University of Technology, Sydney

Molecular interactions between *Mycoplasma hyopneumoniae* and host cells

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CERTIFICATE OF ORIGINAL AUTHORSHIP

I certify that the work in this thesis has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree except as fully acknowledged within the text.

I also certify that the thesis has been written by me. Any help that I have received in my research work and the preparation of the thesis itself has been acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

Signature of Student:

Date:

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List of Publications

[Each of these publications has been included on a separate CD and are labelled (e.g. Publication 1) according to their order here]

1. *__Raymond, B. B.__ and S. P. Djordjevic (2015). "Exploitation Of Plasmin(Ogen) By Bacterial Pathogens Of Veterinary Significance." Vet Microbiol. Accepted for publication, doi:10.1016/j.vetmic.2015.04.008, 9th April 2015.

2. O'Rourke, M. B., __B. B. Raymond__, S. P. Djordjevic, and M. P. Padula (2015). "A Versatile Cost Effective Method For The Analysis Of Fresh Frozen Tissue Sections Via Matrix Assisted Laser Desorption Ionisation Imaging Mass Spectrometry (MALDI-IMS)." Rapid Commun Mass SP 29(7): 637-644.

3. Jarocki, V. M., J. Santos, J. L. Tacchi, __B. B. Raymond__, A. T. Deutscher, C. Jenkins, M. P. Padula and S. P. Djordjevic (2015). "MHJ_0461 is a multifunctional leucine aminopeptidase on the surface of Mycoplasma hyopneumoniae." Open Biol 5(1).

4. *__Raymond, B. B.__, C. Jenkins, L. M. Seymour, J. L. Tacchi, M. Widjaja, V. M. Jarocki, A. T. Deutscher, L. Turnbull, C. B. Whitchurch, M. P. Padula and S. P. Djordjevic (2015). "Proteolytic processing of the cilium adhesin MHJ_0194 (P123J) in Mycoplasma hyopneumoniae generates a functionally diverse array of cleavage fragments that bind multiple host molecules." Cell Microbiol 17(3): 425-444.

5. Woolley, L. K., S. A. Fell, J. R. Gonsalves, __B. B. Raymond__, D. Collins, T. A. Kuit, M. J. Walker, S. P. Djordjevic, G. J. Eamens and C. Jenkins (2014). "Evaluation of recombinant Mycoplasma hyopneumoniae P97/P102 paralogs formulated with selected adjuvants as vaccines against mycoplasmal pneumonia in pigs." Vaccine 32(34): 4333-4341.

6. Tacchi, J. L., **B. B. Raymond**, V. M. Jarocki, I. J. Berry, M. P. Padula and S. P. Djordjevic (2014). "Cilium adhesin P216 (MHJ_0493) is a target of ectodomain shedding and aminopeptidase activity on the surface of Mycoplasma hyopneumoniae." J Proteome Res 13(6): 2920-2930.

7. *__Raymond, B. B.__, J. L. Tacchi, V. M. Jarocki, F. C. Minion, M. P. Padula and S. P. Djordjevic (2013). "P159 from Mycoplasma hyopneumoniae binds porcine cilia and heparin and is cleaved in a manner akin to ectodomain shedding." J Proteome Res 12(12): 5891-5903.

8. Robinson, M. W., K. A. Buchtmann, C. Jenkins, J. L. Tacchi, **B. B. Raymond**, J. To, P. Roy Chowdhury, L. K. Woolley, M. Labbate, L. Turnbull, C. B. Whitchurch, M. P. Padula and S. P. Djordjevic (2013). "MHJ_0125 is an M42 glutamyl aminopeptidase that moonlights as a multifunctional adhesin on the surface of Mycoplasma hyopneumoniae." Open Biol 3(4): 130017.

9. Bogema, D. R., A. T. Deutscher, L. K. Woolley, L. M. Seymour, **B. B. Raymond**, J. L. Tacchi, M. P. Padula, N. E. Dixon, F. C. Minion, C. Jenkins, M. J. Walker and S. P. Djordjevic (2012). "Characterization of cleavage events in the multifunctional cilium adhesin Mhp684 (P146) reveals a mechanism by which Mycoplasma hyopneumoniae regulates surface topography." MBio 3(2).

10. Seymour, L. M., C. Jenkins, A. T. Deutscher, **B. B. Raymond**, M. P. Padula, J. L. Tacchi, D. R. Bogema, G. J. Eamens, L. K. Woolley, N. E. Dixon, M. J. Walker and S. P. Djordjevic (2012). "Mhp182 (P102) binds fibronectin and contributes to the recruitment of plasmin(ogen) to the Mycoplasma hyopneumoniae cell surface." Cell Microbiol 14(1): 81-94.

11. Bogema, D. R., N. E. Scott, M. P. Padula, J. L. Tacchi, **B. B. Raymond**, C. Jenkins, S. J. Cordwell, F. C. Minion, M. J. Walker and S. P. Djordjevic (2011).

"Sequence TTKF↓QE defines the site of proteolytic cleavage in Mhp683 protein, a novel glycosaminoglycan and cilium adhesin of Mycoplasma hyopneumoniae." J Biol Chem 286(48): 41217-41229.

Conference Presentations

1. The Australian Society for Microbiology, Annual Scientific Meeting, 2015 – **Oral**: "A systems approach for the identification of proteins from *Mycoplasma hyopneumoniae* required for biofilm formation"
2. The 3rd Prato Conference On 'The Pathogenesis Of Bacterial Diseases Of Animals', 2014 – **Poster**: "Proteins involved in the adherence of *Mycoplasma hyopneumoniae* to abiotic surfaces and porcine monolayers which may also play a role in biofilm formation"
    a. Recipient of Best Poster Award
3. The 20th Congress of the International Organisation for Mycoplasmology, 2014 – **Oral**: "*Mycoplasma hyopneumoniae* is an invasive pathogen that invades epithelial cells and forms biofilms"
4. The 19th Lorne Proteomics Symposium, 2014 – **Oral**: "Global analysis of protein-protein interactions important for the virulence of *Mycoplasma hyopneumoniae*"
    a. Recipient of Oral Presentation Award
5. BacPath 12: Molecular Analysis of Bacterial Pathogens, 2013 – **Poster**: "Is *Mycoplasma hyopneumoniae* an invasive pathogen?"
6. Elizabeth Agricultural Macarthur Institute (EMAI) Invited Student Speaker, 2013 – **Oral**: "Using high resolution microscopy and proteomics to investigate host-pathogen interactions in *Mycoplasma hyopneumoniae*"
7. The 18th Lorne Proteomics Symposium, 2013 – **Poster**: "Protein promiscuity in *Mycoplasma hyopneumoniae*: not just for show"

8. The 19th Congress of the International Organisation for Mycoplasmology – **Poster**: "P97 is extensively endoproteolytically processed and a multifunctional adhesin: identification of novel binding domains"
9. "Proteomics and Beyond" Symposium, 2012 – **Oral**: "The surfaceome of *Mycoplasma hyopneumoniae* displays an extensive population of multifunctional endoproteolytic cleavage fragments derived from the P97 & P102 adhesin families"
10. The Australian Society for Microbiology, Annual Scientific Meeting, 2012 – **Poster**: "P97 is extensively endoproteolytically processed and a multifunctional adhesin: identification of novel binding domains"
11. The 17th Lorne Proteomics Symposium, 2012 – **Oral**: "Endoproteolytic cleavage of the *Mycoplasma hyopneumoniae* adhesin MHJ_0494 (P159) reveals a TTKF|QE motif which is also the site of cleavage in other adhesins"
12. UTS Science's Research Day, 2011 – **Poster**: "Elucidating Putative Host-Pathogen Interactions of *Mycoplasma hyopneumoniae* Using Super Resolution Fluorescence Microscopy"
13. Royal North Shore Hospital Scientific Research Meeting, 2011 – **Poster**: "Elucidating Putative Host-Pathogen Interactions of *Mycoplasma hyopneumoniae* Using Super Resolution Fluorescence Microscopy"
14. BacPath 11: Molecular Analysis of Bacterial Pathogens, 2011 – **Poster**: "Extensively processed adhesins, lipoproteins and non-classically secreted molecules dominate the surface topography of *Mycoplasma hyopneumoniae*"
15. The 16th Lorne Proteomics Symposium, 2011 – **Poster**: "Biofilm formation in *Mycoplasma hyopneumoniae*, the causative agent of chronic porcine respiratory disease"

16. Royal North Shore Hospital Scientific Research Meeting, 2010 – **Oral**: "Biofilm formation in *Mycoplasma hyopneumoniae*, the causative agent of chronic porcine respiratory disease: a proteome approach"

Abbreviations

3-Dimensional Structured Illumination Microscopy	3D-SIM
3,3´-diaminobenzidine	DAB
4',6-diamidino-2-phenylindole	DAPI
Base pair	Bp
Bronchoalveolar Lavage Fluid	BALF
Bovine Serum Albumin	BSA
Centimetre	cm
Confocal Laser Scanning Microscope/Microscopy	CLSM
Deoxyribonucleic acid	DNA
Extracellular Matrix	ECM
Extracellular DNA	eDNA
Fibronectin	Fn
Hour	h
Immunofluorescence Microscopy	IFM
Isoelectric Point	pI
Isopropyl β-D-1-thiogalactopyranoside	IPTG
Immobilised pH Gradient	IPG
Kilodalton	kDa
Liquid Chromatography – Tandem Mass Spectrometry	LC-MS/MS
Litre	L
Matrix-Assisted Laser Desorption/Ionization	MALDI
Metre	m
Micro; 10-6	µ

Millilitre	mL
Mycoplasma hyopneumoniae (gene name)	Mhp
Open Reading Frame	ORF
Percent	%
Phosphate Buffered Saline	PBS
Plasminogen	Plg
Polyvinylidene fluoride	PVDF
Porcine Epithelial-like	PK-15
Porcine Reproductive and Respiratory Syndrome Virus	PRRSV
Post-Infection	P.I.
Room Temperature	RT
Scanning Electron Microscopy	SEM
Second	s
Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis	SDS-PAGE
Time of Flight	TOF
Tributylphosphine	TBP
Wild Type	WT

Abstract

The Mycoplasmas are a group of wall-less bacteria belonging to the Mollicutes that are believed to have diverged from the Gram-positive Firmicutes. Mollicutes have undergone reductive evolution, losing genes for the biosynthesis of essential biomolecules, subsequently having to form parasite relationships with their hosts in order to acquire these nutrients. They form these relationships as both commensals and pathogens, and a number of Mycoplasma species cause significant clinical and agricultural diseases. *Mycoplasma hyopneumoniae* is the causative agent of porcine enzootic pneumonia, a chronic respiratory disease that affects swine populations worldwide. *M. hyopneumoniae* colonises the upper respiratory tract by adhering to the rapidly beating cilia where it causes ciliostasis and eventual cilial death [1]. *M. hyopneumoniae* possesses a family of surface adhesins referred to as the P97 and P102 paralog family that it utilises to adhere to the cilia [2-10]. A hallmark of *M. hyopneumoniae* infection is a potent inflammatory response which is believed to be one of the contributing factors to the gross lung lesions observed in infected swine [11-13].

M. hyopneumoniae is described as a strict extracellular pathogen that only adheres to cilia and knowledge is lacking on additional receptors that *M. hyopneumoniae* binds to. Recent studies have however, shown that viable *M. hyopneumoniae* cells can be cultured from the liver, spleen, kidneys and lymph nodes of infected swine [14-16]. These observations suggest that *M. hyopneumoniae* has the capability to invade through the epithelial barrier and disseminate to distal tissue sites. In addition to this, large microcolonies have been observed in the respiratory tract of swine infected with *M. hyopneumoniae* [17]. These microcolonies are reminiscent of biofilms, and although biofilm formation has never been investigated in *M. hyopneumoniae* it is likely that they play a role in the chronicity of disease. Notably, even when lung lesions in *M.*

hyopneumoniae-infected swine are cleared, bronchial swabs can still test positive for *M. hyopneumoniae* up to 185 days post-infection (P.I.) [18] and pigs can act as convalescent carriers for up to 200 days P.I. [19]. This suggests that *M. hyopneumoniae* possesses mechanisms in which it can remain dormant within its host whilst remaining infectious. Vaccines against *M. hyopneumoniae* can successfully reduce lung lesions but they are unable to prevent transmission in swine herds [20]. In order to create vaccines that inhibit the transmission of *M. hyopneumoniae*, a better understanding of the disease process is required.

This PhD project has thus been devised in order to address the problems outlined above. This work has investigated the ability of adhesins to undergo extensive endoproteolytic processing; demonstrating that proteolytic processing in the P97 and P102 adhesins occurs much more extensively than what has previously been shown. I also show that these adhesins can bind to a myriad of host components such as heparin, fibronectin (Fn) and plasminogen (Plg) and investigate the domains responsible. Additionally, this work presents a number of novel receptors that *M. hyopneumoniae* targets within its host as well as a comprehensive list of putative adhesins that it utilises to do so.

This work has also investigated the ability of *M. hyopneumoniae* to form biofilms on abiotic surfaces, host cells and within the swine respiratory tract and further demonstrate that surface adhesins play a role in biofilm formation. A number of putative biofilm-associated genes have been identified by screening a transposon mutant library, these genes being potential vaccine candidates. Finally, this work has investigated the ability of *M. hyopneumoniae* to become internalised by host cells and reside within the cytoplasm. *M. hyopneumoniae* becomes internalised by vacuole-like structures, and that internalised cells appear to escape from lysosomes to reside free within the cytoplasm.

Overall, this PhD project has contributed significantly to understanding how *M. hyopneumoniae* causes disease. Future work on the novel mechanisms described in this thesis will aid in future vaccine development programs and potentially aid in the control of this important veterinary disease.

Table of Contents

List of Figures

List of Tables