

**Exploration of the Basis of Virulence Attenuation and
Development of Recombinant Derivatives in a *Mycoplasma*
synoviae Live Vaccine**

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A thesis submitted to the University of Melbourne in total fulfilment of the
requirement for the degree of Doctor of Philosophy

December 2017

**Asia-Pacific Centre for Animal Health
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ABSTRACT

Mycoplasma synoviae is an important avian pathogen that is responsible for significant economic losses in the poultry industry worldwide. It is able to infect chickens, turkeys and other avian species, causing subclinical respiratory tract infections, synovitis, and egg abnormalities. A live attenuated temperature-sensitive (*ts*+) vaccine strain, MS-H, is widely used in many countries to prevent infection with *M. synoviae*. Due to its *ts*+ phenotype, MS-H does not grow at the core body temperature of birds, but colonises the upper respiratory tract and establishes a solid protection against wild-type *M. synoviae*. In rare cases, the *ts*+ MS-H strain may revert to a non-temperature-sensitive (*ts*−) phenotype, but this is not associated with a significant increase in the pathogenicity in MS-H re-isolates, indicating that factors other than temperature sensitivity may be involved in the attenuation of the vaccine. To better characterise the molecular basis of the vaccine attenuation, MS-H and its parent 86079/7NS were subjected to whole genome sequencing.

Comparative analysis of the genome organisations of the MS-H, 86079/7NS and other published *M. synoviae* strains revealed a number of features unique to MS-H that can be used as novel genetic markers in routine diagnostic tests. In *M. synoviae* strains MS-H and 86079/7NS, a 50-kb genomic inversion was observed for the first time amongst *M. synoviae* strains. Two newly identified horizontally transferred genes that encode a deoxyribose-phosphate aldolase and an ATP-dependent DNA helicase were noted for MS-H and 86079/7NS.

Genomic comparison of MS-H and its parent strain 86079/7NS revealed 33 single nucleotide polymorphisms (SNPs) (excluding the highly variable *vlhA* gene), including 11 nonsynonymous SNPs that may have functional consequences. A frameshift

mutation causing a premature termination of translation was found in *oppF-1*, a gene that encodes an ATP-binding protein that is a component of an oligopeptide transporter. This frameshift is likely to have inactivated the OppF protein function, suggesting that this mutation may play a significant role in the attenuation of MS-H.

A nested PCR combined with high-resolution melting (HRM) curve analysis, based on the frameshift mutation within *oppF-1* gene, was developed. This assay could reliably differentiate MS-H from all field strains tested in this study and was efficient for both cultures and swabs from clinical cases.

In addition, this study investigated the potential for use of the MS-H vaccine as a delivery vector for foreign antigens using infectious bronchitis virus (IBV) as a model, aiming at the development of recombinant poultry vaccine candidates, especially for those pathogens affecting the upper respiratory system. To achieve this, expression vectors were constructed based on an *oriC* plasmid (pMAS-LoriC), which is capable of freely replicating in *M. synoviae*. Gibson assembly was used as a versatile strategy for constructing the vectors. The variable lipoprotein haemagglutinin gene (*vlhA*) promoter was used to drive expression of partial S1 and N IBV genes in MS-H. The expression of IBV N or S1 mRNA in MS-H recombinants was detected by quantitative real-time PCRs (qPCRs). Western blotting confirmed the expression of IBV partial S1 and N proteins using polyclonal antibodies specific for S1 and IBV respectively. This is the first report of successful expression of a non-native gene in *M. synoviae*.

Thus the studies reported in this thesis have contributed to (1) development of our understanding of putative virulence factors in *M. synoviae*, (2) development of an assay to discriminate MS-H from field strains, and (3) laid the foundation for the development of novel recombinant vaccines to control other upper respiratory pathogens.

DECLARATION

This is to certify that:

- (i) The thesis comprises only my original work towards the PhD degree.
- (ii) Due acknowledgement has been made in the text to all material used.
- (iii) This thesis is less than 100,000 words in length, exclusive of tables, figures and bibliography.

Ling Zhu

December 2017

PREFACE

The work described in this thesis was performed at the Asia-Pacific Centre for Animal Health (APCAH), Melbourne Veterinary School, Faculty of Veterinary and Agricultural Sciences, the University of Melbourne, between September 2013 and December 2017 under the supervision of Dr. Marc S. Marends, Professor Amir H. Noormohammadi, Professor Glenn F. Browning and Dr. Andrew W. Stent. The author was a recipient of a Melbourne International Fee Remission Scholarship (MIFRS) and a Melbourne International Research Scholarship (MIRS) from the University of Melbourne. Scientific papers published or presented at conferences and manuscripts in preparation for publication derived from this thesis are listed below.

PEER REVIEWED PAPERS

Zhu, L., Konsak, B.M., Olaogun, O.M., Agnew-Crumpton, R., Kanci, A., Marends, M.S., Browning, G.F. and Noormohammadi, A.H., 2017. Identification of a new genetic marker in *Mycoplasma synoviae* vaccine strain MS-H and development of a strategy using polymerase chain reaction and high-resolution melting curve analysis for differentiating MS-H from field strains. *Vet. Microbiol.* 210, 49-55.

Zhu, L., Shahid, M.A., Markham, J., Browning, G.F., Noormohammadi, A.H. and Marends, M.S. Genome analysis of *Mycoplasma synoviae* strain MS-H, the most common *M. synoviae* strain with a worldwide distribution. *BMC Genomics*, 19, 117.

Zhu, L., Shahid, M.A., Markham, J., Browning, G.F., Noormohammadi, A.H. and Marends, M.S. Comparative genomic analyses of *Mycoplasma synoviae* vaccine strain MS-H and its parent strain 86079/7NS: implications for virulence factors and applications in diagnosis of *M. synoviae* (in preparation).

Zhu, L., Shahid, M.A., Browning, G.F., Noormohammadi, A.H. and Marenda, M.S. Expression of infectious bronchitis virus spike and nucleocapsid proteins in *Mycoplasma synoviae* vaccine strain MS-H (in preparation).

CONFERENCE PRESENTATIONS

Zhu, L., Shahid, M.A. and Noormohammadi, A.H., 2017. Molecular Mechanisms Behind the Loss of Virulence of MS-H vaccine. Australasian Veterinary Poultry Association Conference, Geelong, Victoria, Australia, November 15-17. Oral presentation.

Zhu, L., Shahid, M.A., Marenda, M.S., Browning, G.F. and Noormohammadi, A.H., 2016. Expression of IBV genes in *Mycoplasma synoviae* strain MS-H. XXV World's Poultry Congress, Beijing, China, September 5-9. Poster presentation.

Zhu, L., Shahid, M.A., Marenda, M.S., Browning, G.F. and Noormohammadi, A.H., 2016. Determination of the virulence factors of *Mycoplasma synoviae*. 21st Congress of the International Organization for Mycoplasmology, Brisbane, Queensland, Australia, July 3-7. Poster presentation.

Zhu, L., Shahid, M.A., Marenda, M.S., Browning, G.F. and Noormohammadi, A.H., 2014. Determination of the virulence factors of *Mycoplasma synoviae* and development of recombinant vaccines for its control. Postgraduates of Veterinary Science Symposium, the University of Melbourne, Werribee, Victoria, Australia, November 13-14. Poster presentation.

ACKNOWLEDGMENTS

We all have dreams. But in order to make dreams come into reality, it takes an awful lot of determination, dedication, self-discipline and effort.

–Jesse Owens

It is my pleasure to acknowledge my sincere gratitude to all the people who were instrumental in completion of my PhD research.

I owe a deep sense of gratitude to Dr. Marc S. Marenda for his constant supervision, meticulous guidance, timely suggestions with kindness and enthusiasm, which enabled me to finish my thesis on time.

My sincere thanks to Professor Amir H. Noormohammadi who contributed to many discussions that helped to shape this project. His scholarly advice and scientific approach have helped me to accomplish my research in a beautiful manner.

I thank profusely Professor Glenn F. Browning who helped me to fulfill my dream of pursuing a PhD at the University of Melbourne (UoM). His kind help and cooperation throughout my study period require special mention. I would like to acknowledge the role of Associate Professor Joanne M. Devlin, Advisory Committee Chair, and Dr. Andrew W. Stent for their valuable guidance and support throughout my study period.

My thanks to Dr. Muhammad Akbar Shahid, who gave a strong foundation for this research. I appreciate all the technical suggestions and materials provided to me. I am especially grateful to Barbara Ilievski, Rebecca Agnew, Pollob Shil, Olusola Martins, Denise O'Rourke, Penelope Steer, Anthony Chamings, Hayley Smith, Anna Kanci, Rhys

Bushell, and Robin Geyer for their outstanding technical support. My special thanks to Maria Sare and Andrea Merrall for providing me all the laboratory materials needed for my research work.

These acknowledgements would not be complete without mentioning the support from Deepthi Leelamany, Nadeeka Wawegama and Christina Marth. I appreciate and cherish the friendship I share with them.

I would also like to acknowledge Dr. Jagoda Ignjatovic, Dr. Sang-Won Lee, Dr. Neil Young, and Professor James Gilkerson for their scientific and technical assistance during my candidature. The past and present members of the Veterinary Microbiology and Virology group, Khridiana Maria Putri, Somayeh Kordafshari, Kanishka Indiwari, Shumoos Al Riyami, Ahmad Jawad, Harun Rashid, Hagos Gebremikael, Zeeshan Akbar, Oluwadamilola Samuel Omotainse, Dinidu Wijesurendra, Jose Quinteros, James Adamu, and Alistair Legione - thank you for your friendship, moral support and technical assistance during my candidature.

My PhD journey would not have materialised without the support from the Melbourne Scholarship Office of a Melbourne International Research Scholarship (MIRS) and a Melbourne International Fee Remission Scholarship (MIFRS). Thanks for the financial support.

My profound gratitude to Ms. Constance Lucietto, who stood with me as a good friend and a grandmother. Thanks for all the hospitality and care you rendered me during the good and the bad times.

Kendall Hall – A cozy home for four years! My heart felt thanks to Ms. Stacey

Wardrop, Kendall Hall Coordinator, for her cordial nature, instilling in me the flames of unbeatable optimism and for helping me to perform my job in a diligent way. A word of thanks to Dennis Wormald, for being a good colleague in managing Kendall Hall together.

I would like to extend my deep sense of gratitude to Professor Jingyun Ma, Professor Qingmei Xie and Professor Yingzuo Bi from South China Agricultural University and Dr. Pengju Guo from Guangdong Laboratory Animals Monitoring Institute for checking on me always. Special thanks for seeding a research aptitude in me and also for the constant motivation, care and concern.

My deepest appreciation goes to my most beloved mom and dad for believing in my dreams, helping me to chase it and for accepting me for what I am. Without their prayers and blessings, I could not have finished my thesis successfully. Thank you and love to you both!

TABLE OF CONTENTS

ABSTRACT	I
DECLARATION	III
PREFACE	IV
ACKNOWLEDGMENTS	VI
TABLE OF CONTENTS	IX
CHAPTER 1. Literature Review	1
1.1 General features of mycoplasmas.....	1
1.2 <i>Mycoplasma synoviae</i> infection	2
1.2.1 Epidemiology and forms of the disease.....	2
1.2.2 Clinical signs	5
1.3 Pathogenesis of mycoplasma species	6
1.3.1 Cytadherence	6
1.3.2 Invasion and damage to the host.....	7
1.3.3 Phenotypic variation and immune evasion.....	9
1.3.4 Pathogenicity and the oligopeptide permease transport system	10
1.4 Immune modulation and immune response.....	11
1.5 Factors contributing to the virulence of <i>M. synoviae</i>	12
1.5.1 Variable lipoprotein haemagglutinin	13
1.5.2 Sialidase activity.....	15
1.5.3 Other potential virulence factors identified in <i>M. synoviae</i>	16
1.6 Diagnosis of <i>M. synoviae</i> infections.....	17
1.6.1 Isolation and identification	18
1.6.2 Serological tests.....	18
1.6.3 Detection of DNA and genotyping of strains	20
1.7 Control of <i>M. synoviae</i> infections	22
1.7.1 Antibiotics.....	22
1.7.2 Live attenuated vaccines.....	22
1.8 Genetic tools for deciphering the pathogenesis mechanisms of mycoplasmas	24
1.8.1 Next generation sequencing (NGS).....	24
1.8.2 Molecular tools for manipulating the mycoplasma genome	27
1.9 Expression of foreign genes in mycoplasmas	29
1.10 Thesis aims	31
CHAPTER 2. Genome Analysis of <i>Mycoplasma synoviae</i> Strain MS-H, the Most Common <i>M. synoviae</i> Strain with a Worldwide Distribution	32
2.1 Abstract.....	32
2.2 Introduction	33
2.3 Materials and Methods	35
2.3.1 Preparation of genomic DNA and sequencing	35

2.3.2 <i>De novo</i> sequence assembly, gene prediction and annotation of MS-H.....	36
2.3.3 Genome comparison and analysis	36
2.4 Results and Discussion	37
2.4.1 Assembling MS-H genome sequence and resolving the highly repeated <i>vlhA</i> region.....	37
2.4.2 General comparisons of <i>M. synoviae</i> genomes and identification of a large chromosomal inversion in MS-H	38
2.4.3 Large mobile genetic elements	40
2.4.4 Strain-variable sequences in <i>M. synoviae</i> genomes and vaccine-specific genetic markers.....	41
2.4.5 Pseudogenisation of transport systems and their potential impact on attenuation of virulence	43
2.4.6 Clustered regularly interspersed palindromic repeats and evidence of horizontal gene transfer	46
2.5 Conclusion.....	48
CHAPTER 3. Comparative Genomic Analyses of <i>Mycoplasma synoviae</i> Vaccine Strain MS-H and Its Parent Strain 86079/7NS: Implications for the Identification of Virulence Factors and Applications in Diagnosis of <i>M. synoviae</i>	62
3.1 Abstract.....	62
3.2 Introduction	63
3.3 Material and Methods.....	65
3.3.1 Strain, culture conditions and DNA extraction.....	65
3.3.2 Sequencing and assembly	65
3.3.3 <i>De novo</i> sequence assembly, gene prediction and annotation	66
3.3.4 Genome comparison and analysis	67
3.4 Results and Discussion	67
3.4.1 A combination of Illumina and Nanopore sequence reads was used for assembly of the highly repetitive <i>vlhA</i> pseudogene locus	67
3.4.2 MS-H and 86079/7NS share almost identical genomic sequences	68
3.4.3 A few genetic mutations found in MS-H may have functional significance .	70
3.4.4 A frameshift mutation in <i>oppF-1</i> may result in the loss of function of its product.....	73
3.4.5 SNPs for discriminating infected from vaccinated animals (DIVA assays) ..	75
3.5 Conclusion.....	76
CHAPTER 4. Identification of A New Genetic Marker in the <i>Mycoplasma synoviae</i> Vaccine Strain MS-H and Development of a Strategy using Polymerase Chain Reaction and High-Resolution Melting Curve Analysis for Differentiating MS-H from Field Strains	84
4.1 Abstract.....	84
4.2 Introduction	85

4.3 Materials and Methods	87
4.3.1 MS strains and growth media	87
4.3.2 DNA extraction.....	87
4.3.3 Oligonucleotide primers	88
4.3.4 PCR amplifications.....	88
4.3.5 Nested <i>oppF</i> PCR-HRM.....	89
4.3.6 Nucleotide sequencing and analysis	89
4.4 Results	90
4.4.1 Identification and selection of SNPs from MS genomic sequences	90
4.4.2 PCR amplification of selected regions of <i>oppF-1</i> from different MS strains	90
4.4.3 HRM curve analysis of the nested PCR amplicons of <i>oppF-1</i> can distinguish between 86079/7NS and MS-H cultures	91
4.4.4 Nested <i>oppF-1</i> PCR-HRM curve analysis distinguishes MS-H from a collection of field strains from diverse geographical locations.....	92
4.4.5 Nested PCR HRM curve analysis of <i>oppF-1</i> was able to distinguish vaccine and field strains/isolates of MS directly in clinical specimens.....	92
4.4.6 Deletion of <i>oppF-1</i> A468 is consistent in MS-H after at least five passages in vivo in SPF birds	93
4.4.7 Additional results.....	93
4.5 Discussion.....	94
4.6 Conclusion.....	96

CHAPTER 5. Expression of Infectious Bronchitis Virus Spike and Nucleocapsid Proteins in *Mycoplasma synoviae* Vaccine Strain MS-H

.....	106
5.1 Abstract.....	106
5.2 Introduction	106
5.3 Materials and Methods	109
5.3.1 Ethics statement.....	109
5.3.2 Bacterial strains and culture conditions.....	109
5.3.3 Gibson assembly of overlapping PCRs fragments	110
5.3.4 Construction of the rMSH-IBVS1 and rMSH-IBVN vaccine vectors	112
5.3.5 Transformation of MS	113
5.3.6 Detection of MS transformants.....	114
5.3.7 Extraction of total RNA.....	115
5.3.8 cDNA synthesis	116
5.3.9 Quantitative real-time PCR (qPCR).....	116
5.3.10 Antigenic peptides selection and synthesis for IBV S1.....	117
5.3.11 IBV purification.....	117
5.3.12 Preparation of rat anti-IBV polyclonal antibodies.....	118
5.3.13 Indirect ELISA to assess antibody response in rats.....	118
5.3.14 SDS-PAGE and Western immunoblotting.....	119

5.4 Results	120
5.4.1 Two recombinant <i>M. synoviae</i> , rMSH-IBVS1 and rMSH-IBVN, were generated.....	120
5.4.2 Polyclonal antisera against IBV were produced.....	122
5.4.3 IBV S1 and N mRNAs were transcribed in recombinant MS-H.....	122
5.4.4 IBV S1 and N proteins were expressed in MS-H recombinant.....	123
5.5 Discussion.....	124
5.6 Conclusion.....	128
CHAPTER 6. General Discussion	146
6.1 Introduction	146
6.2 Genomic characteristics of <i>M. synoviae</i> MS-H.....	146
6.3 Potential factors involved in attenuation of <i>M. synoviae</i> MS-H.....	148
6.4 Implications of the mutations in <i>oppF-1</i> of MS-H.....	150
6.5 Expression of heterogenous genes in <i>M. synoviae</i>	152
6.6 Conclusions	154
REFERENCES	155

LIST OF FIGURES

- Figure 2.1** - MAUVE alignment analysis of *M. synoviae* genomes, using MS-H as a reference
- Figure 2.2** - Comparative structural analysis of the genomic island (GI) in MS-H, WVU-1853 and MS53
- Figure 2.3** - **(a)** Location of the *deoC* gene in *M. gallisepticum* corresponding to the *deoC* fragment found in MS-H. **(b)** Locations of the ATP-dependent DNA helicase genes in MS-H and *M. gallinaceum* strain B2096 8B. **(c, d, e)** Comparison of unique genomic loci identified in *M. synoviae* strains MS-H, WVU-1853 and MS53.
- Figure 2.4** - Organisation of the *opp* operons in MS-H, MS53 and WVU-1853
- Figure 2.5** - Organisation of the CRISPR/Cas system genes in the three *M. synoviae* genomes
- Figure 3.1** - Mapping of complete genomic sequence of 86079/7NS against its optical map
- Figure 3.2** - MAUVE alignment of complete genomic sequences of *M. synoviae* strains 86079/7NS and MS-H
- Figure 3.3** - Phenotypes of *oppF-1* and *obg* genes in 86079/7NS, MS-H and MS-H re-isolates
- Figure 4.1** - Analysis of different *oppF* PCR products by agarose gel electrophoresis
- Figure 4.2** - **(a)** Comparison of partial *oppF-1* nucleotide sequences (corresponding to nt 375-526 of MS53 *oppF-1*) of *M. synoviae* strains 86079/7NS and MS-H. **(b)** Conventional and **(c)** normalised high-resolution melting-curve analysis of *M. synoviae* strains 86079/7NS and MS-H using *oppF*-F3R3 nested PCR
- Figure 4.3** - **(a)** Comparison of partial *oppF-1* nucleotide sequences (corresponding to nt 375-526 of MS53 *oppF-1*) of *M. synoviae* strains MS-H and overseas field strains. The SNP (nt468) is highlighted with a black box. **(b)** Conventional and **(c)** normalised high-resolution melting-curve analysis of *M. synoviae* strains using *oppF*-F3R3 nested PCR
- Figure 4.4** - **(a)** Comparison of partial *oppF-1* nucleotide sequences (corresponding to nt 375-526 of MS53 *oppF-1*) of *M. synoviae* strain MS-H and Australian field strain/isolates. The SNP (nt468) is highlighted with a

black box. **(b)** Conventional and **(c)** normalised high-resolution melting-curve analysis of *M. synoviae* strains using *oppF*-F3R3 nested PCR

- Figure 5.1** - A schematic diagram showing procedures for generating pGA-IBVS1 (Vector construction: step I)
- Figure 5.2** - A schematic diagram showing procedures for generating pGA-IBVN (Vector construction: step I)
- Figure 5.3** - A schematic diagram showing procedures for generating the final vector construct pMS-IBVS1 (Vector construction: step II)
- Figure 5.4** - A schematic diagram showing procedures for generating the final vector construct pMS-IBVN (Vector construction: step II)
- Figure 5.5** - Agarose gel (0.9%) electrophoresis of **(a)** PCR products generated from pBluescript II KS (+), pMA-T-IBVS1 and pKS-VOTL, and **(b)** pMS-IBVS1 digested with *Bam*HI and *Sal*I
- Figure 5.6** - Agarose gel (1%) electrophoresis of PCR products generated by primers IBVS1-opt F/R and tetM F1/R1 from *M. synoviae* transformant rMSH-IBVS1 C1
- Figure 5.7** - Agarose gel (0.9%) electrophoresis of **(a)** PCR products generated from pMS-IBVS1 and pMA-T-IBVN, and **(b)** of pMS-IBVN digested with *Bam*HI and *Sal*I
- Figure 5.8** - Agarose gel (1%) electrophoresis of PCR products generated by primers IBVN-opt F/R and tetM F1/R1 from *M. synoviae* transformants rMSH-LoriC and rMSH-IBVN C8
- Figure 5.9** - Agarose gel (1%) electrophoresis of PCR products generated by primers IBVS1qPCR-F2/R2 from *M. synoviae* transformants rMSH-LoriC and rMSH-IBVS1 C1
- Figure 5.10** - Quantitation of mRNA transcripts of IBV S1 gene in *M. synoviae* transformant rMSH-IBVS1 C1
- Figure 5.11** - Agarose gel (1%) electrophoresis of the PCR product generated by primers IBVNqPCR-F2/R2 from *M. synoviae* transformants rMSH-LoriC and rMSH-IBVN C8
- Figure 5.12** - Quantitation of mRNA transcript of IBV N gene in *M. synoviae* transformant rMSH-IBVN C8
- Figure 5.13** - Western blots of the whole cell proteins of rMSH-LoriC and rMSH-IBVS1 C1 immunostained with **(a)** rat anti-IBV 691666-1 and **(b)** anti-IBV 691666-2 peptide antisera

Figure 5.14 - Western blots of the whole cell proteins of rMSH-LoriC, rMSH-IBVN C8 and VicS IBV immunostained with **(a)** polyclonal chicken anti-IBV antiserum and **(b)** polyclonal rat anti-IBV antiserum

LIST OF TABLES

Table 2.1 -	Nucleotide positions of the unique gene locus tags in MS-H genome
Table 2.2 -	General characteristic of the genomes of three <i>M. synoviae</i> strains MS-H, MS53 and WVU-1853
Table 2.3 -	Unique amino acid deletions/insertions in <i>M. synoviae</i> strains MS-H, MS53 and WVU-1853
Table 2.4 -	Composition of the inverted region in MS-H
Table 2.5 -	Summary of variable genes across <i>M. synoviae</i> strains MS-H, MS53 and WVU-1853
Table 3.1 -	Nucleotide and coding differences identified between genomes of MS-H and 86079/7NS
Table 3.2 -	Unique single nucleotide variations detected in <i>M. synoviae</i> strains MS-H, 86079/7NS, WVU-1853 and MS53
Table 4.1 -	<i>Mycoplasma synoviae</i> strains used in this study
Table 4.2 -	Primers used in this study
Table 4.3 -	Melting points and genotype identity confidence percentages (C%) in the <i>oppF</i> -F3R3 HRM for <i>M. synoviae</i> strains from diverse geographical locations
Table 4.4 -	Melting points and genotype identity confidence percentages (C%) in <i>oppF</i> -F3R3 HRM for Australian <i>M. synoviae</i> field strains
Table 4.5 -	The <i>oppF</i> -1 and <i>obg</i> sequence type, and temperature sensitivity phenotype of several <i>M. synoviae</i> clones isolated from vaccinated field chickens
Table 5.1 -	<i>Mycoplasma synoviae</i> strains and plasmids used in this study
Table 5.2 -	List of primers used in this study
Table 5.3 -	Synthetic peptides used in this study

CHAPTER 1. Literature Review

1.1 General features of mycoplasmas

Mycoplasmas are a group of bacteria in the class *Mollicutes*, order *Mycoplasmatales*, family *Mycoplasmataceae*, and genus *Mycoplasma* (Johansson and Pettersson, 2002). The organisms are distinguished phenotypically from other bacteria by their very small size and the absence of a cell wall, a characteristic responsible for their resistance to antibiotics that affect cell wall synthesis. Mycoplasmas are widespread in nature as obligate parasites and pathogens of humans, animals, insects and plants. In general, the mycoplasmas that infect animals are relatively host specific. Due to their strict nutritional requirements, human and animal mycoplasmas primarily colonise the mucosal surfaces of the respiratory tract and genital tracts, while some species are also found in the eyes, alimentary canal, mammary glands and joints (Razin, 2006). Most animal mycoplasmas can persist in their eukaryotic hosts and cause chronic infections. Mild only or no clinical signs are sometimes seen in infected animals, which often be an obstacle in the control and eradication of disease (Stipkovits and Kempf, 1996).

Mycoplasmas are considered to be the simplest and smallest self-replicating organisms, with genomes of 580-2200 kbp (Johansson and Pettersson, 2002). Their genomes are characterised by AT nucleotide bias and a low number of genes involved in recombination and repair (Citti and Blanchard, 2013). The massive loss of gene content in mycoplasmas is believed to be a consequence of the metabolic complementarity of their hosts (Andersson and Kurland, 1998). Phylogenetic studies of mycoplasmas can be conducted based on analysis of 5s rRNA (Hori et al., 1981), 16S rRNA (Weisburg et al., 1989) and tRNA (Maniloff, 1983) sequences. It is hypothesised that mycoplasmas are derived from gram-positive bacteria through reductive evolution and a close phylogenetic relationship between mycoplasmas and a branch of the gram-positive

bacteria, including lactobacilli, bacilli and streptococci, has been established (Maniloff, 2002). Three translational start codons (ATG, GTG, and TTG) are used by mycoplasmas (Razin et al., 1998), while TGA is translated as tryptophan, rather than as a translational stop codon, in most mycoplasmas. *Acholeplasma laidlawii* is an exception to this rule, as it is known to use the universal genetic code, in which TGA is a stop codon (Dybvig and Voelker, 1996).

Avian mycoplasmas were first isolated from chickens in 1935 (Jordan, 1979). To date, at least 25 avian mycoplasma species (*A. laidlawii*, *M. anatis*, *M. anseris*, *M. buteonis*, *M. cloacale*, *M. columbinasale*, *M. columbinum*, *M. columborale*, *M. corogypsi*, *M. falconis*, *M. gallinarum*, *M. gallinaceum*, *M. gallisepticum*, *M. gallopavonis*, *M. glycyphilum*, *M. gypis*, *M. imitans*, *M. iners*, *M. iowae*, *M. lipofaciens*, *M. meleagridis*, *M. pullorum*, *M. sturni*, *M. synoviae* and *U. gallorale*) have been isolated from birds (Kleven and Ferguson-Noel, 2008), of which *M. gallisepticum*, *M. synoviae* and *M. meleagridis* are the most economically important pathogens in the poultry industry. *M. gallisepticum* and *M. synoviae* are pathogenic in chickens and turkeys, while *M. meleagridis* only infects turkey (Kleven and Ferguson-Noel, 2008). Avian mycoplasma infections are widespread globally. The infection rate among parent breeder flocks can reach 20-30% in some countries with highly developed poultry industries (Stipkovits and Kempf, 1996).

1.2 *Mycoplasma synoviae* infection

1.2.1 Epidemiology and forms of the disease

M. synoviae is regarded to be the second most important avian mycoplasma species from the clinical and economic point of view (Ferguson-Noel and Noormohammadi, 2013). It infects mainly chickens and turkeys, but can also be isolated from ducks,

geese, guinea fowl, pigeons, Japanese quail, pheasants, red-legged partridge and house sparrows (Kleven and Ferguson-Noel, 2008). Disease occurs worldwide in commercial poultry flocks and may result in severe economic losses due to retarded growth and downgrading at slaughter (Kleven and Ferguson-Noel, 2008).

The disease caused by *M. synoviae* is described as “infectious synovitis”, which mainly occurs in chickens and turkeys. The organism was first reported in 1954 (Olson et al., 1954) as an unidentified agent causing synovitis in chickens and subsequently named *M. synoviae* in 1964 (Olson et al., 1964). Infection with *M. synoviae* does not usually result in mortality, but most frequently causes subclinical upper respiratory tract disease, synovitis (Ferguson-Noel and Noormohammadi, 2013), and eggshell apex abnormalities (EAA) in layer chicken flocks (Feberwee et al., 2009a). *M. synoviae* can cause damages to the respiratory mucosa, which predispose the birds to subsequent infection with other pathogens, such as infectious bronchitis virus (IBV), Newcastle disease virus (NDV), *Escherichia coli*, and *M. gallisepticum*, resulting in more severe clinical respiratory disease.

All ages of chickens and turkeys are susceptible to *M. synoviae*. Natural infection in chickens occurs as early as 1 week of age, but acute infection is generally observed in chickens of 4-16 weeks old and turkeys of 10-24 weeks old. Chronic disease is commonly seen in infected flocks at any age (Kleven and Ferguson-Noel, 2008).

M. synoviae is transmitted horizontally via direct contact and infectious aerosols coughed and sneezed by infected birds (Kleven et al., 1972; Landman et al., 2004), as well as vertically via fertile eggs (Kleven, 1998). *M. synoviae* is thought to spread faster than *M. gallisepticum* through a population. Transmission through the respiratory tract usually results in a 100% rate of infection without development of obvious joint lesions.

The egg transmission rate is highest in the first 4-6 weeks after infection of breeder flocks (Kleven and Ferguson-Noel, 2008). Indirect transmission of *M. synoviae* through contaminated material (dust, feathers and food) can cause delayed infections with remarkably long silent periods in chickens (Marois et al., 2005). As asymptomatic *M. synoviae* infections may be easily overlooked, less attention has been paid to routine monitoring for the pathogen, resulting in a higher prevalence in a number of countries, especially in layer flocks, compared with that of *M. gallisepticum*, against which better control and eradication programmes have been applied (Landman, 2014).

The incubation period of disease caused by *M. synoviae* after direct contact is generally longer (11-21 days) than is seen in birds infected by egg transmission (6 days). In experimentally infected birds, the incubation period varies between routes of inoculation, and titres and levels of pathogenicity of the inoculum (Kleven and Ferguson-Noel, 2008).

M. synoviae strains vary in tissue tropism and virulence, leading to different forms of disease, although this may also depend on the route of infection. Many isolates cause little or no clinical disease. However, these differences cannot be detected by serological examination (Stipkovits and Kempf, 1996). The recovery rate of mycoplasmas from lesions doesn't always correlate with the clinical and histopathological findings in birds (Kawakubo et al., 1980), but strains isolated from air sac lesions are more likely to cause airsacculitis, while strains isolated from synovial tissues are more likely to produce synovitis (Kleven et al., 1975). *M. synoviae*-associated amyloid arthropathy occasionally occurs in brown layer chicken flocks (Landman and Feberwee, 2001). Air sac infection may occur at any age, and often leads to higher carcass condemnation rates in broilers (King et al., 1973; MacOwan et al., 1982).

More recently, specific strains have been isolated from the oviducts of hens producing abnormal eggs (Feberwee et al., 2009a). Occasionally systemic lesions with vasculitis have also been observed and are associated with specific isolates of *M. synoviae* (Kerr and Olson, 1970; Senties-Cué et al., 2005).

1.2.2 Clinical signs

In chickens and turkeys, *M. synoviae* frequently causes subclinical infections of the upper respiratory tract and in some cases may disseminate from this site to cause systemic infection. Infectious synovitis is believed to result from the spread of the organism through the bloodstream (Senties-Cué et al., 2005), while deposition in the air sacs can cause airsacculitis (Kleven et al., 1972; Landman and Feberwee, 2004). Observable clinical signs associated with infectious synovitis include a pale comb, lameness and retarded growth, and later in the course of the disease ruffled feathers and a shrunken comb. Typically lameness is the most prominent form of the disease in turkeys (Kleven and Ferguson-Noel, 2008). Similar signs can be seen in poultry species infected with other avian pathogens, including avian reovirus, *M. gallisepticum*, *Staphylococcus aureus*, *E. coli*, *Pasteurella multocida* and *Salmonella* spp. (Kleven and Bradbury, 2008; Kleven and Ferguson-Noel, 2008)

Under field conditions, *M. synoviae* sometimes cause air sac lesions, which can be exacerbated by vaccination with attenuated strains of Newcastle disease virus and infectious bronchitis virus, or co-infection with various respiratory pathogens, including IBV, NDV and *E. coli* (Kleven et al., 1972; Springer et al., 1974). Adverse environmental factors and/or immunosuppression can result in a higher incidence and greater severity of airsacculitis (Giambrone et al., 1977; Yoder Jr et al., 1977).

A correlation between EAA and the presence of *M. synoviae* in the oviducts has been seen in flocks of layers (Kleven and Ferguson-Noel, 2008) and broiler breeder hens (Feberwee and Landman, 2010). The ability to induce EAA varies between strains (Catania et al., 2016a). EAA is defined as a structural weakness of the egg that promotes the development of cracks and breaks, and is characterised by an altered shell surface, and thinning and increased translucency of the shell (Feberwee et al., 2009a).

1.3 Pathogenesis of mycoplasma species

Mycoplasmas infect the respiratory epithelial surface, resulting in loss of ciliary activity, erosion of the ciliated epithelium, and hypertrophy of the non-ciliated basilar epithelial cells (Dykstra et al., 1985; Lockaby et al., 1998). Although the molecular mechanisms underlying the pathogenesis of mycoplasmoses haven't been well elucidated, the resulting diseases are believed to be complex and multifactorial, involving interactions between the organism, host tissues and the immune response (Davidson et al., 1988; Krause et al., 1983; Razin et al., 1998).

1.3.1 Cytadherence

The majority of human and animal mycoplasmas are typical extracellular parasites, and therefore adherence to mucosal epithelial cells is a prerequisite for successful colonisation and infection of a host (Rottem, 2003). Cytadherence is usually followed by ciliostasis, which is the first cytopathic effect of infection (Noormohammadi, 2007). The intimate cytadherence is mediated by adhesins present on the mycoplasma cell surface. Mycoplasmal adhesins bind receptors on host cell membranes such as sialoglycoconjugates and sulfated glycolipids (Razin and Jacobs, 1992; Razin et al., 1998; Zhang et al., 1994), and have been extensively characterised in some members of the pneumoniae phylogenic group (Razin and Jacobs, 1992). *M. pneumoniae* possesses

a specialised terminal structure, which functions as an attachment organelle (Meng and Pfister, 1980). The cytaherence process is multifactorial, involving the coordinated action of primary adhesion molecules (P1 and P30) and several accessory membrane proteins (P65, B/C, HMW1-HMW3), which are localised in the terminal organelle (Krause, 1998; Krause and Balish, 2004). The attachment organelle also functions in the motility of *M. pneumoniae*, and probably plays a role in the penetration of the organism through mucus layer to the base of the cilia and the membrane of the epithelial cells (Hasselbring and Krause, 2007; Krause, 1996). This tip-mediated adherence to eukaryotic target cells is also observed in *M. gallisepticum* (Maniloff et al., 1965) and *M. imitans* (Bradbury et al., 1993). *M. gallisepticum* shares similar pathogenic mechanisms with *M. pneumoniae*, and several cell surface exposed proteins, such as Mgc1 (or GapA), a homologue of *M. pneumoniae* P1 (Keeler et al., 1996), Mgc2, a homologue of *M. pneumoniae* P30 and *M. genitalium* P32 (Hnatow et al., 1998), and the integral membrane protein PvpA, a homologue of *M. gallisepticum* Mgc2, *M. pneumoniae* P30 and HMW3, and *M. genitalium* P32 (Boguslavsky et al., 2000), have been identified as primary cytaadhesins. It has been suggested that coexpression of GapA and the cytaadherence-related molecule CrmA are required for cytaadherence and virulence in *M. gallisepticum* (Papazisi et al., 2002). Although *M. gallisepticum* and *M. synoviae* belong to different phylogenic groups, both species produce phase variable lipoprotein haemagglutinins (VlhA), which mediate cytaadherence by binding to host-cell receptors that contain sialic acid residues (Bencina, 2002). VlhA is also a determinant of the avidity and specificity of attachment of *M. synoviae* to erythrocytes and chicken embryo cells (Dušanić et al., 2009).

1.3.2 Invasion and damage to the host

Although mycoplasmas were considered to be extracellular, surface parasites, several species have been reported to invade non-phagocytic cells (Abramovitz et al., 1987;

Franzoso et al., 1992; Hegde et al., 2014; Kornspan et al., 2010; Lo et al., 1993; van der Merwe et al., 2010; Winner et al., 2000), an action that is possibly facilitated by incorporation of cholesterol from host cell membranes (Tarshis et al., 1993). *M. synoviae* has been shown to be able to invade into chicken erythrocytes, chondrocytes and embryonic cells (Dušanić et al., 2009), and the human epithelial cell line HEP-2, *in vitro* (Buim et al., 2011). Internalisation of mycoplasmas by host cells may represent a crucial role in passing through the mucosal barrier, and may account for their resistance to antibiotic therapy and contribute to their evasion of the host immune response, therefore resulting in long-term persistence and chronic infection (Buim et al., 2011). Invasion of lower respiratory tract by mycoplasmas can be assisted by the presence of other respiratory pathogens (Hopkins and Yoder Jr, 1984; Springer et al., 1974).

Localisation of mycoplasmas in tissues and accumulation of their metabolic products may contribute to changing the pH in the local environment, depletion of cell resources, interference with host cell receptors, and elicitation of specific immune responses, resulting in tissue damage and development of clinical disease (Razin et al., 1998). Some mycoplasmas possess the potential to disrupt the host cell membrane through the action of their membrane enzymes, such as phospholipase (Rottem, 2003), while the presence of mycoplasmas on the respiratory tract epithelial cells may cause ciliostasis, possibly through the depolarisation of the epithelial cell membrane (Izutsu et al., 1996).

The ability to induce immune-mediated host injury is another property of pathogenic mycoplasmas. During infection with *M. synoviae*, up-regulated levels of rheumatoid factor were detected in the sera of chickens, suggesting autoimmune disease (Sells, 1976; Walker et al., 1978). The articular lesions caused by *M. synoviae* are characterised by infiltration of lymphoid cells, plasma cells, heterophils and fibrin into the joint

spaces and along the tendon sheaths (Kawakubo et al., 1980; Kleven and Ferguson-Noel, 2008). Lesions in the air sacs can be mild, with oedema, capillary proliferation and the accumulation of heterophils and necrotic debris on the surface, or more severe, with hyperplasia of the epithelial cells, a diffuse infiltrate of mononuclear cells and caseous necrosis (Kleven and Ferguson-Noel, 2008).

1.3.3 Phenotypic variation and immune evasion

Phenotypic variation, resulting from high-frequency phase variation in expression, as well as size variation, of surface antigens is a mechanism that microorganisms use to generate extensive diversity on the cell surface to respond and adapt to environmental changes (Deitsch et al., 2009). Switching expression of individual gene between ON and OFF statuses, combined with the ability of each gene to generate variants with different sizes, can give rise to a large number of combinatorial antigenic repertoires (Razin et al., 1998). The high frequency of phase and antigenic variation in some pathogenic mycoplasma species is thought to assist in their evasion of the host immune system, resulting in persistent infection. In *M. gallisepticum*, the putative cytoadhesin-related proteins PvpA and GapA are known to be phase variable, while size variation between strains has also been seen for PvpA (Boguslavsky et al., 2000; Winner et al., 2003). While VlhA (or pMGA) switching can be induced *in vitro* in response to antibody specific for the expressed VlhA, switching off can occur in the absence of detectable VlhA antibodies *in vivo* (Pflaum et al., 2016), while switches to different VlhAs occurs later in infection, suggesting its role in immune evasion during chronic infection (Glew et al., 2000).

Different mechanisms have been shown to be involved in controlling phenotypic variation in mycoplasmas. Spontaneous mutations resulting from nucleotide insertion or deletion in promoter regions prone to DNA slippage, causing on-off switching of

expression of individual genes, have been seen in the *vlp* genes of *M. hyorhinis* (Citti and Wise, 1995) and *vlhA* gene of *M. gallisepticum* (Glew et al., 1998). A reversible mutation involving a single nucleotide insertion/deletion in a short poly-A tract at the beginning of the mature *vaa* gene, which creates a translational frameshift, resulting in either a complete ORF or an internal stop codon, has been shown to be correlated with phase variation of the Vaa lipoprotein of *M. hominis* (Zhang and Wise, 1997). A similar mechanism is also seen in *M. gallisepticum*, controlling phase variation of GapA, which further correlates with the concomitant expression of *crmA*, which is located downstream of *gapA* in the same transcription unit (Winner et al., 2003). Another type of mechanism using silent pseudogenes in mycoplasmas is based on DNA rearrangements (Citti et al., 2010). High-frequency phase variation of surface lipoproteins is known to occur through site-specific recombination within the *vsp* gene family of *M. bovis* (Lysnyansky et al., 2001a; Lysnyansky et al., 2001b), the *vsa* family of *M. pulmonis* (Bhugra et al., 1995) and the *vpma* system of *M. agalactiae* (Czurda et al., 2010; Glew et al., 2002). In *M. synoviae*, variation in the haemagglutinin lipoprotein VlhA has been shown to result from multiple unidirectional recombinations between the expressed copy of the *vlhA* gene and its pseudogenes, resulting in very extensive size and antigenic variation (Noormohammadi et al., 2000).

1.3.4 Pathogenicity and the oligopeptide permease transport system

The oligopeptide permease (Opp) is an ABC transporter encoded by the *oppABCD* operon, and is widely distributed among mycoplasmas (Wium et al., 2015). It is composed of a membrane lipoprotein OppA that binds extracellular peptides, two transmembrane proteins (OppB and OppC), which form the translocation channel, and two membrane-bound cytoplasmic ATPases (OppD and OppF) that bind and hydrolyse ATP, supplying energy for peptide transport (Monnet, 2003). Peptide transport serves as a mechanism for nutrient uptake, but is also involved in sensing environmental signals

which can modulate the pathogenesis of bacteria (Detmers et al., 2001; Lamarque et al., 2011). In *M. hominis*, the oligopeptide-binding protein OppA can function as a cytoadherence molecule and can also affect cell viability, as demonstrated by OppA-deficient mutants (Henrich et al., 1999). An OppD deficient mutant in *Corynebacterium pseudotuberculosis* had impaired growth and a reduced ability to adhere to and to infect macrophages (Moraes et al., 2014). In the intracellular pathogen *Listeria monocytogenes*, OppA is required for survival inside macrophages as well as for import of nutrients (Borezee et al., 2000). Oligopeptide-binding proteins may also have an indirect effect on adhesion properties of bacteria by modulating the expression of adhesion genes (Cundell et al., 1995; McNab and Jenkinson, 1998; Pearce et al., 1994). The Opp system in *Mycobacterium tuberculosis* was shown to be required for the expression of virulence-associated surface lipids and plays a role in the development of chronic infections in patients (Flores-Valdez et al., 2009). Deletion of the *oppB* gene in *Streptococcus agalactiae* impairs adherence to epithelial cells, fibrinogen and fibronectin, and causes a significant reduction in expression of the *fbfA* gene, which encodes a fibrinogen-binding protein (Samen et al., 2004). In *M. gallisepticum*, signature-tagged transposon mutagenesis has been used to show that the loss of *oppD* results in attenuation, with the mutant strain inducing very mild air sac and tracheal lesions in infected birds, suggesting a role in colonisation and virulence (Tseng et al., 2017a). This mutant was assessed as a vaccine candidate (administered as eye-drop), and was shown to protect chickens against the effects of infection with virulent *M. gallisepticum* (Tseng et al., 2017a).

1.4 Immune modulation and immune response

The interactions between mycoplasmas and their host's immune system are complex, involving both suppression of T lymphocytes and polyclonal stimulation of B and T lymphocytes, secretion of various cytokines, enhanced expression of cell receptors, and

activation of the complement cascade (Razin et al., 1998).

Amongst the proteins produced by mycoplasmas, lipoproteins are probably the most active stimulants of humoral responses and inflammation, especially during the early phases of infection (Chambaud et al., 1999). The localisation of lipoproteins on the cell surface, as well as the presence of a distinct amino-terminal lipoylated structure in these molecules, contribute to these properties (Bessler and Jung, 1992; Brenner et al., 1997). Lipoproteins play crucial roles in activating macrophages through recognition by toll-like receptor (TLR) 6, in cooperation with TLR2 (Ozinsky et al., 2000; Takeuchi et al., 2001), which triggers inflammatory responses via activation of the NF- κ B signalling pathway (Into et al., 2004; Muneta et al., 2003). In *M. synoviae*, the lipoprotein MSPB has been shown to activate TLR15 and stimulate chicken macrophages, inducing secretion of proinflammatory cytokines, such as IL-1 β , IL-6, CXCL1 (K60), CCL5 (ah294), and CCL4L1 (MIP-1 β), and effector molecules, such as NO (Lavrič et al., 2007; Lavrič et al., 2008; Oven et al., 2013). MSPB is also known to induce strong local and systemic antibody responses during infectious synovitis (Narat et al., 1998). It has been suggested that thymus-dependent lymphocytes may be necessary for development of macroscopic synovial lesions (Kume et al., 1977). Resistance to lesions induced by *M. synoviae* is controlled by bursal lymphocytes (Kawakubo et al., 1981; Vardaman et al., 1973). The significant reduction in antibody titre in bursectomised chicks, accompanied by more severe lesions in the air sacs, may suggest the necessity of antibody for protection against exposure to *M. synoviae* (Kawakubo et al., 1980; Kawakubo et al., 1981).

1.5 Factors contributing to the virulence of *M. synoviae*

Mycoplasma synoviae strains vary in tissue tropism and invasiveness, and therefore their ability to cause disease. There is evidence of strains with selective tropism for the

epithelial membranes of the lower respiratory tract or the joints, tendon sheaths and bursae, but no single factor has been identified to explain this tropism (Hinz et al., 2003). The pathogenicity of *M. synoviae* is thought to be multifactorial, involving the attachment and colonisation of the upper respiratory tract and factors associated with systemic infection (Kang et al., 2002; Lockaby et al., 1998; Lockaby et al., 1999). The majority of previous studies have been focused on cytoadherence and/or haemadsorption, which only partially examines the mechanisms involved in invasion and systemic dissemination, but may contribute to understanding varying tissue tropisms.

1.5.1 Variable lipoprotein haemagglutinin

The variable lipoprotein haemagglutinin, a major surface antigen subject to phase-variable expression, is abundantly expressed and involved in mediating cell attachment and immune evasion (Bencina, 2002; Narat et al., 1998). The *vlhA* gene is a member of a multigene family (Noormohammadi et al., 1998) and horizontal transfer of the *vlhA* gene family appears to have occurred between *M. synoviae* and *M. gallisepticum*, possibly as a result of coinfection of a shared avian host (Vasconcelos et al., 2005). Unlike the *vlhA* genes of *M. gallisepticum*, which are located in several loci around the genome (Glew et al., 1998; Glew et al., 2000), the functional *vlhA* gene and the *vlhA* pseudogenes in *M. synoviae* are confined to the same genomic region (Allen et al., 2005), with diversity within the expressed *vlhA* allele generated by site-specific DNA recombination between the single complete *vlhA* gene and one or more of the multiple pseudogene (Noormohammadi et al., 2000). Several B cell and T cell epitopes have been identified, mainly in the conserved N-terminal of VlhA, which may be associated with the host immune response during infection (Camargo et al., 2007). Phase variable expression of VlhA and switching of the haemagglutination phenotype between “on” and “off” (Noormohammadi et al., 1999; Rhoades, 1985) can be driven by the selective pressure of anti-VlhA antibodies *in vitro* suggesting involvement in evasion of the

adaptive immune system of the host (Bencina, 2002). Strains with a haemagglutination-positive (HA⁺) phenotype have been found to be more likely to induce infectious synovitis than those with a haemagglutination-negative (HA⁻) phenotype in experimentally infected chickens (Narat et al., 1998).

In most *M. synoviae* strains, VlhA is post-translationally cleaved into an N-terminal portion (MSPB) and a C-terminal portion (MSPA), both of which exhibit high frequency antigenic variation (Noormohammadi et al., 1997). The post-translational cleavage was shown to occur immediately after amino acid residue 344 in strain WVU-1853 (Benčina et al., 1999). In *M. gallisepticum* strain S6, a similar cleavage site has also been detected in the 82-kDa protein encoded by the pMGA1.9 gene, leading to the production of a 45-kDa subunit (Markham et al., 1998d). MSPA, rather than MSPB, is involved in haemagglutination (HA) and haemadsorption (HAD) of chicken erythrocytes (Noormohammadi et al., 1997). A functional motif located near the C-terminus of VlhA facilitates binding to sialylated host cell receptors (May et al., 2014). MSPB is strongly immunogenic and induces local and systemic antibody responses, especially during early stages of the infection by *M. synoviae* (Narat et al., 1998). It contains an amino terminal proline rich region (PRR). PRRs are thought to interact with the eukaryotic cell surface (Dramsi et al., 1993; Okahashi et al., 1993), and to be important in cytodherence and virulence in other mycoplasmas (Boguslavsky et al., 2000; Dallo et al., 1996; Keeler et al., 1996; Layh-Schmitt et al., 1995). Insertions or deletions in the PRR correlate with length variation of the corresponding MSPB proteins (Benčina et al., 2001). Although truncation of MSPB may result in the loss of HA⁺ and/or HAD⁺ phenotypes (Benčina et al., 1999; Noormohammadi et al., 1997), the complete absence of MSPB didn't affect haemagglutinin activity in strain WVUCC (May and Brown, 2011).

1.5.2 Sialidase activity

Sialic (neuraminic) acid is important in the process of infection of host cells, as *M. synoviae* utilises sialylated glycoproteins on the host cell surface, including those on erythrocytes, as receptors for cytoadherence, which is mediated by adhesins such as VlhA (Noormohammadi et al., 2000). Adjacent to the *vlhA* locus, in *M. synoviae* genome there is a cluster of genes that are involved in sialic acid scavenging and degradation, including genes encoding a sialidase, an *N*-acetylneuraminate lyase, an *N*-acetylmannosamine kinase, an *N*-acetylmannosamine-6-phosphate epimerase, an *N*-acetylglucosamine-6-phosphate deacetylase, and a glucosamine-6-phosphate deaminase (May et al., 2015; Vasconcelos et al., 2005). Sialidases are considered to be virulence factors in many bacterial species, because of their association with bacterial colonisation, nutrition, dissemination, and induction of apoptosis (Corfield, 1992; Hunt and Brown, 2007). Sialidase activity varies significantly between strains (Berčič et al., 2008b), which may be explained by sequence differences between the genes encoding the enzymes (Berčič et al., 2011; May and Brown, 2008). The activity was higher in strains isolated from clinically affected birds than in strains isolated from asymptomatic birds, suggesting that sialidases might be associated with invasiveness and virulence (May et al., 2007). Sialidase activity was detected in *M. gallisepticum* strain R_{low}, and horizontal transfer of a sialidase gene from *M. gallisepticum* to *M. synoviae* has been suggested (May and Brown, 2008). Although the sialidase of *M. alligatoris* has been shown to induce apoptosis of pulmonary fibroblasts *in vitro* (Hunt and Brown, 2007), incubation with purified sialidase from *Clostridium perfringens* alone failed to induce apoptosis, suggesting the involvement of other virulence factor(s) in addition to the sialidase in induction of apoptosis of pulmonary fibroblasts during infection with *M. alligatoris* (Hunt and Brown, 2005). The retention of the sialidase gene by strains with an impaired *N*-acetylneuraminate catabolism suggests a non-nutritional function in the ecology of *M. synoviae* (May and Brown, 2008). Since receptor desialylation can reduce

or abolish cytodherence by mycoplasmas (Manchee and Taylor-Robinson, 1969), a potential role of sialidase in modulating adherence to sialylated host cell receptors has also been suggested (May and Brown, 2008). The sialidase gene appears to have been subject to diversifying selection, resulting in variation in non-nutritive sialidase activity between strains of *M. synoviae* (May and Brown, 2009). In a recent study, a strong correlation was detected between haemagglutination and sialidase activity in 14 *M. synoviae* strains, suggesting that the host-induced adaptive allele switching in *vlhA* can indirectly drive sequence variation in the sialidase gene (May and Brown, 2011). Desialylation of chicken immunoglobulin G heavy chain and glycoproteins in chicken tracheal mucus by *M. synoviae* has also been demonstrated, which may facilitate colonisation of the respiratory tract and systemic dissemination in the host (Berčič et al., 2011).

1.5.3 Other potential virulence factors identified in *M. synoviae*

As cytodherence does not completely correlate with pathogenicity, the involvement of additional virulence factors, such as production of toxins, uptake of crucial nutrients, or stimulation of cytokines that result in host tissue damage, has been suggested (Lockaby et al., 1999).

M. synoviae has receptors that can bind to the Fc region of chicken IgG (Lauerman and Reynolds-Vaughn, 1991). Although the Fc receptors do not influence antigen recognition (Boyle and Reis, 1987), this binding may have an affect on effector functions of IgG, which relies on its Fc region to interact with both effector molecules and cells. *M. synoviae* also encodes a putative cysteine protease, CysP, which has the capacity to cleave chicken IgG into Fab and Fc fragments, possibly resulting in the loss of function of IgG and resistance of *M. synoviae* to host antibody (Cizelj et al., 2011).

In the pathogenic *M. synoviae* strain 53, a number of ATP-binding cassette systems (ABC transporters) have been identified and are thought to potentially play a role in the pathogenicity of *M. synoviae* (Nicolás et al., 2007), because they are involved in nutrient uptake and/or secretion of a variety of toxic proteins in other bacteria (Omori and Idei, 2003), and hence many are essential for bacterial survival, colonisation and invasion.

Enolase, an enzyme that catalyses the interconversion of phosphoenolpyruvate and 2-phosphoglycerate (Bao et al., 2014), is a major immunogenic protein of *M. synoviae* (Berčič et al., 2008a). As antibodies against bacterial enolase can cross react with the host enolase, *M. synoviae* enolase has been suggested as a potential contributor to an autoimmune response (Menegatti et al., 2010; Wegner et al., 2009). Moreover, it has been demonstrated that *M. synoviae* enolase is capable of binding to chicken plasminogen and human fibronectin (Bao et al., 2014). Rabbit anti-rMSEno inhibited the adherence of *M. synoviae* to DF-1 cells pre-treated with plasminogen, indicating that enolase may be involved in adhesion (Bao et al., 2014).

It has been shown that *M. synoviae* induce increased production of nitric oxide *in vitro*, leading to apoptosis in infected chicken chondrocytes. This process was accompanied by increased transcription for several pro-apoptotic genes, including *NOS2*, *Mapk11*, *CASP8*, *Casp3*, *AIFM1*, *NFκB1*, *htrA3* and *BCL2*. It is believed that chondrocyte apoptosis might induce tissue destruction and contribute to the development of arthritis in chickens infected with *M. synoviae* (Dusanic et al., 2012).

1.6 Diagnosis of *M. synoviae* infections

Rapid and early diagnostic detection of *M. synoviae* infections is important to prevent the spread of infection and to limit economic losses to the poultry industry. Currently,

the methods recommended by World Organization for Animal Health (OIE) for detection of *M. synoviae* are bacterial isolation, serological assays, and polymerase chain reaction (PCR) assays (OIE, 2015).

1.6.1 Isolation and identification

Recovery of *M. synoviae* from the upper respiratory tract is more reliable than from other sites, especially in chronically infected birds (Ferguson-Noel and Noormohammadi, 2013). Samples can be inoculated into Mycoplasma Broth (MB) or plated onto Mycoplasma Agar (MA) (Whithear, 1993), followed by incubation at 37 °C. Replication of *M. synoviae* in MB to the late log phase results in a colour change of the pH indicator phenol red, generally in 3 to 5 days. A minimum of one week is required for the appearance of detectable “fried egg” colonies on MA. As clinical samples are often contaminated with other organisms, antibiotics to which mycoplasmas are resistant, such as penicillin, and thallium acetate, can be added to media to control the growth of some contaminants. Direct fluorescent-antibody staining can be performed on colonies on agar to distinguish between mycoplasma species (Del Giudice et al., 1967), while other techniques, such as growth and metabolism inhibition assays, may require further broth cultures for species identification (Clyde, 1964; Purcell et al., 1967). Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS), which allows a characteristic mass spectral fingerprint to be obtained from whole inactivated mycoplasmal cells, has recently been introduced as a rapid technique for the identification of mycoplasmas (Goto et al., 2012; Pereyre et al., 2013).

1.6.2 Serological tests

Serological tests are important in monitoring infection. Several assays can be used to detect a serological response to *M. synoviae*, including serum-plate agglutination (SPA), haemagglutination inhibition (HI), and enzyme-linked immunosorbent assay (ELISA).

Antibodies can be detected in both sera and egg yolk by HI and ELISA (Stipkovits and Kempf, 1996).

SPA is considered a screening test, and is relatively easy to perform. However, its low sensitivity and non-specificity have made it necessary to adopt additional techniques to confirm the presence of a serological response, and this has limited the application of this assay in routine diagnostic work (Ewing et al., 1996; Glisson et al., 1984). The HI test is more specific, but it may not detect antigenic variants that differ from the strain used as antigen in the test (Avakian et al., 1988).

Several ELISA assays have been developed using antigens prepared from membrane proteins of lysed cells for the detection of antibodies against *M. synoviae* (Higgins and Whithear, 1986). Treatment of *M. synoviae* whole cell antigens with Triton X-100 has shown to be effective in reducing the cross-reactivity between *M. synoviae* and *M. gallisepticum* in ELISAs (Opitz and Cyr, 1986). A region of the *vlhA* gene from strain WVU-1853 encoding the MSPB protein, which contains a highly antigenic and species-specific domain, was cloned and expressed, and used as the antigen in an indirect ELISA (Noormohammadi et al., 1999). The use of recombinant MSPB protein might enhance the reproducibility of the ELISA because it overcomes the effect of phase variation on the yield and/or immunoreactivity of *M. synoviae* native antigens (Noormohammadi et al., 1999). The MSPB-ELISA was further optimised by using MS-H MSPB as antigen, which was shown to be more sensitive than an ELISA using WVU-1853 MSPB for detection of *M. synoviae* antibodies in sera from MS-H vaccinated SPF and field chickens (Noormohammadi et al., 2002). This study revealed the importance of selecting antigens prepared from homologous *M. synoviae* strains (the same strains used to infect the birds) to detect *M. synoviae* antibodies by ELISA, an observation also made for other serological tests (Kleven et al., 2007; Kleven et al., 2001;

Noormohammadi et al., 2002). In another study, three species-specific recombinant fusion proteins derived from *M. synoviae*, *M. gallisepticum* and *M. meleagridis*, GST-MS2/28, GST-pMGA1.2 and GST-Mm14, respectively, were expressed in *E. coli* and used to develop an ELISA for simultaneous detection of antibodies to these three highly cross-reactive avian mycoplasma species in chicken sera. The MS2/28 protein contained an additional species-specific antigen site within the MSPA region of *vlhA* in *M. synoviae* (Mardassi et al., 2008).

1.6.3 Detection of DNA and genotyping of strains

PCR testing is a rapid, sensitive, and specific method for detection of mycoplasmal DNA, and is considered to be an extremely important confirmation test when a weak or delayed serological response occurs in flocks (Ewing et al., 1998; Kleven et al., 2001). The early PCR assay for detection of *M. synoviae* was developed based on the 16S RNA gene and was applicable to broth cultures and swab specimens. It had high specificity and sensitivity, with a detection limit of 100 colony-forming units (CFU) (Lauerman et al., 1993). A multi-species PCR using a single set of primers targeting the 16S rRNA genes, followed by restriction fragment length polymorphism (RFLP), was developed for identification of *M. gallisepticum*, *M. synoviae* and *M. iowae*. However, the sensitivity of detection of *M. gallisepticum* decreased when a mixture of *M. synoviae* and *M. gallisepticum* DNA was targeted, possibly due to the formation of secondary structure within the 16S rRNA gene of *M. gallisepticum*, which may have reduced the affinity of annealing to the *M. gallisepticum* template (Garcia et al., 1995). This method was further optimised to improve sensitivity by hybridization of the PCR product with a digoxigenin (DIG)-labelled probe (Garcia et al., 1996). The intergenic spacer region (ISR) located between the 16S and 23S rRNA genes was also found to be a good target for PCR detection of *M. synoviae* (Ramírez et al., 2006). To improve on conventional PCR techniques, real-time PCR assays targeting the 16S rRNA, the 16S–23S ISA,

or the *vlhA* gene have been developed to quantify the loads of *M. synoviae* in clinical samples (Sprygin et al., 2010) and to differentiate between *M. synoviae* and other avian mycoplasmas, such as *M. gallisepticum* (Ehtisham-ul-Haque et al., 2015; Fraga et al., 2013; Huang et al., 2015; Jarquin et al., 2009; Raviv and Kleven, 2009).

Because the virulence and tropism of *M. synoviae* strains varies considerably, genotyping of a specific *M. synoviae* strain is important for epidemiological investigations. In addition, differentiation of vaccine strains from field strains provides valuable information to poultry farms on which the vaccination is used to control infection with *M. synoviae*. Analysis of the single-copy domain of the N-terminal *vlhA* region is commonly used for *M. synoviae* sequence typing (Bayatzadeh et al., 2014; Dijkman et al., 2014; El-Gazzar et al., 2012; Hammond et al., 2009; Harada et al., 2009; Hong et al., 2004; Jeffery et al., 2007; Wetzel et al., 2010), and has replaced several other molecular typing techniques, like restriction endonuclease cleavage analysis of DNA (Ley and Avakian, 1992; Morrow et al., 1990b). However, given that several Australian and two European strains share the same *vlhA* sequence type, this region is unlikely to be reliable for differentiation of the MS-H vaccine from field strains (Catania et al., 2016a; Dijkman et al., 2014). Recently, single nucleotide polymorphisms (SNPs) in the *obg* gene of *M. synoviae* field isolates and the MS-H vaccine (nt367 and nt629) have been described (Shahid et al., 2013b). Based on this finding, a strategy of using four PCRs, followed by high-resolution melting (HRM)-curve analysis, was developed for differentiation of the temperature sensitive (ts^+) vaccine strain from rare non-temperature sensitive (ts^-) MS-H re-isolates and field strains (Shahid et al., 2014b). Some of these SNPs have also been targeted to distinguish the MS-H vaccine strain from field strains using melt-curve and agarose gel based mismatch amplification mutation assays (Kreizinger et al., 2015) or quantitative real-time PCR (Dijkman et al., 2017). However, because of the occasional reversion of MS-H re-isolates from a ts^+ to

ts⁻ phenotype and the presence of Australian wild-type isolates that have the same *obg* gene profile as the MS-H vaccine strain, the differentiation of MS-H from field strains based on the *obg* gene may not always be reliable. Two multilocus sequence typing (MLST) assays, targeting 7 or 5 genes, have been developed for *M. synoviae* (Dijkman et al., 2016; El-Gazzar et al., 2017). However, whether the MLST assays enable differentiation of Australian *M. synoviae* field strains from the MS-H vaccine strain needs to be further investigated.

1.7 Control of *M. synoviae* infections

1.7.1 Antibiotics

Antimicrobial treatment and vaccination are commonly used to control the disease caused by *M. synoviae*. *M. synoviae* is sensitive to a number of antimicrobials, including tetracyclines (chlortetracycline, oxytetracycline), fluoroquinolones (danofloxacin, enrofloxacin), aminoglycosides (spectinomycin), and macrolides, lincosamides and pleuromutilins (spiramycin, tylosin, tilmicosin, lincomycin, tylvalosin and tiamulin) (Kleven and Ferguson-Noel, 2008). However, antimicrobial treatment doesn't eliminate *M. synoviae* and the selection of resistant bacteria, as well as the accumulation of antimicrobial residues, may occur in birds (Gautier-Bouchardon et al., 2002; Lysnyansky et al., 2013).

1.7.2 Live attenuated vaccines

Killed vaccines have been considered as an alternative to antimicrobial therapy, in order to prevent or reduce clinical signs, but such vaccines do not protect against infections by wild-type field strains (Noormohammadi et al., 2007), raising interest in live vaccines. At present, two attenuated live vaccines against *M. synoviae* are commercially available: the temperature-sensitive strain MS-H (Vaxsafe[®] MS, Bioproperties Pty Ltd.), which was produced by chemical mutagenesis of an Australian virulence field strain

86079/7NS; and the NAD-independent MS1 vaccine (Nobilis® MS Live, MSD Animal Health Inc.), which was developed by *in vitro* passaging of the type strain (WVU-1853, ATCC 25204, NCTC 10124)(Kreizinger et al., 2017; Morrow et al., 1998).

The MS-H vaccine has been approved in Australia since 1996 and is now the most widely used vaccine for control of disease caused by *M. synoviae*. This strain has been shown to be safe when administered by eye drop and efficacious in protecting chickens and turkeys from the experimental infection with *M. synoviae*, and also in preventing egg shell abnormalities in experimentally infected chickens (Feberwee et al., 2009b; Markham et al., 1998a; Morrow et al., 1998). The *ts*⁺ phenotype of MS-H prevents it growing at the core body temperature of birds, but it can colonise the upper respiratory tract and induce solid immunological protection against infection with wild type *M. synoviae*. The *ts*⁺ phenotype and the avirulent character of the vaccine strain were retained after five *in vivo* and 10 *in vitro* passages (Markham et al., 1998a). MS-H has been shown to transmit laterally by direct or indirect contact, but this mode of transmission can't be relied upon as a means of passive vaccination (Markham et al., 1998a; Markham et al., 1998c). There is no evidence that MS-H is vertically transmitted. It has been shown that an MS-H dose of 4.8×10^5 colour changing units (CCU) is required to elicit an antibody response in birds and protect against air sac lesions (Jones et al., 2006a). Protective immunity against air sacculitis develops 7-14 days after the appearance of serum antibodies (3-4 weeks after vaccination) (Jones et al., 2006c) and is maintained for at least 40 weeks after vaccination (Jones et al., 2006b). The antibody response could be detected in SPF chickens using the SPA test as early as 10 days after vaccination (Markham et al., 1998a; Markham et al., 1998b), while under field conditions antibody responses may only be detectable 40 days after vaccination, with the highest proportion of birds with a detectable antibody response seen at around 100 days after vaccination (Noormohammadi et al., 2002).

Culture of *M. synoviae* at the permissive (33 °C) and non-permissive (39.5 °C) temperatures for MS-H, followed by microtitration (Morrow et al., 1998) or quantitative real-time polymerase chain reaction (Q-PCR) assays targeting the *vlhA* gene (Shahid et al., 2013a) can be used to differentiate *ts*⁺ MS-H from field strains.

In rare cases, re-isolates of the MS-H strain that are no longer temperature-sensitive mutants have been obtained from vaccinated birds, possibly as a result of selective pressure *in vivo*. However, reversion to a *ts*⁻ phenotype has not been accompanied by an increase in infectivity or virulence (Noormohammadi et al., 2003). Recently, a comparison of the partial genome sequence of MS-H, its parent strain 86079/7NS and several MS-H *ts*⁻ re-isolates revealed a mutation in the gene encoding the GTP-binding protein Obg in MS-H that was postulated to influence the temperature-sensitive phenotype (Shahid et al., 2013b). In this study, it was demonstrated that the replacement of glycine with arginine at position 123 might affect the stability of an alpha helix within the MS-H Obg, resulting in a temperature-sensitive phenotype. While an additional substitution of alanine to valine at position 210 (within the GTP-binding domain) observed in several MS-H *ts*⁻ re-isolates could stabilise the Obg structure and therefore restore the wild-type *ts*⁻ phenotype (Shahid et al., 2013b). However, as the MS-H *ts*⁻ re-isolates retained the attenuated virulence phenotype, it is believed that the basis for attenuation is not only temperature sensitivity, but also some other mutations, which are as yet undefined.

1.8 Genetic tools for deciphering the pathogenesis mechanisms of mycoplasmas

1.8.1 Next generation sequencing (NGS)

Comparison of the complete genome sequences of virulent and avirulent bacterial strains can allow the identification of genomic determinants involved in virulence and pathogenicity. Several high-throughput, or next generation, sequencing (NGS) approaches have been developed to replace the Sanger method, with an ultimate goal of deciphering complete genomes of pathogenic bacteria by *de novo* assembly of reads without the use of a reference sequence. The approaches commonly used for whole genome sequencing can be grouped into two broad categories: short-read and long-read platforms. Whole genome sequencing using NGS technologies, followed by comparative genomic analysis, has been conducted for several mycoplasma species, and these studies have contributed to our understanding of genome evolution in *Mollicutes* (Lluch-Senar et al., 2013; Lo et al., 2013; Xiao et al., 2015) and/or their virulence-associated factors (Liu et al., 2013a).

Illumina sequencing

Compared to other short-read platforms, such as 454/Roche or SOLid/ABI, the Illumina system has been increasingly used in NGS research because it provides the best balance between read length, error rate and cost (Loman et al., 2012; Van Dijk et al., 2014). Like the other two platforms, the Illumina sequencing workflow requires that the DNA carrying the sequences to be determined is converted into a special sequencing library (Kircher and Kelso, 2010). First, the sample nucleic acids are sheared to a size compatible with sequencing. Then two different adapters are added to the 5' and 3' ends of all DNA fragments. Single-stranded DNA fragments are generated by melting the double-stranded library using sodium hydroxide, and then hybridised to two populations of complementary oligonucleotides immobilised onto the surface of glass flow cells (Fedurco et al., 2006; Kircher and Kelso, 2010). A reverse strand can be synthesised from the hybridised region, which may then bend over and attach to another oligonucleotide complementary to the second adapter on the other end of this strand,

initiating synthesis of a second reverse strand. After repeating this process for a few cycles (bridge amplification) a cluster of ~1000 DNA molecules can be generated, and then the reverse strands can be selectively removed, leaving only the forward strands for sequencing. A sequencing-by-synthesis approach is used in the Illumina system, which relies on detection of fluorescently labelled single nucleotides with a reversible-terminator to sequence these clonally amplified DNA clusters by adding a single base each round and detecting the base that has been added to the clustered identical sequences (Bentley et al., 2008; Fedurco et al., 2006; Quail et al., 2012). Although Illumina sequencing reads have low error rates, they are only short, and thus the data generated from them cannot resolve highly complex regions containing repetitive elements, as a commonly found in bacterial genomes.

Oxford Nanopore sequencing

Long-read sequencing platforms potentially offer read length of several kilobases, which can span complex or repetitive regions of bacterial genomes with a single continuous read, therefore eliminating ambiguity in the position or size of these genomic elements (Goodwin et al., 2016). The Oxford Nanopore Technologies (ONT) single-molecule sequencing platform has reduced the costs of reagents and instrumentation, increasing the accessibility of long read sequencing. During ONT DNA library preparation, leader structures are attached to the end of the double stranded fragments. After denaturation, single stranded DNA composed of the forward strand, possibly followed by the reverse strand, is analysed (Laver et al., 2015; Quick et al., 2014). The leader adapter interacts with a pore and a motor protein to direct the forward strand to pass through the pore, possibly followed by the structure that links the two strands of the original double-stranded molecule so the reverse strand is read through as well, generating a complementary read. This strategy allows for bidirectional sequencing, resulting in a consensus sequence that is referred to as a Two Direction

read. Unlike other platforms that monitor incorporation or hybridisation of nucleotides guided by a template DNA strand, nanopore sequencers directly detect the DNA composition without the need for labelling. To carry out sequencing, modulations of the ionic current passing through the pore is recorded as the single stranded DNA molecule is passed through an α -haemolysin pore (Clarke et al., 2009). Mikheyev and Tin sequenced the lambda phage genome and reported a low sequencing accuracy (Mikheyev and Tin, 2014). However, despite the high error rates, ONT (or MinION) reads may assist genome assembly by generating a scaffold, that when combined with other types of data, such as Illumina reads, may enable complete assembly of genome sequences (Laver et al., 2015).

1.8.2 Molecular tools for manipulating the mycoplasma genome

Manipulation of the genome is required to gain further understanding of the role of putative virulence genes in the pathogenesis of bacteria in their hosts, and also for the development of novel vaccine candidates. A variety of techniques using transposons, *oriC*-based plasmids, or clustered regularly interspaced short palindromic repeats (CRISPR) have been described for the manipulation of mycoplasma genome.

Transposons, also known as jumping genes, are short specific DNA sequences that can insert into different locations in a bacterial genome, thus inducing a chromosomal mutation by insertion into, and thus disruption of, a gene of interest (Choi and Kim, 2009). Transposon-based genomic engineering techniques have been used in studies of a variety of mycoplasma species. Large-scale transposon mutagenesis can allow identification of essential genes, contributing to our understanding of the basic biology and pathogenic mechanisms of mycoplasmas (French et al., 2008; Glass et al., 2006). In *M. gallisepticum*, transposon mutagenesis has been used in several studies to identify a number of virulence determinants, which are believed to be involved in cytoadherence,

metabolism or nutrient transport (Hudson et al., 2006; May et al., 2012; Mudahi-Orenstein et al., 2003; Tseng et al., 2017a; Tseng et al., 2013).

The identification of the origin of replication (*oriC*) in mycoplasmas has led to the development of shuttle vectors, which are capable of self-replicating in mycoplasmas or integrating into the mycoplasma genome by recombination (Maglennon et al., 2013). The *oriC*-based plasmids have been demonstrated to be effective to generate mutations by targeting specific sites in the mycoplasma genome by homologous recombination (Cordova et al., 2002; Lee et al., 2008). Recently, an *oriC*-based vector, pMAS-Loric, has been developed for *M. synoviae* (Shahid et al., 2014a). This vector, carrying the complete *dnaA* gene along with upstream and downstream *dnaA* box regions, has been shown to be able to freely replicate in *M. synoviae*, but also integrated into the chromosomal *oriC* region. It was anticipated that this *oriC*-based vector could be used for gene complementation studies in the MS-H vaccine strain for elucidation of the role of genes putatively involved in the attenuation of virulence in this strain (Shahid et al., 2014a).

CRISPR and CRISPR-associated systems (Cas) have been identified in bacteria and archaea. These systems use RNA-guided nuclease activity to provide adaptive immunity against invasion of foreign nucleic acids. CRISPR/Cas9 nucleases can be used to achieve highly efficient genome editing in a large number of organisms (Hsu et al., 2014; Jiang et al., 2013; Kleinstiver et al., 2016). Genome engineering using the CRISPR-Cas9 system was achieved by using the system from *Streptococcus pneumoniae* to edit the genome of *E. coli*, suggesting that the method could be applicable to other microorganisms. However, a limitation of the strategy was the presence of cells that survived CRISPR-induced cell death and lacked the desired mutations (Jiang et al., 2013). The procedure was further improved to successfully

create single or multiple gene deletions in *E. coli* (Jiang et al., 2015). CRISPR/Cas9 genome editing tools have also been applied to mutagenesis of yeast, and can thus be adapted to manipulate bacterial genomes cloned in yeast (DiCarlo et al., 2013; Tsarnopoulos et al., 2015). Rideau et al. reported a study in which the full-length genome of *M. hominis* PG21 was introduced into *Saccharomyces cerevisiae* using transformation-associated recombination (TAR) cloning (Kouprina et al., 2004), and the *M. hominis* PG21 genome was then modified in yeast by deletion of the adhesion related gene *vaa* using the CRISPR/Cas9 system (Rideau et al., 2017). Back transplantation of the *M. hominis* PG21 *vaa*-deleted genomes from the yeast clones into a mycoplasma recipient cell will be required to obtain the final mutated *M. hominis* PG21 clones (Lartigue et al., 2009; Rideau et al., 2017). Recently, this system has been shown to be a rapid and efficient method for editing the genome of *Staphylococcus aureus*, by gene deletion, single-base substitution mutation and gene insertion (Chen et al., 2017).

1.9 Expression of foreign genes in mycoplasmas

Foreign gene expression in mycoplasmas has been limited by the lack of knowledge about genetic regulatory elements in these organisms and uncertainty about the identification of promoter sequences (Shil et al., 2011). The first foreign gene functionally expressed in a mycoplasma was the β -galactosidase gene (*lacZ*) of *E. coli* (Knudtson and Minion, 1994), which was used as a reporter for studying promoter function and gene regulation in mycoplasmas by measuring β -galactosidase activity (Knudtson and Minion, 1993, 1994). The spiralin gene, encoding the major membrane lipoprotein of *Spiroplasma citri*, could be expressed from the pNZ18-related plasmid in *A. laidlawii*, although the level of expression was low (Jarhede et al., 1995).

Selection of an appropriate promoter is essential to drive gene expression. The *vlhA*

promoter sequence without the 5' trinucleotide (GAA) repeat was shown to be able to drive expression of foreign genes in *M. gallisepticum* (Kordias, 2003). In another study, the *M. gallisepticum* elongation factor Tu (EF-Tu) promoter, together with the *vlhA* signal sequence was used to successfully express *E. coli* alkaline phosphatase and chicken IFN-gamma in the *M. gallisepticum* vaccine strain ts-11, and this recombinant vaccine could induce greater mucosal cellular immune responses when inoculated into chickens by eye drop than the parental ts-11 strain (Muneta et al., 2008). A similarly constructed vector containing the *M. gallisepticum* *tuf* promoter with an additional 183 bp upstream region was shown to drive the expression of the IBV S1 protein and chicken IL-6 in the *M. gallisepticum* GapA⁺ ts-11 vaccine strain. The growth rate of chickens inoculated with the recombinant vaccine and subsequently challenged with IBV strain N2-75 was significantly greater than unvaccinated chickens and the vaccinated chickens were partially protected against damage caused by pathogenic IBV. It is therefore tempting to attempt the expression of protective antigens from IBV in MS-H, with the aim of producing a recombinant vaccine that offers protection against both these pathogens.

It is known that codon bias has a significant influence on protein expression levels and is also a highly conserved feature in different microorganisms (Lithwick and Margalit, 2003). Similarities between codon usage in a novel gene and the typical codon usage of the host is an important determinant of optimal heterologous protein expression. Expression of foreign genes in mycoplasmas may be impeded when transcripts containing rare codons are generated (Gustafsson et al., 2004). Another factor of importance is that the TGA codon is translated into a tryptophan amino acid in mycoplasmas rather than being used as a stop codon, as it is in most eukaryotic and prokaryotic hosts (Dybvig and Voelker, 1996). Therefore, codon usage optimisation is essential to improve the expression level of novel gene in a mycoplasma. This involves

altering the rare codons in the novel gene sequence to make them more closely reflect the codon usage preference of the host (Hannig and Makrides, 1998).

1.10 Thesis aims

The aims of the studies described in this thesis included:

- 1) To sequence the complete genome of the *M. synoviae* live attenuated vaccine strain MS-H and its parent strain 86079/7NS using next-generation sequencing;
- 2) To analyse the genome of MS-H and compare the whole genome sequences of MS-H and 86079/7NS to identify the factors contributing to the attenuation of the vaccine strain MS-H;
- 3) To develop a novel strategy for differentiation of the MS-H vaccine strain from field strains; and
- 4) To develop a recombinant MS-H vaccine candidate expressing a gene from an upper respiratory pathogen such as IBV.

CHAPTER 2. Genome Analysis of *Mycoplasma synoviae*

Strain MS-H, the Most Common *M. synoviae* Strain with a Worldwide Distribution

The text in this chapter has been published in a peer-review journal (Zhu, L., Shahid, M.A., Markham, J., Browning, G.F., Noormohammadi, A.H. and Marenda, M.S. Genome analysis of *Mycoplasma synoviae* strain MS-H, the most common *M. synoviae* strain with a worldwide distribution. BMC Genomics, 19, 117). Minor corrections in this chapter have been made and further information related to this chapter is given in footnotes.

2.1 Abstract

The bacterial pathogen *Mycoplasma synoviae* can cause subclinical respiratory disease, synovitis, airsacculitis and reproductive tract disease in poultry and is a major cause of economic loss worldwide. The *M. synoviae* strain MS-H was developed by chemical mutagenesis of an Australian isolate and has been used as a live attenuated vaccine in many countries over the past two decades. As a result, it may now be the most prevalent strain of *M. synoviae* globally. Differentiation of the MS-H vaccine from local field strains is important for epidemiological investigations and is often required for registration of the vaccine. Here, the complete genomic sequence of the MS-H strain was determined using a combination of Illumina and Nanopore methods and compared to WVU-1853, the *M. synoviae* type strain isolated in the USA 30 years before the parent strain of MS-H, and MS53, a more recent isolate from Brazil. The vaccine strain genome had a slightly larger number of pseudogenes than the two other strains and contained a unique 55 kb chromosomal inversion partially affecting a putative genomic

island ¹. Variations in gene content were also noted, including a deoxyribose-phosphate aldolase (*deoC*) fragment and an ATP-dependent DNA helicase gene found only in MS-H. Some of these sequences may have been acquired horizontally from other avian mycoplasma species. Overall, MS-H was somewhat more similar to WVU-1853 than to MS53. The genome sequence of MS-H will enable identification of vaccine-specific genetic markers for use as diagnostic and epidemiological tools to better control *M. synoviae*.

2.2 Introduction

The avian pathogen *M. synoviae* is a member of the class *Mollicutes*, a group of bacteria that are characterised by their very small size, lack of a cell wall, complex nutritional requirements and ability to persist in their hosts and establish chronic infections (Kleven, 1998). *M. synoviae* strains appear to have varying tissue tropisms and virulence, although these characteristics may depend on the route of infection (Lockaby et al., 1998). It causes subclinical upper respiratory tract infections in chickens, turkeys and other birds (Olson et al., 1954). It can also disseminate further into the host, leading to synovitis or egg defects (Feberwee et al., 2009a). It is transmitted horizontally via direct contact, or vertically via fertile eggs (Kleven, 1998). Although *M. synoviae* is rarely associated with bird mortality, its impact on avian health is significant. As a result of the implementation of adequate control programs for *M. gallisepticum*, the other major mycoplasmal pathogen of poultry, *M. synoviae* may now be the most important bacterial cause of economic loss in the poultry industry (Landman, 2014). Vaccination is commonly used to control *M. synoviae* infection in commercial flocks in many

¹ Genomic islands are large genomic regions that are thought to have horizontal origins in bacteria. These regions can contain genes that are related to virulence or antibiotic resistance of an organism and may contribute to the adaptation of the organism. Compared with the rest of the bacterial genome, a genomic region that has abnormal sequence composition, such as GC% and codon bias, can be detected by various tools (including SIGI-HMM and IslandPath-DIMOB) as a genomic island.

countries with significant commercial poultry industries. The temperature-sensitive strain MS-H was produced by *N*-nitro-*N*'methyl-*N*-nitrosoguanidine (NTG) chemical mutagenesis of an Australian field isolate, 86079/7NS (Markham et al., 1998b; Markham et al., 1998c). MS-H does not grow at the core body temperature of birds, colonises only their upper respiratory tract and establishes solid protection against wild type *M. synoviae*. MS-H was first registered as a live attenuated vaccine in Australia in 1996 (Vaxsafe MS; Bioproperties Pty. Ltd., Ringwood, Victoria, Australia) and is now formally registered and used for vaccination of commercial poultry in 26 different countries across 6 continents. In addition, MS-H is used in several other countries where formal registration is not required (personal communications with Dr Ross Henderson and Dr Chris Morrow, Bioproperties Australia Pty. Ltd.). The international use of the MS-H vaccine suggests that it may now be the most common strain of *M. synoviae* globally and highlights the importance of development of assays to differentiate local endemic strains from the vaccine (Ogino et al., 2011). However, little information is available about the genetic relatedness of MS-H and other *M. synoviae* field strains found in poultry. Moderately virulent *M. synoviae* strains have been isolated from flocks previously vaccinated with MS-H (Noormohammadi et al., 2003), raising the question of the origin of these organisms and prompting interest in the sequence of the MS-H genome. Such knowledge would help to define molecular markers for tracking MS-H in vaccinated flocks and assessing any variation in the level of cross-protection against local strains of *M. synoviae*. The sequences of the *M. synoviae* field strain MS53, isolated around 2003 from a broiler flock in Brazil (Vasconcelos et al., 2005), and the type strain WVU-1853, isolated in 1957 from a hock joint of chicken in the USA (May et al., 2015), are the only two complete genomes determined thus far for this species. This is possibly due to difficulties experienced in assembling next generation sequencing (NGS) data for *M. synoviae*, which contains large, high complexity, repeat-rich regions. Here, MS-H was completely sequenced by combining short, accurate

Illumina sequence data with long reads generated using the Oxford Nanopore technology. This genome sequence was compared to the complete genome sequences of strains WVU-1853 and MS53 with the aim of identifying features unique to MS-H, WVU-1853 or MS53, and assessing the degree of overall similarity between the three genomes and identifying MS-H specific features that could be targeted to develop genotyping assays and differentiate the vaccine from field strains, enabling improved assessment of disease control strategies.

2.3 Materials and Methods

2.3.1 Preparation of genomic DNA and sequencing

M. synoviae strain MS-H was inoculated into mycoplasma culture medium containing 10% swine serum and 0.01% (w/v) nicotinamide adenine dinucleotide (Whithear, 1993) and grown until late logarithmic phase (pH of approximately 6.8) at 37°C in a 50 mL final volume. Cells were collected by centrifugation at 10,000 ×g for 30 min. Genomic DNA was prepared by proteinase K digestion of the pellet, phenol-chloroform extraction and ethanol precipitation (Green and Sambrook, 2016, 2017). An Illumina paired-end (300 bp insert size) genomic DNA library was prepared and sequenced by the Micromon DNA Sequencing Facility (Monash University, Australia) using a Genome Analyzer IIx system. An Oxford Nanopore long read genomic DNA library was prepared with the sequencing kit SQK-NSK007 (Oxford Nanopore Technologies, Oxford, OX4 4GA, UK) according to the manufacturer's instructions, using a mixture of 1.2 µg of genomic DNA sheared using a Covaris-g Tube and 0.8 µg of unsheared genomic DNA. Sequencing data were generated on a MinION MK-I device fitted with a FLO-MIN104 flowcell (R9 chemistry) and processed using the cloud-based Metrichor workflow 2D base caller RNN SQK-NSK_007 rev 1.107. Oligonucleotides (Geneworks, Australia) were designed using Primer3 version 2.3.4 and PCR amplicons

were sequenced ² using the Sanger method at the Micromon DNA Sequencing Facility (Monash University, Melbourne, Australia).

2.3.2 *De novo* sequence assembly, gene prediction and annotation of MS-H

Paired-end Illumina reads were filtered to select those with quality values above 20 and a minimal read length of 91 after trimming of adapter sequences. The quality of the filtered reads was confirmed with FastQC (Andrews, 2010). The Illumina reads were *de novo* assembled using Velvet (Zerbino and Birney, 2008) (version 1.2.10) using k-mer values of 81 and 91 and coverage cut-off values of 5, 10, 20, 50, 100 and 200. Nanopore 2D reads with lengths >2,500 bp were extracted from the set of fast5 files returned in the “pass” folder by the Metrichor basecaller and converted into .fasta format using Poretools (Loman and Quinlan, 2014). The nanopore reads were *de novo* assembled with Canu 1.2 (Berlin et al., 2015) using the parameters genomesize=0.9m and errorRate=0.1. The Illumina read datasets were mapped against the Nanopore assembly and the consensus sequence extracted using the Geneious version 7.0.6 sequence manipulation suite. Automatic annotation of the MS-H sequence was performed on the RAST webserver with default parameters for mycoplasmas (Aziz et al., 2008). Provisional locus tag numbers were assigned and the corresponding nucleotide positions are listed in Table 2.1.

2.3.3 Genome comparison and analysis

To ensure consistency in the analysis, the genome sequences of *M. synoviae* strains MS53 and WVU-1853 (GenBank accession numbers AE017245 and CP011096) were re-annotated using the RAST website with default parameters as above. Genome characteristics of each *M. synoviae* strain were analysed using Artemis (Rutherford et

² As a number of genomic regions, especially those containing mobile genetic elements, could not be assembled from Illumina contigs, PCRs which could fill the gaps within/between those contigs were designed for gap-closing purpose.

al., 2000). Whole genome sequence alignments were performed using the Mauve Aligner and LASTZ tools in Geneious version 7.0.6. Pairwise alignments of nucleotide and amino acid sequences of individual genes were performed using the generic Geneious alignment tool with the default parameters. The search for genomic pathogenicity islands was conducted using the IslandViewer3 server (<http://www.pathogenomics.sfu.ca/islandviewer>) and the SIGI-HMM and IslandPath-DIMOB methods (Langille and Brinkman, 2009). The PHAge Search Tool (PHAST) (<http://phast.wishartlab.com>) was used to detect complete and/or incomplete prophage sequences (Zhou et al., 2011). Clustered regularly interspersed short palindromic repeats (CRISPR) sequences were detected using CRISPRfinder (<http://crispr.i2bc.paris-saclay.fr>) (Grissa et al., 2007). Insertion sequences (IS) were analysed using the ISFinder tool (<http://www-is.biotoul.fr>) (Siguier et al., 2006).

2.4 Results and Discussion

2.4.1 Assembling MS-H genome sequence and resolving the highly repeated *vlhA* region

Extensive assembly attempts using Velvet with a set of 30,345,486 MS-H Illumina paired-end reads with various K-mer and coverage cutoff values produced 659 contigs with sizes ranging from 181 bp to 134,673 bp. These contigs were aligned to the strain MS53 genome to obtain assembly parameters ³. *De novo* assembly of the MS-H genome was then attempted. However, many genomic regions could not be assembled solely from the Illumina datasets. Sanger sequencing of 28 PCR products and scaffolding of the Velvet data produced a 766,314 bp contig, representing an almost complete genome, but failed to assemble the *vlhA* pseudogene region, a 50 kb locus containing a cluster of highly repeated sequences. To resolve the *vlhA* region and

³ Clarification: MS53 was only used for parameter optimisation purpose. This does not amount to an MS53 reference-guided assembly.

complete the MS-H genome, a set of 29,015 Nanopore reads containing a total of 209,108,300 nt were independently assembled and circularised into a single 810,924 bp contig. Because Nanopore sequences are error-prone, the Illumina reads were then mapped against the contig, providing an average coverage depth of 3661 ± 829 , and the consensus sequence was then extracted from the aligned reads. This approach generated a fully assembled genome encompassing the entire *vlhA* cluster and all the other regions that were difficult to assemble solely from the Illumina reads. The final MS-H genome was then verified by aligning the previously obtained Velvet contigs and Sanger sequenced PCR products, where available, with the new assembly. All nucleotide discrepancies between the two datasets were resolved manually by inspecting the alignments of Illumina reads to verify the quality of the consensus. The results from the Illumina/Nanopore hybrid approach was found to be accurate in all cases, except for a dinucleotide repeat sequence, (CT)₁₅, which was correctly re-interpreted as (CT)₁₃⁴. These results demonstrate that a strategy combining error-prone but long single-molecule reads (Nanopore) with accurate short next generation sequencing data (Illumina) can generate a high quality, complete mycoplasma genome sequence. This approach is particularly well adapted to *Mollicutes*, which often contain repeated regions that are difficult to assemble. As expected, the MS-H-specific mutation in the *obg* gene (Shahid et al., 2013b), previously reported as a marker of the vaccine strain, was identified. The whole-genome sequence of MS-H has been deposited in the National Centre for Biotechnology Information (NCBI) database under accession number CP021129.

2.4.2 General comparisons of *M. synoviae* genomes and identification of a large chromosomal inversion in MS-H

⁴ The region was confirmed by manually checking the accurate illumina sequencing reads. External validation can be performed in the future.

The general features of the MS-H, MS53 and WVU-1853 genomes ⁵ are listed in Table 2.2. The *M. synoviae* MS-H genome was 818,848 bp long with an overall mol% G+C content of 28.2%. The DNA-DNA sequence identities between MS-H and WVU-1853 and between MS-H and MS53 (excluding the *vlhA* locus region) were 92.1% and 91.3% respectively. All 3 strains had similar average gene lengths, ranging from 992 to 1002 bp, average gene densities of approximately 90%, and numbers of putatively encoded proteins, ranging from 723 to 764, of which 541 to 573 had predicted functions. In each strain, 34 tRNAs and 7 rRNAs, consisting of three copies of 5S, two copies of 23S, and two copies of the 16S rRNA subunits, were identified. The strains had most of their genes or gene products in common, sharing 92.5% - 100% nucleotide sequence identities and 88.0% - 100% amino acid sequence identities. Comparisons of open reading frames (ORFs) revealed that 5 of the MS-H, 7 of the WVU-1853 and 3 of the MS53 ORFs had insertions or deletions in multiples of 3 bp, resulting in slightly longer or shorter proteins without frameshifts (Table 2.3). Analysis of the genomes of the 3 strains using MAUVE revealed that their chromosomes were collinear, with the remarkable exception of a unique 55 kb inversion in MS-H (Figure 2.1) (Table 2.4). Large chromosomal inversions have not been reported before in *M. synoviae*, but have been seen occasionally in other mollicutes, including *M. hyopneumoniae* (Vasconcelos et al., 2005) and *M. bovis* (Li et al., 2011). The molecular mechanism underlying this inversion, which is delimited by a tRNA^{Gly} and an IS30 (Figure 2.2), is unclear. It has been proposed that the partial lack of DNA replication and repair functions in mycoplasma species could prevent the formation of large genomic inversions (Suyama and Bork, 2001). The NTG-mutagenesis used to create the MS-H vaccine generates

⁵ The previously published sequences of MS53 and WVU-1853 were annotated using different approaches. A large number (more than fifty) of discrepancies over the entire genome were noticed between the annotations of MS53 and WVU-1853, including some regions that are poorly annotated. Therefore, to ensure consistency in the analysis, RAST re-annotation was performed for both MS53 and WVU-1853.

point mutations and is therefore unlikely to have caused this rearrangement. Whether this genomic inversion occurred naturally in the lineage of MS-H and is a common feature amongst Australian field strains, or was induced by mutagenesis, will require further genomic sequencing of the parental strain of the vaccine, namely 86079/7NS⁶. Repeated sequences can contribute to chromosomal rearrangements, including inversions (Ohtsubo and Sekiya, 1996; Rocha and Blanchard, 2002). The genomic structures of *Mycoplasma bovis* and *Mycoplasma agalactiae* have high synteny except for a 142 kb inversion, which may be related to an *ISMbov1* element adjacent to this region in *M. bovis* (Li et al., 2011). A similar mechanism may have generated the chromosomal rearrangement in MS-H. Apart from the inversion, the genomic organisation of MS-H was generally similar to those of MS53 and WVU-1853.

2.4.3 Large mobile genetic elements

Several large mobile genetic elements⁷ were identified in all three genomes. Two distinct clusters of phage-related sequences were found in MS-H, MS53 and WVU-1853. These two clusters were similarly organised across the strains and were located at nucleotide positions 446358-459743 and 126056-134674 in MS-H, 442958-456345 and 123762-132823 in MS53, and 472072-485459 and 124910-133971 in WVU-1853. Their mol% G+C contents were similar ($28.72 \pm 0.11\%$ for the first cluster and $29.24 \pm 0.05\%$ for the second cluster) and slightly higher than the average mol% G+C content of the host genomes. In contrast, a third region with significantly lower mol% G+C content (22.2%-24.2%) was predicted by IslandViewer3 analysis to be a putative genomic island (GI) in MS53 (nucleotide positions 302828-327410) and WVU-1853 (nucleotide positions 329954-356584). In MS-H, a related region was identified by sequence alignment with the two other strains; this region was adjacent to, and partially

⁶ “Further genomic sequencing” results have been shown in Chapter 3.

⁷ In this paragraph the description of mobile genetic elements in MS-H is based on in-silico predictions and has not been confirmed experimentally by phage transduction or by mating attempts.

affected by, the 55 kb [tRNA^{Gly} - *IS30*] genomic inversion (Figure 2.2) on its right side. Specifically, the left portion of the GI was located at nucleotide positions 303839-321538 while the right portion was found at 360049-367358 as a result of the genomic inversion. The GI encoded lipoproteins, hypothetical proteins and transposases of the *IS30* and *IS1634* families. Three coding sequences (CDSs) adjacent to the GI, encoding an oligoribonuclease A, an aspartate-ammonia ligase and a lichenan-specific IIA (LicA) component of a phosphotransferase system (PTS), were also found at another chromosomal locus. In the GI, these CDSs were flanked by an *IS30* and an *IS1634* (Figure 2.2). In MS53, the GI-encoded aspartate-ammonia ligase was present as a pseudogene, while the other chromosomal copy was intact. In all 3 strains, the much lower mol% G+C content of the GI suggests that it was horizontally acquired by *M. synoviae* from another organism.

2.4.4 Strain-variable sequences in *M. synoviae* genomes and vaccine-specific genetic markers

Comparison of the gene repertoires of the three strains (excluding the *vlhA* locus, which has complex patterns of variability within *M. synoviae*) revealed that 18 predicted gene products were variably distributed amongst the 3 strains (Table 2.5). These genes were either present in only one of the strains or present in two strains, but absent from the third. Strain-variable sequences are of particular interest because they could be used as molecular markers for differentiating the MS-H vaccine from field strains and understanding the recent evolution of *M. synoviae* and other avian mollicutes. Remarkably, most of the loci containing strain-variable genes were associated with, and often flanked by, IS elements. The comparative organisation of the major strain-variable loci is illustrated in Figure 2.3. Only a few of the putative gene products from these loci had a predicted function at the time of analysis. They include a deoxyribose-phosphate aldolase (*deoC*) fragment (MSH_03520) (Table 2.1), an ATP-dependent DNA helicase

(MSH_07190), an N-acetylneuraminate lyase (*nanA*) variant (MSH_00300), an integrase (MSH_07560, VY93_03735), a DNA methylase (MSH_02150, VY93_01015/VY93_02585), and several type I, II and III related restriction-modification proteins. The *deoC* fragment and the ATP-dependent DNA helicase sequences were located at two distinct chromosomal sites in the MS-H genome, and were absent in WVU-1853 and MS53 (Figure 2.3a and 2.3b). While a complete copy of *nanA* was found in MS-H, WVU-1853 and MS53 at a conserved locus, a second truncated sequence variant lacking the first 134 nt and adjacent to an *IS1634* family transposase gene was located between the conserved *glpQ* and *rpiR* genes in MS-H only (Figure 2.3c). NanA is involved in sialic acid scavenging and degradation and is proposed to be associated with virulence in *M. synoviae* (May and Brown, 2008; May et al., 2007). Whether the *nanA* variant found in MS-H influences the sialic acid metabolism of the strain remains to be explored. A locus containing an integrase gene, several of hypothetical protein CDSs and sequences related to type I and type III restriction-modification systems was found in MS-H and WVU-1853 but not in MS53 (Figure 2.3d). The type I and type III restriction-modification related sequences were located at both ends of the locus, in opposite orientations. These sequences were pseudogenes (see below) and had similarities with CDSs for two restriction S subunits, one restriction R subunit and one DNA-methyltransferase M subunit for the type I system, and two restriction-modification system methylation subunits for the type III system. Genes encoding type I restriction modification (RM) systems are known to be unstable and to display allelic variability (O' Neill et al., 1997). Accordingly, no type I RM system was found in MS53 and significant sequence variations were seen between the specificity subunits; MS-H and WVU-1853 had four type III RM system methylation subunits, while only one was seen in MS53. Moreover, a locus encoding a type II restriction endonuclease homologous to *MjaIII* (prototype *MboI*) and a methyl-directed repair DNA adenine methylase, flanked by *IS1634* copies, was detected in MS-

H and WVU-1853, but not in MS53 (Figure 2.3e). Overall, the analysis of the strain-variable gene repertoires indicated that strains MS-H and WVU-1853, which have distinct geographical and historical origins, were more closely related to each other than strain MS53, which was isolated from a geographic location closer to WVU-1853. Whether these findings are representative of strain diversity in North and South America compared to Australia remains to be explored.

2.4.5 Pseudogenisation of transport systems and their potential impact on attenuation of virulence

Excluding the IS elements and the *vlhA* locus, a total of 27 pseudogenes were identified in MS-H, WVU-1853 and MS53, with some differences in pseudogene repertoires between strains (Table 2.5). MS-H, WVU-1853 and MS53 had 17, 15 and 9 pseudogenes, respectively. Of these pseudogenes, 8 were unique to MS-H, 4 to WVU-1853 and 3 to MS53. Only 3 of the 27 were caused by a point mutation creating a TAA stop codon within the same frame. The majority of the pseudogenes were the result of single nucleotide insertions or deletions resulting in frameshifts. Overall, the numbers of pseudogenes were relatively low and similar across all strains, albeit slightly higher in MS-H. Active pseudogenisation in a live vaccine could lower its immunogenic capacity and efficacy by disrupting the expression of protective antigens. Based on our analysis, pseudogenisation does not seem to be significantly more prevalent in MS-H, and is unlikely to affect its repertoire of expressed antigens. The potential impact of pseudogenisation on the attenuation of virulence in MS-H was also considered. A remarkable example of pseudogenisation was found in the oligopeptide permease (Opp) system. Multiple copies of *opp* operons are commonly seen in both Gram-negative and Gram-positive bacteria, including *M. gallisepticum*, and can play a role in virulence (Eitinger et al., 2011; Jones et al., 2014; Moraes et al., 2014; Tseng et al., 2017a). The Opp systems comprise an extracellular substrate binding protein, OppA, two

transmembrane proteins, OppB and OppC, which form the pore, and two cytoplasmic ATPases, OppD and OppF, which provide the energy for peptide translocation (Gardan et al., 2009; Monnet, 2003). As in many mycoplasmas, all 3 *M. synoviae* strains possessed two Opp systems, hereafter named *opp*-I and *opp*-II. The phylogenetic relationships between *opp* operons within the same species are complex. It has been suggested that in the Hominis group the *opp* operons underwent duplication and divergence. In *M. gallisepticum*, one of the *opp* operons has been proposed to have been horizontally acquired from a member of the Hominis group (Tseng et al., 2017a). The correct annotation of *oppA* sequences in *Mycoplasma* spp. genomes is problematic because the gene is often identified only as encoding a lipoprotein (Wium et al., 2015). In this study, we found a putative *oppA* gene in the MS-H *opp*-I cluster, based on the 28% similarity of the product to *M. canadense oppA*. Unlike *M. gallisepticum*, where the two operons are found in tandem, the *M. synoviae opp*-I and *opp*-II systems were distant from each other within the chromosome. Moreover, the order of the *opp* genes differed between the two operons in MS-H and WVU-1853, as has been previously noted for MS53 (Wium et al., 2015). Homologous proteins encoded by the two gene clusters share low sequence similarity, possibly indicating that they are involved in the acquisition of different nutrients. The *M. synoviae opp*-I operon (Figure 2.4a) is similar to the *opp*-1 subtype of *Staphylococcus aureus* (Yu et al., 2014) but contains 2 pseudogenes, *oppF* in MS-H⁸ and *oppA* in WVU-1853. In WVU-1853, a premature stop codon in *oppA* was close to 3' end of the gene, while the frameshift mutation in MS-H *oppF* allows only the translation of 157 of 797 amino acids, most likely resulting in loss of function of the protein. In contrast, the *opp*-II operon (Figure 2.4b) is intact and identically organised in all three strains, but differs from other avian mycoplasma species with respect to its gene arrangement (Wium et al., 2015). Co-existing *opp* systems with different, but partially redundant, substrate specificity have been described

⁸ The pseudogenization of *oppF* in *opp*-I was confirmed by PCR and sequencing.

in *Bacillus subtilis*, in which the inactivation of one system may be compensated for by the presence of an active second operon (Koide and Hoch, 1994; Koide et al., 1999). It is tempting to speculate that in WVU-1853 the *oppA* pseudogene from *opp-I* is at least partially complemented by the full *oppA* copy from the *opp-II* operon, while in MS-H the pseudogenisation of *oppF* in *opp-I* is not compensated by the *opp-II* copy, which has resulted in attenuation of virulence. Experimental verification of this hypothesis is required to establish whether the two OppA transporters can co-operate or display broad substrate specificity, whereas OppF and other components of the Opp system have more exacting interactions in *M. synoviae*. Alternatively, as has been hypothesized for OppD1 in *M. gallisepticum* (Tseng et al., 2017a), it is possible that the two OppF proteins form dimers in the transport complex and that the product of the partial copy of *oppF-I* in MS-H may retain the capacity to form a heterodimer with the product of full-length copy of *oppF-II*, resulting in a functional reduction and thus a decrease in virulence.

Other transport system operons were also found to contain pseudogenes. Advanced pseudogenisation was noted for the *sgaABT* gene cluster, which is predicted to encode an ascorbate-specific PTS and is located next to a strain-variable region containing Type I and Type III RM systems, as well as an integrase gene (see Figure 2.3d). Sequences encoding the subunits EIIC (*sgaT-2*), IIB (*sgaB*) and IIA (*sgaA*) appeared to be intact in MS53, but various degrees of pseudogenisation were observed in the 2 other strains. In MS-H and WVU-1853, *sgaT-2* was split into four fragments. In MS-H, *sgaB* contained a frameshift very close to the start codon, but this gene was intact in MS53 and WVU-1853. The integrity of the PTS transporter locus in MS53 might be correlated with the absence of a RM system and associated sequences in its vicinity (see Figure 2.3d). As with the *opp-I* and *opp-II* operons, a second ascorbate-specific PTS locus containing intact sequences for the transport subunits IIA, IIB and EIIC was identified in the genome of all three *M. synoviae* strains. This second locus may compensate for the

pseudogenisation seen in MS-H and WVU-1853. Multiple copies of the ascorbate-specific PTS are often seen in bacteria (Barabote and Saier, 2005), and are involved in acquisition of L-ascorbate by the organism under anaerobic conditions (Hvorup et al., 2003; Zhang et al., 2003). However, orphan IIA and/or IIB and/or IIC homologues of these systems are also often found, and may be residues of genomic minimisation (Moran, 2002). In MS-H, the *rpiR*-like transcriptional regulator gene, lying downstream of the *nanA* variant and the *IS1634*, was also split into two pseudogenes, but was intact in MS53 and WVU-1853 (Figure 2.3c). It is known that NanA is involved in the breakdown and utilisation of sialic acid (Walters et al., 1999), and RpiR belongs to a family of transcriptional regulators, some of which are able to repress or activate the expression of *nan* genes (Afzal et al., 2015; Johnston et al., 2007; Kim et al., 2011; Therit et al., 2015). As transcriptional regulatory systems in mycoplasmas are not well understood, it is not possible to predict whether the pseudogenisation of the *rpiR* gene might modify sialic acid metabolism in *M. synoviae*. In addition, restriction modification sequences are often present as pseudogenes in bacterial genomes. In MS-H, pseudogenisation affects a type I restriction-modification specificity subunit S and a type III restriction-modification system methylation subunit.

2.4.6 Clustered regularly interspersed palindromic repeats and evidence of horizontal gene transfer

All three *M. synoviae* strains harbor a typical CRISPR-associated Cas system, with *csn1* sequentially followed by *cas1*, *cas2* and a putative *csn2* in a contiguous operon (Figure 2.5). Homologues of these proteins are also encoded in the genomes of other *Mycoplasma* species, including *M. ovipneumoniae*, *M. arthritidis*, *M. hyosynoviae* and *M. gallisepticum* (Bumgardner et al., 2015; Leclercq et al., 2014). The operon structure was consistent with a type II (Nmni subtype) CRISPR/Cas system (Bumgardner et al., 2015; Haft et al., 2005; Makarova et al., 2011). However, MS53 was the only *M.*

synoviae strain found to possess a CRISPR array, formed by 11 spacers separated by 36-bp repeat units, suggesting a record of past foreign DNA invasions in this strain. Small duplicated fragments of *csn1* and *cas1* were positioned downstream of the CRISPR array (Figure 2.5). Among the CRISPR-associated genes, *csn1* appeared intact in MS53, but was present as a pseudogene in WVU-1853 and MS-H. In WVU-1853, *csn1* was disrupted by a single nucleotide deletion, resulting in a frameshift (May et al., 2015). In MS-H, *csn1* was disrupted by an 11 nucleotide insertion. In both strains these mutations were positioned within the 5' region of the coding sequence and were therefore likely to result in the loss of CRISPR function, as has been observed previously in *Streptococcus thermophilus* (Deveau et al., 2008). An IS element was found downstream of the CRISPR-associated genes in all three strains. Genome alignments of all three *M. synoviae* strains with *M. gallisepticum*, another mycoplasma species infecting chickens, detected 11 regions sharing similarity of more than 90%, suggesting that some horizontal gene transfer has occurred between these two species. The majority of these regions have been previously reported as putatively transferred (Vasconcelos et al., 2005), and notably include the *vlhA* haemagglutinin genes (Bencina, 2002; Noormohammadi et al., 1998). In addition, the deoxyribose-phosphate aldolase (*deoC*) sequence fragment (MSH_03520), which was located upstream of an *IS1634* in MS-H (Figure 2.3a) and was not found in WVU-1853 and MS53, was very similar to a sequence in *M. gallisepticum* strain R, sharing 94% amino acid similarity with it. In *M. gallisepticum* strain R this sequence is also flanked by IS-related sequences. Moreover, while MS53 and WVU-1853 have only one ATP-binding helicase CDS, a second helicase gene was found in MS-H (MSH_07190) (Table 2.5). This second copy, flanked by two *IS30* family transposase genes (Figure 2.3b), is also detected in other mycoplasma species, including *M. gallinaceum* strain B2096-8B, with which it had 93% amino acid similarity. *M. gallinaceum* is a mycoplasma of low pathogenicity that shares the same habitat (Abolnik and Beylefeld, 2015; Jordan et al., 1982) and

phylogenetic group (Ramírez et al., 2008) as *M. synoviae*. Additionally, two siderophore-mediated iron transport proteins were also identified as putatively horizontally transferred in a single *M. synoviae* genome region (536617-551599 in MS-H). Siderophores are specific Fe(III)-binding agents produced by many microorganisms that mediate iron scavenging from the environment of the host (Ratledge and Dover, 2000). Siderophore-mediated iron transport system related genes are considered virulence factors in many bacterial pathogens because of their critical role in adaption to iron-limited conditions within the host (Payne and Lawlor, 1990; Richardson and Park, 1995). Although the iron-acquisition mechanisms in mycoplasmas remain largely unknown (Madsen et al., 2006), this putative siderophore-associated iron transport system raises the possibility that siderophores may contribute to the pathogenesis of infection with *M. synoviae*.

2.5 Conclusion

The MS-H live vaccine has been used to prevent infection with virulent *M. synoviae* in the poultry industry for more than two decades and is now used in many countries around the world. As a result, it is possible that MS-H is the most prevalent strain of *M. synoviae* globally. Comparative genome analyses revealed a number of features unique to MS-H, WVU-1853 or MS53, but the genomes of these three strains were largely similar to each other. In particular, striking similarities were found between strains MS-H and WVU-1853, despite their distinct geographical origins and dates of isolation. This apparently low genomic variability within the species supports the evidence from the field that MS-H protects against *M. synoviae* in a wide range of countries. Differentiation of vaccine strains from field strains is always challenging because of the limited number of genetic markers available for development of routine tests. In this study, we identified genetic features unique to the MS-H vaccine strain that may be able to be targeted in the future for diagnostic purpose. Strain-variable sequences, putatively

acquired by horizontal transfer, and pseudogenes play a critical role in the genomic plasticity of *M. synoviae*. This is exemplified by the discovery of a large inversion in the vaccine strain MS-H adjacent to a short region potentially transferred from *M. gallisepticum*, and an ATP-binding helicase gene putatively acquired from *M. gallinaceum*. Although the three *M. synoviae* strains had similar numbers of pseudogenes, the slightly more advanced pseudogenisation of MS-H could be explained by the chemical mutagenesis used to produce the vaccine. It is not yet clear whether the pseudogenisation of some of these genes is associated with attenuation of virulence that is characteristic of the MS-H vaccine. The complete genomic sequencing of MS-H is the first step towards a thorough comparison with its parental strain 86079/7NS, currently underway in our laboratory, that will help address this question.

Table 2.1. Nucleotide positions of the unique gene locus tags in the MS-H genome

Locus tag	Nucleotide position	Gene product
MSH_00300	28019 -> 28492	N-acetylneuraminate lyase variant
MSH_00310	28955 -> 28536	Transcriptional regulator
MSH_00330	29383 -> 29156	Transcriptional regulator
MSH_00500	43386 -> 42931	Translation elongation factor G
MSH_00560	50164 -> 49772	Choline kinase family
MSH_01200	107067 -> 106285	Multiple sugar ABC transporter, ATP-binding protein
MSH_01840	175663 -> 173771	Hypothetical protein
MSH_01930	184582 -> 183302	Hypothetical protein
MSH_01960	186751 -> 186948	Hypothetical protein
MSH_01970	188503 -> 187223	Hypothetical protein
MSH_02140	207173 -> 206346	Type II restriction enzyme <i>Mja</i> III
MSH_02150	208962 -> 207925	Methyl-directed repair DNA adenine methylase
MSH_02310	221997 -> 227897	Hypothetical protein
MSH_02940	305844 -> 305725	3'-to-5' oligoribonuclease A
MSH_02950	306074 -> 306421	3'-to-5' oligoribonuclease A
MSH_02960	306479 -> 307435	3'-to-5' oligoribonuclease A
MSH_02990	308913 -> 309719	Aspartate-ammonia ligase
MSH_03480	364422 -> 364087	Hypothetical protein
MSH_03490	365713 -> 364859	Hypothetical protein
MSH_03500	366730 -> 365717	Asparaginyl-tRNA synthetase-related protein
MSH_03520	368566 -> 368291	Deoxyribose-phosphate aldolase
MSH_03740	397534 -> 395855	Oligopeptide transport ATP-binding protein OppF
MSH_03750	398244 -> 397774	Oligopeptide transport ATP-binding protein OppF
MSH_03790	404758 -> 401783	Lipoprotein
MSH_04020	423554 -> 427795	Hypothetical protein
MSH_04620	489742 -> 487808	Hypothetical protein
MSH_04790	507373 -> 507642	Hypothetical protein
MSH_04880	513817 -> 514188	Hypothetical protein
MSH_04960	522769 -> 534519	Hypothetical protein
MSH_05040	538680 -> 536617	Siderophore-mediated iron transport protein
MSH_05850	637916 -> 638287	Hypothetical protein
MSH_05860	638473 -> 638802	Hypothetical protein
MSH_05940	649545 -> 648349	Hypothetical protein
MSH_06430	686373 -> 686516	CRISPR-associated protein, Csn1
MSH_06440	686627 -> 690139	CRISPR-associated protein, Csn1
MSH_07180	762003 -> 760981	<i>IS</i> 30 family transposase
MSH_07210	766383 -> 765667	<i>IS</i> 30 family transposase
MSH_07190	765087 -> 762259	ATP-dependent DNA helicase
MSH_07360	780322 -> 780450	Hypothetical protein
MSH_07370	780478 -> 780834	Hypothetical protein
MSH_07380	780812 -> 781477	ABC transporter ATP-binding and permease protein
MSH_07610	809290 -> 808670	Hypothetical protein
MSH_07640	810593 -> 810363	PTS system enzyme IIB component

Table 2.1. Continued

MSH_07650	811270 -> 810704	Ascorbate-specific PTS system, EIIC component
MSH_07660	811730 -> 811542	Ascorbate-specific PTS system, EIIC component
MSH_07670	811882 -> 811763	Ascorbate-specific PTS system, EIIC component
MSH_07680	812098 -> 811931	Ascorbate-specific PTS system, EIIC component
MSH_07490	795353 -> 795622	Type III restriction-modification system
MSH_07500	795853 -> 796308	Type III restriction-modification system
MSH_07510	796998 -> 797807	Hypothetical protein
MSH_07520	797795 -> 798205	Hypothetical protein
MSH_07530	798227 -> 798664	Hypothetical protein
MSH_07540	798775 -> 799170	Hypothetical protein
MSH_07560	800370 -> 799453	Integrase
MSH_07570	801887 -> 800385	Type I restriction-modification system, specificity
MSH_07580	802665 -> 802075	Type I restriction-modification system, specificity
MSH_07590	805898 -> 802701	Type I restriction-modification system, restriction
MSH_07600	808458 -> 805906	Type I restriction-modification system, DNA-methyltransferase subunit M

Table 2.2. General characteristics of the genomes of three *M. synoviae* strains MS-H, MS53 and WVU-1853

Characteristic	MS-H	MS53	WVU-1853
Total length (base pairs)	818848	799476	846495
G+C content (%)	28.2	28.5	28.3
Gene density (%)	90.0	90.4	90.2
Average gene length (base pairs)	1002	994	992
No. features (genes)	775	764	805
No. coding sequences	734	723	764
No. CDSs with predicted function	541	543	573
No. rRNAs			
16S	2	2	2
23S	2	2	2
5S	3	3	3
No. of tRNAs	34	34	34

Table 2.3. Unique amino acid deletions/insertions in *M. synoviae* strains MS-H, MS53 and WVU-1853

Features (locus tag)	MS-H	MS53	WVU-1853
Cell division protein FtsZ (MS53_0340)		– 7	
Conserved hypothetical protein (MS53_0547)		– 4	
Conserved hypothetical protein (MS53_0590)		– 10	
Hypothetical protein (VY93_01870)			– 4
Hypothetical protein (VY93_02525)			+ 3
Hypothetical protein (VY93_02550)			– 29
Peptidase C1 (VY93_03235)			– 13
Signal peptidase I (VY93_00225)			– 6
PTS sugar transporter subunit IIABC (VY93_00785)			+ 8
ABC transporter ATP-binding protein (VY93_03350)			– 2
Choline kinase family (MSH_00560)	– 5		
Hypothetical protein (MSH_02310)	– 3		
Hypothetical protein (MSH_04020)	– 5		
Siderophore-mediated iron transport protein (MSH_05040)	– 20		
Hypothetical protein (MSH_05940)	+ 4		

Table 2.4. Composition of the inverted region in MS-H

Locus tag	Gene start	Gene end	Strand	Product
MSH_03130	321756	321829	-1	tRNA-Gly
MSH_03140	322042	422893	-1	DegV-like protein
MSH_03150	322976	324418	1	Hypothetical protein
MSH_03160	324427	324897	1	Transcription termination protein NusB
MSH_03170	324869	325369	1	Hypothetical protein
MSH_03180	325373	326536	1	Cysteine desulfurase, SufS subfamily
MSH_03190	326523	326951	1	Putative iron-sulfur cluster assembly scaffold protein, SufE2
MSH_03200	326938	328179	1	DNA polymerase IV
MSH_03210	328181	329266	1	Nicotinate-nucleotide adenylyltransferase, YqeK
MSH_03220	329266	329873	1	Ribosomal large subunit pseudouridine synthase B
MSH_03230	330083	332680	1	DNA gyrase subunit A
MSH_03240	332752	334923	-1	Hypothetical protein
MSH_03250	335203	335685	-1	Hypothetical protein
MSH_03260	335660	335923	-1	Hypothetical protein
MSH_03270	336106	336756	-1	Hypothetical protein
MSH_03280	337459	340131	-1	ATP-dependent protease La Type I
MSH_03290	340172	342469	-1	Leucyl-tRNA synthetase
MSH_03300	342478	343248	-1	Putative membrane integrated oxidoreductase
MSH_03310	343248	343868	-1	Uracil phosphoribosyltransferase
MSH_03320	344026	344967	1	Phenylalanyl-tRNA synthetase alpha chain
MSH_03330	344971	345645	1	Uracil-DNA glycosylase, family 1
MSH_03340	345629	347809	1	Phenylalanyl-tRNA synthetase beta chain
MSH_03350	347812	349077	1	Serine hydroxymethyltransferase
MSH_03360	349105	349401	-1	hypothetical protein
MSH_03370	349403	350959	-1	Methionyl-tRNA synthetase
MSH_03380	350961	351752	-1	tRNA (adenine37-N(6))-methyltransferase TrmN6
MSH_03390	351727	353934	-1	3'-to-5' exoribonuclease RNase R
MSH_03400	354021	354440	-1	Ferroxidase
MSH_03410	354464	355546	-1	Endo-1,4-beta-glucanase
MSH_03420	355673	358171	1	Valyl-tRNA synthetase
MSH_03430	358159	358995	1	Methenyltetrahydrofolate cyclohydrolase
MSH_03440	358995	359972	1	6-phosphofructokinase
MSH_03450	360049	360627	-1	Mobile element protein
MSH_03460	360729	361808	-1	Mobile element protein
MSH_03470	362172	364103	-1	Hypothetical protein
MSH_03480	364087	364422	-1	Hypothetical protein
MSH_03490	364859	365713	-1	Hypothetical protein
MSH_03500	365717	366730	-1	Asparaginyl-tRNA synthetase-related protein
MSH_03510	367191	367358	1	IS30 element

Table 2.5. Summary of variable genes across *M. synoviae* strains MS-H, MS53 and WVU-1853

Gene product	ORF (locus tag ^a)		
	MS-H	MS53	WVU-1853
N-acetylneuraminate lyase variant	MSH_00300	–	–
Transcriptional regulator	MSH_00310 ^b MSH_00330 ^b	MS53_0024	VY93_00145
Translation elongation factor G	MSH_00500	MS53_0039 ^b	VY93_00220 ^b
Multiple sugar ABC transporter, ATP-binding protein	MSH_01200 ^c	MS53_0102	VY93_00560
Hypothetical protein	MSH_01840 ^c	–	VY93_00865
Hypothetical protein	MSH_01960	–	VY93_00910
Hypothetical protein	MSH_01930 MSH_01970	–	VY93_00915
Type II restriction enzyme <i>Mja</i> III	MSH_02140	–	VY93_01010 VY93_02580
Methyl-directed repair DNA adenine methylase	MSH_02150	–	VY93_01015 VY93_02585
3'-to-5' oligoribonuclease A	MSH_02940 ^b MSH_02950 ^b MSH_02960 ^b	MS53_0278 ^b MS53_0279 ^b	VY93_01505 ^b VY93_01510 ^b
Aspartate-ammonia ligase	MSH_02990	MS53_0281 ^b	VY93_01515
Hypothetical protein	MSH_03480 ^b MSH_03490 ^b	MS53_0293	VY93_01595 ^b
Asparaginyl-tRNA synthetase-related protein	MSH_03500	MS53_0291 ^b MS53_0700 ^b	VY93_01590 ^b
Deoxyribose-phosphate aldolase	MSH_03520	–	–
Oligopeptide transport ATP-binding protein OppF	MSH_03740 ^b MSH_03750 ^b	MS53_0345	VY93_01875
Lipoprotein	MSH_03790	MS53_0349	VY93_01895 ^b VY93_01900 ^b
Hypothetical protein	MSH_04620	MS53_0430 ^b	VY93_02305
Hypothetical protein	MSH_04790 ^b	MS53_0442	
Hypothetical protein	MSH_04880		VY93_02420
Hypothetical protein	MSH_04960	MS53_0458 ^b MS53_0459 ^b	VY93_02465
Hypothetical protein	MSH_05850 ^b MSH_05860 ^b	MS53_0713 ^b MS53_0540 ^b	VY93_02935 ^b
CRISPR-associated protein, Csn1	MSH_06430 ^b MSH_06440 ^b	MS53_0582	VY93_03200 ^b VY93_03205 ^b
IS30 family transposase	MSH_07180 MSH_07210	–	VY93_00200 VY93_00900 VY93_01540 VY93_02375

Table 2.5. Continued

ATP-dependent DNA helicase	MSH_07190	–	–
Hypothetical protein	MSH_07360b	MS53_0668	VY93_03635
Hypothetical protein	MSH_07370b	MS53_0669b	VY93_03640
ABC transporter ATP-binding and permease protein	MSH_07380	MS53_0670b	VY93_03645b
Hypothetical protein	MSH_07610 ^c	MS53_0680	VY93_03760 ^b
PTS system enzyme IIB component	MSH_07640 ^b	MS53_0682	VY93_03770
Ascorbate-specific PTS system, EIIC component	MSH_07650 ^b MSH_07660 ^b MSH_07670 ^b MSH_07680 ^b	MS53_0683	VY93_03775 ^b
Type III restriction-modification system methylation subunit	MSH_07490 ^b MSH_07500 ^b	–	VY93_03700 ^b VY93_03705 ^b
Hypothetical protein	MSH_07510	–	VY93_03710 ^b
Hypothetical protein	MSH_07520	–	VY93_03720 ^b
Hypothetical protein	MSH_07530	–	VY93_03725 ^b
Hypothetical protein	MSH_07540 ^b	–	VY93_03730
Integrase	MSH_07560	–	VY93_03735
Type I restriction-modification system, specificity subunit S	MSH_07570 ^b MSH_07580 ^b	–	VY93_03740 ^b VY93_03745 ^b
Type I restriction-modification system, restriction subunit R	MSH_07590	–	VY93_03750
Type I restriction-modification system, DNA-methyltransferase subunit M	MSH_07600	–	VY93_03755

^a Pseudogenes that have lost more than 30% of their full length; nucleotide positions corresponding to the locus tags of MS-H are listed in Table 2.1. IS elements and the *vlhA* locus were not included in pseudogenisation analysis. ^b Pseudogenes that result from a frameshift mutation. ^c Pseudogenes that result from acquisition of an internal stop codon. “–” indicates the absence of the gene in a strain. Genes without a specified locus tag are not indicated in this table.



Figure 2.1. MAUVE alignment analysis of *M. synoviae* genomes, using MS-H as a reference. Similarly coloured blocks with connecting lines represent homologous regions. A block below the centre line indicates a region that aligned in reverse complement (inverse) orientation.

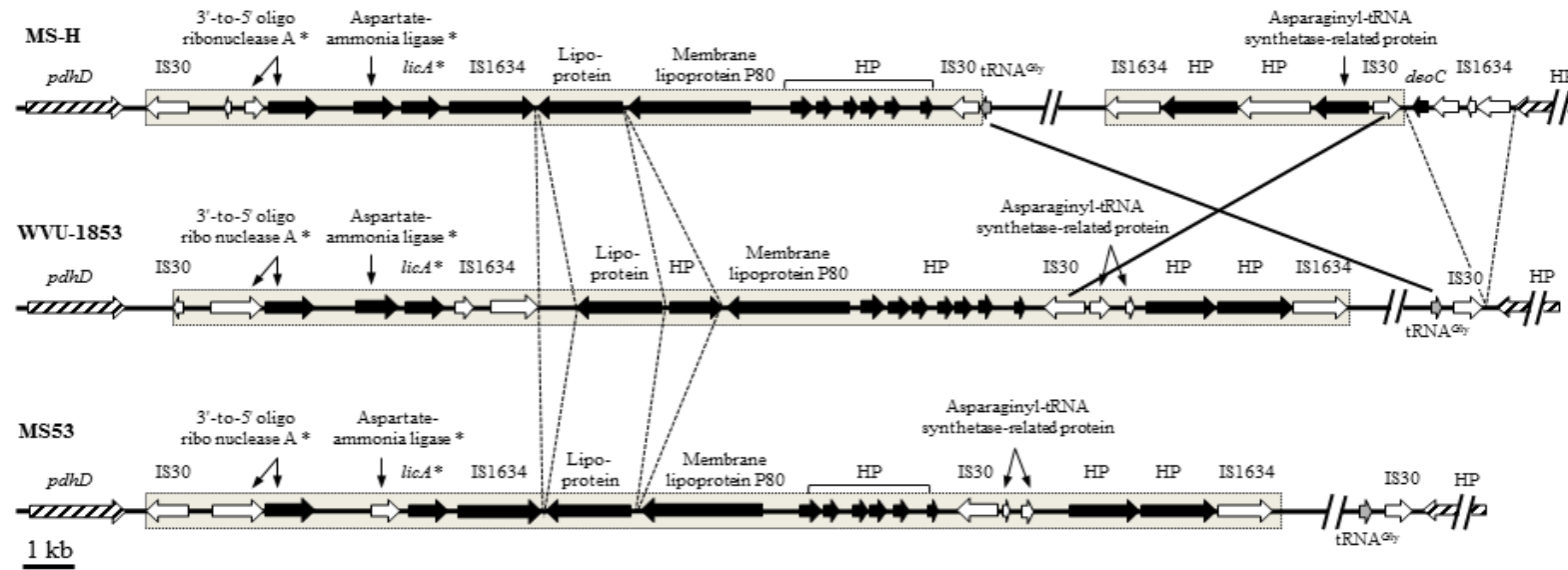


Figure 2.2. Comparative structural analysis of the genomic island (GI) in MS-H, WVU-1853 and MS53. The grey boxes with dotted lines indicate genes identified as part of the genomic island. Hatched arrows indicate genes flanking the GI. The inverted region in MS-H relative to MS53 and WVU-1853 is indicated by crossed solid lines. Inserted/deleted sequences are indicated by dashed lines. Genes with a complete predicted CDS are depicted in black; pseudogenes are depicted in white; tRNA genes are depicted in grey. HP, hypothetical protein. The asterisks indicate sequences duplicated elsewhere in the host genome.

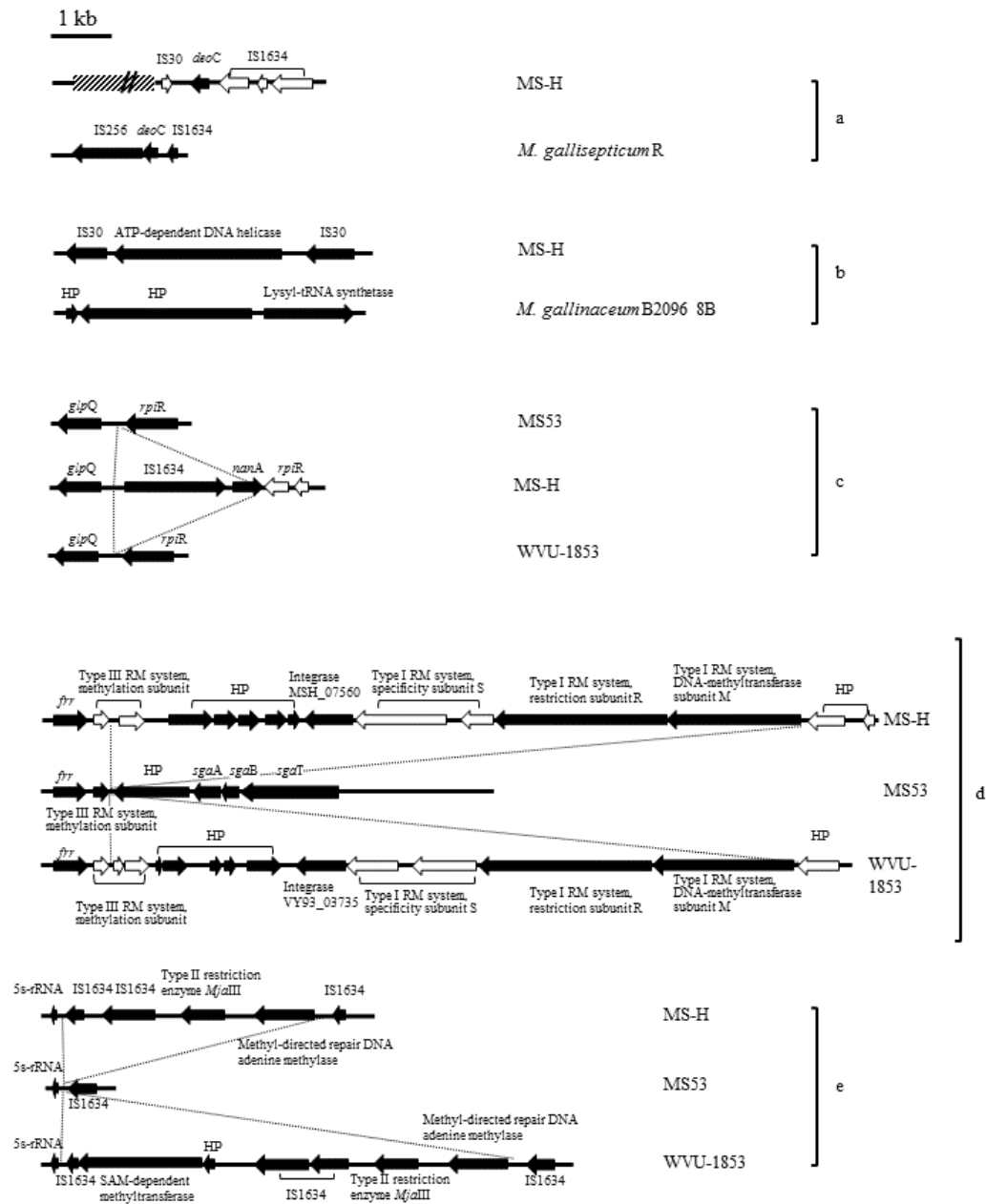


Figure 2.3. (a) Location of the *deoC* gene in *M. gallisepticum* corresponding to the *deoC* fragment found in MS-H. **(b)** Locations of the ATP-dependent DNA helicase genes in MS-H and *M. gallinaceum* strain B2096 8B. **(c, d, e)** Comparison of unique genomic loci identified in *M. synoviae* strains MS-H, WVU-1853 and MS53. The inserted/deleted sequences are indicated by dotted lines. Genes with complete predicted CDSs are depicted in black; pseudogenes are depicted in white. HP, hypothetical protein.

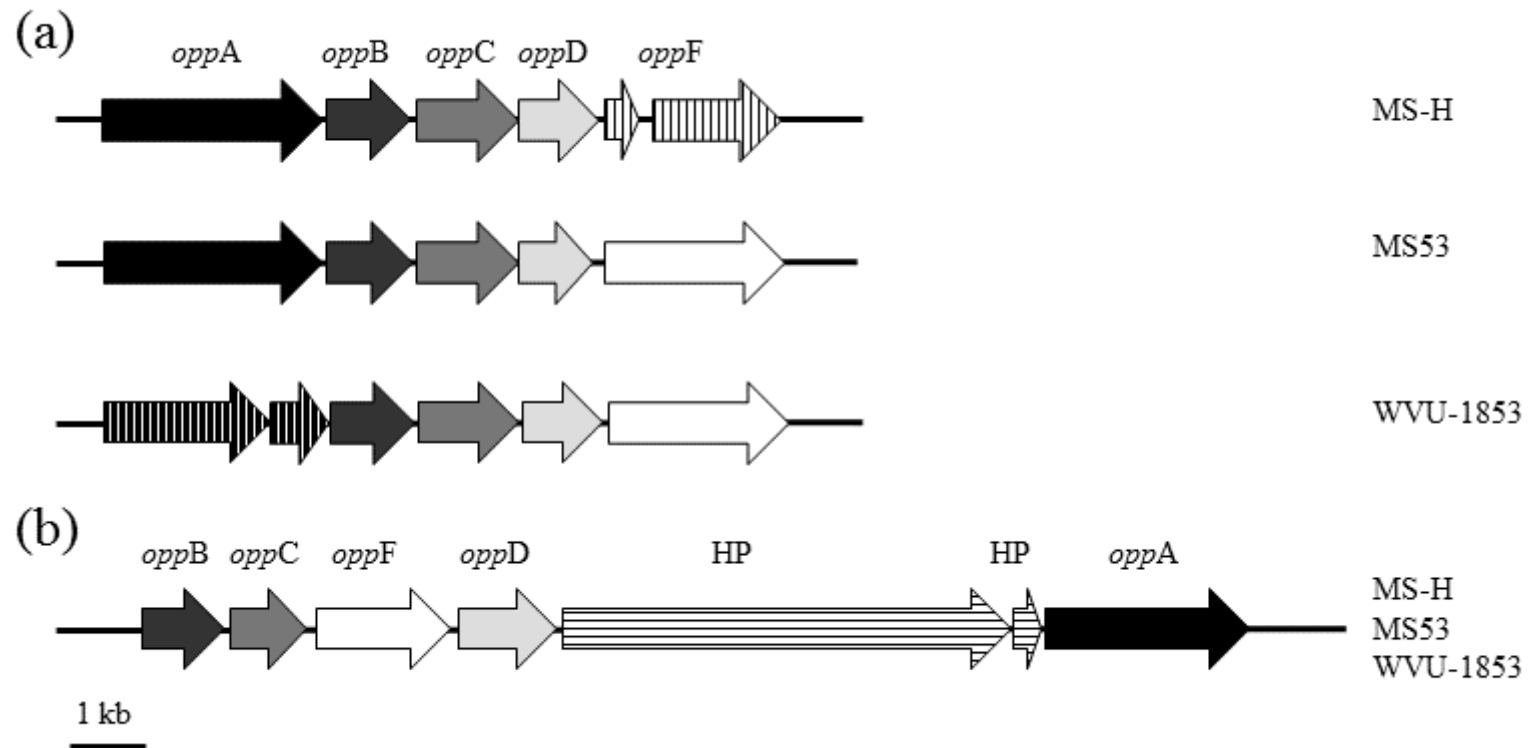


Figure 2.4. Organisation of the *opp* operons in MS-H, MS53 and WVU-1853. **(a)** Comparison of operon I in strains MS-H, MS53 and WVU-1853; **(b)** organisation of operon II in all three strains. Solid arrows indicate functional genes, while vertically hatched arrows indicate pseudogenes. Horizontally hatched arrows indicate hypothetical proteins (HP).

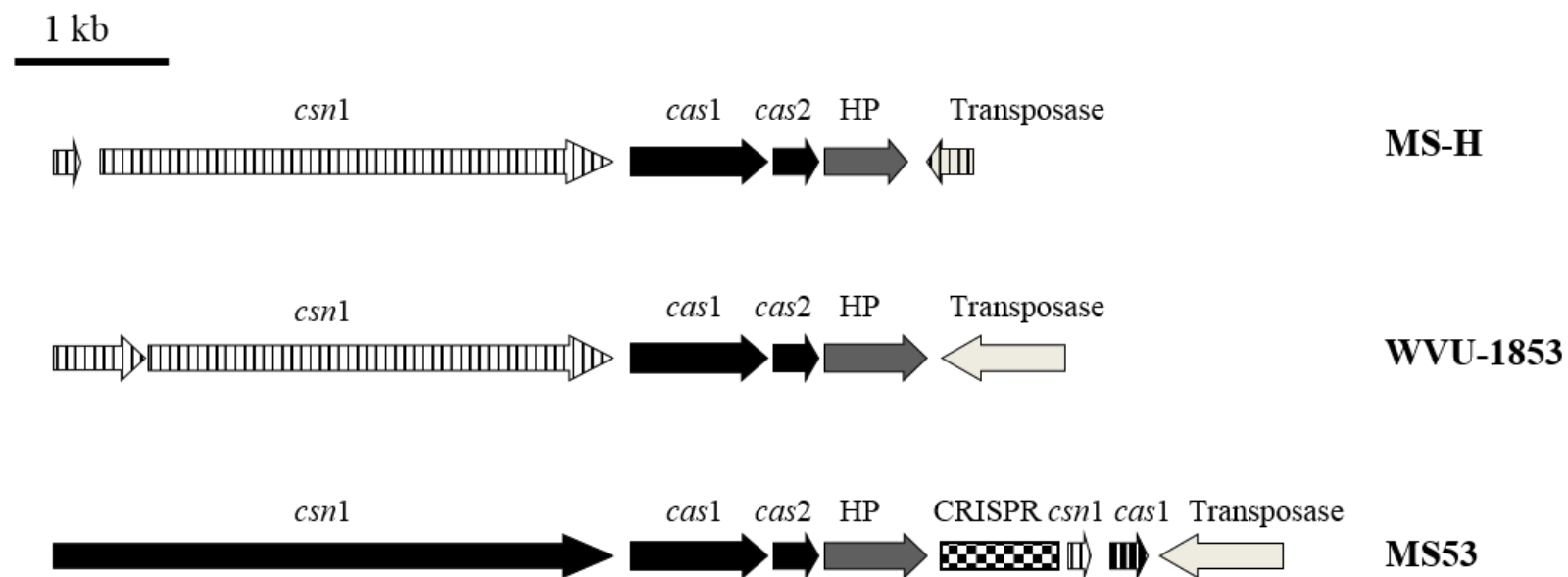


Figure 2.5. Organisation of the CRISPR/Cas system genes in the three *M. synoviae* genomes. Solid arrows represent genes. Vertically hatched arrows represent pseudogenes. The checked rectangle indicates the CRISPR repeats.

CHAPTER 3. Comparative Genomic Analyses of *Mycoplasma synoviae* Vaccine Strain MS-H and Its Parent Strain 86079/7NS: Implications for the Identification of Virulence Factors and Applications in Diagnosis of *M. synoviae*

3.1 Abstract

Mycoplasma synoviae is an economically important pathogen in the poultry industry worldwide, causing respiratory disease and infectious synovitis in chickens and turkeys. The *M. synoviae* strain MS-H was developed by chemical mutagenesis of an Australian isolate and has been used as a live attenuated vaccine in many countries over the past two decades. Despite the widespread use of MS-H, the precise molecular basis for attenuation of its virulence is still poorly understood. To explore genetic mechanisms behind the attenuation of MS-H vaccine, the whole genome sequencing of the vaccine parent strain 86079/7NS was performed using a combination of Illumina and Nanopore methods, followed by a comparative genomic analysis of these two strains. The genome (excluding the *vlhA* expressed region) of the vaccine parent 86079/7NS, was shown to be almost identical to that of MS-H, exhibiting no insertions, deletions, or rearrangements of genes. A total of 33 single nucleotide polymorphisms (SNP) were identified in MS-H (excluding the *vlhA* locus), including 11 nonsynonymous mutations that were predicted by the tool Emboss Garnier to have disrupted the secondary structures of the deduced proteins, one leading to a truncation of *oppF-1*, which encodes the ATP-binding protein of an oligopeptide permease transporter. Other nonsynonymous mutations mapped to predicted coding sequences involved in amino acid metabolism, environmental sensing, central metabolism, transport, tRNA metabolism, and nucleic acid metabolism. This study has identified a range of SNPs with putative functional effects and yielded some intriguing insights that suggest that the attenuation observed in

the MS-H strain is not the result of a single mutation but rather the cumulative and complex effect of several. In addition, the SNPs identified in MS-H were further analysed by comparing the MS-H and 86079/7NS sequences with another two published strains, WVU-1853 and MS53. A large number of insertions, deletions and genomic rearrangements were detected, and 30 SNPs were found to be unique to MS-H, and were located in regions conserved between the strains. These results have provided a foundation for the development of novel biomarkers for the detection of virulence in *M. synoviae* and also for designing new genotyping assays for discrimination of MS-H from field strains.

3.2 Introduction

Mycoplasmas are a group of bacteria that are distinguished phenotypically from other bacteria by their very small size and the lack of a cell wall. The organisms belong to the class *Mollicutes*, order *Mycoplasmatales*, family *Mycoplasmataceae*, and genus *Mycoplasma* (Stipkovits and Kempf, 1996). *Mycoplasma synoviae* (MS) is an important avian pathogen worldwide. Infection with MS was first described in 1954 (Olson et al., 1954), before isolation the causative agent in 1956 (Olson, 1956). The first isolation of MS in Australia was reported in 1990 (Morrow et al., 1990a).

M. synoviae can cause subclinical upper respiratory infection, infectious synovitis (Ferguson-Noel and Noormohammadi, 2013), airsacculitis (Kleven et al., 1972), and eggshell apex abnormalities (EAA) (Feberwee et al., 2009a) in poultry. The severity of the clinical signs may vary between different strains and routes of infection (Kang et al., 2002; Lockaby et al., 1998). The organism is transmitted horizontally through direct contact, or vertically via fertile eggs (Kleven, 1998). Although the disease is rarely associated with bird mortality, it can cause a significant economic impact in the poultry industry due to condemnation of carcasses, decreased weight gain and reduced egg production.

Vaccination is a common strategy used to control infection with *M. synoviae* in commercial flocks. The MS-H live attenuated vaccine (Vaxsafe MS®; Bioproperties Pty. Ltd., Ringwood, Victoria, Australia) has been widely used in a large number of countries since its registration in Australia in 1996. The MS-H strain is a temperature-sensitive (ts^+) mutant obtained by *N*-nitro-*N'*-methyl-*N*-nitrosoguanidine (NTG) mutagenesis of the virulent Australian field strain 86079/7NS (Morrow et al., 1998), isolated from the choanal cleft of a commercial layer hen with respiratory disease (Morrow, 1990). NTG mainly causes mutations by methylation of nucleotides, especially of guanine, which can then pair with thymine or cytosine (Lucchesi et al., 1986) and give rise to GC-to-AT conversions. Treatment of 86079/7NS with NTG produced a panel of 15 ts^+ mutants, from which MS-H was selected as a vaccine on the basis of its consistent colonisation and temperature-sensitivity (Morrow et al., 1998). MS-H grows well at 33 °C but its growth is significantly reduced at 39 °C (the core body temperature of birds). Therefore MS-H colonises only the upper respiratory tract of its host and induces long-term protective mucosal immunity (Jones et al., 2006b; Jones et al., 2006c). Despite the widespread use of the MS-H vaccine, the genetic basis for its attenuation is still poorly understood, owing to the random nature of NTG mutagenesis. The first study that attempted to identify mutations linked to attenuation described an alteration, G123R, in Obg (a GTP binding protein), which could be a potential factor contributing to the ts^+ phenotype of MS-H (Shahid et al., 2013b). As the ts^+ MS-H strain can revert to a non-temperature-sensitive (ts^-) phenotype in rare cases without resulting in reversion to the more virulent phenotype of the parent strain 86079/7NS, it is believed that more factors may be involved in the attenuation of virulence seen in the vaccine.

Recently, MS-H was completely sequenced in our laboratory using a combination of

Illumina sequence data and long reads generated by Oxford Nanopore Technology. A major step towards defining the molecular basis of attenuation in MS-H was the completion of the genome sequence of the virulent parent strain 86079/7NS. In this study, the same sequencing strategies were applied to 86079/7NS, followed by a comparative genomic analysis of these two strains. As expected, both of the strains shared almost identical genomic sequences, but a large number of single nucleotide polymorphisms (SNPs) were detected in MS-H. A few of them resulted in nonsynonymous or frameshift mutations. These specific genomic differences may provide insights into determinants responsible for the avirulent nature of MS-H and the genetic basis of virulence in *M. synoviae*.

3.3 Material and Methods

3.3.1 Strain, culture conditions and DNA extraction

Mycoplasma synoviae strain 86079/7NS (the 4th passage) was grown in mycoplasma culture medium supplemented with 10% swine serum and 0.01% (w/v) of nicotinamide adenine dinucleotide (NAD) (Whithear, 1993) at 37 °C in a 50 mL final volume until late logarithmic phase (approximately pH 6.8). Cells were collected by centrifugation at 10,000 ×g for 30 min. Genomic DNA was prepared by proteinase K digestion of the pellet, phenol-chloroform extraction and ethanol precipitation (Green and Sambrook, 2016, 2017). The DNA concentration was determined by measuring the optical density at 260 nm (OD₂₆₀), and the purity was determined by calculating the OD_{260/280} ratio using a NanoDrop™ 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

3.3.2 Sequencing and assembly

An Illumina MatePaired (3-kbp insert size) genomic DNA library was prepared and sequenced by the Micromon DNA sequencing Facility (Monash University, Australia)

using a Genome Analyzer IIX system (Illumina, San Diego, CA, USA). Two Oxford Nanopore long read genomic DNA libraries were independently prepared using the sequencing kit 2D SQK-LSK208 (Oxford Nanopore Technologies, Oxford, OX4 4GA, UK) according to the manufacturer's instructions using 1.6 or 2.3 µg of DNA sheared using a Covaris-g Tube. Sequencing data was generated on a MinION MK-I device fitted with a FLO-MIN106 flowcell (R9.4 chemistry) and processed using the ONT Albacore Sequencing Pipeline (version 1.2.1). To improve the accuracy of *de novo* assembly, 86079/7NS genomic DNA was digested with the restriction endonuclease *Afl*II using the OpGen enzyme kit and the MapCard kit and subjected to ArgusTM optical mapping (BGI, Hongkong). Optical mapping data were analysed using MapSolver (version 3.1). Oligonucleotides (Geneworks, Australia) were designed using Primer3 (version 2.3.4) to perform PCR amplifications to close gaps in the assembly and PCR amplicons were sequenced using the Sanger method at the Micromon DNA Sequencing Facility (Monash University, Australia).

3.3.3 *De novo* sequence assembly, gene prediction and annotation

MatePaired Illumina reads were filtered using Neson 0.122 to trim bases below Phred quality 20, and remove the adapter sequences and reads with a length less than 91 bp (<https://pypi.python.org/pypi/neson/0.122>). The quality of the filtered reads was confirmed using FastQC (Andrews, 2010). The Illumina reads were then *de novo* assembled using Velvet (Zerbino and Birney, 2008) (version 1.2.10) using k-mer values of 81 and 91 and coverage cut-off values of 5, 10, 20, 50, 100 and 200.

The Nanopore 2D reads with a minimal length of 1000 bp were extracted from a set of fast5 files returned by the Albacore basecaller and converted into '.fasta' format using the Poretools program (Loman and Quinlan, 2014). The Nanopore reads were then *de novo* assembled using Canu 1.4 (Berlin et al., 2015) with the parameter

genomesize=0.9m and errorRate=0.1. The Illumina short reads were mapped against the Nanopore contig using the Geneious[®] v.7.0.6 (Kearse et al., 2012) sequence manipulation suite to generate the consensus sequence. Automatic annotation of the MS-H sequence was performed on the RAST webserver with default parameters for mycoplasmas (Aziz et al., 2008).

3.3.4 Genome comparison and analysis

Whole genome alignment of MS-H and 86079/7NS was performed using MAUVE (Darling et al., 2004). General nucleotide sequence alignments were conducted with MAFFT v.7.017. Screening for synonymous and non-synonymous SNPs was conducted with Find Variations/SNPs in Geneious[®]. Secondary structures of the deduced protein sequences were predicted using EMBOSS 6.5.7 (Rice et al., 2000). The genome sequences of *M. synoviae* strains MS53 and WVU-1853 (GenBank accession numbers AE017245 and CP011096) were also included as references for analysis of SNPs.

3.4 Results and Discussion

3.4.1 A combination of Illumina and Nanopore sequence reads was used for assembly of the highly repetitive *vlhA* pseudogene locus

A set of 12,817,968 Illumina MatePaired reads from strain 86079/7NS were *de novo* assembled using Velvet with a series of K-mer and coverage cutoff settings. With the best K-mer and cutoff values of 91 and 10, 55 contigs were produced, with sizes ranging from 181 bp to 569,106 bp. The contig sequences were converted into *in silico* *Afl*II restriction endonuclease cleavage maps and aligned with the 86079/7NS optical map to determine their locations and orientations during assembly. However, the high similarity and repetitive nature of insertion sequences (IS), prevented assembly of many of the IS-carrying genomic regions solely from the Illumina datasets. Sanger sequencing of 38 PCR products combined with scaffolding of the Velvet data generated a 766,256

bp contig, representing an almost complete genome, but failed to assemble the *vlhA* pseudogene region, a 50 kb locus containing a large number of highly repetitive sequences. To resolve the *vlhA* pseudogene region and circularise the 86079/7NS genome, a set of 12,991 Nanopore reads with a minimal length of 1000 bp were independently assembled. After trimming two short overlapping regions at both ends, the assembly was circularised into a single 809,647 bp contig. Due to the high error rate of Nanopore sequences, the Illumina reads were mapped against the contig, revealing an average depth of 1776 ± 305 . The extraction of the consensus sequence resulted in a fully assembled genome encompassing the entire *vlhA* cluster and all the other regions that couldn't be assembled solely from Illumina reads. The previous Velvet contigs were then aligned with the new Illumina/Nanopore hybrid assembly. Most discrepancies were found in the highly repeated transposase genes, to which the misassembly of the Velvet contigs was attributable. All nucleotide discrepancies between these two datasets were resolved manually by inspecting the alignments of Illumina reads or Sanger sequences of PCR products to produce a finished genome assembly. Single molecule restriction endonuclease cleavage fragments generated using *Afl*III were converted into a circular optical map using the OpGen optical map assembler, with a total size of 0.803 Mb, covering 97.69% of the genome. Using OpGen MapSolver (v.3.1), the complete 86079/7NS genome sequence was converted *in silico* into an *Afl*III restriction map and aligned to its optical map (Figure 3.1). The complete genomic sequence of 86079/7NS was in complete agreement with the optical map, confirming the accuracy of the assembly.

3.4.2 MS-H and 86079/7NS share almost identical genomic sequences

The genome of the vaccine parent strain, 86079/7NS, was 818,795 bp in length and highly similar to that of MS-H, with no multinucleotide insertions or deletions, or genomic rearrangements (except for the *vlhA* expressible region) (Figure 3.2). In our

previous study, a 55 kb genomic inversion delimited by a tRNA^{Gly} and an *IS30* was identified in MS-H (see Chapter 2). The same inverted region was also noted in 86079/7NS, which confirmed that the genomic inversion was not introduced by chemical mutagenesis, but occurred naturally at an earlier stage. A number of genes thought to be unique to MS-H (see Chapter 2), including a deoxyribose-phosphate aldolase (*deoC*) fragment, an ATP-dependent DNA helicase, and an N-acetylneuraminate lyase variant (*nanA*), were also found in 86079/7NS, suggesting that these genes were horizontally acquired by the vaccine parental strain or its ancestor. Whether the presence of these genes is a common feature of Australian *M. synoviae* strains remains to be explored. The most distinctive region in the MS-H and 86079/7NS genomes was located in the expressed *vlhA* gene (Bencina, 2002; Narat et al., 1998). The *vlhA* gene from MS-H shared 81% nucleotide and 75.4% amino acid sequence identity with 86079/7NS, which further highlights the variable nature of this gene (Noormohammadi et al., 2000). The predicted VlhA protein sequence diverged from residue 224 to the carboxyl terminus, similar to those previously described in a variant *vlhA* gene of strain WVU-1853 (Noormohammadi et al., 2000). Clonal variations of the VlhA protein are common in *M. synoviae* and may influence cytoadherence in the host and therefore contribute to disease (May et al., 2014). In *M. synoviae*, *vlhA* pseudogenes are used as a repertoire to generate sequence diversity in the expressed *vlhA* gene (Noormohammadi et al., 2000). The *vlhA* pseudogenes are highly conserved between MS-H and its parent, except for a 'G→A' mutation at nucleotide 255672 and a deletion of two bases (TT) before nucleotide 258581 in MS-H. In addition, a total of 33 mutations were distributed across the remainder of the genome of MS-H. The majority of the mutations (85%) were caused by transitions, with a strong preference for 'G→A' substitutions (Table 3.1). The extreme bias in single base substitutions confirmed one of the properties of the mutagen, NTG, which mainly leads to transition mutations, as observed in previous studies (Gordon et al., 1988; Ohnishi et al., 2008).

3.4.3 A few genetic mutations found in MS-H may have functional significance

Of the 33 SNPs identified between virulent strain 86079/7NS and MS-H, 26 were located in putative coding regions, and 7 were found in intergenic spaces where a coding sequence with an unknown function was located downstream. No coding sequence had a SNPs resulting in more than one amino acid change. While synonymous SNPs (sSNPs) may have functional consequences in rare cases (Kimchi-Sarfaty et al., 2007), nonsynonymous SNPs (nsSNPs), frameshifts, or intergenic SNPs that affect gene expression or protein structure are the most likely causes of phenotypic variation. An apparent bias in the numbers of nsSNPs was noted, in favour of those that introduced amino acid substitutions or frameshifts that might cause differences in protein functions between MS-H and 86079/7NS (Table 3.1). These affected genes were grouped into categories involved in amino acid metabolism, environmental sensing, central metabolism, transport, tRNA metabolism and nucleic acid metabolism. A total of 11 substitutions were predicted to have disrupted the secondary structures of the deduced proteins and were therefore subjected to further sequence analysis to predict the potential effects of the 11 substitutions on the deduced proteins described in this chapter (Table 3.1).

In *M. synoviae*, the *vlhA* locus was flanked by identical homologues of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which were putatively transferred from *M. gallisepticum* (Vasconcelos et al., 2005) and share 100% amino acid similarity amongst *M. synoviae* strains 86079/7NS, WVU-1853 and MS53. However, each of the GAPDH coding genes in MS-H was found to carry a mutation that introduced an amino acid change in the product. GAPDH is a NAD⁺ dependent glycolytic enzyme that catalyses the conversion of glyceraldehyde 3-phosphate to 1, 3-diphosphoglycerate, and can be located in the cytoplasm as well as on the cell surface (Tunio et al., 2010). In addition to its metabolic role, surface-localised GAPDHs from several bacterial species,

including *Streptococcus pneumoniae* (Ling et al., 2004), *Staphylococcus aureus*, *Staphylococcus epidermidis* (Modun and Williams, 1999), *Listeria monocytogenes* (Schaumburg et al., 2004) and *Escherichia coli* (Egea et al., 2007), have also been demonstrated to have a role in pathogenesis, including facilitating host immune evasion (Terao et al., 2006) and colonisation and invasion of host tissues (Alvarez et al., 2003). It is worth noting that the conservative substitutions, A185V and R306K, caused by the two nsSNPs, each in one of the two copies of the GAPDH gene (nt 242451 and nt 296526, respectively), were located in the catalytic domain of the enzyme, which encompasses residues 149 to 333 (Branlant and Branlant, 1985; Walker et al., 1980). One substitution was found located in the S loop (residues 178-201), which is believed to be associated with the thermal stability of the enzyme (Walker et al., 1980). However, as the biological structure of GAPDH has not been well characterised in *M. synoviae*, whether such amino acid changes can disrupt the core binding motifs and therefore affect function still need to be explored.

In MS-H, a single base pair substitution within a gene coding for a protein homologous to haemolysin C (HlyC) resulted in a change from alanine to valine in the C-terminal region of the protein. Haemolysins are potential virulence factors in mollicutes (Chambaud et al., 2001). Membrane-associated haemolysin activity is usually dependent on the conversion of a nontoxic prohemolysin, proHlyA, to toxic hemolysin, HlyA, by an internal protein acyltransferase, HlyC, using the acyl-acyl carrier protein (ACP) as the essential acyl donor (Minion and Jarvill-Taylor, 1994; Trent et al., 1999). These proteins are known to be present in *Mycoplasma pulmonis* (Chambaud et al., 2001), *Ureaplasma urealyticum* (Glass et al., 2000) and *Mycoplasma penetrans* (Sasaki et al., 2002). However, although orthologues of HlyC and ACP synthase were found in *M. synoviae*, a CDS for HlyA was not identified and thus a catalyst performing the function of HlyC remains elusive.

M. synoviae encodes a VACB-like ribonuclease II (RNase R, the gene product of *vacB*), the orthologues of which have been implicated in virulence in several pathogens (Cheng et al., 1998; Erova et al., 2008; Tobe et al., 1992; Tsao et al., 2009). In MS-H, a nsSNP (nt 352952) was found in the *vacB* gene. BLAST-P analysis indicated that the *vacB* mutation occurred at a serine residue in MS-H that was highly conserved across the orthologues from other mycoplasmal species. Even though a role for this protein in virulence in *M. synoviae* is yet to be established, RNase R has been shown to be essential for the growth of *M. genitalium* (Hutchison et al., 1999; Lalonde et al., 2007).

Non-conservative amino acid substitutions were also identified in three other putative proteins associated with nucleic acid metabolism, including *uvrB* (G26E), *rpoC* (R1270I) and *dnaE* (S103F), which are involved in DNA repair, transcription of RNA, and replication/repair, respectively. Mutations in the *uvrB* gene of *Mycobacterium tuberculosis* have been shown to increase sensitivity to acidified nitrite, a reactive nitrogen intermediate in the phagosome that helps protect the host during infection (Darwin et al., 2003) and an insertion mutation in this gene attenuated virulence in mice (Darwin and Nathan, 2005). A mutation (G to A) in the *rpoC* gene of *E. coli* leading to the substitution D1033G in the RNA polymerase beta' subunit causes a temperature-dependent increase in chromosome copy number (Rasmussen et al., 1983) through effects on chromosomal replication control by altering the expression of the *dnaA* gene (Petersen and Hansen, 1991). DnaE is the principal chromosomal replication enzyme in many bacteria, although it is also involved in DNA repair in some mycoplasmas (Hutchison et al., 1999; Koonin and Bork, 1996). In *E. coli* the N-terminal region of DnaE has been shown to be responsible for its catalytic activity (Kim et al., 1997). A single amino acid substitution within the N-terminal region of DnaE results in a *ts*⁺ phenotype in both *E. coli* (Flett et al., 1999) and *Caulobacter crescentus*, possibly by

affecting the initiation of DNA replication (Lo et al., 2004). As very little is known about the mechanism of chromosomal replication and its regulation in *M. synoviae*, it would be interesting to find out whether the mutations detected in nucleotide metabolism related genes in MS-H had an influence on its chromosomal replication and therefore its growth rate under permissive/non-permissive temperatures.

In MS-H, a non-conservative H275Y substitution was identified within the gene encoding KtrB, the membrane-spanning subunit of the Na⁺/K⁺ symporter KtrAB, which mediates Na⁺-dependent high affinity K⁺ uptake in many prokaryotes (Durell et al., 1999; Johnson et al., 2009; Nakamura et al., 1998). K⁺ transport is especially important for cytoplasmic pH homeostasis for bacteria exposed to an alkaline external pH (Nakamura et al., 1984). A substitution in the P-loop selectivity filter in KtrB, resulting in decrease in affinity for K⁺, has been seen in *Vibrio alginolyticus* (Lü and Miller, 1995; Tholema et al., 1999). Alignment with the consensus sequence of KtrB of *B. subtilis* (Albright et al.) revealed that the H275Y mutation of MS-H was not located within the TM-P-loop-TM domain, indicating that this mutation might be tolerated in *M. synoviae* and was less likely to be linked with the attenuation of virulence seen in MS-H.

3.4.4 A frameshift mutation in *oppF-1* may result in the loss of function of its product

The deletion of one nucleotide (T) at position 397721 led to a codon frameshift in the *oppF* gene in MS-H. OppF is proposed to act as the ATP-binding component of an ABC transporter involved in uptake of oligopeptides. The frameshift is likely to have inactivated protein function by interrupting the coding sequence, allowing translation of only the amino-terminal 156 amino acids. Among the molecular factors associated with the virulence of pathogenic bacteria, the importance of oligopeptide permease (Opp)

transporters has been highlighted in both Gram negative and Gram positive pathogens (Eitinger et al., 2011; Jones et al., 2014; Moraes et al., 2014). These multi-subunit protein complexes belong to the ABC transporter family, and are typically composed of an extracellular substrate binding lipoprotein, OppA, two transmembrane proteins, OppB and OppC, forming the pore, and two ATP-binding proteins, OppD and OppF (Garai et al., 2017). Studies on several pathogenic bacteria have indicated that Opp proteins are involved not only in importing peptides as nutrients, but also in adhesion to the host, acting as colonisation and virulence factors (Cundell et al., 1995; Moraes et al., 2014; Podbielski et al., 1996; Samen et al., 2004; Tseng et al., 2017a). In the case of *M. tuberculosis*, the Opp system was shown to be required for chronic infection and for expression of virulence-associated surface lipid (Flores-Valdez et al., 2009) and *opp* mutants exhibited *in vivo* survival defects, indicating diminished virulence of the organism (Flores-Valdez et al., 2009; Sassetti and Rubin, 2003). Partially impairment of OppD₁ function in *M. gallisepticum* strain Ap3AS, reduced colonisation and virulence. A mutant that could express the N-terminal 34% of OppD₁ was less pathogenic than a mutant that could express only the N-terminal 7.9% of OppD₁ (Tseng et al., 2017a). *M. synoviae* possesses two Opp systems that are located distantly from each other within the chromosome, hereafter named *opp-I* and *opp-II*. The phylogenetic relationships between *opp* operons within the same species are complex, although it has been suggested that the *opp* operons in the Hominis group underwent duplication and divergence. Homologous proteins encoded by the two gene clusters share low sequence similarity, possibly indicating that they have different biological functions. It is tempting to speculate that in MS-H the pseudogenisation of *oppF* in *opp-I* is not compensated for by the *opp-II* copy, which could result in attenuation of virulence. Alternatively, as has been hypothesized for OppD₁ in *M. gallisepticum* (Tseng et al., 2017a), it is possible that, as the two OppF proteins form dimers in the transport complex, the product of the partial copy of *oppF-I* in MS-H may retain the capacity to

form a less functional heterodimer with the product of full-length copy of *oppF-II*, resulting in less efficient transportation and thus a decrease in virulence.

An substitution (G123R) within Obg has been predicted to be one of the causes of the *ts*⁺ phenotype of MS-H (Shahid et al., 2013b). However, the *ts*⁺ MS-H strain can occasionally revert to a *ts*⁻ phenotype, possibly due to the selection pressure in the field (Markham et al., 1998c). Although the loss of the *ts*⁺ phenotype didn't result in full reversion to the level of virulence of the original parent strain 86079/7NS, a partial increase in virulence was seen in different MS-H *ts*⁻ clones (Noormohammadi et al., 2003), suggesting that factors other than the *ts*⁻ phenotype are also associated with the attenuation of the MS-H vaccine strain. The *ts*⁻ reisolate MS-H⁴ caused upper tracheal mucosal thicknesses that were significantly greater than those caused by *ts*⁻ MS-H⁵, although they were both less pathogenic than the parental strain (Markham et al., 1998c; Noormohammadi et al., 2003). Comparative sequence analyses of the *oppF-1* gene revealed that the frameshift mutation within *oppF-1* was present in MS-H⁵, but not found in MS-H⁴ (Figure 3.3). It is tempting to speculate that the mutation in *oppF-1* could have resulted in a greater reduction of virulence observed in MS-H⁵, indicating that the mutation in *oppF-1* could be another factor that might slow down growth without affecting survival, and therefore contribute to the attenuation of the vaccine strain.

3.4.5 SNPs for discriminating infected from vaccinated animals (DIVA assays)

As MS-H has been used widely to prevent disease caused by virulent *M. synoviae*, differentiation of the vaccine strain from field isolates would be useful during vaccination and eradication programs. Comparative genomic sequence analyses of strains MS-H, 86079/7NS, combined with two other field strains, WVU-1853 and MS53 (the only two field strains of *M. synoviae* for which full genome sequences are

available in GenBank), revealed 30 SNPs unique to the MS-H vaccine strain, which were located in regions conserved between the strains (Table 3.2). These SNPs could be used as novel vaccine markers to develop diagnostic assays, such as PCR-HRM, for discriminating MS-H from wild-type *M. synoviae* strains. As the MS-H vaccine is now widely used in a large number of countries and back mutation within MS-H may occasionally occur under selective pressure in the field, it would be ideal to perform complete genome sequencing of MS-H reisolates from different geographical locations to confirm the stability of the SNPs unique to MS-H. Several molecular techniques, based on sequence analyses of the conserved region of the *vlhA* gene, are currently available for the discrimination of *M. synoviae* strains (Bayatzadeh et al., 2014; Dijkman et al., 2014; El-Gazzar et al., 2012; Hammond et al., 2009; Harada et al., 2009; Hong et al., 2004; Jeffery et al., 2007; Wetzel et al., 2010). However, given that several Australian and possibly two European isolates share the same *vlhA* sequence type as MS-H (Catania et al., 2016b; Dijkman et al., 2014), the discriminatory power of *vlhA* sequence typing for molecular epidemiological studies of *M. synoviae* isolates has limitations. A number of studies have therefore used the mutations found in the *obg* gene for discriminating MS-H from field strains (Dijkman et al., 2017; Shahid et al., 2014b). As there are Australian field strains that are identical to the MS-H vaccine, as well as rare cases where *ts* MS-H re-isolates are identical in the targeted region of the *obg* gene, the differentiation of the MS-H vaccine from field strains using these techniques may not be totally reliable, especially in Australia. Therefore, development of a typing technique with improved discriminatory power based on novel genetic markers of the MS-H vaccine is desirable.

3.5 Conclusion

Few virulence determinants or virulence-associated determinants have been identified in *M. synoviae*. Elucidation of the genetic basis of attenuation of the MS-H vaccine will contribute to understanding the pathogenic mechanisms employed by *M. synoviae*, and

to the development of improved vaccines and better strategies for control of the disease it causes. The genomic comparison of MS-H and its wild-type progenitor 86079/7NS was a first step in the identification of potential *M. synoviae* virulence factors. The SNPs with putative functional effects identified by comparison of the two strains have confirmed that the attenuation observed in MS-H is likely to be the result of the cumulative and complex effects of several mutations. As predicting the impact of intergenic SNPs or nsSNPs is problematic without experimental validation, we have adopted a conservative approach in the interpretation of the SNP data and instead see them as the basis for facilitating future focused studies on the basis of attenuation of MS-H. A total of 30 SNPs were found to be unique to MS-H. These results have provided a rich source of novel biomarkers that can be used to develop new genotyping assays for discrimination of MS-H from field strains.

Table 3.1. Nucleotide and coding differences identified between the genomes of MS-H and 86079/7NS*

Position (nt) ^a		Nucleotide difference (MS-H/86079/7NS) ^b	Protein/region	Functional category	Effect on MS-H coding ^c
MS-H	86079/7NS				
14181	14181	A/G	tRNA (Guanine-N1) - methyltransferase	tRNA metabolism	Val→Ile, hydrophobic to hydrophobic
36932	36932	T/A	Non-coding region		
61685	61685	A/G	Topoisomerase IV subunit A	Nucleic acid metabolism	Glu→Lys, acidic to basic
62874	62874	A/G	Excinuclease ABC subunit B	Nucleic acid metabolism	Gly→Glu, hydrophobic to acidic ^d
67028	67028	A/G	tRNA-Trp	tRNA metabolism	
104704	104704	A/G	Non-coding region		
107765	107765	A/G	Multiple sugar ABC transporter, ATP-binding protein	Transport	Ala→Val, hydrophobic to hydrophobic
193771	193773	A/G	GTP-binding protein Obg	Environmental sensing	Gly→Arg, hydrophobic to basic
201094	201096	A/G	p80-related protein	Unknown	Pro→Ser, hydrophobic to polar
203205	203207	A/C	Non-coding region		
242451	242453	A/G	NAD-dependent glyceraldehyde-3-phosphate dehydrogenase	Glucose metabolism	Ala→Val, hydrophobic to hydrophobic ^d
296526	296469	A/G	NAD-dependent glyceraldehyde-3-phosphate dehydrogenase	Glucose metabolism	Arg→Lys, basic to basic ^d
352952	352895	A/G	VACB-like ribonuclease II	Nucleic acid metabolism	Ser→Phe, polar to hydrophobic
389629	389571	A/G	Aspartate-ammonia ligase	Amino acid metabolism	Ala→Thr, hydrophobic to hydrophobic
397778	397721	—/T	Oligopeptide transport ATP-binding protein OppF	Transport	Frameshift ^d
433343	433287	A/G	Alanyl-tRNA synthetase	tRNA metabolism	Asp→Asn, acidic to polar
438657	438601	A/G	Triacylglycerol lipase	Lipid metabolism	Synonymous substitution
481287	481231	A/G	Haemolysin C	Structural Protein	Ala→Val, hydrophobic to hydrophobic ^d
498421	498365	C/T	Histidyl-tRNA synthetase	tRNA metabolism	Thr→Ala, polar to hydrophobic ^d

Table 3.1. Continued

502506	502449	C/—	Non-coding region		
502827	502770	—/TA	Non-coding region		
522899	522844	A/G	Hypothetical protein	Unknown	Gly→Glu, hydrophobic to acidic ^d
563391	563336	A/G	Hexosephosphate transport protein	Transport	Leu→Phe, hydrophobic to hydrophobic
567729	567674	A/C	DNA-directed RNA polymerase beta' subunit	Nucleic acid metabolism	Arg→Ile, basic to hydrophobic ^d
584838	584783	A/G	Hypothetical protein	Unknown	Synonymous substitution
615741	615686	A/G	Potassium uptake protein KtrB	Transport	His→Tyr, basic to polar ^d
628272	628217	A/G	DNA polymerase III alpha subunit	Nucleic acid metabolism	Ser→Phe, polar to hydrophobic
679814	679759	A/G	Cation-transporting P-type ATPase	Transport	Synonymous substitution
686265	686210	A/G	Non-coding region		
716623	716568	A/G	Hypothetical protein	Unknown	Ser→Leu, polar to hydrophobic ^d
737180	737125	A/G	Hypothetical protein	Unknown	Synonymous substitution
780237	780182	—/AT	Non-coding region		
794057	794004	A/G	Uridylate kinase	Central metabolism	Val→Ile, hydrophobic to hydrophobic

* *vlhA* locus was excluded from SNP analysis.

^a nt, nucleotide

^b Specific nucleotides; '—' indicates absence of nucleotide(s)

^c aa, amino acids

^d Amino acid substitution putatively affecting predicted secondary structure of the protein

Table 3.2. Unique single nucleotide variations detected in *M. synoviae* strains MS-H, 86079/7NS, WVU-1853 and MS53

Nucleotide position ^a	MS-H	86079/7NS	WVU-1853	MS53	Gene ^b	Protein/region
14181	A	G	G	G	<i>trmD</i>	tRNA (guanine-N1)-methyltransferase
36932	T	A	C	A		Non-coding region
61685	A	G	G	G	<i>parC</i>	Topoisomerase IV subunit A
62874	A	G	G	G	<i>uvrB</i>	Excinuclease ABC subunit B
67028	A	G	G	G		tRNA-Trp
104704	A	G	G	G		Non-coding region
107766	A	G	G	G		Multiple sugar ABC transporter, ATP-binding protein
193771	A	G	G	G	<i>obg</i>	GTP-binding protein Obg
201094	A	G	G	G		p80-related protein
203205	A	C	C	C		Non-coding region
242451	A	G	G	G	<i>gap</i>	NAD-dependent glyceraldehyde-3-phosphate dehydrogenase
296526	A	G	G	G	<i>gap</i>	NAD-dependent glyceraldehyde-3-phosphate dehydrogenase
352952	A	G	G	G	<i>vacB</i>	VACB-like ribonuclease II
389629	A	G	G	G		Aspartate--ammonia ligase
397778	-	T	T	T	<i>oppF-1</i>	Oligopeptide transport ATP-binding protein OppF
433343	A	G	G	G	<i>alaS</i>	Alanyl-tRNA synthetase
438657	A	G	G	G	<i>lip3</i>	Triacylglycerol lipase
481287	A	G	G	G	<i>hlyC</i>	Haemolysin C
498421	C	T	T	T	<i>hisS</i>	histidyl-tRNA synthetase
522899	A	G	G	G		Hypothetical protein
563391	A	G	G	G		Hexosephosphate transport protein
567729	A	C	C	C	<i>rpoC</i>	DNA-directed RNA polymerase beta' subunit
584838	A	G	G	G		Hypothetical protein
615741	A	G	G	G	<i>ptrB</i>	Potassium uptake protein KtrB
628272	A	G	G	G	<i>dnaE</i>	DNA polymerase III alpha subunit
679814	A	G	G	G	<i>mgta</i>	cation-transporting P-type ATPase
686265	A	G	G	G		Non-coding region
716623	A	G	G	G		Hypothetical protein
737180	A	G	G	G		Hypothetical protein
794057	A	G	G	G	<i>pyrH</i>	uridylate kinase

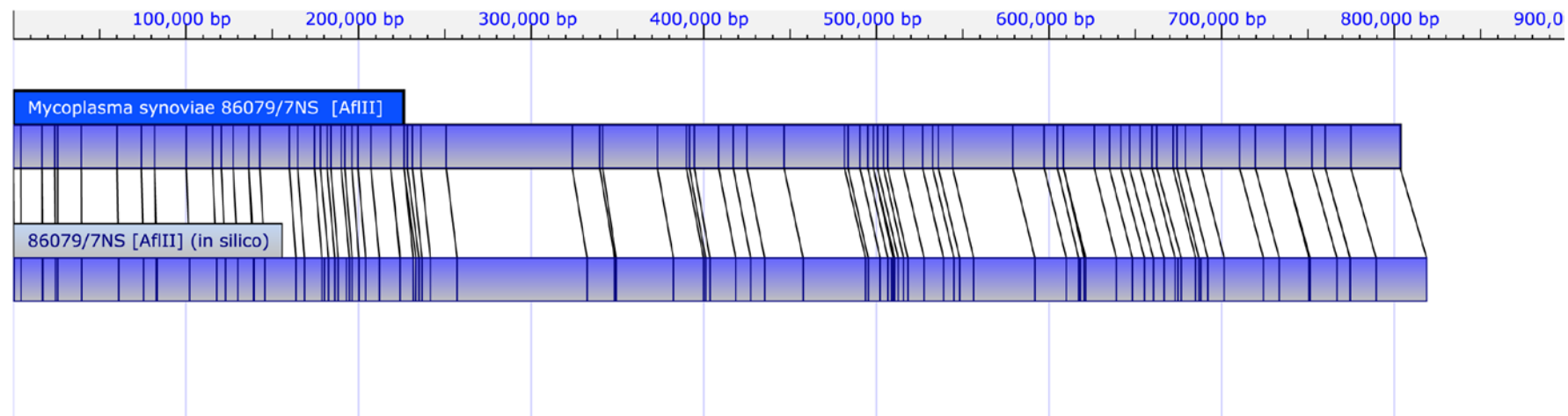


Figure 3.1. Mapping of complete genomic sequence of 86079/7NS against its optical map



Figure 3.2. MAUVE alignment of the complete genomic sequences of *M. synoviae* strains 86079/7NS and MS-H

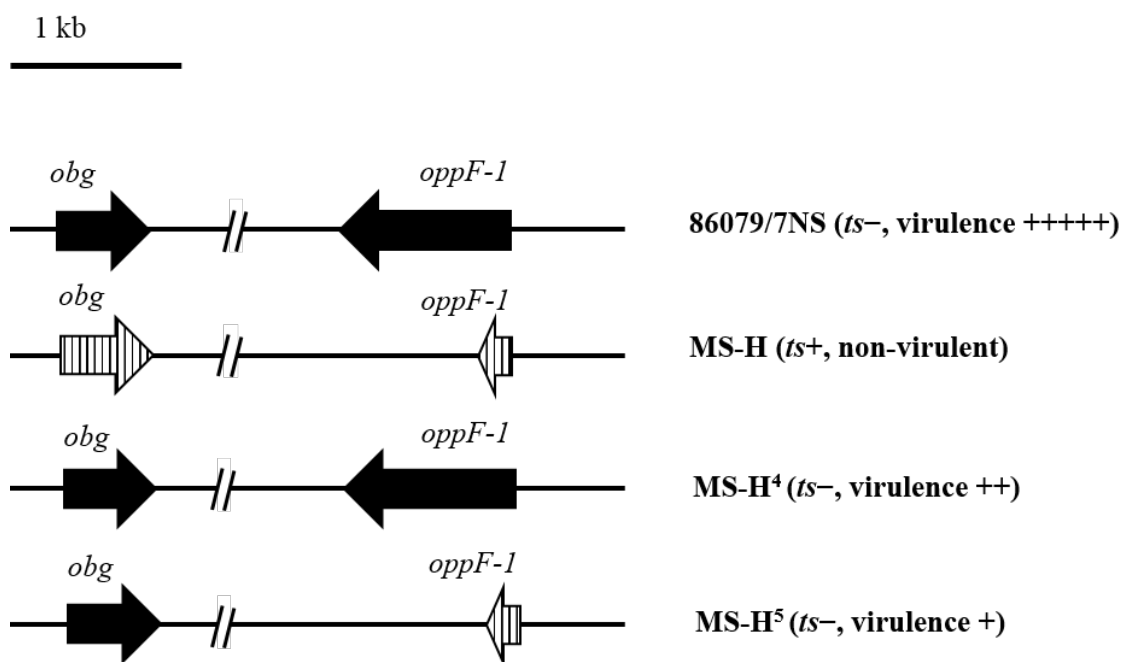


Figure 3.3. Genotypes of *oppF-1* and *obg* genes in 86079/7NS, MS-H and MS-H re-isolates. Solid arrows represent wild-type genes. Vertically hatched arrows represent vaccine-type genes.

CHAPTER 4. Identification of A New Genetic Marker in the *Mycoplasma synoviae* Vaccine Strain MS-H and Development of a Strategy using Polymerase Chain Reaction and High-Resolution Melting Curve Analysis for Differentiating MS-H from Field Strains

This chapter has been published in a peer-review journal (Zhu, L., Konsak, B.M., Olaogun, O.M., Agnew-Crumpton, R., Kanci, A., Marenda, M.S., Browning, G.F. and Noormohammadi, A.H., 2017. Identification of a new genetic marker in *Mycoplasma synoviae* vaccine strain MS-H and development of a strategy using polymerase chain reaction and high-resolution melting curve analysis for differentiating MS-H from field strains. Vet. Microbiol. 210, 49-55). Supplementary information related to this chapter is given in footnotes.

4.1 Abstract

Mycoplasma synoviae (MS) is an economically important avian pathogen worldwide, causing subclinical respiratory tract infection and infectious synovitis in chickens and turkeys. A temperature-sensitive (ts^+) live attenuated vaccine MS-H, derived from the Australian field strain 86079/7NS, is now widely used in many countries to control disease induced by MS. Differentiation of the MS-H vaccine from field strains is crucial for monitoring vaccination programs in commercial poultry. Comparison of genomic sequences of MS-H and its parent strain revealed an adenine deletion at nucleotide position 468 of the MS-H *oppF-1* gene. This mutation was shown to be unique to MS-H in further comparative analyses of *oppF-1* genes of MS-H re-isolates and field strains from Australia and other countries. Based on this single nucleotide, a combination of

nested PCR and high-resolution melting (HRM) curve analysis was used to evaluate its potential for use in differentiation of MS-H from field strains. The mean genotype confidence percentages of 99.27 and 48.20 for MS-H and field strains, respectively, demonstrated the high discriminative power of the newly developed assay (*oppF* PCR-HRM). A set of 13 tracheal swab samples collected from MS-H vaccinated specific pathogen free birds and commercial chicken flocks infected with MS were tested using the *oppF* PCR-HRM test and the results were totally consistent with those obtained using *vlhA* genotyping. The nested-PCR HRM method established in this study proved to be a rapid, simple and cost-effective tool for discriminating the MS-H vaccine strain from Australian and international strains in pure cultures and on tracheal swabs.

4.2 Introduction

Mycoplasma synoviae (MS) is an economically important avian pathogen worldwide, causing subclinical respiratory tract infection, synovitis in chickens and turkeys (Ferguson-Noel and Noormohammadi, 2013), and eggshell apex abnormalities (EAA) in layer chicken flocks (Feberwee et al., 2009a). A temperature-sensitive (*ts*⁺) vaccine, MS-H (Vaxsafe MS[®]; Bioproperties Ltd., Ringwood, Victoria, Australia), is widely used in many countries to prevent infection with virulent strains of MS. The MS-H vaccine was derived by chemical mutagenesis of the Australian field isolate 86079/7NS (Morrow et al., 1998) and only colonises the upper respiratory tract of the birds. Differentiation of the MS-H vaccine from field strains is important for evaluation of the efficacy of the MS-H vaccine, especially when field strains may be present.

Several genotyping techniques have been developed to differentiate MS strains. Restriction fragment length polymorphism (RFLP) of whole genomic DNA has been used for classification of MS strains (Morrow et al., 1990b). However, RFLP is time consuming and can sometimes be unreliable because of high frequency DNA rearrangements in some regions of the genome (Noormohammadi et al., 2000). Several

polymerase chain reaction (PCR)-based assays have been developed for differentiation of MS strains. Earlier PCRs were based on 16S rRNA genes (Buim et al., 2010) or the 16S-23S rRNA intergenic spacer region (Ramírez et al., 2011), but more recently the conserved region of the variable lipoprotein and hemagglutinin (*vlhA*) gene has been targeted for MS genotyping (Hammond et al., 2009; Hong et al., 2004; Wetzel et al., 2010). However this region cannot differentiate MS-H from a number of Australian field strains (Jeffery et al., 2007). Recently, single nucleotide polymorphisms (SNPs) found in the *obg* gene of the MS-H vaccine (Shahid et al., 2013b) have been used in a nested PCR, followed by HRM curve analysis, to differentiate the *ts*⁺ MS-H vaccine strain from rare non-temperature sensitive (*ts*⁻) re-isolates and field strains (Shahid et al., 2014b). Some of these SNPs have also been targeted for differentiation of the vaccine from field strains using melting curve and agarose gel based mismatch amplification mutation assays (Kreizinger et al., 2015) or real time quantitative PCR (Dijkman et al., 2017). However, because there are occasional *ts*⁻ MS-H re-isolates and Australian field strains that are identical to the MS-H vaccine in the sequence targeted by these assays, differentiation of the MS-H vaccine from field strains based on the *obg* gene may not be totally reliable, especially in Australia.

We have recently compared partial genome sequences of MS-H and its parent strain 86079/7NS, and detected an adenine deletion at nucleotide position 468 of the *oppF-1* gene in MS-H (unpublished data). To confirm whether this mutation also occurred in all MS-H re-isolates, MS-H re-isolates and field strains from Australia and other countries were examined. A simple and rapid technique based on PCR and HRM curve analysis was developed and evaluated for its capacity to differentiate the MS-H vaccine from field strains.

4.3 Materials and Methods

4.3.1 MS strains and growth media

All MS strains used in this study are listed in Table 4.1. A total of 13 cloned MS strains and 13 swabs from clinical cases were used. Tracheal swabs (MS-Hv1-5) were collected from specific pathogen free (SPF) chickens used for serial *in vivo* passage of MS-H. The origins of strains F10-2AS, K1938, FMT, K870, K1858, 1853M, WVU-1853, YA, K1968 and K1723 were described previously (Jeffery et al., 2007). DNA extracted from 8 field birds infected with MS field strains were also used. These specimens were diagnostic clinical samples submitted to our laboratory. The *vlhA* genotype was determined as described previously (Jeffery et al., 2007) using PCR-HRM curve analysis followed by sequencing of the 5' conserved region of the *vlhA* gene. All MS cultures used in this study were grown in mycoplasma broth (MB) containing 10% swine serum and 0.01% (w/v) nicotinamide adenine dinucleotide (NAD), as described previously (Whithear, 1993).

4.3.2 DNA extraction

DNA was extracted from cultures of MS by centrifuging 500 µl of culture at $12,000 \times g$ for 5 min and subjecting the resultant cell pellet to DNA extraction as described below for tracheal samples. Tracheal swabs taken from vaccinated or infected birds were placed in 500 µl of RLT lysis buffer (Qiagen, Chadstone, VIC, Australia) containing 1% β -mercaptoethanol and incubated at 4°C overnight. A 15 µl volume of Qiaex II matrix (Qiagen) and 300 µl of 70% ethanol were added to the lysate, and the solution mixed by vortexing. This suspension was then loaded onto a Multispin MSK-II column (Axygen, Union City, CA, USA) placed in a 1.5 ml microfuge tube. Columns were centrifuged at $10,000 \times g$ for 30 sec and the flow-through discarded. The matrix was washed once with 600 µl of RW1 buffer and twice with 500 µl of RPE buffer. For each wash, columns were centrifuged at $10,000 \times g$ for 30 sec. The columns were dried by centrifugation at

14,000 × g for 1 min, then 50 µl of nuclease-free water was loaded onto the columns. After 5 min incubation at room temperature, columns were centrifuged at 10,000 × g for 1 min and the eluted DNA was collected. The DNA samples were used immediately or stored at -20 °C prior to use in PCR assays.

4.3.3 Oligonucleotide primers

The oligonucleotide primers (Geneworks, Thebarton, SA, Australia) used in this study were designed using Primer3 (version 2.3.4) (Rozen and Skaletsky, 1999) and AmplifX 1.7.0 (<http://crn2m.univ-mrs.fr/pub/amplifx-dist>). Their sequences are listed in Table 4.2 and their locations are shown on Figure 4.1a. The specificity of primer pairs was verified individually using Primer-BLAST (Ye et al., 2012) with default settings and then tested for specificity by PCR using genomic DNA of MS and *M. gallisepticum* (MG).

4.3.4 PCR amplifications

Two sets of primers, oppF-F1/R1 and oppF-F2/R2, were designed and used to amplify a region carrying the SNP detected in *oppF-1* for sequencing and nested PCR, respectively. PCR reactions were performed in an iCycler thermal cycler (Bio-Rad, Gladesville, NSW, Australia). For both oppF-F1/R1 and oppF-F2/R2 PCRs, the 25 µl reaction mixture contained 0.4 µl of 25 µM solutions of each of the forward and reverse primers, 1 µl of 50 mM MgSO₄, 4 µl of a 1.25 mM solution of each dNTP (Promega, Alexandria, NSW, Australia), 0.1 µl (1 U) of High Fidelity Platinum[®] Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA), 2.5 µl of 10 × Platinum Taq DNA polymerase buffer (Invitrogen), 2 µl of extracted MS genomic DNA (~50 ng/µl) and 14.6 µl of nuclease free water. The reaction mixture was incubated at 94°C for 2 min, and then through 35 cycles of 94°C for 15 sec, 56°C for 30 sec and 68°C for 40 sec (oppF-F1/R1 PCR) or 10 sec (oppF-F2/R2 PCR). In each set of reactions, MG DNA and nuclease-free water were included as negative controls. All PCR products were analysed by electrophoresis through 1.5% agarose gels stained with GelRed[™] (Biotium,

Fremont, CA, USA) and visualisation by UV transillumination using the ChemiDoc™ Imaging System (Bio-Rad, Gladesville, NSW, Australia).

4.3.5 Nested *oppF* PCR-HRM

Oligonucleotide primers oppF-F3 and oppF-R3 were used to amplify a 40/41 bp internal region of *oppF-1* (harboring the SNP targeted in this study) from PCR products oppF-F2R2 (Figure 4.1a). PCR was performed using High Fidelity Platinum® Taq DNA polymerase (Invitrogen). The 25 µl reaction mixture contained 0.4 µl of 25 µM solutions of each of the oligonucleotide primers, 1 µl of 50 mM MgSO₄, 4 µl of a 1.25 mM solution of each dNTP (Promega), 0.1 µl (1 U) of High Fidelity Platinum Taq DNA polymerase (Invitrogen), 2.5 µl of 10 × Platinum Taq DNA polymerase buffer (Invitrogen), 1 µl of a 100 µM solution of SYTO 9 green fluorescent nucleic acid stain (Invitrogen), 2 µl of a 10 fold dilution of the oppF-F2R2 PCR product as template and 13.6 µl of nuclease-free water. The reaction was incubated at 94°C for 2 min, then through 35 cycles of 94°C for 15 sec, 45°C for 30 sec and 68°C for 5 sec. All reactions were carried out in duplicate or triplicate. In each set of reactions, a negative control containing nuclease-free water, instead of template, was included. After completion of PCR, HRM curve analysis was carried out using a RotorGene 6000 (Corbett Research, Mortlake, NSW, Australia) with optical measurements in the green channel using an excitation wavelength of 470 nm and detection at 510 nm. Melting-curve data were generated by increasing the temperature from 60°C to 80°C and recording fluorescence. Different ramp speeds of 0.05, 0.1, 0.3, and 0.5 °C/sec were used to optimise melting conditions for maximal differentiation of sequence differences. HRM curve analysis was performed using a Rotor-Gene 1.7.27 and the HRM algorithm provided within the software.

4.3.6 Nucleotide sequencing and analysis

The nucleotide sequences of the *oppF-1* regions of the MS strains MS53 and WVU-

1853 were retrieved from GenBank (Accession Nos. AE017245 and CP011096). For the other strains used in this study, *oppF*-F1R1 PCR products were purified using the AxyPrep DNA gel extraction kit (Axygen), eluted in 30 µl nuclease-free water and then subjected to Sanger sequencing in both directions using primers *oppF*-F1 and *oppF*-R1 at the Micromon DNA Sequencing Facility (Monash University, Australia). The nucleotide sequences were compared with each other and multiple sequence alignments were created using the ClustalW tool (Thompson et al., 1994) within Geneious® (version 7.0.6) (Kearse et al., 2012).

4.4 Results

4.4.1 Identification and selection of SNPs from MS genomic sequences

Comparative genomic analysis of the MS-H vaccine, its parent strain, 86079/7NS, and the MS strains MS53 and WVU-1853 revealed a number of SNPs (unpublished data). Only the SNPs that were detected in MS-H but absent from 86079/7NS, MS53 and WVU-1853 were targeted, based on their potential relevance to the attenuation of the vaccine. One SNP (A→del at position 468, according to GenBank accession no. AE017245), located in the gene *oppF-1*, resulted in a frameshift mutation in the predicted coding sequence in MS-H (Figure 4.2a). Therefore, this SNP was targeted for further examination with the aim of developing a novel marker for differentiation of the MS-H vaccine and field strains.

4.4.2 PCR amplification of selected regions of *oppF-1* from different MS strains

The specificity of the three primer pairs was verified by performing PCRs using genomic DNA of MS and MG ⁹. Amplicons of the anticipated size were generated from

⁹ Only DNA of MS and MG was used to confirm the specificity of primers in PCR assays. However, the specificity of primers against other avian mycoplasmas was checked using Primer-BLAST, as mentioned in 4.3.3.

all MS strains, but not from MG strain ts-11 nor the non-template negative control (Figure 4.1b). Two sets of oligonucleotide primers, oppF-F2/R2 and oppF-F3/R3 (Table 4.2), targeting 152 (151 in MS-H) and 41 (40 in MS-H) bp regions of *oppF-1*, were used in PCRs and the resultant amplicons were subjected to HRM curve analysis. Conventional melting curve analysis of the oppF-F2/R2 PCR amplicons using a ramp of 0.3 °C/sec showed that MS-H and 86079/7NS generated similar single peak curves at $74.22 \pm 0.1^{\circ}\text{C}$ and $74.16 \pm 0.03^{\circ}\text{C}$, respectively. The very small difference in melting temperature between the amplicons from two strains made it difficult to differentiate MS-H from other strains (data not shown). Therefore, oppF-F2/R2 HRM curve analysis was not examined further as a tool for differentiation of MS-H from other strains.

4.4.3 HRM curve analysis of the nested PCR amplicons of *oppF-1* can distinguish between 86079/7NS and MS-H cultures

The 40/41 bp oppF-F3R3 PCR products of 86079/7NS and MS-H harbouring the SNP (nt 468) were subjected to HRM curve analysis as described above. Conventional melting curves generated with different ramps revealed that a ramp rate of 0.3°C/sec yielded the most distinct curves, so this rate was used in all subsequent HRM curve analyses in this study. The conventional melting curve analysis detected a major peak at $66.07 \pm 0.05^{\circ}\text{C}$ for MS-H and of $66.62 \pm 0.07^{\circ}\text{C}$ for 86079/7NS (Figure 4.2b). When normalization¹⁰ was applied to the regions of 64.60-64.82°C and 68.14-68.31°C, the normalised curves separated MS-H and 86079/7NS into two distinct genotypes (Figure 4.2c). When genotyping was applied to the normalised HRM curve using MS-H as a reference genotype, the identity confidence percentages (C%) $\pm$ SD were $98.37 \pm 1.25\%$ for MS-H and $41.41 \pm 9.8\%$ for 86079/7NS, respectively. When a genotype C% threshold of 90% was applied, 86079/7NS could be auto-called as a ‘variation’ from

¹⁰ In each assay, the pre- and post-melt regions were adjusted manually, aiming at most effectively differentiating MS vaccine strain from wild-type strains.

MS-H.

4.4.4 Nested *oppF-1* PCR-HRM curve analysis distinguishes MS-H from a collection of field strains from diverse geographical locations

The nucleotide sequences of the *oppF-1* region of the MS53 and WVU-1853 strains were retrieved from GenBank. For twelve other field strains used in this study (Table 4.1), the *oppF-1* sequences were determined by PCR followed by Sanger sequencing. Alignment of the partial nucleotide sequences of the *oppF-1* gene from MS-H and 12 other field strains of MS demonstrated that the A-deletion at position 468 within *oppF-1* was only found in the MS-H vaccine strain. Two additional SNPs (G→A at position 487 and A→G at position 525) were found to be unique to MS-H (Figure 4.3a). HRM curve analysis of the nested-*oppF-F3R3* PCR products yielded a single peak for all the strains that were analysed (Figure 4.3b). The melting curve peak for MS-H was at $66.06 \pm 0.00^{\circ}\text{C}$, while those for the other strains were between $66.38 \pm 0.02^{\circ}\text{C}$ and $66.45 \pm 0.00^{\circ}\text{C}$ (Table 4.3). Visual examination of the melting curves revealed that all non-Australian MS strains generated curve profiles similar to that of 86079/7NS. The normalised curves generated from MS-H were also distinct from those of the field strains (Figure 4.3c). When genotyping with a C% threshold of 90% was applied using MS-H as the reference genotype, all field strains were genotyped as a ‘variation’ from MS-H (Table 4.3).

4.4.5 Nested PCR HRM curve analysis of *oppF-1* was able to distinguish vaccine and field strains/isolates of MS directly in clinical specimens

Swabs taken from 8 clinical submissions from commercial chicken flocks on different farms in Australia were examined using nested *oppF-1* PCR HRM curve analysis (Table 4.1). Based on the HRM curve and sequence analyses of the 5’ conserved region of the *vlhA* gene, all these samples were identified as containing field strains with *vlhA* sequences distinct from that of type A Australian MS strains/isolates, which includes the

MS-H vaccine (Jeffery et al., 2007). Partial nucleotide sequences of the *oppF-1* gene from these strains revealed two SNPs (nt 468 and nt 487) within *oppF-1*, with the adenine at position 468 found to be missing only in MS-H (Figure 4.4a). The 40/41 bp *oppF-F3R3* PCR products generated from these Australian field strains/isolates were subjected to HRM curve analysis. Conventional melting curve analysis revealed a single peak of $65.85 \pm 0.00^{\circ}\text{C}$ for MS-H, and of $65.96 \pm 0.06^{\circ}\text{C}$ for MSHv1 (a swab collected from an SPF chicken vaccinated with MS-H), while the field strains/isolates (except 101462.1) had melting curves identical to that for 86079/7NS (Figure 4.4b) (Table 4.4). A minor T_m shift of the main curve and a shoulder were seen for specimen 101462.1. Application of genotyping on normalised HRM curves (Figure 4.4c) using MS-H as the reference and a C% threshold of 90% classified each of the field strains/isolates as a 'variation' (Table 4.4).

4.4.6 Deletion of *oppF-1* A468 is consistent in MS-H after at least five passages *in vivo* in SPF birds

Five tracheal swabs, MSHv1-5, collected from SPF chickens after five consecutive *in vivo* passages of the MS-H vaccine were examined (Table 4.1). The nucleotide sequences of the *oppF-1* regions of each of the five MS-H re-isolates were identical with that of MS-H.

4.4.7 Additional results

To examine whether the A-deletion at position 468 within *oppF-1* was present in MS-H strains isolated from the vaccinated field chickens, partial *oppF-1* sequences of MS-H re-isolates with different temperature sensitivity (*ts*) phenotypes were further analysed. Comparison of the *oppF-1* sequences revealed that the A-deletion at position 468 within *oppF-1* was present in seven of nineteen cloned MS-H re-isolates with either a ts^{+} or ts^{-}

phenotype (Table 4.5) ¹¹. (see discussion in Chapter 6)

4.5 Discussion

The live attenuated MS-H vaccine is now used in many countries to prevent infection with virulent MS. The increasing use of this vaccine has resulted in a need for a reliable technique to discriminate infected from vaccinated animals. HRM has become one of the most common techniques used for detection of SNPs, and can also be used for strain identification in bacteria.

In this study, partial *oppF-1* gene sequences of the MS-H vaccine and its parent strain, 86079/7NS, were initially compared, and an adenine-deletion at position 468 was detected in MS-H. Therefore, this SNP was selected for further analysis with the aim of developing a tool for differentiation of the MS-H vaccine and field strains. Two SNPs (nt 468 and nt 487) were detected when the *oppF-1* genes of other Australian field strains were examined, while the adenine-deletion (nt 468) was found only in MS-H, indicating that this mutation was specific for the vaccine strain. Further analysis was performed on international field strains/isolates, and this confirmed that the SNP at nt 468 was unique to MS-H, with two additional variations (G→A at position 487 and A→G at position 525) detected in all international strains.

In order to differentiate the MS-H vaccine from field strains/isolates, a new technique was designed, consisting of two PCR assays followed by a HRM curve analysis, targeting a 40/41 bp region of the *oppF-1* gene containing the SNP at nt 468. The reliability of the nested *oppF* PCR-HRM was first evaluated using cultures of MS-H, its parent strain 86079/7NS and several international field strains, including a new live

¹¹ Please refer to Chapter 10 (General discussion) for further information on the concordance between *oppF* and *obg* types described in this section. Please note that the *obg* type was confirmed experimentally in this study and is not taken from published data.

attenuated MS vaccine strain, MS1. To further assess the application of this nested *oppF* PCR-HRM directly to clinical specimens, a set of swabs collected from commercial chicken flocks and from SPF birds vaccinated with MS-H were tested. The consistent melting curve pattern generated by MS-H and re-isolates of it after repeated passage *in vivo* suggested that the technique was robust and reliable for both cultures and swabs from clinical cases. All Australian field strains/isolates (except 101462.1) generated HRM curve profiles identical to that of 86079/7NS, demonstrating that this assay was capable of differentiating MS-H from field strains of MS. The shift in the main curve for the product amplified from submission 101468.1 could have been caused by the presence of a small population of a second MS strain in the clinical specimen, but this was not detected in sequencing chromatograms. Dideoxynucleotide sequencing can only reliably detect mutations when at least 10-20% of the alleles in the sample contain the genetic difference (Koelt et al., 1998). In contrast HRM curve analysis has been shown to be more sensitive for identification of mixed populations of mutations (Nomoto et al., 2006). However, HRM curve analysis may not be reliable if a very small population of a second organism is present in a mixed specimen. For such specimens, alternative SNP detection techniques, such as Melt Analysis of Mismatch Amplification Mutation Assay (Melt-MAMA) (Birdsell et al., 2012), TaqMan probe-based assays (Foster et al., 2008), or Allele-Specific (AS) PCR assays (Mokrousov et al., 2003), need to be used.

To determine whether the A-del mutation at position 468 in *oppF-1* of the vaccine strain was reversible, MS-H re-isolates obtained after 5 consecutive *in vivo* passages were examined. All MS-H re-isolates collected after passage *in vivo* in SPF chickens had the same *oppF-1* sequence as the vaccine strain. The number of passages (×5) used in this study was in accordance with the requirements of most regulatory authorities that five successive *in vivo* passages are needed to demonstrate the absence of a risk of reversion

to virulence.

Analysis of the single-copy domain of the conserved region of the *vlhA* gene is commonly used in diagnostic laboratories for classification of MS strains (Bayatzadeh et al., 2014; Dijkman et al., 2014; El-Gazzar et al., 2012; Hammond et al., 2009; Harada et al., 2009; Hong et al., 2004; Jeffery et al., 2007; Wetzel et al., 2010). However, as several Australian isolates, and possibly two European isolates, have been found to have the same *vlhA* sequence type (Catania et al., 2016b; Dijkman et al., 2014), this region may not be totally reliable for the discrimination of MS-H from field strains. In our study, the mean genotype identity confidence percentages of 99.27 ± 0.80 for MS-H and 48.20 ± 13.63 for field strains demonstrated high discriminatory power of the nested *oppF* PCR HRM. Therefore, our novel test can be used as a complementary assay to those targeting the *vlhA* gene for the differentiation of the MS-H vaccine from field strains.

4.6 Conclusion

This study identified a new genetic marker, a SNP at nt 468 in the *oppF-1* gene of MS strains. Based on this finding, a rapid, simple and cost-effective method was developed for the discrimination of the MS-H vaccine from Australian and international field strains/isolates. This assay can be reliably used to identify MS isolates directly from clinical samples, which will be particularly advantageous in countries where the MS-H vaccine is widely used in commercial poultry.

Table 4.1. *Mycoplasma synoviae* strains used in this study

Strain/isolate	<i>vlhA</i> Genotype * (where available)	Origin	Sample form	Reference	GenBank accession no. (where available)
86079/7NS	A	Australia, parent strain of MS-H vaccine, palatine cleft	Pure culture	Morrow et al., 1998	
MS-H	A	Australia, vaccine strain derived from 86079/7NS	Pure culture	Morrow et al., 1998	
MSHv1, 2, 3, 4, 5	A	Australia	Swabs from MS-H vaccinated SPF chickens	This study	
101468.2	Distinct from A-J	Australia	Swab	This study	
101428.1	Distinct from A-J	Australia	Swab	This study	
101428.2	Distinct from A-J	Australia	Swab	This study	
101411.B2	Distinct from A-J	Australia	Swab	This study	
101462.1	Distinct from A-J	Australia	Swab	This study	
101520.4	Distinct from A-J	Australia	Swab	This study	
101488-C	Distinct from A-J	Australia	Swab	This study	
101278-SR	Distinct from A-J	Australia	Swab	This study	
MS53		Brazil	NA		AE017245
F10-2AS	E	USA, NC, field strain, air sac	Pure culture	(Yoder Jr et al., 1977) (Yogev et al., 1988)	
K1938	F	USA, AR, field strain	Pure culture	(Morrow et al., 1990b)	
FMT	F	USA, MS, field strain, trachea	Pure culture	(Vardaman and Drott, 1980) (Yogev et al., 1988)	
K870	F	USA, ME, field strain	Pure culture	(Hong et al., 2004)	
K1858	G	USA, field strain, trachea	Pure culture	(Weinack et al., 1983)	
1853M	H	USA, WV, via MN, joints	Pure culture	(Olson, 1956)	
WVU-1853	H	USA, WV, joints	Pure culture	(Olson, 1956)	CP011096
YA	H	Source unknown	Pure culture	(Noormohammadi et al., 1997)	
K1968	I	USA, CO, field strain, turkey, joints	Pure culture	(Hong et al., 2004; Morrow et al., 1990b)	
K1723	J	USA, AR, field strain, trachea	Pure culture	(Hong et al., 2004)	
MS1		USA, vaccine strain derived from 86079/7NS	Pure culture		
<i>M. gallisepticum</i> ts-11		Vaccine strain	DNA stock	(Whithear et al., 1990)	

* *vlhA* was genotyped using the method described by Jeffery et al. (2007).

Table 4.2. Primers used in this study

Primers	Sequence (5' to 3')	Position ^a	PCR product size (bp) ^b	Application
oppF-F1	TCAAGTATGCTTTTTCCAATTGCT	893-870	627/628	Sequencing
oppF-R2	GTCTTAAACACATGCGTAGAAACA	266-289		
oppF-F2	ACTTTGTATAAAACGGTTCAAAAAGTG	526-500	151/152	oppF-F2R2
oppF-R2	TGGCGTTATCCAAGAAAAAGTTAAA	375-399		PCR
oppF-F3	TTTATCTTCTAGTTCTAG	486-469	40/41	Nested-oppF
oppF-R3	CTTTTTTAGAACTAGCTA	446-463		HRM

^a Nucleotide positions of primers within the nucleotide sequence of *oppF-1* of MS53, GenBank accession number AE017245.

^b PCR products obtained from MS-H were one nucleotide shorter.

Table 4.3. Melting points and genotype identity confidence percentages (C%) in the *oppF*-F3R3 HRM for *M. synoviae* strains from diverse geographical locations

Strain/isolate	<i>oppF</i> -F3R3 HRM		
	Melting point (°C) (Mean $\pm$ SD)	HRM genotype	C% Mean $\pm$ SD [#]
MS-H	66.06 $\pm$ 0.00	MS-H	99.96 $\pm$ 0.00
86079/7NS	66.38 $\pm$ 0.02	Non-MS-H	50.33 $\pm$ 0.99
F10-2AS	66.42 $\pm$ 0.04	Non-MS-H	45.32 $\pm$ 7.23
K1938	66.45 $\pm$ 0.00	Non-MS-H	40.66 $\pm$ 1.35
FMT	66.45 $\pm$ 0.00	Non-MS-H	35.37 $\pm$ 1.13
K870	66.45 $\pm$ 0.00	Non-MS-H	43.75 $\pm$ 8.78
K1858	66.45 $\pm$ 0.00	Non-MS-H	37.73 $\pm$ 1.29
1853M	66.45 $\pm$ 0.00	Non-MS-H	46.08 $\pm$ 5.57
WVU-1853	66.41 $\pm$ 0.06	Non-MS-H	46.83 $\pm$ 4.99
YA	66.42 $\pm$ 0.04	Non-MS-H	45.52 $\pm$ 2.26
K1968	66.45 $\pm$ 0.00	Non-MS-H	43.70 $\pm$ 7.71
K1723	66.42 $\pm$ 0.04	Non-MS-H	44.62 $\pm$ 5.42
MS1	66.45 $\pm$ 0.00	Non-MS-H	38.21 $\pm$ 3.62

[#] Normalised regions of 64.60-64.82 and 68.14-68.31 were applied.

Table 4.4. Melting points and genotype identity confidence percentages (C%) in *oppF*-F3R3 HRM for Australian *M. synoviae* field strains

Strain/isolate	<i>oppF</i> -F3R3 HRM		
	Melting point (°C) (Mean $\pm$ SD)	HRM genotype	C% Mean $\pm$ SD [#]
MS-H	65.85 $\pm$ 0.00	MS-H	98.58 $\pm$ 0.10
MSHv1	65.96 $\pm$ 0.06	MS-H	99.85 $\pm$ 0.00
86079/7NS	66.35 $\pm$ 0.06	Non-MS-H	41.73 $\pm$ 5.30
101468.2	65.93 $\pm$ 0.53	Non-MS-H	61.03 $\pm$ 25.27
101428.1	66.33 $\pm$ 0.04	Non-MS-H	54.59 $\pm$ 1.62
101411.B2	66.36 $\pm$ 0.00	Non-MS-H	42.89 $\pm$ 1.63
101462.1	66.08 $\pm$ 0.02	Non-MS-H	85.22 $\pm$ 2.65
101520.4	66.45 $\pm$ 0.00	Non-MS-H	26.94 $\pm$ 0.03
101428.2	66.33 $\pm$ 0.04	Non-MS-H	41.05 $\pm$ 1.73
101488.C	66.50 $\pm$ 0.06	Non-MS-H	16.90 $\pm$ 0.47
101278-SR	66.39 $\pm$ 0.00	Non-MS-H	42.01 $\pm$ 0.49

[#] Normalised regions of 65.08-65.38 and 68.48-68.60 were applied.

Table 4.5. The *oppF-1* and *obg* sequence type, and temperature sensitivity phenotype of several *M. synoviae* clones isolated from vaccinated field chickens

Strains	<i>oppF-1</i> ^a	<i>obg</i> ^a	<i>ts</i> phenotype ^b
86079/7NS	F	F	—
MS-H	V	V	+
MS-H ⁴	F	F	—
MS-H ⁵	V	F	—
93198 1-24b	V	V+C	—
93198 3-13b	V	V	+
93198 3-15a	F	V	+
93198 4-19a	F	V	+
93198 5-10a	V	V	+
93198 6-1a	V	V+C	—
93198 6-5b	V	V	+
93205 1-2a	F	V	+
93205 2-9a	F	F	—
93205 2-13a	F	F	—
93205 9-3a	V	V	+
94036 2-1a	F	V+C	+
94036 2-2a	F	V	+
94036 6-3a	F	F	—
93025 8-9c	F	F	—
94036 9-2a	F	V+C	—
94036 10-5a	F	F	—

^a “V” indicates vaccine-type mutations (adenine-deletion at position 468 in *oppF-1* and G→A at position 367 in *obg*). “F” indicates genotype consistent with 86079/7NS. “C” indicates complementary mutation in *obg* (C→T at position 629).

^b *ts* phenotype was determined in previous studies (Markham et al., 1998c; Shahid et al., 2013a).

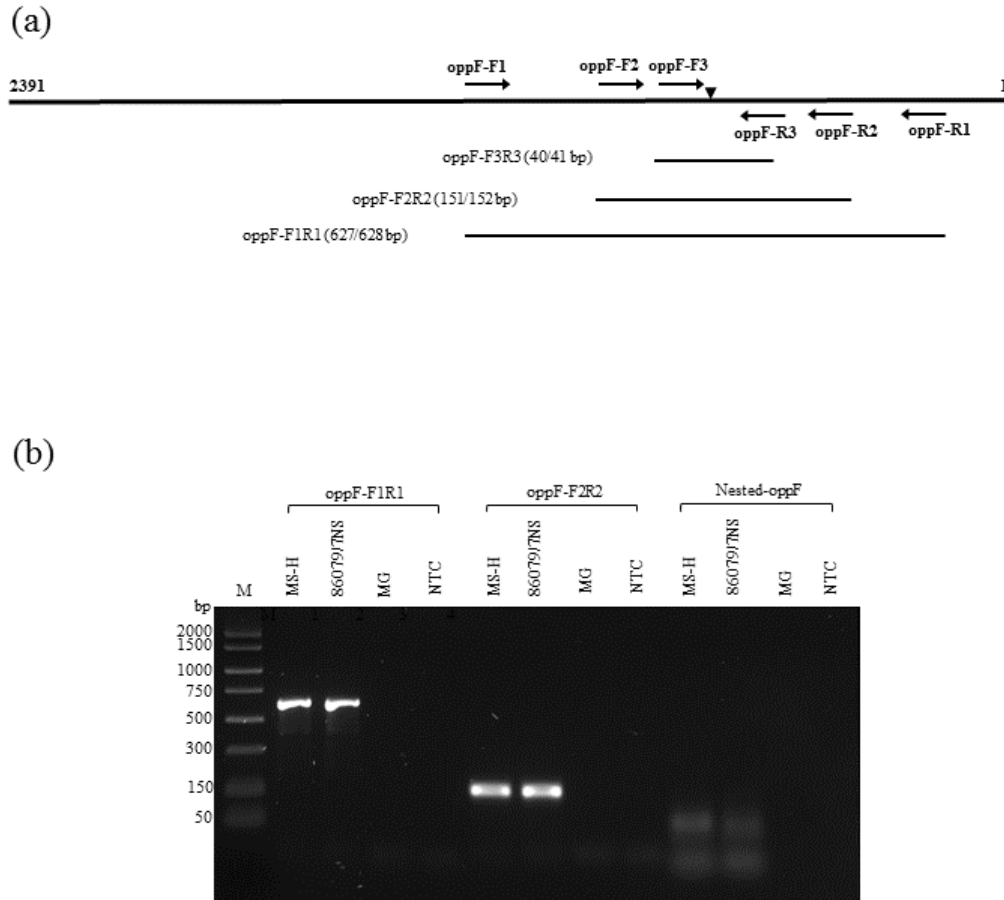


Figure 4.1. Analysis of different *oppF* PCR products by agarose gel electrophoresis. **(a)** Schematic presentation of *oppF-1* PCRs and the location of primers. The thick line indicates the full length *oppF-1*, encoded in the reverse orientation, and thin lines indicate different *oppF-1* PCR products. Arrows represent primer locations and the vertical arrow head indicates the location of the SNP at position 468. **(b)** Agarose gel electrophoresis of products from *oppF-1* PCRs from MS-H, 86079/7NS, *M. gallisepticum* ts-11 (MG) and non-template control (NTC). Molecular weight marker (PCR Marker; Sigma, Missouri, USA)

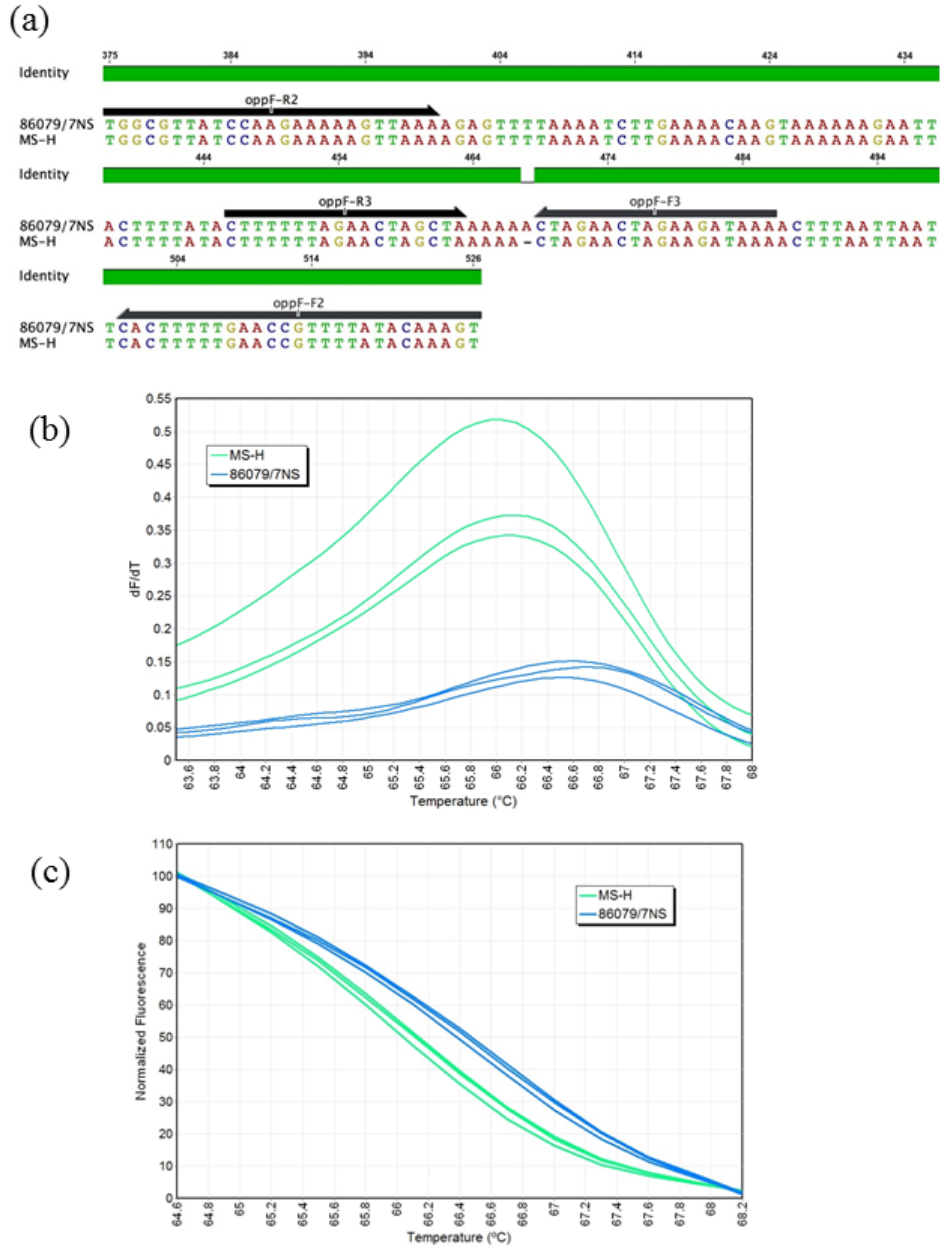


Figure 4.2 (a) Comparison of partial *oppF-1* nucleotide sequences (corresponding to nt 375-526 of MS53 *oppF-1*) of *M. synoviae* strains 86079/7NS and MS-H. **(b)** Conventional and **(c)** normalised high-resolution melting-curve analysis of *M. synoviae* strains 86079/7NS and MS-H using *oppF-F3R3* nested PCR.

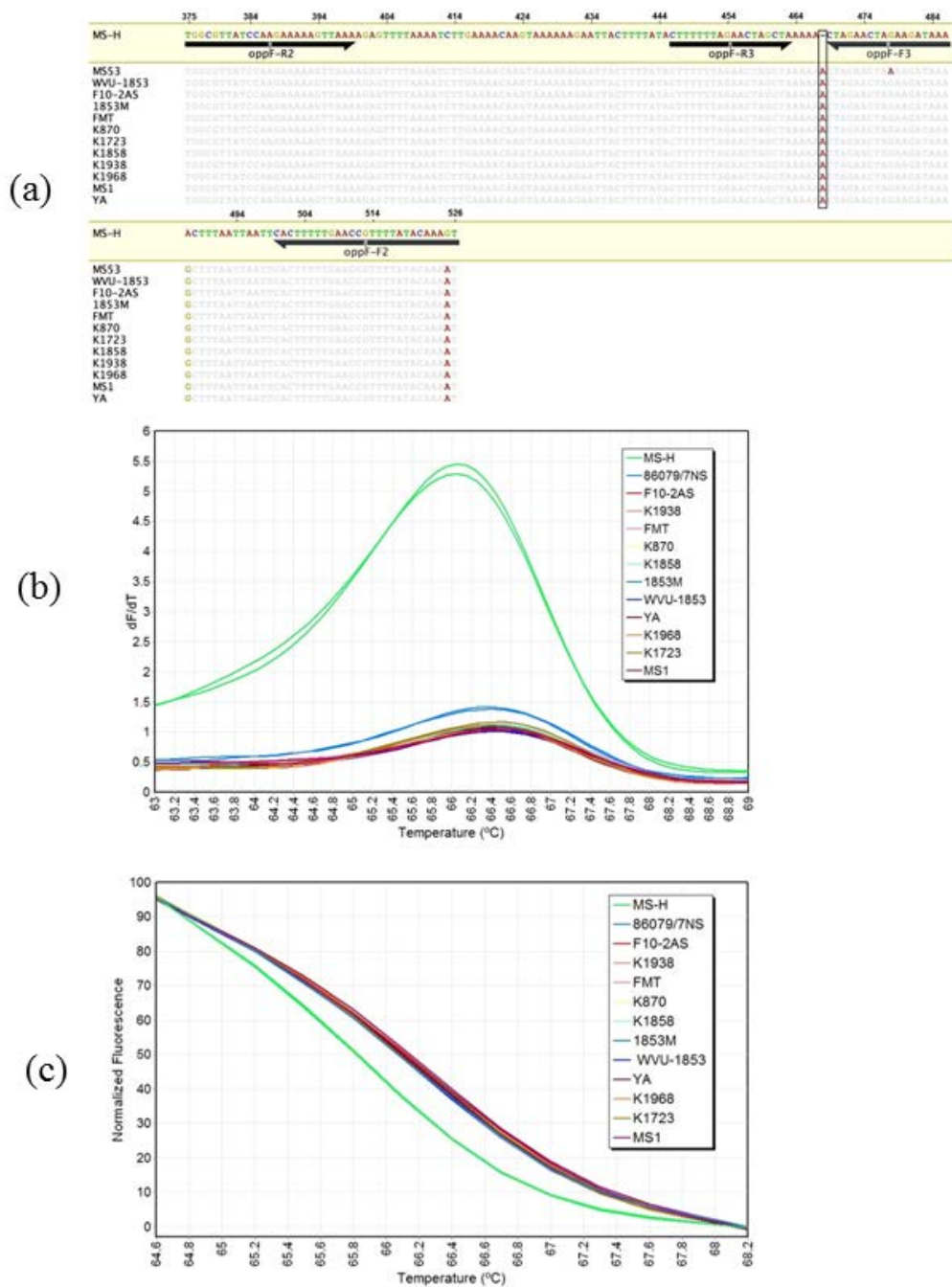


Figure 4.3. (a) Comparison of partial *oppF-1* nucleotide sequences (corresponding to nt 375-526 of MS53 *oppF-1*) of *M. synoviae* strains MS-H and overseas field strains. The SNP (nt468) is highlighted with a black box. (b) Conventional and (c) normalised high-resolution melting-curve analysis of *M. synoviae* strains using *oppF-F3R3* nested PCR.

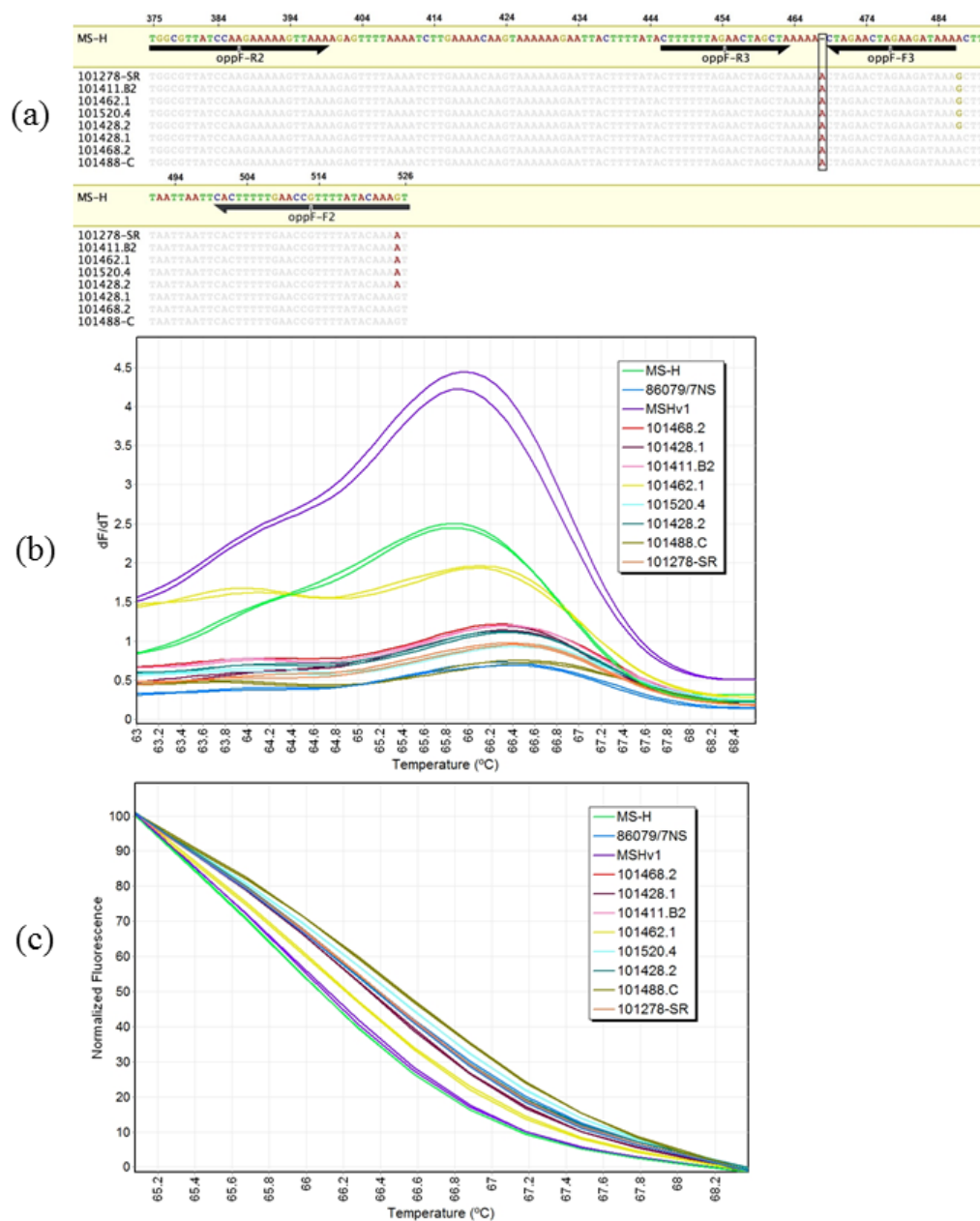


Figure 4.4. (a) Comparison of partial *oppF-1* nucleotide sequences (corresponding to nt 375-526 of MS53 *oppF-1*) of *M. synoviae* strain MS-H and Australian field strain/isolates. The SNP (nt468) is highlighted with a black box. (b) Conventional and (c) normalised high-resolution melting-curve analysis of *M. synoviae* strains using *oppF-F3R3* nested PCR.

CHAPTER 5. Expression of Infectious Bronchitis Virus Spike and Nucleocapsid Proteins in *Mycoplasma synoviae* Vaccine Strain MS-H

5.1 Abstract

Mycoplasma synoviae (MS) is a major pathogen of poultry and causes chronic respiratory disease and synovitis in chickens and turkeys. A live attenuated vaccine, MS-H, has been used for the control of MS in many countries worldwide. The impact of MS on the respiratory tract mucosae is magnified by co-infections with other respiratory pathogens, such as the infectious bronchitis virus (IBV). With the ultimate goal of developing recombinant vaccines that provide protection against both MS and IBV, two MS-H transformants expressing the IBV spike protein (S1) and nucleocapsid (N) proteins were developed in this study. The IBV S1 gene (798 bp) and N gene (1230 bp) from the VicS-v strain were separately inserted into pMAS-LoriC, a previously constructed *oriC* plasmid capable of replicating in MS, and introduced into MS-H cells by transformation. Expression of the partial S1 and N genes in MS-H transformants was confirmed by quantitative real-time PCR and Western blotting. This study is the first report of successful expression of foreign genes in MS. The rMSH-IBV clones developed in this study could potentially serve as bivalent vaccines to induce protection against both virulent MS and IBV infections in chickens.

5.2 Introduction

Mycoplasma synoviae causes subclinical respiratory tract diseases, infectious synovitis (Ferguson-Noel and Noormohammadi, 2013; Olson, 1956), airsacculitis (Kleven et al., 1972) and eggshell apex abnormalities (EAA) (Feberwee et al., 2009b) in chickens, resulting in considerable economic losses in the poultry industry due to condemnation

of carcasses and decreased weight gains and egg production.

M. synoviae colonises the respiratory epithelial lining and can predispose the host to secondary infection with other respiratory pathogens, such as infectious bronchitis virus (IBV), Newcastle disease virus (NDV) and *Escherichia coli*, resulting in more severe clinical respiratory disease (Kleven et al., 1972; Springer et al., 1974).

To prevent infection with MS, the MS-H live attenuated vaccine (Vaxsafe MS[®]; Bioproperties Pty. Ltd.) has been registered and used in many countries since its introduction to Australia in 1996. MS-H is a temperature-sensitive (*ts*⁺) mutant generated by chemical mutagenesis of a virulent Australian field strain 86079/7NS (Morrow et al., 1998). MS-H grows normally at 33°C, but its growth is significantly reduced at 39°C. MS-H only colonises the upper respiratory tract of its host and induces long-term protective immunity without significant gross or microscopic lesions (Jones et al., 2006a, b).

IBV is a highly infectious pathogen, causing bronchitis, nephritis and/or reproductive tract disease, leading to huge economic losses in poultry industry worldwide. It replicates primarily in epithelial cells of the respiratory tract (Cavanagh and Naqi, 2003), causing clinical signs characterised by sneezing, coughing, tracheal râles, nasal discharge and laboured breathing in chickens (Hodgson et al., 2004). Strains of IBV differ in their virulence and pathogenicity and cause a range of diseases from mild respiratory signs to severe disease affecting the oviduct and kidneys, and mortality (Cavanagh and Gelb Jr., 2008).

IBV is an enveloped, positive-stranded RNA virus that belongs to the genus *Gammacoronavirus*, subfamily *Coronavirinae*, family *Coronaviridae*, order *Nidovirales*

(Carstens, 2010). Like all coronaviruses, IBV contains four structural proteins: the spike protein (S), which is post-translationally cleaved into two subunits (amino-terminal S1 and carboxyl-terminal S2), the nucleocapsid protein (N), the membrane protein (M), and the small envelope protein (E) (Lai and Cavanagh, 1997). The S1 protein is one of the immunogenic proteins and contains the receptor-binding region and is involved in the induction of virus neutralizing (VN), haemagglutination inhibiting (HI) and serotype specific antibodies (Cavanagh et al., 1986; Karaca et al., 1992). The S1 protein has been considered the most likely inducer of protective immunity and therefore is commonly targeted for the design of recombinant vaccines against IBV (Johnson et al., 2003; Tomley et al., 1987; Wang et al., 2002; Zhao et al., 2017a). However, the S1 gene of IBV is highly variable across different viral strains and this is responsible for the diversity of IBV serotypes/genotypes, resulting in poor cross-protection (Cavanagh, 2007; Cavanagh et al., 1992; Cavanagh et al., 1988). The N protein carries epitopes for induction of cross-reactive systemic antibodies (Ignjatovic and Galli, 1994) and cell-mediated immunity (CMI) (Seo et al., 1997), and therefore is also important in IBV immunity. Cellular immune responses elicited by a live IBV vaccine have been found to be cross-reactive (Raj and Jones, 1997a).

Vaccination with attenuated and/or inactivated IBV vaccines is used to prevent disease in commercial chickens (Al-Tarcha and Sadoon, 1990; Andrade, 1983; Box et al., 1980; Burke and Luginbuhl, 1972a, b; Ladman et al.), but the reversion to virulence of attenuated vaccines (Hopkins and Yoder Jr, 1986) and the emergence of new field viruses remains a major risk for the poultry industry throughout the world (Estevez et al., 2003). Thus, the development of new vaccines that induce broad cross-protection would be ideal. Foreign gene expression in mycoplasmas has been limited by the lack of knowledge about genetic regulatory elements in these organisms and uncertainty in identifying promoter sequences. The β -galactosidase (*lacZ*) gene of *E. coli* was the first

foreign gene functionally expressed in a mycoplasma (Knudtson and Minion, 1994). A recombinant *Mycoplasma gallisepticum* vaccine strain based on GapA⁺ ts-11, expressing the IBV S1 protein and chicken interleukin-6, was generated using a transposon-based delivery vector (Shil et al., 2011). An *oriC* plasmid capable of replicating and expressing *tetM* in MS that was developed recently in our laboratory (Shahid et al., 2014a) was proposed as a potential vector for stable expression of foreign genes. In this study, we constructed a derivative of this *oriC* plasmid to express a conserved region of the IBV S1 gene and the N gene using the MS *vlhA* promoter in the MS vaccine strain MS-H. The ultimate goal of this project was to develop novel recombinant vaccines that could provide protection against MS and IBV in birds.

5.3 Materials and Methods

5.3.1 Ethics statement

Production of polyclonal antibodies for use in this study was approved by Animal Ethics Committee of the University of Melbourne (Ethics ID: 1413074.1).

5.3.2 Bacterial strains and culture conditions

M. synoviae strain MS-H (Table 5.1) was grown in mycoplasma broth (MB) supplemented with 0.01% (w/v) nicotinamide adenine dinucleotide (NAD) and 10% swine serum (Whithear, 1993) at 37°C until late logarithmic phase (approximately pH 6.8). MS-H transformants were either grown in MB or on mycoplasma agar (MA), which had the same composition as MB except that glucose and phenol red were omitted and the medium was solidified with 1% agar (Difco, Sparks, Maryland, USA). For the selection of MS-H transformants, tetracycline (Sigma, St. Louis, Missouri, USA) was added to the liquid or solid medium to a concentration of 2.5 µg/ml.

E. coli α -Select Gold Efficiency competent cells (Bioline, Alexandria, New South Wales, Australia) were used for cloning and were cultured at 37°C in Luria-Bertani (LB) broth (1% w/v tryptone (Oxoid, England) 0.5% w/v yeast extract (Oxoid, England), and 0.5% w/v NaCl) with shaking or on LB agar plates (LB with 1% agar (Difco, Sparks, Maryland, USA)). *E. coli* DH5 α transformants containing the plasmid construct were selected and grown in LB broth or on LB agar plates containing 100 μ g ampicillin/ml (Sigma) and/or tetracycline (2.5 μ g/ml).

5.3.3 Gibson assembly of overlapping PCR fragments

Codon-optimised IBV vaccine strain VicS-v S1 (GenBank accession no. KF460437, nt 817-1611, 795 bp) and N (GenBank accession no. KF460437, 1230 bp) genes were synthesised and inserted into a pMA-T vector (Invitrogen, Thermo Fisher Scientific, Scoresby, Victoria, Australia).

To construct a vector capable of expressing the IBV S1 gene in MS (pGA-IBVS1) by Gibson assembly (New England Biolabs, La Jolla, California, USA), four pairs of primers, pKS-VOTL F/R, MCS-lacZ F/R, pVlha-ATG F/R and IBVS1-opt F/R (Table 5.2), were designed (NEBuilder[®] Assembly Tool, <http://nebuilder.neb.com>) and synthesised (Geneworks, Thebarton, SA, Australia) and used to amplify four DNA fragments with overlapping flanking sequences for the backbone and the inserts (Table 5.2) (Figure 5.1a). The construct backbone containing *oriC* (*M. synoviae* origin of replication) and a fraction of pBluescript II KS (+) was amplified from a template plasmid pKS-VOTL. Briefly, PCR was conducted in a 25 μ l reaction containing 0.4 μ l of 25 μ M of each of the forward and reverse primers, 1 μ l of 50 mM MgSO₄, 4 μ l of 1.25 mM dNTP mixture (Promega), 0.1 μ l (1 U) of Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA, USA), 2.5 μ l of 10 $\times$ Platinum Taq DNA polymerase buffer (Invitrogen), 2 μ l of DNA template (~50 ng/ μ l) and 14.6 μ l of

nuclease free water (Invitrogen). The resultant reaction mixture was incubated at 94°C for 2 min, and then through 35 cycles of 94°C for 15 sec, 60°C for 30 sec and 68°C for 4 min 15 sec. The other fragments were amplified using the same reagents and conditions as were used for the construct backbone PCR, except that an extension time of 40 sec was used.

Similarly, to construct a vector capable of expressing the IBV N gene in MS (pGA-IBVN) by Gibson assembly, two pairs of primers, pMS-IBV-bb F/R and IBVN-opt F/R (Table 5.2) were designed, synthesised (Geneworks, Thebarton, SA, Australia) and used to amplify two DNA fragments with overlapping flanking sequences for the backbone and the insert (Table 5.2) (Figure 5.2a). The backbone construct was amplified from the template plasmid pMS-IBVS1. Briefly, PCR was conducted in a 25 µl reaction containing 0.4 µl of 25 µM of each of the forward and reverse primers, 1 µl of 50 mM MgSO₄, 4 µl of 1.25 mM dNTP mixture (Promega), 0.1 µl (1 U) of Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA, USA), 2.5 µl of 10 × Platinum Taq DNA polymerase buffer (Invitrogen), 2 µl of DNA template (~50 ng/µl) and 14.6 µl of nuclease free water (Invitrogen). The resultant reaction mixture was incubated at 94°C for 2 min, then through 35 cycles of 94°C for 15 sec, 59°C for 30 sec and 68°C for 5 min 40 sec. The IBV N gene was amplified using the same reagents and conditions as were used for the backbone construct PCR, except that an annealing temperature of 62°C and an extension time of 80 sec were used. The concentration of PCR products was determined in a 1% agarose gel by comparison with a DNA molecular weight marker (Bioline, Alexandria, New South Wales, Australia) and analysed using a ChemiDocTM MP Imaging System (Bio-Rad, Gladesville, New South Wales, Australia).

The cloning strategy used to construct pGA-IBVS1 from overlapping PCR products is described in Figure 5.1b. Briefly, four overlapping PCR fragments were assembled

according to the manufacturer's recommendations (New England BioLabs). Approximately 0.22 pmol of each DNA fragment were pooled in a 5 µl volume and this was mixed with 5 µl of nuclease-free water and 10 µl of NEBuilder® High-Fidelity Master Mix (New England BioLabs, La Jolla, California, USA) was added. The mixture was incubated at 50°C for 1 h and then 2 µl of this was used for transformation of α -Select Gold Efficiency competent *E. coli* cells (Bioline).

The Gibson cloning strategy used to assemble the construct pGA-IBVN is described in Figure 5.2b. Briefly, two overlapping PCR fragments were assembled according to the manufacturer's recommendations (New England BioLabs). Approximately 0.039 pmol of backbone and 0.078 pmol of insert PCR products were pooled in an 8.5 µl volume and this was mixed with 5 µl of nuclease-free water and 10 µl of NEBuilder® High-Fidelity Master Mix (New England BioLabs). The mixture was incubated at 50°C for 1 h and then 2 µl of this was used for transformation of α -Select Gold Efficiency competent *E. coli* cells (Bioline).

Bacterial plasmid DNA extraction was performed using the AxyPrep™ Plasmid Miniprep kit (Axygen, USA) according to the manufacturer's instructions. *E. coli* transformants were first screened by PCRs using primers MCS-lacZ F/R, pVlhA-ATG F/R, or IBVS1-opt F/R (or IBVN-opt F/R), and the DNA sequence of the construct was then verified by sequencing (Micromon, Monash University, Australia).

5.3.4 Construction of the rMSH-IBVS1 and rMSH-IBVN vaccine vectors

To introduce a selection marker suitable for *Mycoplasma* spp., a DNA fragment containing *tetM* (tetracycline resistance gene) was excised from pKS-VOTL using the restriction endonucleases *Bam*HI and *Sal*I. The excised *tetM* fragment was cloned into *Bam*HI and *Sal*I digested plasmids pGA-IBVS1 and pGA-IBVN, which were then

designated pMS-IBVS1 (Figure 5.3) and pMS-IBVN (Figure 5.4), respectively. Plasmids pMS-IBVS1 and pMS-IBVN were propagated in *E. coli* and extracted using the PureYield™ Plasmid Midiprep kit (Promega, Alexandria, New South Wales, Australia) according to the manufacturer's instructions.

5.3.5 Transformation of MS

To prepare MS cells for transformation, a serial dilution of a culture of MS strain MS-H working seed was prepared by inoculating 25, 50, 100, 200 and 400 µl into 15 ml of MB separately and incubating these dilutions at 37°C. Cultures were harvested after approximately 36 h of growth when a gradient of colour change from the lowest to highest dilution of culture was seen. The cells were harvested by centrifugation at $10,000 \times g$ for 30 min at 4°C. The pelleted cells were pooled and washed with 10 ml of ice-cold HEPES-sucrose buffer (8 mM HEPES, 272 mM sucrose, pH 7.4). After centrifugation at $10,000 \times g$ for 30 min at 4°C, the cell pellet was resuspended in 1.5 ml of ice-cold HEPES-sucrose buffer, transferred to a 1.5 ml Eppendorf tube and washed a further two times as before. After the final wash, the cell pellet was resuspended in 2 ml of ice-cold HEPES-sucrose buffer containing 10% glycerol and the suspension was distributed into 100 µl aliquots.

Transformation of MS-H was performed by electroporation. For each transformation, a frozen aliquot of MS-H cells was thawed on ice for 5 min and diluted with ice-cold HEPES-sucrose buffer. An 8 µg sample of the pMS-IBVS1 or pMS-IBVN plasmid (500 ng/µl) was added to and mixed with undiluted and diluted (1:2, 1:4 and 1:8) cells, the cells then transferred to a pre-chilled electroporation cuvette (0.2 cm gap) (Molecular BioProducts, Scoresby, Australia) and incubated on ice for a further 20 min. Transformation was performed by electroporation using a Gene Pulser Xcell™ (Bio-Rad, Gladesville, New South Wales, Australia) at 2,500 V, 100 Ω and 25 µF with an

exponential decay pulse. The electroporated cells were resuspended immediately in 1 ml of cold MB, incubated on ice for 10 min, and at 37°C for 3-4 h, and then plated onto MA containing 2.5 µg tetracycline/ml and incubated at 37°C for 7-10 days in an airtight jar. Individual tetracycline resistant colonies were selected and subcultured in 1 ml MB containing 2.5 µg tetracycline/ml and incubated at 37°C until an acid colour change was seen. For each transformation, an empty vector, pMAS-LoriC (Shahid et al., 2014a), which is capable of replication in MS, was included as a control.

5.3.6 Detection of MS transformants

Colonies that had grown on plates were picked using a Pasteur pipette and placed in 500 µl of MB containing tetracycline (2.5 µg/ml) followed by incubation at 37°C until the medium showed a colour change. Subculture was performed several times to eliminate the residual plasmid from the medium. A 100 µl volume of culture was pelleted by centrifugation in a 1.5 ml Eppendorf tube at 10,000 × g for 10 min at room temperature, and the cell pellet was resuspended in 25 µl of nuclease-free water. The cell resuspension was then heated at 100°C for 5 min and 2 µl of this used as DNA template for PCR to confirm the presence of the pMS-IBVS1 or pMS-IBVN plasmid. The PCR assays were performed in a 25 µl reaction containing 0.4 µl of 25 µM of each of the forward and reverse primers (IBV-S1-opt F/R and tetM F1/R1 for pMS-IBVS1, and IBV-N-opt F/R and tetM F1/R1 for pMS-IBVN) (Table 5.1), 1.25 µl of 25 mM MgCl₂, 1.6 µl of 1.25 mM dNTP mixture (Promega), 0.25 µl (1.25 U) of GoTaq (Promega), 5 µl of 5 × GoTaq Green Flexi buffer (Promega), 2 µl of crude DNA template and 14.1 µl of nuclease-free water. The tetracycline resistance gene PCR was performed using an iCycler thermocycler (Bio-Rad). The reaction was incubated at 95°C for 2 min, then through 35 cycles of 95°C for 30 sec, 56°C for 30 sec and 72°C for 30 sec, with a final extension at 72°C for 5 min. The resultant products were visualised in a 1.5% agarose gel.

5.3.7 Extraction of total RNA

M. synoviae transformant cells containing pMS-IBVS1, pMS-IBVN or pMAS-LoriC grown at 37°C until late logarithmic phase were harvested for RNA extraction. Briefly, 20 ml of culture was pelleted by centrifugation at $10,000 \times g$ for 30 min at room temperature, mixed with 1 ml of Trizol (Invitrogen), immediately resuspended by vortexing and vigorous pipetting, and then kept at room temperature for 10 min. A 200 μ l volume of chloroform was added and the mixture vigorously shaken for 15 sec. After incubation at room temperature for 5 min, phase separation was conducted by centrifugation at $12,000 \times g$ for 15 min at 4°C. The colourless aqueous upper phase containing RNA was transferred into a new tube, and mixed gently with 500 μ l of isopropanol and the mixture incubated for 10 min at room temperature. RNA was precipitated by centrifugation at $12,000 \times g$ for 15 min at 4°C. The pellet was washed with 500 μ l of 70% ethanol (prepared with nuclease-free water) and the tube was centrifuged at $12,000 \times g$ for 10 min at 4 °C. The final RNA pellet was air dried for 5 min and dissolved in 100 μ l of nuclease-free water. The nucleic acid concentration and purity were assessed by spectrophotometry using a NanoDrop® ND-1000 (Thermo Fisher Scientific, Scoresby, Australia). A 10 μ g sample of total extracted RNA was resuspended in 1 $\times$ DNase I Reaction Buffer (New England BioLabs) to a final volume of 100 μ l, which also contained 4 μ l (8 U) of DNase I (RNase-free) (New England BioLabs). The reaction mixture was incubated at 37°C for 90 min, then 1 μ l of 0.5 M ethylenediaminetetraacetic acid (EDTA) was added and the enzyme was heat inactivated at 75°C for 10 min. The absence of residual plasmid DNA in the extract was confirmed by PCR. The reaction was performed in a 25 μ l reaction containing 1 μ l of 10 μ M solutions of each of the forward and reverse primers (tetM-F1/tetM-R1) (Table 5.2), 1.25 μ l of 25 mM MgCl₂, 1.6 μ l of 1.25 mM dNTP mixture (Promega), 0.25 μ l (1.25 U) of GoTaq (Promega), 5 μ l of 5 $\times$ GoTaq Green Flexi buffer (Promega), 2 μ l of DNase I treated RNA and 12.9 μ l of nuclease free water. The mixture was incubated at 95°C for

2 min, followed by 35 cycles of 95°C for 30 sec, 58°C for 30 sec and 72°C for 30 sec, with a final extension at 72°C for 5 min. The reaction products were subjected to electrophoresis in a 1% agarose gel.

5.3.8 cDNA synthesis

The cDNA was synthesised using 5 µl of DNase I treated RNA, 1 µl of IBVqPCR-R2 (S1 mRNA) or IBVNqPCR-R2 (N mRNA) primer (Table 5.1), 0.25 µl (200 U) of M-MLV reverse transcriptase (Promega), 0.25 µl (40 U) of Recombinant RNasin® ribonuclease inhibitor (Promega), 4 µl of 1.25 mM dNTP mixture (Promega), 5 µl of 5 × M-MLV 5 × Reaction Buffer (Promega), and 9.5 µl nuclease-free water (Invitrogen) to a total volume of 25 µl.

5.3.9 Quantitative real-time PCR (qPCR)

To examine mRNA transcription of the IBV S1 or N fragment in MS-H transformants, quantitative real-time PCRs were performed on cDNA samples using a Rotor-GENE Q PCR machine (Qiagen, Chadstone, Australia). To quantify the absolute copy number of IBV S1 or N cDNA molecules, serial tenfold dilutions of pMS-IBVS1 or pMS-IBVN plasmid DNA were prepared in nuclease-free water and standard curves ranging from 3×10^1 to 3×10^6 copies per 2 µl were created. The plasmid copy number was calculated using the formula provided by the URI Genomic & Sequencing Centre (<http://www.uri.edu/research/gsc/resources/cndna.html>). Primers used to detect the IBV S1 and N genes were designed using the Primer-BLAST web-based tool (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>). The initial evaluation and optimisation were performed for each pair of primers using the plasmids (pMS-IBVS1 or pMS-IBVN) harbouring the target fragment. In the initial evaluation, the best primer pair was selected for use in quantitative real-time PCR. Each PCR was conducted in a 25 µl reaction containing 0.4 µl of 25 µM solutions of each of the forward and reverse primers (IBVqPCR-F2 and IBVqPCR-R2, or IBVNqPCR-F2 and IBVNqPCR-R2)

(Table 5.1), 1.25 µl of 25 mM MgCl₂, 1.6 µl of 1.25 mM dNTP mixture (Promega), 2 µl of 100 µM SYTO 9 green fluorescent nucleic acid stain (Invitrogen), 0.25 µl (1.25 U) of GoTaq (Promega), 5 µl of 5 × GoTaq Green Flexi buffer (Promega), 2 µl of cDNA and 12.1 µl of nuclease-free water. A negative control (pMAS-LoriC), a non-template control (nuclease-free water) and the appropriate standard curves were included in each run. All samples were tested in triplicate. The reactions were incubated at 95°C for 2 min, then through 35 cycles of 95°C for 30 sec, 63°C (for IBV S1) or 60°C (for IBV N) for 30 sec and 72°C for 17 sec. A melting curve analysis was performed between 72°C and 95°C with a ramp of 1.0°C/sec to confirm the identity of the amplicons. All reactions were carried out in triplicate.

5.3.10 Antigenic peptides selection and synthesis for IBV S1

Several antigenic epitopes within IBV S1 gene were predicted using the GenScript OptimumAntigenTM design tool. Based on the criteria for hydrophilicity, hydrophobicity, surface probability, transmembrane, homology to mouse or rat proteins, flexible region, helix region, sheet region, signal peptide and modification, two epitopes were selected. Peptides were synthesised with or without conjugation (Genscript, Piscataway, New Jersey, USA) (Table 5.3). Two peptides conjugated to keyhole limpet haemocyanin (KLH) were used to raise polyclonal antibodies in rats.

5.3.11 IBV purification

Allantoic fluid (AF) containing VicS-v viral particles at 2×10^5 EID₅₀/ml was used as a source of IBV. Viral culture was performed by inoculating 100 µl of AF into the allantoic cavity of four 9-day-old embryonated specific-pathogen-free (SPF) chicken eggs. After 48 h incubation, the AF was collected aseptically. A 30 ml volume of the AF was clarified by centrifugation at $2,500 \times g$ for 20 min at 4°C. The supernatant (clear AF) was collected using a 10 ml syringe with a 1 inch 18 gauge needle, transferred to a

sterile 50 ml Falcon tube, and centrifuged at $100,000 \times g$ for 2 h at 4°C. The pellet containing viral particles was then resuspended in 200 µl of Tris-buffered saline (TBS) (150 mM NaCl, 50 mM Tris-HCl, pH 7.4). This viral suspension was layered on a 30% to 55% continuous sucrose gradient in TBS and centrifuged at $100,000 \times g$ for 4 h at 4°C. The virus band was removed using a 1 inch 18 gauge needle from the side of the tube, transferred into a separate tube and resuspended in 500 µl of TBS. The viral particles were then pelleted by centrifugation at $90,000 \times g$ for 1 h, resuspended in 250 µl of TBS and stored at -80°C until used.

5.3.12 Preparation of rat anti-IBV polyclonal antibodies

To provide sufficient volume of serum for subsequent experiments, antisera against IBV strain VicS-v were raised in 2 rats. A blood sample was taken from the tail vein (~1 ml) of each rat prior to immunisation for use as a control. Each rat was immunised subcutaneously with a total of 0.25 ml of an emulsion (0.125 ml of IBV S1 peptide or purified IBV strain VicS-v emulsified with 0.125 ml of Freund's complete adjuvant (Sigma)). Three weeks later, a second inoculation was performed using the same inoculum volumes but with the antigens emulsified in Freund's incomplete adjuvant (Sigma). Ten days later, a third inoculation was performed using Freund's incomplete adjuvant. Three weeks after the third inoculation, the rats were anaesthetised in a chamber with isoflurane and blood samples were collected by cardiac puncture. Euthanasia was performed using an overdose of pentobarbitone (300 mg/kg).

5.3.13 Indirect ELISA to assess antibody response in rats

Each well of a Maxisorp ELISA plate (Nunc) was coated overnight at 4 °C with 100 µl 0.1 M carbonate-bicarbonate buffer (pH 9.6) containing unconjugated peptide 691666-3 or 691666-4 (0.3-10 µg/ml). After removal of the peptide coating solution, the wells of

the ELISA plates were washed three times with PBS-T (PBS containing 0.1% Tween-20 (Thermo Fisher Scientific, Taren Point, NSW, Australia)) before blocking with 1% bovine serum albumin (BSA) (Sigma) in PBS-T at 37°C for 1 h. The plates were then washed as described above and 100 µl of diluted test sera (1/100 to 1/800 in blocking buffer) was added to the wells of the ELISA plates. The plates were incubated for 1 h at room temperature, washed as described above, 100 µl of rabbit anti-rat immunoglobulin G conjugated to horseradish peroxidase (HRP) (Abcam, Melbourne, Victoria, Australia) at a dilution of 1/2000 was added, the plates were incubated at 37°C for 1 h and then washed again three times. A 100 µl volume of TMB substrate solution (Life Technologies, Waltham, MA, USA) was added to each well and the plate was incubated at room temperature for 5 min. The reaction was stopped by addition of 50 µl of stop buffer (1 M HCl) to each well. The absorbance of each well was read at 450 nm using a microplate reader (Thermo LabSystems Multiskan RC, Beverly, MA, USA).

5.3.14 SDS-PAGE and Western immunoblotting

The MS-H transformants were grown in 20 ml MB to late log phase at 37°C (containing tetracycline (2.5 µg/ml) and harvested by centrifugation at $10,000 \times g$ for 30 min at room temperature. After washing with sterile phosphate buffered saline (PBS) (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄), the cell pellet was resuspended in 200 µl of PBS and mixed with 200 µl of 2 × protein lysis buffer (2% SDS, 10% glycerol, 62.5 mM Tris-HCl (pH 6.8), 5% β-mercaptoethanol, and bromophenol blue (0.5 µg/ml)). The whole cell lysis proteins were separated by electrophoresis in 12.5% polyacrylamide gels and transferred onto PVDF membrane (Roche Diagnostics, Indianapolis, IN, USA) using a Trans-Blot® Turbo™ RTA Mini PVDF Transfer Kit (Bio-Rad) and the Trans-Blot® Turbo™ Transfer System (Bio-Rad) according to the manufacturer's instructions. The membrane was blocked with 5% skim milk (Coles, Australia) in distilled water overnight at 4°C, and then washed three times (5 min each) with PBS-T (PBS

containing 0.1% Tween-20). The membrane was incubated with rat anti-peptide polyclonal antibody (rat anti-691666-1 or rat anti-691666-2) or rat anti-IBV polyclonal antibody diluted 1:200 in 1% skim milk in PBS-T for 2 h at room temperature with gentle rocking, then washed three times as described above. The membrane was then incubated in goat anti-rat IgG HRP conjugate (Merck Millipore, Bayswater, Victoria, Australia) diluted 1:2000 in 1% skim milk in PBS-T for 1 h at room temperature. Following incubation, the membrane was washed in PBS-T for 15 min followed by three washes for 5 min each. The blot was developed using a solution of SIGMAFAST™ 3-3' diaminobenzidine (DAB) and urea hydrogen peroxide tablets (Sigma) dissolved in distilled water, then washed with distilled water and air dried at room temperature.

5.4 Results

5.4.1 Two recombinant *M. synoviae*, rMSH-IBVS1 and rMSH-IBVN, were generated

Four divergent overlapping PCR fragments (IBV S1, *vlhA* promoter, MCS-lacZ and backbone) of 842 bp, 685 bp, 694 bp and 4108 bp were amplified (Table 5.1) (Figure 5.5a). A plasmid (pGA-IBVS1) was constructed using Gibson assembly by ligation of the overlapping PCR fragments. The coding sequence for the IBV S1 gene was located immediately following the *vlhA* promoter in pGA-IBVS1. The construct was introduced into competent *E. coli* cells. Blue colonies were picked after growth on LB plates containing ampicillin and X-gal, cultured overnight and the plasmid was extracted. The presence of the insert sequences and correct order of assembly of each fragment were examined using overlapping PCRs and a series of pairs of primers (results not shown).

The sequence of the pGA-IBVS1 construct was confirmed by Sanger sequencing ¹². A *tetM* DNA fragment (~5.3 kbp) released from plasmid pKS-VOTL was further ligated into the construct pGA-IBVS1 between the cleavage sites for *Bam*HI and *Sal*I (Figure 5.5b). After transformation, *E. coli* transformants resistant to both ampicillin and tetracycline were selected, cultured overnight and plasmid was extracted. The final construct (pMS-IBVS1) was used to transform MS-H cells. Twenty-six tetracycline resistant transformants were selected from cultures on MA and subcultured in MB containing tetracycline. After 5 passages, the construct was still present in the transformants, as confirmed by IBV S1 gene and *tetM* gene PCRs (Figure 5.6). A pMAS-LoriC transformant (rMSH-LoriC) passaged 5 times in MB was used as a control for transformation. A single pMS-IBVS1 MS-H transformant colony, C1 (rMSH-IBVS1), was selected for further investigation ¹³.

Two overlapping PCR fragments of 1268 bp (IBV N) and 5434 bp (backbone) were amplified (Table 5.1) (Figure 5.7a) and ligated together using Gibson Assembly. The construct (pGA-IBVN) was introduced into competent *E. coli* cells. Blue colonies were picked after growth on LB plates containing ampicillin and X-gal, cultured overnight and plasmid was extracted. The presence of the coding sequence for the IBV N gene was confirmed using PCRs (results not shown). The sequence of the pGA-IBVN construct was confirmed by Sanger sequencing. A *tetM* DNA fragment (~5.3 kbp) released from pMS-IBVS1 vector was then ligated into pGA-IBVN between the cleavage sites for *Bam*HI and *Sal*I (Figure 5.7b). The final construct (pMS-IBVN) was then introduced into competent *E. coli* cells. A number of colonies resistant to both ampicillin and tetracycline were selected, cultured overnight and plasmid was extracted.

¹² The entire plasmid of pGA-IBVS1 and pGA-IBVN was sequenced respectively excepted for backbone.

¹³ Two clones were randomly selected in each transformation. No difference in expression of the foreign gene between these two clones was observed. The results represented here refer to only one clone for each case.

The final construct (pMS-IBVN) was used to transform MS-H cells. Forty tetracycline resistant transformants were selected from cultures on MA and subcultured in MB containing tetracycline. After 5 passages, the construct was still present in the transformants, as confirmed by IBV N gene and *tetM* gene PCRs (Figure 5.8). The pMAS-LoriC transformant (rMSH-LoriC) passaged 5 times in MB was used as a control for transformation. A single pMS-IBVN MS-H transformant colony, C8 (rMSH-IBVN), was selected for further investigation.

5.4.2 Polyclonal antisera against IBV were produced

To assess whether a specific antibody response was induced against IBV S1 peptides (691666-1 and 691666-2) in immunised rats, blood samples were collected from each rat and an ELISA was performed. Unconjugated peptides (691666-3 and 691666-4) were used to coat the ELISA plate to test the rat anti-peptide polyclonal antibody. The antibodies in the test sera bound to the different concentrations of coated peptides, and reached peak levels within seven to eight weeks after the first inoculation (results not shown).

5.4.3 IBV S1 and N mRNAs were transcribed in recombinant MS-H ¹⁴

The expression of IBV S1 and N gene mRNA in MS-H recombinants was assessed using quantitative real-time PCR (RT-qPCR). Total RNA was extracted and, after DNase I treatment, was confirmed to be free of any contaminant plasmid DNA by *tetM* PCR (result not shown).

The presence of IBV S1 mRNA in rMSH-IBVS1 C1 was demonstrated by both conventional RT-PCR (Figure 5.9) and RT-qPCR (Figure 5.10). A PCR product of 232 bp (IBV S1 mRNA) was only detected in rMSH-IBVS1. The IBV S1 mRNA was

¹⁴ Untransformed MS-H vaccine was not included here. The comparative profiles of rMSH-IBVS1 C1 and rMSH-IBVN C8 against MS-H will be established in our future study.

transcribed in rMSH-IBVS1 at a level of 9518 ± 1704 copies per reaction. No IBV S1 mRNA transcript was detected in rMSH-LoriC by conventional or quantitative PCR.

The presence of IBV N mRNA in rMSH-IBVN C8 was demonstrated by both conventional RT-PCR (Figure 5.11) and RT-qPCR (Figure 5.12). A PCR product of 239 bp (IBV N mRNA) was only detected in rMSH-IBVN. The IBV N mRNA was transcribed in rMSH-IBVN at a level of $88,720 \pm 2057$ copies per reaction. No IBV N mRNA transcript was detected in rMSH-LoriC by conventional or quantitative PCR.

5.4.4 IBV S1 and N proteins were expressed in MS-H recombinant

Expression of the IBV S1 gene using the *vlhA* promoter in rMSH-IBVS1 C1 was initially examined by SDS-PAGE, followed by staining with Coomassie blue. No difference in protein bands was detectable between rMSH-IBVS1 C1 and rMSH-LoriC (result not shown). Immunostaining of the proteins of rMSH-IBVS1 C1 and rMSH-LoriC with polyclonal rat antisera against IBV peptides 691666-1 or 691666-2 detected a band of ~40 kDa in rMSH-IBVS1 C1, but not in rMSH-LoriC (Figure 5.13). However, the rat anti-IBV-peptide sera was unable to detect the complete IBV S1 protein in Western blots of lysates of the VicS vaccine (result not shown). Expression of the IBV N gene using the *vlhA* promoter in rMSH-IBVN C8 was also examined using SDS-PAGE and staining with Coomassie blue. No difference in protein bands was detectable between rMSH-LoriC and rMSH-IBVN C8 (result not shown). Immunostaining of the proteins of rMSH-IBVN C8 and rMSH-LoriC with the polyclonal rat antiserum against IBV strain VicS-v detected a band of ~50 kDa in both rMSH-IBVN and lysates of the VicS vaccine, but not in rMSH-LoriC. A band of similar size was detected by sera from VicS vaccinated birds in rMSH-IBVN C8 and lysates of the VicS vaccine, but not in rMSH-LoriC (Figure 5.14).

5.5 Discussion

Since it was first described in 1931 (Schalk and Hawn, 1931), IBV has continued to be a major economic concern for the poultry industry worldwide. Despite extensive vaccination, virulent IBVs, in some cases resulting from genomic recombination, continue to emerge and circulate in the field (Zhao et al., 2017b). Live vaccines can recombine with other IBV strains, resulting in novel, virulent recombinant strains (Estevez et al., 2003; Quinteros et al., 2016) and can also sometimes cause disease in chickens themselves (Liu et al., 2013b). This suggests that a vectored IBV vaccine may be a safer option. Both IBV and MS-H colonise the upper respiratory system of chickens. Therefore, the MS-H vaccine could be an ideal vehicle for the delivery of IBV subunit vaccines. In this study, the MS-H vaccine, which has proven safety and efficacy, was used as a vector to develop a novel IBV vaccine. Protective antigens of IBV, partial S1 and N proteins derived from VicS, a vaccine strain of IBV consisting of two viral subpopulations VicS-v and VicS-del (Hewson et al., 2012), were expressed in MS-H to induce immunity against viral infection. As codon bias could be a significant underlying problem in heterologous gene expression in mycoplasmas (Kanaya et al., 1999), the codons of the IBV S1 or N genes were optimised for MS.

To generate the vector expressing S1 or N gene in MS, Gibson Assembly was used instead of other conventional plasmid vector construction strategies. Three enzymes, including T5 exonuclease, thermostable DNA polymerase and DNA ligase, are used together in Gibson Assembly in a single reaction. These enzymes can be simultaneously active without interfering with each other's performance (Gibson et al., 2009). Briefly, the T5 exonuclease first removes nucleotides at the 5' end of double-stranded DNA molecules, creating single-stranded 3' terminal overhangs. Then complementary single-stranded DNA overhangs anneal and Phusion DNA polymerase fills the gaps and *Taq* DNA ligase seals the nicks. This approach has simplified and accelerated the DNA

assembly workflow and therefore been used in constructing gene expression systems (Liu et al., 2016) and full-length infectious cDNA clones (Bordat et al., 2015). A key point for this strategy was the design of the overlapping regions in the PCR amplicons, because single stranded 5' ends produced by T5 exonuclease in each PCR fragment can form secondary structures such as hairpins, which can interfere with annealing of the overlapping fragments and therefore decrease the efficiency of the construct assembly (Bordat et al., 2015). In this study, Sanger sequencing of the assembled constructs revealed nucleotide mismatches in or near the overlapping zone of several clones (results not shown). This is likely to have been caused during 5' end gap filling by DNA polymerase. In addition, the β -galactosidase promoter was included upstream of the IBV S1 and N genes in the pGA-IBVS1 and pGA-IBVN constructs. This design allowed the expression of the S1 and N proteins in the intermediate *E. coli* host.

The S1 glycoprotein is known to be a major inducer of protective immune responses because it carries antigenic regions that induce neutralising antibodies associated with protection (Ignjatovic and Galli, 1994; Ignjatovic and Galli, 1995). Therefore, S1 has been widely used as a target in IBV recombinant vaccine candidates, such as those using fowl adenovirus (Johnson et al., 2003), fowlpox virus (Wang et al., 2002; Wang et al., 2009), Newcastle disease virus (Zhao et al., 2017a) and *M. gallisepticum* (Shil et al., 2011) as vectors. A number of conformation-dependent epitopes that are involved in protection have been identified within or outside the hyper-variable region of S1 (Ignjatovic and Sapats, 2005; Kant et al., 1992; Koch et al., 1990). The region of the S1 glycoprotein used to generate rMSH-IBVS1 is immunogenic and contains B cell epitopes that are conserved in all Australian IBV strains (Ignjatovic and Sapats, 2005), and also encompasses cytotoxic T lymphocyte (CTL) epitopes (Tan et al., 2016) that are involved in protective immunity against IBV. When expressed in *M. gallisepticum*, this IBV S1 gene region was also shown to reduce pathological changes induced by IBV

challenge (Shil et al., 2011).

Detection of S1 expression by recombinant IBV vaccine candidates has always been challenging when conventional immunological assays such as Western blotting have been used. Therefore, a number of previous studies have attempted to detect RNA transcripts of the S1 gene (Chiu, 2006; Johnson et al., 2003; Shil, 2014; Shil, 2010). In this study, RNA transcripts of the IBV S1 gene were detected by RT-qPCR, and the IBV S1 protein could be detected in rMSH-IBVS1 transformant C1 whole cell proteins by Western blotting using mono-specific rat anti-S1-peptide polyclonal antibodies. However, the S1 protein was not detectable on an SDS-PAGE gel of the whole cell proteins of the transformants stained with Coomassie blue, indicating a relatively low level of expression of the S1 protein. Interestingly, the S1 protein could not be detected in Western blots of lysates of the VicS vaccine, suggesting that the rat polyclonal sera may be insufficiently potent to detect the amount of virus in the vaccine.

There was also a discrepancy between the predicted (27 kDa) and observed molecular weight (40 kDa) of S1 in the Western blot of rMSH-IBVS1 C1 cell lysates. This effect may be due to “gel shifting”, a phenomenon where membrane proteins migrating unusually slowly in SDS-PAGE (Rath et al., 2009). Anomalous SDS-PAGE migration of S1 might be influenced by incomplete unfolding of the protein and/or less binding of SDS due to amino acid composition. Chicken anti-IBV serum was also used to detect the presence of IBV S1 in rMSH-IBVS1. However, a large number of non-specific bands were detected in both the negative control and rMSH-IBVS1, which obscured the detection of IBV S1 expression in rMSH-IBVS1 C1 (result not shown). Therefore, further analysis of the band detected by the mono-specific rat antisera against the S1 protein (possibly by immunoprecipitation followed by mass spectrometry) may be required to confirm the identity of the protein.

Given that S1 is highly variable between IBV strains, and that it may fail to provide cross protection across different serotypes (Ignjatovic and Galli, 1995; Pei et al., 2003), other IBV antigens have been targeted for the development of recombinant vaccines against IBV. The N protein is a phosphoprotein bound to genomic RNA in a helical ribonucleoprotein complex (Jayaram et al., 2005) and plays diverse roles in the replication and transcription of IBV (Stern and Sefton, 1982). Abundant N protein is produced during viral replication, and it is highly conserved across IBV strains (Siddell et al., 1983; Williams et al., 1992) and therefore may induce cross-reactive immune responses. The N protein possesses T-cell epitopes (Boots et al., 1991), and has been the protein most often associated with the induction of cell mediated immune responses (Boots et al., 1992; Ignjatovic and Galli, 1995). Specific CTL epitopes have been mapped in the carboxyl end of N protein and are associated with initial elimination of the virus in the absence of S1 neutralising antibodies (Seo et al., 1997), which are induced at later stages of viral infection (Ignjatovic and Galli, 1994). The role of cell mediated immune responses in protection against IBV was also demonstrated by the finding that transfer of either primary effector CD4⁻/CD8⁺ T cells bearing the $\alpha\beta$ T cell receptor (TCR) at 10 days post-infection (p.i.) or memory CD8⁺ T cells collected from 3 to 6 weeks p.i. could protect chicks from acute IBV infection (Collisson et al., 2000; Seo et al., 2000). The role of the N protein in humoral immune responses has also been investigated. Several B-cell epitopes have been identified in the N protein (Ignjatovic and Sapats, 2005; Seah et al., 2000; Yu et al., 2010). The N protein can also accelerate induction of the antibody response to the S protein, as measured by the HI and VN assays (Boots et al., 1992). These indicate the essential role of N protein in development of neutralising antibodies against IBV.

In rMSH-IBVN C8, mRNA transcripts of the N gene were detected by RT-qPCR, revealing that the gene was transcribed at a relatively high abundance, at least compared

to S1. A protein band of about 50 kDa was expected for the IBV N protein, but it was not visible on a Coomassie blue stained SDS-PAGE gel, suggesting only a moderate level of expression in rMSH-IBVN C8. However, a band of the expected size was detected on Western blots probed with rat anti-IBV antiserum and chicken anti-IBV antiserum, demonstrating that the protein was expressed at a detectable level in rMSH-IBVN C8.

5.6 Conclusion

The study described here is the first attempt to develop a vaccine vector to prevent infection against both *M. synoviae* and IBV. *In vivo* studies will be required to examine the safety of rMSH-IBVS1 and rMSH-IBVN in chickens and to determine if the expression of the IBV N or/and S1 gene fragment is sufficient to induce an immune response, and if so, whether the immune response is protective against *M. synoviae* and IBV infection.

Table 5.1. *Mycoplasma synoviae* strains and plasmids used in this study

Strains or plasmids	Relevant genotype/ Characteristics	Reference
<i>M. synoviae</i> strains		
MS-H	<i>M. synoviae</i> live attenuated vaccine strain	(Morrow et al., 1998)
rMSH-IBVS1	MS-H transformed with pMS-IBVS1, clone 1	This study
rMSH-IBVN	MS-H transformed with pMS-IBVN, clone 8	This study
MSH-LoriC	MS-H transformed with pMAS-LoriC, clone 1	This study
Plasmids		
pBluescript II KS (+)	Cloning vehicle	Thermo Fisher Scientific
pMA-T-IBVS1	Vector harbouring optimised IBVS1 gene	This study
pMA-T-IBVN	Vector harbouring optimised IBVN gene	This study
pMAS-LoriC	LoriC-pGEM-T- <i>tet</i> (vector capable of replicating in <i>M. synoviae</i>)	(Shahid et al., 2014a)
pKS-VOTL	Vector expressing Obg protein in <i>M. synoviae</i>	Unpublished result
pMS-IBVS1	LoriC-pBluescript II KS (+)- <i>tet</i> -P _{vlhA} -IBVS1 (vector used as backbone for construction of pMS-IBVN)	This study
pMS-IBVN	LoriC-pBluescript II KS (+)- <i>tet</i> -P _{vlhA} -IBVN (vector used to express IBVN gene in <i>M. synoviae</i>)	This study

Table 5.2. List of primers^a used in this study

Primer name	Template ^a	Amplified fragment/ region	Sequence (5'-3') ^b	Product size (bp)
pKS-VOTL F	pKS-VOTL	Backbone	TAAT CAAAAGAATCTGATGAATCAGTGTAATTTATAAG	4108
pKS-VOTL R	plasmid		AGGTGGCACTTTTCGGGG	
MCS-lacZ F	pBluescript II	MCS- <i>lacZ</i>	TTCCCCGAAAAGTGCCACCT TAGGCACCCCAGGCTTTA	694
MCS-lacZ R	KS (+) plasmid		CAAGCTATT ACTAATCAAGTTTTTTGGGGTCG	
pVlhA-ATG F	MS-H genome	<i>vlhA</i>	ACTTGATT AGTAATAGCTTGCCTTAAAGCG	685
pVlhA-ATG R	DNA	promoter	TAGTTAACGT CATAAATAAATCCTTTTATTTTACGCATC	
IBVS1-opt F	pMA-T-IBVS1	IBV S1	AAATAAAAGGATTTATTTAT GACCCTTACCAACTTTACTTTTC	842
IBVS1-opt R	plasmid		GATTCATCAGATTCTTTTG ATTATTAGCTTCCGTTGGTAAG	
pMS-IBV-bb F	pMS-IBVS1	pMS-IBV	TCAAAAGAATCTGATGAATCAG	5,434
pMS-IBV-bb R	plasmid	backbone	CATAAATAAATCCTTTTATTTTACGCATC	
IBVN-opt F	pMA-T-IBVN	IBV N	AAATAAAAGGATTTATTTAT GGCTTCAGGTAAAGCTGCTG	1268
IBVN-opt R	plasmid		GATTCATCAGATTCTTTTG ATTATTATAATTCGTTTTCTCCAAGAGC	
tetM-F1	NA	<i>tetM</i>	GCAGTTATGGAAGGGATACG	340
tetM-R1			TTCTTGAATACACCGAGCAG	
IBVqPCR-F2	NA	IBV S1	CCACCCAATTCTGGTGGTGT	232
IBVqPCR-R2			AACCGCCTTGAATAGGACCG	
IBVNqPCR-F2	NA	IBV N	AGCTGATGAAATGGCTCACAGA	239
IBVNqPCR-R2			GGTGAAGTCCGTCTGGTTGT	

^a Used for Gibson assembly PCRs.^b Overlapping regions between PCR fragments are in bold letters; NA, not applicable.

Table 5.3. Synthetic peptides used in this study

Name	Sequence	Description	Usage
691666-1	CTKSDGSRIQTATTP	KLH conjugated to cysteine	Raising polyclonal antibody in rats
691666-2	GFEYKESNFMYGSYC	KLH conjugated to cysteine	Raising polyclonal antibody in rats
691666-3	CTKSDGSRIQTATTP	Unconjugated	ELISA to detect antibody response
691666-4	GFEYKESNFMYGSYC	Unconjugated	ELISA to detect antibody response

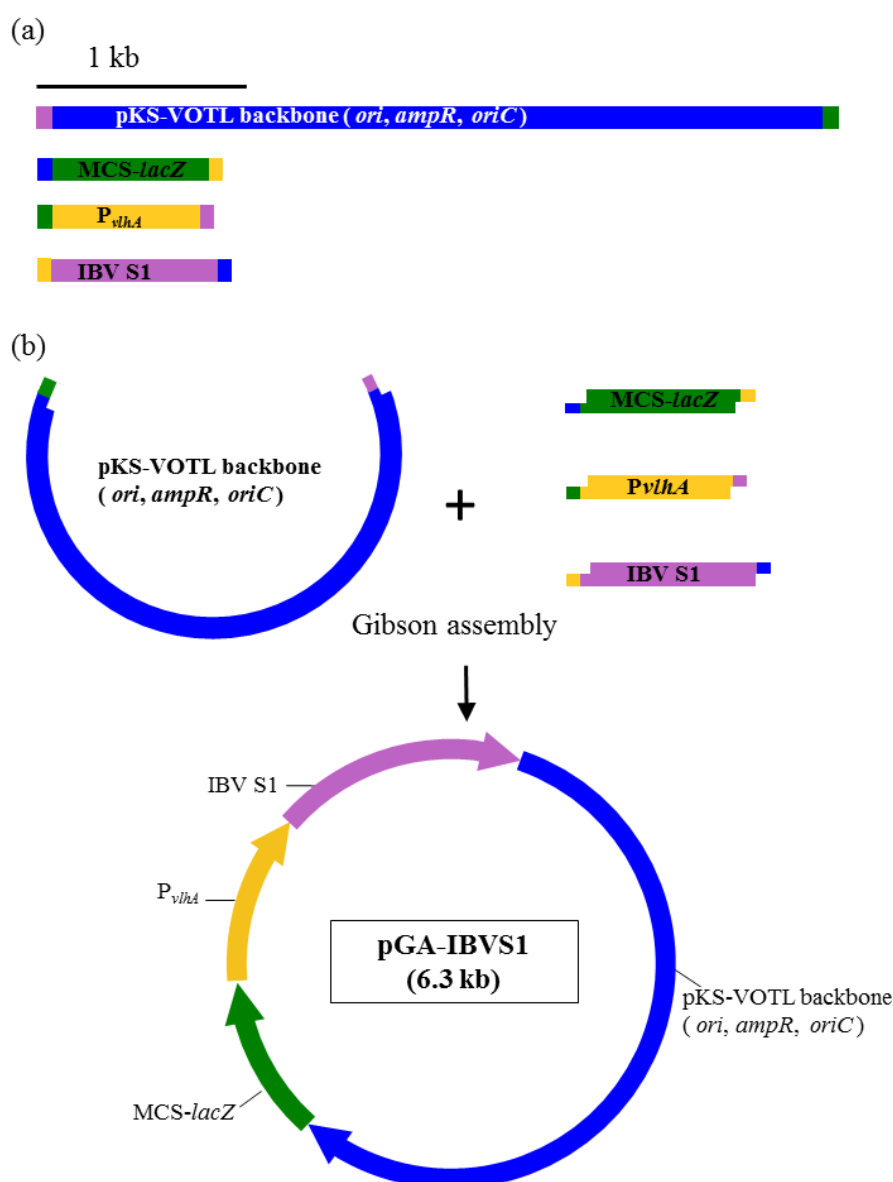


Figure 5.1. A schematic diagram showing procedures for generating pGA-IBVS1 (vector construction, step I). **(a)** Four DNA fragments, namely pMS-VOTL backbone, MCS-*lacZ*, *vlhA* promoter and IBV S1 were amplified from pKS-VOTL, pBluescript II KS (+), MS-H genome DNA and pMA-T-IBVS1, respectively, using specific primers. **(b)** The four modular segments were then joined using Gibson Assembly[®] Master Mix (New England Biolabs, La Jolla, California, USA) to form a circular plasmid. *E. coli* transformation was performed according to the manufacturer's instructions, and the plasmid was selected on LB agar plates containing ampicillin (100 µg/ml), IPTG (0.2 mM) and X-gal (40 µg/ml). Vector elements: *ori*, origin of replication of *E. coli*; *oriC*, origin of replication of *M. synoviae*; *ampR*, ampicillin resistance gene; P_{*vlhA*}, promoter sequence of *vlhA* gene; MCS-*lacZ*, multiple cloning sites and *lacZ* operon gene; IBV S1, S1 gene fragment of IBV.

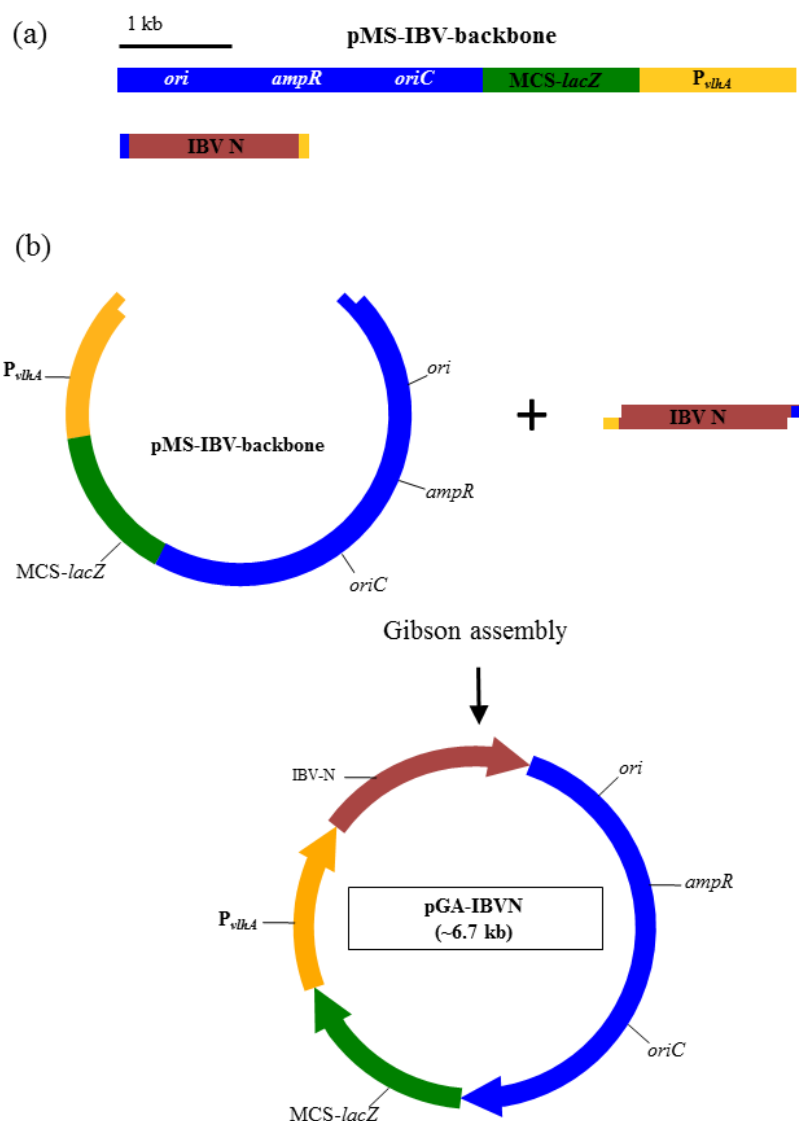


Figure 5.2. A schematic diagram showing procedures for generating pGA-IBVN (vector construction, step I). **(a)** Two DNA fragments, namely pMS-IBV-backbone and IBV N were amplified from pMS-IBVS1 and pMA-T-IBVN, respectively, using specific primers. **(b)** The two modular segments were then joined to each other using Gibson Assembly[®] Master Mix (New England Biolabs, La Jolla, California, USA) to form a circular plasmid. *E. coli* transformation was performed according to the manufacturer's instructions, and the plasmid was selected on LB agar plates containing ampicillin (100 µg/ml), IPTG (0.2 mM) and X-gal (40 µg/ml). Vector elements: *ori*, origin of replication of *E. coli*; *oriC*, origin of replication of *M. synoviae*; *ampR*, ampicillin resistance gene; P_{*vlhA*}, promoter sequence of *vlhA* gene; MCS-*lacZ*, multiple cloning sites and *lacZ* operon gene; IBV N, N gene of IBV.

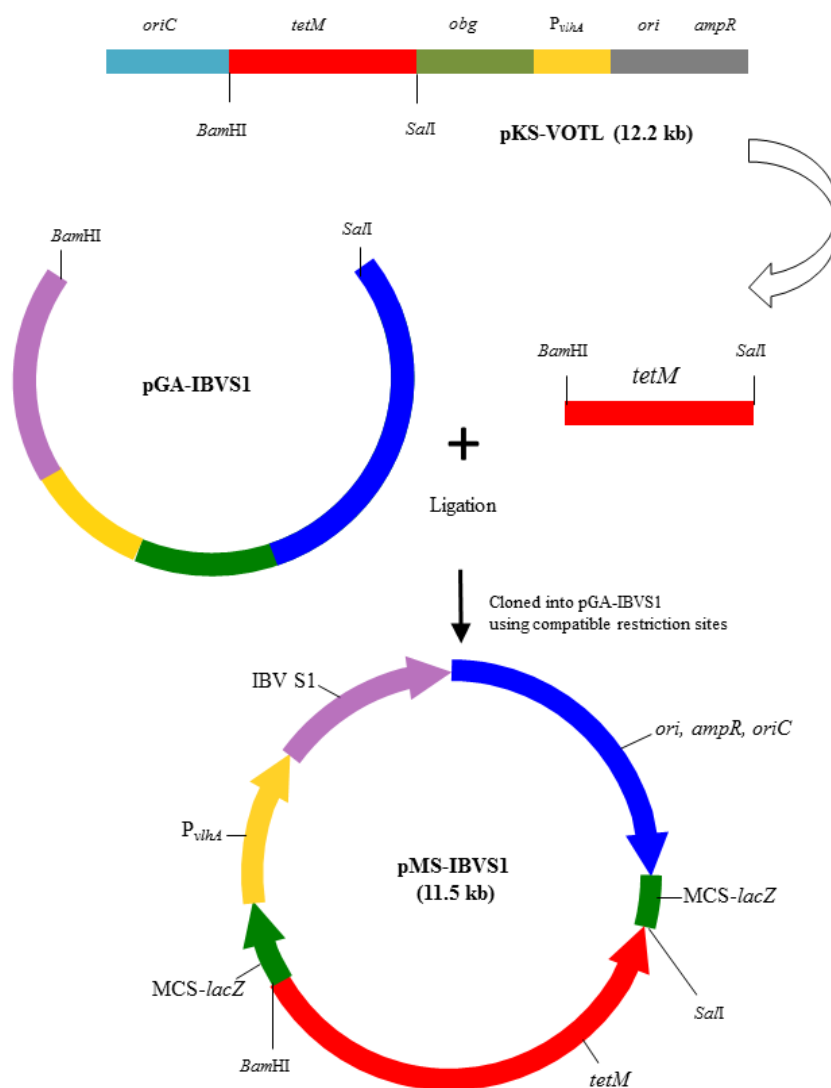


Figure 5.3. A schematic diagram showing procedures for generating the final vector construct pMS-IBVS1 (vector construction, step II). The DNA fragment containing the *tetM* gene was released from pKS-VOTL. This product was subsequently cloned into the vector constructed in step I to generate a circular plasmid, pMS-IBVS1. *E. coli* transformation was performed according to the manufacturer's instructions, and the plasmid was selected on LB agar plates containing ampicillin and tetracycline. Vector elements: *ori*, origin of replication of *E. coli*; *oriC*, origin of replication of *M. synoviae*; *ampR*, ampicillin resistance gene; *obg*, a GTP binding protein gene of *M. synoviae*; IBV S1, S1 gene of IBV; *P_{vlhA}*, promoter sequence of *vlhA* gene; *tetM*, tetracycline resistance gene.

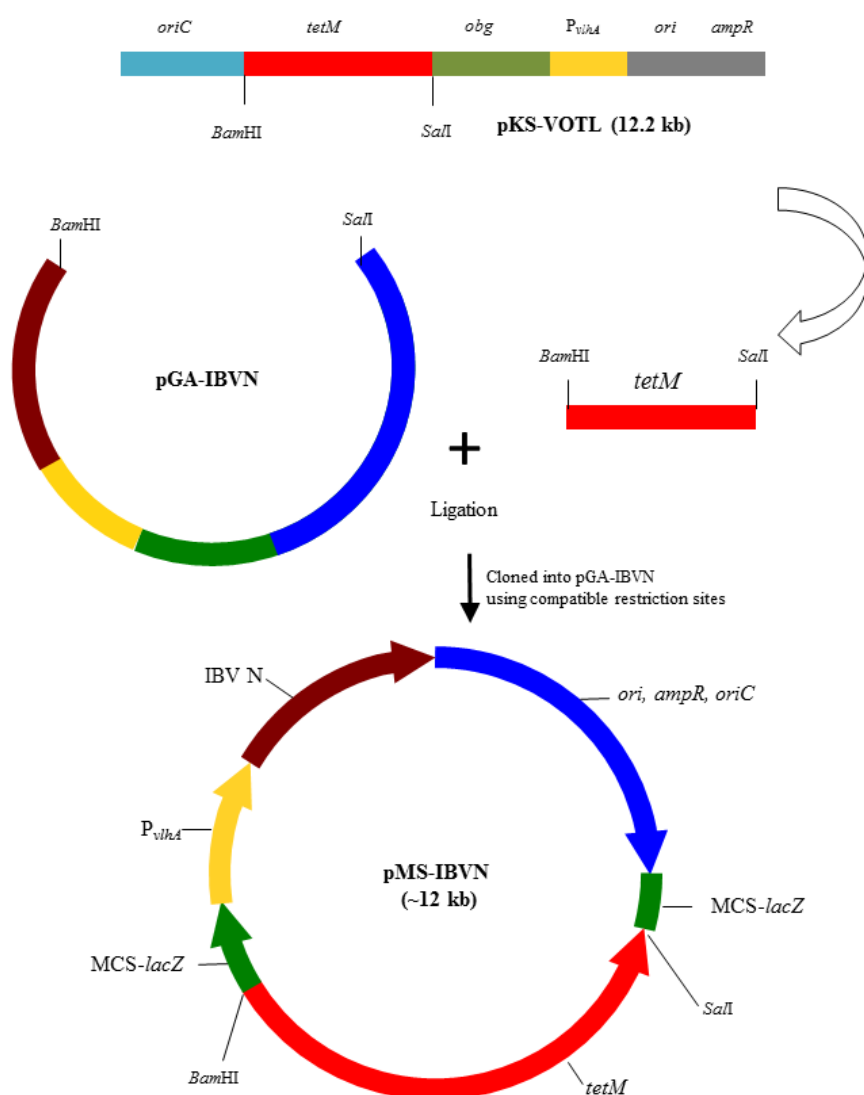


Figure 5.4. A schematic diagram showing procedures for generating the final vector construct pMS-IBVN (vector construction, step II). A DNA fragment containing the *tetM* gene was released from pKS-VOTL. This product was subsequently cloned into the vector constructed in step I to generate a circular plasmid, pMS-IBVN. *E. coli* transformation was performed according to the manufacturer's instructions, and the plasmid was selected on LB agar plates containing ampicillin (100 µg/ml) and tetracycline (2.5 µg/ml). Vector elements: *ori*, origin of replication of *E. coli*; *oriC*, origin of replication of *M. synoviae*; *ampR*, ampicillin resistance gene; *obg*, a GTP binding protein gene of *M. synoviae*; IBV N, N gene of IBV; *P_{vlhA}*, promoter sequence of *vlhA* gene; MCS-*lacZ*, multiple cloning sites and *lacZ* gene; *tetM*, tetracycline resistance gene.

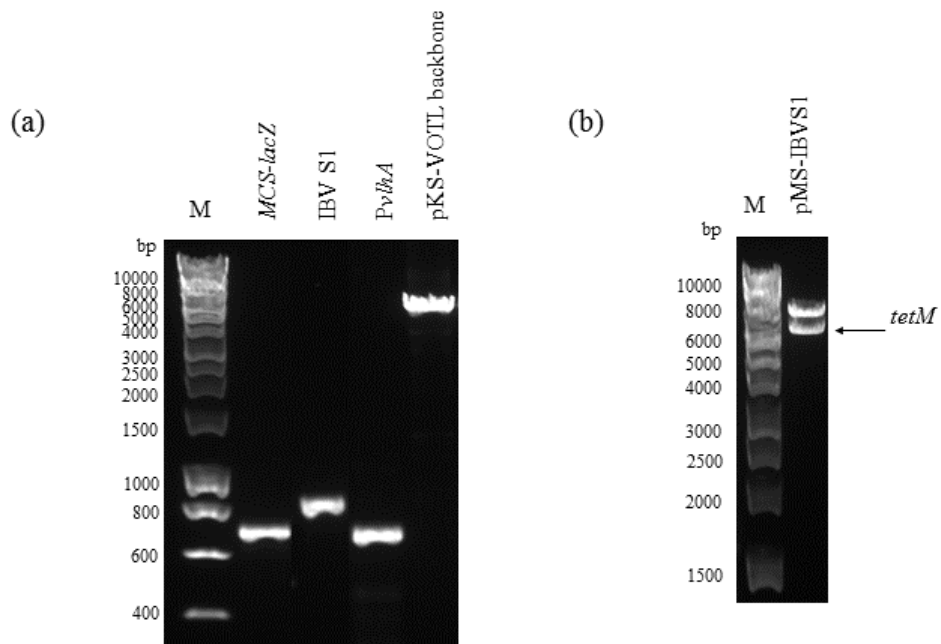


Figure 5.5. Agarose gel (0.9%) electrophoresis of **(a)** PCR products generated from pBluescript II KS (+), pMA-T-IBVS1 and pKS-VOTL, and **(b)** pMS-IBVS1 digested with *Bam*HI and *Sal*II. M, molecular weight markers (HyperLadder™ 1 kb; Bioline, Alexandria, Australia).

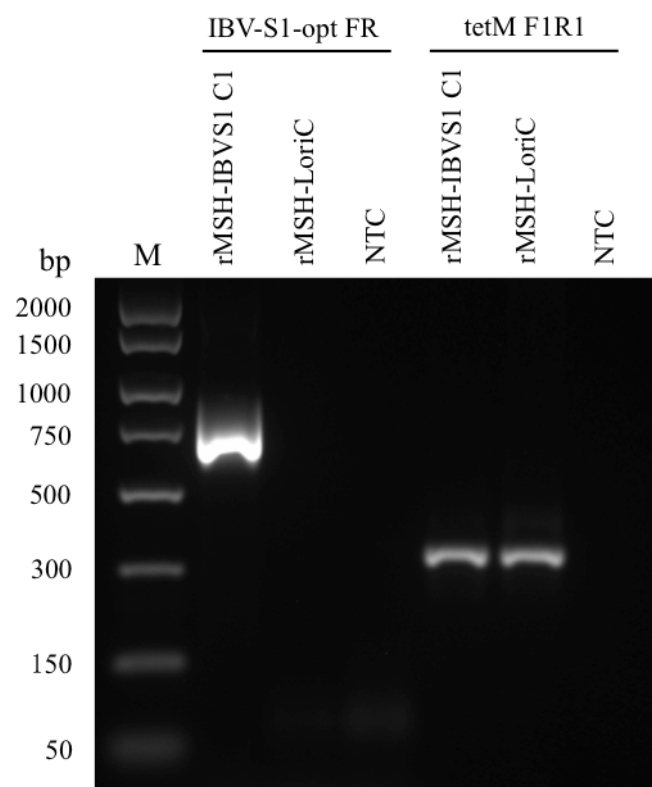


Figure 5.6. Agarose gel (1%) electrophoresis of PCR products generated by primers IBVS1-opt F/R and tetM F1/R1 from *M. synoviae* transformant rMSH-IBVS1 C1. M, molecular weight marker (PCR marker; Sigma, Missouri, USA). NTC, no template control.

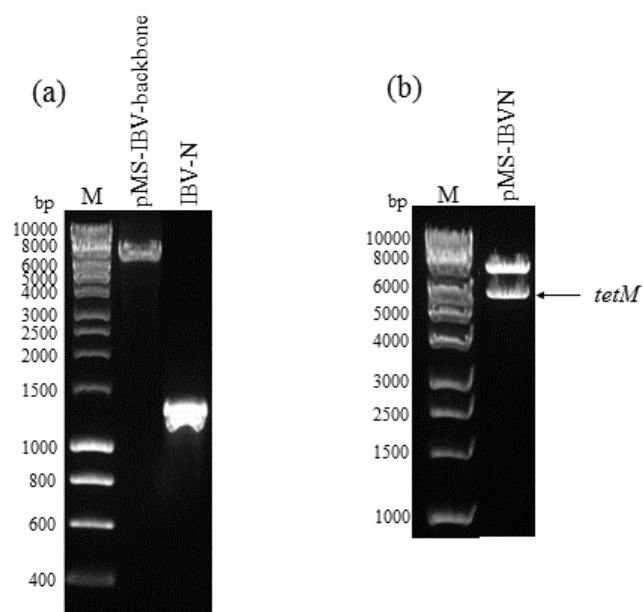


Figure 5.7. Agarose gel (0.9%) electrophoresis of **(a)** PCR products generated from pMS-IBVS1 and pMA-T-IBVN, and **(b)** of pMS-IBVN digested with *Bam*HI and *Sal*I. M, molecular weight markers (HyperLadderTM 1 kb; Bioline, Alexandria, Australia).

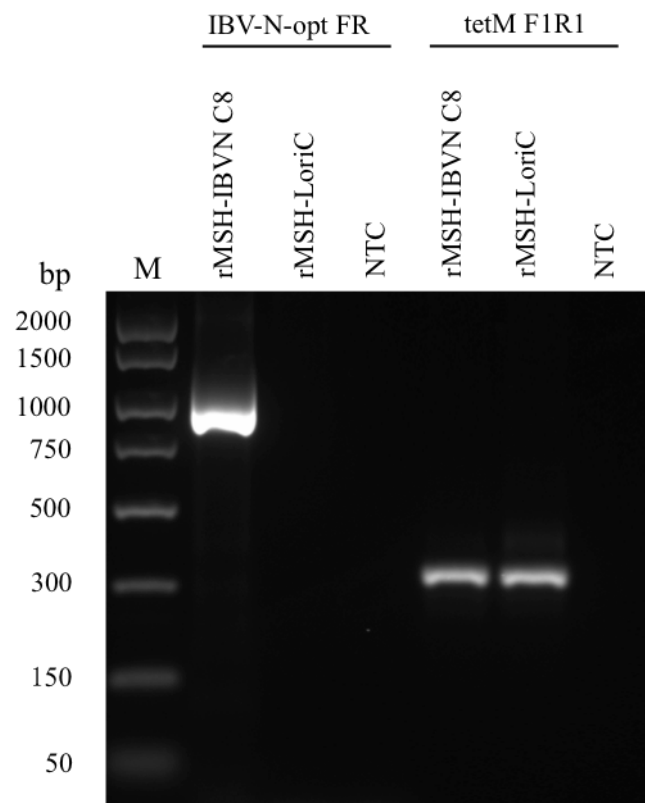


Figure 5.8. Agarose gel (1%) electrophoresis of PCR products generated by primers IBVN-opt F/R and tetM F1/R1 from *M. synoviae* transformants rMSH-LoriC and rMSH-IBVN C8. M, molecular weight markers (PCR marker; Sigma, Missouri, USA).

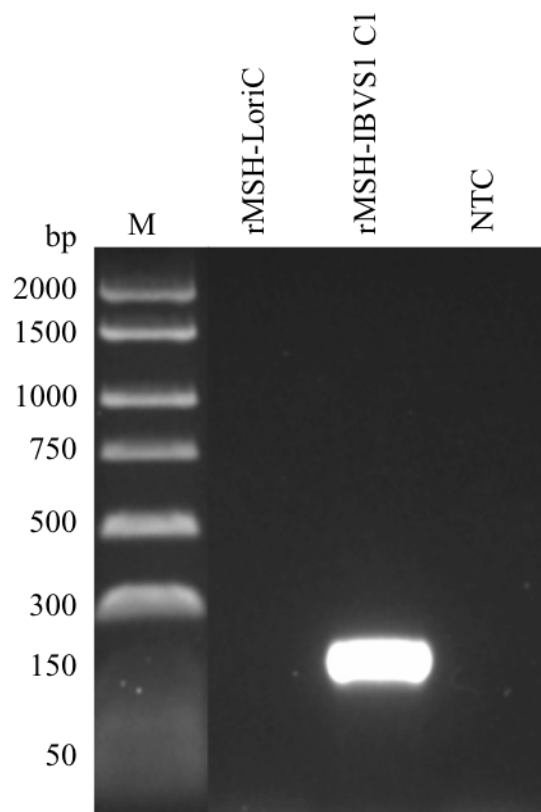
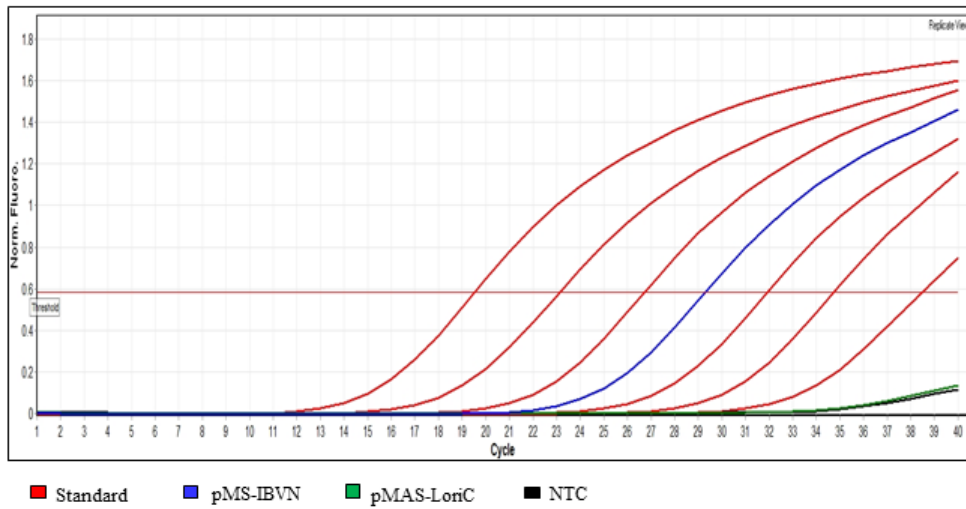


Figure 5.9. Agarose gel (1%) electrophoresis of PCR products generated by primers IBVS1qPCR-F2/R2 from *M. synoviae* transformants rMSH-LoriC and rMSH-IBVS1 C1; cDNA was used as a template. M, molecular weight markers (PCR marker; Sigma, Missouri, USA). NTC, no template control.

(a)



(b)

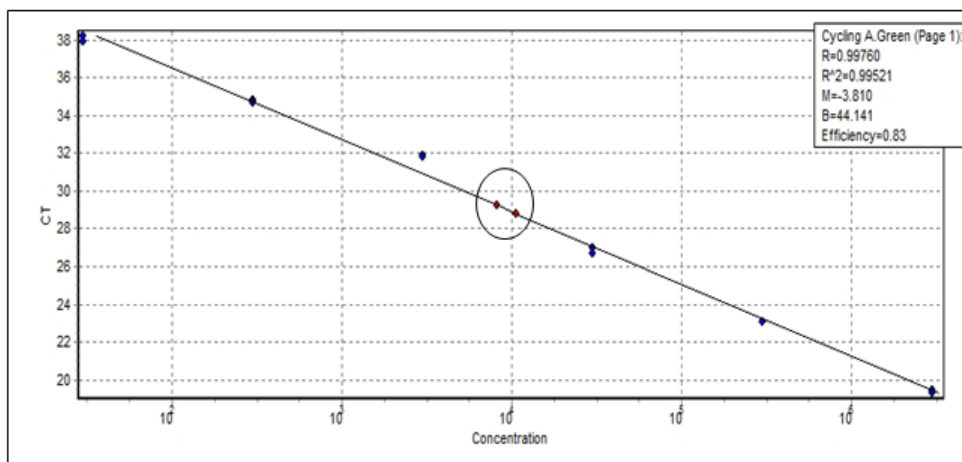


Figure 5.10. Quantitation of RNA transcripts of IBV S1 gene in *M. synoviae* transformant rMSH-IBVS1 C1. **(a)** Linear view of amplification curve generated from serial 10-fold dilutions of pMS-IBVS1 plasmid DNA. Auto-scale was used to set the threshold for analysis. **(b)** Standard curve, with the IBV S1 mRNA level indicated by a circle.

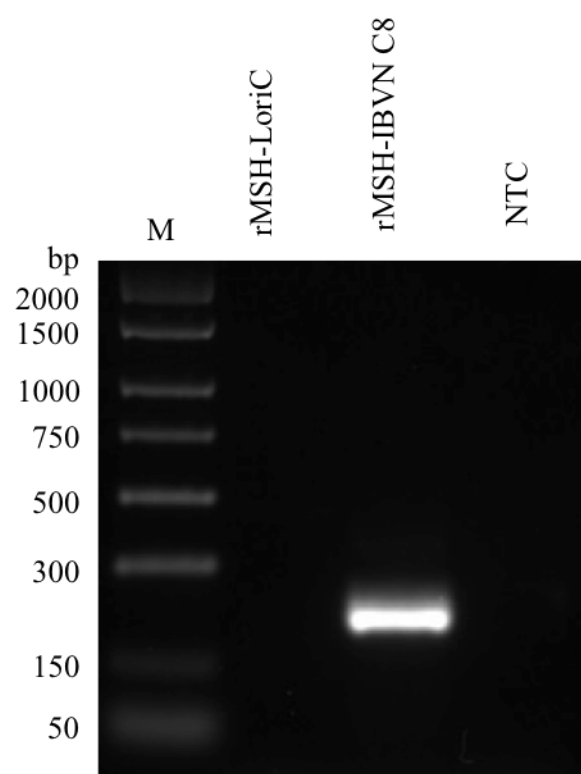
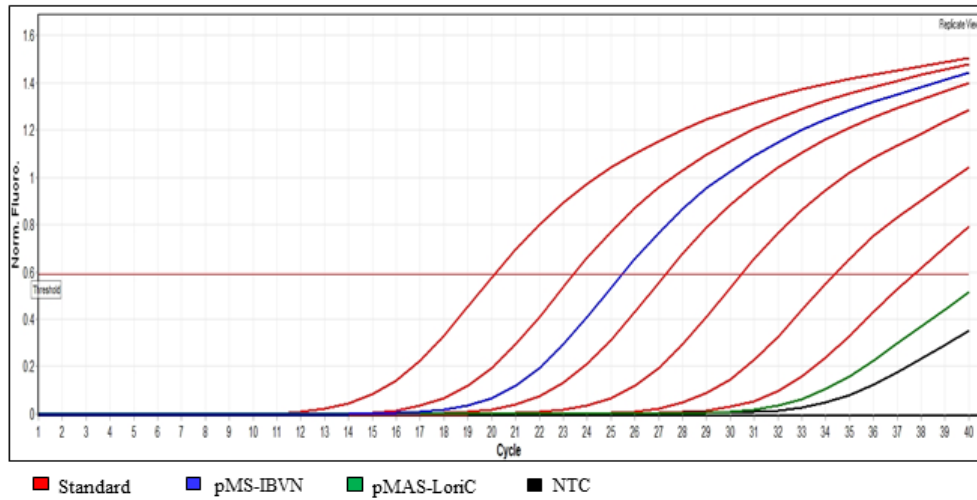


Figure 5.11. Agarose gel (1%) electrophoresis of the PCR product generated by primers IBVNqPCR-F2/R2 from *M. synoviae* transformants rMSH-LoriC and rMSH-IBVN C8. cDNA was used as a template; M, molecular weight markers (PCR marker; Sigma, Missouri, USA). NTC, no template control.

(a)



(b)

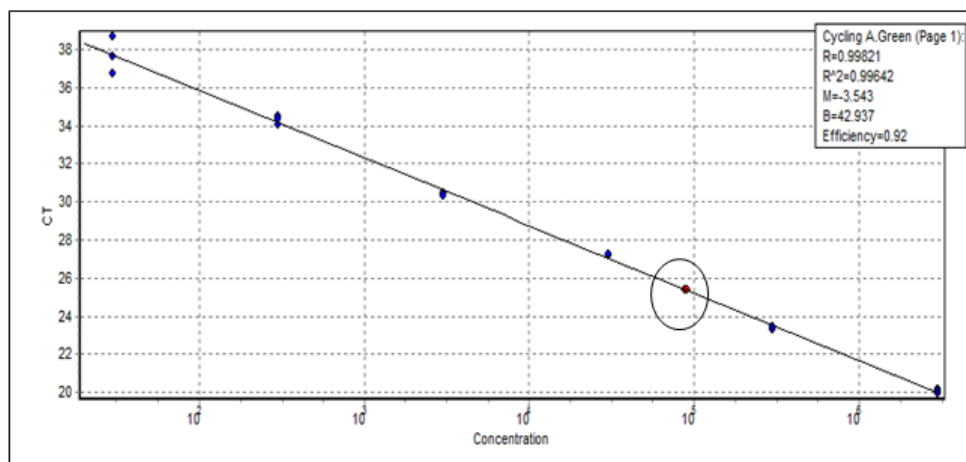


Figure 5.12. Quantitation of RNA transcript of IBV N gene in *M. synoviae* transformant rMSH-IBVN C8. **(a)** Linear view of amplification curve generated from serial 10-fold dilutions of pMS-IBVN DNA. The auto-scale was used to set the threshold for analysis. **(b)** Standard curve, with the IBV N mRNA level indicated by a circle.

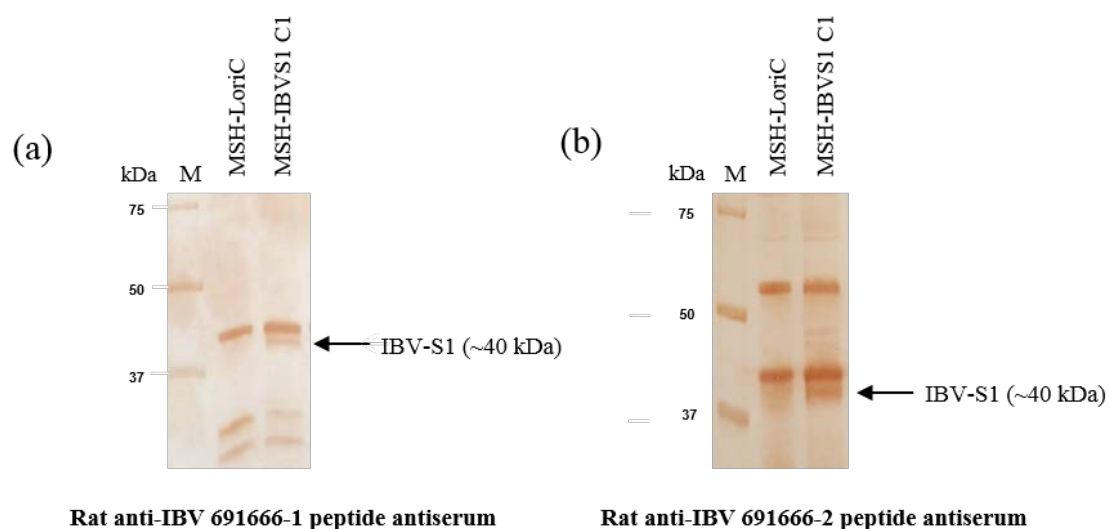


Figure 5.13. Western blots of the whole cell proteins of rMSH-LoriC and rMSH-IBVS1 C1 immunostained with **(a)** rat anti-IBV 691666-1 and **(b)** anti-IBV 691666-2 peptide antisera. M, molecular weight markers (Precision Plus ProteinTM Dual Colour Standard, Biorad, USA).

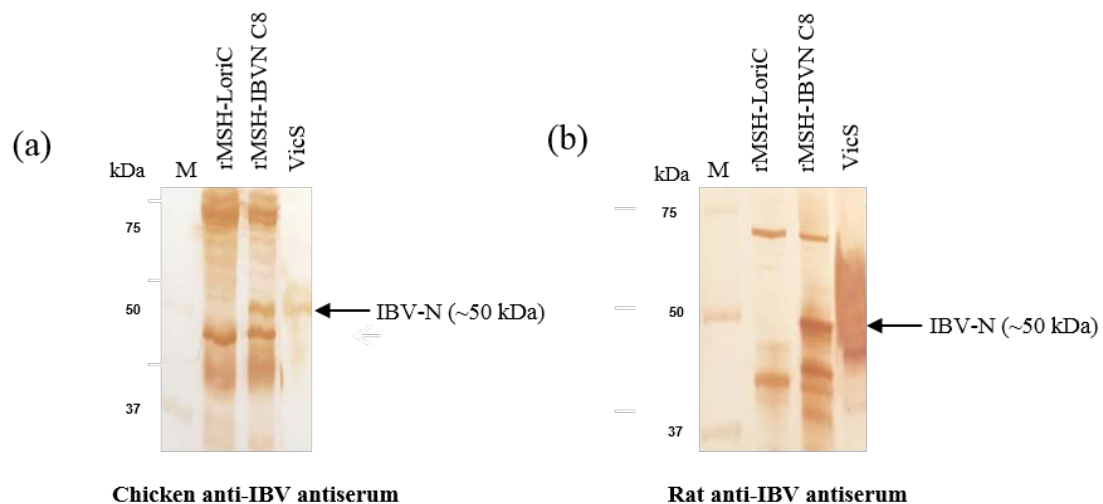


Figure 5.14. Western blots of the whole cell proteins of rMSH-LoriC, rMSH-IBVN C8 and VicS IBV immunostained with **(a)** polyclonal chicken anti-IBV antiserum and **(b)** polyclonal rat anti-IBV antiserum. M, molecular weight markers (Precision Plus ProteinTM Dual Colour Standard, Biorad, USA).

CHAPTER 6. General Discussion

6.1 Introduction

Mycoplasma synoviae is considered one of the most pathogenic avian mycoplasmas, causing significant economic impact in intensive chicken production, due to decreased egg production, inefficient food conversion, and downgrading of carcasses at slaughter because of airsacculitis and arthritis (Ferguson-Noel and Noormohammadi, 2013; Marois et al., 2002). In recent years, diseases associated with *M. synoviae* have been reported to be more apparent than those associated with *M. gallisepticum* in countries where more aggressive *M. gallisepticum* eradication programs have been introduced (Landman, 2014). The MS-H vaccine, a temperature-sensitive (ts^+) live attenuated *M. synoviae* strain that was derived by chemical mutagenesis of a virulent Australian field strain 86079/7NS, has been used in a large number of countries to prevent disease caused by *M. synoviae*. However, the molecular bases for the attenuation of MS-H are still unknown. There is also a need for rapid and reliable tests for the differentiation of the MS-H vaccine and field strains. This required full characterisation of MS-H genome. Finally, the possible use of MS-H as a vaccine vector to confer protection against other avian pathogens had not been explored previously. The studies performed during this project aimed to address these knowledge gaps, and to establish a solid foundation for understanding the attenuation mechanism of MS-H vaccine or the virulence genes of *M. synoviae*, development of a DIVA test for MS-H vaccine and also recombinant respiratory tract vaccine candidates for poultry.

6.2 Genomic characteristics of *M. synoviae* MS-H

As a result of recent advances in DNA sequencing technologies, whole genome sequencing has become a relatively inexpensive and convenient approach to improve our understanding of the evolution and pathogenic potential of many bacteria. In recent

years, the genomes of many mycoplasma species have been fully sequenced (Abolnik and Beylefeld, 2015; Kenri et al., 2012; Li et al., 2011; Liu et al., 2011; May et al., 2015; Siqueira et al., 2013; Wise et al., 2011). In the first study described in this thesis, *M. synoviae* strain MS-H and its wild type-progenitor 86079/7NS were subjected to next generation sequencing, combining Illumina and Nanopore data, to determine their complete genome sequences. Comparative analysis of organisation of the genomes of MS-H and 86079/7NS, and those of other *M. synoviae* strains that have been published, revealed a number of features unique to MS-H, which may be used as novel genetic markers in future routine diagnostic tests.

In both MS-H and 86079/7NS, a 50 kb genomic inversion was seen. This may have been mediated by insertion sequences (ISs), the most abundant class of mobile elements in mycoplasma genomes. Mobile genetic elements are agents of gene instability and chromosomal rearrangement, causing insertions, deletions, duplications and inversions of genomic loci, and consequently contributing to the genetic variability of bacteria (Nevers and Saedler, 1977; Ohtsubo and Sekiya, 1996). The horizontal transfer of fourteen regions between *M. synoviae* and *M. gallisepticum* (Vasconcelos et al., 2005) has been suggested, with nine regions associated with ISs (Loreto et al., 2007). The results presented in this thesis confirmed and expanded on these findings. As an example of transposable elements acting as agents of horizontal gene transfer in *M. synoviae*, in both 86079/7NS and MS-H genes coding for the deoxyribose-phosphate aldolase and ATP-dependent DNA helicase were found to have been transferred from *M. gallisepticum* and *M. gallinaceum*, respectively, most likely by flanking ISs. The, genomic rearrangements and/or horizontal gene transfers also has been seen in some other mycoplasma species (Citti and Blanchard, 2013; Sirand-Pugnet et al., 2007).

6.3 Potential factors involved in attenuation of *M. synoviae* MS-H

Virulence or virulence-associated determinants of *M. synoviae* have not been well characterised. Adherence of bacteria to host cells is known to be the initial step in, and a prerequisite for, successful colonisation and further invasion. Factors that are associated with cytodherence have been studied intensively in several mycoplasma species (Boguslavsky et al., 2000; Keeler et al., 1996; Krause, 1996, 1998; Razin and Jacobs, 1992; Zhang et al., 1994). In *M. synoviae*, cytodherence mediated by the primary adhesion VlhA is thought to be associated with virulence (Noormohammadi, 2007). Genomic comparisons of MS-H and its parental strain 86079/7NS established a preliminary profile of genes related to virulence. One of the most noticeable differences between MS-H and 86079/7NS was found in the C-terminal region of the expressible *vlhA* gene, a highly variable region associated with haemagglutination and haemadsorption (Khiari and Mardassi, 2012; Noormohammadi et al., 1997). However variations in the VlhA C-terminal region are not uncommon, even between isolates/clones of the same *M. synoviae* strain (Noormohammadi et al., 2000). Further studies are required to establish whether this difference has an impact on the haemagglutination activity of the vaccine strain compared to that of its parent strain. Strains that are able to agglutinate erythrocytes have been found to induce acute synovitis more frequently than haemagglutination-negative variants, highlighting the important role of the haemagglutinin in the pathogenicity of *M. synoviae* (Narat et al., 1998). Clonal variations in VlhA may influence cytodherence of *M. synoviae* to host cells and therefore its ability to induce disease (May and Brown, 2011; May et al., 2012). In rare cases, non-temperature-sensitive (*ts*⁻) MS-H isolates that are less virulent than the parent strain 86079/7NS have been recovered in the field (Markham et al., 1998c; Noormohammadi et al., 2003). Therefore, determination of the *vlhA* gene sequence and the haemagglutination phenotype in MS-H re-isolates expressing different

VlhA alleles may help to understand the role of this gene in the attenuation of *M. synoviae*.

Apart from the difference in the *vlhA* gene, 33 single nucleotide polymorphisms (SNP) were identified between the virulent strain 86079/7NS and the MS-H vaccine strain. Predictions of the potential effect of these non-synonymous SNPs on protein function can be made using a number of bioinformatic methods (Biasini et al., 2014; Bromberg and Rost, 2007; Worth et al., 2011). An amino acid alteration (G123R) within Obg has been suggested to induce the *ts*⁺ phenotype of the MS-H vaccine (Shahid et al., 2013b). However, the loss of the *ts*⁺ phenotype in MS-H *ts*⁻ clones reisolated from vaccinated birds was not associated with a significant increase in pathogenicity, indicating that the attenuation of the MS-H vaccine is multifactorial (Noormohammadi et al., 2003).

In the studies described here, a deletion was detected in a polyA tract of the MS-H *oppF-1* gene (encodes the ATP-binding component of an ABC transporter). This deletion causes a frameshift, leading to premature stop codon TAA in this gene. Slipped-strand mispairing can occur during bacterial chromosomal replication, resulting in insertions or deletions in polynucleotide tracts, which can affect gene expression by creating frameshifts leading to premature termination of translation (Levinson and Gutman, 1987; Moxon et al., 1994). The oligopeptide transporter OppABCDF is an important nutrient uptake system in mycoplasmas. It is noteworthy that peptide transporters have been found to be associated with virulence in many pathogenic bacteria (Flores-Valdez et al., 2009; Moraes et al., 2014; Tseng et al., 2017a; Wang et al., 2005). In addition to their nutritional role, peptide transporters are involved in sensing environmental signals in the form of peptides, allowing bacteria to modulate expression of specific genes (Serror and Sonenshein, 1996), including those encoding surface-exposed molecules (Flores-Valdez et al., 2009). The interruption of the *oppD*₁ gene by a transposon has been shown to result in a reduction in the capacity to colonise

and in virulence in *M. gallisepticum* (Tseng et al., 2017a; Tseng et al., 2017b). Therefore, it was predicted that the truncation of OppF-1 in MS-H could have a similar effect and may play a significant role in the attenuation of MS-H. To confirm the role of the *oppF-1* gene in virulence in *M. synoviae*, studies involving inactivation of the gene in 86079/7NS or complementation of it in MS-H need to be conducted. In a recent unpublished study in our laboratory, the wild-type *oppF-1* gene was cloned into an *oriC* plasmid vector (Shahid et al., 2014a) and expressed in MS-H under the control of the *M. synoviae* *vlhA* promoter. The effect of OppF-1 complementation on the *in vitro* and *in vivo* characteristics of MS-H is under investigation.

6.4 Implications of the mutations in *oppF-1* of MS-H

Prior to this study, the conserved region of the *vlhA* gene and the SNP in the *obg* gene were most commonly targeted for sequence typing for *M. synoviae* (Jeffery et al., 2007; Shahid et al., 2014b). Several multi-locus sequence typing (MLST) assays have also been developed recently (Cizelj et al., 2015; Dijkman et al., 2016; El-Gazzar et al., 2017). The inter-strain differences in the *obg* gene have been the basis of a number of assays to differentiate the MS-H vaccine strain from field strains (Kreizinger et al., 2015; Shahid et al., 2014b). However, these assays either cannot differentiate MS-H from a number of Australian (Jeffery et al., 2007) or European (Catania et al., 2016b; Landman, 2014) field strains, or have limitations in discriminating between wild type field strains and occasional *ts⁻* MS-H re-isolates from vaccinated birds (Kreizinger et al., 2015; Shahid et al., 2014b). In this project, the *oppF-1* gene from a number of *M. synoviae* field strains isolated from diverse geographical locations were sequenced, confirming that the adenine deletion at nt468 in *oppF-1* was unique to MS-H. To explore whether the A-del mutation (nt468) in *oppF-1* was reversible, *M. synoviae* strains isolated from SPF chickens vaccinated with MS-H under controlled conditions and also from field chickens vaccinated with MS-H were examined. All MS-H re-

isolates collected after passage *in vivo* in SPF chickens had an *oppF-1* sequence identical to the vaccine strain. Based on this finding, a nested PCR, combined with high-resolution melting (HRM) curve analysis, was developed and was shown to have potential for differentiation of MS-H from field strains. However, the *oppF-1* gene sequence analysis of “vaccine re-isolates” from field chickens found that a number of these isolates had a vaccine-type *obg* and a wild-type *oppF-1*. This finding indicated a possibility of a reversion mutation in *oppF-1* under field conditions (see Table 4.5). Therefore, differentiation of field strains from MS-H field re-isolates may require further investigation. The observation of this *oppF-1* mutation in both *ts*⁺ and *ts*⁻ MS-H re-isolates indicated that this mutation is less likely to contribute to the temperature sensitivity of the MS-H vaccine. In a few isolates, including MS-H⁴, 93205 2-9a, 94036 6-3a, 93025 8-9c, 94036 10-5a and 93205 2-13a, revertant mutations were simultaneously observed in both *oppF-1* and *obg* genes (Shahid et al., 2013b), which has raised the question of whether these are true “MS-H re-isolates”. In a previous study, it was also noted that infection with MS-H⁴ induced increases in upper tracheal mucosal thicknesses that were significantly greater than those induced by infection with MS-H⁵, although they were both less pathogenic than the parental strain 86079/7NS (Noormohammadi et al., 2003). Given that the MS-H clones with revertant mutations were collected from field chicken flocks (Markham et al., 1998c), there is a possibility that these isolates were actually field strains indistinguishable from the vaccine parent strain 86079/7NS by *vlhA* sequencing and RFLP analysis of genomic DNA (Markham et al., 1998c). Therefore, as the *oppF-1* mutation may have limitations in discriminating MS-H field re-isolates, whole genome sequencing and analysis of the MS-H field re-isolates, especially those MS-H clones with revertant mutations in *oppF-1* or/and *obg* genes, are required in the future to further investigate their identity. This investigation is also likely to contribute to our understanding of *M. synoviae* virulence-associated genes.

6.5 Expression of heterogenous genes in *M. synoviae*

In this study, IBV was used a model to investigate the potential of the MS-H vaccine to act as a delivery vector for foreign antigens, aiming for development of recombinant poultry vaccine candidates for those pathogens affecting the respiratory tract. Infectious bronchitis is a highly contagious disease, and can exacerbate the severity of clinical respiratory signs associated with *M. synoviae*. The economic impact of IB includes production losses due to poor weight gains, mortality, growth retardation, condemnation at processing and mortality in broiler chickens, and diminished egg production and reduced hatchability in layers and breeders (Awad et al., 2014; Broadfoot et al., 1954; Ignjatovic and Sapats, 2000; Raj and Jones, 1997b). Bacteria that replicate and persist in the chicken's respiratory tract are excellent vector candidates for expression of IBV protective antigens and may allow the generation of recombinant vectored vaccines as alternative approaches for the control of IBV infection (Johnson et al., 2003; Shil et al., 2011; Wang et al., 2009; Zhao et al., 2017a). The IBV S1 protein is involved in viral tropism (Casais et al., 2003; Wickramasinghe et al., 2011) and in the induction of virus neutralizing, haemagglutination inhibiting and serotype specific antibodies (Cavanagh et al., 1986; Karaca et al., 1992) and therefore has commonly been targeted for vectored vaccine development. The N protein is immunogenic and highly conserved among IBV strains. It carries epitopes for induction of high titres of cross-reactive ELISA antibodies (Ignjatovic and Galli, 1994) and stimulation of cytotoxic lymphocytes (Collisson et al., 2000; Seo et al., 1997). Because of the continuously evolving antigenic variation of S1 among IBV strains, the N protein is a suitable alternative candidate for development of an effective subunit vaccine.

The recombinant MSH-IBV clones rMSH-IBVS1 and rMSH-IBVN developed as part of this study were shown to express IBV S1 and N genes, respectively. Although wild-type *obg* was previously expressed in MS-H (Shahid, 2013), this is the first report of successful expression of exogenous genes in *M. synoviae*. The expression of an

exogenous protein(s) in a recombinant host cell or organism often utilises significant resources of the host cell, and therefore can challenge host cells with an increased metabolic burden or toxicity (Glick, 1995), leading to a deterioration in cell physiology (Chou, 2007). The ability to grow the MS-H vaccine to high titres *in vitro* is important because a minimal dose of 4.8×10^5 colour changing units is required to elicit a protective immune response (Jones et al., 2006a). Preliminary observations have indicated that both rMSH-IBVS1 and rMSH-IBVN grow slightly slower than the MS-H cells transformed with the empty vector (pMAS-LoriC), although their titre at late log-phase was high. Whether the reduced growth rate *in vitro* would defer the onset of protective immunity of the MSH-IBV vaccine candidates in birds will need to be explored in future studies. The efficacy of the MSH-IBV recombinant vaccine candidates needs to be explored in an *in vivo* experiment using challenge protocols with both *M. synoviae* and IBV.

The MSH-IBV recombinant vaccine candidates developed in this study carry an *oriC* expression plasmid with a *tetM* gene for their maintenance through antibiotic selection. Without this selection pressure, plasmids could potentially be lost from a proportion of cells during cell division (Gill et al., 2009). To experimentally evaluate the stability of the tetracycline resistant MS-H vaccine vector *in vivo*, specific pathogen free chickens subjected to chlortetracycline medication at different doses will need to be inoculated (by eye drop) with rMSH-IBVS1 and rMSH-IBVN and examined for colonisation. Groups of birds inoculated with the MS-H vaccine and MS-H transformed with an “empty” version of the *oriC* plasmid will need to be used as controls. To examine colonisation of the respiratory tract by rMSH-IBVS1, rMSH-IBVN, MS-H and MS-H carrying the empty *oriC* plasmid, swabs need to be taken from the palatine cleft and upper trachea of the birds and examined for the presence of the respective organisms by culture and PCR. In addition, experimental infection of chickens needs to be conducted to examine the efficacy of the recombinant MSH-IBV strains in inducing protection

against challenge with a virulent strain of IBV. For this, groups of specific pathogen free chickens will need to be inoculated with the recombinant MSH-IBV clones, rMSH-IBVS1 and rMSH-IBVN, as well as MS-H with the empty *oriC* plasmid, and challenged with virulent strains of IBV and *M. synoviae*. Systemic humoral and cell-mediated immune responses will need to be examined. The safety of the clones can also be evaluated by inclusion of inoculated unchallenged groups and examination of air sac lesions, the thickness of the tracheal mucosa and reisolation of the organisms.

6.6 Conclusions

Overall, this project has advanced our understanding of the mechanisms underlying the attenuation of the *M. synoviae* MS-H vaccine by identifying potential virulence genes of *M. synoviae* based on comparison of the whole genome sequence of MS-H and its parent strain 86079/7NS. By further examining a mutation found in the *oppF-1* gene of MS-H, a rapid and effective PCR-HRM strategy was established to discriminate MS-H from *M. synoviae* field strains. Moreover, recombinant MS-H vectors expressing IBV antigens were developed, which lay the foundation for the development of novel vaccines for the control of other respiratory tract pathogens, such as *M. gallisepticum*, *E. coli*, *Avibacterium paragallinarum* and infectious laryngotracheitis virus.

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