vaccinated with *M. gallisepticum* ts-11. Examination of gross air sac lesions has been a valuable method to assess infection and pathogenicity of *M. gallisepticum*. The *in vivo* studies described in Chapter 5 showed that chickens infected with ST mutant 26-1 had only very minor lesions 2 weeks after inoculation. There were no visible air sac lesions in birds inoculated with this mutant at 4 weeks after exposure in this study. In contrast, the parental strain caused severe air sac

lesions in 20% of the unvaccinated chickens (Group 2). These results further support the previous observations that ST mutant 26-1 has lower pathogenicity than the parent strain. The capacity of organisms to colonise the trachea can be examined by re-isolation with ST mutant 26-1 re-isolated from the tracheas of birds 2 weeks after infection. The studies described resulted in very few re-isolations of ST mutant 26-1 from the air sacs or the tracheas at 4 weeks after vaccination. Previous studies have shown that bacterial genome titres in trachea reach a peak 3 weeks after infection with virulent *M. gallisepticum* strain Ap3AS and then decrease quickly. This may explain why ST mutant 26-1 could be re-isolated from so few chickens in Group 3 (vaccinated only) and none in Group 4 (vaccinated and challenged). In contrast, wild-type strain Ap3AS could be recovered from the tracheas of around half the inoculated birds in Groups 2 (challenged only) and 4 (vaccinated and challenged), even though it could only be re-isolated from the air sacs of a few birds in Group 2 and none of the birds in Group 4. It is well accepted that *M. gallisepticum* can persist in flocks even though it is sometimes hard to re-isolate from individual birds. The findings from this study might suggest that ST mutant 26-1 retains a capacity to persistently colonise birds for at least 4 weeks, albeit at low concentrations, and that this mutant can induce effective protective immunity. However, as the study did not include quantitation of *M. gallisepticum* in the trachea or in-contact controls, the concentrations of Ap3AS in the tracheas of vaccinated birds after challenge and the possibility of horizontal transmission to other birds requires further investigation.

Histopathological examination of tracheal sections indicated only mild damage in some chickens vaccinated with ST mutant 26-1 and showed that there was no difference in the severity of lesions or the upper tracheal mucosal thickness between the vaccinated, the vaccinated and challenged, and the unchallenged control groups. In contrast, extensive inflammatory cell infiltration was seen in the tracheas of most of the unvaccinated, challenged birds. This further supports the contention that vaccination with ST mutant 26-1 can effectively prevent the damage caused by infection with wild type *M. gallisepticum*.

Body weight gain is another method to evaluate the protection afforded by vaccination. At 14 days after vaccination, the weight gains of all groups were similar. After infection with strain Ap3AS, the weight gain of the vaccinated birds was significantly greater than that of unvaccinated, challenged birds. This further suggests that vaccination with ST mutant 26-1 offers protection against the effects of infection with virulent *M. gallisepticum*.

In ST mutant 26-1, the transposon was introduced into the gene that encodes OppD, a protein likely to be the ATP-binding component of an ABC transporter. The superfamily of ABC (ATP-binding cassette) transporters is responsible for the uptake of essential substrates, including sugars and peptides, as well as the export of polysaccharides, haemolysins and proteases, in both prokaryotes and eukaryotes. Studies of *M. pneumoniae, M. genitalium* and *M. hominis* have indicated that the Opp ABC transporter may be responsible for the transportation of oligopeptides. This transporter system has been well characterised in *M. hyorhini*s and is similar to that found in other bacteria. There are four domains of ABC transporters. Domains OppB and OppC are predicted to be integral membrane proteins and to be required for the movement of oligopeptides across cell membrane. The genes *opp*D and *opp*F encode predicted ATP-binding proteins (OppD and OppF). The ATP-binding P-loops have been found in all OppD domains examined thus far. The insertion in ST mutant 26-1 is predicted to block the expression of OppD and thus is likely to influence the transport of oligopeptides into *M. gallisepticum*, but this appears to have only a minor impact on colonisation.

Vaccine Potential of STM26 Mutation

The experimental studies described in this chapter showed that eye-drop vaccination with ST mutant 26-1 not only induced a detectable antibody response, but also protected against the effect of infection with virulent *M. gallisepticum* on weight gain, which may be an important attribute for the poultry industry. It also protected chickens against the development of air sac lesions and damage to the trachea. These results indicate that ST mutant 26-1 has potential as a vaccine, although it is at an early stage in the vaccine development.

Implications

The results from the microarray analysis indicate the most highly expressed VlhA peptide of *M. gallisepticum* R strain is VlhA3.0. The finding that there are 12 GAA trinucleotide repeats preceding *vlhA*3.03 strengthens the hypothesis that expression is enhanced when 12 repeats are present. Detection of a serological response to *M. gallisepticum* infection is primarily to VlhA as it is abundant and surface located. Indeed the homologous VlhA peptide is more sensitive in detection of an antibody response than that of a heterologous VlhA peptide such as that observed for *M. gallisepticum* VlhA when the homologous antigen is used in an ELISA. The ability to detect and identify the expressed VlhA peptide of a particular *M. gallisepticum* strain will be enhanced using the microarray and if configured correctly may provide a signature-binding pattern suitable for strain identification.

The Signature Tagged Mutagenesis approach was used to identify a number of strains that were attenuated and one strain was selected for testing its ability to protect against virulent challenge. Signature Tagged mutant 26 was tested in a challenge model and provided protection. The ability to identify the target for attenuation lends itself to producing vaccines in other strains by interrupting similar or identical genes and provides a method for identifying the vaccine.

Recommendations

To further test the microarray chip to identify *M. gallisepticum* strains by creating a signature binding pattern using labelled *vlh*A gene sequences for a particular strain. As there is variability in the number and of *vlh*A genes and DNA sequence this approach would be an interesting epidemiological tool, perhaps more suited to a research laboratory.

The identification of an attenuating mutation in the OppD gene of ST mutant 26 is worthwhile testing in other bacterial strains, indeed not only *Mycoplasma spp* but also other bacterial strains that produce disease of chickens. This approach was undertaken in the following research project using *Escherichia coli* as a model organism to prevent colibacillosis.

Introduction

Development of *oppD/dppD* knockouts in pathogenic *Escherichia coli*

In a PhD project directed towards achieving the milestones in "Detection of Virulence Genes of *M. gallisepticum*", Mr Kert Tseng used signature tagged mutagenesis (STM) to randomly knockout genes of the most virulent strain of *M. gallisepticum* (strain Ap3AS) in the University of Melbourne culture collection. This strain is used as a wild-type challenge strain in vaccine efficacy studies.

Tseng's studies identified a number of attenuating gene mutations including the *dpp*D/*opp*D gene, which is homologous to other bacterial genes involved in the transport of oligopeptides into the cell. One knockout strain containing this mutation (known as STM26) was tested for immunogenicity as part of Tseng's PhD project and was found to be highly efficacious.

As all bacterial species possess near identical transport systems, new vaccines against other avian pathogens, as well as pathogens of other animal species, could be developed by producing *dpp*D knockouts.

In this project we produced knockouts of the *dpp*D and *opp*D genes in the avian pathogenic *Escherichia coli* strain E956 (APEC E956) and tested each of these mutants for safety and efficacy using a challenge system employed by Dr Kelly Tivendale during her PhD within Project 03-9

Objectives

To produce a *oppD/dppD* knockout in a colibacillosis causing strain of *E. coli* and test the safety and efficacy of these knockouts.

Methodology

Development of oppD/dppD gene knockout constructs and vectors

The avian pathogenic *Escherichia coli* strain E956 (APEC E956) was originally isolated from a day-old chick at a broiler breeder farm) and was found to be sensitive to the antibiotics ampicillin, sulphafurazole, trimethoprim, chloramphenicol, tetracycline, and kanamycin. The organism was propagated in Luria-Bertani broth (LB) or on LB agar at 30°C or 37°C overnight with the appropriate antibiotic selection (ampicillin, 50 μg/ml; kanamycin, 100 μg/ml) unless otherwise stated. The *E. coli* DH5α strain (kind gift of Nino Ficorilli) was used for standard cloning and plasmid production.

The red recombinase system was used to promote homologous recombination between the knockout constructs and the gene knockout target using the temperature sensitive pKD46 plasmid carrying an ampicillin resistance gene, the three "Red system" genes γ, β, and *exo* coding for Gam, Bet, and Exo respectively. The knockout constructs were assembled by PCR and ligated to the pGEM-T vector (Promega). The plasmid containing each construct was used as template in PCR to produce linear DNA for transformation.

The kanamycin gene was amplified from the transposon Tn*phoA* using the oligonucleotide primers TXkanFor and TYkanRev (Table 14). The resultant 1.064 kbp PCR product was purified using the UltraClean PCR purification kit (MoBio, California USA) following the manufacturer's instructions and ligated to pGEM-T vector (Promega) following the manufacturer's protocols. The ligation mixture was used to transform *E. coli* DH5α by electroporation using a Gene Pulser (BioRad) with the settings 2,500 V, 200 Ω, 250 μF and recombinants selected on LB agar containing 25 μg/ml of kanamycin. Transformants were grown overnight in LB broth containing 25 μg/ml of kanamycin and plasmid extracted using kit (Promega) following the manufacturer's instructions. Cloning of the kanamycin resistance gene was verified by restriction endonuclease analysis. Linear DNA was used for transformation: for dppD knockouts the DNA was prepared by PCR using the primer pair WE and WF each containing 40 bp 5' regions complementary to regions 4384-4423 bp and 4829-4868 bp respectively within the *dppD* gene of the GenBank Accession L08399. The WE and WF oligonucleotide primers contained 20 bp 3'regions complementary to the kanamycin gene and when used in PCR amplified the kanamycin gene with 40 bp ends complementary to the dppD DNA.

Table 14. Oligonucleotides used in this study

Oligonucleotides	Sequence (5'-3')	PCR product
TXkanFor	TAGACTGGGCGGTTTTATGG	Kanamycin
TYkanRev	CGAAGCCCAACCTTTCATAG	Kanamycin
TZ5'dppD	CGTAGACCGCATCAGCTACA	dppD
UA3'dppD	GTGGGTAATGGCATTTGGAC	dppD
WAKanFshort	CGTTGGCTACCCGTGATATT	Kanamycin
WE_dppD5'_kanF	CCGTCAGCGAGCGATTGACCTGCTGAATCAGGTCGGTATTtagactgggcggttttatgg	dppD/Kan 5'
WF_dppD3'_kanR	GCGGGTTAAGCAGGCAGCCGTTCGGGCGGTCGTACTTGCCcgaagcccaacctttcatag	dppD/Kan 3'
WSoppDFor	GCACTGCTGAACGTGAAAGA	oppD
WToppDRev	CTCCACCGGTTTAAAGCAAG	oppD
WU1oppDRevKan	CCATAAAACCGCCCAGTCTAtttttaccgcatcgagcatc	oppD arm 5'
WV2oppDForKan	CTATGAAAGGTTGGGCTTCGTCGACCTAAGCTGCTGATTG	oppD arm 3'
Sidfwdseq	CTTTCCACCCTGCACCTAAG	iucA
bwd siderophore	CTCACGGGTGAAAATATTTT	iucA
16Sfwd	GCTGACGAGTGGCGGACGGG	16s RNA
16Srev	TAGGAGTCTGGACCGTGTCT	16s RNA

The APEC strains E956 was transformed with pKD46 by electroporation using the Bio-Rad Gene Pulser (Bio-Rad) set at 2,500 V, 200 Ω, 250 µF. Recombinants were selected on Luria-Bertani agar containing 100 µg/ml of ampicillin following incubation at 30°C overnight. We produced gene knockouts through homologous recombination using previous methods with modifications. A single fresh colony of APEC E956/pKD46 was placed into 5 ml LB containing 100 µg/ml ampicillin and incubated with shaking (200 rpm) at 30°C for 16 hours. The following day, a 1:50 dilution of an overnight culture was made in a volume of 20 ml of LB containing 100 µg/ml ampicillin and the cultures shaken (200 rpm) at 30°C in a 125 ml conical flask. At a concentration of 10^7 cells/ml, L-arabinose (Sigma) was added to a final concentration of 1 mM. Cultures were induced for approximately 1 hour at 30° C and the cells then heat shocked for 15 minutes at 42°C and immediately transferred to an ice-water bath for 10 mins, then collected by centrifugation at 10,000 x g for 10 mins at 4°C. The cells were resuspended in 1 ml of ice-cold 1 mM MOPS containing 20% glycerol, transferred to an 1.5 ml centrifuge tube and centrifuged at 16,000 x g for 30 s at 4°C in a bench-top microfuge. The supernatant was removed and cells resuspended in 1 ml of 1 mM MOPS containing 20% glycerol and centrifuged as before. This step was repeated once more and the cells were finally resuspended in 100 µl 1 mM MOPS containing 20% glycerol. The cells were then added to pre-cooled electroporation cuvettes (2 mm gap, Bio-Rad) and 50 µl of cells with 300 ng of *Dpn*I digested DNA was transformed using the previous settings. Immediately following electroporation, the cells were recovered by suspension in 0.3 ml of LB, diluted into 2.7 ml LB and incubated at 37°C with shaking for 1.5 h. The culture was 5x concentrated in LB and 0.2 ml inoculated onto LB plates containing 20 µg/ml kanamycin and incubated overnight at 37°C. Transformants were selected and grown in LB containing 40 µg/ml kanamycin and gene knockouts confirmed by PCR and Southern blotting.

To verify the insertion of the kanamycin resistance gene in *dppD* or *oppD*, PCR was conducted using the primer pair for *dppD* (TZ/UA) and *oppD* (WS/WT) to amplify the respective gene region. The predicted size of the PCR product for APEC E956 parent for *dppD* and *oppD* would be 0.89 kbp and 0.951 kbp respectively. The predicted size increase with insertion of the kanamycin gene (1.064 kbp) would be 1.774 kbp and 1.924 kbp for *dppD* and *oppD* respectively.

Genomic DNA of APEC E956, Δ*dppD*, and Δ*oppD* strains was prepared using phenol/chloroform as previously described. Genomic DNA from *E. coli* strains was digested with *Pst*I, and the fragments together with molecular weight markers were separated by agarose gel electrophoresis. Following agarose gel electrophoresis genomic DNA fragments was transferred from the gel to a nylon membrane (Hybond-N^+, GE Healthcare) by capillary transfer. DNA probes were labeled with [γ^{-32}P]dATP using a random-primed DNA-labeling kit (Roche). Prehybridization and hybridization were carried out in Church buffer (0.5 M Na2HPO4 [pH 7.4], 7% sodium-dodecyl sulfate, 1 mM EDTA, 1% bovine serum albumin BSA) overnight at 54 °C. Membranes were washed in 2 x SSC (1 x SSC is 0.15 M NaCl plus 0.015M sodium citrate)–0.1% sodium dodecyl sulfate twice at 54°C for 5 min each and then

once in 0.5 x SSC, 0.1% SDS for 15 min at 65°C and autoradiographed with Kodak BioMax MS film at -70°C.

Experimental Infection of Chicks with *E. coli* Δ*dppD* and Δ*oppD* strains

One-day-old chicks were infected with APEC E956, Δ*dppD*, and Δ*oppD* strains to assess the pathogenicity of each mutant compared to uninfected and APEC E956 infected. A total of 95 one-day-old chicks were purchased (SPAFAS, Woodend Vic) and allocated to three groups of 20 chicks each and one group of 35. The birds were housed in positive pressure isolators and fed ad libetum on irradiated commercial starter feed. Cloacal swabs were taken from two chicks each from each group and streaked onto MacConkey agar to assess commensal *E. coli*. Overnight cultures of E956 Δ*dppD,* Δ*oppD* in nutrient broth with 40 µg/ml kanamycin together with E956 without kanamycin were prepared to give a concentration of 1E+10 colony forming units/ml (cfu/ml). All groups were inoculated by eye-drop with 10 times the normal immunising dose of the commercial infectious bronchitis virus vaccine Vic S (Websters, Australia) following the method developed by Ginns *et al* (1998). Groups 2 and 3 of 20 chicks each received 20 ml of an aerosol containing 1E+10 cfu/ml of ΔdppD and ΔoppD respectively whilst group 4 of 35 birds received 20 ml of 1E+10 cfu/ml of E956. Chicks in Group 1 were left untreated and served as the negative control. The chicks from Groups 2, 3 and 4 were exposed a further two times to the same APEC strain at 3 and 5 days of age. All birds were subjected to post mortem at 10 days of age. Disease was assessed by gross pathology, airsac lesions were scored according to previous criteria Ginns *et al* (1998). Swabs were taken from the left and right posterior and anterior airsacs, the trachea and aseptically from the liver. The swabs were inoculated onto MacConkey agar with and without kanamycin (40 µg/ml) and incubated overnight at 37°C. The next day the plates were observed for the presence of "brick-red" colonies (typical E. coli phenotype) their numbers were counted and recorded. If the number of colonies was less than 30 then PCR was conducted to identify *E. coli* strain. The APEC strains were identified using the primer pairs: for *dppD* WA/UA, for *oppD* WA/WT, for E956 Sidfwdseq/bwd siderophore (Table 14) which amplifies the iucA gene present on pVM01 and for commensal *E. coli* the 16s rDNA was amplified with 16Sfwd/16Srev.

To assess the efficacy of the E. coli vaccine candidate, sixty one-day-old birds were divided into three groups of 20 birds each. Groups 2 and 3 were aerosol vaccinated with E956 Δ*dppD,* Δ*oppD* at day one as above whilst group 1 remained unvaccinated as a control and housed separately in isolators as above. On day 12-post vaccination all groups were separately challenged with 20 ml of an aerosol containing 1E+10 cfu/ml of APEC E956 as above and replaced in their isolators. After 4 days all birds were euthanased by inhalation of halothane and subjected to post mortem. Airsac lesion scores were assessed as described above and swabs taken for re-isolation of infecting organisms were cultured on MacConkey agar with and without kanamycin (40 µg/ml).

Statistical analysis was performed on the rate and degree of airsac lesion scores, isolation rate of organisms and weight gain of birds. The tests used were the Fisher's Exact Test for reisolation rates and the Mann-Whitney U Test for lesion scores and weight change.

Results

Production of *E. coli* Δ*dppD* and Δ*oppD* strains

Linear DNA constructs for specific gene knockouts produced by PCR were used to transform APEC E956/pKD46. Transformants were selected on LB agar containing 40 µg/ml of kanamycin and then PCR conducted to confirm clones were carrying the kanamycin gene. The size of the amplicon from E956 Δ*dppD* using the oligonucleotide primer pair TZ/UA was predicted to be 1.774 kbp due to the insertion of the kanamycin DNA (1.064 kbp) as compared to the E956 parental strain (0.89 kbp) Figure 17b, Panel A, lanes 1 and 2 respectively. Similarly, the size of the amplicon for E956 Δ*oppD* using the oligonucleotide primer pair WS/WT was close to the predicted size of 1.924 kbp with the E956 parent strain producing an amplicon of 0.951 kbp, Figure 17b, Panel B, lanes 1 and 2 respectively. Genomic DNA of APEC strains E956, Δ*dppD* and Δ*oppD* were digested with the *Pst*I restriction enzyme, this enzyme was chosen as the kanamycin resistance gene has a single

*Pst*I site in the middle of the gene. The *dppD* and *oppD* radiolabeled probes detected bands in APEC E956, Δ*dppD* and Δ*oppD* strains (Figure 18). The *dppD* probe bound to a similar sized fragment in E956 and Δ*oppD* strains (Figure 18, lanes 1 and 3) but to a slightly lower high molecular weight band and a lower band of 2 kbp.

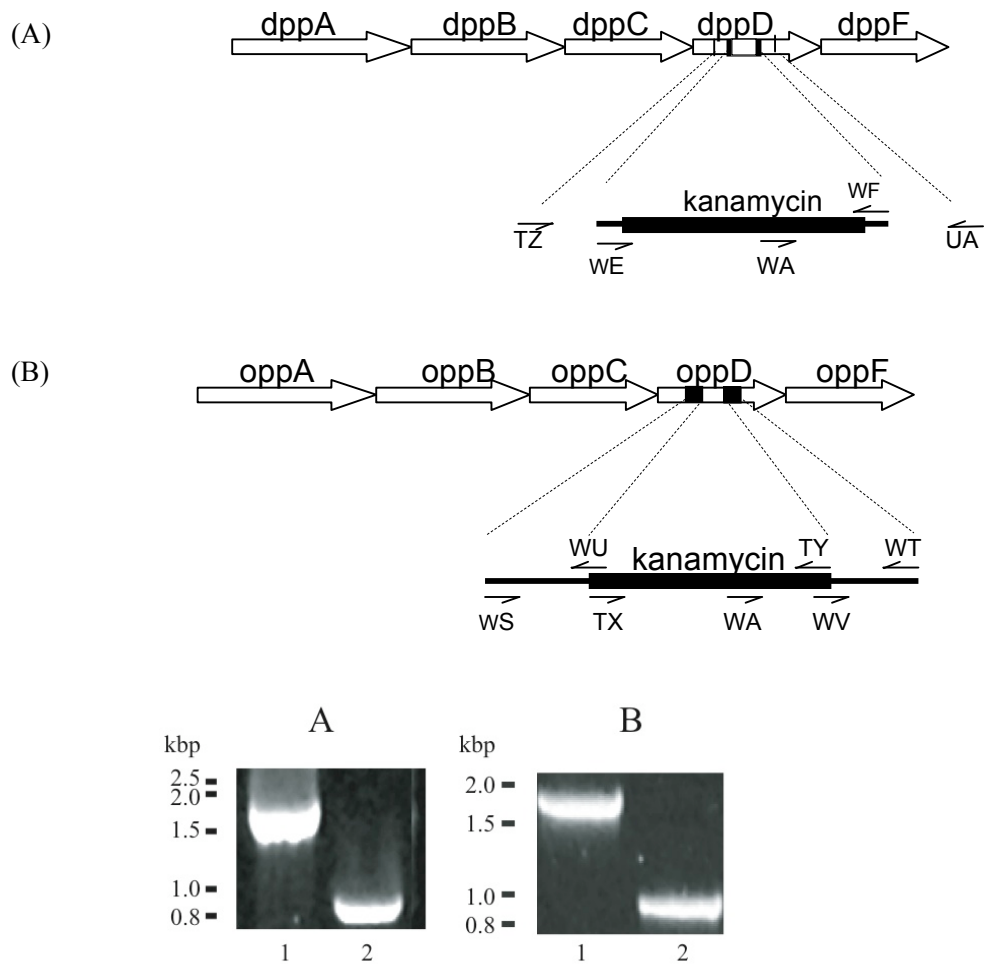


Figure 17. Schematic diagram of PCR constructs used for homologous recombination and detection of homologous recombination by PCR. The PCR products were generated with the oligonucleotide primers as described. Panel A and B: The kanamycin gene was amplified for both *oppA* and *dppD* gene constructs using the primer pair TX/TY. Construct arms were generated and added by additional PCR products using WS/WU and WY/WT for oppD or by using 60 mer primers WE/WF in PCR for dppD. Panel C: The oligonucleotide primers for dppD (A) and oppD (B) genes were used in PCR to amplify the respective regions in *E. coli* strains using the primer pair WS/WT for oppD and TZ/UA for dppD: E956 (lanes 1 of A and B), ΔdppD (A lane 1), and ΔoppD (B lane 1 panel).

The *oppD* probe bound to a similar sized fragment in E956 and Δ*dppD* strains (Figure 18, lanes 1 and 2) and to bands of 9.8 and 7 kbp in Δ*oppD* (Figure 18, lane 3). The kanamycin gene probe did not bind to E956 but to the same bands bound by the *dppD* and *oppD* probes in Δ*dppD* and Δ*oppD* strains (Figure 18, lanes 1 and 2).

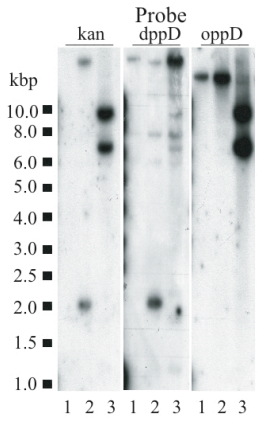


Figure 18. Southern blot of *E. coli* E956 *dppD* and *oppD* knockouts. The kanamycin, *dppD* and *oppD* probes were radiolabelled and used to probe *Pst*I restricted genomic DNA of the *E. coli* E956, Δ*dppD*, and Δ*oppD*, lanes 1, 2, and 3 respectively.

Table 15. Weight gain of chickens in pathogenicity and efficacy experiments

Challenge strain (No. birds)	**% Weight gain**		
	Pathogenicity Exp't	**Efficacy Exp't**	
	PM-vaccination	**Challenge-vac'n**	**PM-challenge**
None (20)^	174.5±30.6[a]	-	-
E956 (23)^	143.0±40.5[b]	-	-
Challenge only (20)*	-	269±29.6[a]	17.9±3.7[a]
Δ*dppD* (19)^, (19)*	143.0±36.2[b]	255±54.0[a]	19.3±3.1[a]
Δ*oppD* (20)^, (20)*	155.0±31.1[ab]	255±49.0[a]	18.4±2.4[a]

^Pathogenicity experiment. *Efficacy experiment. Values with the same subscript symbols in the same column are not significantly different (P≥0.05, Student's T-Test 2-tailed)

Table 16. Probability (P) values for statistical analysis of weight gains from table 15.

	Pathogenicity Experiment		
	Δ*dppD*	**Δ*oppD***	**E956**
Non-vacc	0.006	0.053	0.007
Δ*dppD*		0.237	0.995
Δ*oppD*			0.291

	Efficacy Experiment	
	Δ*dppD*	**Δ*oppD***
Non-vacc	0.302	0.257
Δ*dppD*		0.990

Student's T-Test 2-tailed

The chickens were examined at post mortem for signs of colibacillosis, the six airscacs were examined for lesions and a sample swab taken from the left and right anterior airsac, the trachea and liver of each bird for the reisolation of vaccine, parental or commensal organisms. The swabs were plated onto MacConkey agar with or without kanamycin added.

The results from these studies are discussed below, summarised in Tables 15, 17 and 20 with statistical comparisons made between each of the groups summarised in Tables 16, 18, 19, 21 and 22.

Table 17. Safety of *oppD* and *dppD* knockouts compared with *E. coli* E956 strain

Challenge strain (No. birds)	Re-isolation rate No antibiotics				Median no. isolated (Log_{10}+1) no antibiotics (range)		Airsac Lesion rate	Median Lesion Score
	LPTas	RPTas	Trachea	Liver	Left+Right Airsac	Trachea	(>0.5)	(range)
None (20)	2/20	3/20	11/20	4/20	0.00 (0-0.48)[a]	0.00 (0-3.70)[a]	0/20[a]	0.0 (0-0.0)[a]
E956 (23)	11/23	11/23	21/23	3/23	0.48 (0-4.00)[c]	2.48 (0-3.70)[c]	17/23[b]	3.0 (0-20)[b]
Δ*dppD* (19)	10/19	11/19	16/19	8/19	2.08 (0-4.00)[b c]	1.93 (0-3.70)[b]	11/19[bc]	1.0 (0-16)[bc]
Δ*oppD* (20)	9/20	7/20	10/20	4/20	0.00 (0-4.00)[a b]	0.85 (0-3.70)[a]	9/20[c]	0.0 (0-20)[c]

Values with the same subscript symbols in the same column are not significantly different (P≥0.05 by Mann-Whitney test, Fischer's exact 2-tailed)

Table 18. Probability (P) values for statistical analysis of airsac and tracheal re-isolation rates from table 17.

	Airsac				Trachea		
Strain	**E956**	**Δ*dppD***	**Δ*oppD***		**E956**	**Δ*dppD***	**Δ*oppD***
Non-vacc	0.017	0.0105	0.0399		7e-06	0.0005	0.3589
E956		0.4299	0.1696			0.0238	0.00012
Δ*dppD*			0.1231				0.0089

Mann-Whitney test

Table 19. Probability (P) values for statistical analysis of airsac lesion score and rate from table 17.

	Lesion Score*				Lesion Rate^		
Strain	**E956**	**Δ*dppD***	**Δ*oppD***		**E956**	**Δ*dppD***	**Δ*oppD***
Non-vacc	1e-05	0.0003	0.0154		0.008	0.00001	0.000003
E956		0.092	0.0065			0.0561	0.112
Δ*dppD*		-	0.1415				1.0

*Mann-Whitney test, ^Fischer's exact 2-tailed

Table 20. Efficacy studies using *E. coli* E956 strain, *oppD* and *dppD* knockouts

Challenge (No. birds)	Re-isolation rate No antibiotics				Median no. isolated (Log_{10}+1) No antibiotics (range)		Airsac Lesion rate	Median Lesion score
	LPTas	RPTas	Trachea	Liver	Left+Right Airsac	Trachea	(>0.5)	(range)
E956 (20)	2/20	1/20	8/20	0/20	0.00 (0-2.25) [a]	0.15 (0-1.88) [a]	13/20 [a]	1.25 (0-4.5) [a]
Δ*oppD* (20)	1/20	0/20	9/20	0/20	0.00 (0-1.43) [a]	0.00 (0-2.80) [a]	6/20 [b]	0.0 (0-14) [b]

Values with the same subscript symbols in the same column are not significantly different (P≥0.05 by Mann-Whitney test or Fischer's exact 2-tailed test)

Table 21. Probability (P) values for statistical analysis of airsac and tracheal re-isolation rates from table 17.

	Airsac		Trachea
Strain	**Chall only**		**Chall only**
Δ***oppD***	0.130		0.0004

Mann-Whitney test

Table 22. Probability (P) values for statistical analysis of airsac lesion scores and rates from table 17.

	Lesion Score*		Lesion Rate^
Strain	**Chall only**		**Chall only**
Δ***oppD***	0.011		0.052

*Mann-Whitney test, ^Fischer's exact 2-tailed

Analysis of Chick Experiment

The difference between the percentage weight gains in the pathogenicity experiment between E956 (143%), Δ*dppD* (143%) and non-vaccinated chickens (174%) was marginally significant whilst no significant difference was seen with Δ*oppD* (155%) to that of non-vaccinated birds (Table 15 and 16). In the efficacy experiment there was no statistically significant difference in the percentage weight gain of any bird group before challenge or at post mortem (Table 15 and 16).

There was no significant difference between the median numbers of organisms reisolated from the airsac or trachea of control and Δ*oppD* vaccinated birds in the pathogenicity experiment (Table 17). There were higher numbers of organisms isolated from those birds vaccinated with E956 or Δ*dppD* than non-vaccinated birds (Table 17). The results for the efficacy experiment (Table 20) show no difference between the E956 challenged and Δ*oppD* vaccinated birds. There was no significant difference in the rate of isolations made from the trachea versus airsacs in birds vaccinated with either Δ*dppD* or Δ*oppD* though a significant increase in isolation of organisms from the trachea compared to the airsacs was observed with birds vaccinated with E956 ($P<0.05$).

Examination of the airsacs and organs at post mortem showed signs of colibacillosis in some birds with perihepatitis and pericarditis observed. In the pathogenicity experiment there was no statistically significant difference between birds vaccinated with either Δ*dppD* or Δ*oppD* in the rate and lesion score of the airsacs, there were less lesions observed between Δ*oppD* and E956 but no statistical difference between Δ*dppD* and E956 vaccinated birds (Table 17 and 19). In the protection experiment, comparison of lesion rate and scores of Δ*oppD* vaccinated and E956 challenged birds showed a statistically significant protective effect from prior vaccination with Δ*oppD* with a median lesion rate of 0.0 for vaccinates and 1.25 for the non-vaccinated and challenged group (Table 20 and 22).

During the efficacy trial (following the pathogenicity trial) the facility housing the birds suffered a power failure due to flooding in the early hours of the morning. Emergency response teams that included the fire brigade and later on animal house personnel arrived at the facility. Inspection of the facility showed several of the isolators were without power for a period of time and there was concern the experiment had been compromised. It was decided to bring forward the post mortem. Due to these events we performed extra analysis using both PCR and Southern blot; results showed the presence of the Δ*oppD* vaccine in our non-vaccinated control and Δ*dppD* vaccinated groups. The absence of Δ*oppD* in the E956 group provided us with the opportunity to use these data in our analysis.

Discussion of Results

In this study we produced two knockout clones in the *dppD* and *oppD* genes within the dipeptide and oligopeptide operons respectively. This was accomplished using the red recombinase system using the kanamycin resistance gene to select for recombinants. The two gene knockouts were then tested in *vivo* for attenuation and efficacy.

The red recombinase system constructs were prepared using PCR (Figure 17 and Table 14). The *dppD* construct contained 40 bp DNA stretches 5' and 3' that were homologous to the *dppD* region to be knocked out. Numerous attempts were made to produce knockouts in the APEC E3/3.4 strain, changing many of the original parameters but to no avail. We did produce *dppD* gene knockouts in the *E. coli* laboratory strain 1821 and in APEC E956 strain with this construct though only a single clone was obtained for each strain. Through increasing the length of the homologous regions at the 5' and 3' ends to 400 bp we successfully produced numerous knockouts in APEC E956, suggesting that by increasing these regions to 400 bp regions we improved homologous recombination.

The insertion of each of these constructs into their respective genes producing E956 Δ*dppD and* Δ*oppD* strains is shown in Figures 17C.

The Δ*dppD and* Δ*oppD* strains were tested for attenuation and their ability to protect chickens against challenge from wildtype infection. Two experiments were run in conjunction to assess these characteristics. Vaccination and challenge cultures were administered by aerosol as per previous methods developed in our facility. The results of attenuation for each strain were accessed using the following parameters: percentage weight gains, re-isolation rates of organisms from the airsacs and trachea and lesion rate and score; all were analysed statistically. The Δ*dppD* and E956 strains were statistically different to non-vaccinates in all parameters tested. The Δ*oppD* strain appeared to be intermediate in pathogenicity with no significant difference to non-vaccinated birds in weight gain or re-isolation rates (Table 16 and 17). Whilst no difference

was observed with lesion rates and scores of Δ*oppD* compared to Δ*dppD* strain there were significant differences to the parent strain E956 (Table 17).
There appeared to be some benefit associated with Δ*oppD* vaccination as revealed from results from the efficacy experiment. Δ*oppD* vaccination resulted in reduction in the rates of organism re-isolation, reduced airsac lesion score and rate (Table 20). Vaccination with either Δ*dppD* or Δ*oppD* did not significantly change weight gain compared to non-vaccinated (Table 15) nor was there any affect seen following challenge though this was using the pathogenic E956 strain and as such there was no benefit in weight gain from vaccination.
The efficacy experiment was compromised from the power failure following flooding resulting in premature ending of the experiment and resultant breakdown in hygiene control. The occurrence of the Δ*oppD* strain as identified by PCR in the non-vaccinated and non-challenged group and in the Δ*dppD* vaccinated group has compromised the findings surrounding Δ*dppD* and non-vaccinated groups. The E956 group appeared to be unaffected and remained free of the two-vaccine strains Δ*dppD* and Δ*oppD*

Implications

By inactivating the *oppD* gene/operon we were able to attenuate an *E. coli* strain which was capable of producing colibacillosis disease in chickens. This methodology and approach would be useful for producing live attenuated vaccine strains for a number of bacterial diseases of chickens and or other animal species. The methodology and intellectual property generated in this project has resulted in a patenting this technology.

Recommendations

Attention should be paid to other bacterial species that cause disease in poultry, investigated for there susceptibility to the same methodology and then vaccines produced. There is also a need to have a means to remove the antibiotic resistance marker and perhaps incorporate another attenuating mutation for regulatory acceptance.

Introduction

Genome Sequencing of *M. gallisepticum* parent and vaccine strains

Differentiation and detection of the *M. gallisepticum* ts-11 vaccine strain from wild-type infection is a goal of the poultry industry. An approach to achieve this goal is to determine the genome sequence of the *M. gallisepticum* strain ts-11 and 80083 (vaccine and parent strains respectively) and by comparing the genomes determine the unique attributes of the vaccine strain. It will be possible to determine the temperature sensitive genotype and also determine the unique VlhA antigen expressed by the vaccine. Once identified the antigen can then be cloned and expressed so as to improve the serological detection of vaccinated flocks by ELISA.

Objectives

Genomic sequencing of *M. gallisepticum* strain ts-11 and 80083 to identify genetic attenuation and improve and develop diagnostic tests.

Methodology

Commercial Sequencing of *M. gallisepticum* parent and vaccine strains

M. gallisepticum strain ts-11 and 80083 was cultured at 37°C in broth medium until late logarithmic phase. The cells were harvested by centrifugation, washed three times in PBS and DNA extracted using phenol-chloroform. The DNA amount was quantified by gel electrophoresis and 10 µg of each strain shipped on dry-ice to 454 Life Sciences Corporation, Branford, CT 06405 USA.
Sequencing was conducted on a 454's GS20 instrument.

Results

Analysis of DNA sequence

The machine was reported to be able to read up to 25 million bases of a bacterial genome in a single four-hour run. The beads were deposited with 30% efficiency on a glass Picotitre Plate with approximately 1.6 million wells, a well center-to-center distance of 50 µm, and a well depth of 55 µm. The GS20 was able to obtain average sequencing read lengths of 110 bases with a raw read accuracy of 96%.

The assembled data that was returned comprised 330 and 376 DNA stretches (contigs) of the Parent and Vaccine strains respectively. To assemble the genomes of the Parent and Vaccine the *M. gallisepticum* R strain genome was used as a scaffold, the contigs were aligned to the R strain genome using the CodonCode Aligner commercially available computer program. The size of the contigs from each strain varied with the vast number of contigs in the 1000-5000 bp range (Fig. 19) though three contigs for the Parent strain were greater than 90,000 bp in length.

There were a number of assembly problems to contend with as the *M. gallisepticum* R strain reference genome contained 43 *vlhA* genes with 50-98% DNA identity located in five loci, twelve transposons, one complete and one incomplete rRNA operon. The total size of the R strain genome is 996,442 bp including *vlhA* genes that comprise 99,754 bp (around 10% of the genome).

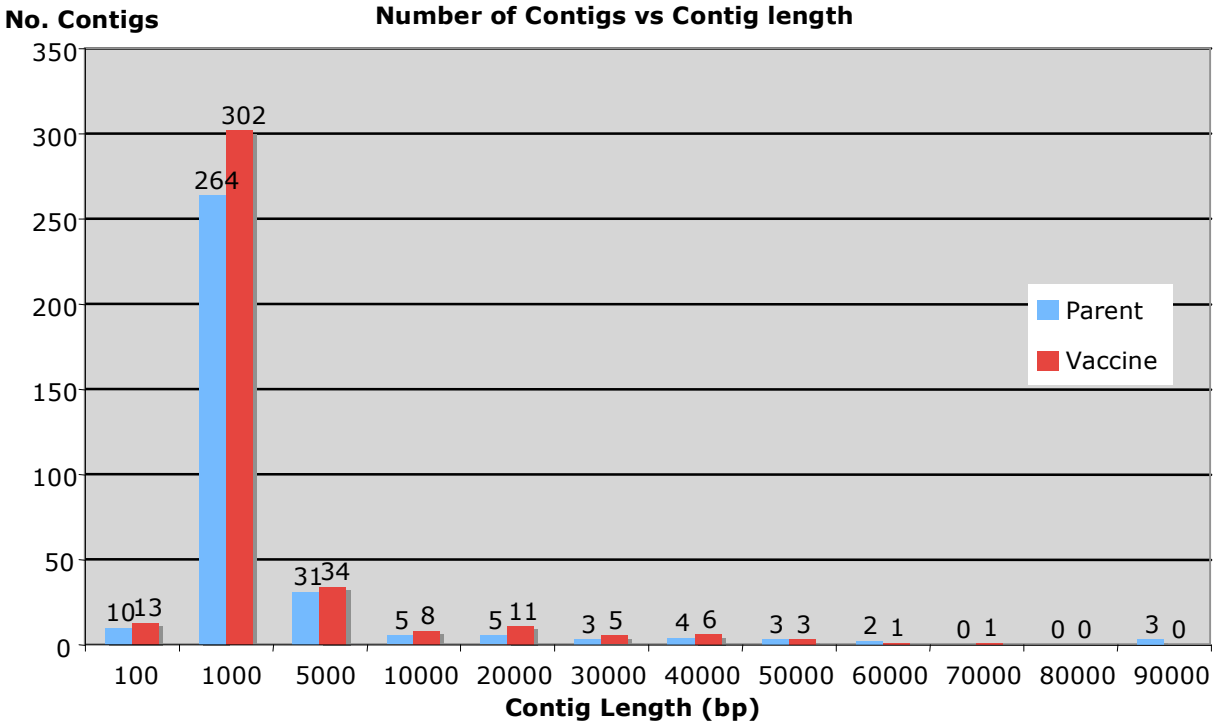


Figure 19. Contig length versus number of contigs for Parent and Vaccine strains. Number above bars indicate number of contigs found.

The alignment of contigs to the *M. gallisepticum* R strain genome used as the reference genome sequence is shown graphically in Figure 20. The *M. gallisepticum* R strain is comprised of 747 open reading frames (ORFs), most Parent and Vaccine strain contigs cover the ORFs with the exception of vlhA genes and some transposons. There were a number of base substitutions (both synonomous and non-synonomous) and indels between the *M. gallisepticum* R strain ORFs and those of the Parent and Vaccine strains. The number of base substitutions (both synonomous and non-synonomous) are presented as percentage base pair difference for individual genes in Figure 21, the average was 2.16 % bp per ORF. The occurrence of indels between the *M. gallisepticum* R strain ORFs and those of the Parent and Vaccine strains was investigated further and are presented in Table 23. The occurrence of a 20 bp duplication in the vaccine strain is a known indel, no other large indels were observed between *M. gallisepticum* R strain or the parent or vaccine strains.

Following alignment of the Parent and Vaccine contigs to the *M. gallisepticum* R strain genome a number of contigs remained unaligned

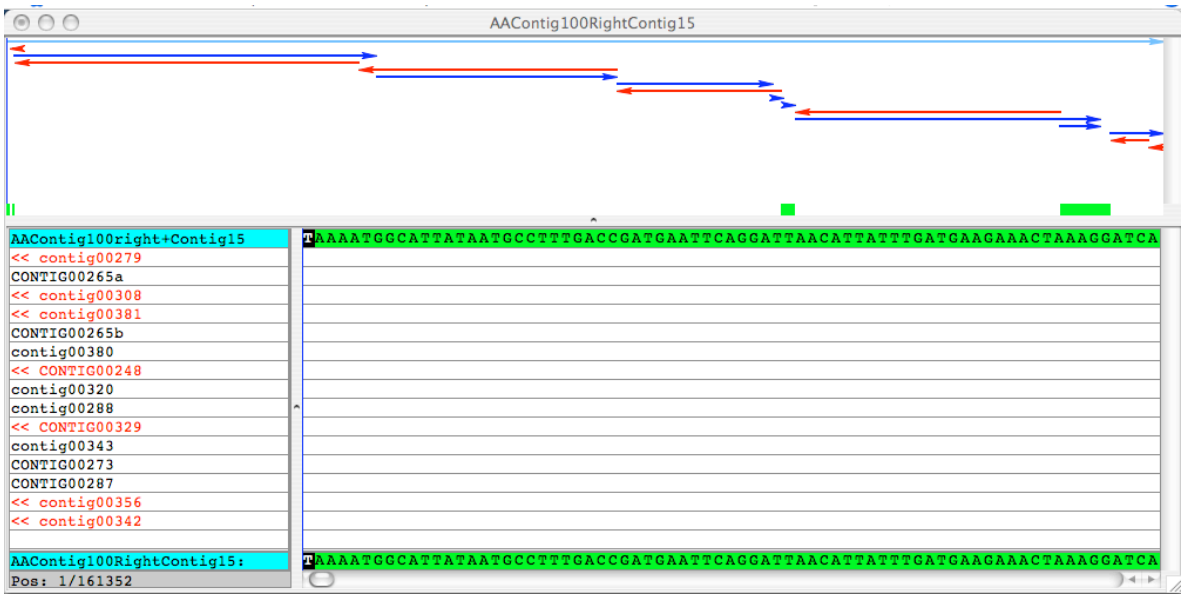


Figure 20. Graphical representation of both Parent and Vaccine contigs aligned to the *M. gallisepticum* R strain genome.

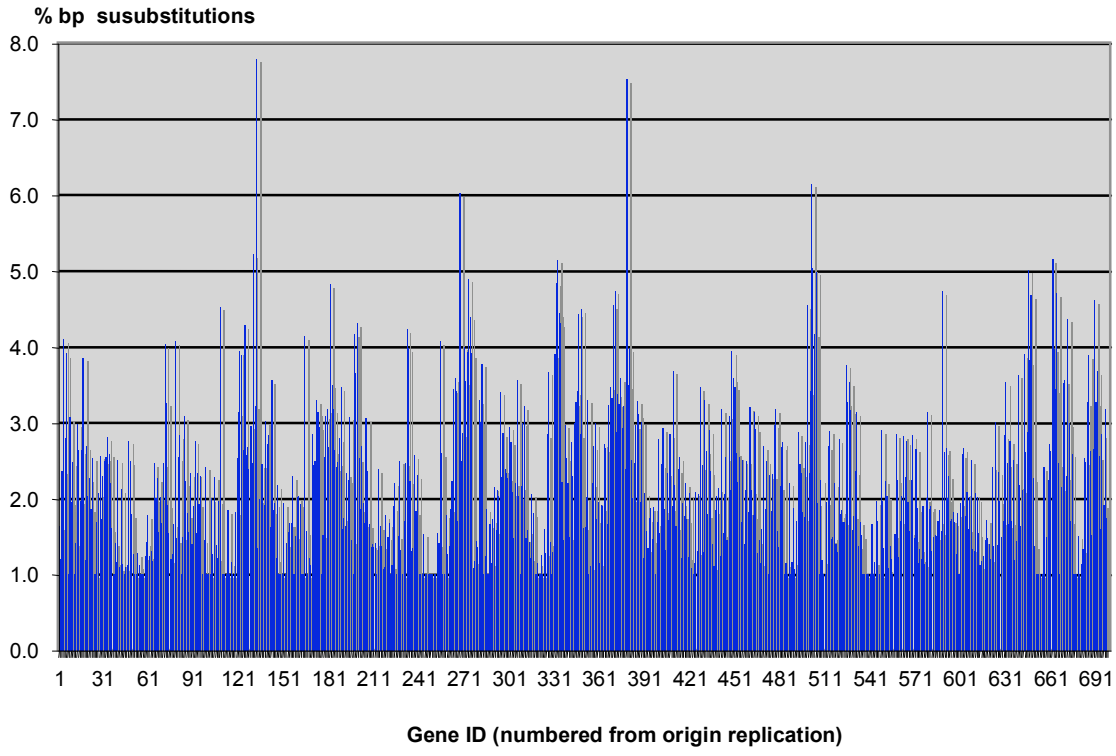


Figure 21. Percentage of base substitutions versus ORF (Gene ID number).

Table 23. Large indels found between *M. gallisepticum* R strain and parent and vaccine strains

Assigned Name	Gene ID	DNA Differences
UH	MG_1071	24 bp duplication in R strain
gapA	MG_0934	20 bp duplication in vaccine
CH	MG_1122	21 bp duplication in R strain

CH	MG_1199	18 bp duplication in R strain
rpoD	MG_0012	9 bp duplication in R strain
CH	MG_0023	9 bp duplication in R strain
CH lipoprotein	MG_0267	12 bp duplication in R strain
CH lipoprotein	MG_0274	42 bp duplication in P&V
HMW1	MG_0306	18 bp duplication in P&V
CH	MG_0337	9 bp duplication in P&V
UH	MG_0403	9 bp duplication & 6 bp duplication
UH	MG_0566	21 bp addition in R strain
rnpA	MG_0630	42 bp duplication in P&V
rpsE	MG_0737	15 bp duplication in R strain
cdsA	MG_0785	6 bp addition in R strain

UH: Unique hypothetical
CH: Conserved hypothetical
P&V: Parent and Vaccine

Comparison of the ORFs of *M. gallisepticum* parent and vaccine strains revealed 74 indels, each producing a frame shift resulting in premature truncation of the peptide, most of these indels occurred following a single nucleotide repeat which is an artefact of the sequencing technique used. No indels occurred in a gene of the vaccine that may produce the Ts phenotype. A PCR was designed to amplify the indel region of 18 genes of the vaccine strain. The DNA sequence of the PCR product was determined and compared to that of the Parent and R strain and in all cases the indel in the vaccine contig was not present, reaffirming the problems associated with DNA sequencing artefacts.
To improve coverage and linkage of the contigs of the vaccine strain a total of 162 oligonucleotide primers were synthesised and 252 DNA sequencing reactions to "fill-in" missing DNA regions performed. The number of contigs remaining following these "fill-in" reactions resulted in 9 contigs that were aligned to the *M. gallisepticum* R strain genome, interspersed between the contigs were 5 vlhA loci and several gene repeats and transposons (Fig. 22).

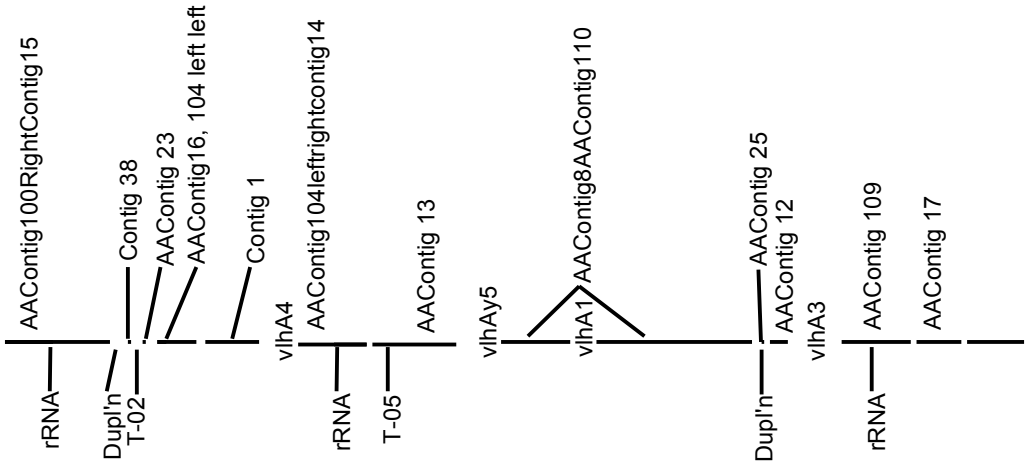


Figure 22. Schematic representation of the *M. gallisepticum* vaccine strain contigs aligned to the *M. gallisepticum* R strain genome. Shown are the *vlhA* loci 1 to 5, rRNA regions, transposons (Tn) and DNA repeats. rRNA: ribosomal DNA sequence, T-02 or T-05: transposase genes, vlhA1-5: vlhA gene loci, Dupl'n: DNA dupication

Identification of expressed *vlhA* gene

The DNA regions bordering each of the vlhA loci were identified and DNA clones obtained by PCR, random genome cloning and targeted sequencing produced 19 full-length *vlhA* genes.
The monoclonal antibody affinity purified VlhA gene was subjected to peptide mass fingerprinting. Following *in silico* searches of the vlhA gene sequences, a single VlhA sequence was identified with 15

peptides matching in MW to that determined by PMF. The amino acid sequence of the VlhA peptide is shown in Figure 23.

>VlhAVaccine
VKRKNILKFVSLLGIGSFVMLAAASCTPTPNPNPPSNGGMNGGNTNPGDG
QGMMNATSQELAAARMGLTTVFDSKAKNLGLYVDYKKTQDTLTKAYDAAK
TVLDNSSSTTQNLNEAKTRLETAIRTAATSK**QTFDEQHAELVKVYEELKT**
TLSNETAALAPYADAQYAGIKMHLSGLYDAGKAITTKTLEPVEGDPLTAN
AVMMANTK**IVEAIKDEVLNPQK**ENATKLADSFVKQVLVKEKVTGVEEAHN
KVQPANYSFVGYSVDITGTTNGQTSIPNWDYAQRTIFTNGDEPR**SVSNTP**
VDGQTMAQPLSNVSWIYSLAGTGAKYTLEFTYYGPSTGYLYFPYKLVNTS
DQMKLGLEYK**LNDATEPSAITFGSEQTMNGK**TPTVNDINVAKVTLANLK**F**
GSNKIEFSVPAEKVSPMIGNMYLSSSPNNWNKIYDDIFGNSVTTKNNRTI
ISVDALNGYSLASDWSTFIAEYSGTGLTLDNQNVSNQK**YYLIGYVGGTSS**
RNDMMVSKNNVQK**FPLASNTTNRNYTFYVNAPK**AGAYYIKGVFASRDPRD
LKFSTGDMSSNNNVTIK**QLSTDNLTTLRTFDTFATTEPTR**DTTVSDRKTL
TLVEGLNKIVVSGATANIGSAPNFGYLEFILNETQPESSNVSSPSY

Figure 23. Amino acid sequence of expressed VlhA peptide of *M. gallisepticum* vaccine strain. Matching tryptic PMF peptide mass with those determined by *in silico* analysis of the VlhA peptide are in bold. Occurrence of match by chance alone: 5e-017

Discussion of Results

The DNA sequencing technology available at the time limited the results from genome sequencing of the *M. gallisepticum* parent and vaccine strains. Since then high thru-put sequencing is now offered by a number of companies each using different technologies. The G20 analyzer originally used produced 100-150 bp reads which in themselves are unable to bridge larger DNA repeats such as transposons, rDNA sequences, repeat DNA sequences and *vlhA* genes. It was thought the use of the *M. gallisepticum* R strain genome would overcome this problem but due to the inherent short reads and incorrect DNA sequence resulted in incomplete genome sequences of *M. gallisepticum* parent and vaccine strains. Alignment of the ORFs between the *M. gallisepticum* R, parent and vaccine strains was completed with the aim to identify an ORF responsible for producing the Ts phenotype. The alignment revealed a number of indels and initial attempts to identify "real" indels focussed on genes with a predicted function. None of the indels were real and occurred after a run of single nucleotide repeats, a problem that is know to occur in this type of sequencing. The use of NTG to produce the Ts phenotype is thought to create mutations around the replication fork of the bacteria and as such a cluster of mutations may be expected to be present. This "mutation cluster" was not observed in the aligned contigs suggesting either the mutation may occur outside an ORF and affect gene regulation, the mutation is present in an ORF and we have not resequenced this region or it may lie in an area that remains unsequenced or unplaced in a contig perhaps a vlhA gene. Another method such as pair-ended sequencing of the vaccine may produce a more complete genome picture and capitalise on the DNA sequence already obtained.

The *M. gallisepticum* vaccine VlhA gene used as an antigen in the ELISA was identified using peptide mass fingerprinting. The *vlhA* gene was cloned using PCR and is now expressed in an expression vector and is undergoing experiments to determine how suitable it is as an ELSA antigen. It may be that certain regions of the gene may need to be trimmed to remove cross reactivity with antibodies to *M. synoviae*.

Implications

Though the DNA mutation(s) responsible for producing the Ts phenotype of the *M. gallisepticum* vaccine strain was not determined there is now the basis to use more current technologies to capitalise on the DNA sequence determined so far. There is a relatively long DNA sequence present that differentiates the *M. gallisepticum* parent and vaccine strains from the R strain though not the AP3AS challenge strain, this region could be exploited to differentiate strains.

The VlhA peptide present in the *M. gallisepticum* vaccine strain was identified and is cloned and expressed. This antigen would form the basis of an ELISA for the sensitive and specific serological detection of *M. gallisepticum* vaccine strain vaccinated flocks.

Recommendations

To capitalise on the DNA sequence already produced for this project and use paired-end sequencing to produce complete genome maps of the *M. gallisepticum* parent and vaccine strain. The ability to cheaply DNA sequence small genomes is now widely available with machines located at numerous sites in Australia. To support the initial production of VlhA antigen for commercial ELISA use such as BioCheck MG ELISA.

Acknowledgements

UNSW Project leader
Dr Wendy Muir

PhD students
SangWon Lee
ChiWenTseng

Research Assistants
Anna Kanci
Douglas Craick

Visiting Research Fellows
Dr Joanne Chen
Dr Yoshihiro Muneta

Collaborators
AsProf Amir Noormohammadi
Prof Glenn Browning
Dr Robert Moore
Dr Tamsyn Crowley
Dr Andrew Bean
Dr Pat Blackall
Dr Christine Citti

Laboratory Personnel
Cynthia Brown
Tony Belfiore
Cheryl Colson

References

Avakian, A. P. & Ley, D. H. (1993). Protective immune response to Mycoplasma gallisepticum demonstrated in respiratory-tract washings from Mycoplasma gallisepticum infected chickens. *Avian Diseases* **37**, 697-705.

Cole, B. C. & Wells, D. J. (1990). Immunosuppressive properties of the Mycoplasma arthritidis T-Cell Mitogen in vivo. Inhibition of proliferative responses to T-cell mitogens. *Infect Immun* **58**, 228-236.

Collett, S. R., Thomson, D. K., York, D. & Bisschop, S. P. (2005). Floor pen study to evaluate the serological response of broiler breeders after vaccination with ts-11 strain Mycoplasma gallisepticum vaccine. *Avian Dis* **49**, 133-137.

Gaunson, J. E., Philip, C. J., Whithear, K. G. & Browning, G. F. (2000). Lymphocytic infiltration in the chicken trachea in response to Mycoplasma gallisepticum infection. *Microbiology* **146**, 1223-1229.

Ghorashi, S., Noormohammadi, A. & Markham , P. (2009). Differentiation of *Mycoplasma gallisepticum* strains using PCR and High Resolution Melting Curve Analysis. *Microbiology*.

Kidney, J. C. & Proud, D. (2000). Neutrophil transmigration across human airway epithelial monolayers: mechanisms and dependence on electrical resistance. . *American Journal of Respiratory Cellular and Molecular Biology* **23**, 389–395.

Kogut, M. H., Pishko, E. J., Kaspers, B. & Weining, K. C. (2001). Modulation of functional activities of chicken heterophils by recombinant chicken IFN-gamma. *Journal of Interferon and Cytokine Research* **21**, 85–92.

Kogut, M. H., Rothwell, L. & Kaiser, P. (2005). IFN-gamma priming of chicken heterophils upregulates the expression of proinflammatory and Th1 cytokine mRNA following receptor-mediated phagocytosis of *Salmonella enterica* serovar *enteritidis*. *J Interferon Cytokine Res* **25**, 73–81.

Lee, S.-W., Browning, G. F. & Markham, P. F. (2008). Development of a replicable oriC plasmid of *Mycoplasma gallisepticum* in *Mycoplasma imitan*s and gene disruption through homologous recombination in *Mycoplasma gallisepticum*. *Microbiology* **154**, 2571-2580.

Ley, D. L. (2008). *Mycoplasma gallisepticum* infection. In *Diseases of Poultry*, 12th edition edn, pp. 807-834. Edited by Y. M. Saif, Fadly, A.M., Glisson, J.R., McDougald, L.R., Nolan, L.K., Swayne, D.E. Ames: Blackwell Publishing Ltd.

Markham, P. F., Kanci, A., Czifra, G., Sundquist, B. G., Hains, P. & Browning, G. F. (2003). Homologue of macrophage-activating lipoprotein in Mycoplasma gallisepticum is not essential for growth and pathogenicity in tracheal organ cultures. *Journal of Bacteriology* **185**, 2538-2547.

Muneta, Y., Panicker, I. S., Kanci, A., Craick, D., Noormohammadi, A. H., Bean, A., Browning, G. F. & Markham, P. F. (2008). Development and immunogenicity of recombinant Mycoplasma gallisepticum vaccine strain ts-11 expressing chicken IFN-gamma. *Vaccine* **26**, 5449-5454.

Noormohammadi, A., Browning Glenn, F., Cowling Peter, J., O'Rourke, D., Whithear Kevin, G. & Markham Philip, F. (2002a). Detection of antibodies to Mycoplasma gallisepticum vaccine ts-11 by an autologous pMGA enzyme-linked immunosorbent assay. *Avian Diseases* **46**, 405-411.

Noormohammadi, A. H., Jones, J. E., Underwood, G. & Whithear, K. G. (2002b). Poor systemic antibody response after vaccination of commercial broiler breeders with Mycoplasma gallisepticum vaccine ts-11 not associated with susceptibility to challenge. *Avian Diseases* **46**, 623-628.

Papazisi, L., Gorton, T. S., Kutish, G., Markham, P. F., Browning, G. F., Nguyen, D. K., Swartzell, S., Madan, A., Mahairas, G. & Geary, S. J. (2003). The complete genome sequence of the avian pathogen Mycoplasma gallisepticum strain R(low). *Microbiology* **149**, 2307-2316.

Roussan, D. A., Haddad, R. & Khawaldeh, G. (2008). Molecular survey of avian respiratory pathogens in commercial broiler chicken flocks with respiratory diseases in Jordan. . *Poultry Science* **87**, 444–448.

Striz, I., Mio, T., Adachi, Y., Carnevali, S., Romberger, D. J. & Rennard, S. I. (2000). Effects of interferons alpha and gamma on cytokine production and phenotypic pattern
of human bronchial epithelial cells. . *International Journal of Immunopharmacology* **22**, 573–585.

Sykes, J. E., Allen, J. L., Studdert, V. P. & Browning, G. F. (2001). Detection of feline calicivirus, feline herpesvirus 1 and Chlamydia psittaci mucosal swabs by multiplex RT-PCR/PCR. *Veterinary Microbiology* **81**, 95-108.

Teh, H. S., Ho, M. & Williams, L. D. (1988). Suppression of cytotoxic responses by a supernatant factor derived from *Mycoplasma hyorhinis*-infected mammalian cell lines. *Infection and Immunity* **56**, 197–203.

Whithear, K. G. (1996). Control of avian mycoplasmoses by vaccination. *Revue Scientifique et Technique* **15**, 1527-1553.

Appendices 1-4

Microbiology (2008), **154**, 2571–2580 DOI 10.1099/mic.0.2008/019208-0

Development of a replicable *oriC* plasmid for *Mycoplasma gallisepticum* and *Mycoplasma imitans*, and gene disruption through homologous recombination in *M. gallisepticum*

S.-W. Lee, G. F. Browning and P. F. Markham

School of Veterinary Science, The University of Melbourne, Parkville, Victoria 3010, Australia

Correspondence
P. F. Markham
pmarkham@unimelb.edu.au

Received 9 April 2008
Revised 8 June 2008
Accepted 9 June 2008

The genome of *Mycoplasma gallisepticum* strain R_{low} has been sequenced completely, but subsequent genetic studies have been limited by the lack of a replicable vector system. In this study, replicable plasmids were constructed for *M. gallisepticum* and *Mycoplasma imitans* using the *oriC* region upstream from the *soj* gene. The *oriC* plasmids of *M. gallisepticum* (pGTLori) and *M. imitans* (pMIori) replicated in both species, but *Mycoplasma pneumoniae* could not support replication of pGTLori. A 180 bp section of the *oriC* region of *M. gallisepticum* was found to be the minimal region required for plasmid replication in *M. gallisepticum* strain S6, the shortest *oriC* region defined for mycoplasmas. Targeted gene disruption of *vlhA1.1* of *M. gallisepticum* S6 was attempted using these *oriC* plasmids. Constructs made in pPLoriC7 integrated into the *M. gallisepticum* genomic *oriC* region, not into the targeted gene, whereas those made in pMIori disrupted the *vlhA1.2* gene, which has 97 % DNA sequence identity with the *vlhA1.1* gene. During *in vitro* passages, antimicrobial selection pressure did not influence the rate of chromosomal integration. These *oriC* plasmids will thus be useful for genetic studies, including inactivation or expression of selected genes, in *M. gallisepticum* and *M. imitans*, and will lead to a better understanding of their molecular biology. They are, to our knowledge, the first replicable plasmids developed for the Pneumoniae phylogenetic group of mycoplasmas.

INTRODUCTION

Mycoplasma gallisepticum is an important pathogen of poultry, causing chronic respiratory disease in chickens and infectious sinusitis in turkeys. It is responsible for considerable economic losses in poultry production worldwide due to downgrading of carcasses, reduced feed conversion efficiency, decreased egg production and increased medication costs (Ley & Yoder, 1997). Recently the genome of *M. gallisepticum* was sequenced completely (Papazisi *et al.*, 2003), allowing prediction of the function of specific genes. However, genetic studies of *M. gallisepticum* have been limited by the lack of genetic tools for its manipulation, with most studies using transposons Tn*916* (Dybvig & Cassell, 1987; Dybvig & Alderete, 1988; Whetzel *et al.*, 2003) and Tn*4001* (Bearson *et al.*, 2003; Hedreyda *et al.*, 1993; Hudson *et al.*, 2006; Mahairas & Minion, 1989; Mudahi-Orenstein *et al.*, 2003; Papazisi *et al.*, 2002; Whetzel *et al.*, 2003; Winner *et al.*, 2003) or suicide vectors (Markham *et al.*, 2003) to study gene function. However, the random insertion of the transposon in the genome of the organism does not allow specific targeting of a gene of interest, and random integration can confound analyses of gene expression (Dybvig & Cassell, 1987; Mahairas & Minion, 1989). There has been some success in development of vectors for mollicutes using homologous origins of replication (*oriC*) and selectable antibiotic resistance markers. These plasmids are able to replicate extrachromosomally and have been used to inactivate target genes by homologous recombination (Cordova *et al.*, 2002; Duret *et al.*, 1999; Janis *et al.*, 2005; Lartigue *et al.*, 2002). In many cases *oriC* plasmids containing larger *oriC* regions have been found to integrate into the *oriC* region in the genomic DNA by homologous recombination following *in vitro* passage. Plasmids containing a shorter *oriC* region are more stable and have been used to generate targeted homologous recombination (Cordova *et al.*, 2002; Lartigue *et al.*, 2002; Renaudin *et al.*, 1995). Most of the *oriC* plasmids of mollicutes show host specificity, but plasmids containing the *oriC* of *Mycoplasma mycoides* subsp. *mycoides* LC and SC replicate in the closely related species *Mycoplasma capricolum* subsp. *capricolum* (Lartigue *et al.*, 2003). *Mycoplasma imitans*, which has been isolated from ducks and geese, is phylogenetically closely related to *M. gallisepticum*. DNA–DNA hybridization studies show

The GenBank/EMBL/DDBJ accession number for the *oriC* region sequence of *M. imitans* is EF028085.

that *M. gallisepticum* and *M. imitans* share 40–46 % genetic identity (Bradbury *et al.*, 1993), whilst PFGE and random amplified polymorphic DNA studies suggest a DNA sequence identity of 53–60 % (Bradbury *et al.*, 1993; Marois *et al.*, 2001). Papazisi *et al.* (2003) reported that the predicted *oriC* region of *M. gallisepticum* was located between the *dnaN* and *dnaA* genes. The predicted *oriC* region contains five DnaA box sequences, with the consensus sequence 5′-TTWTMHAMA-3′ identical for each DnaA box, and the region between *dnaN* and *soj* contains a higher than average content of adenosine and thymidine (80 %). However, the DNA sequence of the *M. imitans oriC* region has not been determined. The aims of this study were to determine the DNA sequence of the *M. imitans oriC* region and to produce vectors containing different regions of the *oriC* of *M. gallisepticum* and *M. imitans* to assess their replication and stability during passage. We also attempted to inactivate a target gene in *M. gallisepticum* using these *oriC* plasmids. We chose to knockout the expressed VlhA1.1 gene of *M. gallisepticum* as the genome sequence is available and the target gene is not essential for the survival of the organism (Glew *et al.*, 2000).

METHODS

Bacterial strains and growth media. *M. gallisepticum* strain S6 (Markham *et al.*, 1992) and *M. imitans* strain 4229 (Bradbury *et al.*, 1993) were grown in modified Frey's medium to late exponential phase at 37 °C (Whithear, 1993). *Mycoplasma pneumoniae* strain FH (ATCC No. 15531) (donated by V. Peters, Department of Virology, Royal Children's Hospital, Melbourne) was grown in modified Hayflick's medium in tissue-culture flasks. For the growth and selection of antibiotic-resistant mycoplasma transformants, tetracycline was added to a concentration of 4 μg ml^{-1} in broth or agar medium (Markham *et al.*, 2003). *Escherichia coli* strain DH5α was used as the host for gene cloning and was grown in Luria–Bertani (LB) broth at 37 °C and subjected to standard molecular biological techniques (Sambrook & Russell, 2001).

Amplification and DNA sequencing of the *oriC* region of *M. imitans* strain 4229. Part of the *oriC* region and the *dnaA* gene of *M. imitans* was amplified using the primer pairs SW15for and MIsojrev-1, and SW6for and dnaArev, respectively (Table 1), and cloned into pGEM-T for DNA sequencing. The remainder of the *oriC* region was sequenced directly from genomic DNA. The DNA sequence was aligned with the homologous region from the complete genome of *M. gallisepticum* strain R_{low} (GenBank accession no. NC_004829).

Table 1. Oligonucleotides used in this study

Oligonucleotides	Sequence* (5′–3′)	PCR product
SW4 for/SW5 rev	GAGCTTTTGCATGCAAAAAATTTC/ TTGTTAATCCATGGAATGATCATAA	Long *oriC* region (LoriC; 1.96 kb)
SW6 for/SW7 rev	CCTCAGCTGCAGTAGGTTATG/ GAACTGAAATGTCGACTAAAAATC	Short *oriC* region (SoriC; 0.67 kb)
SW4 for/SW7 rev		Whole *oriC* region (WoriC; 3.16 kb)
SW4 for/SW9 rev	/CTGATGCCGTTAGCATTAGG	Partial LoriC 1 (1.06 kb)
SW15 for/SW16 rev	CGCTTTAATATTGTAACAATA/ GCTGTTATTCAAGAATAATAAATTC	Partial LoriC 2 (565 bp)
SW4 for/PLori-rev-2	/CAGATAGACAAAAGAATGAAAAAG	Partial LoriC 3 (707 bp)
Start DnaN f/PLori-rev-2	GATAAATCTGTGGATAACTC/	Partial LoriC 4 (633 bp)
SW4 for/PLori-rev-4	/CACAATATTTCCGATAAAAATCAC	Partial LoriC 5 (510 bp)
SW4 for/PLori-rev-5	/TTTTACAAACAATATTGTTAC	Partial LoriC 6 (459 bp)
SW4 for/PLori-rev-6	/ACACGTTTTTGTGAAAAAGTGA	Partial LoriC 7 (180 bp)
SW4 for/pPLoriC8rev	/GAGTTATCCTCTGATTTATC	Partial LoriC 8 (96 bp)
pPLoriC9for/PLori-rev-6	CCATGATAATTGTTGATAAATC/	Partial LoriC 9 (119 bp)
Tetfor/Tetrev	GAAAAGATCTGGAGTAATTGGAAG/ ACTAGTCCATATTTATATAACAACTT	Tetracycline resistance gene (2.36 kb)
AmpR for/AmpR rev	CCAATGCTTAATCAGTGAGG/ GTATGAGTATTCAACATTTCCG	Ampicillin resistance gene (862 bp)
SW6 for/dnaA rev	/AAGCATGTCTTAAAGTCTAATTAGAA	*dnaA* gene of *M. imitans* (2.1 kb)
SW15 for/MIsoj rev-1	/TTTTCCATTTTGTTTATTCTC	partial *oriC* region and *soj* gene of *M. imitans* (2 kb)
MIdnaN for/MIsoj rev-2	CAGAAGAGAAGTTGCTTGAAAAC/ CTAGTGTCGTCTTCAAAACAC	*oriC* region of *M. imitans* (2.3 kb)
P3.03-F-SphI/P3.03-R-*Nco*I	CTTTAGCTGCATGCGCAGATTCG/ TTTTCCATGGGTTTTGCTTGG	Internal fragment of the *vlhA3.03* gene (1 kb)
P3.03-F-*Pst*I/P3.03-R-*Sal*I	CTTTAGCTCCATCTGCAGATTCG/ CATTAGGTTTTGCGTCGACGTTTAATG	Internal fragment of the *vlhA3.03* gene (1 kb)
SW13 for/vlhALeader rev	CGACTCACTATAGGGCGAA/ CAAATGAACCAATACCTAATAA	Integration site of pMIoriC/Δ3.03

*Underlined bases are restriction endonuclease cleavage sites.

Construction of *oriC* plasmids. Several regions of the predicted *oriC* region of *M. gallisepticum* were amplified by PCR (Fig. 1a). The long *oriC* region, the short *oriC* region, and the whole *oriC* region were amplified with the specific primer pairs SW4for and SW5rev, SW6for and SW7rev, and SW4for and SW7rev, respectively (Table 1). Each PCR contained 5 µl 10× reaction buffer (Invitrogen), 10 µM each deoxynucleoside triphosphate, 2.5 mM $MgSO_4$, 12.5 µM each primer, 1.25 U Platinum *Taq* High Fidelity DNA polymerase (Invitrogen), 10 ng genomic DNA of *M. gallisepticum* strain R_{low} as template, and water to a final volume of 50 µl. PCRs were performed in a thermocycler (iCycler, Bio-Rad) under the following conditions: 94 °C for 4 min, followed by 35 cycles of 94 °C for 30 s, 45 °C for 2 min, and 68 °C for 4 min, with a final extension at 68 °C for 7 min. PCR products were separated by agarose gel electrophoresis and extracted from gel slices using the QIAEX II kit (Qiagen) according to the manufacturer's instructions. The long and short *oriC* regions were cloned separately into pGEM-T (Promega), into which the tetracycline-resistance gene had already been inserted (*tetM*/pGEM-T) (Markham *et al.*, 2003). The long *oriC* region was inserted between the *Sph*I and *Nco*I cleavage sites, and the short *oriC* region was inserted between the *Pst*I and *Sal*I sites (Fig. 1a). The resulting constructs were designated pGTLori and pGTSori. A further construct was produced that contained both the regions upstream and downstream from the *soj* gene, and was named pGTDori. The whole *oriC* region was amplified similarly and cloned into pGEM-T, and the *tetM* gene was then cloned into the *Spe*I site of the vector, resulting in pGTWori (Fig. 1a). The *oriC* region of *M. imitans* was amplified from the genome of *M. imitans* strain 4229 using the MIdnaNfor and MIsojrev-2 primer pair (Table 1), introduced into the cloning site of pGEM-T, and the *tetM* gene was then ligated into the *Spe*I cleavage site of the vector (Fig. 1b). To determine the minimal *oriC* region for plasmid replication in *M. gallisepticum*, different regions upstream of the *soj* gene were amplified by PCR with specific primer pairs (Table 1). To generate the *oriC* plasmids containing the PCR products shown in Fig. 2, each PCR product was cloned separately into pGEM-T, and the *tetM* gene was then ligated into the *Spe*I site of the vector.

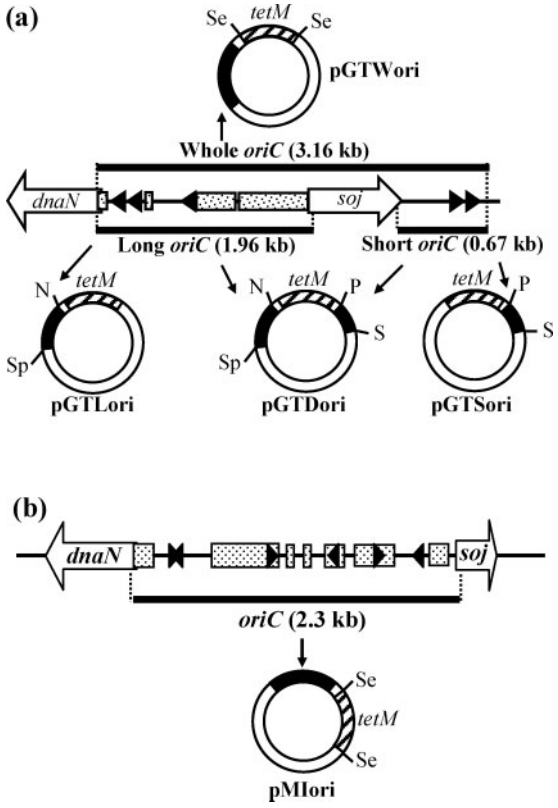


Fig. 1. Construction of *M. gallisepticum* and *M. imitans oriC* plasmids. (a) *M. gallisepticum* genomic *oriC* region and plasmid constructs containing different *oriC* regions. The plasmid backbone was pGEM-T (Promega) containing the tetracycline-resistance gene (*tetM*). The long *oriC* region was inserted between the *Sph*I and *Nco*I sites located in the multicloning site of pGEM-T, and the short *oriC* was inserted between the *Pst*I and *Sal*I sites. The whole *oriC* region was amplified by PCR and cloned into pGEM-T, and the *tetM* gene was then cloned into the *Spe*I site. (b) *M. imitans* genomic *oriC* region and plasmid construct containing the *oriC* region. The *oriC* region was amplified and cloned into pGEM-T and then the *tetM* gene was cloned into the *Spe*I site. The black triangles indicate the locations of the DnaA box consensus sequences and shaded rectangles indicate the locations of AT-rich clusters. Sp, *Sph*I; N, *Nco*I; P, *Pst*I; S, *Sal*I; Se, *Spe*I.

Transformation of *M. gallisepticum* strain S6, *M. imitans* strain 4229 and *M. pneumoniae* strain FH. *M. gallisepticum* strain S6, *M. imitans* strain 4229 and *M. pneumoniae* strain FH were transformed by electroporation, as described by Hedreyda *et al.* (1993). Briefly, 5 ml cultures of mycoplasmas were grown to late exponential phase and harvested by centrifugation at 12 000 ***g*** for 5 min in a bench-top centrifuge at room temperature. The cells were washed twice in 250 µl ice-cold HEPES–sucrose buffer (8 mM HEPES, 272 mM sucrose, pH 7.4). The cell pellet was then resuspended in 100 µl HEPES–sucrose buffer containing ~10 µg plasmid DNA and transferred to a Gene Pulser cuvette with a 0.2 cm electrode gap (Bio-Rad), and the mixture was pulsed using a Gene Pulser (Bio-Rad) set at 2.5 kV, 100 Ω and 25 µF. The cells were resuspended in 1 ml broth and incubated at 37 °C until the medium showed a colour change, after which 500 µl culture was plated onto agar medium containing 4 µg tetracycline ml^{-1}. The plates were incubated at 37 °C for 7–10 days in the dark in an airtight jar for *M. gallisepticum* and *M. imitans*, and incubated for 21 days for *M. pneumoniae*. Individual tetracycline-resistant colonies

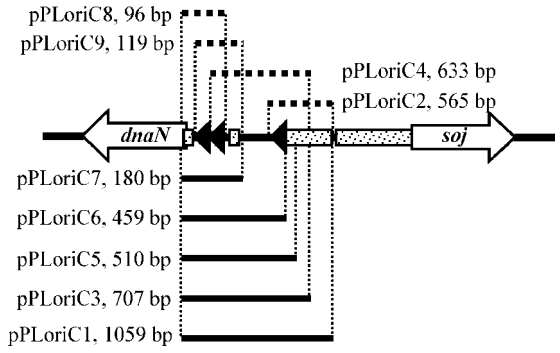


Fig. 2. Schematic representation of reduced *oriC* regions amplified by PCR and cloned into pGEM-T. The solid lines indicate the PCR products which generated plasmids that could be detected following transformation of *M. gallisepticum*, while the dashed lines indicate those that generated plasmids that could not be detected in *M. gallisepticum*. Black triangles indicate DnaA boxes and shaded rectangles indicate AT-rich clusters.

were selected and subcultured in 1 ml broth containing 4 μg tetracycline ml^{-1} and incubated until the medium changed colour. To confirm the presence of the plasmid, *tetM* was amplified by PCR with the Tetfor and Tetrev primer pair (Table 1). Transformants were passaged by inoculating 1 ml late-exponential-phase culture into 19 ml broth containing 4 μg tetracycline ml^{-1}.

Southern blot analysis. *M. gallisepticum* strain S6 and *M. imitans* strain 4229 genomic DNA, and *oriC* plasmid DNA were digested to completion with the restriction endonuclease *Nsi*I (New England Biolabs). The fragments were separated in a 0.7 % agarose gel and blotted onto Hybond-N^+ membranes (Amersham). The DNA was fixed to the membrane by exposure to UV light for 5 min and the blot was prehybridized in a buffer containing 7 % SDS, 1 % BSA, 1 mM EDTA and 0.25 M Na_2HPO_4 (pH 7.2) for 2 h at 58 °C (Church & Gilbert, 1984). A probe containing 50 ng of a 1.96 kb PCR product produced with the primer pair SW4for and SW5rev was radiolabelled with [γ-^{32}P]ATP using a random-primed DNA labelling kit (Roche). Unincorporated nucleotides were removed by passage through a Bio-Spin 30 chromatography column (Bio-Rad), following the manufacturer's instructions. The radiolabelled probe was denatured by incubation at 100 °C for 10 min and then added to the hybridization buffer and incubated with the membrane at 58 °C overnight. The next day the membrane was washed three times in 2× SSC (0.3 M NaCl, 30 mM sodium citrate, pH 7.0), 0.1 % SDS at 58 °C for 20 min each. The membrane was autoradiographed at room temperature overnight on BioMax film (Kodak). The same method was used to produce probes to detect the ampicillin-resistance gene (*ampR*) and the *oriC* region of *M. imitans*. The *ampR* gene was amplified from pGEM-T using the AmpRfor and AmpRrev primer pair, and the partial *oriC* region of *M. imitans* was amplified using the SW15for and Misojrev-1 primer pair (Table 1), together with the products radiolabelled as described above. The hybridization and washing conditions were the same as those used for the *oriC* probe.

Targeted gene inactivation by homologous recombination using *oriC* plasmids. To construct an *oriC* plasmid that could integrate into a target gene by homologous recombination, an internal fragment of the *vlhA3.03* gene (which also has 96 % DNA identity with *vlhA1.1* and *vlhA1.2* of *M. gallisepticum* strain S6), including regions that diverged significantly from most other *vlhA* genes, was amplified from the genome of *M. gallisepticum* strain R_{low} using the P3.03-F-*Pst*I and P3.03-R-*Sal*I primer pair or the P3.03-F-SphI and P3.03-R-*Nco*I primer pair (Table 1). The PCR products were digested with the appropriate restriction endonucleases and cloned separately into the *Pst*I and *Sal*I restriction sites in pPLoriC7, which contained 180 bp of the *M. gallisepticum oriC* region, and into the *Sph*I and *Nco*I restriction sites in the *M. imitans oriC* plasmid pMIori (Fig. 3). Approximately 10 μg of these plasmids was introduced into *M. gallisepticum* strain S6 by electroporation, as described above. Seven days after electroporation, individual tetracycline-resistant colonies were selected from the agar plate and incubated in 500 μl medium containing 4 μg tetracycline ml^{-1} until a colour change was observed in the medium. The cultures were then screened for the presence of the *tetM* gene by PCR. To promote homologous recombination, each transformant was passaged 10 times in medium containing tetracycline, and then an additional five times without tetracycline. Transformants were spread on agar containing tetracycline and individual tetracycline-resistant colonies were selected 7 days after plating. Insertion of the construct into *vlhA3.03* was assessed by Southern blotting. The genomic DNA from transformants and plasmid DNA were digested with *Pst*I (New England Biolabs) and Southern-transferred. The membrane was hybridized to radiolabelled *ampR* and the amplified fragment of *vlhA3.03*, and binding of the probes was detected as described above. The integration of pMIori/Δ3.03 was confirmed by DNA sequencing of the PCR product that was obtained using the primer pair SW13 for and vlhALeader rev (Table 1).

RESULTS

Functional analysis of *M. gallisepticum oriC* plasmids for *M. gallisepticum*, *M. imitans* and *M. pneumoniae*

Several *oriC* plasmids containing different extents of the putative *oriC* region of *M. gallisepticum* were produced and investigated for their ability to replicate in *M. gallisepticum* strain S6. The pGTLori plasmid contained a 1.96 kb region upstream of the *soj* gene, which included three DnaA boxes and four AT-rich clusters; the pGTSori plasmid contained a 0.64 kb DNA region downstream from the *soj* gene, which included two DnaA boxes but no AT-rich cluster; and pGTDori contained both the 1.96 kb region upstream and the 0.64 kb region downstream from the *soj* gene, which was replaced in the plasmid by the *tetM* gene. The pGTWori plasmid contained the 3.16 kb putative *oriC* region, including the *soj* gene (Fig. 1a). All plasmid constructs were introduced into *M. gallisepticum* strain S6 by electroporation and all, with the exception of

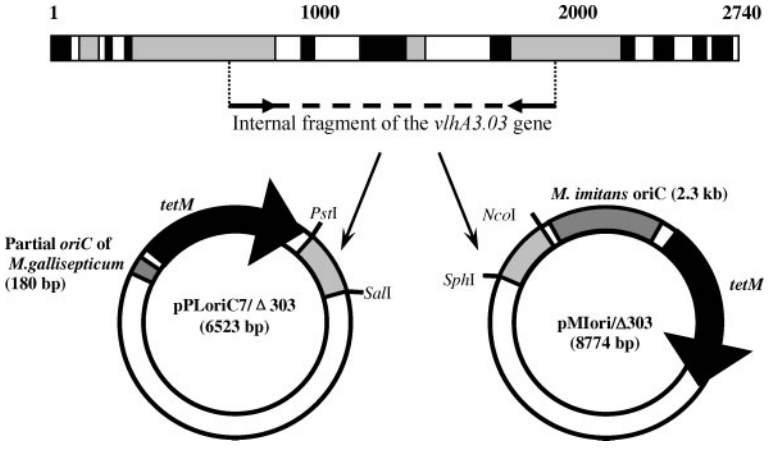


Fig. 3. Schematic representation of alignment of the *vlhA* multigene family and plasmid constructs containing an internal part of the *vlhA3.03* gene. Black rectangles indicate regions of conserved DNA, grey rectangles indicate regions of variable DNA sequence and white rectangles indicate regions of highly variable DNA sequence; results were obtained through DNA sequence alignment of the *vlhA* multigene family. The amplified internal part of the *vlhA3.03* gene was cloned separately into the *Pst*I and *Sal*I restriction sites in pPLoriC7 and into the *Sph*I and *Nco*I restriction sites in the *M. imitans oriC* plasmid pMIori.

pGTSori, could be detected in cultures of tetracycline-resistant transformants; the frequency of transformation was $\sim 6 \times 10^{-7}$ transformants per c.f.u. These results suggested that the AT-rich regions were important for plasmid replication, and in addition that the *dnaA* and *soj* genes were not essential for the replication of the *oriC* plasmid. In order to determine the replicability of the *oriC* plasmid of *M. gallisepticum* in *M. imitans* strain 4229 and *M. pneumoniae* strain FH, plasmid pGTLori, harbouring a 1.96 kb region from *oriC* of *M. gallisepticum*, was introduced into *M. imitans* and *M. pneumoniae* by electroporation. Seven days after transformation, several tetracycline-resistant *M. imitans* colonies were observed on mycoplasma agar plates containing 4 μg tetracycline ml^{-1}, but no tetracycline-resistant colonies of *M. pneumoniae* were detected up to 21 days of incubation, in spite of repeated attempts to introduce pGTLori into *M. pneumoniae* strain FH.

The minimal functional *oriC* region in *M. gallisepticum*

Previous studies have reported that *oriC* plasmids containing larger *oriC* regions can easily integrate into the *oriC* region of genomic DNA through homologous recombination after *in vitro* passage (Cordova *et al.*, 2002; Lartigue *et al.*, 2002; Renaudin *et al.*, 1995). In order to reduce the likelihood of this, a number of plasmids containing different lengths of the *oriC* region were tested for replication in *M. gallisepticum*. We amplified the partial *oriC* regions (oriC1 to oriC9) (Fig. 2) using specific primer pairs (Table 1) and cloned the products into *tetM*/pGEM-T, and these plasmids were then used to transform *M. gallisepticum* strain S6. Following transformation, the constructs pPLoriC1, pPLoriC3, pPLoriC5, pPLoriC6 and pPLroiC7, but not pPLoriC2, pPLoriC4, pPLoriC8 or pPLoriC9, were able to replicate in *M. gallisepticum* (Fig. 2). These results suggested that the minimal *oriC* region that was functional in *M. gallisepticum* strain S6 was around 180 bp and included two DnaA boxes and two AT-rich regions.

Determination of the DNA sequence of the *oriC* region in, and development of an *oriC* plasmid from, *M. imitans*

Part of the *oriC* region and the *dnaA* gene of *M. imitans* were amplified using the corresponding *M. gallisepticum* primer pairs. PCR products and the remainder of the *oriC* region were sequenced. The 2.17 kb region upstream from the *soj* gene of *M. imitans* contained six DnaA boxes, which had a 9 nt consensus sequence (5′-TTWTMHAMA-3′), and was 90 % A+T (Fig. 1b). Alignment of the *oriC* regions of *M. imitans* and *M. gallisepticum* strain R revealed 56 % DNA sequence identity, whilst their *dnaA* genes had 85 % DNA sequence identity. The pMIori plasmid contained the 2.3 kb region upstream from the *soj* gene (Fig. 1b). The pMIori plasmid was introduced into *M. imitans* strain 4229 and *M. gallisepticum* strain S6 by electroporation and was found to replicate in both species.

Stability of *M. gallisepticum oriC* plasmids in *M. gallisepticum* and *M. imitans*

We investigated the stability of *oriC* plasmids in transformants by Southern blot analysis after repeated passage. DNA was extracted from cells of transformants after passaging and, together with the genomic DNA of the untransformed organism and the *oriC* plasmid purified from *E. coli*, was digested with *Nsi*I; the fragments were separated by gel electrophoresis, Southern-blotted and hybridized to the appropriate radiolabelled probe. Following *Nsi*I digestion, the *M. gallisepticum oriC* probe is predicted to bind to an extrachromosomal plasmid with a predicted size of 7.0 kb (Fig. 4a). The 1.96 kbp oriC probe bound a 3.9 kb fragment in *M. gallisepticum* strain S6 (Fig. 4b, lane 1) and to a 7 kb fragment in pGTLori transformants (Fig. 4b, lane 2); a similar-sized fragment was also detected after five passages of the transformants (Fig. 4b, lane 3). The detection of 4.8 and 6 kb fragments by the probe at the 10th passage (Fig. 4b, lane 4) indicated integration of the plasmid into the genome in the *oriC* region. In transformants obtained with pPLoriC1, which contained a 1.06 kb *oriC* region, the *oriC* probe hybridized to the 4.5 kb fragment predicted for an extrachromosomal plasmid (Fig. 4b, lanes 5 and 6). The detection of 3.4 and 4.8 kb fragments at the 10th passage indicated that the plasmid had integrated into the genome (Fig. 4b, lane 7). To determine the stability of pPLoriC7, the replicative plasmid containing the shortest section of the *oriC* region, we performed Southern blot analysis on two transformants passaged under antibiotic selection pressure (4 μg tetracycline ml^{-1}) after 5, 10 and 15 passages. Due to the low sensitivity of the *oriC* probe for the 180 nt *oriC* region, a longer probe that detects the *ampR* gene, which would indicate the location of the *oriC* plasmid, was used. It was expected that the probe would not hybridize to DNA from untransformed *M. gallisepticum* but would hybridize with a 3.5 kb DNA fragment after *Nsi*I digestion of the DNA extracted from transformants containing extrachromosomal plasmid, whilst hybridization to a 4.0 kb fragment would suggest that the plasmid had integrated into the chromosome (Fig. 5a). The probe did not hybridize to untransformed *M. gallisepticum* (Fig. 5b, lane 1), but bound to a 3.5 kb fragment derived from the *oriC* plasmid, and to a similar-sized band in both transformants at the fifth passage (Fig. 5b, lanes 2, 3 and 6). At the 10th passage a 4.0 kb fragment was detected in all clones, but extrachromosomal plasmid was also detected (Fig. 5b, lanes 4 and 7). By the 15th passage, extrachromosomal plasmid could not be detected and the plasmid appeared to have integrated into the chromosome of each transformant (Fig. 5b, lanes 5 and 8). One possible reason for the integration of pPLoriC7 was the selection pressure exerted by the antibiotic. To investigate if this was the cause, we passaged both parental *M. gallisepticum* and a transformant

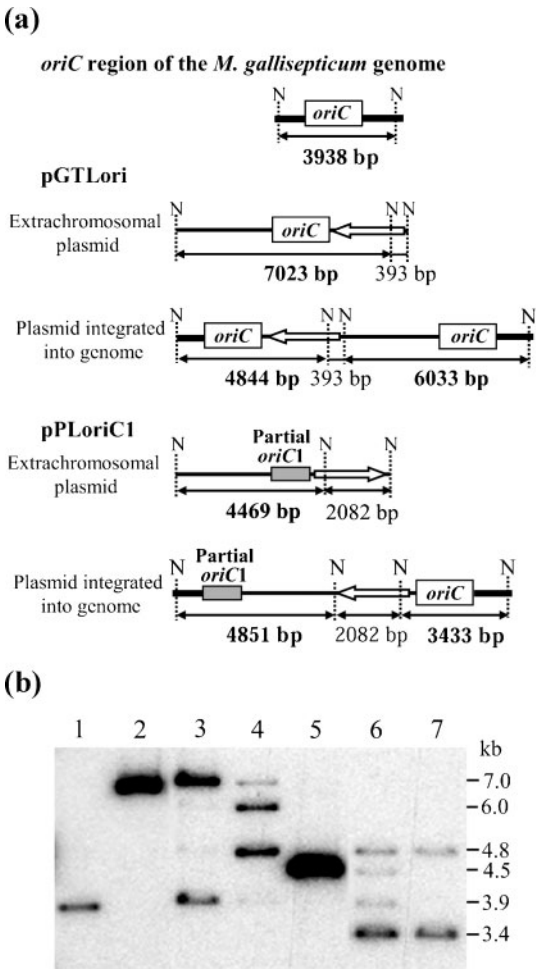


Fig. 4. Stability of *oriC* plasmids in *M. gallisepticum*. (a) Schematic diagram showing the *Nsi*I (N) cleavage sites in the extrachromosomal plasmid and the predicted integrated forms of pGTLori and pPLoriC1 in the *M. gallisepticum* chromosome. The predicted sizes of fragments generated by *Nsi*I cleavage that are expected to hybridize with the radiolabelled *oriC* probe are shown in bold type. The unshaded arrow indicates the *tetM* gene. (b) Southern blot analysis of *Nsi*I-digested DNA. Lanes: 1, untransformed *M. gallisepticum*; 2, pGTLori; 3 and 4, *M. gallisepticum* transformed with pGTLori after 5 and 10 passages, respectively; 5, pPLoriC1; 6 and 7, *M. gallisepticum* transformed with pPLoriC1 after 5 and 10 passages, respectively.

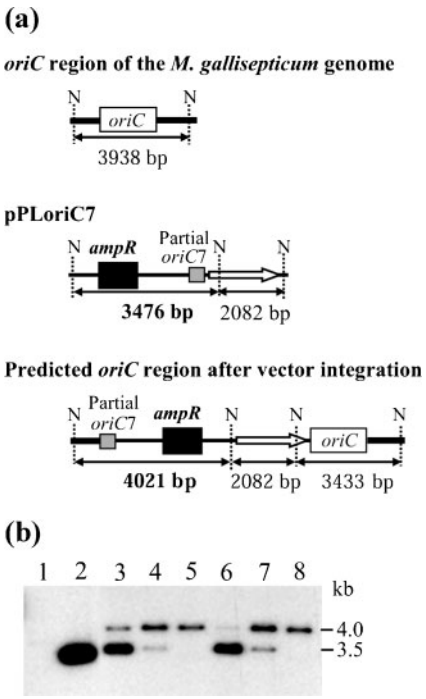


Fig. 5. Stability of the plasmid pPLoriC7, containing a 180 bp *oriC* region, in *M. gallisepticum*. (a) Schematic representation of the integration of pPLoriC7 into the *M. gallisepticum* genome. The predicted sizes of fragments generated by *Nsi*I cleavage that would hybridize with the radiolabelled *ampR* probe are shown in bold type. The unshaded arrow indicates the *tetM* gene. (b) Southern blot analysis of *Nsi*I-digested DNA. Lanes: 1, untransformed *M. gallisepticum* strain S6; 2, pPLoriC7; 3–8, fifth, 10th and 15th passages of *M. gallisepticum* clones 1 (lanes 3–5) and 2 (lanes 6–8) transformed with pPLoriC7.

containing pPLoriC7 in broth containing tetracycline at 0.4 or 1 μg ml^{-1}. Parental *M. gallisepticum* could only grow in broth containing 0.4 μg tetracycline ml^{-1}. Neither extrachromosomal nor integrated forms of the plasmid could be detected in the transformant after five passages in media containing 0.4 μg tetracycline ml^{-1}, suggesting that 0.4 μg tetracycline ml^{-1} was an inadequate concentration to select for transformants. In the transformant grown in 1 μg tetracycline ml^{-1}, pPLoriC7 had completely integrated into the genome by the 15th passage. There appeared to be no difference in the rate of integration of the plasmid into the chromosome in transformants cultured in low concentrations of tetracycline, suggesting that antimicrobial selection pressure did not influence the rate of chromosomal integration. The stability of pGTLori was tested in two transformants of *M. imitans* strain 4229, which were selected on solid media containing 4 μg tetracycline ml^{-1} and then passaged 15 times in broth with tetracycline. Southern blot analysis was performed at the fifth, 10th and 15th passages of each transformant using the radiolabelled *M. gallisepticum oriC* probe. The *oriC* probe did not hybridize with the *oriC* region of untransformed *M. imitans* (results not shown). At all passage levels examined, a 7 kb DNA band, indicative of the extrachromosomal form of pGTLori, was detectable in both transformants (results not shown).

Stability of *M. imitans oriC* plasmids in *M. gallisepticum* and *M. imitans*

The *oriC* plasmid pMIori replicated in both *M. gallisepticum* strain S6 and *M. imitans* strain 4229. The stability of this *oriC* plasmid was investigated following passage of

transformants. *Nsi*I-digested DNA was hybridized with the radiolabelled 2 kb *M. imitans oriC* probe, which contained part of the *oriC* region and the *soj* gene. A 3.5 kb band indicative of the genomic *oriC* region of *M. imitans* was detected in untransformed *M. imitans* and all passaged transformants (Fig. 6a, lanes 2–8). At the 15th passage the 7.4 kb extrachromosomal form of plasmid was still detectable in both transformants (Fig. 6a, lanes 5 and 8). In *M. gallisepticum*, the *oriC* probe, which contained part of the *M. imitans soj* gene, hybridized to a 3.9 kb band containing the *M. gallisepticum soj* gene (Fig. 6b, lane 2). A 7.4 kb band, corresponding to the extrachromosomal plasmid, was detectable until the 15th passage in transformant C1 (Fig. 6b, lane 5). However, the plasmid had integrated into the genome by the 10th passage, although not into the *oriC* region (Fig. 6b, lanes 4, 5, 7 and 8).

Targeted inactivation of a gene by homologous recombination using *oriC* plasmids

pPLoriC7/Δ3.03-transformed *M. gallisepticum* S6 produced several tetracycline-resistant colonies after 7 days of incubation. To investigate the integration site of the plasmid, Southern blot analysis was performed using DNA extracted from 16 pPLoriC7/Δ3.03 transformants that had been passaged 15 times. Identical membranes were hybridized to the radiolabelled *ampR* gene or the internal fragment of the *vlhA3.03* gene. The *ampR* probe bound to a 6.5 kb band in the digest of the plasmid pPLoriC7/Δ3.03 and in most passaged transformants to a 10.5 kb band, corresponding to the predicted size of the fragment containing the chromosomal *oriC* region of *M. gallisepticum* S6 after the plasmid had integrated into this region. However, no band indicative of targeted homologous recombination was detected (results not shown).

Tetracycline-resistant colonies of the pMIori/Δ3.03 transformants of *M. gallisepticum* S6 were passaged 15 times and the integration site of the plasmid was investigated by hybridizing Southern blots with radiolabelled *ampR* gene and *vlhA3.03* probes. Several bands were detected in all passaged transformants, indicating that the plasmid had integrated at least twice into the *M. gallisepticum* genome. The *vlhA3.03* probe detected bands of a different size in one of the transformants. This transformant was passaged an additional five times in media that did not contain tetracycline, to allow the transformant to cure itself of the extrachromosomal plasmid, and then inoculated onto mycoplasma agar containing 4 μg tetracycline ml^{-1}. Two single tetracycline-resistant colonies were selected for Southern blot analysis. The *ampR* probe detected an 8.77 kb band in the digest of pMIori/Δ3.03, while an ~15 kb band was detected in these transformants (Fig. 7 lanes P, 2 and 3). The gene that was predicted to have been interrupted was amplified using the primer pair SW13 for and vlhALeader rev, generating a PCR product of ~2 kb. DNA sequencing of the PCR product and searches of the databases determined that the amplicon was identical to part of the *vlhA1.2* gene (previously *pMGA1.2*) of *M. galliseptiucm* S6 and the section of the *vlhA3.03* gene that was contained within the plasmid construct. The product also contained part of the pGEM-T vector. This suggested

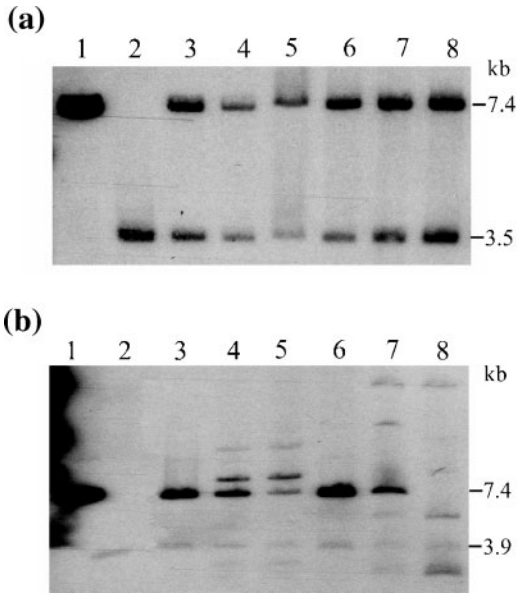


Fig. 6. Stability of *oriC* plasmid pMIori in *M. gallisepticum* and *M. imitans*. (a) Southern blot analysis with *Nsi*I-digested DNA hybridized with *ampR* radiolabelled probe. Lanes: 1, pMIori; 2, untransformed *M. imitans* strain 4229; 3–8, fifth, 10th and 15th passages of *M. imitans* clones 1 (lanes 3–5) and 2 (lanes 6–8) of pMIori transformants. (b) Southern blot analysis with *Nsi*I-digested DNA. Lanes: 1, pMIori; 2, untransformed *M. gallisepticum* strain S6; 3–8, 5th, 10th and 15th passages of clones 1 (lanes 3–5) and 2 (lanes 6–8) of *M. gallisepticum* transformed with pMIori.

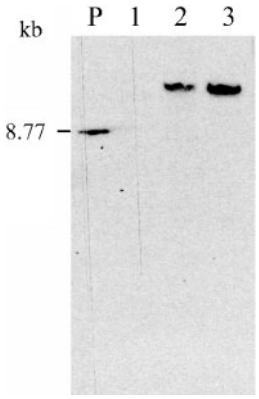


Fig. 7. Integration of pMIori/Δ3.03 into the *M. gallisepticum* strain S6 chromosome. Southern blot analysis of *Pst*I-digested DNA. The blot was hybridized to radiolabelled *ampR* DNA. Lanes: P, pMIori/Δ3.03; 1, untransformed *M. gallisepticum* strain S6; 2 and 3, 20th passage of *M. gallisepticum* pMIori/Δ3.03 transformants 1 and 2, respectively.

that pMIori/Δ3.03 had integrated into the *vlhA1.2* gene rather than the *vlhA1.1* gene.

DISCUSSION

In this study, we produced functional *oriC* plasmids containing the putative *oriC* regions of *M. gallisepticum* and *M. imitans*. In earlier studies, *oriC* plasmids have been constructed for *Spiroplasma citri*, *M. mycoides* subspecies *mycoides* LC and SC, *M. capricolum* subsp. *capricolum*, *Mycoplasma agalactiae* and *Mycoplasma pulmonis* (Chopra-Dewasthaly *et al.*, 2005; Lartigue *et al.*, 2003). Apart from *M. pulmonis*, which belongs to the Hominis phylogenic group, all these species belong to the Spiroplasma phylogenic group, and all have a conserved gene order within the *oriC* region (Fig. 8), which contains the *dnaA* gene. The functional *oriC* plasmids for these species require regions upstream and downstream from the *dnaA* gene, with the exception of the smallest *oriC* plasmid for *S. citri*, which contains only a 163 bp region downstream from the *dnaA* gene (Cordova *et al.*, 2002; Lartigue *et al.*, 2002, 2003). The gene order in the *oriC* regions of *M. gallisepticum* and *M. imitans* is similar to those of *M. pneumoniae* and *Mycoplasma genitalium* (Fig. 8). These mycoplasmas belong to the Pneumoniae phylogenetic group and the putative *oriC* regions surround the *soj* gene but lie upstream of the *dnaA* gene (Cordova *et al.*, 2002; Papazisi *et al.*, 2003). We produced four *oriC* plasmid constructs that contained the *soj* gene and regions upstream and downstream of the *soj* gene in *M. gallisepticum*. Only the plasmids containing the region upstream from the *soj* gene could be detected in *M. gallisepticum*, indicating that only the AT-rich sequences found in this region were essential for plasmid replication, even though the region downstream from the *soj* gene included two DnaA boxes. This suggests that, at least in *M. gallisepticum*, the *soj* gene is not required for replication of an *oriC* plasmid. The *M. gallisepticum oriC* plasmid, pGTLori, and the *M. imitans oriC* plasmid pMIori were able to replicate in both species. Alignment of the *oriC* region of *M. imitans* with that of *M. gallisepticum* showed that the sequences were very different, but that the surrounding genes had high levels of DNA sequence identity. Both species contained similar DnaA box consensus sequences. Therefore, *M. gallisepticum* and *M. imitans* might be expected to support replication of heterologous *oriC* plasmids. In contrast to *M. imitans*, *M. pneumoniae* did not appear to support replication of the *M. gallisepticum oriC* plasmid pGTLori. Though both species have a conserved gene order in the *oriC* region (Fig. 8), the DNA sequence of their *oriC* regions appears to be poorly conserved, and alignment of the peptide sequence of the DnaA proteins of *M. gallisepticum* and *M. pneumoniae* revealed only 23 % peptide sequence identity. Several AT-rich clusters were identified in the putative *oriC* region of *M. pneumoniae*, but consensus DnaA box nonamers were not found. This suggests that the DnaA boxes of *M. pneumoniae* may have a more relaxed consensus sequence. The minimal *oriC* region of *M. gallisepticum* that was found to be functional in *M. gallisepticum* strain S6 was 180 bp in size. This region was upstream from the *soj* gene and included two DnaA boxes and two AT-rich regions of 31 and 22 bases (Fig. 2). The size of the minimal *oriC* region in *M. gallisepticum* was similar to that required in the functional *oriC* vector for *S. citri*, which was a 163 bp region downstream from the *dnaA* gene containing three DnaA boxes and two AT-rich regions (Lartigue *et al.*, 2002). For *M. gallisepticum* strain S6, we found that only two DnaA boxes were necessary for plasmid replication, and this is the smallest *oriC* region capable of supporting plasmid replication in mycoplasmas. Both pGTLori and pPLoriC1, which contained larger sections of the *oriC*

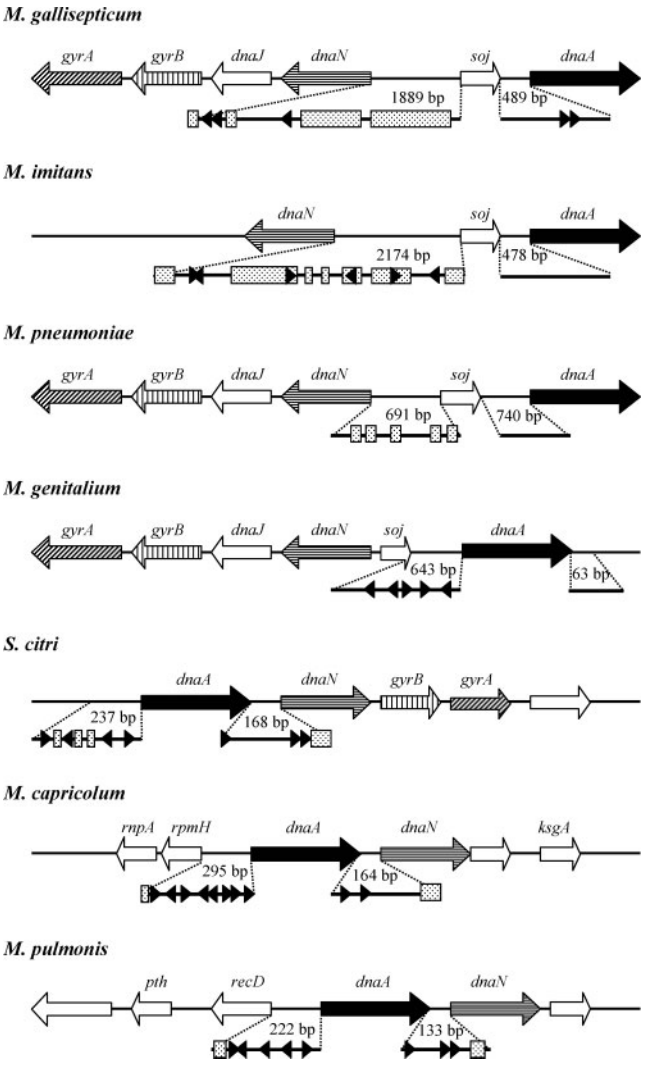


Fig. 8. Gene order surrounding the chromosomal *oriC* regions of mollicutes and the structure of their putative *oriC* regions. Triangles indicate the locations of the DnaA box consensus sequences and shaded rectangles indicate the locations of AT-rich regions.

region, integrated readily into the chromosome during passage, as has been seen with the *oriC* plasmids pBOT1 in *S. citri* and pMPO1 in *M. pulmonis* (Cordova *et al.*, 2002; Renaudin *et al.*, 1995). We produced a smaller *oriC* plasmid in an attempt to generate a vector that would not integrate readily into the genome. While pPLoriC7 did not integrate into the chromosome as rapidly as pGTLori and pPLoriC1, it had integrated completely into the chromosome by the 15th passage. In *M. pulmonis*, the minimal *oriC* regions necessary for plasmid replication are 262 bp upstream and 327 bp downstream from the *dnaA* gene. This *oriC* plasmid (pMPO5) remains extrachromosomal for at least 15 passages (Cordova *et al.*, 2002). The *oriC* plasmid pGTLori could replicate in *M. imitans* strain 4229 and appeared to be more stable in *M. imitans* than in *M. gallisepticum*. In Southern blot analysis, free plasmid was detectable until the 15th passage, with only limited integration into the chromosome. This suggested that the *oriC* plasmid of *M. imitans* might be more stable in *M. gallisepticum* than the homologous *oriC* plasmid. However, while some plasmid remained extrachromosomal until the 15th passage, a portion had integrated into the genome at sites outside the *oriC* region by the 10th passage. Interestingly, an extrachromosomal form of pMIori was found in all passaged transformants of *M. imitans*. These results suggest that the homologous recombination system in *M. gallisepticum* has a higher efficiency than that of *M. imitans*.

Targeted gene inactivation was attempted in *M. gallisepticum* using the pPloriC7 and pMIori plasmids. In a previous study, only five of 16 transformants containing the smallest *S. citri oriC* plasmid (containing 163 bp of the *oriC* region) had integration of the plasmid into the chromosomal *oriC* region after 15 passages, and this construct was successfully used to inactivate *scm1* (Lartigue *et al.*, 2002). However, pPLoriC7, containing the 180 bp *M. gallisepticum oriC* region, integrated readily into the chromosomal *oriC* region by the 15th passage, and attempts to inactivate *vlhA3.03* using pPLoriC7 were unsuccessful, with the plasmid integrating into the chromosomal *oriC* region in all transformants rather than into the target gene region, even though the *vlhA3.03* fragment in the plasmid was 986 bp in size.

Of the 16 *M. gallisepticum* transformants containing pMIori/Δ3.03, only one showed evidence of an interruption of a *vlhA* gene in Southern blot analysis. The inactivated gene was identified as *vlhA1.2*. At the DNA level, *vlhA1.2* and *vlhA1.1* are 98 % identical, so this result is not unexpected. Targeted gene inactivation has, to our knowledge, only been achieved once before in *M. gallisepticum*, but use of the *M. imitans oriC* plasmid could improve the efficiency of recombination. Thus, in this study, we constructed several *oriC* plasmids that could replicate successfully in *M. gallisepticum* and *M. imitans*. This is the first report, to our knowledge, of *oriC* plasmids for members of the Pneumoniae phylogenetic group. In other mollicutes, *oriC* plasmids have been used to inactivate genes or to express exogenous genes (Cordova *et al.*, 2002; Duret *et al.*, 1999; Janis *et al.*, 2005; Lartigue *et al.*, 2002), so these *oriC* plasmids are likely to be useful tools for genetic research in *M. gallisepticum* and *M. imitans*.

ACKNOWLEDGMENTS

This research was funded by grants from the Australian Poultry Cooperative Research Centre (APCRC) to P. F. M. and G. F. B., and S.-W. L. received an APCRC scholarship. S.-W. L. was also in receipt of an International Postgraduate Research Scholarship and a Melbourne International Research Scholarship.

REFERENCES

Bearson, S. M., Collier, S. D., Bearson, B. L. & Branton, S. L. (2003). Induction of a *Mycoplasma gallisepticum* pMGA gene in the chicken tracheal ring organ culture model. *Avian Dis* **47**, 745–749.

Bradbury, J. M., Abdul-Wahab, O. M., Yavari, C. A., Dupiellet, J. P. & Bove, J. M. (1993). *Mycoplasma imitans* sp. nov. is related to *Mycoplasma gallisepticum* and found in birds. *Int J Syst Bacteriol* **43**, 721–728.

Chopra-Dewasthaly, R., Marenda, M., Rosengarten, R., Jechlinger, W. & Citti, C. (2005). Construction of the first shuttle vectors for gene cloning and homologous recombination in *Mycoplasma agalactiae*. *FEMS Microbiol Lett* **253**, 89–94.

Church, G. M. & Gilbert, W. (1984). Genomic sequencing. *Proc Natl Acad Sci U S A* **81**, 1991–1995.

Cordova, C. M., Lartigue, C., Sirand-Pugnet, P., Renaudin, J., Cunha, R. A. & Blanchard, A. (2002). Identification of the origin of replication of the *Mycoplasma pulmonis* chromosome and its use in *oriC* replicative plasmids. *J Bacteriol* **184**, 5426–5435.

Duret, S., Danet, J. L., Garnier, M. & Renaudin, J. (1999). Gene disruption through homologous recombination in *Spiroplasma citri*: an *scm1*-disrupted motility mutant is pathogenic. *J Bacteriol* **181**, 7449–7456.

Dybvig, K. & Alderete, J. (1988). Transformation of *Mycoplasma pulmonis* and *Mycoplasma hyorhinis*: transposition of Tn916 and formation of cointegrate structures. *Plasmid* **20**, 33–41.

Dybvig, K. & Cassell, G. H. (1987). Transposition of Gram-positive transposon Tn916 in *Acholeplasma laidlawii* and *Mycoplasma pulmonis*. *Science* **235**, 1392–1394.

Glew, M. D., Browning, G. F., Markham, P. F. & Walker, I. D. (2000). pMGA phenotypic variation in *Mycoplasma gallisepticum* occurs *in vivo* and is mediated by trinucleotide repeat length variation. *Infect Immun* **68**, 6027–6033.

Hedreyda, C. T., Lee, K. K. & Krause, D. C. (1993). Transformation of *Mycoplasma pneumoniae* with Tn*4001* by electroporation. *Plasmid* **30**, 170–175.

Hudson, P., Gorton, T. S., Papazisi, L., Cecchini, K., Frasca, S., Jr & Geary, S. J. (2006). Identification of a virulence-associated determinant, dihydrolipoamide dehydrogenase (*lpd*), in *Mycoplasma gallisepticum* through i*n vivo* screening of transposon mutants. *Infect Immun* **74**, 931–939.

Janis, C., Lartigue, C., Frey, J., Wroblewski, H., Thiaucourt, F., Blanchard, A. & Sirand-Pugnet, P. (2005). Versatile use of *oriC* plasmids for functional genomics of *Mycoplasma capricolum* subsp. *capricolum*. *Appl Environ Microbiol* **71**, 2888–2893.

Lartigue, C., Duret, S., Garnier, M. & Renaudin, J. (2002). New plasmid vectors for specific gene targeting in *Spiroplasma citri*. *Plasmid* **48**, 149–159.

Lartigue, C., Blanchard, A., Renaudin, J., Thiaucourt, F. & Sirand-Pugnet, P. (2003). Host specificity of mollicutes *oriC* plasmids: functional analysis of replication origin. *Nucleic Acids Res* **31**, 6610–6618.

Ley, D. H. & Yoder, H. W. J. (1997). *Mycoplasma gallisepticum* infection. In *Diseases of Poultry*, pp. 194–207. Edited by B. W. Calnek, H. J. Barnes, C. W. Beard, L. R. McDougald & Y. M. Saif. London: Mosby-Wolfe.

Mahairas, G. G. & Minion, F. C. (1989). Random insertion of the gentamicin resistance transposon Tn*4001* in *Mycoplasma pulmonis*. *Plasmid* **21**, 43–47.

Markham, P. F., Glew, M. D., Brandon, M. R., Walker, I. D. & Whithear, K. G. (1992). Characterization of a major hemagglutinin protein from *Mycoplasma gallisepticum*. *Infect Immun* **60**, 3885–3891.

Markham, P. F., Kanci, A., Czifra, G., Sundquist, B., Hains, P. & Browning, G. F. (2003). Homologue of macrophage-activating lipoprotein in *Mycoplasma gallisepticum* is not essential for growth and pathogenicity in tracheal organ cultures. *J Bacteriol* **185**, 2538–2547.

Marois, C., Dufour-Gesbert, F. & Kempf, I. (2001). Molecular differentiation of *Mycoplasma gallisepticum* and *Mycoplasma imitans* strains by pulsed-field gel electrophoresis and random amplified polymorphic DNA. *J Vet Med B Infect Dis Vet Public Health* **48**, 695–703.

Mudahi-Orenstein, S., Levisohn, S., Geary, S. J. & Yogev, D. (2003). Cytadherence-deficient mutants of *Mycoplasma gallisepticum* generated by transposon mutagenesis. *Infect Immun* **71**, 3812–3820.

Papazisi, L., Frasca, S., Jr, Gladd, M., Liao, X., Yogev, D. & Geary, S. J. (2002). GapA and CrmA coexpression is essential for *Mycoplasma gallisepticum* cytadherence and virulence. *Infect Immun* **70**, 6839–6845.

Papazisi, L., Gorton, T. S., Kutish, G., Markham, P. F., Browning, G. F., Nguyen, D. K., Swartzell, S., Madan, A., Mahairas, G. & Geary, S. J. (2003). The complete genome sequence of the avian pathogen *Mycoplasma gallisepticum* strain R_{low}. *Microbiology* **149**, 2307–2316.

Renaudin, J., Marais, A., Verdin, E., Duret, S., Foissac, X., Laigret, F. & Bove, J. M. (1995). Integrative and free *Spiroplasma citri oriC* plasmids: expression of the *Spiroplasma phoeniceum* spiralin in *Spiroplasma citri*. *J Bacteriol* **177**, 2870–2877.

Sambrook, J. & Russell, D. W. (2001). *Molecular Cloning: a Laboratory Manual*, 3rd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Whetzel, P. L., Hnatow, L. L., Keeler, C. L., Jr & Dohms, J. E. (2003). Transposon mutagenesis of *Mycoplasma gallisepticum*. *Plasmid* **49**, 34–43.

Whithear, K. G. (1993). Avian mycoplasmosis. In *Australian Standard Diagnostic Techniques for Animal Diseases*, pp. 1–12. Edited by L. A. Corner & T. J. Bagust. East Melbourne: Australian Agricultural Council (Standing Committee on Agriculture and Resource Management).

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

Edited by: C. Citti

Vaccine 26 (2008) 5449–5454

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Development and immunogenicity of recombinant *Mycoplasma gallisepticum* vaccine strain ts-11 expressing chicken IFN-γ

Yoshihiro Muneta[a,b,*], Indu S. Panicker[a], Anna Kanci[a], Douglas Craick[a], Amir H. Noormohammadi[a], Andrew Bean[c], Glenn F. Browning[a], Philip F. Markham[a]

[a] Department of Veterinary Science, Veterinary Preclinical Centre, The University of Melbourne, Parkville, Victoria 3010, Australia
[b] Research Team for Advanced Biologicals, National Institute of Animal Health, 3-1-5 Kannondai, Tsukuba, Ibaraki 305-0856, Japan
[c] Australian Animal Health Laboratory, CSIRO Livestock industries, Geelong, Victoria 3220, Australia

ARTICLE INFO

Article history:
Received 14 March 2008
Received in revised form 15 July 2008
Accepted 27 July 2008
Available online 14 August 2008

Keywords:
Mycoplasma gallisepticum
Mucosal vaccine
ts-11
Chicken
IFN-γ

ABSTRACT

Mycoplasma gallisepticum (MG) is a poultry pathogen that causes respiratory disease and loss of egg production worldwide. A live attenuated vaccine, ts-11, has been used for the control of MG in several countries. To improve the functionality of the vaccine and investigate its potential as a delivery vector for host immune molecules and foreign antigens, we have developed ts-11 as a vector to express and secrete chicken IFN-γ (ts-11 C3) using a transposon-based delivery vector. Following administration of ts-11 C3 in chickens by eye drop, up to 2 weeks post-vaccination, neither significant systemic IFN-γ expression nor an antibody response as determined by the rapid serum agglutination (RSA) could be detected, while moderate RSA scores were detected in birds vaccinated with ts-11. However, the MG-specific IFN-γ response in spleen cultures was significantly enhanced in ts-11 C3 vaccinated chickens and, more interestingly, significant heterophil infiltration was detected in the tracheal epithelium in ts-11 C3 vaccinated birds, but not in ts-11 vaccinated birds. These results indicate that the IFN-γ expressed by ts-11 C3 enhanced host cellular immunity rather than humoral immunity and may also have stimulated mucosal heterophil infiltration. These results also suggest that ts-11 is a promising vector for protective antigens of other chicken respiratory pathogens.

1. Introduction

Mycoplasma gallisepticum (MG) is the etiological agent of avian respiratory mycoplasmosis, which causes chronic respiratory disease in chickens and infectious sinusitis of turkeys [1,2]. MG infection also causes a reduction in egg production and significant economic losses in the poultry industry [1,2]. Vaccination is an option for controlling MG infection when the prevention of exposure of poultry flocks to wild type challenge, even by strict biosecurity, is impossible [3]. Both killed vaccines and live vaccines are currently in commercial use worldwide [3].

The MG vaccine strain ts-11 used in this study is a temperature-sensitive mutant, which was generated by chemical mutagenesis of a moderately virulent Australian field isolate (strain 80083) [4,5]. It has a temperature-sensitive phenotype, with normal growth at 33 °C but reduced growth at 39.5 °C [4,5]. This strain is avirulent for chickens and turkeys and a single dose of ts-11 by eye drop application results in colonization of the upper respiratory tract and induces long-term immunity [4,5]. The ts-11 vaccine strain has been successfully used to control and eradicate MG infections worldwide and can be used safely in combination with other respiratory virus vaccines. The genome sequence of *M. gallisepticum* strain R_{low} has recently been determined [6]. As there is a relatively large amount of genetic redundancy in MG, as demonstrated by the *vlhA* gene family of MG (which occupies over 10% of the genome), the organism has the capacity to accommodate large amounts of foreign DNA and these findings, together with the recent advances in development of molecular tools for genetic modification of mycoplasmas [7], make it worthwhile investigating whether it would be a useful vaccine vector.

One of the central cytokines in viral immunity is IFN-gamma (IFN-γ). It plays important roles in enhancing the development of cellular immunity in animals, including in the development of Th1 cells, CD8+ T cell responses, and natural killer cell cytotoxicity [8]. IFN-γ also plays important roles in mucosal immune responses [9]. Recent studies have suggested that cellular immune responses in the tracheal mucosa, including natural killer and cytotoxic T cell responses, are important contributors to the protective

* Corresponding author at: Research Team for Advanced Biologicals, National Institute of Animal Health, 3-1-5 Kannondai, Tsukuba, Ibaraki 305-0856, Japan. Tel.: +81 298 38 7790; fax: +81 298 38 7880.
E-mail address: ymuneta@affrc.go.jp (Y. Muneta).

0264-410X/$ – see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2008.07.076

immunity afforded by MG vaccines [10]. Generally speaking, one of the characteristics of respiratory disease caused by mycoplasmas is a lymphoproliferative response in the respiratory tract [11,12]. Over-expression of inflammatory cytokines has been reported in many mycoplasmoses [12]. However, we have recently reported on cytokine expression during *Mycoplasma hyopneumoniae* (MHP) infection in pigs, and found decreased IFN-γ production during MHP infection [13]. Chicken IFN-γ (chIFN-γ) has been well-characterized [14,15] and has been shown to enhance heterophil activity and promote growth in chickens [16–18]. Therefore, the introduction of IFN-γ into the MG ts-11 vaccine strain is a promising approach for enhancement of cellular and mucosal immune responses to the vaccine, as well as promoting growth in chickens.

In this study, we developed a vaccine strain of MG ts-11 that expresses and secretes chIFN-γ using a transposon-based delivery vector. We examined the cellular immune response following vaccination with MG expressing chIFN-γ and its effect on modulation of host immune responses and ts-11 immunogenicity.

2. Materials and methods

2.1. Mycoplasma gallisepticum strain and culture

M. gallisepticum strain ts-11 was used in this study [4]. Culture of the organism was performed in modified Frey's broth media (MB broth) containing 10% swine serum [19] or by plating on mycoplasma agar (MB agar), which has the same formula as MB broth except glucose and phenol red were omitted and the medium was solidified with 1% Noble agar (Difco). All of the experiments were done under The Office for Gene Technology Regulator guidelines and approval.

2.2. Plasmid construction

The transposon Tn4001-based vector pISM2062.2 [20] was used for the construction of the chIFN-γ expression vector. The primers used for the construction of the plasmid are shown in Table 1. The MG tuf promoter, signal sequence, and *E. coli* alkaline phosphatase gene (*phoA*) fused with the cleavage signal (QASETQ) for MG VlhA1.9 [21] were amplified by PCR and cloned into pGEM-T (Promega) using TA cloning and this used to transform *E. coli* DH5α (pGEMt-tufp-sig-phoA-QASETQ). The mature part of chIFN-γ cDNA fused with FLAG sequence at the 3′ end was amplified from a plasmid containing chIFN-γ cDNA [14] and cloned into pGEMt-tufp-sig-phoA-QASETQ using the *Spe*I and *Nsi*I endonuclease cleavage sites of the vector, and this used to transform DH5α (pGEMt-tufp-sig-phoA-QASETQ-IFNγ-FLAG). The entire sequence containing the tuf promoter, signal sequence, *phoA*, secretion signal, chIFN-γ, and FLAG was amplified and ligated into the *Bam*HI site of pISM 2062.2 and used to transform *E. coli* XL10-Gold (Stratagene). The resulting plasmid (pISM-tufp-sig-phoA-QASETQ-IFNγ-FLAG) was used for the transformation of MG. The DNA sequence of the construct was confirmed by cycle sequencing using the ABI PRISM Big Dye Terminator reagent (Applied Biosystems) following the manufacturer's instructions.

2.3. Transformation of Mycoplasma gallisepticum

Transformation of MG was performed by electroporation. Briefly, MG ts-11 was cultured overnight till the pH of the media was judged to be 7.2 as determined by color change of the phenol red indicator and cells were collected by centrifugation at 16,000 × *g* for 20 min at 4 °C. The cells were washed two times in cold HEPES–sucrose buffer (8 mM HEPES and 272 mM sucrose, pH 7.4) and resuspended in the same buffer with 10 μg of plasmid DNA (pISM-tufp-sig-phoA-QASETQ-IFNγ-FLAG). The mixture was transferred to a pre-chilled electroporation cuvette (0.2 cm, Bio-Rad) and immediately pulsed (2.5 kV, 100 Ω, 25 μF) using a Gene pulser (Bio-Rad). The cells were resuspended gently in 1 ml of cold MB broth and incubated on ice for 10 min and incubated at 37 °C for 2–3 h. The cells were then plated onto MB agar containing 20 μg gentamicin (Gibco)/ml and incubated at 33 °C for 4–7 days.

2.4. Characterization of ts-11 C3

Colonies of putative gentamicin-resistant transformants were selected randomly from agar plates and inoculated into 1 ml fresh MB broth containing gentamicin. Following growth (judged by a change in pH detected as a color change in the medium), the supernatants were collected and screened for chIFN-γ secretion using a chIFN-γ ELISA as described previously [15]. A clone positive for chIFN-γ secretion (ts-11 C3) was further sub-cultured three times and stored at −70 °C. Cells in a 1 ml volume of ts-11 C3 culture were pelleted by centrifugation at 16,000 × *g* for 5 min at room temperature and the cell pellet re-suspended in 50 μl of water and heated at 95 °C for 5 min and 0.5 μl was used as template in a PCR to verify the presence of the gene construct. The oligonucleotide primers used to determine the presence of the gentamicin gene, a 20 bp insertion in the *gapA* gene found in ts-11, and the chIFN-γ gene are shown in Table 1. PCR was performed in a total volume of 50 μl using GoTaq

Table 1
Primers used in this study

Primer name	Sequence (5′–3′)	PCR conditions	Size of the product (bp)
BglII-tufp-sig-PhoA-For	GATCAGATCTTTTAGGGGTGTAGTTCAA	95 °C 4 min and 35 cycles of 95 °C 30 s, 60 °C 30 s, 68 °C 90 s and 1 cycle of 68 °C 7 min	1717 bp
QASETQ-PhoA-Rev-2	GTGTTTCAGAAGCTTGTTTCAGCCCCAGAGCGGCT		
SpeI-IFNγ-For-2	GATCACTAGTGCATACTGCAAGTAGT	95 °C 3 min, and 35 cycles of 95 °C 30 s, 50 °C 30 s, 68 °C 30 s and 1 cycle of 68 °C 7 min	486 bp
NsiI-BglII-FLAG-IFNγ-Rev	TCATGCATAGATCTTTACTTGTCATCGTCATCCTT-GTAATCGCAATTGCATCTCCTCTGA		
Gm-For	CCAAGAGCAATAAGGGCATAC	95 °C 2 min and 30 cycles of 95 °C 30 s, 55 °C 30 s, 72 °C 30 s	223 bp
Gm-Rev	ACACTATCATAACCACTACCG		
GapA-For	AGATAAATTCACTCAAGAAAATAAT	94 °C 3 min, and 40 cycles of 94 °C 30 s, 50 °C 40 s, 72 °C 15 s	120 bp
GapA-Rev	TGTTAATCATGTGCTGTCTTT		

Gm, gentamicin-resistant gene; GapA, *gapA* gene.

polymerase (Promega) as described in the manufacturer's instructions. The PCR conditions and the sizes of the products are shown in Table 1. Expression of alkaline phosphatase and its activity in transformants were assessed using an anti-alkaline phosphatase monoclonal antibody (Sigma) to detect the peptide in Western blots and by using the substrate BCIP/NBT (Sigma) directly on colonies or colony lifts.

2.5. Immunogenicity of ts-11 C3 in chickens

Thirty 2-week-old White Leghorn specific-pathogen-free chickens (SPAFAS Pty Ltd., Woodend, Victoria, Australia) were divided into three treatment groups (Groups 1–3). Group 1 received 30 μl MB broth only, whilst birds in Group 2 and 3 received 30 μl of broth containing 5.0×10^7 ccu of ts-11 or 7.5×10^7 ccu of ts-11 C3, respectively, by eye drop instillation.

Blood was collected weekly and examined for systemic antibody responses against MG using the rapid serum agglutination test (RSA) and scored on a scale of 0–4 as described previously [22]. Serum IFN-γ concentrations were estimated by chIFN-γ ELISA. Average daily weight gain was calculated using the following formula: (Bird weight at post mortem − Bird weight at vaccination)/duration of the experiment in days. Re-isolation of MG was attempted from swabs taken from the choanal cleft, which were then inoculated into culture medium. Strain identity was confirmed by PCR.

At one or two weeks after immunization 15 birds (5 from each group) were subjected to post mortem examination and their spleens were collected. The spleen tissue was passed through a sterile metal mesh and then filtered through sterile gauze. The cell suspension was then washed twice using minimum essential medium (MEM). Approximately 5×10^7 spleen cells were cultured in 1 ml of DMEM containing 10% fetal calf serum with antibiotics and stimulated with either 10 μg Concanavalin A (ConA, Sigma)/ml, 10 μg/ml lipopolysaccharide (LPS) from *E. coli* O55B5 (Sigma) or heat-killed ts-11 whole antigen (cell:ts-11 = 1:1). Following stimulation, cells were incubated in 5% CO_2 at 39 °C for 5 days and the IFN-γ concentration in the supernatant was measured using the chIFN-γ ELISA.

2.6. Measurement of tracheal mucosal thickness

Upper and lower tracheal sections were taken and fixed in 10% neutral buffered formalin, embedded in paraffin and 2-μm sections were prepared and stained with haematoxylin and eosin. The mean mucosal thickness of the trachea of each bird was determined by measuring the thickness at six random points in sections from the anterior and posterior ends of the trachea as described previously [23].

2.7. Assessment of heterophil infiltration

The number of heterophils that had infiltrated into the tracheal epithelium was estimated by microscopy using 1000× magnification. The number of heterophils in six randomly selected microscopy fields in each tracheal section were counted and the mean was calculated.

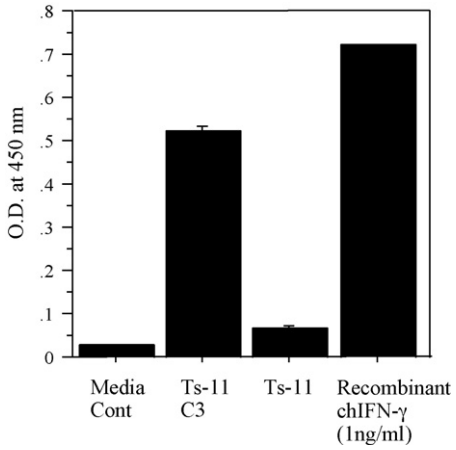


Fig. 1. IFN-γ secretion into the supernatants of cultures of MG ts-11 transformants. The supernatants (diluted twofold) of cultures of 27 gentamicin-resistant MG transformants were screened in duplicate using the chIFN-γ ELISA. A positive clone (ts-11 C3) was obtained. Recombinant chIFN-γ and MG broth only were used as a positive and a negative control, respectively. ELISA ODs at 450 nm are shown.

2.8. Statistical analysis

The differences between each group were analyzed using Student's *t*-test in Statview (Abacus Concept, Berkeley, CA). $P < 0.05$ was considered to be significant.

3. Results

3.1. Development of ts-11 C3 and its characterization

Following two separate transformation experiments, a total of 27 gentamicin-resistant MG ts-11 transformants were obtained. Among these transformants, one clone, ts-11 C3, was positive for chIFN-γ secretion into the culture supernatant after screening by chIFN-γ ELISA. Although ChIFN-γ expression in ts-11 C3 was unable to be detected by Western blotting (data not shown), it was detected by chIFN-γ ELISA in the culture supernatant at concentrations of approximately 1 ng/ml (Fig. 1). The presence of IFN-γ and gentamicin-resistance genes, and a 20 bp insertion in the *gapA* gene in ts-11 C3 was confirmed by PCR, and alkaline phosphatase activity and expression of this protein were detected by Western blotting and immunstaining, and using the BCIP/NBT substrate (results not shown).

3.2. Serum IFN-γ, daily weight gain and re-isolation

Table 2 shows the results from assays for detection of serum IFN-γ, the daily weight gains and the re-isolation of mycoplasmas

Table 2
Serum IFN-γ, daily weight gain, and re-isolation of MG

Group	Serum IFN-γ	Daily weight gain	Re-isolation of MG		
			MG colonies on MB agar	Color change in MB broth	PCR positive cultures
1	0/10	13.25 ± 1.139	0/10	0/10	0
2	0/10	12.72 ± 0.769	10/10 (1–<100)	10/10	9
3	0/10	12.51 ± 1.021	1/10 (1)[a]	0/10	0/10

Values in parenthese indicate the range in the number of MG colonies. Group 1: media; Group 2: ts-11; Group 3: ts-11 C3.
[a] This colony was unable to be sub-cultured. Results from birds 1 and 2 weeks after vaccination.

Table 3
RSA scores in chickens vaccinated with ts-11 and ts-11 C3

Group	Number of chickens	Mean RSA score (range)		
		0 week	1 week	2 weeks*
1	10	0 (0,0)	0 (0,0)	0 (0,0)[b]
2	10	0 (0,0)	0 (0,0)	1.4 (1,2)[a]
3	10	0 (0,0)	0 (0,0)	0 (0,0)[b]

Values with different superscript letters are significantly different ($P < 0.01$).
* RSA scores of 5 birds from each group.

from vaccinated birds. IFN-γ was unable to be detected by ELISA in any serum sample examined by ELISA over the duration of the experiment. There was no significant difference in daily weight gain between the three groups. No MG was re-isolated from birds in Group 1, but ts-11 was re-isolated from all birds in Group 2 by broth culture and confirmed by PCR. However, only one MG-like colony was obtained from 1 of 10 chickens in Group 3, but this clone was unable to be sub-cultured.

3.3. Systemic antibody responses

Table 3 shows the RSA scores of chickens at 1 and 2 weeks after vaccination. One week after vaccination, no RSA score was detected in any of the birds in any of the groups. All the chickens in Group 2 had a moderate RSA score of between 1 and 2 at 2 weeks after immunization, whilst the birds in Groups 1 and 3 did not produce any detectable RSA antibodies.

3.4. IFN-γ production in spleen cell cultures

Spleen cell cultures were stimulated with either MG ts-11, ConA or LPS and the amounts IFN-γ produced are shown in Fig. 2. Group 3 produced significantly higher amounts of IFN-γ in response to MG ts-11 compared with Groups 1 or 2. Non-specific IFN-γ production after ConA stimulation was lower in Group 2, but this reduction was not seen in Group 3. No significant difference was found in IFN-γ production after LPS stimulation.

3.5. Trachea l mucosal thickness and histology

Table 4 shows the mean tracheal mucosal thicknesses and the mean number of heterophils within the tracheal epithelium. There was no significant difference in tracheal mucosal thickness between the three groups. However, the number of heterophils that had infiltrated the tracheal epithelium was significantly increased in the ts-11 C3 vaccinated group (Group 3) compared to Groups 1 or 2 (Table 4). Histological examination of the tracheas also demonstrated significant infiltration of heterophils into the tracheal epithelium in Group 3 (Fig. 3).

4. Discussion

In this study, a strain of MG ts-11 expressing chIFN-γ (ts-11 C3) was generated using a transposon expression vector. This is the first report of successful expression of a cytokine in any mycoplasma species. The ts-11 C3 strain secreted chIFN-γ into the culture supernatant, but still retained its temperature-sensitive phenotype. PCR analysis showed that the 20 bp insertion in the *gapA* gene found in ts-11 was also preserved in ts-11 C3, indicating that this clone possessed similar genotypic and phenotypic properties to the ts-11 vaccine.

ChIFN-γ expression in ts-11 C3 was unable to be detected by Western blotting (data not shown), and was only detected in the supernatants of ts-11 C3 cultures by ELISA (Fig. 1) and at a con-

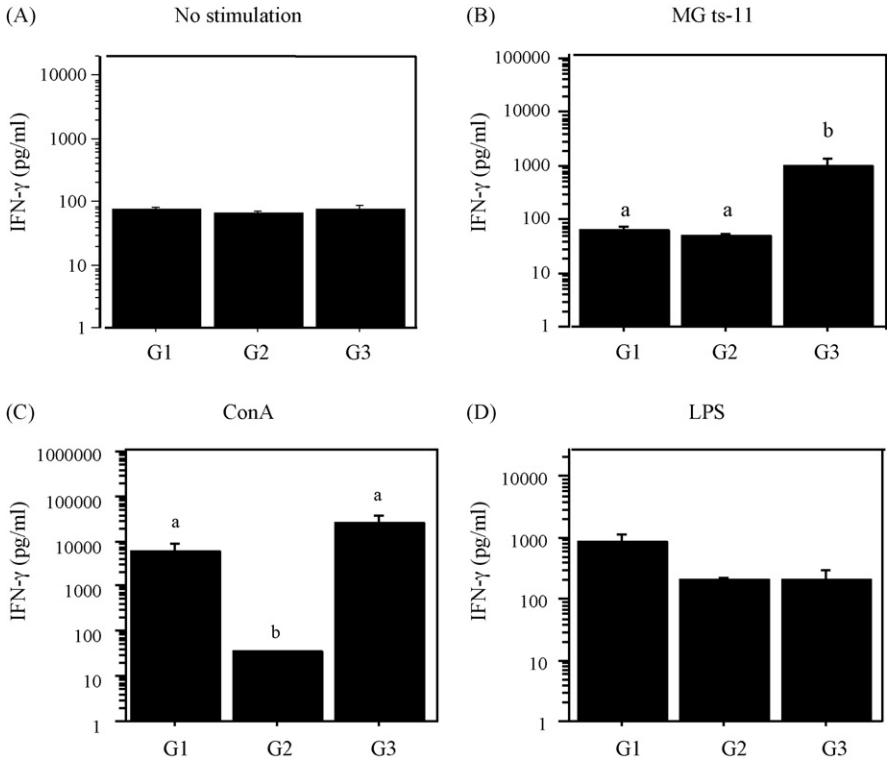


Fig. 2. IFN-γ production by stimulated spleen cell cultures. Fifteen chickens (5 from each group) were killed at 2 weeks after immunization, spleens were collected and the spleen cells were isolated. A total of 5×10^7 cells were either left unstimulated (A) or stimulated with either heat-killed MG ts-11 antigen (B), 10 μgConA/ml (C), or 10 μg LPS/ml (D) and cultured at 39 °C. After 5 days, the IFN-γ concentration in the supernatant was measured by chIFN-γ ELISA. a vs. b Columns labelled with different lower case letters are significantly different ($P < 0.05$).

Table 4
Trachea l mucosal thickness and the number of heterophils in the tracheal epithelium

Group	Trachea l mucosal thickness (μm ± S.E.)		Number of heterophils per high powered field (mean ± S.E.)	
	Upper trachea	Lower trachea	Upper trachea	Lower trachea
1	50.1 ± 3.6	33.3 ± 1.8	0.60 ± 0.22[a]	0.25 ± 0.15[a]
2	53.1 ± 3.2	32.3 ± 1.8	0.40 ± 0.14[a]	0.32 ± 0.13[a]
3	52.5 ± 1.8	33.4 ± 2.1	8.77 ± 2.18[b]	6.00 ± 1.32[b]

The data from birds killed at both 1 and 2 weeks after vaccination were pooled for comparison. Values with a different superscript letter are significantly different ($P < 0.05$).

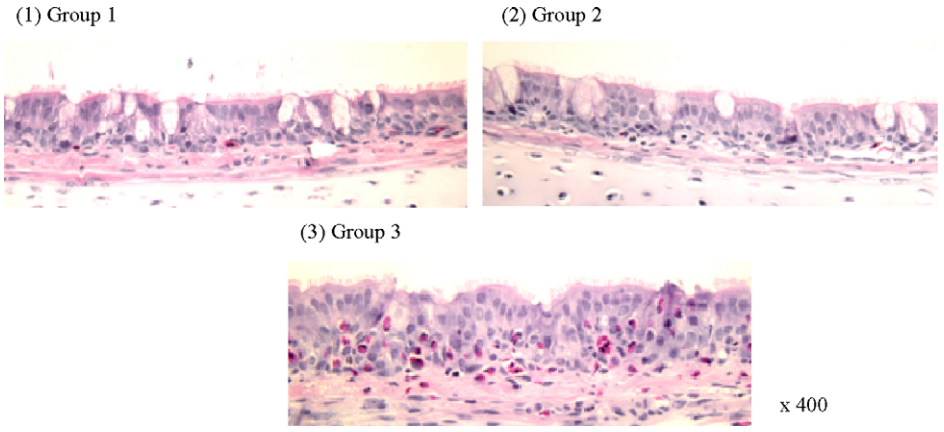


Fig. 3. Heterophil infiltration into the tracheal epithelium. Hematoxylin and eosin stained sections of trachea from immunized chickens were prepared and examined as described in Section 2. (1) Group 1; (2) Group 2; (3) Group 3. Significant heterophil infiltration was observed in the tracheal epithelium of birds in Group 3 (400× magnification).

centration of approximately 1 ng/ml. This level of expression is considered very low for a foreign gene, but is enough to modulate the immune response *in vivo*, as most cytokines work at ng and/or pg levels.

Indeed, when ts-11 C3 was inoculated into chickens by eye drop, the host immune response was altered compared with immunization with the parent ts-11 strain. However, no IFN-γ was detected in the serum and no daily body weight change was observed in ts-11 C3 vaccinated chickens (Table 2), suggesting there were no adverse side effects of expression of IFN-γ at this level by ts-11 C3 under these conditions.

Interestingly, ts-11 C3 immunized chickens did not produce detectable RSA antibodies, even at 2 weeks after vaccination (Table 3), although all ts-11 immunized chickens had a moderate RSA score by this time. Strain ts-11 usually induces sero conversion detectable by the RSA test at 2 weeks after immunization [3]. Moreover, ts-11 C3 immunization of chickens induced significantly higher MG ts-11 specific IFN-γ responses in spleen cells (Fig. 2B). The suppression of T cell responses has been reported in other mycoplasmas [24,25], and may be related to the effects of co-infection with MG on disease caused by viral respiratory pathogens [26]. Indeed, IFN-γ production after ConA stimulation, a non-specific T cell mitogen, was suppressed in ts-11 immunized birds 2 weeks after immunization. However, the amount of IFN-γ produced by spleen cells from ts-11 C3 immunized chickens after ConA stimulation was similar to that seen in unvaccinated chickens (Figs. 2C). These results, when considered together with those seen in spleen cells stimulated with ts-11, indicate that IFN-γ production by ts-11 C3 stimulates a host cellular immune response that overcomes the immunosuppressive effects of ts-11.

Chickens immunized with ts-11 C3 also had significant heterophil infiltration of the tracheal epithelium (Fig. 3 and Table 4). Heterophils are the avian equivalent of mammalian neutrophils. IFN-γ stimulates IL-8 production by human bronchial epithelial cells (HBECs) and enhances ICAM-1 expression on HBECs [27], which is critical for neutrophil migration and adherence. IFN-γ also enhances neutrophil transmigration across the human airway epithelium through its effect on IL-8 production [28]. Previous studies with recombinant chIFN-γ have shown that it can activate and enhance phagocytosis of *Salmonella enteritidis* by heterophils [17] as well as cytokine gene expression in heterophils [18]. These results suggest that IFN-γ production by ts-11 C3 stimulates the mucosal cellular immune response and recruits heterophils into the tracheal epithelium. The increased concentration of heterophils may account for the difficulty experienced in isolating ts-11 C3 from the mucosa after immunization even though ts-11 was readily re-isolated from the mucosa of all birds after immunization (Table 2) as ts-11 C3 cells might be expected to be phagocytosed and killed by the heterophils.

In conclusion, a strain of MG ts-11 expressing chIFN-γ was successfully created and was examined for its immunogenicity in chickens. The strain ts-11 C3 induced greater cellular immunity and less humoral immunity compared to the parent ts-11 strain, and was also able to induce greater mucosal heterophil infiltration. These results indicate that IFN-γ produced by ts-11 C3 can alter the host's immune response and Th1/Th2 balance, and can also induce greater mucosal cellular immune responses. These results also suggest that ts-11 C3 is a promising vaccine vector, capable of delivering protective antigens from other chicken respiratory pathogens such as avian influenza virus as well as generating non-specific protection against mucosal bacterial pathogens of chickens such as *E. coli*, and *S. enteritidis*. Challenge studies are currently underway to explore the protective effect of vaccination with ts-11 C3 against a virulent strain of MG.

Acknowledgements

This work was supported by a Zoonosis Control Project grant (ZCP-16) from the Ministry of Agriculture, Forestry, and Fisheries of Japan and a 2005–2006 from the Japanese Society for the Promo-

tion of Science Postdoctoral Fellowship for Research Abroad. This work was also supported by funding from the Australian Poultry CRC (Project no. 03-11).

References

[1] Stipkovits L, Kempf I. Mycoplasmoses in poultry. Rev Sci Tech 1996;15:1495–525.
[2] Levisohn S, Kleven SH. Avian mycoplasmosis (*Mycoplasma gallisepticum*). Rev Sci Tech 2000;19:425–42.
[3] Whithear KG. Control of avian mycoplasmoses by vaccination. Rev Sci Tech 1996;15:1527–53.
[4] Whithear KG, Soeripto, Harrigan KE, Ghiocas E. Safety of temperature-sensitive mutant *Mycoplasma gallisepticum* vaccine. Aust Vet J 1990;67:159–65.
[5] Whithear KG, Soeripto, Harrigan KE, Ghiocas E. Immunogenicity of a temperature-sensitive mutant *Mycoplasma gallisepticum* vaccine. Aust Vet J 1990;67:168–74.
[6] Papazisi L, Gorton TS, Kutish G, Markham PF, Browning GF, Nguyen DK, et al. The complete genome sequence of the avian pathogen *Mycoplasma gallisepticum* strain R (low). Microbiology 2003;149:2307–16.
[7] Halbedel S, Stulke J. Tools for the genetic analysis of mycoplasma. Int J Med Microbiol 2007;297:37–44.
[8] Boehm U, Klamp T, Groot M, Howard JC. Cellular responses to interferon-γ. Annu Rev Immunol 1997;15:749–95.
[9] Solid LM, Kvale D, Brandtzaeg P, Markussen G, Thorsby E. Interferon-gamma enhances expression of secretory component, the epithelial receptor for polymeric immunoglobulins. J Immunol 1987;138:4303–6.
[10] Gaunson JE, Philip CJ, Whithear KG, Browning GF. The cellular immune response in the tracheal mucosa to *Mycoplasma gallisepticum* in vaccinated and unvaccinated chickens in the acute and chronic stages of disease. Vaccine 2006;24:2627–33.
[11] Gaunson JE, Philip CJ, Whithear KG, Browning GF. Lymphocytic infiltration in the chicken trachea in response to *Mycoplasma gallisepticum* infection. Microbiology 2000;146:1223–9.
[12] Razin S, Yogev D, Naot Y. Molecular biology and pathogenicity of mycoplasmas. Microbiol Mol Biol Rev 1998;62:1094–156.
[13] Muneta Y, Minagawa Y, Shimoji Y, Nagata R, Markham PF, Browning GF, et al. IL-18 expression in pigs following infection with *Mycoplasma hyopneumoniae*. J Interferon Cytokine Res 2006;26:637–44.
[14] Digby MR, Lowenthal JW. Cloning and expression of the chicken IFN-γ gene. J Interferon Cytokine Res 1995;15:939–45.
[15] Lowenthal JW, Lambrecht B, van den Berg TP, Andrew ME, Strom AD, Bean AG. Avain cytokines—the natural approach to therapeutics. Dev Comp Immunol 2000;24:355–65.
[16] Johnson MA, Pooley C, Lowenthal JW. Delivery of avian cytokines by adenovirus vectors. Dev Comp Immunol 2000;24:343–54.
[17] Kogut MH, Pishko EJ, Kaspers B, Weining KC. Modulation of functional activities of chicken heterophils by recombinant chicken IFN-gamma. J Interferon Cytokine Res 2001;21:85–92.
[18] Kogut MH, Rothwell L, Kaiser P. IFN-gamma priming of chicken heterophils upregulates the expression of proinflammatory and Th1 cytokine mRNA following receptor-mediated phagocytosis of *Salmonella enterica* serovar enteritidis. J Interferon Cytokine Res 2005;25:73–81.
[19] Markham PF, Glew MD, Brandon MR, Walker ID, Whithear KG. Characterization of a major hemagglutinin protein from *Mycoplasma gallisepticum*. Infect Immun 1992;60:3885–91.
[20] Knudtson KL, Minion FC. Construction of Tn4001lac derivatives to be used as promoter probe vectors in mycoplasmas. Gene 1993;137:217–22.
[21] Markham PF, Glew MD, Browning GF, Whithear KG, Walker ID. Expression of two members of the pMGA gene family of *Mycoplasma gallisepticum* oscillates and is influenced by pMGA-specific antibodies. Infect Immun 1998;66:2845–53.
[22] Whithear KG. Avian mycoplasmosis. In: Bagust TJ, Corner LA, editors. Australian Standard Diagnostic Techniques for Animal Diseases. Melbourne: CSIRO for the Standing Committee on Agriculture and Resource Management; 1993. p. 1–12.
[23] Nunoya T, Tajima M, Yagihashi T, Sannai S. Evaluation of respiratory lesions in chickens induced by *Mycoplasma gallisepticum*. Jpn J Vet Sci 1987;49:621–9.
[24] Teh HS, Ho M, Williams LD. Suppression of cytotoxic responses by a supernatant factor derived from *Mycoplasma hyorhinis*-infected mammalian cell lines. Infect Immun 1988;56:197–203.
[25] Cole BC, Wells DJ. Immunosuppressive properties of the Mycoplasma arthritidis T-cell mitogen *in vivo*: inhibition of proliferative responses to T-cell mitogens. Infect Immun 1990;58:228–36.
[26] Roussan DA, Haddad R, Khawaldeh G. Molecular survey of avian respiratory pathogens in commercial broiler chicken flocks with respiratory diseases in Jordan. Poult Sci 2008;87:444–8.
[27] Striz I, Mio T, Adachi Y, Carnevali S, Romberger DJ, Rennard SI. Effects of interferons alpha and gamma on cytokine production and phenotypic pattern of human bronchial epithelial cells. Int J Immunopharmacol 2000;22:573–85.
[28] Kidney JC, Proud D. Neutrophil transmigration across human airway epithelial monolayers: mechanisms and dependence on electrical resistance. Am J Respir Cell Mol Biol 2000;23:389–95.

Differentiation of *Mycoplasma gallisepticum* strains using PCR and High Resolution Melting Curve Analysis

Running title: HRM curve analysis for MG strain differentiation

Seyed A. Ghorashi, Amir H. Noormohammadi* and Philip F. Markham

Faculty of Veterinary Science, The University of Melbourne, Werribee, Victoria, 3030, Australia

*Corresponding author. Mailing address: Faculty of Veterinary Science, The University of Melbourne, 250 Princes Highway, Werribee, Victoria, 3030, Australia
Phone: +61 3 9731 2275, Fax: +61 3 9731 2366, Email: amrih@unimelb.edu.au

Mycoplasma gallisepticum **(MG) is an economically important pathogen of poultry worldwide causing chronic respiratory disease in chickens and turkeys. Differentiation of MG strains is critical, especially in countries where poultry flocks are vaccinated with live vaccines. In this study, oligonucleotide primers were designed based on a region preceding the trinucleotide repeat of a member of the *vlhA* gene family and amplicons of 145-352 bp were generated from cultures of 10 different MG strains including the ts-11, F and 6/85 vaccine strains. High resolution melting (HRM) curve analysis of the resultant amplicons could differentiate all MG strains. Analysis of the nucleotide sequence of the amplicons from each strain revealed that each melting curve profile related to a unique DNA sequence. The HRM curve profiles (for ts-11) remained consistent after at least five passages under laboratory condition. PCR HRM curve analysis of 33 DNA extracts derived from respiratory swabs, or mycoplasma cultures grown from respiratory swabs, of ts-11 vaccinated commercial or specific pathogen free chickens identified all these specimens, according to their sequences, as ts-11. The potential of the PCR-HRM curve analysis was also shown for genotyping of 30 additional MG isolates from Europe, USA and Israel. The results presented in this study indicate that PCR followed by HRM curve analysis provides a rapid and robust technique for genotyping of MG isolates/strains using both MG cultures and clinical swabs.**

Key words: high resolution melting curve analysis, *Mycoplasma gallisepticum*, MG, PCR, strain differentiation, genotyping

INTRODUCTION

Mycoplasma gallisepticum (MG) is an important pathogen of poultry worldwide causing chronic respiratory disease in chickens and turkeys, reduction in egg production and considerable economic losses to the poultry industry (Ley, 2008). Diagnosis of MG infection can be made by various methods but the gold standard test for confirmation of diagnosis is isolation and identification of the organism (Ley, 2008). Such examinations typically require 2-3 weeks to complete. PCR and recently, real-time PCR have been used for rapid detection and/or identification of MG from cultures or directly from clinical samples (Callison *et al.*, 2006; Feberwee *et al.*, 2005b; Grodio *et al.*, 2008; Hess *et al.*, 2007; Nascimento *et al.*, 1991). Since live vaccines are used in many countries, differentiation of MG strains has become increasingly important. The live vaccine strains F and 6/85 (originated from USA), and ts-11 (originated from Australia) are used for control of MG infection in several countries (Ley, 2008). PCR followed by RFLP (Kiss *et al.*, 1997; Lysnyansky *et al.*, 2005) and amplified fragment length polymorphism (AFLP) (Cherry *et al.*, 2006; Hong *et al.*, 2005) have been used for identification of MG strains. However, these techniques may suffer from low reproducibility, are labour intensive and time consuming and often require extensive interpretation leading to a relatively expensive diagnostic test. Recently real-time PCR was used for differentiation of one of five vaccine strains from a challenge strain but restricted to comparison of only paired strains in each test (Raviv *et al.,* 2008). Differentiation of MG strains based on multiple surface protein genes has also been reported (Ferguson *et al.*, 2005). However, this technique requires nucleotide sequencing of amplified genes and subsequent analysis and interpretation of the results. Recent studies in our laboratory have found that a combination of PCR and high resolution melting (HRM) curve analysis provides a rapid and cost-effective alternative to the direct analysis of nucleotide sequence variation, particularly when large numbers of samples are to be analysed (Hewson *et al.,* 2009; Jeffery *et al.,* 2007; Steer *et al.,* 2009).

The *vlhA* intergenic region in MG genome contains conserved and variable regions (Papazisi *et al.*, 2003). Preliminary examinations of the *vlhA* intergenic region through sequences previously available in the GenBank database, suggested the potential of this region for detection and differentiation of MG strains.

The aim of the present study was to investigate the potential of HRM curve analysis of PCR amplicons generated from *vlhA* intergenic region to establish a single closed-tube test method for differentiation of MG strains. Both conventional and normalised dissociation plots were generated and genotyping applied to differentiate the MG isolates/strains, and the results compared with DNA sequencing of PCR amplicons.

METHODS

MG strains. Twelve MG strains including three vaccine strains (ts-11, 6/85 and F), the reference strain S6 and eight MG strains (from Australia or the USA) available in our laboratory were used initially in this study (Table 1). All cultures were grown from a single colony in modified mycoplasma broth (Morrow *et al.,* 1998). All mycoplasma cultures were grown in mycoplasma broth containing 10% swine serum, 0.01% (w/v) nicotinamide adenine dinucleotide (NAD) and 0.002% (w/v) herring sperm DNA (Sigma, Castle Hill, Australia). The media base consisted of: 7.5 g trypticase peptone, 2.5 g phytone peptone, 0.5 g thiotone peptone (all

from BBL, North Ryde, Australia), 5 g yeast extract (Difco, North Ryde, Australia), 5 g sodium chloride, 0.4 g potassium chloride, 0.35 g magnesium sulphate heptahydrate, 0.05 g disodium hydrogen orthophosphate, 0.1 g potassium dihydrogen phosphate, 1 g glucose, 1.5 ml of a 1.6% phenol red solution, and 10 ml of a yeast autohydrolosate solution prepared from bakers' yeast per liter. Mycoplasma agar was prepared as for mycoplasma broth, with the omission of glucose and phenol red, and solidified with 1.0% (w/v) Special Noble agar (Difco).
Also three groups of MG strains from USA, Europe and Israel were used to evaluate the newly developed PCR-HRM technique for its potential to differentiate MG strains from a wider geographical locations (Table 4). These specimens were compared with each other and with MG strains analysed earlier in this study (Table 1).

DNA extraction. Total genomic DNA was extracted from mycoplasma cultures and from swabs taken from birds using a DNA extraction kit (QIAGEN) according to the manufacturer's instructions. Genomic DNA was extracted from a commercial batch of Vaxsafe MG (Bioproperties Australia Pty Ltd) and from the ts-11 master seed which was previously passaged at least five times *in vitro*.
A group of fifteen specific pathogen free (SPF) chickens and two separate commercial layer chicken flocks were vaccinated with ts-11 vaccine strain according to the manufacturer's instructions at 28 days of age within a positive pressure isolator or under field condition respectively. DNA was extracted from fifteen tracheal swabs taken from SPF chickens two weeks postvaccination and from eighteen cultures grown from swabs taken from commercial layer chickens 7-12 weeks post vaccination. Briefly, 0.5 ml of mycoplasma culture was pelleted by centrifugation at 20000 ***g*** for 5 min. The cells were washed twice in phosphate buffered saline (PBS) and resuspended in 500 µl RLT lysis buffer (QIAGEN) and incubated for two hours at room temperature or overnight at 4 ºC. Swabs taken from birds were also placed in 0.5 ml lysis buffer (QIAGEN) and incubated at 4 ºC overnight. Then 15 µl of Qiaex II matrix (QIAGEN) and 300 µl 70% ethanol were added and mixed, and the lysate was loaded into a multispin MSK-100 column (Axygen Inc., Hayward, CA, USA), centrifuged for 30 s at 10000 ***g*** and the flow-through discarded. The column was washed with 600 µl RW1 buffer (QIAGEN) and twice with 500 µl RPE buffer (QIAGEN) and subjected to centrifugation at 18000 ***g*** for 90 s. The DNA was eluted from the matrix using 50 µl distilled water and used in PCR immediately or stored at -20 ºC for future use.

PCR. The region preceding the trinucleotide repeat of a member of the *vlhA* gene family was chosen for amplification. This was based on the observation of poor conservation of nucleotides within this region between *vlhA* family members (Papazisi *et al.*, 2003). A pair of oligonucleotide primers, ts-11-F (5'-GTTTGGAGTTGGTGTATAGTTAG -3') and ts-11-R (5'-TCTTCTTCGAAAACAAAAGG -3'), flanking the target region was designed with the PCR amplicon expected to yield a product of 226 bp. Amplification of DNA was performed in 25 µl reaction volume on I-Cycler thermal cycler (Bio-Rad). The reaction mixture contained 3 µl extracted genomic DNA, 25 µM of each primer, 1.5 mM $MgCL_2$, 1250 µM of each dNTp, 5 µM SYTO® 9 green fluorescent nucleic acid stain (Invitrogen), 1× GoTaq® Green Flexi Reaction Buffer (Promega) and 1 U of Go Taq DNA polymerase (Promega). PCR conditions were one cycle of 94 ºC for 60 s, 35 cycles of 94 ºC for 10 s, 50 ºC for 10 s and 72 ºC for 10 s, and a final cycle of 72 ºC for

one min. In each set of reactions, MG ts-11 genomic DNA and distilled H_2O were included as positive and negative controls, respectively. The PCR products together with standard molecular weight marker, were analysed by electrophoresis in 1.7% agarose gels stained with GelRed™ (Biotium, Hayward, CA, USA), and visualised by UV transillumination.
DNA from *Mycoplasma synoviae* (strain WVU-1853), a field isolate of *Mycoplasma meleagridis*, *Mycoplasma anatis*, *Mycoplasma gallinaceum* and *Mycoplasma gallinarum* were tested in PCR (as described above) did not produce any detectable band on agarose gel.

High-resolution melting curve acquisition and analysis. HRM curve analysis was performed in a Rotor-Gene™ 6000 thermal cycler (Corbett Life Science Pty Ltd). In order to determine the optimal melting condition for differentiation of MG strains, the PCR products were subjected to three different rampings of 0.1 °C, 0.2 °C and 0.3 °C/s between 72 °C and 82 °C. All specimens were tested in triplicate and their melting profiles analysed using Roter Gene 1.7.27 software and the HRM algorithm provided. Normalisation regions of 73.5-74.0 and 79.5-80.0. Each isolate was set as a 'genotype' and the average HRM genotype confidence percentages (GCP), (the value attributed to each isolate/strain being compared to the genotype with a value of 100% indicating an exact match) for the replicates were predicted by the software. The GCPs for MG ts-11 known isolates were averaged and the standard deviation (SD) calculated and used to establish the GCP range for ts-11 vaccine strain cut off point. The cut off point was applied in HRM analysis to evaluate the differentiation power of the test to discriminate ts-11 strain.

Sequencing and nucleotide sequence analysis of PCR amplicons. PCR amplicons were gel purified using the QIAquick® Gel Purification Kit (QIAGEN) following manufacturer's instructions. Purified amplicons were subjected to automated sequencing (BigDye® Terminator v3.1, Applied Biosystems) in both directions, using the same primers as used for PCR. The sequences were analysed using ClustalW (Thompson *et al.*, 1994) and DNAdist (Felsenstein, 1989) in BioManager (Australian National Genomic Information Service, Sydney Bioinformatics) and BioEdit Sequence Alignment Editor (version 6.0.9.0). GenBank accession numbers were assigned to the nucleotide sequences of the MG isolates and reference strains (Table 1).

RESULTS

PCR amplicons of different sizes generated from MG strains using oligonucleotide primers ts-11-F and ts-11-R

Amplified PCR products from different MG strains were analysed by gel electrophoresis (Fig. 1). MG strains ts-11, S6, F and K1453 generated one major band while 6/85, Ap3AS, K1659, 86134, 87006 and 87081 produced a major band with one or two minor bands visible on agarose gel. The major band amplicons ranged in size from 145 (87006) to 352 bp (S6).

Conventional and normalised HRM curve analysis differentiates MG strains

The PCR amplicons from 10 different MG strains (Table 1) were subjected to HRM curve analysis (Fig. 2). Visual examination of the conventional melt curves at different ramp temperatures revealed that 0.3 °C/s resulted in most strains showing distinct conventional melt curves for MG strains. In the conventional melt curve, ten distinct curve profiles were detected (Fig. 2a). MG vaccine strains ts-11 and 6/85 each generated a single peak at 76.5 and 74.8 ºC respectively (Table 2). The MG strain 86134 and Ap3As generated only two major peaks at 75.3 and 77.3 ºC, and 75.7 and 77.1 ºC respectively. The MG strains S6 and K1453 generated a major peak at 78.2 and 77.7 ºC respectively and a shoulder peak at a lower temperature than their major peak. MG strains 87006 and K1659 generated a major peak at 74.9 and 75.2 ºC respectively and a minor peak at higher temperatures than their major peak. The MG strain 87081 was the only strain that generated one major peak at 74.8 ºC and two minor peaks at higher temperatures than the major peak.

All MG strains also generated ten distinct normalised HRM curves (Fig. 2b). Replicates of each MG strain were genotyped with defined genotypes in normalised HRM graph with equal or greater than 95 GCP (Table 2). HRM curve analysis for PCR amplicons using templates from DNA extractions and/or PCRs run on different days showed slight shifts in melting temperature; however, the conventional melt curve shapes and normalised HRM graphs were unchanged. The mean and standard deviation of the melting points for the different peaks, and GCP of standard errors resulting from several runs of PCR and HRM curve analysis are shown in Table 2.

Consistent HRM curve profile of MG after passage *in vitro* and *in vivo*

In order to evaluate the consistency of the newly-developed PCR HRM curve analysis technique following MG strain passage *in vitro* and *in vivo*, the MG ts-11 vaccine strain was used as a model. Highly similar conventional and normalised HRM curve profiles were generated from Vaxsafe MG (Bioproperties Australia Pty Ltd) and the master seed (results not shown). To assess *in vivo* sensitivity and specificity of the developed PCR test, DNA from swabs taken from SPF and commercial layer chickens were subjected to PCR and HRM curve analysis. Both conventional and normalised curves were highly similar to those of ts-11 (Fig. 3) with a mean of 94.5 ± 2.0 and 87.0 ± 14.3 GCP for the fifteen SPF and eighteen commercial layer chicken specimens, respectively.

Detection of minor variations in *vlhA* nucleotide sequence by the newly-developed PCR HRM curve analysis technique

To confirm if classification of MG strains by HRM curve analysis is correlated with variation in nucleotide sequence of the amplicons, nucleotide sequences of amplicons from each distinct curve profile as well as from one MG isolate, from a SPF chicken and one from a commercial layer chicken specimen were determined and compared. Some of MG strains generated more than one DNA band on agarose gel (Fig. 1). In order to compare the nucleotide sequences of the amplicons from different MG strains, the major DNA band of each strain was gel purified, sequenced and used for calculation of sequence identities. Note, only major DNA bands were examined here since contribution of the other (minor) bands towards the overall sequence identities was difficult to determine especially because the intensity of staining varied between the bands most likely due to different number of amplicons per band.

The PCR amplicon for S6 reference strain was composed of 352 bp and in comparison, ts-11 amplicon was 226 bp in length with a 126 bp deletion at the 5’ end (Fig. 4). Other strains with a distinct HRM curve profile demonstrated contained deletion of 33 to 207 nucleotides in their

sequences with a number of nucleotide substitutions through out each of sequences (Fig. 4). Comparison of the nucleotide sequences revealed that PCR amplicons generated from isolates from the SPF and commercial layer chickens vaccinated with ts-11 strain were identical to the MG ts-11 vaccine strain sequence (data not shown). The extent of sequence variability in PCR amplicons for the representative of each distinct group was determined and compared with the mean GCP of HRM analysis (Table 3).

The highest sequence identity (100%) was observed between amplicon sequences from 6/85, Ap3AS and 86134 strains while the lowest (36.1%) belonged to those of K1659 and 87081 strains. The GCPs for the HRM curves related to 6/85/Ap3AS, 6/85/86134 and 86134/Ap3AS were 0.0, 0.0 and 13.2 respectively. The GCP for the HRM curves of K1659 and 87081 was 0.4. MG strains F and 87081 also had high sequence identity (99.1%) however, generated two different conventional melt curves with three peaks and one peak respectively. This difference also was reflected in their normalised melt curves and GCP (0.1). Since MG F strain produced one amplicon band while MG 87081 generated two, the difference in the shape of the normalised and conventional melt curves or the number of peaks in conventional melt curves was likely to be due to the contribution of additional DNA bands.

The highest GCP (66.1) was found between S6 and F strain while the lowest (0.0) was between ts-11 and a number of specimens including F, S6 and 6/85 strains. The sequence identitiy for the S6 and F strains was 98.7% while those for the ts-11 and other strains such as F, S6 and 6/85 were 79.2, 81.0 and 56.4% respectively.

A low correlation (0.2) was observed when GCP and sequence identity of all MG strains were compared. The correlation between sequence identity and GCP of MG strains (ts-11, S6, F and K1453) (that generated a single amplicon) was found to be 0.78. Thus the low correlation between sequence identities and GCPs when all strains were used in comparison was due to the contribution of extra amplicons generated by MG strains 6/85, Ap3AS, K1659, 86134, 87006 and 87081.

A mathematical calculation for non-subjective grouping of MG strains using GCPs

Using GCPs for ts-11 vaccine strain, a cut-off value was generated as a mathematical model to assess the relationship of the field isolates without visual interpretation by the operator (non-objective). The average of the 125 genotype confidence values for ts-11 strain was 93.7 with a SD of 10.7. A value of 3 SD (32.1) was then calculated and subtracted from the average GCP to determine a cut off point. Thus the GCP range for ts-11 vaccine strain and related field samples was determined to be 61.6 - 100. The cut off point of 61.6 was then applied for genotyping MG strains and isolates. All amplicons generated from ts-11 vaccinated SPF and commercial layer chickens had GPCs between 63.1-100 and were therefore genotyped automatically as ts-11. All the other MG strains had GCPs between 0.0-19.5 and were therefore automatically identified as “variation” (Fig. 5). Thus the gap between the highest non-ts-11 and lowest ts-11 specimens was approximately 43 GCP.

Assessment of the potential of the PCR-HRM technique for differentiation of MG isolates from diverse geographical locations

The PCR amplicons from each three groups of MG isolates from USA, Europe and Israel (Table 4) were subjected to HRM curve analysis and isolates with conventional/normalised curves

similar to each other or to those characterised earlier in this study (Table 1) were selected for further examinations by nucleotide sequencing. The shape of the curves and number, relative height and temperature of the peaks and subsequently the GCPs were considered in this initial screening.

Visual examination of the conventional and normalised melt curves of the MG isolates from USA (Fig. 6a) revealed that MG isolate K6216D had a single peak at 76.8 °C highly similar to the ts-11 and was genotyped as ts-11 with 95 GCP. The nucleotide sequence of the amplicon (226 bp) was also identical to that of ts-11. MG isolates K5917 and K6096 had two similar conventional melt curve peaks at 76.5 and 79.7 °C and similar normalisation curves at GCP of 99. The sequence of the PCR amplicons (323 bp) was also identical. The MG isolates K5037A and K5792D also generated similar normalised and conventional melt curves with one peak at 78.3 °C and a shoulder peak at a lower temperature. These two isolates produced a major DNA band (319 bp) with identical nucleotide sequences. The MG isolate K4181C and K4094 generated slightly different pattern to those of K5917 and K6096 with two peaks at 76.6 and 79.5 °C, and 76.6 and 79.8 °C respectively. The nucleotide sequence analysis of these two isolates revealed 99.7% identity to each other and 93.7-100% identity to K5917/K6096. The rest of MG isolates from USA (K4158C, K4355, K4705, K503 and K5104) each had curve shapes that were distinct to the other USA and Australian MG strains/isolates used in this study.

Visual examination of conventional and normalised melt curves of MG isolates from Europe revealed that MG isolate B40/95 and F strain generated amplicons with similar melt curve with one peak at 78.4 °C and a shoulder peak at lower temperature. Nucleotide sequences of amplicons revealed 99.7% sequence identity (one nucleotide substitution in 319 bp). This similarity was also reflected in similar normalised melt curves with GCP of 88. All other MG isolates from Europe did not have similarity to each other (Fig. 6b) or to any other reference strains/isolates characterised earlier in this study.

Examination of the Israeli MG isolates revealed that the isolate MSA-9 and MKT-6 generated highly similar conventional melt curves with two peaks at 74.6 and 77.8 °C (Fig. 6e). The normalized melt curves for the amplicons of these isolates also were highly similar and had GCP of 92 (Fig. 6f). The nucleotide sequence of the respective amplicons (355 bp) were found to be identical. Similarly the MG isolates BCV-6 and VR-5 generated amplicons with similar conventional melt curves with one peak at 78.0 °C and a shoulder peak at a lower temperature. Nucleotide sequences (319 bp) of amplicons were identical to each other and to the MG strain K1453 (examined earlier in this study). This similarity was also reflected in similar normalised melt curves with GCP of 90.

DISCUSSION

This study describes a rapid and reliable technique for differentiation of MG isolates/strains. Increasing use of the MG live vaccines in poultry has led to a need for a reliable technique that can differentiate MG vaccine strains from field isolates. This is primarily for epidemiological investigation but may also be a requirement of registration authorities when a new MG vaccine is introduced to a country.

The use of PCR alone (Evans & Leigh, 2008; Feberwee *et al.*, 2005b) or combined with sequencing (Ferguson *et al.*, 2005; Raviv *et al.*, 2007), RFLP (Khan & Yamamoto, 1989; Kleven *et al.*, 1988a; Lysnyansky *et al.*, 2005) or AFLP (Feberwee *et al.*, 2005a; Hong *et al.*, 2005) and of RAPD (Feberwee *et al.*, 2005a; Ferguson *et al.*, 2005; Geary *et al.*, 1994), and PFGE (Marois

et al., 2001; Mettifogo *et al.,* 2006) have been described for differentiation of MG isolates/strains. Although these techniques may currently be used in some laboratories, they have limitations such as low reproducibility, lengthy procedure and need for extensive interpretation, particularly when a large number of specimens are to be tested. In this study, initial PCR demonstrated a discriminatory power to differentiate ts-11 vaccine strain and MG field isolates based solely on the amplicon size. The ts-11 vaccine strain produced a single DNA band (226 bp) while some isolates produced two (Ap3AS, K1659) or three bands (86134, 87006 and 87081). It has been shown that MG has a large number of *vlhA* gene copies distributed in five distinct loci throughout the genome (Baseggio *et al.*, 1996; Papazisi *et al.*, 2003). Thus, generation of more than one amplicon is expected since a relatively high sequence identity exists between promoter regions of the *vlhA* gene family members. Irrespective of the basis for amplification of more than one amplicon, variation in the number of the amplicons may indeed have contributed to the power of the HRM technique in differentiating different MG strains. Variation in size and number of amplicons could have sufficed for differentiation of a number of MG strains by examination of the PCR products on an agarose gel. However, some strains were found to generate the same number of bands with similar sizes. For example strain K1453 and F both generated a single band of similar size (Fig.1). Thus discrimination of these strains using agarose gel electrophoresis may not be possible. In contrast, these strains were readily distinguishable from each other in conventional and normalised HRM curve analysis.

The melting profile of a PCR product is dependent upon length, sequence, GC content, and heterozygosity (Reed *et al.*, 2007). There was no correlation between the number of *vlhA* bands and the number of peaks. Although the number of peaks could be influenced by the number of bands and distribution of the nucleotides in the amplicon. The first shoulder peak of S6, F and K1453 is likely to be due to A-T rich region which melts at lower temperature and the second peak is likely to be due to melting G-C rich region at a higher temperature. For specimens such as 87006 with 3 PCR bands, the major band produces one peak and the 2 extra bands may perhaps contribute to the shape of the curve.

In this study, ts-11 was used as a model to demonstrate the consistency of HRM curve profiles after passage *in vitro* and *in vivo*. All ts-11 vaccine reisolates generated one peak at 76.5 ± 0.3 in conventional melt curve and genotyped as ts-11 with 63.1 or higher GCP. In contrast the highest GCP between ts-11 and other strains used in this study was 19.5%. This illustrates the discriminatory power of HRM curve analysis for differentiation of ts-11 from other MG strains. The PCR HRM curve analysis could also differentiate MG strains that generated amplicons with high sequence identities. For example, the MG strains 6/85, 86134 and Ap3AS had a 100% sequence identity but had distinct conventional and normalised melt curves. Likewise, strains 87081 and K1453 generated distinct melt curve profiles although had only a relatively small difference (7.2%) in their similar size (319 bp) amplicons.

The capacity of PCR-HRM technique in differentiating MG strains/isolates was further evaluated by testing additional thirty MG isolates from different geographical locations including Europe, USA and Israel. Interestingly a field isolate from USA (K6216D) was found to generate similar conventional and normalised melt curves to those of ts-11 with 95 GCP. This isolate had been isolated from progenies of ts-11 vaccinated broiler breeders and found to be indistinguishable from ts-11 by RAPD (personal communication with Dr Naola Ferguson, University of Georgia, USA). The USA MG isolates K5917 and K6096 were found identical by HRM curve analysis. These two isolates had similar mgc2 sequences and were found undistinguishable in the host laboratory (personal communication with Dr Naola Ferguson, University of Georgia, USA). The

MG isolates K5037A and K5792D were also found to have similar HRM curves however were reportedly different in their IGSR sequences (Raviv *et al.*, 2007). The cause of this discrepancy is unknown and may require further examination by nucleotide sequencing of other genes. The two Israeli MG strains VR5 and BCV-6 that were found indistinguishable by HRM melt curve analysis, had been isolated from broiler breeders located at different farms but, epidemiologically appeared to be linked (personal communication with Dr Inna Lysnyansky, Kimron Veterinary Institute Israel). No apparent epidemiological links existed between the other two Israeli strains (MSA-9 and MKT-6) that were also indistinguishable by HRM curve analysis. These isolates are currently under further examinations in the host laboratory.
The newly developed PCR HRM technique provides a rapid and reliable means for detection and differentiation of MG strains from both culture and clinical swabs.

ACKNOWLEDGMENTS

Funding for this project was provided by the Australian Poultry Cooperative Research Centre. The authors also like to thank Professor Janet Bradbury (University of Liverpool, UK), Dr Naola Ferguson-Noel (University of Georgia, USA) and Dr Lysnyansky Inna (Kimron Veterinary Institute, Israel) for providing mycoplasma isolates and also Mr Garry Anderson for providing advice on statistical analysis of the data.

REFERENCES

Adler, H. E., Yamamoto, R. & Berg, J. (1957). Strain differences of pleuropneumonialike organisms of avian origin. *Avian Dis* **1**, 19-27.

Baseggio, N., Glew, M. D., Markham, P. F., Whithear, K. G. & Browning, G. F. (1996). Size and genomic location of the pMGA multigene family of *Mycoplasma gallisepticum*. *Microbiology* **142 (Pt 6)**, 1429-1435.

Callison, S. A., Riblet, S. M., Sun, S., Ikuta, N., Hilt, D., Leiting, V., Kleven, S. H., Suarez, D. L. & Garcia, M. (2006). Development and validation of a real-time Taqman polymerase chain reaction assay for the detection of *Mycoplasma gallisepticum* in naturally infected birds. *Avian Dis* **50**, 537-544.

Carpenter, T. E., Miller, K. F., Gentry, R. F., Schwartz, L. D. & Mallinson, E. T. (1979). Control of *Mycoplasma gallisepticum* in commercial laying chickens using artificial exposure to Connecticut F strain *Mycoplasma gallisepticum*. *Proc Annu Meet U S Anim Health Assoc*, 364-370.

Cherry, J. J., Ley, D. H. & Altizer, S. (2006). Genotypic analyses of *Mycoplasma gallisepticum* isolates from songbirds by random amplification of polymorphic DNA and amplified-fragment length polymorphism. *J Wildl Dis* **42**, 421-428.

Evans, J. D. & Leigh, S. A. (2008). Differentiation of *Mycoplasma gallisepticum* vaccine strains ts-11 and 6/85 from commonly used *Mycoplasma gallisepticum* challenge strains by PCR. *Avian Dis* **52**, 491-497.

Evans, R. D. & Hafez, Y. S. (1992). Evaluation of a *Mycoplasma gallisepticum* strain exhibiting reduced virulence for prevention and control of poultry mycoplasmosis. *Avian Dis* **36**, 197-201.

Feberwee, A., Dijkstra, J. R., von Banniseht-Wysmuller, T. E., Gielkens, A. L. & Wagenaar, J. A. (2005a). Genotyping of *Mycoplasma gallisepticum* and *M. synoviae* by Amplified Fragment Length Polymorphism (AFLP) analysis and digitalized Random Amplified Polymorphic DNA (RAPD) analysis. *Vet Microbiol* **111**, 125-131.

Feberwee, A., Mekkes, D. R., de Wit, J. J., Hartman, E. G. & Pijpers, A. (2005b). Comparison of culture, PCR, and different serologic tests for detection of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* infections. *Avian Dis* **49**, 260-268.

Felsenstein, J. (1989). Mathematics vs. Evolution: Mathematical Evolutionary Theory. *Science* **246**, 941-942.

Ferguson, N. M., Hepp, D., Sun, S., Ikuta, N., Levisohn, S., Kleven, S. H. & Garcia, M. (2005). Use of molecular diversity of *Mycoplasma gallisepticum* by gene-targeted sequencing (GTS) and random amplified polymorphic DNA (RAPD) analysis for epidemiological studies. *Microbiology* **151**, 1883-1893.

Geary, S. J., Forsyth, M. H., Aboul Saoud, S., Wang, G., Berg, D. E. & Berg, C. M. (1994). *Mycoplasma gallisepticum* strain differentiation by arbitrary primer PCR (RAPD) fingerprinting. *Mol Cell Probes* **8**, 311-316.

Grodio, J. L., Dhondt, K. V., O'Connell, P. H. & Schat, K. A. (2008). Detection and quantification of *Mycoplasma gallisepticum* genome load in conjunctival samples of experimentally infected house finches (Carpodacus mexicanus) using real-time polymerase chain reaction. *Avian Pathol* **37**, 385-391.

Hess, M., Neubauer, C. & Hackl, R. (2007). Interlaboratory comparison of ability to detect nucleic acid of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* by polymerase chain reaction. *Avian Pathol* **36**, 127-133.

Hewson, K., Noormohammadi, A. H., Devlin, J. M., Mardani, K. & Ignjatovic, J. (2009). Rapid detection and non-subjective characterisation of infectious bronchitis virus isolates using high-resolution melt curve analysis and a mathematical model. *Arch Virol*.

Hong, Y., Garcia, M., Levisohn, S., Savelkoul, P., Leiting, V., Lysnyansky, I., Ley, D. H. & Kleven, S. H. (2005). Differentiation of *Mycoplasma gallisepticum* strains using amplified fragment length polymorphism and other DNA-based typing methods. *Avian Dis* **49**, 43-49.

Jeffery, N., Gasser, R. B., Steer, P. A. & Noormohammadi, A. H. (2007). Classification of *Mycoplasma synoviae* strains using single-strand conformation polymorphism and high-resolution melting-curve analysis of the vlhA gene single-copy region. *Microbiology* **153**, 2679-2688.

Khan, M. I. & Yamamoto, R. (1989). Differentiation of the vaccine F-strain from other strains of *Mycoplasma gallisepticum* by restriction endonuclease analysis. *Vet Microbiol* **19**, 167-174.

Kiss, I., Matiz, K., Kaszanyitzky, E., Chavez, Y. & Johansson, K. E. (1997). Detection and identification of avian mycoplasmas by polymerase chain reaction and restriction fragment length polymorphism assay. *Vet Microbiol* **58**, 23-30.

Kleven, S. H., Browning, G. F., Bulach, D. M., Ghiocas, E., Morrow, C. J. & Whithear, K. G. (1988a). Examination of *Mycoplasma gallisepticum* strains using

restriction endonuclease DNA analysis and DNA-DNA hybridisation. *Avian Pathol* **17**, 559-570.

Kleven, S. H., Morrow, C. J. & Whithear, K. G. (1988b). Comparison of *Mycoplasma gallisepticum* strains by hemagglutination-inhibition and restriction endonuclease analysis. *Avian Dis* **32**, 731-741.

Ley, D. L. (2008). *Mycoplasma gallisepticum* infection. In *Diseases of Poultry*, pp. 807-834. Edited by Y. M. Saif, Fadly, A.M., Glisson, J.R., McDougald, L.R., Nolan, L.K., Swayne, D.E. Ames: Blackwell Publishing Ltd.

Lysnyansky, I., Garcia, M. & Levisohn, S. (2005). Use of mgc2-polymerase chain reaction-restriction fragment length polymorphism for rapid differentiation between field isolates and vaccine strains of *Mycoplasma gallisepticum* in Israel. *Avian Dis* **49**, 238-245.

Lysnyansky, I., Gerchman, I., Perk, S. & Levisohn, S. (2008). Molecular characterization and typing of enrofloxacin-resistant clinical isolates of *Mycoplasma gallisepticum*. *Avian Dis* **52**, 685-689.

Marois, C., Dufour-Gesbert, F. & Kempf, I. (2001). Molecular differentiation of *Mycoplasma gallisepticum* and *Mycoplasma imitans* strains by pulsed-field gel electrophoresis and random amplified polymorphic DNA. *J Vet Med B Infect Dis Vet Public Health* **48**, 695-703.

Mettifogo, E., Buzinhani, M., Buim, M. R., Piantino Ferreira, A. J., Kleven, S. H. & Timenetsky, J. (2006). Molecular characterization of MG isolates using RAPD and PFGE isolated from chickens in Brazil. *J Vet Med B Infect Dis Vet Public Health* **53**, 445-450.

Morrow, C. J., Markham, J. F. & Whithear, K. G. (1998). Production of temperature-sensitive clones of *Mycoplasma synoviae* for evaluation as live vaccines. *Avian Dis* **42**, 667-670.

Nascimento, E. R., Yamamoto, R., Herrick, K. R. & Tait, R. C. (1991). Polymerase chain reaction for detection of *Mycoplasma gallisepticum*. *Avian Dis* **35**, 62-69.

Papazisi, L., Gorton, T. S., Kutish, G. & other authors (2003). The complete genome sequence of the avian pathogen *Mycoplasma gallisepticum* strain R(low). *Microbiology* **149**, 2307-2316.

Raviv, Z., Callison, S., Ferguson-Noel, N., Laibinis, V., Wooten, R. & Kleven, S. H. (2007). The *Mycoplasma gallisepticum* 16S-23S rRNA intergenic spacer region sequence as a novel tool for epizootiological studies. *Avian Dis* **51**, 555-560.

Raviv, Z., Callison, S. A., Ferguson-Noel, N. & Kleven, S. H. (2008). Strain differentiating real-time PCR for *Mycoplasma gallisepticum* live vaccine evaluation studies. *Vet Microbiol* **129**, 179-187.

Reed, G. H., Kent, J. O. & Wittwer, C. T. (2007). High-resolution DNA melting analysis for simple and efficient molecular diagnostics. *Pharmacogenomics* **8**, 597-608.

Soeripto, Whithear, K. G., Cottew, G. S. & Harrigan, K. E. (1989). Virulence and transmissibility of *Mycoplasma gallisepticum*. *Aust Vet J* **66**, 65-72.

Steer, P. A., Kirkpatrick, N. C., O'Rourke, D. & Noormohammadi, A. H. (2009). Classification of fowl adenovirus serotypes by use of high-resolution melting-curve analysis of the hexon gene region. *J Clin Microbiol* **47**, 311-321.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673-4680.

Whithear, K. G., Soeripto, Harringan, K. E. & Ghiocas, E. (1990). Safety of temperature sensitive mutant *Mycoplasma gallisepticum* vaccine. *Aust Vet J* **67**, 159-165.

Table 1. *Mycoplasma gallisepticum* strains/isolates used in this study and their origin

Isolate/strain	Origin	Reference	GenBank Accession No.
ts-11	Australia	(Whithear *et al.*, 1990)	FJ654144
F	USA	(Carpenter *et al.*, 1979)	FJ654142
S6	USA	(Adler *et al.*, 1957)	FJ654143
6/85	USA	(Evans & Hafez, 1992)	FJ654146
Ap3AS	Australia	(Soeripto *et al.*, 1989)	FJ844437
K1659	USA	(Kleven *et al.*, 1988b)	FJ654145
K1453	USA	(Kleven *et al.*, 1988b)	FJ654137
86134	Australia	This study	FJ654139
87006	Australia	This study	FJ654140
87081	Australia	This study	FJ654141
94001	Australia †	This study	N/A*
93148	Australia †	This study	N/A

* Not applicable: nucleotide sequence was identical to ts-11

† Flock vaccinated with ts-11

Table 2. Mean ± SD of the melting points and genotype confidence percentage (GCP) for each strain following PCR and HRM curve analysis

Genotype/Number of times tested	No. of isolates/batches tested	Peak 1 (ºC)	Peak 2 (ºC)	Peak 3 (ºC)	GCP ± SD
ts-11 (125)	3	76.5 ± 0.3			96.1 ± 3.6
K1659 (30)	1	75.2 ± 0.1	76.9 ± 0.1		97.9 ± 2.5
K1453 (28)	1	77.7 ± 0.1			98.1 ± 1.5
87006 (21)	1	74.9 ± 0.1	79.1 ± 0.1		98.9 ± 0.3
87081 (18)	1	74.8 ± 0.9	76.4 ± 0.7	77.6 ± 0.4	95.0 ± 2.1
F (58)	1	78.2 ± 0.1			98.4 ± 1.6
S6 (28)	1	78.2 ± 0.1			99.2 ± 0.7
6/85 (38)	1	74.8 ± 0.1			96.9 ± 2.2
86134 (42)	1	75.3 ± 0.8	77.3 ± 0.3		98.4 ± 1.4
Ap3AS (21)	1	75.7 ± 0.1	77.1 ± 0.4		98.2 ± 1.2

Table 3. Percentage of sequence identity of MG isolates/strains compared with confidence percentage of HRM analysis

Sequence Identity (%)

Strain	S6	F	K1453	87081	ts-11	6/85	K1659	86134	87006	Ap3AS
S6	-	98.7	95.0	98.4	81.0	56.4	44.9	56.4	47.6	56.4
F	66.1	-	93.7	99.1	79.2	55.7	47.6	55.7	46.9	55.7
K1453	36.2	22.1	-	92.8	88.9	56.4	44.9	56.4	47.6	56.4
87081	1.5	0.1	0.8	-	79.2	56.4	36.1	56.4	42.8	56.4
ts-11	0.0	0.0	0.0	0.4	-	56.4	44.9	56.4	47.6	56.4
6/85	0.0	0.0	0.0	0.0	0.00	-	66.7	100.0	68.3	100.0
K1659	0.0	0.0	0.0	0.4	0.08	1.9	-	68.7	77.9	68.7
86134	4.8	0.4	5.6	64.2	0.57	0.0	0.0	-	68.3	100.0
87006	0.0	0.0	0.0	0.3	0.00	3.9	28.3	0.0	-	66.9
Ap3AS	0.1	0.0	0.0	39.2	0.69	0.0	2.3	13.2	3.2	-

Genotype confidence percentage

Table 4. *Mycoplasma gallisepticum* isolates obtained from USA, Europe and Israeil

Isolate	Host	Origin	GenBank Accession No./Reference
K415830	Turkey	USA	NRA*
K4181C	Chicken	USA	(Ferguson *et al.*, 2005)
K4355	Chicken	USA	(Ferguson *et al.*, 2005)
K4705	Chicken	USA	(Ferguson *et al.*, 2005)
K5037A	Turkey	USA	(Raviv *et al.*, 2007)
K503	Chicken	USA	(Ferguson *et al.*, 2005)
K6216D	Chicken	USA	NRA
K5792D	Chicken	USA	(Raviv *et al.*, 2007)
K5917	Chicken	USA	NRA
K6096	Chicken	USA	NRA
K5104	Turkey	USA	(Ferguson *et al.*, 2005)
K4049	Housefinch	USA	NRA
B18/86	Turkey	England	NRA
B5/94	Pheasant	England	NRA
B40/95	Turkey	England	NRA
B41/02	Chicken	England	NRA
B134/03	Pheasant	England	NRA
B7/04/50	Not known	Germany	NRA
B19/04/5	Turkey	Germany	NRA
B90/05	Turkey	England	NRA
B114/06	Partridge	Scotland	NRA
B40/07/6	Chicken	England	NRA
MYZ-8	Chicken	Israel	(Lysnyansky *et al.*, 2008)
MSA-9	Chicken	Israel	(Lysnyansky *et al.*, 2008)
RV-6	Chicken	Israel	NRA
VR-5	Chicken	Israel	NRA
BCV-6	Chicken	Israel	NRA
DSD-14	Turkey	Israel	NRA
BNC-10	Turkey	Israel	NRA
MKT-6	Turkey	Israel	NRA

* No reference available

FIGURE LEGENDS

Fig. 1. Agarose gel electrophoresis of PCR products of the *vlhA* gene promoter region from different MG isolates/strains. MW, molecular weight marker (PCR Marker, Sigma).

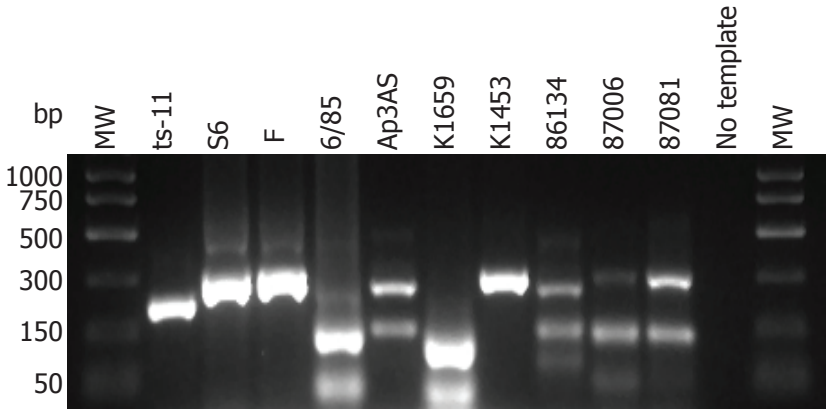
Fig. 2. (a) Conventional melting-curve analysis of PCR products of the *vlhA* gene promoter region from different MG isolates/strains. (b) Normalised HRM curve analysis of PCR amplicons of the *vlhA* gene promoter region from different MG isolates/strains.

Fig. 3 (a) Conventional melting-curve analysis of PCR products of the *vlhA* gene promoter region from ts-11 and MG isolates from ts-11 vaccinated birds. (b) Normalised HRM curve analysis of PCR amplicons of the *vlhA* gene promoter region from ts-11 and MG isolates from ts-11 vaccinated birds. The isolates 93148a, b, c and 94001a, b, c were field specimens collected 7-12 weeks postvaccination with ts-11.

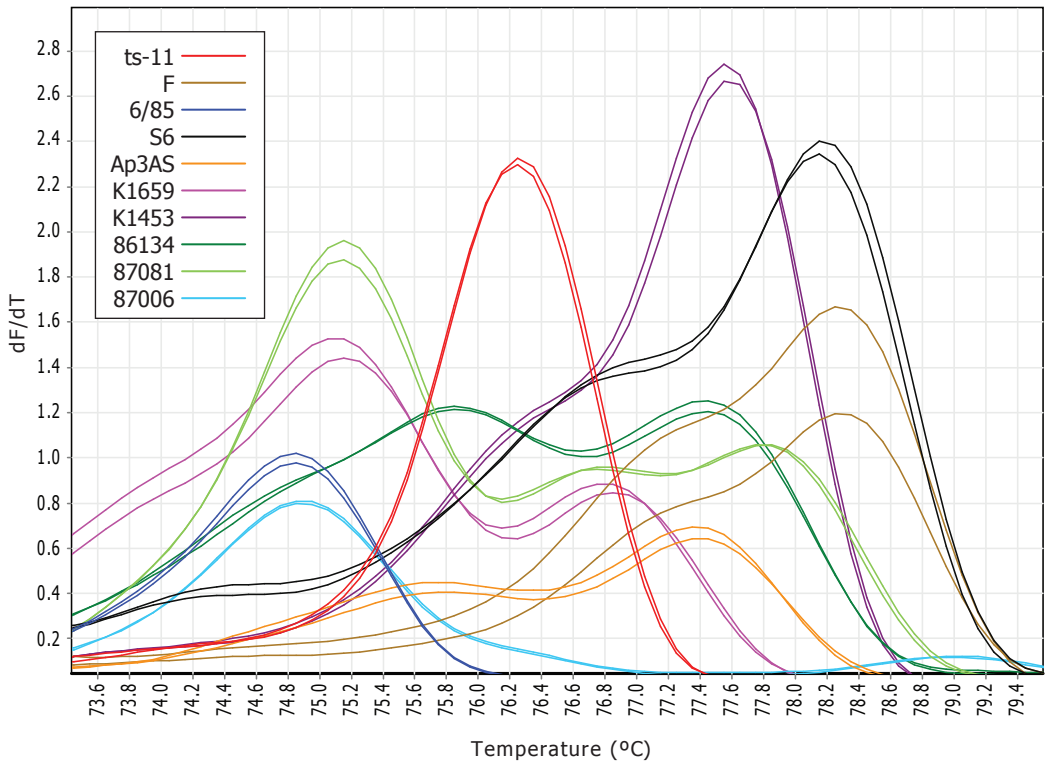
Fig. 4. Comparison of the nucleotide sequences of MG isolates/strains *vlhA* gene promoter region amplicons using CLUSTALW. Identical nucleotides and deletions are shown by '.' and '-', respectively.

Fig. 5. Comparison of the distribution of genotype confidence percentages (GCP) from ts-11 and non-ts-11 strains/isolates using dot plot.

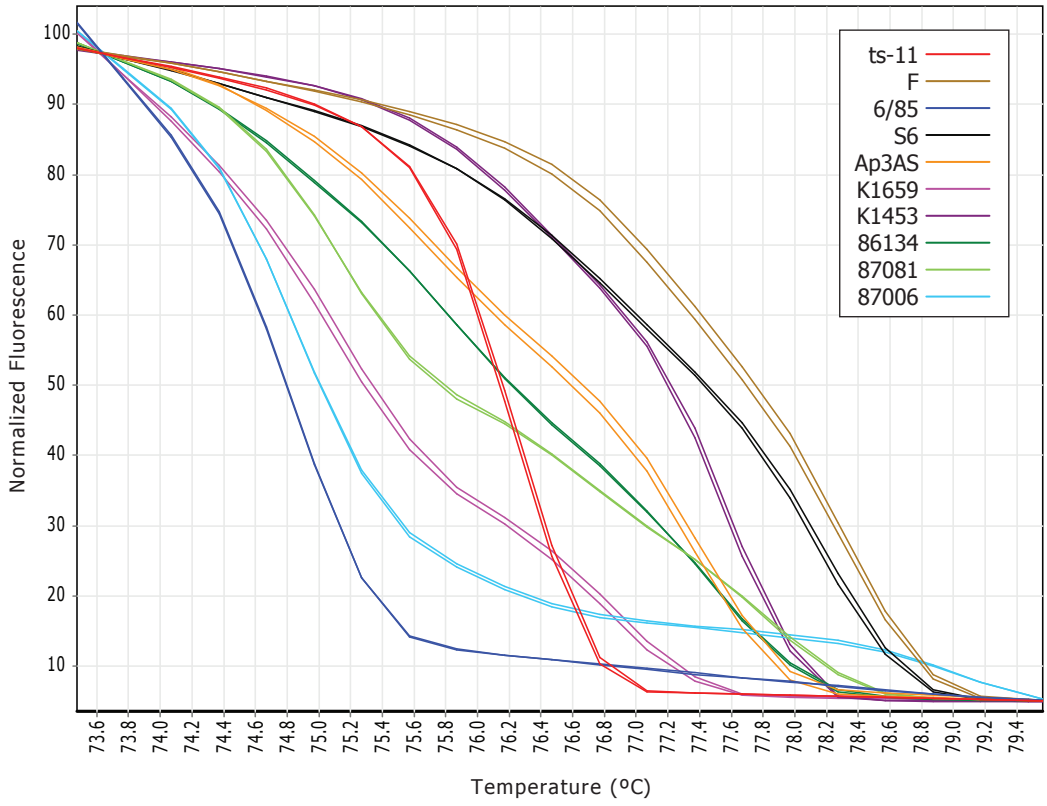
Fig. 6 (a, c and e) Conventional melting-curve and (b, d and f) normalised HRM curve analysis of PCR products of the *vlhA* gene promoter region from ts-11 and MG isolates from USA (a and b), Europe (c and d) and Israel (e and f).


bp
MW
ts-11
S6
F
6/85
Ap3AS
K1659
K1453
86134
87006
87081
No template
MW
1000
750
500
300
150
50

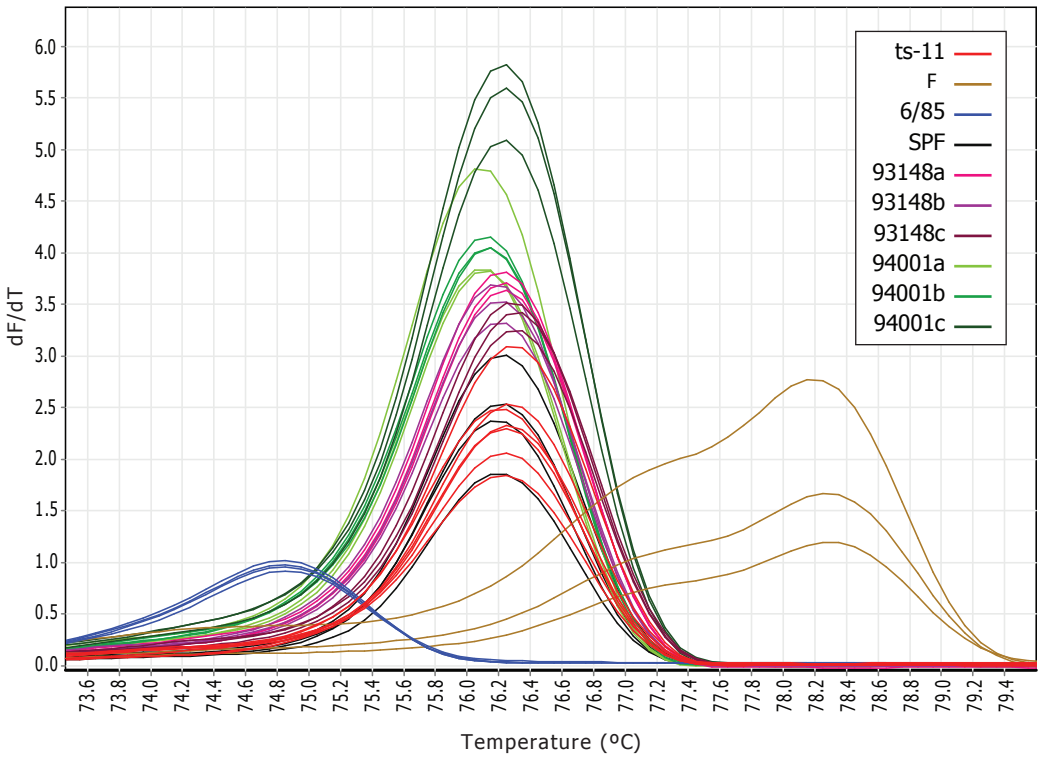
(a)


ts-11
F
6/85
S6
Ap3AS
K1659
K1453
86134
87081
87006
dF/dT
Temperature (°C)

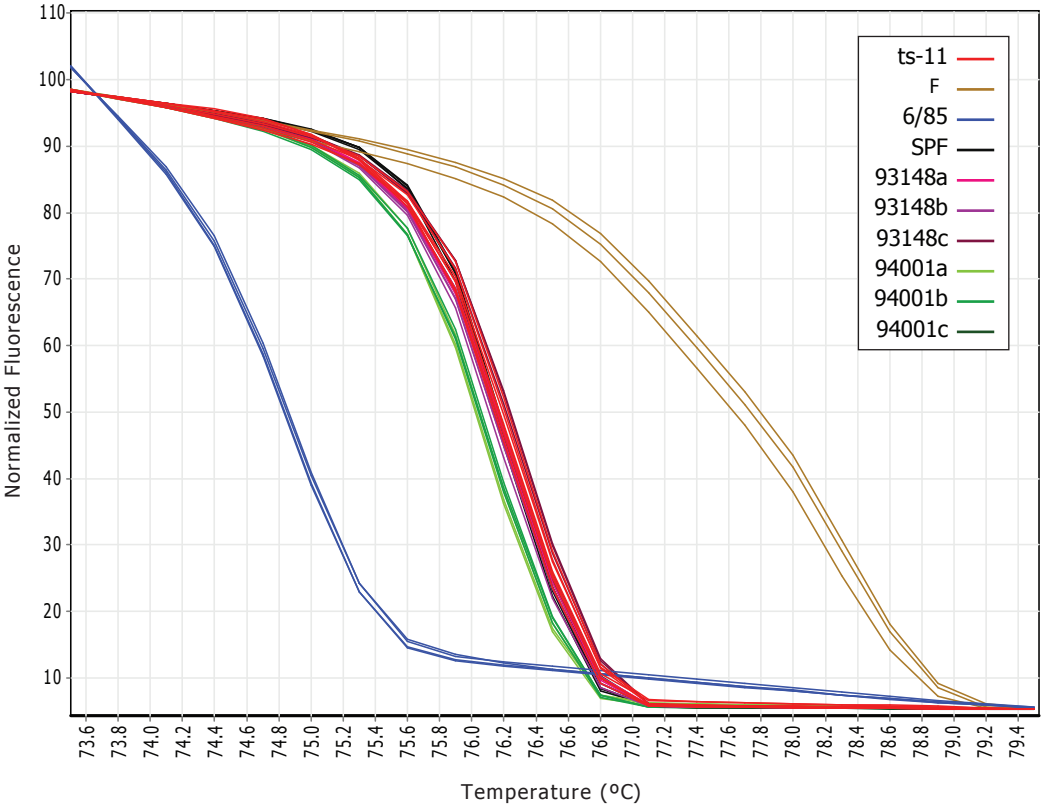
(b)


ts-11
F
6/85
S6
Ap3AS
K1659
K1453
86134
87081
87006
Normalized Fluorescence
Temperature (°C)

(a)


dF/dT
Temperature (°C)
ts-11
F
6/85
SPF
93148a
93148b
93148c
94001a
94001b
94001c

(b)

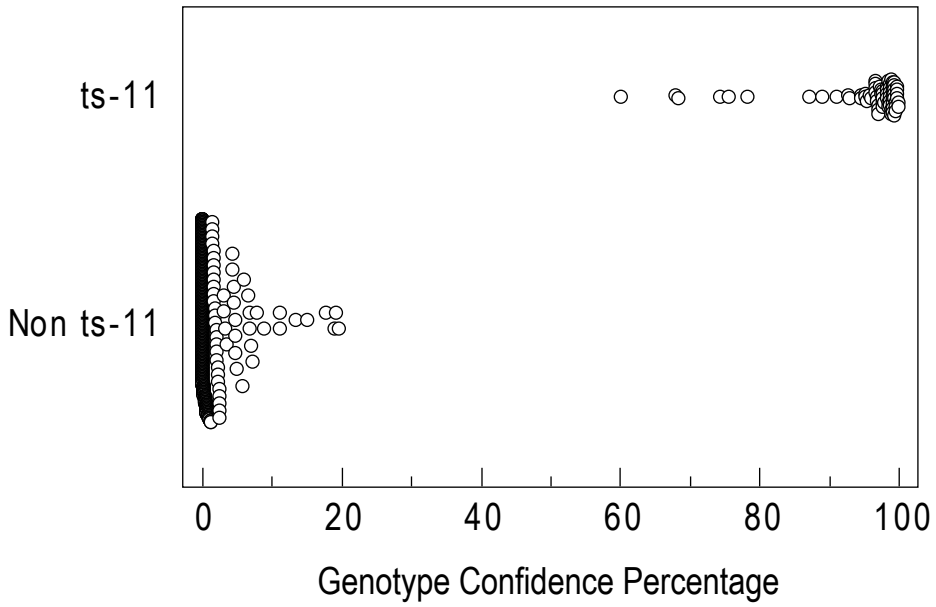

Normalized Fluorescence
Temperature (°C)
ts-11
F
6/85
SPF
93148a
93148b
93148c
94001a
94001b
94001c

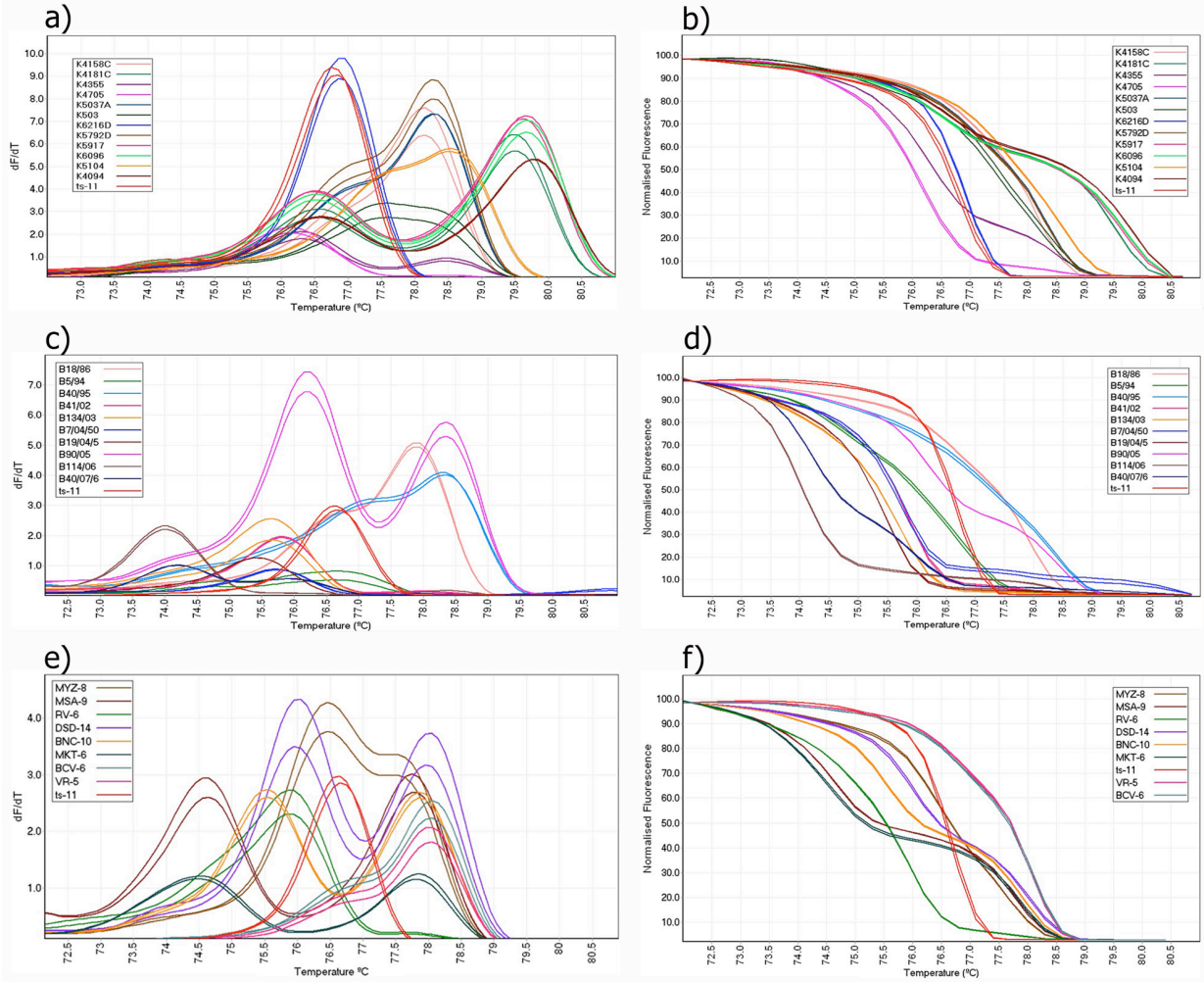
```
                10        20        30        40        50        60        70        80        90       100
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
S6         GTTTGGAGTTGGTGTATAGTTAGTTTAGGTTTATCAATTGTTATGTTTAGGTTGCGGGCTGGTTGTTTTATGATGGTGTTGAGTTAAAGGGTTGGTTGTT
87081      ..................................................................................................------
F          ..................................................................................................------
K1453      ..........................................................T.......................................------
ts_11      ----------------------------------------------------------------------------------------------------
86134      ----------------------------------------------------------------------------------------------------
6_85       ----------------------------------------------------------------------------------------------------
Ap3AS      ----------------------------------------------------------------------------------------------------
K1659      ----------------------------------------------------------------------------------------------------
87006      ----------------------------------------------------------------------------------------------------

               110       120       130       140       150       160       170       180       190       200
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
S6         TTATGATGGTGTTGAGTTAAAGGGTTGTTAGTTTTAGAGAGCTGTTT--TAAGATTTAGTTAAGCTATAGATCTAGTTAGGGTTATGGGTT-AGAAATGA
87081      --------------------------.................--.................................A.........-.........
F          --------------------------.................--.....................................-.........
K1453      --------------------------...T.....-...TG.T...-....G.........................A....G......T.......G
ts_11      ------------------------------....GGAGTT.G...A.AG.T..T..AG...T.T.A..T.T.A.-...TA....G......T.......G
86134      ----------------------------------------------------------------------------------------------------
6_85       ----------------------------------------------------------------------------------------------------
Ap3AS      ----------------------------------------------------------------------------------------------------
K1659      ----------------------------------------------------------------------------------------------------
87006      ----------------------------------------------------------------------------------------------------

               210       220       230       240       250       260       270       280       290       300
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
S6         GTAGG-TAGTAAAAA-TAGGATAACTTATCCTTTTATTTTTATGTTTAGGTTATGGGAAC-GGT---TCTATTGTTCTTATATGATTTATTTAAAATTAA
87081      .....-.........-.....................................................T-...---.............................
F          .....-.........-.....................................................-...---.............................
K1453      T....G.........A.G.............G.............T.................-...---.............................
ts_11      T....G.........A.G.............G.............T.................-...---.............................
86134      ---------..TTGG.G.T..TG----...AG..AGGG..G.GG...AG...AG.AAA...A...ATA.T....A..T..C.....AG.G..A........
6_85       ---------..TTGG.G.T..TG----...AG..AGGG..G.GG...AG...AG.AAA...A...ATA.T....A..T..C.....AG.G..A........
Ap3AS      ---------..TTGG.G.T..TG----...AG..AGGG..G.GG...AG...AG.AAA...A...ATA.T....A..T..C.....AG.G..A........
K1659      ---------..TTGG.G.T..TG----...AG..AGT.ACA..AAA...---...GCACC.A-.ACCGA.AA.CG...T.GG.G.TT...TA..T...AAGG
87006      ----------.TTGG.G.T..TG----...AG..AGT.AAA..AAA.C.-----GT.CC.G-AACCGA.GA.CG...T.GG.T.TT.A.TAA.T...AAG.

               310       320       330       340       350       360
           ....|....|....|....|....|....|....|....|....|....|....|..
S6         CCAAGATAA-GTAGTTGTAAGTACGATAAAAACAAGGTAAAACCTTTTGTTTTCGAAGAAGA
87081      .........-.........C.............AC.....................
F          .........-.........C.............AC.G...................
K1453      .........-.....................................................
ts_11      .........-.....................................................
86134      .........-.......................AG.....................
6_85       .........-.......................AG.....................
Ap3AS      .........-.......................AG.....................
K1659      .TG.T...TTA.TTC..A.TCA..TG.....G.G.ACGG..C...................
87006      ....A..GT-AG.TC..GTTTAGAT.....T.TCTATA...T...................
```


ts-11
Non ts-11
0
20
40
60
80
100
Genotype Confidence Percentage


a)
b)
c)
d)
e)
f)
dF/dT
Normalised Fluorescence
Temperature (ºC)
K4158C
K4181C
K4355
K4705
K5037A
K503
K6216D
K5792D
K5917
K6096
K5104
K4094
ts-11
B18/86
B5/94
B40/95
B41/02
B134/03
B7/04/50
B19/04/5
B90/05
B114/06
B40/07/6
MYZ-8
MSA-9
RV-6
DSD-14
BNC-10
MKT-6
BCV-6
VR-5

Plain English Compendium Summary

Project Title:	Development of New Generation Mycoplasma Based Vaccines
Project No.:	UM 03-11
Researcher:	Dr Philip Markham
Organisation:	The University of Melbourne
Phone:	03 8344 7422
Fax:	03 8344 7374
Email:	pmarkham@unimelb.edu.au
Objectives	1. New approaches for monitoring and understanding the efficacy of vaccination with attenuated live *Mycoplasma gallisepticum* vaccine (strain ts-11) 2. Development of an antigenically marked *Mycoplasma gallisepticum* vaccine 3. Development of *Mycoplasma gallisepticum* ts-11 as a vaccine vector 4. Microarray based detection of virulence genes in mycoplasmas
Background	Serology has been traditionally used to detect infection of flocks with *M. gallisepticum* (MG). Infection with wild-type MG usually produces a serological response that is detectable using tests such as rapid serum agglutination (RSA), haemagglutination inhibition and ELISA. With the introduction of live attenuated vaccine strains, such as ts-11, producers have required an indicator that their flocks have been effectively vaccinated and hence protected against any future challenge with wild-type MG. However, strain ts-11 is highly attenuated and does not elicit a strong serological response, the attenuation is unknown. An objective of this project is to discover and evaluate alternatives to serology that correlate with protective immunity, ideally using a simple sampling technique such as a swab from trachea or choanal cleft. The development of a polymerase chain reaction (PCR) test to determine the presence of the vaccine would benefit the industry. The use of vectors to deliver antigens would alleviate the problems associated with handling and dosing of animals. Using the current vaccine as a vector to deliver foreign antigens has not been explored due to the lack of genetic tools, which are being developed in our laboratory. The ability to deliver a vaccine carrying other antigens would improve the functionality of the vaccine. The identification of genes involved in virulence is important in the understanding of the disease and development of vaccines. This can be achieved by DNA sequencing the genome of the organism, in this case the vaccine which is attenuated or use a system to knock-out individual genes and test these knock-out strains in the animal for their safety and efficacy.
Research	Methods were developed for the identification of MG vaccine strain ts-11 using quantitative PCR and High Resolution Melt curve analysis. A microarray chip to detect genes expressed by MG was produced that included all 43 members of the *vlhA* gene family. A vector system was produced that carried the avian cytokine gamma-interferon; the vaccine strain was then used as a vector to express this gene in the chicken to alter its immune response. A series of Signature Tagged mutants carrying gene knockouts were tested in the chicken and an individual ST mutant was selected to assess its ability to protect against wild-type challenge. The success of the ST mutant experiment led to the same methodology

	being developed for the colibacillosis *E. coli* strain E956. The genomes sequences of the *M. gallisepticum* parent and vaccine strain were determined using a new high thru-put sequencing technology.
Outcomes	A new HRM curve test was developed that can distinguish the MG vaccine strain from other mycoplasma isolates. Using the test the vaccine could be detected from tracheal swabs, field isolates or laboratory grown cultures. The MG vaccine was successfully used to express chicken gamma-interferon and alter the chickens' immune response. An attenuating gene knock-out was identified though STM studies and used successfully to attenuate the colibacillosis *E. coli* strain E956 leading to a patenting of the technology. The genome sequencing of the MG vaccine strain identified the VlhA gene that is expressed. This antigen has now been clonedfor expression in a bacterial expression system and ready for testing as an antigen in an MG ELISA.
Implications	New improved methods for detection of the MG vaccine by HRM curve analysis and when available an ELISA based on the expressed VlhA peptide. A method to produce a vectored vaccine system capable of stimulating and altering the chickens' immune response. A "platform technology" based on defined attenuating mutations that can be used to produce attenuating mutations in other bacteria.
Publications	**Thesis** "Improving mycoplasma vaccines targets for defined attenuation," **Chi-Wen Tseng**, University of Melbourne, 2007 "Development of a replicable *oriC* plasmid for *Mycoplasma gallisepticum* and *Mycoplasma imitans,"* **Sang-Won Lee**, University of Melbourne, Submitted 2009 **Published Manuscripts** Differentiation of *Mycoplasma gallisepticum* strains using PCR and High Resolution Melting Curve Analysis. Ghorashi SA, Noormohammadi AH, Markham PF. **Microbiology**. 2009 Dec 24. PMID: 20035007 Development and immunogenicity of recombinant *Mycoplasma gallisepticum* vaccine strain ts-11 expressing chicken IFN gamma. Muneta Y, Panicker IS, Kanci A, Craick D, Noormohammadi AH, Bean A, Browning GF, Markham PF. **Vaccine**. 2008 26:5449-54 Development of a replicable oriC plasmid for *Mycoplasma gallisepticum* and *Mycoplasma imitans*, and gene disruption through homologous recombination in *M. gallisepticum*. Lee SW, Browning GF, Markham PF. **Microbiology**. 2008 154:2571-80 **Manuscripts in preparation** Expression of GapA or CrmA in *Mycoplasma gallisepticum* Australian isolate are important but may be not essential for colonization Chi-Wen Tseng, Anna Kanci, Christine Citti, Zheng-Hong Chen, Steve. J. Geary, Glenn F. Browning and Philip F. Markham

Conference Posters

Czifra,G., Zohari, S., Kanci,A., Browning,G.F and Markham, P.F. Characterisation of four monoclonal antibodies reactive with *Mycoplasma synoviae*, World Veterinary Poultry Congress, Istanbul, Turkey, 22-26 August 2005. p402

Hemmatzadeh, F., Ngyuen, H.D.T., Browning, G.F., Markham, P.F. and Noormohammadi, A.H. (2006) Detection of antibodies to *Mycoplasma gallisepticum* vaccine strain ts-11 using an autologus recombinant VlhA. 16th IOM International Congress, Cambridge. p221

Kanci, A., Browning, G.F., Geary, S.J., Papazisi, L., Gorton, T.S. and Markham, P.F. (2004) Is an unstable repeated sequence responsible for the attenuation of the *Mycoplasma gallisepticum* strain ts-11? IOM Letters Vol VI:118

Lee, S-W., Browning, G.F. and Markham. P.F., (2004) Development of a replicapable OriC plasmid for *Mycoplasma gallisepticum*. 15th IOM International Congress, Athens, Georgia. p75

Lee, S-W., Browning, G.F. and Markham. P.F., (2006) Replicability of oriC plasmid of *Mycoplasma gallisepticum* in *Mycoplasma imitans* strain 4229. 16th IOM International Congress, Cambridge. p112

Lee, S-W., Browning, G.F. and Markham. P.F., (2008) Development of a replicable oriC plasmid of *Mycoplasma gallisepticum* in *Mycoplasma imitans* and gene disruption through homologous recombination in Mycoplasma gallisepticum. 17th IOM International Congress, Tienjing. p174

Markham, P.F., Sang-Won Lee, Wilton, J., Collins, A., Crowley, T., Geary, S.J., Browning G.F. and Djordjevic. S., (2006) Analysis of vlhA gene expression in *Mycoplasma gallisepticum* utilising two-dimensional gel electrophoresis and microarrays. 16th IOM International Congress, Cambridge. p106

Markham, P.F., Zohari, S., Kanci,A., Bradbury, J., Hellman, U. and Czifra,G. Development of a blocking ELISA for detection of antibodies to *Mycoplasma synoviae*, World Veterinary Poultry Congress, Istanbul, Turkey, 22-26 August 2005. p403

Nguyen. H.D.T., Noormohammadi. A.H., Markham. P.F., Whithear. K.G. and Browning G.F. (2004) Identification, characterisation and expression of a specific gene to improve detection of antibodies to the *Mycoplasma gallisepticum* vaccine ts-11. 15th IOM International Congress, Georgia. p145

Tseng, Chi-Wen., Kanci, A., Browning, G.F., Citti, C., Rosengarten, R. and Markham, P.F. (2004) Transposon mutagenesis of *Mycoplasma gallisepticum*: defining virulence genes IOM Letters Vol VI:118

	Oral Presentations Markham, P.F., (2006) Derrick Edward Award Lecture. Progress in understanding virulence factors of *Mycoplasma gallisepticum*: from in vivo to in vitro. 16th IOM International Congress, Cambridge. p47