AUSTRALIAN EGG CORPORATION LIMITED

Improving mycoplasma vaccines-targets for defined attenuation

A report for the Australian Egg Corporation Limited

Dr P.F. Markham, Prof G.F. Browning,
Mr C-W. Tseng, Ms A. Kanci.

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Researcher Contact Details

Name:	Dr Philip F Markham]
Address:	School of Veterinary Science, The University of Melbourne, Parkville 3010]
Phone:	61 3 8344 7422
Fax:	61 3 8344 7374
Email:	pmarkham@unimelb.edu.au

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

AECLContact Details:
Australian Egg Corporation Limited
A.B.N: 6610 2859 585
Suite 4.02, Level 4
107 Mount Street
North Sydney NSW 2060

Phone:	02 9409 6999
Fax:	02 9954 3133
Email:	irene@aecl.org
Website:	http://www.aecl.org

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Foreword

Mycoplasma gallisepticum (MG) is the primary cause of chronic resiratory disease in poultry world-wide The disease is typically controlled though improved biosecurity or vaccination with bacterins or live attenuated organisms. The vaccine currently used in the Australian poultry industry is MG ts-11, a temperature sensitive isolate developed at the Department of Veterinary Science, The University of Melbourne and funded by the RIRDC.
Although mycoplasmosis has been controlled with the current generation of vaccines, future control programs may need to focus on improvements in these vaccines so they are compatible with eradication or so that they are effective in different strain of bird. In addition the considerable capacity of avian mycoplasmas to evade the immune response of the chicken implies that current control measures that rely on blanket vaccination may eventually lead to varient strains that cannot be controlled by current vaccines. The aims of this project are to improve the efficacy and functionality of mycoplasma vaccines by identifying novel targets for attenuation of pathogenic mycoplasmas, with the strategic potential to apply this to developing new vaccines capable of providing protection against variant strains that may not be controlled by the current generation of vaccines. The outcomes will be improved control of mycoplasmosis and other respiratory diseases of chickens, with the potential to respond rapidly to alterations in the efficacy of current control measures. This project will also develop techniques that will be able to be readily adapted for the rapid development of vaccines against other emerging bacterial diseases of chickens. The benefits of improved vaccines will include improved profitability of poultry production and reduced use of antimicrobial therapy for respiratory disease, with consequent reductions in development of antimicrobial resistance in bacteria in poultry and contamination of the environment with antimicrobial drugs. Also, deletion of individual genes enables vaccinated birds to be distinguished from those exposed to wild-type strains, permitting closer epidemiological surveillance of mycoplasmosis and an assessment of the potential for eradication.

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This report is an addition to AECL's range of research publications and forms part of our R&D program, which aims to support improved efficiency, sustainability, product quality, education and technology transfer in the Australian egg industry.

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Irene Gorman
Research Manager
Australian Egg Corporation Limited

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Executive Summary

The vaccine strain ts-11 is currently used to control chronic respiratory disease caused by *M. gallisepticum*. With the genome of *M. gallisepticum* being sequenced through a RIRDC grant there is the opportunity to improve our understanding of the virulence factors of *M. gallisepticum* and develop new methodologies to produce a new generation of avian mycoplasma vaccines.

There were two main approaches taken to determine those genes responsible for producing the attenuated *M. gallisepticum* vaccine strain ts-11. The first approach used Representational Difference Analysis (RDA) to determine differences in mRNA expression between the parent of the vaccine strain and the ts-11 vaccine. This approach yielded a number of candidate genes, though closer examination and DNA sequencing revealed that they were unlikely to be involved in attenuation. The second approach used proteomic analysis, one and two-dimensional gel electrophoresis was carried out on the parent and ts-11 strains to separate cellular proteins. There were a number of differences observed between the parent and vaccine strains, one in particular was due to a genetic mutation in the *gapA* gene. The GapA protein is involved in attachment of the organism to the host cells and is a prerequisite for colonisation and infection of the host. Analysis of vaccine reisolates showed the *gapA* genetic mutation was reversible. Whether this mutation is responsible for producing the vaccine ts-11 is still unclear.

A collection of clones produced by Signature Tagged Mutagenesis (STM) of the virulent *M. gallisepticum* strain AP3 were used to infect chickens to determine those genes involved in infectivity and virulence. The gene(s) disrupted by the STM process were determined and those clones not recovered from infected birds were further analysed for infectivity and attenuation. Several genes were identified as potential knockout candidates for producing vaccines from wild-type *M. gallisepticum* strains. Though we have identified candidate genes there is still a need to improve the vectors and methodology used to introduce and produce gene knockouts by homologous recombination in the *M. gallisepticum* host.

Introduction

Mycoplasma gallisepticum (MG) is the primary agent of chronic respiratory disease in poultry worldwide. The organism predominantly causes a disease of the respiratory system producing rhinitis, sinusitis, tracheitis and airsacculitis. The organism is disseminated by lateral transmission from bird to bird or by vertical transmission by infecting ova. Infection with MG may predispose the chicken to infection with other organisms or viruses leading to increased morbidity and in some cases mortality. The disease in chickens is responsible for poor feed conversion, decreased egg production and down grading of carcases at slaughter causing economic losses to the poultry industry worldwide. Control of the disease by antimicrobial therapy is expensive and their use is facing increased public opposition. Alternative strategies in controlling the disease include bio-security with improved management practices and vaccination with bacterins or live attenuated organisms.

MG ts-11 Vaccine

The ts-11 vaccine strain of MG was developed in the Veterinary Microbiology Laboratory at The University of Melbourne. It has been extensively tested for safety and efficacy in both chickens and turkeys (Whithear, *et al*., 1990; Whithear, *et al*., 1990). It establishes a chronic infection in the respiratory tract of inoculated birds that persists for the life of the bird. The ts-11 vaccine is marketed worldwide and has proven to be effective in the control of chronic respiratory disease in chickens (Barbour, *et al*., 2001; Branton, *et al*., 2000; Whithear, 1996).

Future Control of MG

The use of blanket vaccination brings with it the possibility of vaccine breaks, as it has been shown that MG possesses the genetic capacity to rapidly alter its cell surface antigens (Markham, *et al*., 1998; Markham, *et al*., 1994), thus creating new variants with altered immunogenic phenotypes. There is potential for vaccines to lose their ability to control disease as new or more virulent strains emerge. The capacity to create new vaccines from virulent isolates allows a rapid response when vaccine breaks occur. To create new vaccines the genes responsible for virulence need to be identified and made non-functional, for example by homologous recombination. Several methods can be employed to identify those genes responsible for virulence.

Identifying Translational Differences between Vaccine and Parent Strains

2-D Gel Electrophoresis

One method is to analyse and compare proteins expressed by virulent strains and non-virulent or vaccine strains. Recent developments driven in part by whole genome sequencing has resulted in techniques for proteome analysis. Proteins of an organism are separated by two-dimensional gel electrophoresis and individual proteins visualised as spots are extracted and analysed by mass spectrometry. Mass spectrometry data together with the genomic sequence can readily identify genes encoding specific proteins (Yates, 2000). This method has been successfully employed in *Mycoplasma pneumoniae* where a large number of proteins and their corresponding genes have been identified (Regula, *et al*., 2000). This technique has the potential to identify virulence associated proteins found only in the wild-type strain 80083 from which the vaccine ts-11 was derived.

Identifying Transcriptional Differences between Vaccine and Parent Strains

Representational Difference Analysis

Though 2-D gel electrophoresis is a powerful technique for identifying proteins, it is unable to distinguish between two unrelated proteins with identical charge and molecular weight (gel mobility). This would result in missing important protein differences between MG strains. Also, 2-D gel electrophoresis identifies only proteins and not RNA species that may be present in one strain and not another. As such it is important to use another method to analyse RNA transcriptional differences. One method that identifies RNA transcriptional differences is representational difference analysis (RDA) (Lisitsyn and Wigler, 1993). RDA belongs to the general class of DNA subtractive methodologies, in which one DNA population (known as the "Driver") is hybridised in excess against a second population (the "Tester"), to remove common (hybridising) sequences, thereby enriching for "target" sequences unique to the Tester population. This is achieved by producing cDNA from the RNA derived from the tester and driver organisms. Oligonucleotide adaptors are ligated to the 5' end of the cDNA molecules and PCR used to generate double stranded DNA representations of the RNA. A different set of oligonucleotide adaptors are ligated to the 5' end of the tester cDNA representations. Due to the abundance of rRNA a representational library of the 16s and 23s rDNA sequences are made. The tester, driver and rDNA representations are annealed together. Only tester molecules that have annealed to other tester-originating sequences, will yield molecules with double-stranded adaptor sequences at both the 5' and 3' ends of the double stranded sequences. Accordingly, only these molecules will be exponentially amplifiable by PCR (using the specific oligonucleotides as primer), thus facilitating enrichment of Tester-specific sequences. This method is widely used in studies to identify differences in gene content or expression of eukaryotic cells and has recently been adapted to the prokaryotes *Haemophilus influenzae* and *Neisseria meningitidis* (Bowler, *et al*., 1999; Duim, *et al*., 1997). In this situation RDA identifies genetic differences in two RNA pools using subtractive PCR methods. The technique appears to be readily applicable to the study of genetic differences between MG 80083 and ts-11.

Identifying Virulence Associated Genes

Signature Tagged Mutagenesis

Though the above two methods will look at differences that contribute to the ts-11 vaccine phenotype, they do not address whether other genes may be involved in MG infection that are suitable for attenuation. Genes involved in virulence have been identified in *Salmonella typhimurium* using signature-tagged mutagenesis (STM) in a mouse model whereby mutants attenuated for virulence were revealed by use of tags that were present in the inoculum but not in bacteria recovered from infected mice. This approach resulted in the identification of new Salmonella virulence genes (Hensel, *et al*., 1995). Though transposon mutagenesis has been used to identify mycoplasma binding proteins and redundant genes (Hutchison, *et al*., 1999; Krause, *et al*., 1997; Reddy, *et al*., 1996) STMs have not been used in *in vivo* studies.

Methods for Producing New Vaccines

Homologous Recombination

The genome sequence of MG was published in 2003 (Papazisi, 2003). This project resulted from collaboration of scientists from the Universities of Connecticut, Washington and Iowa and is part funded by an EIRDC grant CME99-07. There have been a limited number of genetic studies in mycoplasmas due to the difficulty of transforming them and still fewer studies on homologous recombination. *M. pneumoniae* is phylogenetically related to MG and has been transformed using

transposon vectors. Recent studies have used transposon mutagenesis to disrupt virulence genes and determine functionality of others (Hutchison, *et al*., 1999; Kenri, *et al*., 1999; Krause, *et al*., 1997; Reddy, *et al*., 1996). There are a limited number of reports using homologous recombination that either interrupt genes or introduce foreign DNA into mycoplasmas (Dhandayuthapani, *et al*., 1999; King, *et al*., 1994; Marais, *et al*., 1996). Recent studies in our laboratory have identified p47, a cell surface protein recognised by monoclonal antibody B3 and the target for a MG blocking ELISA ((Czifra, *et al*., 1993, Czifra, *et al*., 1995, Markham, *et al*., 2000). A plasmid construct carrying the p47 gene interrupted by the gene encoding tetracycline resistance was used to transform MG and inserted into the p47 gene resulting in the first reported success of homologous recombination in MG.

Objectives

- To improve the efficacy and functionality of mycoplasma vaccines by identifying novel targets for attenuation of pathogenic mycoplasmas.
- Apply this knowledge to developing new vaccines capable of providing protection against variant strains that may not be controlled by the current generation of vaccines.
- Develop techniques that will be able to be readily adapted for the rapid development of vaccines against other emerging bacterial diseases of chickens.

Methodology

Proteome Analysis

The aim of these experiments will be to identify proteins that differ between ts-11 and its parent strain. The genes encoding these proteins will then be specifically disrupted in a virulent strain to determine if they are a basis for a rational attenuation. The MG strains ts-11 and 80083 will be grown under the same conditions (at 33ºC) harvested and immediately treated for iso-electric focusing (the first step in 2D-gel electrophoresis). Initially cell proteins will be separated using a linear pH gradient of pH 2 to 10 followed by electrophoretic separation by size in a linear gradient 8-20% sodium dodecylsulphate polyacrylamide gel (SDS-PAGE). The cell proteins will be stained with colloidal Coomassie brilliant blue R250 or silver stained to identify any protein differences between the vaccine strain ts-11 and parent strain 80083. Refinement of the pH gradient and/or SDS-PAGE gradients will depend on observations of the stained gels. To further assess immunoreactive antigens, 2-D separated proteins will be blotted onto nitrocellulose or poly-vinyldifluoride (PVDF) membrane and immunostained with hyperimmune chicken antisera raised against MG strains ts-11 or 80083 and with specific antisera or monoclonal antibodies to MG. Once conditions have been optimised for identifying protein differences large scale preparations of either ts-11 or 80083 cellular proteins will be separated by 2-D gel electrophoresis and stained as before. Protein(s) of interest will be excised from the gel or the gel transferred to PVDF membrane, stained as before and the protein(s) excised. The protein will be subjected to amino terminal sequencing or alternatively digested with approximately 5% w/w of the endoproteinase trypsin and prepared for matrix-assisted laser desorption/ionisation time of flight (MALDI TOF) spectroscopy. Results from amino terminal sequencing will be used to identify the relevant gene in the genome sequence of MG whilst MALDI TOF results will be used to scan *in silico* tryptic digests of gene products of the MG genome. Once the relevant gene is identified PCR primers will be constructed to amplify the gene sequence together with upstream and downstream areas from the genome of ts-11 and 80083. The PCR product will be subjected to DNA sequencing and the results analysed for DNA differences. Those genes determined to be involved in producing the ts-11 phenotype will be engineered by oversew PCR to contain *Sal*I endonuclease restriction sites and cloned into the pGEM-T vector (pINACT1). The tetracycline resistance gene (already cloned in the plasmid pGEM-T) will be released by digestion with *Sal*I and

ligated into pINACT1 and the construct used to transform our in-house challenge strain MG AP3 using either electroporation or polyethylene glycol transformation protocols.

Transformation and *in vivo* studies

Transformed MG clones will be selected for by growth on tetracycline containing agarose medium and recovered in mycoplasma broth. Clones will be analysed by PCR and Southern blot hybridisation for homologous recombination and disruption of the relevant gene. Phenotypic characteristics such as growth rate and the temperature optimal for growth will be determined. To determine infectivity 3 groups of 5 chickens will be aerosol challenged with either mycoplasma broth (control), 10^8 organisms of transformed MG clone or 10^8 organisms of AP3 parent strain. The birds will be bled before and at the conclusion of the experiment and sera tested in the MG ELISA and by Western blot and birds examined for differences in the lesions induced by infection.

Representational Difference Analysis

The aim of these experiments will be to identify genes that are expressed in the virulent parent strain but not in the ts-11 vaccine strain. These genes will be then disrupted in a second virulent strain to access their potential as targets for rational attenuation. The basic procedure for RDA is adapted from Bowler *et al* (1999). The MG strains ts-11 and 80083 will be both grown at 33ºC till late log phase, harvested and cellular RNA extracted. cDNA synthesis will be performed using Pharmacia TimeSaver cDNA synthesis kit (Pharmacia) followed by second strand DNA synthesis. In this case the "Tester" population is 80083 and the "Driver" population ts-11. To increase the specificity of RDA due to over representation of rRNA sequences, synthetic oligonucleotides to the published rRNA gene sequences of MG will be used on genomic DNA to produce 16s and 23s DNA products by the PCR. The rRNA gene and cDNA products (generated by cDNA synthesis) will be digested with *Dpn*II and ligated to R-12/24 mer adapters. At least 17 cycles of PCR will be conducted on rRNA gene and mRNA products to produce representations using R-24 mer primer. The "Tester" population will be digested with *Dpn* II and ligated to a new adapter J-12/24 mer whilst the rRNA gene and driver products are again digested with *Dpn*II. Several rounds of subtractive hybridisation and selective hybridisation PCR containing the J-12/24 mer primer will be conducted with the tester, driver and rRNA representational populations at different tester:driver ratios. This will produce the first difference product DP1. A second round of subtractive hybridisation and selective hybridisation PCR will be conducted using DP1. For this DP1 is digested with *Dpn*II followed by ligation of N-12/24 mer adapter, incubated with the driver representational populations and the PCR conducted using the N-24 mer primer. The unique products of the tester population produced by PCR will be cloned into the pGEM–T vector and the DNA sequence determined and compared to the MG genome gene sequences. The gene will then be amplified by the PCR and used to generate pINACT constructs for *in vivo* testing as described.

Signature tagged mutagenesis

The aim of these experiments will be to identify MG genes necessary for infection (survival in the birds for 10 days after infection) or for persistence (survival in the bird for 28 days after infection), which will then be used as targets for directed disruption and rational attenuation. To create an STM library of MG clones, oligonucleotide signature tags comprised of a unique 40 bp DNA sequence with two invariable sequence arms (see Fig. 1) will be cloned into the *Kpn*I restriction site of the plasmid pISM2062.2, carrying the transposon Tn4001 and gentamicin resistance gene (Knudtson and Minion, 1993). The MG STM will be pooled and inoculated into chickens.

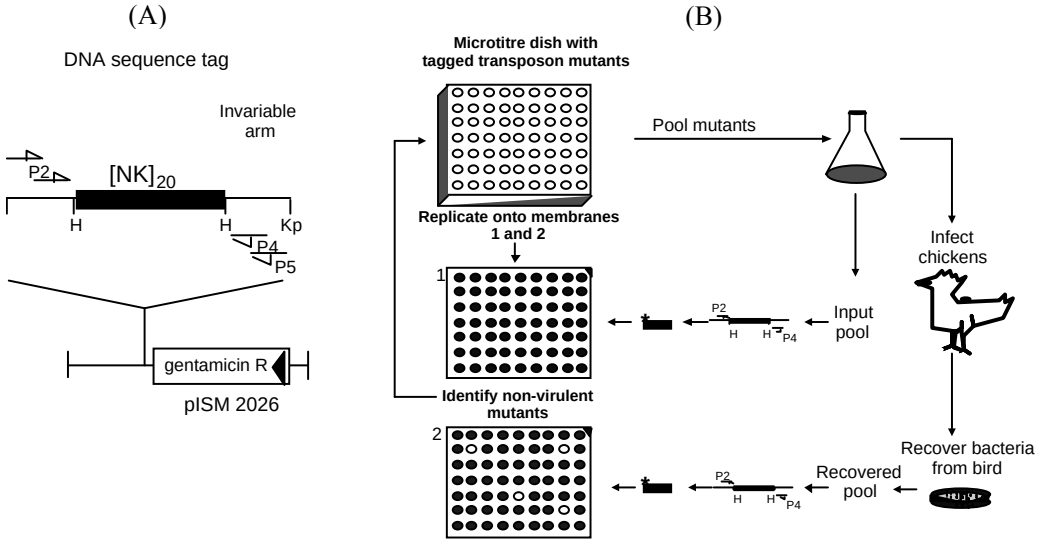


Figure 1 Schematic design of components and experimental protocol

(A) Design of transposon tags. Oligonucleotide synthesis and the PCR will generate a complex mixture of double stranded DNA tags. Each tag comprises a different sequence of 40 bp $[NK]_{20}$ flanked by arms of 20 bp, which are common to all of the tags. The arms allow the sequence tags to be amplified in a PCR with the use of primers P3 and P5. The tags are digested with *Kpn*I (Kp) and ligated into the unique Kp site of transposon Tn 4001 of pISM2026. The vector pISM2062.2 is able to replicate in *Escherichia coli* and allows the amplification of each of the TN 4001 signature tags. **(B)** Virulence gene screen. A bank of MG transposon tagged mutants is arrayed in 96 well microtiter dishes. The mutants in each microtiter dish are pooled and an aliquot is removed for DNA extraction (input pool). The pooled MG cells are used to inoculate chickens. After 10 and 28 days the birds are sacrificed and organisms recovered from the chicken and used to inoculate broth or plated onto solid media. The tags within the recovered pools are amplified using digoxigenin labelled oligonucleotide primers by PCR. The labelled amplified tags are used to probe blots containing oligonucleotide primers corresponding to the tag DNA sequence. Colonies that hybridise to the probe from the input pool but not to the probe from the recovered pool represent mutants with attenuated virulence. (Figure adapted from Hensel *et al* (1995)).

Virulence attenuated mutants will be grown and DNA extracted and the presence and genomic location of the transposon identified by restriction fragment polymorphism and Southern blotting. The mutated gene and transposon DNA will be cloned into the pUC18 vector and subjected to DNA sequencing. The mutated gene will be identified and the PCR used to amplify the gene from MG AP3 and engineered by oversow PCR to contain *Sal*I endonuclease restriction sites and cloned into the pGEM-T vector (pINACT). The gene conferring tetracycline resistance with *Sal*I restriction sites 5' and 3' will be digested with *Sal*I and ligated into pINACT and used to transform MG strain AP3.
Transformed MG clones will be selected for on tetracycline containing agarose medium and recovered in mycoplasma broth. Clones will be analysed by the PCR and Southern blot hybridisation for homologous recombination and disruption of the relevant gene. To determine infectivity 3 groups of 5 chickens will be aerosol challenged with either mycoplasma broth (control), 10^8 organisms of transformed MG clone or 10^8 organisms of AP3 parent strain. The birds will be sacrificed on days 10 and 28, tissues will be taken to assess disease and mycoplasma presence and recovered organisms assessed for the relevant gene mutation. The birds will be bled during the experiment and sera tested by MG ELISA and Western blot.

Detailed Results

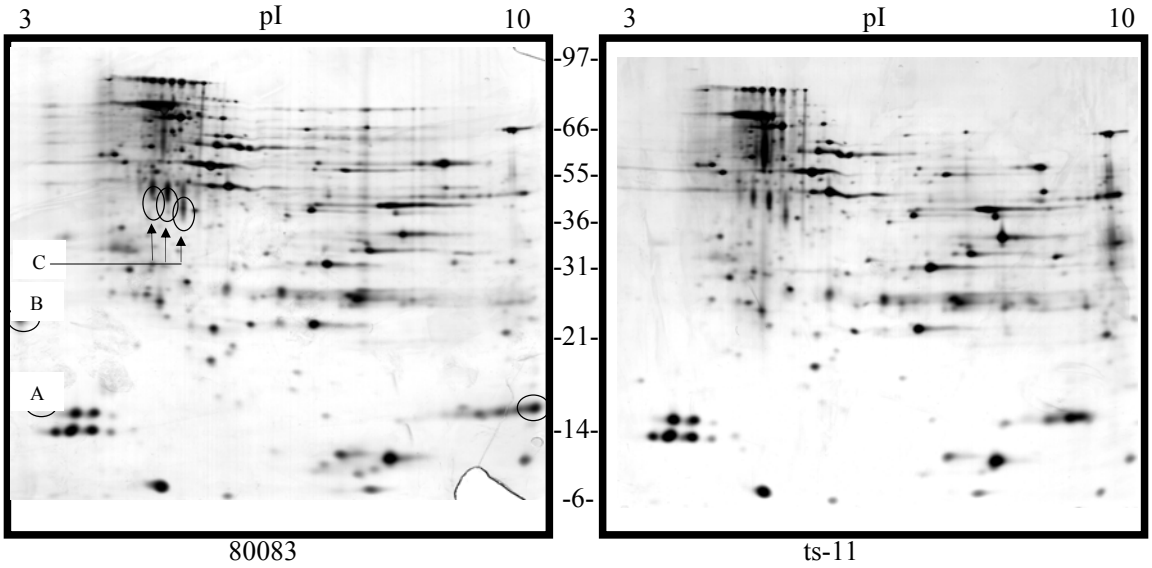
Mycoplasma Culture and 2-Dimensional Gel Electrophoresis

M. gallisepticum strains ts-11 and 80083 were grown at 33°C and harvested when the media reached pH 6.8. The cells were washed extensively and whole cell proteins or hydrophobic and hydrophilic fractions (following Triton X-114 treatment) were prepared for 2-D gel electrophoresis as previously described (Regula *et al*., 2000). Using the IPGphor system (Amersham Pharmacia Biotec) proteins were separated in immobilised pH gradient strips of pH 3-10L or pH 4-7L. The proteins in the strip were then separated on a 12.5 % gel by SDS-PAGE and gels were either silver stained or colloidal Coomassie blue stained directly or Western blotted onto PVDF membrane. Serum taken from chickens vaccinated by eyedrop with strain 80083 or ts-11 was used at a dilution of 1/2000 for the immunostaining of Western blots as were monoclonal antibodies 71 or 86 and rabbit antisera produced against VlhA. Reactivity of monoclonal antibodies and antisera was detected by a 1/2000 dilution of rabbit anti-mouse (DAKO), swine anti-rabbit (DAKO) or rabbit anti-chicken (Nordic Immunologicals) HRPO conjugates respectively and visualised by chemiluminescence (Amersham Pharmacia Biotec) and images were captured and manipulated using Adobe Photoshop software.

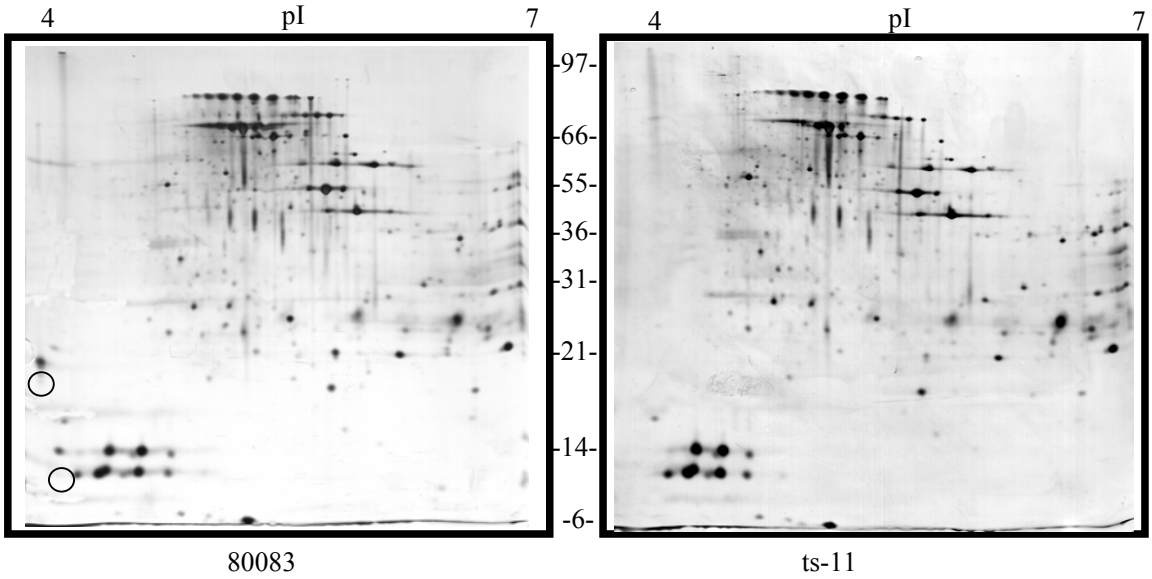
Analytical gels revealed several protein differences between MG strains 80083 and ts-11 grown at 33ºC. In Figs.2 A and B several protein spots with molecular weights of 18 and 25 kDa were identified as present in 80083 but not in ts-11. Further analysis of hydrophilic fractions showed the 25 kDa spot was present in 80083 but absent in ts-11 (Fig. 2C). The peptides from Figure 2a labelled A, B, and C were excised and digested with trypsin. Single peptide species were purified using reverse-phase chromatography and amino-acid peptide sequence determined.

Figure 2 Analytical 2-D gels of cell proteins of MG 80083 and ts-11

A. Cell proteins separated on 18 cm IPG 3-10L IPG strip 1st dimension and on 12.5% polyacrylamide SDS gel 2nd dimension and silver stained. B. Cell proteins separated on 18 cm IPG 4-7L IPG strip 1st dimension and on 12.5% polyacrylamide SDS gel 2nd dimension and silver stained. C. Hydrophilic cell protein phase from TX-114 fractionation separated on 18 cm IPG 4-7L IPG strip 1st dimension and on 12.5% polyacrylamide SDS gel 2nd dimension and silver stained. O= protein differences between 80083 and ts-11.



(A)



(B)

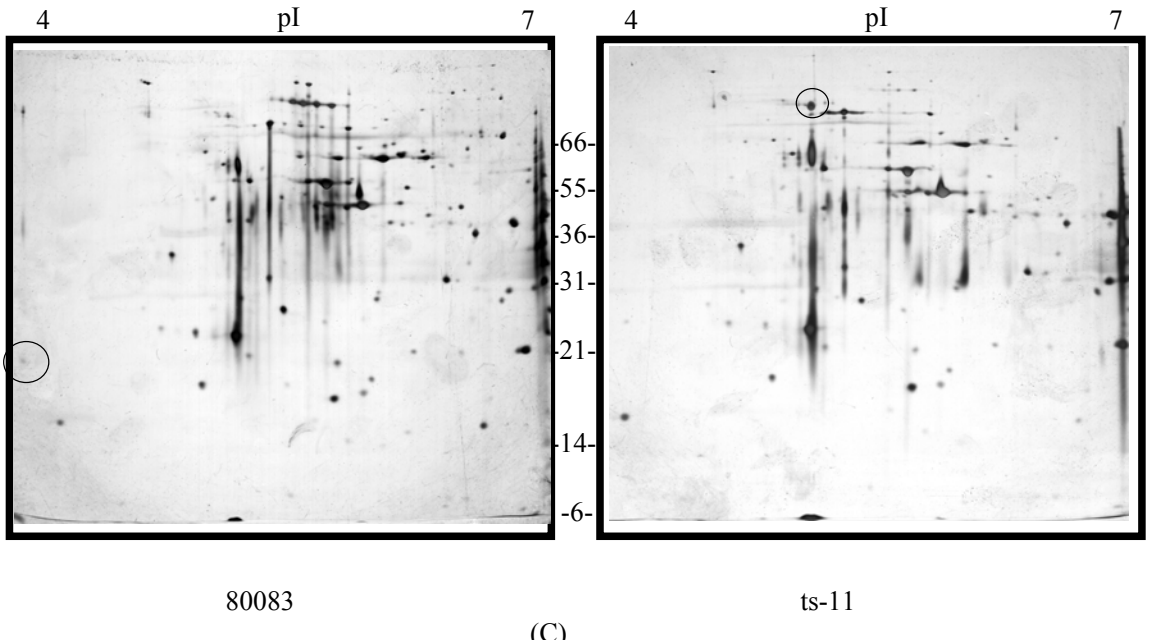
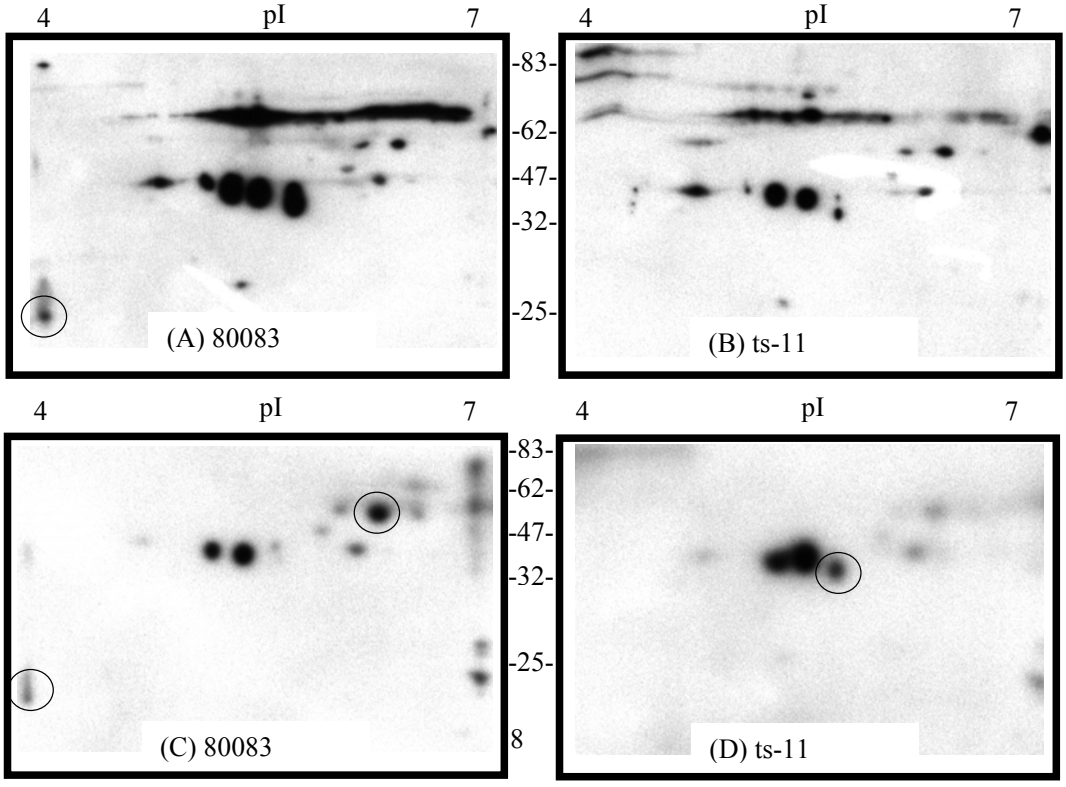


Figure 3 Immunostaining of Western transferred proteins of 80083 and ts-11

Identical gels of Fig. 2 B and C above were Western blotted and immunostained with antisera from chickens infected with 80083. (A and B) Cell proteins separated on 7 cm IPG 4-7L strip 1st dimension and on 12.5% polyacrylamide SDS gel 2nd dimension and silver stained. (C and D). Hydrophilic cell protein phase from TX-114 fractionation separated on 18 cm IPG 4-7L IPG strip 1st dimension and on 12.5% polyacrylamide SDS gel 2nd dimension and silver stained. **O**= protein differences between 80083 and ts-11



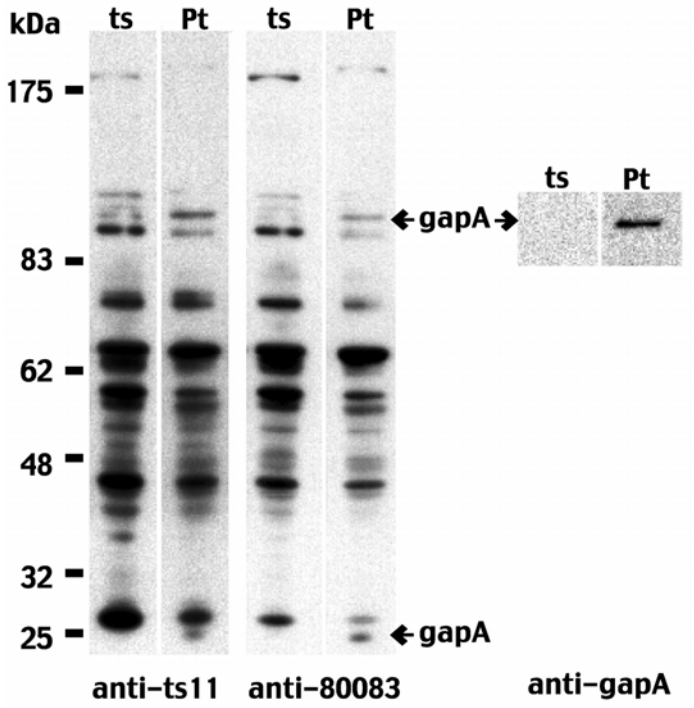
The immunostaining patterns each strain in Fig 3 A and 2 B were similar. There are a series of antigenic spots corresponding to VlhA that span the membrane at around 70 kDa (Fig. 3 A). A major antigenic difference corresponding to a 25 kDa peptide with pI of around 4 that is present in 80083 but not ts-11. There are two antigenic spots showing different intensity levels in antibody binding. Analysis suggests the circled protein in Fig. 3 panel D may be an immunogenic media contaminant (see later in peptide analysis) whilst the other immunogenic circled peptide in panel C of around 50 kDa is MGA_0162 AceF (dihydrolipoamide acetyltransferase).

Peptide differences between 80083 and ts-11

The identity of proteins excised from 2-D gels were determined using MALDI TOF as outlined in Figure 4. Proteins excised from several 2-D gels were identified because of their immunogenic reactivity. Of particular interest was a 25 kDa protein that was present in 80083 but absent from the ts-11 strain. This peptide was identified as the amino-terminal fragment of the gapA protein with a molecular weight of the mature form of 105 kDa. The gapA protein is involved in attachment of MG to the cell surface of host tissues and is necessary for the organism to successfully colonise the chicken. Anti-sera to gapA (kindly provided by Dr S Geary) reacted with a band of 105 and 25 kDA in 80083 but absent in ts-11 as seen by Western blot of 1-D gels in Figure 4. Further analysis revealed anti-sera from chickens vaccinated with ts-11 detected both the 25 kDa and 105 kDa gapA proteins (Figure 4).

Figure 4 Western blot of ts-11 and 80083 strains immunostained with rabbit or chicken sera

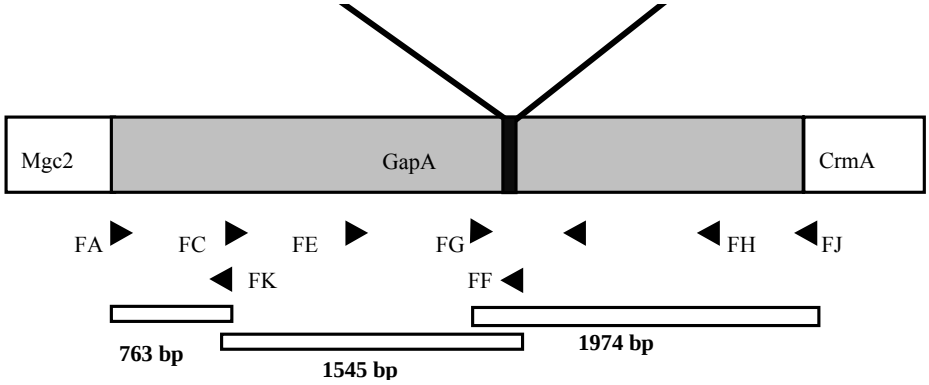
Whole cell proteins of ts-11 or 80083 were separated by SDS-PAGE, transferred to PVDF membrane and probed with anti-sera (1/2000) from chickens infected with ts-11 or 80083. Antisera from a rabbit immunised with a gel extracted 105 kDa peptide corresponding to gapA was also used at 1/10,000 to immunostain whole cell proteins of ts-11 and 80083. Bound antibodies were detected with a 1/2000 dilution of HRPO labelled conjugated antibodies appropriate for each species and visualised using chemiluminescence and autoradiography. ts: ts-11 cell proteins, Pt: 80083 cell proteins



From these results the genomic region of DNA encoding gapA from ts-11 and 80083 strains was amplified by PCR using the oligonucleotides FA and FJ (Appendix 1) yielding an amplicon of around 4.2 kbp. A series of oligonucleotide primers were designed and used in determining the DNA sequence of this region (Figure 5).

Figure 5 Schematic representation of gapA gene

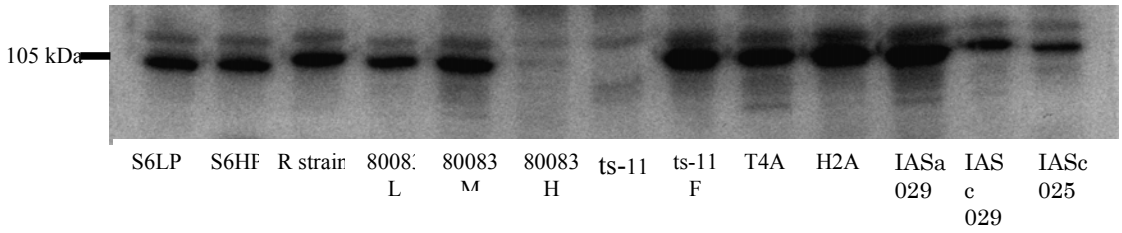
Arrows represent PCR primers used for cloning FA/FK, FC/FF and FG/FJ and sequencing of the gene. Nucleotide repeat shown above *gapA* gene. The DNA sequence of oligonucleotides may be found in the Appendices.



Alignment of the gapA sequence from ts-11 and 80083 revealed a 20 bp DNA duplication in the ts-11 strain that would cause premature truncation of the gapA peptide accounting for the absence in the ts-11 strain. It was possible that the 20 bp insertion would be useful in differentiating the ts-11 strain from 80083 and other MG strains and reisolates of the vaccine/ts-11 strain were tested by Western blot. The immunostaining band corresponding to gapA was found in all vaccine isolates and with the exception of high passage 80083 (80083H) in all other strains tested (Figure 6).

Figure 6 Western blot of *M. gallisepticum* strains

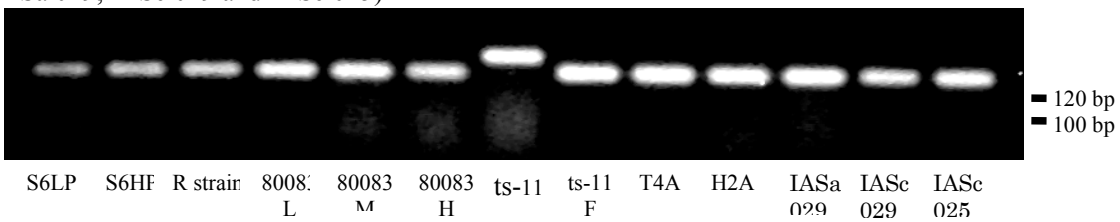
M. gallisepticum strains S6 low passage (S6LP) and high passage (S6HP), low passage R, vaccine parent strain 80083L (9th passage), 80083M (40th passage), 80083H (100th passage) and H2A (non-vaccine field isolate) were grown at 37°C. The ts-11 vaccine and five field re-isolates of ts-11, (ts-11 F, T4A, IASa 029, IASc 029 and IASc 025) were grown in broth medium at 33°C. Proteins were separated in 7.5 % polyacrylamide gels, then transferred onto PVDF membrane and probed with rabbit anti-GapA serum diluted 1:10,000. The bound antibodies were detected with swine anti-rabbit HRPO conjugate (DAKO) at a final concentration of 1:2,000 and antibody binding visualised by chemiluminescence.



To investigate whether the 20 bp insert was also found in ts-11 field isolates or other strains a PCR was developed that amplified the region surrounding and including the 20 bp insertion. Analysis of the PCR products by agarose gel electrophoresis (Figure 7) revealed that only ts-11 contained a higher molecular weight band corresponding to the 20 bp insertion. We were unable to differentiate ts-11 field isolates from 80083 using the DNA duplication and this line of investigation was not pursued any further.

Figure 7 Detection of *gap*A by PCR

A pair of oligonucleotide primers were designed to amplify the region of the *gapA* gene containing the 20 bp duplication in ts-11. Cells of MG strains were used as template in the PCR and products were separated by gel electrophoresis in 3% agarose and stained with ethidium bromide. The lanes contained: *M. gallisepticum* strains S6 low passage (S6LP) and high passage (S6HP), low passage R, vaccine parent strain 80083L (9th passage), 80083M (40th passage), 80083H (100th passage), H2A (non-vaccine field isolates) and the ts-11 vaccine and five field re-isolates of ts-11, (ts-11 F, T4A, IASa 029, IASc 029 and IASc 025)



Further comparison of 2-D gel electrophoresis of MG strains 80083 and ts-11 revealed a number of absent proteins or differences in intensity of protein staining. Protein spots of interest were excised from gels containing hydrophilic cell proteins (Figure 8), prepared for MALDI-TOF and the encoding gene predicted from genome sequence analysis (Table 1). Several of the genes would encode proteins with higher molecular weights than that detected by 2-D, this data and the absence of MALDI-TOF peaks of tryptic peptides of the amino or carboxyl regions suggest premature truncation of the protein, post translational cleavage events or indicate DNA differences to the sequenced MG R strain. These events may be responsible for detection of the carboxyl region of the ts-11 proteins: peptide I encoding AcoB thiamine pyrophosphate dependant dehydrogenases E1 component beta, peptide V encoding DnaK heat shock protein 70 or the amino terminal region of peptide IV encoding AceF dihydrolipoamide acyltransferase. The DnaK peptide of ts-11 was also spread over a small pI range (peptide X of Figure 8) with a similar molecular weight (MW). Several of the peptides were investigated using quantitative PCR to compare messenger RNA expression levels between strains, also DNA sequencing of the gene promoter regions and gene sequences was also carried out.

Figure 8 Preparative 2-D gels of hydrophilic cell proteins of MG 80083 and ts-11.

Hydrophilic cell proteins separated on 18 cm IPG 3-10L IPG strip 1st dimension and on 12.5% polyacrylamide SDS gel 2nd dimension and stained with colloidal Coomassie blue. Protein spots showing increased staining intensity or only present in one strain (circled) were excised and digested with approximately 5% w/w of the endoproteinase trypsin and prepared for MALDI TOF spectroscopy. Results from MALDI TOF were used to scan *in silico* tryptic digests of gene products of the MG genome.

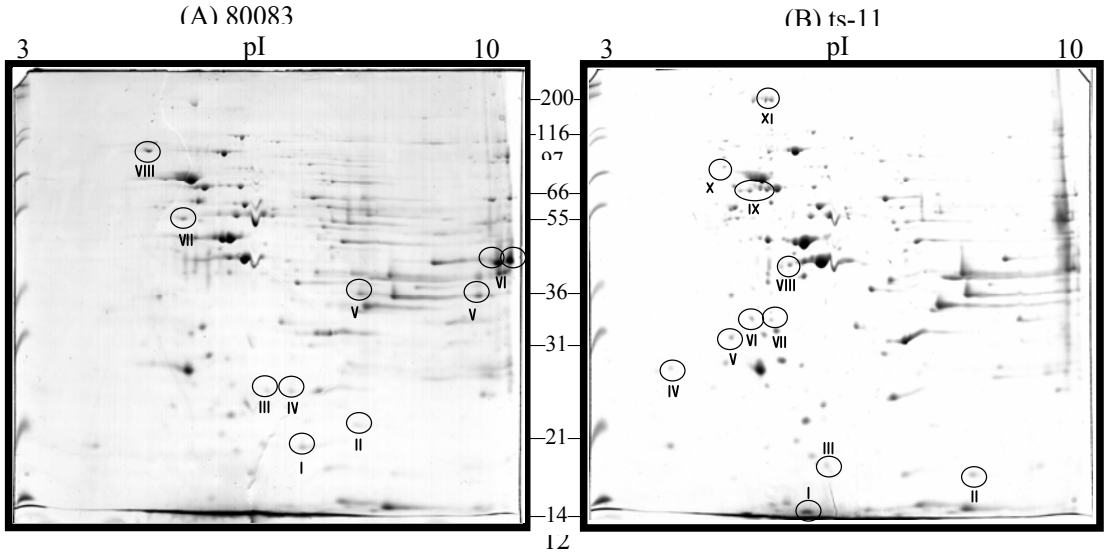


Table 1 Differentially expressed proteins determined by MALDI_TOF

	80083 strain		ts-11 strain	
Protein ID*	Gene ID	Gene Name	Gene ID	Gene Name
I	MGA_0480	Unique hypothetical	MGA_0164	AcoB Thiamine pyrophosphate dependant dehydrogenases E1 component beta subunit COG0022 (fragment)
II	MGA_0462	Gmk Guanylate kinase COG0194	MGA_0696	NrdI ribonucleotide reduction COG1780
III	MGA_0091	Phospholipid-binding protein COG1881	MGA_0294	Similar to ArsC Arsenate reductase and related proteins glutaredoxin family COG1393
IV	MGA_1121	Inorganic pyrophosphatase COG0221	MGA_0162	AceF Dihydrolipoamide acyltransferases COG0508 (fragment)
V	MGA_0164	AcoB Thiamine pyrophosphate dependant dehydrogenases E1 component beta subunit COG0022	MGA_0279	DnaK heat shock protein 70 COG0443 (fragment)
VI	MGA_1186	GAPDH Glyceraldehyde-3-P dehydrogenase COG0057	MGA_1033	TufB GTPase Elongation factor TU COG0050
VII	MGA_1177	AtpD F0F1-type ATP synthase beta subunit COG0055	MGA_1033	TufB GTPase Elongation factor TU COG0050
VIII	MGA_0818	NusA-homolog Transcription elongation factor COG0195	MGA_0165	AcoA Thiamine pyrophosphate dependent dehydrogenases E1 component alpha subunit COG1071
IX			MGA_0279	DnaK heat shock protein 70 COG0443
X			MGA_1261	Conserved hypothetical prokaryotic lipoprotein signal
XI			MGA_0241	Unique hypothetical

* Peptide spots annotated in Fig. 8.

Quantitative PCR

Quantitative PCR was used to investigate the level of mRNA expression for differentially expressed genes identified through MALDI-TOF. Oligonucleotide primers were designed to amplify genomic regions of around 80 bp for each gene. The PCR products were detected by binding of SYBR green fluorescent dye to double stranded DNA. Total RNA was isolated from MG 80083 and ts-11 strains grown at 33ºC or in some instances 37ºC using RNeasy Minikit columns (QIAGEN) according to the manufacturer's instructions. The quantity of purified RNA was determined by spectrophotometer and quality determined by subjecting 2 µg to non-denaturing gel electrophoresis, staining with ethidium bromide and examining using fluorescence. To prepare RNA for qPCR it was first treated with Dnase enzyme to remove contaminating DNA. To 2 µg of total RNA of 2 µL 10x Dnase 1 reaction buffer (Invitrogen) was added followed by 2 µL Dnase 1 enzyme (Invitrogen) and RNAse free water to a final volume of 20 µL. The mixture was incubated for 15 min at room temperature after which 2 µL of 25 mM EDTA was added and the mixture heated at 65ºC for exactly 10 min. To make cDNA for qPCR 10 µL of Dnase treated RNA was added to 100 ng random hexamers (Invitrogen), 10 pmol of each dNTP (BioRad) and heated to 65 °C for 5 min and then immediately placed on ice. To this was added 4 µl 5x 1st strand reaction buffer (Invitrogen), 2 µL 0.1 M DTT and incubated at 25 °C for 2 min. To the mixture 1 µL of the reverse transcriptase SuperScript II (Invtrogen) was added and incubated at 25°C for 10 min To a no reverse transcription control only water was added. The mixture was incubated at 42°C for a further 50 min and inactivated by heating to 70°C for 15 min and water added to a final volume of 600 µL.

The concentration for each oligonucleotide pair was optimised using genomic DNA as template in the PCR reaction. In most cases a 1:1 ratio of each oligonucleotide produced the lowest number of cycles before crossing an arbitrary threshold (Cycle threshold or Ct). The reaction efficiency for each

oligonucleotide pair was determined using a 10-fold dilution series of genomic DNA as template. To determine the difference in expression levels for given genes in either MG 80083 or ts-11 strains a mixture of cDNA was used as the calibrator and expression levels for a house-keeping gene determined in each strain. The expression level for each gene can be determined relative to the normalised levels of the house-keeping gene for each strain. The sites used in QPCR several of the genes are shown in Figure 9.

Figure 9 Schematic diagram of QPCR sites and DNA sequencing

Unidirectional block open arrows show transcriptional orientation of genes whilst short solid arrows show quantitative PCR oligonucleotide regions. Solid lines indicate PCR amplicons used in DNA sequencing and long solid arrows show DNA sequencing range.

(a) Gene organisation for pyrophosphate dependant dehydrogenases E1 component alpha and beta subunits.

(b) Gene organisation for unique hypothetical proteins MG0477-0487.

(c) Gene organisation of the ribonucleotide reductase gene region.

Analysis of genes with altered RNA levels

The RNA (cDNA) levels of selected genes in MG 80083 and ts-11 were normalised to either the tuf or GAPDH RNA levels for each strain and calibrated to a standard sample containing pooled cDNA. The PCR efficiency for each primer pair was used in the analysis software (Stratagene) to calculate an RNA expression ratio for each gene. In most cases when the RNA ratios of the MG parent and ts-11 strains was 2 or less the genes were not assessed further as this was considered within the experimental error. The results for selected genes are shown below normalised to the tuf gene (Table 2).

Table 2 RNA transcription ratios of selected genes of MG strains 80083 and ts-11

Gene ID	Gene Name	PCR product Size bp	PCR Efficiency %	Ratio of RNA expression levels (strains grown at different temperatures) 37ºC	33ºC	39.5ºC
MG1186	GAPDH	53	100	same[†]	same	same
MG0162	AceF	53	70	nt	x1.3 Pt*	nt
MG0218	OppF	53	69	nt	x1.2 Pt	nt
MG0477	uh^	53	80	nt	x7.0 Pt	x3.6 Pt
MG0480	uh	53	105	x8.2 Pt	x5.75 Pt	x4.3 Pt
MG0487	uh	59	82	x6.4 Pt	x7.3 Pt	x6.2 Pt
MG0487[#]	uh	53	100	nt	x7.1 Pt	x3.8 Pt
MG0696	NrdI	53	98	x5.9 ts	x3.0 ts	nt
MG0698	NrdF	56	52	nt	x2.0 ts	nt
MG1155	RpsI	53	97	same	x2.38 Pt	nt
MG1033	tuf	72	106	normalised[¥]	normalised	normalised

[†]Same: no difference in RNA level between 80083 and ts-11, [∂]nt: not tested, * Pt: Ratio of 80083/ts-11 RNA levels, [¶]ts: Ratio of ts-11/80083 RNA levels, ^Unique hypothetical protein, [¥]normalised: RNA levels of ts-11 and 80083 normalised using this gene, [#]Oligonucleotide primers IX/IW used in QPCR

Several of the genes appeared to be within an "operon-like" gene structure with genes overlapping or separated by only a few nucleotides, this is most likely the case with the gene regions depicted in Figures 9a, b and c. To further investigate the reasons why changes had occurred in RNA expression levels, selected regions of the genome and regions upstream of these genes were subjected to DNA sequencing in the MG 80083 and ts-11 strains (shown in Figures 9 b and c). Alignment of the DNA sequences revealed that they were identical in most cases or contained 1 or 2 nucleotide changes in regions that would not necessarily affect mRNA expression levels. These results suggest other genetic factors may influence mRNA expression or stability of these genes or other post-translational modifications may affect peptide presence.

Homologous Recombination and Transformation Studies

A construct previously used to successfully transform MG strain S6 was introduced into MG ts-11 by electroporation. The construct carried the p47 gene interrupted by the tetracycline resistance gene *tet*M located midpoint of the p47 gene. Recombinants were selected for following transformation by resistance to the antibiotic tetracycline. The experiments were repeated a number of times but no recombinants were ever successfully isolated. These results suggest the MG strain S6 is particularly susceptible to homologous recombination possibly due to its rate of growth and titre being much higher than those MG strains recently isolated. It may well be that whilst p47 is not needed for *in vitro* growth in MG strain S6 there may be a requirement for it to be present and functional in strain ts-11.

Representational Difference Analysis (RDA)

As part of an alternative approach to determine mRNA differences between MG strains 80083 and ts-11 the relatively new methodology of RDA was employed. As the original method for RDA was devised for eukaryotic organisms and involved using relatively large amounts of RNA several areas of the procedure were modified to accommodate the sample size and bacterial origin or the RNA. The schematic diagram shown in Figure 10 gives a pictorial overview of the technique. The technique used for our prokaryotic system was originally developed by Bowler et al (1999). The RDA methodology uses DNA subtraction and PCR amplification to enrich for unique RNA sequences found in one strain, the "Tester", cDNA from the "Driver" strain is hybridised in excess to that of the "Tester" strain and unique DNA sequences from the tester amplified by PCR. The

"Tester" and "Driver" strains in our system are the pathogenic parent strain 80083 and the vaccine strain ts-11. The method uses commercially available kits to generate cDNA from bacterial mRNA. In brief cDNA is digested using the restriction enzyme *Dpn*II to generate short stretches of DNA containing 5' "GATC" overhangs to which oligonucleotides R12 and R24 adapters are added. PCR products from both the driver and tester are produced using the R24 oligonucleotide. Following digestion of the driver and tester with *Dpn*II the J12/J24 oligonucleotides are ligated to the tester. A first round of hybridisation and subtraction with the driver in excess of the J12/24 ligated tester is performed and PCR amplification done using the J24 oligonucleotide. The first round products consisting of unique representations of mRNA from the tester are again digested with *Dpn*II and the R12/24 linkers added. Another round of hybridisation, subtraction and amplification is undertaken as before using oligonucleotide J24 in the PCR and the resultant products run on a gel, excised, cloned and the DNA sequence determined.

One of the modification introduced used the "phenol emulsion re-association technique (PERT) developed by Kohne *et al.* (Biochemistry, 16:5329-5341, 1977) whereby the amount of product used in the hybridisation and substraction procedure is reduced. Following on from one RDA experiment the difference products detected using 80083 or ts-11 as the driver and tester or visa versa a 16s rDNA sequence was detected. This is not surprising as the rRNA genes are highly expressed in bacterial species. To overcome this artefact the corresponding 16s DNA sequence was included in the driver of all subsequent hybridisation and substraction procedures to reduce the presence of the corresponding sequence in the tester.

Figure 10 Schematic diagram of RDA procedure

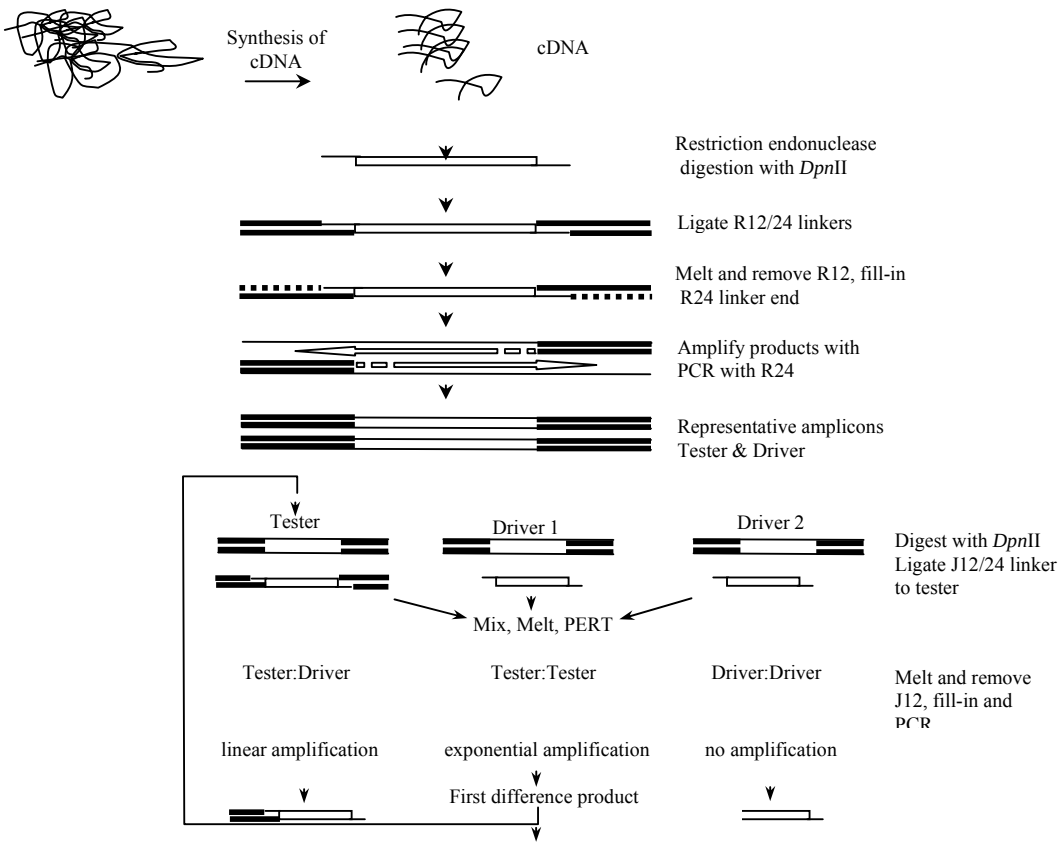


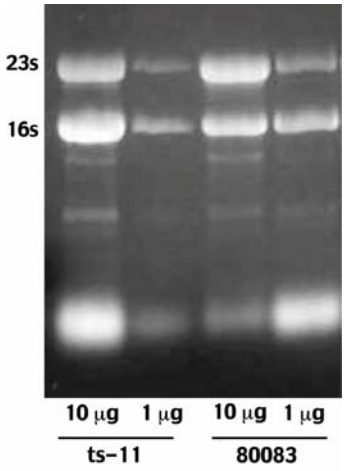
Table 3 Oligonucleotide primers used in Representational Difference Analysis

Oligonucleotide	Application/Target	5'-Sequence-3'
KA1 Forward	RT-PCR p47 PCR	GGTGCTGCTTCTTCACTAAC
KA2 Reverse	RT-PCR p47 PCR	GAATTCGGTTTAGTGCTGATTC
MG16s Forward	cDNA-RDA	AGAGTTTGATCCTGGCTCAG
MG16s Reverse	cDNA-RDA	CTTGTTACGACTTAACTCCAA
MG23s Forward	cDNA-RDA	TAAGGCCTTATGGTGGATGC
MG23s Reverse	cDNA-RDA rev	TCGAATTATTAGTACTAATCAGC
R-Bgl-12	cDNA-RDA	GATCTGCGGTGA
R-Bgl-24	cDNA-RDA	AGCACTCTCCAGCCTCTCACCGCA
J-Bgl-12	cDNA-RDA	GATCTGTTCATG
J-Bgl-24	cDNA-RDA	ACCGACGTCGACTATCCATGAACA
N-Bgl-12	cDNA-RDA	GATCTTCCCTCG
N-Bgl-24	cDNA-RDA	AGGCAACTGTGCTATCCGAGGGAA
Universal M13-for	DNA Sequencing	GTTGTAAAACGACGGCCAGT
Universal M13-rev	DNA Sequencing	CAGGAAACAGCTATGACC

Representational Difference Analysis Results

Total RNA was extracted from MG strains 80083 and ts-11, treated with DNAse to remove contaminating genomic DNA and separated by gel electrophoresis to assess purity and integrity (Fig 11).

Figure 11 Total RNA of MG strains 80083 and ts-11 separated by gel electrophoresis and stained with ethidium bromide

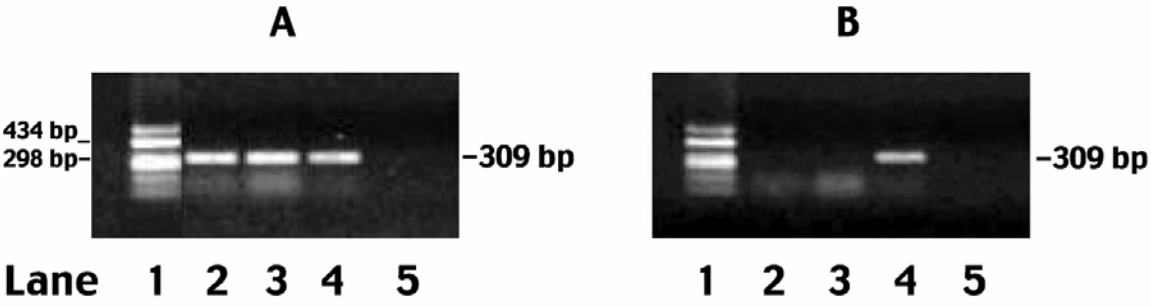


Removal of Genomic DNA

To remove contaminating genomic DNA from each RNA preparation, each preparation was treated with DNase and to confirm the absence of genomic DNA, a PCR based strategy was chosen that would produce a product if genomic DNA were present. To this end DNase treated RNA was either reverse transcribed or not and used as template for PCR. The PCR used KA1 and KA2 (Table 3) oligonucleotide primers to the p47 gene and would amplify a 309 bp product. The results shown in Figure 12 reveal the absence of contaminating DNA, only reverse transcribed RNA yielded the expected sized product whilst RNA not reverse transcribed did not.

Figure 12 Strategy used to determine the presence of contaminating genomic DNA in RNA preparations

Total RNA preparations of MG strains ts-11 and 80083 were DNAse treated and either reverse transcribed (RT) or not and used as template for PCR to detect a 309 bp DNA sequence of the p47 gene. Panel A. Lane 1: DNA size marker, Lane 2: MG strain ts-11 RT-p47 PCR, Lane 3: MG strain 80083 RT-p47 PCR, Lane 4: MG genomic DNA p47 PCR positive control, Lane 5 no template control. Panel B. Lane 1: DNA size marker, Lane 2: MG strain ts-11 p47 PCR, Lane 3: MG strain 80083 p47 PCR, Lane 4: MG genomic DNA p47 PCR positive control, Lane 5 no template control.



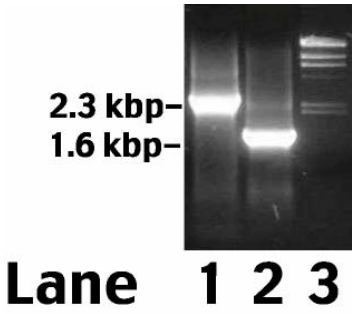
Subtractive Hybridisation of 16s and 23s Sequences

Three rounds of subtractive hybridisation and selective amplification produced a number of products, these were subsequently cloned and their DNA sequence determined. Analysis of these clones showed they were *Dpn*II fragments with several being derived from the 23s and 16s rDNA sequences. Due to the large amounts of rRNA present in the mixture we produced PCR products of the 16s and 23s rDNA genes and used these products in driver mixture in each subtractive hybridisation step. This procedure would then remove and 16s or 23s homologous DNA sequences from the tester after each amplification step.

A PCR reaction was used to amplify the 16s and 23s

Figure 13 PCR amplification of 16s and 23s rRNA gene sequences

Oligonucleotides complimentary to the 23s and 16s gene sequences were used in PCR to produce products of 2.3 kbp and 1.6 kbp respectively. Lane 1: 23s PCR product, Lane 2 16s PCR product, Lane 3 MW DNA standard. MW: *Hin*dIII digested λ DNA



Signature Tagged Mutagenesis

Several variations in experimental protocol were used to produce STMs. Due to the variability in producing unique signature tags in the pISM2062.2 vector the DNA sequence of the each insert was determined for 40 or so separate clones. Of the vectors carrying the correct signature tag, 36 unique pISM4001 clones were selected and used to transform MGAP3. Transformants were selected for their resistance to gentamicin and the presence of the transposon verified by PCR using the P2 and P4 oligonucleotide primers.
An oligonucleotide dot blot hybridisation system was developed using oligonucleotide primers corresponding to the signature tag of each of the 34 ST clones. Recovered STs were amplified using digoxigenin labelled P2 and P4 primers and used to probe the oligonucleotide primers of the dot blot as shown in Fig 1.
A preliminary experiment using 10 STMs to infect birds was conducted to verify the methodology was appropriate and would yield results.

Animal Experiments

Experiment 1

To establish if the proposed experimental procedures would be appropriate a preliminary study was undertaken using twelve 4-week old chickens. The chickens were infected by being placed in an aerosol chamber and exposed for 10 minutes to a measured volume of air into which a log phase culture of a pool of ten STM clones had been aerosolised. The birds were then housed in negative pressure isolators and given water and feed *ad libitum*. Three days after aerosol infection, four same age SPF chickens were placed in the isolator to serve as in-contact controls to assess the dissemination of STM clones. Fourteen days after infection 6 aerosol exposed chickens were euthanased by intravenous administration of pentobarbitone and tissue samples taken from each chicken for histopathology and recovery of STM organisms. Similarly, twenty-eight days after infection the remaining aerosol and in-contact exposed chickens were euthanased and tissue samples taken for histopathology and recovery of STM organisms. Organisms were recovered by swabbing the trachea, airsacs and other organs of the bird. The swab was plated onto MA plates either containing gentamicin or not and then placed into mycoplasma broth medium containing gentamicin and all samples were incubated at 37ºC. The MA plates were incubated for 7 days and the number of colonies estimated. Any mycoplasma broth cultures showing an acid colour change (indicative of growth) were tested by PCR and dot blot hybridisation to determine which of the STMs were present.

Experiment 2

In this study we tested 108 STMs for their ability to colonise and produce disease. Three groups of twenty 3-week old chickens were used. Each group was infected by being placed in an aerosol chamber and exposed for 10 minutes to a measured volume of air into which a log phase culture of a pool of thirty-six unique STM clones had been aerosolised. This was repeated twice more using a further seventy-two unique STM clones. Three days after aerosol infection 10 “same-age” SPF chickens were placed in each isolator to serve as in-contact controls to assess the dissemination of STM clones. At day 14 post infection, 10 aerosol exposed chickens were euthanased by intravenous administration of pentobarbitone. Tissue samples from each chicken were taken for histopathology and for the recovery of organisms. Twenty-eight days post infection the remaining aerosol and in-contact exposed chickens were euthanased by intravenous administration of pentobarbitone. Organisms were recovered by swabbing the trachea and airsacs of the bird. The swab was plated onto MA plates either containing gentamicin or not and then placed into mycoplasma broth medium containing gentamicin and all samples were incubated at 37ºC. The MA plates were incubated for 7 days and the number of colonies estimated. Any mycoplasma broth cultures showing an acid colour change (indicative of mycoplasma growth) were tested by PCR and dot blot hybridisation to determine which STM was present.

Experiment 3

Based on the results from experiment 2 we examined the safety and recovery of 16 STMs containing defined gene knock-outs. A group of twenty 4-week old chickens were infected by being placed in an aerosol chamber and exposed for 10 minutes to a measured volume of air, into which a log phase culture of 8 STM clones had been aerosolised. This was repeated once more using another 8 unique STM clones to infect 20 chickens. The birds were housed in the same isolator to investigate horizontal transfer of organisms. Fourteen days after infection all chickens were euthanased by intravenous administration of pentobarbitone. Gross examination was made of the air sacs and trachea. Organisms were recovered by swabbing the trachea and airsacs of the bird and the swab plated onto MA plates either containing gentamicin or not and then placed into mycoplasma broth medium containing gentamicin, all samples were then incubated at 37ºC. The MA plates were incubated for 7 days and the number of colonies estimated. Any mycoplasma broth cultures showing an acid colour change (indicative of mycoplasma growth) were tested by PCR and/or dot blot hybridisation to determine which STM was present.

Signature Tagged Mutagenesis Results

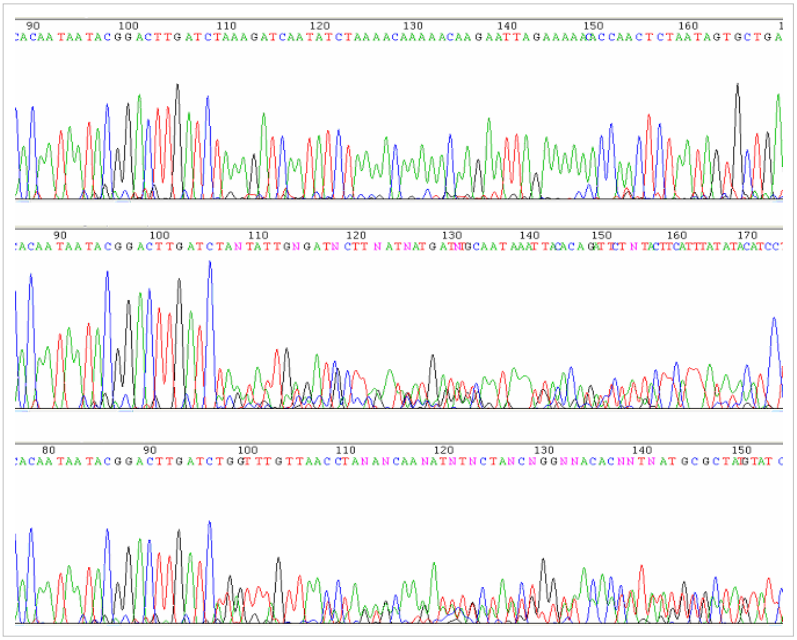
Determination of STM insertion site in the Genome

Over 40 separate transformations with *M. gallisepticum* Appin 3AS strain were performed, each transformation used a unique signature tagged pISM2006.2 vector and recombinants selected for using gentamicin. Individual clones from each transformation were grown in MB media containing gentamicin with at least 3 separate clones chosen from each transformation. This process was time consuming and laborious and continued through the first experiment until an adequate number of clones were obtained. In most cases the genome insertion site for each STM was determined by sequencing out from the transposon into the genome using the unique oligonucleotide primer IG (Appendix 2). In a number of cases the DNA sequencing of several of the STM clones revealed mixed sequence following the transposon indicating multiple copies of this DNA sequence from the transposon in the genome (Figure 14A) this was verified in most cases by Southern blot analysis (Figure 14B). In the case of STM13 the DNA sequence determined from sequencing did not match the MG strain R genome sequence and the surrounding genome insertion site was sequenced using primer walking until the specific genomic location could be accurately determined. The primers used in this are shown in the Appendices.

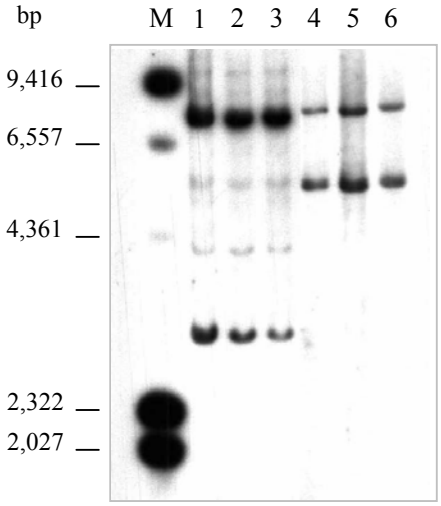
Figure 14 Electropherograms and Southern blot analysis of STM mutants

A. Electropherograms obtained by direct sequencing. I. STM26-2 carrying a single transposon shows a clear genomic read of the host strain whereas mixed sequencing signals are seen starting at the junction of transposon and host strain gemone in both STM15-1 (II) and STM25-1 (III). B. Multiple insertions are shown in the STM transformants. Radiolabelled STM DNA was used to probe *Bam*HI restricted genomic DNA in Southern blot. Lane 1, STM15-1; lane 2, STM15-2; lane 3, STM15-3. Lane 4, STM25-1; lane 5, STM25-2; lane 6, STM25-3. M, DNA size standard contained λDNA digested with *Hind*III.

(A)



(B)



STM Experimental Results

Over time it became apparent that several of the clones derived from the same transformation had identical insertion sites. This was most likely due to the initial single cell transformant replicated during the recovery stage after transformation, thereby producing a number of colonies on MA. These colonies were subsequently cloned and cultured. Once this duplication became apparent we devised several PCRs using sequence information from the genomic STM sites to test whether the sister transformants were identical. As shown in Figure 14b, STM15 and STM25 contained at least two copies of the probe reactive transposon and clones 1,2 and 3 of each were most likely identical as seen by the Southern blot pattern.
The details of individual transformants are detailed in Table 4 and includes the ten clones used in experiment 1.

Experiment 1

The number of organisms used in each ml of inoculum was determined by limiting dilution titration and together with the gene interrupted by the STM are shown in Table 4.

Table 4 STM ID, number of STM organisms contained in the inoculum and gene interrupted

STM name	CCU/ml*	Gene interrupted
¶STM02-1	8.5E+07	MGA_0934, Adherence protein A (GapA)
STM03-1	3.8 E+07	MGA_1102, Conserved hypothetical protein
STM04-1	3.4 E+07	MGA_0680, MalF ABC-type sugar transport system
STM06-1	1.3 E+07	MGA_0934, Adherence protein A (GapA)
STM09-1	6.1 E+07	MGA_0662, Unique hypothetical protein
STM17-1	4.1 E+07	MGA_0379, VlhA3.02 gene product
STM19-1	1.9 E+07	MGA_0216, EF-P, elongation factor-P
¶STM32-1	2.8 E+07	MGA_0817, Unique hypothetical protein
¶STM35-1	1.6 E+08	MGA_0758, Unique hypothetical protein
¶STM37	1.3 E+07	MGA_1087, Unique hypothetical protein

*colour changing units per ml (CCU/ml)
¶ STM reisolated in experiment 1 (see text and also Table 5)

The results of STM detection are shown in Table 5. By 4 weeks post infection all birds showed a serological response in RSA to MG though only 1 bird from the in-contact group showed serological activity in the RSA after 25 days exposure. Most birds with a positive RSA score also showed air sac lesions ranging from 0.5-2.5 indicative of *M. gallisepticum* infection. Only 4 of the 10 STMs from the input pool could be reisolated, these included STM02-1, STM32-1, STM35-1 and STM37, the last three being classified as unique hypothetical proteins whilst in STM02-1 the gapA protein is interrupted. In several cases there were greater than 10x the number of reisolates on MA plates without gentamicin than on MA plates without. This was indicative that not all re-isolates contained the gentamicin resistance gene. Bird 749 showed both clinical signs and serological response though no STM could be reisolated from the trachea or airsacs by broth culture. These cases may represent

an instance whereby withdrawing gentamicin from the STM environment caused the loss of the transposon and reversion to wildtype. Normally the transposon is reasonably stable but if the gene interruption was itself deleterious or unstable then this may lead to its loss.

Table 5 Serology, pathology and STM isolation results from experiment 1

	Summary			Air sacs		Trachea		Lung		Heart		Spleen		Liver		Kidney		Brain	
Bird group and ID	STM detected	RSA	^Lesion Score	MA	MB	MA	MB	MA	MB	MA	MB	MA	MB	MA	MB	MA	MB	MA	MB
14 d post mortem																			
744		0	0	¶-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
745		1	0.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
746		1	0.5			*+	STM37 &			+									
	STM35/7			-	STM37		35	-	-		-	-	-	-	-	-	-	-	-
747	STM32	0	0	-	-	-	STM32	-	-	-	-	-	-	-	-	-	-	-	-
748	STM32	0	0	-	-	+	STM32	-	-	-	-	-	-	-	-	-	-	-	-
751	STM32/7	0	0	-	-	-	STM37& 32	-	-	-	-	-	-	-	-	-	-	-	-
28 d post mortem																			
741		1	0	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-
742	STM35	1	0	-	-	-		-	-	-	-	-	-	-	-	-	-	-	STM35
743	STM32	2	0.5	-	STM32	+	STM32	-	-	-	-	-	-	-	-	-	-	-	-
749		4	2.5	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
750	STM35	1	0	-	-	+	STM35	-	-	-	-	-	-	-	-	-	-	-	-
757	STM37	2	1	-	STM37	+	STM37	-	-	-	-	-	-	-	-	-	-	-	-
In contact controls																			
737		0	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
738	STM02-1	0	0	-	STM02-1	-	STM02-1	-	-	-	-	-	-	-	-	-	-	-	-
739		0	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
740		1	0.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

RSA: Rapid plate Serum Agglutination test, scored 0-4

^Lesion score: Determined by gross examination of air sacs, scored 0-3 MA: Mycoplasma Agar plate MB: Mycoplasma Broth

¶-: No MG organism reisolated

*+: 10x the number of colonies on MA plates as compared to MA plates with gentamicin added

Experiment 2

Results from experiment are detailed in Table 6 and include the serology as shown by RSA score, clinical lesion scores of airsacs and the STM detected from various sites within the host.

Table 6 Experiment 2 serology, clinical score and STMs detected from all birds

	Summary			Air sacs		Trachea		Lung/Heart Spleen	Liver/Kydney Brain
Bird ID	STM detected	RSA	Lesions	MA	MB	MA	MB	MB	MB
14 days post mortem									
76		1			-			-	-
78	STM07-1 STM18-1 STM20-1 STM24-1 STM38-1	1	2		-		STM07-1 STM18-1 STM20-1 STM24-1 STM38-1	-	-
79		0.5			-		-	-	-
80	STM39-1	1			-		STM39-1	-	-
81	STM12-1 STM20-1	1	2		STM20-1		STM12-1 STM20-1	-	-
83		1			-		-	-	-
84	STM07-1 STM12-1 STM19-1 STM24-1	0.5			-		STM07-1 STM12-1 STM19-1 STM24-1	-	-
85		2			-		-	-	-
90	STM12-1 STM19-1	1			-		STM12-1 STM19-1	-	-
*93									
28 days post mortem									
75		1			-			-	-
77		1			-			-	-
82		2			-			-	-
86		2			-			-	-
87	STM28-1	1			STM28-1			-	-
88	STM12-1 STM20-1 STM24-1 STM27-1 STM28-1	1			-		STM12-1 STM20-1 STM24-1 STM27-1 STM28-1	-	-
89	STM20-1	0			-		STM20-1	-	-
91	STM20-1 STM24-1 STM27-1 STM28-1	2			-		STM20-1 STM24-1 STM27-1 STM28-1	-	-
*92									
94	STM20-1 STM24-1	1			-		STM20-1 STM24-1	-	-
In contact controls					-				
48		0			-			-	-
49		0			-			-	-
50		0			-			-	-
51		0			-			-	-

	Summary			Air sacs		Trachea		Lung/Heart Spleen	Liver/Kydney Brain
Bird ID	STM detected	RSA	Lesions	MA	MB	MA	MB	MB	MB
54	STM16-1 STM20-1 STM28-1	0			STM16-1 STM20-1 STM28-1			-	-
*56					-			-	-
62		0			-			-	-
*64					-				
71		0			-			-	-
72		0			-			-	-

RSA: Rapid plate Serum Agglutination test, scored 0-4
Lesion score: Determined by gross examination of air sacs, scored 0-3
MA: Mycoplasma Agar plate
MB: Mycoplasma Broth [3]
[¶]-: No MG organisms reisolated
[*]+: 10x the number of colonies on MA plates as compared to MA plates with gentamicin added

Group 2.

	Summary			Air sacs		Trachea		Lung/Heart Spleen	Liver/Kydney Brain
Bird ID	STM detected	RSA	Lesions	MA	MB	MA	MB	MB	MB
14 days post mortem									
116	-	1	2		STM28-2		-	-	-
117	STM07-2 STM14-2 STM28-2	1			-		STM07-2 STM14-2 STM28-2	-	-
119	STM12-2	0			-		STM12-2	-	-
121	STM16-2 STM20-2 STM27-2	2			STM20-2	+	STM16-2 STM20-2 STM27-2	-	-
122	STM27-2	1			-		STM27-2	-	-
123	STM20-2	0			-		STM20-2	-	-
125	-	0			-		-	-	-
127	-	1			-		-	-	-
131	STM11-2 STM12-2 STM20-2	2	4	+	STM12-2	+	STM11-2 STM12-2 STM20-2	-	-
134	STM20-2	0			-		STM20-2	-	-
28 days post mortem					-		-		
115	-	1			-	+	-	-	-
118	-	2			-		-	-	-
120	STM20-2 STM28-2	2			-		STM20-2 STM28-2	-	-
124	-	2	1		-		-	-	-
126	-	0			-		-	-	-
128	-	1			-		-	-	-

129	-	0	-	-	-	-

	Summary			Air sacs		Trachea		Lung/Heart Spleen	Liver/Kydney Brain
Bird ID	STM detected	RSA	Lesions	MA	MB	MA	MB	MB	MB
132	-	4			-		-	-	-
133	-	4			-		-	-	-
3X	STM12-2	2			-	+	STM12-2	-	-
4X	STM20-2	2			-		STM20-2	-	-
In contact controls									
45	-	0			-		-	-	-
46	-	0			-		-	-	-
52	-	0			-		-	-	-
55	-	0			-		-	-	-
59	-	0			-		-	-	-
61	-	0			-		-	-	-
63	-	0			-		-	-	-
65	-	0			-		-	-	-
67	-	0			-		-	-	-
69	-	0			-		-	-	-

RSA: Rapid plate Serum Agglutination test, scored 0-4
Lesion score: Determined by gross examination of air sacs, scored 0-3
MA: Mycoplasma Agar plate
MB: Mycoplasma Broth
[¶]-: No MG organisms reisolated
[*]+: 10x the number of colonies on MA plates as compared to MA plates with gentamicin added

Group 3

	Summary			Air sacs		Trachea		Lung/Heart Spleen	Liver/Kydney Brain
Bird ID	STM detected	RSA	Lesions	MA	MB	MA	MB	MB	MB
14 days post mortem									
96	STM16-3 STM20-3	4	4	+	STM16-3 STM20-3		-	-	-
98	-	0			-		-	-	-
100	STM02-3	1			-	+	STM02-3	-	-
105	STM14-3 STM20-3 STM27-3	1	0.5	+	STM20-3	+	STM14-3 STM20-3 STM27-3	-	-
106	STM02-3 STM28-3	0		+	STM28-3	+	STM02-3	-	-
108	STM02-3 STM39-3 STM16-3 STM18-3 STM20-3 STM27-3	0	3	+	STM16-3 STM18-3 STM20-3 STM27-3	+	STM02-3 STM39-3	-	-

109	STM02-3 STM13-3 STM24-3 STM27-3 STM38-3	1			-		STM02-3 STM13-3 STM24-3 STM27-3 STM38-3	-	-

	Summary			Air sacs			Trachea	Lung/Heart Spleen	Liver/Kydney Brain
Bird ID	STM detected	RSA	Lesions	MA	MB	MA	MB	MB	MB
110	STM01-3 STM16-3 STM20-3 STM27-3 STM28-3 STM36-3	0			-	+	STM01-3 STM16-3 STM20-3 STM27-3 STM28-3 STM36-3	-	-
111	STM20-3 STM27-3	0			-		STM20-3 STM27-3	-	-
114	STM11-3 STM12-3 STM27-3	1	2	+	STM11-3	+	STM11-3 STM12-3 STM27-3	-	-
28 days post mortem									
95	STM02-3 STM12-3 STM20-3 STM28-3	4	4		STM02-3 STM20-3	+	STM02-3 STM12-3 STM20-3 STM28-3	-	-
97	STM02-3 STM18-3	2			-	+	STM02-3 STM18-3	-	-
99	-	1			-		-	-	-
101	STM12-3 STM20-3 STM24-3 STM28-3	2	0.5	+	STM20-3	+	STM12-3 STM20-3 STM24-3 STM28-3	-	-
102	STM20-3	0			-		STM20-3	-	-
103	STM16-3 STM20-3 STM24-3 STM27-3	2		+	STM20-3	+	STM16-3 STM24-3 STM27-3	-	-
104	STM12-3 STM20-3 STM24-3 STM28-3	1		+	STM20-3	+	STM12-3 STM20-3 STM24-3 STM28-3	-	-
107	STM02-3 STM20-3	2			-	+	STM02-3 STM20-3	-	-
112	STM16-3	1		+	STM16-3		-	-	-
113	STM02-3 STM12-3 STM20-3	4		+	STM12-3	+	STM02-3 STM20-3	-	-
In contact controls									
*53									
57	-	0			-			-	-
58	-	0			-			-	-
60	-	0			-			-	-
66	-	0			-			-	-
68	-	0			-			-	-
70	-	0			-			-	-

*73					
74	-	0	-	-	-

*Died before final postmortem RSA: Rapid
plate Serum Agglutination test, scored 0-4
Lesion score: Determined by gross examination of air sacs, scored 0-3
+: 10x the number of colonies on MA plates as compared to MA plates with gentamicin added
MA: Mycoplasma Agar plate MB: Mycoplasma Broth ¶-: No STM detected*

The STM clones detected in experiment 2 are shown in Table 7. The summary shows that several of the clones were detected more than once indicating there ability to colonise and replicate was not impaired. The interruptions in each STM appear to be in genes not essential for survival in the host. It can be seen from Table 7 that STM 20 was the most readily detectable: a total of 41 times from the trachea and airsacs. Two of the clones that were also readily detectable (STM16 and STM38) showed the transposon had inserted intergenically, whereby gene inactivation would be minimal or to not occur at all. Two other recovered STM clones had insertions in genes predicted to be transposases. Interestingly, a number of insertions were located in several genes involved in transport of oligopeptides across the cell membrane. There was some functional duplication in several of these oligopeptide genes, indicating there may be some form of redundancy. Three genes were annotated as "unique hypothetical" (STM07, STM14 and STM18) and another as "conserved hypothetical" (STM27): their function remains unknown. There were two STM detected that had gene interruptions to CrmB (STM28 and STM39), a cytadhesin related protein. From these and previous results a set of STM clones were selected upon their ability to poorly colonise the host.

Table 7 Summary of STM clones detected in Experiment 2

STM Detected				Gene interrupted	
Airsac	No. times detected	Trachea	No. times detected	Gene ID	Gene description
		STM01	1	MGA_1142	OsmC Stress-induced protein
STM02-3	1	STM02-3	9	MGA_0145/0545	Predicted transposase
		STM07	3	MGA_0554	Unique Hypothetical
STM11	1	STM11	3	MGA_0967	VlhA4.02
STM12	2	STM12	11	16s rRNA	16s
		STM13	1	Genomic seq'ing	Predicted transposase
		STM14	2	MGA_0549	Unique hypothetical
STM16	4	STM16	7	Intergenic	Between MGA_0395/0398 VlhA and Malp
STM18	1	STM18	3	MGA_0578	Unique hypothetical
		STM19	2	MGA_0216	EF-P elongation factor P
STM20	10	STM20	31	MGA_0220	DppD oligopeptide transport ATP-binding protein
		STM24	9	MGA_0221	DppC oligopeptide transport ATP-binding protein
STM27	1	STM27	11	MGA_0226	Conserved hypothetical, lipoprotein
STM28	4	STM28	12	MGA_0943	CrmB cytadherence related protein B
		STM36	1	MGA_0223/0224	Not annotated: OppB oligopeptide transport
		STM38	2	Intergenic	b/n MGA_0518/0519 Unique/conserved
		STM39	2	MGA_0943	CrmB cytadherence related protein B

STM clones not detected STM2-1, STM2-2, STM3, STM4, STM6, STM10, STM11, STM15, STM17, STM21, STM22, STM23, STM25, STM26, STM31, STM32, STM33, STM34, STM35

Experiment 3

Results from experiment 3 are detailed in Table 8 and include the serology as shown by RSA score, clinical lesion scores of airsacs and the STM detected from either the trachea or airacs of the host.

Table 8 Experiment 3 serology, clinical score and STMs detected from all birds

Group 1.

	RSA			Airsac			Trachea
Bird ID	Day 0	Day 14	Lesions	MA	MB	MA	MB
135	0	1	0		-	+	STM02 STM10 STM17
138	0	1	0		-	+	STM17
139	0	1	0		-		-
141	0	1	0		-	+	STM02
143	0	3	1	+	STM10	+	STM10 STM17
144	0	1	0		-	+	STM10 STM17
145	0	0	0		-	+	STM10 STM17
147	0	1	0		-	+	STM02 STM04 STM10
149	0	0	0		-	+	STM02 STM10 STM17
153	0	2	0		-	+	STM03 STM10 STM17
156	0	2	0		-	+	STM17
157	0	2	0		-	+	STM10
158	0	0	0.5		-	+	STM17
159	0	1	0		-	+	STM06 STM09 STM10 STM23
160	0	1	0		-		-
161	0	3	2.5	+	STM03 STM09 STM10 STM15 STM34	+	STM09 STM10 STM15 STM17 STM34
163	0	3	0		-		-
164	0	0	0		-	+	STM09 STM17 STM23 STM25
166	0	1	0		-	+	STM17
*174	0		-		-		-

Birds in Group 1 were infected with pooled mutants containing STM02-2, STM03, STM04-1, STM06, STM09-1, STM10, STM15 and STM17-1.

Group 2.

	RSA			Air sacs		Trachea	
Bird ID	Day 0	Day 14	Lesions	MA	MB	MA	MB
136	0	1	0		-	+	STM09 STM23
137	0	1	0		-	+	STM09 STM17
140	0	0	0		-	+	STM02-2 STM10 STM23
142	0	0	0		-	+	STM09 STM17 STM23
146	0	2	0		-	+	STM09 STM17 STM23
*148	0	-	-		-		-
150	0	2	0		-	+	STM09 STM23 STM25
151	0	3	0		-	+	STM09 STM10 STM15 STM17 STM23 STM34
152	0	2	0		-	+	STM09, STM10 STM17 STM22 STM23
154	0	2	0		STM23	+	STM03 STM09 STM15 STM17 STM23 STM25 STM34
155	0	0	0		-	+	STM10 STM23
162	0	2	0		-	+	STM09 STM10 STM17 STM22 STM23
165	0	1	0		-	+	STM09 STM17 STM23 STM25
167	0	2	1	+	STM09	+	STM09
168	0	0	0		-	+	STM09
169	0	1	0		-	+	STM17 STM23
170	0	0	0		-	+	STM09 STM17 STM23
171	0	2	0		-	+	STM23
172	0	1	0	+	STM09 STM17 STM23		-
173	0	1	0		-	+	STM09 STM23

Birds in Group 2 were infected with pooled mutants containing STM04-3, STM09-3, STM17-3, STM22, STM23, STM25, STM33 and STM34
RSA: Rapid plate Serum Agglutination test, scored 0-4
Lesion score: Determined by gross examination of air sacs, scored 0-3
MA: Mycoplasma Agar plate
MB: Mycoplasma Broth
¶-: No MG organisms resisolated
*+:10x the number of colonies on MA plates as compared to MA plates with gentamicin added

The results showed that all STM clones apart from STM33 were detected by either Southern dot blot or PCR. The experiment also showed that following aerosol infection (Table 9) and intermingling of infected birds from both groups that their pen mates became colonised with STMs from the other group. This may be due to either horizontal transmission or direct physical contact between birds that had been aerosolised: those STM clones transmitted were STM03, STM10, STM15, STM23, STM25 and STM34. These results show that these clones still retained their ability to infect and transmit infection to other birds, indicating the gene(s) interruptions are not deleterious to the organism. Most

birds developed an immune response as shown by the rapid serum agglutination (RSA) test, this ranged from 0.5 to 3. There were few birds with airsac lesions, 3 in the group A and 1 in group B. The scores were relatively low, an indication the infection was relatively mild due to the reduced virulence of the STMs, though no control group of birds infected with the virulent AP3 parental strain was included in this experiment.

Table 9 Summary of results for Experiment 3

STM ID	STM used in Infected Group	No times STM detected in each group		Gene ID	Predicted gene function
		Group 1	Group 2		
STM02-2	1	4	0	MGA_0549	Unique hypothetical
STM03	1	2	1	MGA_1102	Conserved hypothetical
STM04-1	1	1	0	MGA_0680	MalF ABC-type sugar transport permease
STM04-3	2	0	0	MGA_0073/145	Predicted transposase
STM06	1	1	0	MGA_0934	gapA MG adherence protein A (GapA)
STM09-1	1	4	0	MGA_0662	Unique hypothetical
STM09-3	2	0	14	Not determined	
STM10	1	11	5	INTERGENIC	Between MGA_0537/0539, HsdS system
STM15	1	2	2	Not determined	
STM17-1	1	12	0	MGA_0379	VlhA3.02
STM17-3	2	0	11	Not determined	
STM22	2	0	2	MGA_1079	Unique Hypothetical
STM23	2	2	17	Before gene start	Match VlhA3.03 and 5.05
STM25	2	1	3	Not determined	
STM33	2	0	0	MGA_0939	CrmA cytadherence related molecule A
STM34	2	2	2	Not determined	

Discussion

The overall strategy to determine genes responsible for virulence and transmission was successful. We were able to produce a panel of clones containing interruptions to coding sequences of a number of genes, this was accomplished by the random integration of a transposon carrying a unique DNA tag and an antibiotic resistance gene into the genome.

Transformants were selected for their ability to grow in the presence of gentamicin, from there they were propagated in media containing antibiotics. Following the transformation stage any clones containing lethal insertions would not have been able to grow and therefore were not selected. The clones that grew contained transposon insertions that presumably allowed *in vitro* growth and there ability to infect and produce disease at this stage was unknown. The initial experiment proved our methodology was appropriate as we were able to infect birds and recover and detect STMs used to infect the birds. An initial problem that reduced our capacity to identify and select unique STMs was the inability to rapidly determine the insertion site for each STM. It was assumed that each clone taken from the transformation plate was unique but from DNA sequencing of a number of clones we discovered our assertion was incorrect. There were a number of contributing factors/problems: these

involved producing high quality and adequate amounts of genomic DNA for DNA sequencing, insertion of the transposon into more than one site in the genome, insertion of the transposon into DNA regions unique to the *M. gallisepticum* strain AP3 not found in the genomic sequence of R strain. Some of these problems were overcome by using PCR to verify if the STM was identical to that derived from the same transformation. In other cases we used Southern blot to show the transposon had inserted more than once and used DNA sequencing combined with primer walking to determine the region(s) where the transposon had inserted.

Following the success of our first feasibility experiment we used a selection of STMs in experiment 2 to infect three separate groups of birds. In most instances each group received the same STM clone. There were a number of identical STMs isolated from two or all three groups indicating reproducibility of infection and virulence of these STMs had not diminished. Those STMs isolated three times were: STM12, STM16, STM20, STM27 and those isolated twice were STM7, STM11, STM14, STM18, STM24, STM38 and STM39. The inability to isolate a number of STMs could be due to reduced fitness of the clone and/or the ability of other clones to exclude or otherwise occupy sites to establish infection. To avoid the possibility of exclusion occurring a number of infectious STMs were not used any further and a select group of clones that were not reisolated were examined in further experiments.

This select group consisted of 16 STM clones, 8 clones each were used to infect 2 groups of 20 birds each, which were then intermingled and housed in a single isolator. Several STMs that were used to infect one group were also isolated from the other group indicating either physical transmission following vaccination or the possibility of horizontal transmission. The STMs used in the third experiment were able to be isolated at a greater rate and from more individual birds than in previous experiments. These results support the suggestion that more virulent organisms exclude “less fit” STMs from establishing infection. Previous research has shown that chickens infected with one strain of *M. gallisepticum* resist colonisation by a second strain. It should be stressed that in the previous research carried out by Levinson et al (1986) the experiments were not conducted using unique clones derived from a single strain nor were the chickens subjected to simultaneous infection. It is possible that the numbers of a single STM infecting organisms may be dominant and influence the ability of other STMs to colonise successfully. To this extent the number of STM organisms in each primary culture that was used for aerosol infection in experiment 1 varied from 1.3E+07 to 1.6E+08 CCU/ml. Of the reisolated STMs from experiment 1, two had low titres suggesting that the number of organisms used to infect the birds did not unduly influence whether an STM clone was reisolated.

The reiterative experimental approach focuses on those clones that are attenuated and not able to effectively colonise nor cause disease in the host. Whilst these set of experiments have given broad direction to attenuating mutations in the MG genome, further experiments will need to be conducted using birds infected by a single STM clone to be certain that mutation is responsible for attenuation.

Appendices

Oligonucleotides used for PCR and DNA sequencing of gapA gene

Oligo name	Oligonucleotide sequence 5'-3'
FA	AGACCAAACTTCCCTAAC
FC	GCCGGATTGATTTGTATG
FD	TGGATGCTCATATGGAGAG
FF	ACTTGTTTTGTGTTTCC
FG	ATTAGTAAGCCAGCTGGT
FH	CTTACGGTTTTGAGACATTG
FI	TAACGTAATCGGTCAAGGTGC
FJ	CCAGCAAAATCATCACTTAG
FK	TCCAGAAAATGCAGTCAATC
FM	GCTGTCATTGAAGATGCACCTA
FU	TACCGTATCATCTAAATTAAACG
FV	CCTTGGCATTAGCTTTAGGTC

Oligonucleotides used for quantitative PCR and DNA sequencing

Oligo name (gene ID)	Oligonucleotide sequence 5'-3'
GW477For	TAATAAACGTTTTAGGGTTACATA
GX480For	TGAAAAACAAAAATAAAAACAATACT
GY480Rev	ATTACGCGTTAATGATCTCTTTA
GZ1186Rev	TAACCAACTTTAATTCCGAACTT
HA1186For	CGGGTTCGGACGCATTGGT
HB696For	AAACACGCCGAAACTAAATGTT
HC696Rev	GTATTCGTTCACGAACTAATTCT
HL1186For	TGGTAAGTTAAACGGGATCG
HM1186Rev	AATGAACCAGTGATTGTTGGA
HN696For	AATTATGGAAAACACGCCGA
HO696Rev	CCAGTGGGTTTTCTTTTAGGA
HP480For	ACACGGCTTTGTTGCAAAC
HQ480Rev	ACTTGGCTTGAGGTGAACTTT
HR1155For	AACGGTGGTGGGTTTACTGG
HS1155Rev	TGCAATTCCTAAACGGATCG
HU487For	TTGTTGTTACTCAACGCAGTTACTTTATAA
HV487Rev	TGAAAACCCAATTAACACAAGATTTACT
IGSTMGenmeF3	GGACTGTTATATGGCCTTTTTGGATC
IV699Rev	AGATCGTACCTGTGTTAGTTCG
IW487For	TCGACCATTCAACTTGGT
IX487Rev	GCATCACCATTAGCTTGG
JC698For	GAGCTTCTTCGATCTCATCACTTG
JD698Rev	GTTATGGAACTATCTTCTCAACTCTTAATACAA

Oligo name (gene ID)	Oligonucleotide sequence 5’-3’
JE164For	AGAGGAAAACTTAACGCTCCAATC
JF164Rev	TCCTCCACCCATTGGCATT
JG218For	TGAGGGTCTGTAATCAACGCTCTA
JH218Rev	TGGTCAACGTCAAAGAGTGGTG
JI237For	AACGGTCAATCCCTCCACC
JK237Rev	ATCCAGTGAACCGCAGGTACAT
JL477For	CCTGCCAGGTTATTTTTGGTCA
JM477Rev	GCCTGCTGCATCTCTTTGG
JN162For	TGTAGTTGCTCACCCAATTTTCA
JO162Rev	CGGTTGCTAGCTTTGTCGTAGTG
JP223For	GGTTCCAACAAGATATGTCGCA
JQ223Rev	ATACTTGAAAACCCACCCAAACTT
JR226For	AATCAGAACGATTCAAGCCCTC
JS226Rev	AGCCAGTTTATCTGGTCGTGACTT
JT230For	GGTTCCAACAAGATATGTCGCA
JU230Rev	CGCCCAACCGTATAACCATTT
IW487For	TCGACCATTCAACTTGGT
IX487Rev	GCATCACCATTAGCTTGG
STM13-JA-rev	TTAATACGCCTGGGTAAGGTCG
STM13-JB-for	TATCTATCACGTAATTGGAATGC
STM13-KE	TCGCTATAACATTTGATTTAG
STM13-KE-con’d	CATTTTGATGTTTGCGTCA
STM13-KE-C’-1	CTTCAAGTTCTCTGTGG
STM13-KF	CCGTTTTTCTTTTATTCGTATTATC
STM13-KF-con’d	TATAAACCTGGTACGG
STM13-KF-C’-1	CACACTGAAAGTTATATTA
STM13-KF-C’-2	CTCACTATATAAAATAGC
STM13-KG	CAGAAGATAAGTCTTTAACAG
P2	ATCCTACAACCTCAAGCT
P4	ATCCCATTCTAACCAAGC
STM02-B-Rev	TTCAATTCGAAAAATGAGTT
STM03-B-Rev	ACAGCTTGACGTTTTCCA
STM04-B-1	CGGGGACACAGTAAGGCTAA
STM04-C-rev	CGCATTGATTCCTACCAC
STM06-A-rev	AACATGGATTAGACGTTTGC
STM09-B-Rev	TGCCTTTCTTAATAGTGCTCA
STM10-Rev	TTGTGCCCATTCGTTAGT
JX-STM17	CTAGAAGAAGAAGAAGAGGTAACGAAGC
JZ-STM22	CTCAATTGAAGAATTATATGATG
STM23-B-Rev	TTAACCCCATTCTTGCAG
KB-STM33	ACTACACGGTAGTTAGTAC

Table of STM clone, insertion site in genome and gene disrupted.

STM ID	TAG ID	Insertion	Operon	Gene start	No. aa	% of gene at insert	Gene name
STM01-1	Tag5	MGA_1142	N	394983	151	40.5	OsmC Stress-induced protein
STM01-2	" "	" "	" "	" "	" "	" "	" "
STM01-3	" "	" "	" "	" "	" "	" "	" "
STM02-1	R7	MGA_0934	Y	223214	1123	68.3	gapA MG adherence protein A (GapA)
STM02-2	R7A	MGA_0549	N	931683	188	13.3	Unique hypothetical
STM02-3	R7B	MGA_0145/0545	N	645994	412	43.9	predicted transposase
STM03-1	R9	MGA_1102	Y	366321	543	16.0	Conserved hypothetical
STM03-2	" "	" "	" "	" "	" "	" "	" "
STM03-3	" "	" "	" "	" "	" "	" "	" "
STM04-1	R10	MGA_0680	Y	46565	330	0.2	MalF ABC-type sugar transport permease
STM04-2	" "	" "	" "	" "	" "	" "	" "
STM04-3	R10A	MGA_0073/145	N	588939	312	9.6	Putative transposase
STM06-1	R17	MGA_0934	Y	466498	728	24.3	gapA MG adherence protein A (GapA)
STM06-2	" "	" "	" "	" "	" "	" "	" "
STM06-3	" "	" "	" "	" "	" "	" "	" "
STM07-1	Tag20	MGA_0554	?	939971	1202	71.5	Unique Hypothetical
STM07-2	" "	" "	" "	" "	" "	" "	" "
STM07-3	" "	" "	" "	" "	" "	" "	" "
STM09-1	1R1-10	MGA_0662	Y	35414	299	60.3	Unique hypothetical
STM09-2	" "	" "	" "	" "	" "	" "	" "
STM09-3		not determined					
STM10-1	1R120	INTERGENIC	N	925062			Between MGA_0537/0539, HsdS system
STM10-2	" "	" "	" "	" "	" "		
STM10-3	" "	" "	" "	" "	" "		
STM11-1	1R1-29	MGA_0967	N	253111	728	0.9	VlhA4.02
STM11-2	" "	" "	" "	" "	" "	" "	" "

STM ID	TAG ID	Insertion	Operon	Gene start	No. aa	at insert	Gene name
STM11-3	" "	" "	" "	" "	" "	" "	" "
STM12-1	R2-3	16s rRNA	Y	330800			16s
STM12-2	" "	" "	" "	" "			" "
STM12-3	" "	" "	" "	" "			" "
STM13-1	R2-5	no match	N	a			Putative transposase
STM13-2	" "	" "	" "	" "			
STM13-3	" "	" "	" "	" "			
STM14-1	R2-9	MGA_0549	N	931683	188	53.3	Unique hypothetical
STM14-2	" "	" "	" "	" "	" "	" "	" "
STM14-3	" "	" "	" "	" "	" "	" "	" "
STM15-1	R2-18	Not determined					
STM15-2	" "						
STM15-3	" "						
STM16-1	R2-22	INTERGENIC	N	826379			Between MGA_0395/0398 VlhA and Malp
STM16-2	" "	" "	" "	" "			" "
STM16-3	" "	" "	" "	" "			" "
STM17-1	R3-9	MGA_0379	N	809410	660	80.1	VlhA3.02
STM17-2	" "	" "	" "	" "	" "	" "	" "
STM17-3		not determined					
STM18-1	R3-17	MGA_0578	N	965746	225	69.7	Unique hypothetical
STM18-2	" "	" "	" "	" "	" "	" "	" "
STM18-3	" "	" "	" "	" "	" "	" "	" "
STM19-1	R3-23	MGA_0216	Y	692170	188	88.6	EF-P elongation factor P
STM19-2	" "	" "	" "	" "	" "	" "	" "
STM19-3	" "	" "	" "	" "	" "	" "	" "
STM20-1	R3-24	MGA_0220	Y	697324	444	7.9	DppD oligopeptide transport ATP-binding protein
STM20-2	" "	" "	" "	" "	" "	" "	" "
STM20-3	" "	" "	" "	" "	" "	" "	" "
STM21-1	R3-33	MGA_0554	N	939971	1202	12.4	Unique Hypothetical
STM21-2	" "	" "	" "	" "	" "	" "	" "

STM ID	TAG ID	Insertion	Operon	Gene start	No. aa	% of gene at insert	Gene name

STM21-3	" "	" "	" "	" "	" "	" "	" "
STM22-1	R3-35	MGA_1079	N	350161	1579	22.3	Unique Hypothetical
STM22-2	" "	" "	" "	" "	" "	" "	" "
STM22-3	" "	" "	" "	" "	" "	" "	" "
STM23-1	R3-47	Before gene start	N	471597			Match VlhA3.03 and 5.05!!
STM23-2	" "	" "	" "	" "			" "
STM23-3	" "	" "	" "	" "			" "
STM24-1	R3-50	MGA_0221	Y	698460	379	68.0	DppC oligopeptide transport ATP-binding protein
STM24-2	" "	" "	" "	" "	" "	" "	" "
STM24-3	" "	" "	" "	" "	" "	" "	" "
STM25-1		Not determined					
STM25-2							
STM25-3							
STM26-1	R3-57	MGA_0220	Y	697324	444	34.2	DppD oligopeptide transport ATP-binding protein
STM26-2	" "	" "	" "	" "	" "	" "	" "
STM26-3	" "	" "	" "	" "	" "	" "	" "
STM27-1	R3-66	MGA_0226	N	702524	905	51.0	Conserved hypothetical, lipoprotein
STM27-2	" "	" "	" "	" "			" "
STM27-3	" "	" "	" "	" "			" "
STM28-1	1R1-23	MGA_0943	Y	229927	931	67.6	CrmB cytadherence related protein B
STM28-2	" "	" "	" "	" "	" "	" "	" "
STM28-3	" "	" "	" "	" "	" "	" "	" "
STM31-1	1R1-43	INTERGENIC	N	59880			Between Xposase/VlhA1.05
STM31-2	" "	" "	" "	" "			" "
STM31-3	" "	" "	" "	" "			" "
STM32-1	R46	MGA_0817	N	141020	443	52.0	Unique Hypothetical
STM32-2	" "	" "	" "	" "	" "	" "	" "
STM32-3	" "	" "	" "	" "	" "	" "	" "
STM33-1	1R1-52	MGA_0939	Y	226598	1065	79.2	CrmA cytadherence related molecule A
STM33-2	" "	" "	" "	" "	" "	" "	" "

STM ID	TAG ID	Insertion	Operon	Gene start	No. aa	% of gene at insert	Gene name
STM33-3	" "	" "	" "	" "	" "	" "	" "

STM34-1	R56	Not determined					
STM34-2	" "						
STM34-3	" "						
STM35-1	R62	MGA_0758	N	89748	249	76.4	Unique Hypothetical
STM35-2	" "	" "	" "	" "	" "	" "	" "
STM35-3	" "	" "	" "	" "	" "	" "	" "
STM36-1	R75	MGA_0223/0224	Y	698754	249	38.9	not annotated OppB oligopeptide transport
STM36-2	" "	" "	" "	" "	" "	" "	" "
STM36-3	" "	" "	" "	" "	" "	" "	" "
STM37-1	R77	MGA_1087	N	359415	212	20.5	Unique Hypothetical
STM38-1	1R1-79	Intergenic	N	904609			b/n MgGA_0518/0519 Unique/conserved
STM38-2	" "	" "	" "	" "			" "
STM38-3	" "	" "	" "	" "			" "
STM39-1	1R1-80	MGA_0943	Y	229927	931	14.7	CrmB cytadherence related protein B
STM39-2	" "	" "	" "	" "	" "	" "	" "
STM39-3	" "	" "	" "	" "	" "	" "	" "

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Plain English Compendium Summary

Project Title:	
AECL Project No.:	UM-54
Researcher:	Dr Philip Markham
Organisation:	The University of Melbourne Parkville, 3010
Phone:	03 8344 7422
Fax:	03 8344 7374
Email:	pmarkham@unimelb.edu.au
Objectives	• To identify gene targets that can be used to attenuate wildtype *Mycoplasma gallisepticum* field strains and produce vaccine strains.
Background	*Mycoplasma gallisepticum* causes a chronic respiratory disease in chickens and produces substantial economic loss to the poultry industry. The disease is typically treated using antibiotics and controlled by strict biosecurity measures and/or vaccination. Vaccination by live attenuated *M. gallisepticum* strains is standardly used nowadays to prevent disease. With blanket vaccination using the current *M. gallisepticum* vaccines there is the possibility of vaccine breakdown. To ensure a quick and adequate response to this event we must have the ability to quickly and precisely attenuate the infecting wildtype organism. This can be achieved by identifying and attenuating those genes responsible for producing disease.
Research	To identify virulence genes in *M. gallisepticum.* The *M. gallisepticum* vaccine strain ts-11 and its wild-type parent *M. gallisepticum* 80083 were used as prototypes. The approach will determine attenuating gene mutations using three approaches: analysis of gene transcription, analysis of protein expression and creating attenuated *M. gallisepticum* clones by interrupting genes of a virulent strain. The last part will assess the gene interruptions on the ability of the organism to infect and produce disease in chickens.
Outcomes	Analysis of proteome profiles of *M. gallisepticum* ts-11 strain identified a number of missing proteins. Genetic analysis of the genes encoding the proteins revealed an DNA insertion that would cause premature truncation of the GapA peptide involved in attachment of the organism to the host mucosal surface. Previous researchers have shown that *M. gallisepticum* cells cannot colonise the host without the presence of this peptide. Continued analysis of reisolates of *M. gallisepticum* vaccine strain ts-11 from chickens showed re-acquisition of GapA, these findings suggest a reversible genetic switch with off and on. Several other proteins identified as absent or present in the strains were subjected to quantitative polymerase chain reaction (qPCR) to measure the relative level of gene transcription. Whilst there were differences in levels, DNA sequencing showed no major DNA changes that may account for these findings. Several attempts were made to transform *M. gallisepticum* using a gene-knockout plasmid construct without success. Whether this was due to the *M. gallisepticum* strain ts-11 requiring the particular gene for growth as compared to another *M. gallisepticum* strain S6 remains to be determined. Without further development this pathway was not pursued further. A panel of unique *M. gallisepticum* clones containing gene mutations produced by Signature Tagged Mutagenesis (STM) were created and used in bird experiments to identify genes involved in producing disease in

	chickens. A number of clones with gene mutations were not reduced in virulence whilst several clones were attenuated. The genes and/or genomic DNA regions were identified by DNA sequencing for both attenuating and non-attenuating mutations.
Implications	Proteomic analysis of *M. gallisepticum* vaccine strain showed GapA was absent. As the GapA peptide is involved in colonisation of the host and recovered organisms expressed GapA then a genetic switch to restore gene function has taken place. Whether this genetic switch is responsible for attenuation remains to be determined, it is unlikely this mutation will be used to produce vaccines. Vaccines will be produced by targeting specific genes identified by STM. Further experiments to identify the most suitable *M. gallisepticum* clone need to be conducted and a methodology to knockout the corresponding gene in wild-type strains of *M. gallisepticum* still needs to be developed.
Publications	Markham, P. F., Kanci, A., Geary, S.J. and Browning G. F. (2002). Mapping of avian mycoplasma vaccine proteins by two-dimensional gel electrophoresis, *IOM Letters Vol VII:*189 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 VIII:118 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 VIII:118 Browning, G.F. and Markham, P.F. (2004) Determination of the genomic sequence of Mycoplasma gallisepticum RIRDC publication No 04/140