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**CHARACTERISATION OF THE
PRTM MATURASE OF
STREPTOCOCCUS EQUI;
A PROVEN VIRULENCE FACTOR
IN STRANGLES**

FELICIA ADAOBI IKOLO

PhD

2013.

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IN STRANGLES**

FELICIA ADAOBI IKOLO

A thesis submitted in partial fulfilment
of the requirements of the
University of Northumbria at Newcastle
for the degree of
Doctor of Philosophy

Research undertaken in the
School of Life Sciences

October, 2013.

Abstract

Streptococcus equi subspecies *equi* (*S. equi*) is the pathogen responsible for the prevalent and highly contagious equine disease called strangles. Strangles has been reported worldwide as a cause of a high level of animal suffering and economic loss. *S. equi* is susceptible to many antibiotics *in vitro*, but relapse due to insufficient vascularity often renders such treatment ineffective. Getting effective and universally accepted vaccines against *S. equi* have been slow mainly because of safety concerns. It was previously reported that colonization of air interface organ cultures, after inoculation with a mutant strain ($\Delta prtM_{138-213}$) deficient in the putative maturase lipoprotein (PrtM, a homologue of the pneumococcal PpmA) was less than that seen in cultures which were infected with wild-type *S. equi* strain 4047 (Hamilton *et al.*, 2006), indicating that PrtM is a major virulence factor in strangles. It has also been demonstrated by *in vitro* and *in vivo* studies that many streptococcal adhesins, for example, serve as colonization or virulence factors and this makes them attractive targets for therapeutic and preventive strategies against streptococcal infections (Nobbs *et al.*, 2009). *S. equi* adhesins or other colonization factors may be substrates for PrtM.

Understanding PrtM is key to designing drugs or vaccines against the equine *S. equi* infection and strangles. In this research, advanced biomolecular techniques were systematically applied to investigate and characterise PrtM, and to evaluate its potential as a therapeutic or vaccine target. Bioinformatics, microbiological, biochemical and molecular biology techniques were used in screening the *S. equi* WT 4047 and Mutant ($prtM_{138-213}$) strains to evaluate the immunogenicity and conservation of PrtM. Proteomics techniques: two-dimensional gel electrophoresis and mass spectrometry were employed in evaluating the cell-associated and secreted protein extracts of both the *S. equi* WT 4047 and mutant ($prtM_{138-213}$) strains. In this study, genetic engineering technology involving targeted domain knock-out and/or knock-in, was employed in producing the central domain recombinant protein and mutant ($prtM_{138-213}$) revertants. Following cloning, over-expression and purification of the full length and central domain, biochemical data on the PrtM protein (k_{cat}/K_M for *S. equi* 4047 PrtM full length recombinant protein = 5.84×10^6 /M/s) were derived via enzyme (peptidylprolyl isomerase - PPIase) assay; and crystallography was applied in an attempt to derive structural data on the PrtM protein. Advanced biomolecular techniques (including Western blots and Proteomics) were employed in screening the complemented mutants.

It has been proven from this research that the PrtM of *S. equi* 4047 is involved in adaptation to NaCl stress and in regulating sensitivity to antibiotics; PrtM may have roles in speeding up the synthesis of hyaluronic acid and in the folding or remodeling of HPr Kinase. The parvulin-type structure of PrtM elucidated by bioinformatics analysis, the cross reactions of the WT and mutant with a number of antisera, the observation that PrtM may be a multisubstrate foldase due to the detectable and significant differences in the proteomes of the WT, mutant and complemented mutants, the dimeric protein formed by the full length recombinant protein of *S. equi* 4047 WT, and the PPIase-Chaperonine activities of PrtM, all observed from this study, validate PrtM of *S. equi* 4047 as a viable and novel therapeutic target which pharmaceutical industries should extensively evaluate for the prevention and treatment of *S. equi* infection and strangles.

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Above all, I am most grateful to God Almighty for everyone and for everything.

Declaration

I declare that the work contained in this thesis has not been submitted for any other award and that it is all my own work carried out under the supervision of Professors Gary W. Black, Iain C. Sutcliffe, and Dr Meng Zhang.

I also confirm that this work acknowledges the opinions, ideas and contributions from the work of others.

Name: Felicia Adaobi Ikolo

Signature:

Date: 15th October, 2013.

Poster Abstract

Abstract of poster presented at the Society of General Microbiology Autumn Meeting, 6-9 September, 2010; at University of Nottingham Jubilee Campus (ELR1):

NT03/18 **Characterization of the PrtM maturase of *Streptococcus equi*; a proven virulence factor in strangles**

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Streptococcus equi subspecies *equi* is responsible for the prevalent and highly contagious equine respiratory infection known as strangles. Developing effective and universally accepted vaccines against this disease has been slow. The *S. equi* lipoprotein PrtM is a putative maturase, i.e. involved in the folding of proteins to be exported. It has been previously reported that PrtM is a virulence factor of *S. equi*, as demonstrated in both a mouse model and the equine host. Moreover, colonization of air interface organ cultures after inoculation with a maturase-deficient mutant strain was reduced compared to infections with the wild-type strain. In this study, we have performed proteomic analyses of cell-associated and secreted protein extracts from the maturase-deficient mutant strain and the wild type strain *S. equi* 4047. These data have revealed differentially expressed proteins. Specifically, we have found that FNE (a fibronectin binding protein) and IdeE2 (an immunoglobulin G endopeptidase) are present in the secreted protein extract of the wild type strain but not in the mutant strain. This suggests proteolytic degradation of misfolded secreted proteins and that PrtM may not be linked solely to the folding of one specific substrate, but is likely a multi-substrate maturase. These data have allowed us to identify potential maturase substrates which warrant further investigation for their contribution to the virulence of *S. equi*.

Oral Presentation

Ikolo A. Felicia, Sutcliffe C. Iain and Black W. Gary (2010). Characterisation of the PrtM Maturase of *Streptococcus equi*; a Proven Virulence Factor in Strangles. Presented at **St. George's University Phi Zeta Honor Society Research Day**, on Saturday, 27th February, 2010 in Grenada.

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List of Abbreviations

Abbreviation	Name
aa	Amino acid
ABC	ATP Binding Cassette
ACN	Acetonitrile
AGE	Agarose gel electrophoresis
Amp ^r	Ampicillin resistance
APS	Ammonium persulphate
ATP	Adenosine tri phosphate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BLAST	Basic local alignment search tool
bp	Base pair(s)
bromophenol blue	Bromophenol blue
BSA	Bovine serum albumin
CaCl ₂	Calcium chloride
CFE	Cell free extract
CFU	Colony forming unit (s)
CHAPS	3-[(3-chloroimidopropyl)dimethylammino]-1-propane sulfonate
CH ₃ OH	Methanol
CHCl ₃	Chloroform
cm	Centimetre
Cm ^r	Chloramphenicol resistance
CO ₂	Carbondioxide
COOH	Carboxyl group
CSS	Clear strategy screen
C-terminal	Carboxy terminal
3D	Three dimensional
Da	Dalton

Abbreviation	Name
2DE	Two dimensional gel electrophoresis
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
dsDNA	Double stranded DNA
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid, disodium salt
Em ^r	Erythromycin resistance
EtBr	Ethidium bromide
ESI	Electrospray ionization
ExPASy	Expert protein analysis system
FCS	Foetal calf serum
FKBP	FK506-binding protein
FA	Formic acid
<i>g</i>	Gram(s)
<i>glmU</i>	N-acetyl-glucosamine-1-phosphate uridyltransferase.
h(s)	Hour(s)
HA	Hyaluronan/Hyaluronic acid
<i>hasA</i>	hyaluronate synthase
<i>hasB</i>	UDP-glucose dehydrogenase
<i>hasC</i>	glucose-1-phosphate uridyltransferase
HCl	Hydrochloric acid
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid
His ₆ tag	Hexahistidine tag
HMW	High molecular weight
H ₂ O ₂	Hydrogen peroxide
HPr	phosphocarrier protein of the phosphoenolpyruvate:sugar phosphotransferase system
H ₂ SO ₄	Sulfuric acid
IAA	iodoacetamide
IEF	Isoelectric focussing
Ig A	Immunoglobulin A

Abbreviation	Name
Ig G	Immunoglobulin G
Ig M	Immunoglobulin M
IMAC	Immobilised metal affinity chromatography
IPTG	Isopropyl- β -D-thiogalactopyranoside
Kan ^r	Kanamycin resistance
kb	Kilobase pair(s)
k_{cat}	Turnover number
k_{cat}/K_M	Catalytic efficiency
kDa	Kilodalton
KH ₂ PO ₄	Potassium bi phosphate
K_M	Michaelis-Menten constant
kV	Kilo volts
L	Litre(s)
LB	Luria-Bertani medium
LC-MS	Liquid chromatography mass spectrometry
Lgt	Prolipoprotein diacylglycerol transferase
LMW	Low molecular weight
LppC/LppA	Acid phosphatase lipoprotein
Lsp	Signal peptidase II
m	Metre(s)
M	Molar / Methionine
mA	Milliamps
MCS	Multiple cloning site
MES	2-(N-morpholino)-ethanesulphonic acid
mg	Milligram
MgCl ₂	Magnesium Chloride
MIC	Minimum inhibitory concentration
min	Minute(s)
mL	Mili litre

Abbreviation	Name
mm	Millimetre(s)
mM	Milimolar
<i>Mr</i>	Relative molecular mass
MS	Mass spectrometry
MW	Molecular weight
MWCO	Molecular weight concentrator
m/z	Mass-to-charge
NaCl	Sodium chloride
Na ₂ HPO ₄	Sodium bi phosphate
NaOH	Sodium hydroxide
NBT	Nitroblue tetrazolium salt
NCBI	National Centre for Biotechnology Information
NCTC	National Collection of Types cultures
ng	Nanogram
NH ₂	Amino group
nm	Nanometer
nt	Nucleotide(s)
N-terminal	Amino terminal
OD _x	Optical density at x nm
ORF	Open reading frame
PAGE	Polyacryamide gel electrophoresis
PBS	Phosphate Buffered Saline
PBST	PBS, 0.05% Tween-80
PCR	Polymerase chain reaction
p	Plasmid
pDNA	Plasmid DNA
PEG	Polyethylene glycol
<i>Pgi</i>	glucose-6-phosphate isomerase
pH	Potential of hydrogen: measure of hydrogen ions associated with acidity

Abbreviation	Name
PMSF	Phenylmethanesulfonyl flouride
PPIase	Peptidyl-prolyl cis/trans isomerase
PpmA	Putative protein maturase lipoprotein
PPP	pentose phosphate pathway
PrtM	Putative maturase lipoprotein
RNA	Ribonucleic acid
r-plasmid	Recombinant plasmid
r-protein	Recombinant protein
rpm	Revolutions per minute
sec	Second(s)
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
sp.	Specie
ssDNA	Single stranded DNA
SEQ	<i>Streptococcus equi</i>
<i>S. equi</i>	<i>Streptococcus equi</i> subspecies <i>equi</i>
SEQz	<i>Streptococcus equi</i> subspecies <i>zooepidemicus</i>
TAE	Tris-Acetate-EDTA
TEMED	N,N,N',N'-tetramethylethylene diamine
TFA	Trifluoroacetic acid
THA	Todd-Hewitt Agar
THB	Todd-Hewitt Broth
T _m	Melting temperature
Tris	tris(hydroxymethyl)aminomethane
T7 <i>lac</i>	lac operator just downstream of T7 promoter
UV	Ultraviolet
V	Volt(s)
V _{max}	Maximum velocity
v/v	Volume per volume
w/v	Weight per volume

Abbreviation	Name
WT	Wild type
$x g$	Times gravity (centrifugal force)
α	Alpha
β	Beta
Δ	Delta or 'mutant'
ϵ	Molar absorptivity
k	Kilo
λ	Lambda
m	milli
mM	millimeters
n	Nano
$^{\circ}\text{C}$	Degree Celsius
%	Percent
μ	Micro
μL	Micro litre
μg	Micro gram
μM	Micromolar
σ	Sigma
1 $^{\circ}$	Primary
2 $^{\circ}$	Secondary
3'	Three prime
5'	Five prime
18.2 M Ω H ₂ O	18.2 mega ohm water

1 General Introduction

1.1 Strangles:

Streptococcus equi subspecies *equi* (*S. equi*) is the pathogen responsible for the prevalent and highly contagious equine disease called strangles (Hamilton *et al.*, 2006; Jacobs *et al.* 2000; Hoopes *et al.*, 2008; Pirie, 2013). Strangles is characterized by fever, followed by profuse nasal discharge and the formation of abscesses on the lymph nodes of the head and neck (Figure 1.1a), which eventually burst and discharge highly infectious pus (Kelly *et al.*, 2006, Robinson *et al.*, 2013) – Figures 1.1b and 1.1c.



Figure 1.1a: Swelling around the head and neck region of a horse due to inflamed lymph nodes – photo adapted from America's Horse Daily, 2011.



Figure 1.1b: A *S. equi* infected horse showing profuse purulent nasal discharge after rupture of retropharyngeal lymph nodes (Adapted from Brazil, 2005).



Figure 1.1c: Pus draining from a retropharyngeal *S. equi* abscess. (Adapted from Professor P. Dixon in Brazil, 2005).

The disease is called strangles because soon after nasopharyngeal infection, affected horses were sometimes suffocated due to constriction of the pharynx (resulting from lymph node swelling – Figure 1.1d) and airway obstruction (Sweeney *et al.*, 2005;

Hamilton *et al.*, 2006). About 50% of horses with retropharyngeal lymphadenitis develop some degree of empyema after abscess rupture into the guttural pouches (Brazil, 2005).



Figure 1.1d: Pony showing prominent swelling in the throat latch area (black arrowheads) due to chronic guttural pouch empyema (Adapted from Brazil, 2005).

Equine strangles has been reported to occur worldwide (Jacobs *et al.*, 2000; Kelly *et al.*, 2006; Waller *et al.*, 2011), incurring a lot of economic losses from treatment, quarantine measures and occasional death of the animals (Jacobs *et al.*, 2000). In Australia, death due to strangles had been reported to account for a minimum direct loss to the horse industry of \$6.5 million annually (Canfield *et al.*, 2000). It has been reported that *S. equi* was responsible for about 30% of all reported cases of equine infections worldwide (Chanter, 1997). Though mostly reported in equidea, *S. equi* infection has lately been reported in camelids (Yigezu *et al.*, 1997); and a case of canine strangles was recently reported in a dog with enlarged lymph nodes due to infection with *S. equi* (Ladlow *et al.*, 2006). To date there has been one report of human infection with *S. equi* (Duma *et*

al 1969) and one case of a human with a syndrome resembling strangles (Breiman and Silverblatt, 1986).

In premises, outbreak of strangles may be initiated by the introduction of:

- animals that are incubating the disease,
- any horse that is still shedding bacteria after clinical recovery
- chronic carriers that may shed bacteria intermittently (Brazil 2005).

It has been known for about 100 years that *S. equi* can remain in the upper respiratory tract of previously infected horses which presently appear clinically normal and can consequently serve as sources of infection to other animals (Brazil, 2005). The guttural pouches are the main sites of bacterial persistence in chronic carriers (Harrington *et al.*, 2002; Kelly *et al.*, 2006). Although there are no detailed immunological studies, it has been suggested that about seventy five per cent (75%) of animals develop solid immunity after recovery from infection and may be protected from re-infection for several years (Hamlen *et al.*, 1994; Brazil, 2005). Colostrum and milk from immune mares have specific immunoglobulin G (IgG) and immunoglobulin A (IgA) which is normally protective against foalhood strangles till the animals are weaned (Galan *et al.*, 1986).

Younger animals, less than five years old, are more commonly and more severely affected by strangles (Ijaz *et al.*, 2010). *S. equi* is capable of infecting the host by attaching to tonsillar epithelium after inhalation or ingestion of infected secretions (Brazil, 2005). After attachment, the organism quickly migrates to draining lymph nodes where it multiplies, causing lymphadenopathy with surrounding oedema within 7-10 days (Brazil, 2005). This is closely followed by abscessation of the submandibular, parotid and retropharyngeal lymph nodes (Sweeney *et al.*, 2005; Timoney and Kumar,

2008). The hyaluronic acid capsule of *S. equi* and production of antiphagocytic enzymes and toxins by the organism makes it highly resistant to phagocytosis by neutrophils (Holden *et al.*, 2008) The result of this phagocytic resistance is uncontrolled bacterial multiplication which in turn gives rise to intense neutrophil accumulation, resulting in rapid abscess formation (Brazil, 2005; Holden *et al.*, 2008). Nasal shedding of bacteria begins 2-3 days after the onset of pyrexia and persists for 2-3 weeks (Sweeney *et al.*, 2005).

Haematogenous or lymphatic spread results in secondary abscessation of local lymph node chains and metastatic dissemination to remote lymph nodes and other tissues, a phenomenon called bastard strangles (Timoney, 2004; Brazil, 2005). Young horses are more likely to develop severe lymph node abscessation which eventually opens and drains; however outbreaks characterised by mild clinical signs (characterised by nasal discharge, small abscesses and rapid resolution of the disease) have been reported, often in older horses (Sweeney *et al.*, 2005). The severity of the disease depends on the innate immune status (Sweeney *et al.*, 2005) and the general health of the infected animals; as well as on the dose of the bacteria (Brazil, 2005).

1.2 *Streptococcus equi* subspecies *equi* (*S. equi*) 4047

S. equi is a member of the pyogenic group of organisms which are beta-haemolytic, producing true haemolysis on blood agar (Nobbs *et al.*, 2009). A Gram positive bacteria, *S. equi* shares membership of Lancefield's group C *Streptococci* (Anzai *et al.*, 1997) with *Streptococcus zooepidemicus*, *Streptococcus equisimilis* (Brazil, 2005; Harrington *et al.*, 2002) and *S. dysgalactiae* (Efstratiou *et al.*, 1994; Nobbs *et al.*, 2009).

S. equi is susceptible to most antibiotics *in vitro* (Harrington *et al.*, 2002; Jacks *et al.*, 2003). However, treatment of strangles is usually ineffective because of relapse resulting likely from insufficient vascularity (in the abscess) to enable antibiotic penetration to therapeutic levels and/or inability of antibiotic treatment to eliminate mucosal colonization (Harrington *et al.*, 2002). Consequently, after treatment, it is not uncommon for clinical signs to abate, or for onset to be delayed until withdrawal of treatment (Harrington *et al.*, 2002). Generally, there are only a small number of new antibiotic agents for therapy of infections caused by Gram positive organisms, and the pathway for the development of new antibiotics is long and highly expensive (Upton *et al.*, 2012).

Horizontal gene transfer continues to influence the virulence and pathogenicity of Streptococcal species (Upton *et al.*, 1996; Lawrence, 2005). There is strong evidence that genetic exchange between *S. equi*, *S. zooepidemicus* and *Streptococcus pyogenes* continues to influence the pathogenicity of these important bacteria (Waller *et al.*, 2011). *S. equi* and *S. zooepidemicus* share more than 80% DNA sequence identity with *S. pyogenes*, a human pathogen (Waller *et al.*, 2011). Understanding *S. equi* better may shed new light into *S. pyogenes* infections because both organisms apart from having a lot in common, are also the etiologic agents of strangles and tonsillitis (respectively) - diseases that share similarities as well (Waller *et al.*, 2011).

S. zooepidemicus is believed to be the ancestral parent from which *S. equi* evolved (Guss *et al.*, 2009; Webb *et al.*, 2008, Holden *et al.*, 2009). The two organisms share 97% sequence identity (Ladlow *et al.*, 2006). The population of the *S. zooepidemicus*

group is highly diverse and is made up of at least 218 sequence types (Guss *et al.*, 2009). However, isolates of *S. equi* are either ST-179 or a single locus variant (ST-151) that are characteristic of *S. equi* isolates from the USA, Canada, Australia and Europe (Webb *et al.*, 2008). The limited genetic diversity of *S. equi* indicates that it is possible for an effective vaccine to confer broad protection to horses throughout the world (Guss *et al.*, 2009; Flock *et al.*, 2012).

The development of effective preventative vaccines against equine *S. equi* infection has been slow (Guss *et al.*, 2009, Guss *et al.*, 2011). Although not licensed for sale in Europe due to safety concerns, a non-encapsulated strain of *S. equi* (Pinnacle INTM) has been used as a nasal vaccine against strangles (Guss *et al.*, 2009). A second live attenuated vaccine called Equilis StrepE was marketed in Europe but was withdrawn in 2007 due to safety concerns as well (Guss *et al.*, 2009, Guss *et al.*, 2011). The Committee for Medicinal Products for Veterinary Use (a committee of the European Medicine Agency, European Union) has given marketing authorisation for Equilis StrepE, concluding that the benefit of this vaccine exceed the risks for immunisation of horses against *S. equi* to reduce clinical signs and occurrence of lymph-node abscesses (European public assessment report, for European Medicine Agency, 2013).

Regarding the risk associated with Equilis Strep E vaccine, the committee for Medicinal Products for Veterinary Use (European public assessment report, for European Medicine Agency, 2013) did state that “Only healthy horses that are at risk of infection should be vaccinated. After injection, swelling will develop at the injection site within four hours and the injection site may become warm or painful. The reaction is greatest at two to three days after injection, but the area affected should not be larger than 3 x 8 cm. The swelling should disappear within three weeks and should have no effect on the

horse's appetite, nor cause it any discomfort. In very rare cases, an abscess and discharge may develop at the injection site, and the lymph nodes in the head may enlarge, which may be painful for a short while. An increase in body temperature of up to 2°C may occur on the day of vaccination. In rare cases, loss of appetite, fever and shivering may be observed. In very rare cases, depression may develop". According to Meehan *et al.* (1998), a vaccine targeted against M protein (SeM a surface antigen that increases *S. equi* infection) was successful in experiments with mice. However, the same results were not replicated in horses (Libardoni *et al.*, 2013). It is highly desirable that a safe and effective vaccine be developed against *S. equi* (Guss *et al.*, 2009; Waller, 2013).

The virulent strain of *S. equi* 4047 (ST-179 by multilocus sequence typing - MLST) was isolated from a horse with strangles in the New Forest, England in 1990 (Swiss Institute of Bioinformatics, 2010; Holden *et al.*, 2009). Multilocus sequence typing is a molecular technique that is established in typing bacteria via the generation of an allelic profile (Smith *et al.*, 2007).

Availability of data from the *S. equi* genome and proteome projects (Wellcome Trust Sanger Institute, 2013, and Swiss Institute of Bioinformatics, 2010 respectively) is a major development in the study of strangles and *S. equi* (Hamilton *et al.*, 2006). Access to such sequence data (Figure 1.2a) permit the identification of surface exposed and secreted proteins and is anticipated to revolutionize vaccine design – reverse vaccinology (Bambini *et al.*, 2009 and Serruto *et al.*, 2009 in Guss *et al.*, 2009).

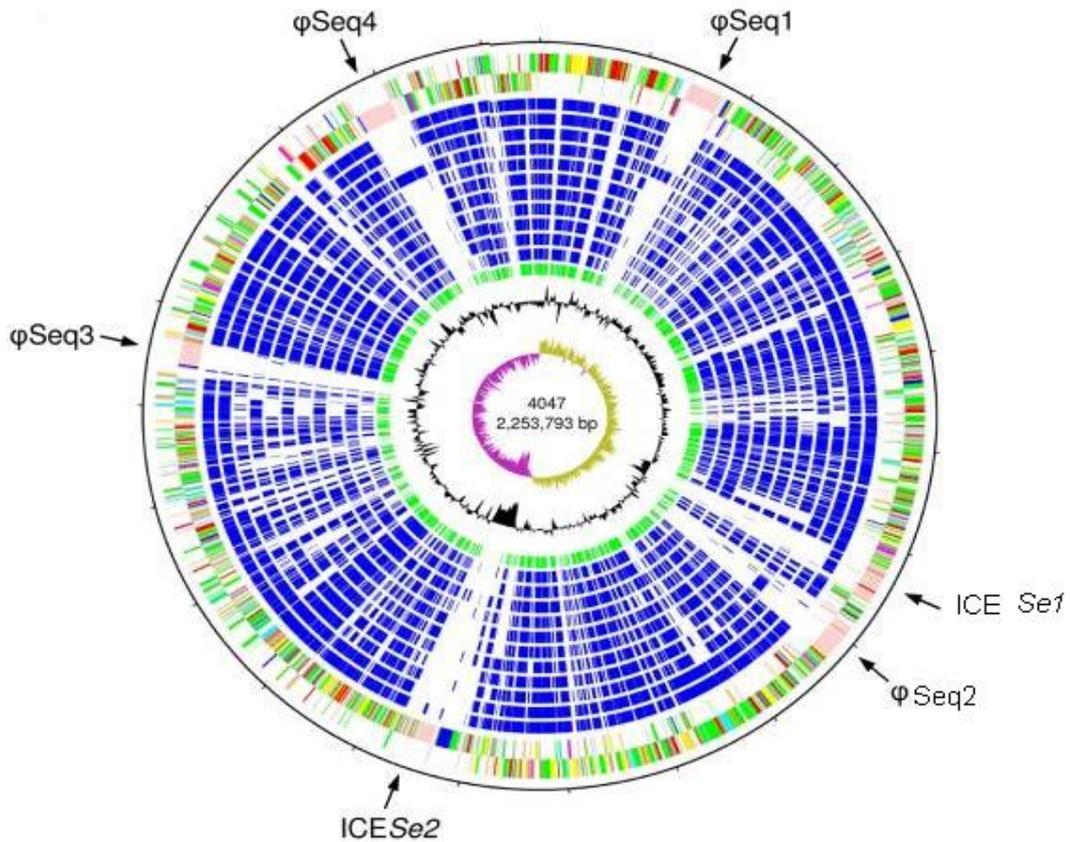


Figure 1.2a: Schematic circular diagram of the *S. equi* 4047 genome (Adapted from Holden *et al.*, 2009). Key (outside to inside): scale (in Mb); annotated coding sequences (*CDSs*) coloured according to predicted function represented on a pair of concentric circles, representing both coding strands; orthologue matches which are shared with the *Streptococcal species*, *S. equi* 4047 or SzH70, SzMGCS10565, *S. uberis* 0140J, *S. pyogenes* Manfredo, *S. mutans* UA159, *S. gordonii* Challis CH1, *S. sanguinis* SK36, *S. pneumoniae* TIGR4, *S. agalactiae* NEM316, *S. suis* P1/7, *S. thermophilus* CNRZ1066, blue; orthologue matches that are shared with *Lactococcus lactis subspecies lactis*, green; G+C% content plot; G+C deviation plot (>0%, olive, <0%, purple). Colour coding for *CDS* functions: dark blue for pathogenicity/adaptation; black for energy metabolism; red for information transfer; dark green for surface-associated; cyan for degradation of large molecules; magenta for degradation of small molecules; yellow for central/intermediary metabolism; pale green for unknown; pale blue for regulators; orange for conserved hypothetical; brown for pseudogenes; pink for phage and *IS* elements; grey, miscellaneous. The positions of the four prophages and two *ICESe*, present in the *S. equi* 4047 genome, are shown (Holden *et al.*, 2009).

It is believed that the evolution of *S. equi* has been shaped by recent gene loss and gain (Holden *et al.*, 2009). In terms of gene loss, compared to *S. zooepidemicus*, *S. equi* produces less hyaluronidase which means that *S. equi* is less able to attach to mucosal surfaces (Holden *et al.*, 2009). Hyaluronidases are enzymes which can break down hyaluronate and are produced by a number of pathogenic Gram-positive bacteria that

initiate infections at the skin or mucosal surfaces (Hynes and Walton, 2006). *S. zooepidemicus* (SzH70) genome has a single coding sequence encoding a putative hyaluronate lyase, SZO06680 (Holden *et al.*, 2009). However, SEQ1479, which is an *S. equi* 4047 orthologue, contains a 4 bp deletion (TCTC) leading to a frameshift at codon 199 (Holden *et al.*, 2009). *S. equi* 4047 has acquired a different hyaluronate lyase (SEQ2045) encoded on a prophage (Holden *et al.*, 2009). This type of phage-encoded enzyme usually has much lower activity and reduced substrate range (Baker *et al.*, 2002) compared to orthologues of SZO06680 (Pritchard *et al.*, 1994) and may be why *S. equi* infection rarely progresses beyond the lymphatic system (Holden *et al.*, 2009). The reduced level of hyaluronidase activity provides an alternative explanation as to why *S. equi* maintains high levels of hyaluronic acid capsule (Holden *et al.*, 2009).

Comparison of the genome sequences of *S. zooepidemicus* and *S. equi*, observed a 5 kb deletion in the *S. equi* 4047 genome that partially deleted *lacD* and *lacG* and deleted *lacE*, *lacF* and *lacT* (Holden *et al.*, 2009). *S. equi* 4047 also contains a deletion of *sorD* gene which is immediately upstream of SEQ0286 and a deletion between SEQ0536 and SEQ0537 which spans the operon required for ribose fermentation (Holden *et al.*, 2009). Specialization of *S. equi* has likely rendered these pathways redundant, resulting in their loss (Holden *et al.*, 2009). Carbohydrate metabolism in streptococci is important in colonization of mucosal surfaces (Shelburne *et al.*, 2008). Carbohydrate fermentation is also used to differentiate *S. equi* strains from *S. zooepidemicus* (Bannister *et al.*, 1985).

Gram-positive bacteria have some cell wall-anchored proteins on their surface and these proteins are attached covalently through a process mediated by sortase enzymes (Marraffini *et al.*, 2006). These cell wall-anchored proteins have been shown to play a

role in modulating host-cell interactions in many cases (Holden *et al.*, 2009). Only two putative sortase coding sequences are present in the *S. equi* 4047 genome: *srtA* (SEQ1171) and *srtC.1* (SEQ0937), whereas *S. zooepidemicus* H70 genome has five: *srtA* (SZO09440), *srtC.1* (SZO11490), *srtC.2* (SZO18270), *srtC.3* (SZO18280) and *srtC.4* (SZO18290) (Holden *et al.*, 2009).

Mutation and gene loss are likely to contribute to decreased fibronectin binding in *S. equi* compared to *S. zooepidemicus* (Holden *et al.*, 2009). Pili play an important role in the adherence of *S. pyogenes* to host tissues (McElroy *et al.*, 2002). The *S. zooepidemicus* H70 genome contains two loci that encode genes required putatively for pilus expression (Holden *et al.*, 2009). The genome of *S. equi* 4047 lacks a putative pilus locus through an ISSeq3 element-mediated deletion (Holden *et al.*, 2009). Diversification of pilus loci may play an important role in *S. zooepidemicus* ability to infect different hosts and tissues (Holden *et al.*, 2009).

The absence of prophage in *S. zooepidemicus* may be due to the presence of clustered regularly interspaced short palindromic repeat (CRISPR) arrays and competence proteins which confer resistance to circulating phage and maintain genome integrity (Holden *et al.*, 2009). In *S. equi*, the acquisition of prophage plays an important evolutionary role through integration of cargo genes; recirculation and secretion of the integrated ϕ Seq1 (Figure 1.2a) may kill susceptible competing bacteria like *S. zooepidemicus*; ϕ Seq2 contains a gene encoding a phospholipase A₂ (SlaA) that may enhance virulence; and ϕ Seq3 and ϕ Seq4 encode superantigens SeeH, SeeI, SeeL, and SeeM that target the equine immune system (Holden *et al.*, 2009).

In *S. equi* 4047, the presence of integrative conjugative elements (ICESe2) locus (Figure 1.2a) may enhance iron acquisition through the production of a potential siderophore, equibactin (Holden *et al.*, 2009). The ICESe2 locus was absent in the *S. zooepidemicus* isolates that were examined (Holden *et al.*, 2009). The key speciation event in the evolution of *S. equi*, due to gene gain, may be the acquisition of ICESe2, which is the first of its kind to be identified in streptococci (Holden *et al.*, 2009).

1.3 Molecular Basis of *S. equi* Infection and Disease

S. equi is able to gain entrance into the horse or pony through the mouth or nose, where it attaches to the cells in the crypt of the lingual and palatine tonsils and to the follicular-associated epithelium of the pharyngeal and tubal tonsils (Sweeney *et al.*, 2005). The factors which are produced by *S. equi* and implicated in its virulence can be broadly grouped into the following categories: adherence, invasion, immune evasion and nutrient acquisition factors (Harrington *et al.*, 2002).

The first step in *S. equi* pathogenesis entails its adhesion to the host epithelium in the upper respiratory tract (Slater, 2003). Some ligands that may be responsible for this adhesion to host tissues may include the exposed surface proteins SzPSe, Se73.9, and Se51.9 (Sweeney *et al.*, 2005). A fibronectin binding protein designated FNZ in *S. zooepidemicus*, is also produced by *S. equi* (FNZ is designated FNE in *S. equi*) but without a C terminal anchor and so may not be functional (Lindmark *et al.*, 2001; Harrington *et al.*, 2002; Sweeney *et al.*, 2005; Waller *et al.*, 2011).

The ability of *S. equi* to interact with and adhere to different surfaces may increase its chances of survival when it gets to surfaces where specific and irreversible interactions occur and conditions are favourable for colonization (Harrington *et al.*, 2002). The hyaluronic acid capsule of *S. equi* may also be involved in mediating adherence to the host (Harrington *et al.*, 2002).

To be able to gain access to the lymphatics and blood vessels of the lamina propria, *S. equi* has to invade the respiratory epithelium of the host (Sting *et al.*, 1990 in Slater, 2003). To achieve this, *S. equi* produces degradative enzymes like hyaluronidase (Sting *et al.*, 1990 in Slater, 2003) and streptolysin-S like haemolysin which is a cytolytic toxin (Flanagan *et al.*, 1998). Before penetration, there is no evidence for colonization; however *S. equi* reaches the deeper tissues of the tonsil within a few hours of gaining entry (Sweeney *et al.*, 2005).

To be able to invade and proliferate, a pathogen has to avoid being recognized by immunoglobulins, which are key components of the adaptive immune response (Lannergard and Guss, 2006). The immune complement of the host interacts with *S. equi* peptidoglycan and attracts large numbers of polymorphonuclear neutrophils.

S. pyogenes produces and secretes IdeS (streptococcal cysteine proteinase an immunoglobulin-degrading enzyme) which has an important impact on the ability of the bacteria to survive in the human host (von Pawel-Rammingen and Bjorck, 2003). *S. equi* encodes two IgG endopeptidases – IdeE and IdeE2 which share amino acid sequence identity with IdeZ and IdeZ2 of *S. zooepidemicus* and IdeS of *S. pyogenes* (von Pawel-Rammingen and Bjorck, 2003). These enzymes cleave the IgG produced by a number of animal species, thereby reducing IgG recognition and targeting of bacteria

by the host immune response (Waller *et al.*, 2011). For *S. equi*, the observed degradation of IgG - in the presence of horse serum, but not when grown with purified IgG - might be due to the expression of an unknown enzyme rather than IdeE (Lannergard and Guss, 2006). IdeE2 and IdeZ2 cleave equine IgG more efficiently than IdeZ and IdeE (Hulting *et al.*, 2009),

The inability of the neutrophils to phagocytose and kill the invading *S. equi* may be due to a combination of the hyaluronic acid capsule, SeM protein [antiphagocytic, acid resistant, fibrillar and fibrinogen binding] (Timoney, 2004) and other undetermined antiphagocytic factors released by the bacteria (Sweeney *et al.*, 2005). The resistance of *S. equi* to phagocytosis is dependent on its level of expression of hyaluronic acid capsule; encapsulated strains were found to be more resistant than nonencapsulated strains (Anzai *et al.*, 1999; Harrington *et al.*, 2002). The antiphagocytic capsule is believed to reduce the numbers of *S. equi* cells that get associated with the surface of neutrophils and are subsequently ingested and killed (Timoney, 2004).

Streptolysin S and streptokinase may have roles to play in abscess development and lysis, by damaging cell membranes and activating the proteolytic properties of plasminogen (Timoney, 2004). *S. equi* has also exhibited some mitogenic activity which may contribute to severe inflammation and abscess formation (Anzai *et al.*, 1999; Harrington *et al.*, 2002). At least four pyrogenic mitogens, SePE-H, SePE-I, SePE-K and SePE-L, are expressed by *S. equi* and these result in non-specific T cell stimulation, proliferation, proinflammatory cytokine release and production of an acute phase response with high fever, neutrophilia and fibrinogenemia (Timoney, 2004). Long chains of *S. equi* accumulate, surrounded by large numbers of degenerating neutrophils (Timoney, 2004; Sweeney *et al.*, 2005). Only the lysis of the abscess capsule and

evacuation of its contents would eventually dispose of the bacteria (Sweeney *et al.*, 2005).

A *S. equi* LppC (a class C acid phosphatase) homologue; and ATP-binding cassette (ABC) transporter MBL (a *S. equi* lipoprotein homologue of the PsaA protein of *Streptococcus pneumoniae*) may be involved in nutrient acquisition (Harrington *et al.*, 2002). Hyaluronidase activity may be involved in the utilization of the hyaluronic acid (an abundant carbon source) of the host and in recycling of released capsular hyaluronic acid (Harrington *et al.*, 2005). HPr (histidine-containing phosphocarrier protein) is an important player in bacterial and streptococcal phosphoenolpyruvate (PEP) phosphotransferase system (PTS). It was speculated by Dixon *et al.*, (2001) that surface-localised and released HPr-1 of *S. equi* may also have roles to play in its (HPr-1) mitogenic activity which had previously been demonstrated in *S. pyogenes* by Gerlach *et al.*, (1992).

The success of *S. equi* pathogenesis is therefore partly attributed to the wide array of proteins expressed by the organism.

1.4 Lipoproteins in Gram-Positive Bacteria

In addition to providing a rigid exoskeleton for protection against mechanical and osmotic lysis, the cell wall of Gram-positive bacteria also serves as an attachment site for proteins which interact with the bacterial environment (Navarre and Schneewind, 1999). Gram-positive bacteria can immobilize proteins on their surface by covalently attaching proteins to the peptidoglycan or by noncovalently binding protein to either the

peptidoglycan or secondary wall polymers such as teichoic acids (Navarre and Schneewind, 1999).

Lipoproteins are proteins which contain lipids that are covalently linked to an N-terminal cysteine residue (Braun and Wu, 1994). In Gram-positive bacteria, lipoproteins are cell envelope proteins that are anchored into the outer leaflet of the plasma membrane (Hutchings *et al.*, 2009). An N-terminal signal sequence that is synthesized with a lipoprotein, directs the protein into the lipoprotein-processing pathway (Denham *et al.*, 2009).

Most exported proteins are transported across the cytoplasmic membrane of prokaryotes by one of either the general secretory (Sec) pathway (which is the predominant route of protein transport) or the twin arginine protein transport (TAT) system which transports folded and even oligomeric proteins (Hutchings *et al.*, 2009) – Figure 1.4a.

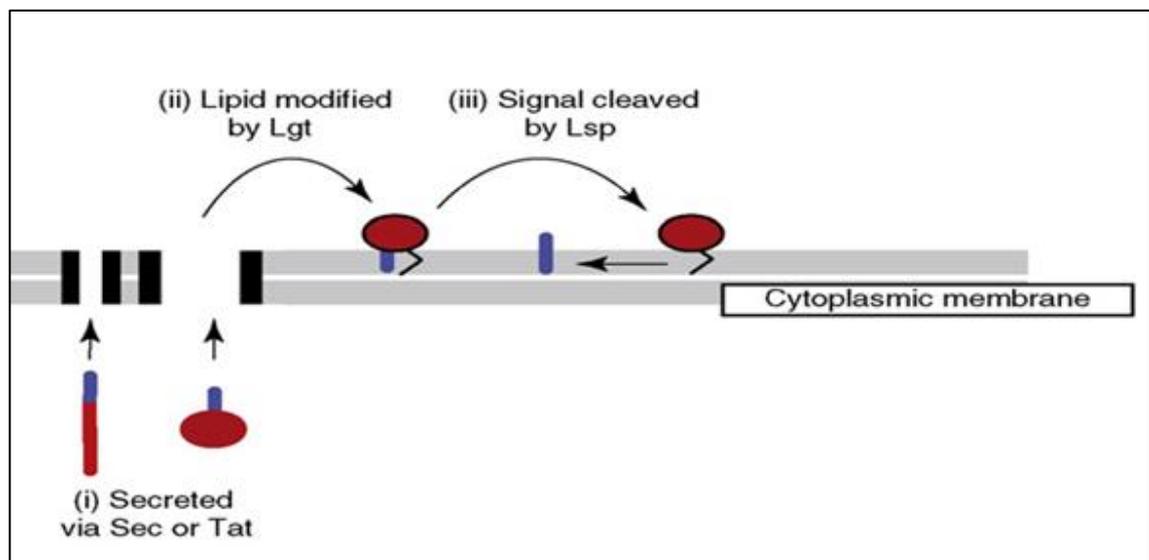


Figure 1.4a: The lipoprotein biogenesis pathway in Gram Positive Bacteria: unfolded (red straight line) or folded (red filled circle) lipoproteins are directed to and translocated across the cytoplasmic membrane by the Sec or Tat pathways by their signal sequences (shown in blue) (i). A lipid group (angled black line) is covalently attached to the sulphhydryl group of the lipobox cysteine by *Lgt* (prolipoprotein diacylglycerol transferase) (ii), and the signal peptide is cleaved by *Lsp* (lipoprotein or type II signal peptidase) (iii). The pathway is conserved in Gram-positive bacteria, but does not necessarily occur in strict order (Hutchings *et al.*, 2009).

In both the Sec and TAT pathways -Figure 1.4b - proteins are targeted by means of the N-terminal signal peptides (Hutchings *et al.*, 2009). Several streptococci lack the TAT pathway (Dilks *et al.*, 2003). It is believed that in Gram-positive bacteria, mutants that are defective in lipoprotein biosynthetic enzymes remain viable possibly because some lipoprotein precursors retain functionality (Hutchings *et al.*, 2009).

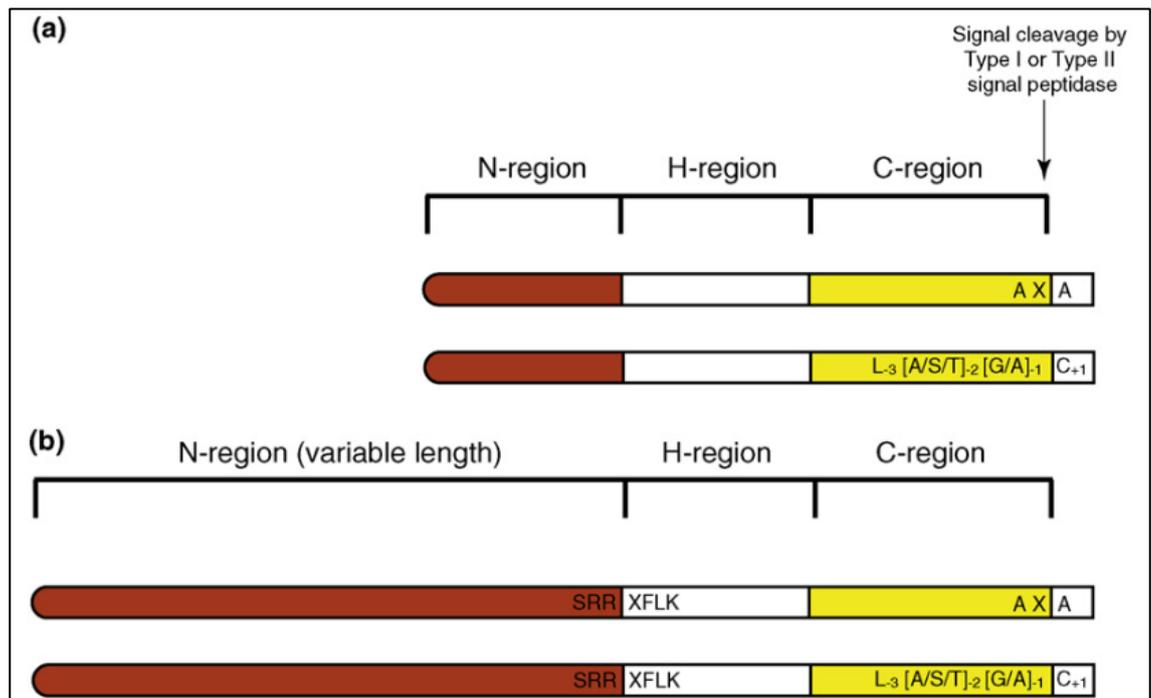


Figure 1.4b: Type I and type II signal peptides for Sec- and Tat-dependent transport (Adapted from Hutchings *et al.*, 2009). Both the *Sec* (a) and the *Tat* (b) signal peptides are tripartite in structure with a positively charged N- (N-terminal) region, an H- (hydrophobic) region and a C- (cleavage) region, that contains the recognition motif for type I (A-X-A, where X is any amino acid) or type II ($L_3[A/S/T]_2[G/A]_1C_{+1}$) signal peptidases. The type II cleavage site is referred to as the lipoprotein ‘lipobox’. *Tat* signal peptides have variable length N-regions and a conserved *SRRXFLK* sequence between the N- and H-regions (Petit *et al.*, 2001 in Hutchings *et al.*, 2009) where the twin arginine (RR) motif is almost completely conserved and gives the transport pathway its name.

In Gram-positive bacteria, lipoproteins are believed to play important roles in substrate binding for ATP-binding Cassette (ABC) transporters; adhesion; antibiotic, lantibiotic and bacteriocin resistance and superinfection exclusion; cell envelope homeostasis; protein secretion, folding and localization, redox processes; and in sensory processes,

including signalling in sporulation and germination (Sutcliffe and Russel, 1995; Hutchings *et al.*, 2009). Bioinformatic evaluation of microbial genomes has revealed that putative lipoproteins represent a notable proportion (~2%) of the typical Gram-positive bacterial proteome; are also notable as cell envelope proteins which interact with membrane associated or exported proteins (Hutchings *et al.*, 2009) and are predicted to have important roles in pathogenic bacteria/host interactions (Hamilton *et al.*, 2006; Chimalapati *et al.*, 2012).

Apart from the surface components directly involved in adhesion and immune invasion, the factors which influence proper protein folding and surface composition are also essential for bacterial virulence (Cron *et al.*, 2009). After translocation and cleavage of a signal peptide, proteins fold into their native conformation (Wahlstrom *et al.*, 2003). Rapid and correct folding of secreted proteins is essential, in particular because (partly) unfolded proteins are very sensitive to proteases several of which are present at the *trans* side of the membrane (Tjalsma *et al.*, 2000).

Many folding factors that assist post-translocational folding have been identified and these include chaperones, peptidylprolyl *cis/trans*-isomerases (PPIase) and thiol-disulphide oxido-reductases (Tjalsma *et al.*, 2000). In *Bacillus subtilis*, one of the proteins (named PrsA) involved in the folding of proteins after their translocation is a lipoprotein that is anchored to the outer leaflet of the cytoplasmic membrane (Kotinen and Sarvas, 1993). PrsA belongs to the parvulin family of PPIases (Rahfeld *et al.*, 1994). Most PrsA-Like proteins from the gram-positive bacteria contain a peptide leader followed by a cysteine, suggesting that they are lipoproteins (Drouault *et al.*, 2002). Some other lipoproteins identified to be PPIases include SIRA of *Streptococcus*

pneumonia (Hermans *et al.*, 2006) and the proteinase maturation protein, PrtM of *Lactococcus lactis* (Haandrikman *et al.*, 1991).

1.5 Peptidyl-prolyl cis/trans Isomerases

To be able to achieve a functional conformation, many proteins require enzymatic assistance (Lazar and Kolter, 1996). Peptidyl-prolyl cis/trans isomerases (PPIases; also termed foldase or maturase) are found in both prokaryotic and eukaryotic cells, where they ensure the conformation (folding and remodeling) of proteins involved in many vital functions, including virulence (Reffuveille *et al.*, 2012). PPIases are folding proteins which catalyze the cis/trans isomerization of peptidylprolyl (Lazar and Kolter, 1996; Reffuveille *et al.*, 2012; Elfaki *et al.*, 2013) – Figure 1.5a. The *cis-trans* isomerization of prolyl residues is a rate-limiting step in protein folding (Lazar and Kolter, 1996).

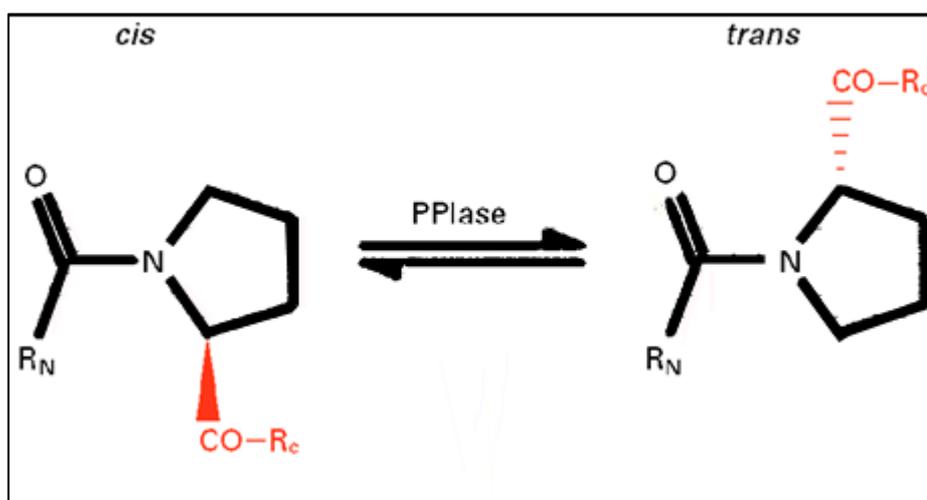


Figure 1.5a: The cis/trans isomerization reaction of PPIases (adapted from Kay, 1996)

PPIases can be broadly divided into four different families which are unrelated in their amino acid sequences (Kouri *et al.*, 2009): FK506 binding proteins (FKBPs),

cyclophilins, parvulins (Heikkinen *et al.*, 2009) which bind to juglone (Gothel and Marahiel, 1999), protein phosphatase 2A (PP2A) and phosphatase activator [PTPA] (Jordens *et al.*, 2006). Each member of the PPIase family has its own fold, substrate specificity and catalytic mechanism (Heikkinen *et al.*, 2009; Jordens *et al.*, 2006).

Cyclophilins were the first PPIases to be discovered, followed by FKBP. Both bind immunosuppressive drugs cyclosporin A and FK-506, respectively (Siekierka *et al.*, 1989; Kofron *et al.*, 1991). Although, cyclophilins and FKBP share no primary sequence similarity, and no similarities in their tertiary structure, their mode of action converges with PPIase activities (Freedman, 1989; Hayano *et al.* 1991; Bose *et al.*, 1994).

PP2A is a major serine/threonine protein phosphatase in eukaryotic cells (Janssens and Goris, 2001). In addition to playing an important role in cell-cycle regulation, cell growth control, development, regulation of multiple signal transduction pathways, cytoskeleton dynamics, and cell mobility (Xing *et al.*, 2006), protein synthesis (Janssens and Goris, 2001), PP2A is also an important tumour-suppressor protein (Janssens *et al.*, 2005). PTPA, a Ppiase-like FKBP12 and cyclophilin, is an essential protein which is involved in the regulation of PP2A and the PP2A-like enzymes (Jordens *et al.*, 2006). The PPIase activity of PTPA is inhibited by a glutamic acid in the -1 position of the prolyl residue (Jordens *et al.*, 2006).

The parvulins are PPIases (named after the *Escherichia coli* protein parvulin) which are not inhibited by the immunosuppressants: cyclosporin A or FK506 (Rahfeld *et al.*, 1994; Hennig *et al.*, 1998; Hani *et al.*, 1999), but can be inactivated by juglone (Hennig *et al.*, 1998). Apart from PPIase activity, the prokaryotic parvulins exhibit chaperone-like

activities (He *et al.*, 2004). For example, *SurA* is found in the periplasm of *E. coli* and partakes in the early stage maturation of outer membrane proteins (Lazar and Kolter, 1996). Parvulins are involved in the folding and trafficking of periplasmic and outer-membrane proteins (Rudd *et al.*, 1995).

Gram-positive bacteria have numerous PPIase lipoproteins that most likely accelerate protein folding outside the cell (Hutchings *et al.*, 2009). The lipoprotein PPIases are thought to be well placed to interact with unfolded substrates emerging from the Sec translocon and could therefore be important in processing virulence factors of Gram-positive bacteria (Hutchings *et al.*, 2009).

For efficient murine colonization, *Streptococcus pneumoniae* requires the SlrA (streptococcal lipoprotein rotamase A) PPIase (Hermans *et al.*, 2006). PpmA (putative proteinase maturation protein A) and SlrA, both belong to the chaperone family of PPIases and play valuable roles in *S. pneumoniae* colonization, avoidance of phagocytosis and pulmonary infection (Overweg *et al.*, 2000a).

In *Bacillus subtilis*, the PrsA lipoprotein (a parvulin foldase) is essential for protein secretion and viability (Kontinen and Sarvas, 1993). PrsA-Like proteins are formed by 3 regions: the central domain which may confer a PPIase activity in certain PrsA-Like proteins, as well as the N-terminal and carboxy-terminal domains whose functions remain unknown (Drouault *et al.*, 2002). In *Lactococcus lactis*, the proteinase maturation protein (PrtM) is a lipoprotein (Haandrikman *et al.*, 1991).

Bray *et al.*, (2009) confirmed that incorrect processing of lipoproteins has pleiotropic effects which may be of significance to *Streptococcus agalactiae* colonization and

pathogenesis. *Listeria monocytogenes* PrsA2 is a chaperone implicated in the secretion of virulence factors and virulence within the cytosol of infected host cells (Alonzo and Freitag, 2010). In *Enterococcus faecalis*, two lipoprotein PPIases:- the parvulin family rotamase EF0685, together with EF1534 (belonging to the cyclophilin family), have been shown to be important for virulence and resistance to sodium chloride (Reffuveille *et al.*, 2012).

Putative lipoproteins perform a wide variety of other predicted functions, including a wide array of enzymatic activities (Hutchings *et al.*, 2009). Genomic analysis has revealed that approximately 30% of these putative lipoproteins are conserved hypothetical proteins or hypothetical proteins of unknown function; it is therefore a challenge for the post-genomic era to assign functions to these proteins (Hutchings *et al.*, 2009).

1.6 Putative Maturase Lipoprotein (PrtM) of *S. equi*

To date, only a few *S. equi* lipoproteins have been characterized, some of which are: a lipoprotein acid phosphatase enzyme, LppC (Hamilton *et al.*, 2000) ; a putative metal-binding lipoprotein (MBL) homologous to pneumococcal PsaA which is believed to be involved in ABC transporter-mediated uptake of manganese (Harrington *et al.*, 2000); and another lipoprotein which was initially identified as hyaluronate (capsule)-associated protein – HAP (Chanter *et al.*, 1999 in Hamilton *et al.*, 2006), but is likely to act as a substrate-binding lipoprotein for ABC transporter-mediated uptake of oligopeptides (Harrington *et al.*, 2002 in Hamilton *et al.*, 2006).

32 other putative lipoproteins have been identified by bioinformatic analysis of the draft of *S. equi* genome sequence including PrtM, a putative maturase lipoprotein (Hamilton *et al.*, 2006). PrtM is a homologue of the pneumococcal vaccine candidate PpmA (putative proteinase maturation protein A) (Hamilton *et al.*, 2006; and Overweg *et al.*, 2000a). It was shown that inactivation of PpmA considerably attenuated pneumococcal virulence in a mouse intranasal challenge model of pneumonia; and antibodies raised to PpmA were opsonophagocytic (Overweg *et al.*, 2000b).

In the study of Overweg *et al.* (2000b), it was demonstrated that PpmA was expressed at higher levels in the transparent phenotype of *S. pneumoniae*, indicating that the substrate for the pneumococcal maturases may be involved in the colonization of the host. In 2009, Cron *et al.* (2009) demonstrated that PpmA contributes to the early stages of infection (colonization) in *S. pneumoniae*. They stated that the contribution of PpmA to virulence can be explained by its strain-specific role in adherence to epithelial cells and contribution to the evasion of phagocytosis. It can therefore be hypothesized that the substrates for both the pneumococcal PpmA and *S. equi* PrtM maturases may be bacterial adhesins or some other colonization factors. Such a role would make *S. equi* PrtM maturase and its substrate/s excellent targets for therapies that are designed to treat or prevent strangles.

Expression studies of *L. lactis* subsp. *cremoris* clones bearing plasmids (having PrtM gene, the PrtP gene, or both) showed that the PrtM gene encoded a trans-acting activity involved in the maturation of the cell envelope located and secreted forms of SK11 proteinase (prtP) (Vos *et al.*, 1989). PrtM (a *trans*-acting protein involved in the processing of precursors of serine protease PrtP into active enzymes) belong to the family of PPIases (Vos *et al.*, 1989, Overweg *et al.*, 2000a).

At present, there is a lack of information about the substrates of most putative streptococcal maturases; and information regarding their nature as true PPIases is not clear. It has been reported that Pneumococcal PpmA lacks significant PPIase activity (Hermans *et al.*, 2006). Drouault *et al.* (2002) also reported that the chromosomally-encoded putative maturase lipoprotein of *L. lactis* and some other streptococcal homologues like *S. pyogenes* PrsA2 and pneumococcal PpmA, do not have a complete PPIase sequence signature. However, an evaluation of the entire length of the proteins, including the unconserved signature region, by secondary structure predictions, suggests that the folding of these PrsA-like proteins is conserved (Drouault *et al.*, 2002). Therefore, the streptococcal enzymes may have a distinctive mechanism of action and/or substrate specificity compared to other parvulins. Defining their PPIase capability will therefore be very important in defining the properties of this subgroup of the parvulin family and may reveal highly specific drug targets.

The deletion of the single specific lipoprotein PrtM was investigated in order to evaluate the contribution of lipoproteins to the virulence of *S. equi* in its natural host (Hamilton *et al.*, 2006). In their study, Hamilton *et al.* (2006) discovered that colonization of the air interface organ cultures after inoculation with a mutant strain deficient in the maturase lipoprotein ($\Delta prtM_{138-213}$, with a deletion of nucleotides 138 to 213, and henceforth referred to as $\Delta PrtM$) was less than that seen in cultures which were infected with wild-type *S. equi* strain 4047 or a mutant strain that was unable to lipidate prelipoproteins $-\Delta lgt_{190-685}$.

The parental *S. equi* 4047 WT strain induced disease in 57% (17 of 30) of mice models during a 5-day study period, as determined by changes in weight gain, rate of sneezing,

and histopathological analysis (Hamilton *et al.*, 2006). It is interesting to note that the deletion of the PrtM gene significantly attenuated *S. equi* 4047 in the mouse model of *S. equi* 4047 infection ($P < 0.001$) such that none of the mice challenged with *S. equi* 4047 Δ PrtM showed signs of disease (either reduced weight gain or sneezing) throughout the study period, and no disease was detected histologically (Hamilton *et al.*, 2006). It is very interesting to note from the study of Hamilton *et al.* (2006) that the virulence of the PrtM-deficient mutant (Δ PrtM) was also significantly attenuated in the Welsh Mountain ponies. The findings of Hamilton *et al.* (2006) suggests that the deletion of the central domain of *S. equi* 4047 PrtM is sufficient to abrogate its function *in vivo*, thereby attenuating this strain. However, further analyses of the molecular consequences of the deletion of PrtM gene in *S. equi* are now required in order to identify those virulence factors which rely on its activity and which are essential to pathogenicity in the horse (Hamilton *et al.*, 2006).

1.7 Aim of Project

S. equi virulence factors have only been partially identified (Anzai *et al.*, 1999). It has been demonstrated by *in vitro* and *in vivo* studies that many streptococcal adhesins serve as colonization or virulence factors and this makes them attractive targets for therapeutic and preventive strategies against streptococcal infections (Nobbs *et al.*, 2009).

Although it has been previously demonstrated that *S. equi* 4047 putative lipoprotein maturase (PrtM) is a major virulence factor (Hamilton *et al.*, 2006), no functional role has been given to it (*S. equi* PrtM). Understanding *S. equi* PrtM therefore, would be key to designing drugs or vaccines against the equine *S. equi* infection and strangles.

This research was meant to systematically apply advanced biomolecular techniques in investigating and characterising PrtM, to identify the virulence factors which rely on its activity and are essential to pathogenicity, as well as evaluate its potential as a therapeutic or vaccine target. This study was embarked on with the hope of generating valuable information which would be useful to pharmaceutical industries in designing therapies against *S. equi* infection and strangles. The outcome of this research would contribute to the body of knowledge about lipoprotein maturases in general. These objectives have been achieved in this study by:

- a) Applying bioinformatics, microbiological, biochemical and molecular biology techniques in screening the *S. equi* 4047 WT and Δ PrtM strains to evaluate the immunogenicity and conservation of PrtM.
- b) Using proteomics techniques: 2D-E and mass spectrometry (methods of Zhang *et al.*, 2007) in evaluating the cell-associated and secreted protein extracts of both the *S. equi* WT 4047 and Δ PrtM strains.
- c) Deriving biochemical data on the PrtM protein via enzyme (peptidylprolyl isomerase) assay, following cloning, over-expression and purification of the full length and central domain.
- d) Attempting to derive structural data (via crystallography) on the PrtM protein after cloning, over-expression and purification of the full length and central domain.
- e) Producing and screening mutant (Δ PrtM) revertants.

This information presented hereafter, shows how from the outcome of this research, the following conclusions were reached:

- some significant proteins are differentially expressed between the *S. equi* 4047 WT and Δ PrtM strains.
- PrtM is a peptidylprolyl isomerase which may not only be linked to the folding of one specific substrate but could be a multisubstrate foldase.
- PrtM is indeed a major virulence factor in strangles and
- PrtM is a robust therapeutic/vaccine candidate.

2 Materials and Methods

The sources of all chemicals and reagents are given in the Appendix A1. Unless stated otherwise, all solutions and buffers were made up with 18.2 M Ω /cm H₂O purified by a Milli – Q Plus 18.2 H₂O purification system, and stored at room temperature.

All solutions and apparatus were sterilised by autoclaving (Appendix B1) at 121°C, with a pressure of 1.05 bar for 20 minutes, unless otherwise stated.

2.1 Bacterial Strains and Growth Media

The bacterial strains and culture media used in this research are described in this Section (2.1)

2.1.1 Bacterial Strains

Bacterial strains were maintained at -80°C in glycerol stocks (section 2.1.2). On a monthly basis, bacterial stocks of *E coli* BL21 (DE3) were streaked onto an agar plate and incubated at 37°C overnight. The plate was stored at 4°C for one month so that in the event of contamination of bacterial stock, this could be used for re-inoculation.

The *Streptococcus equi* subspecies *equi* (*S. equi*) strains used in this study are listed in Table 2.1; while the *E. coli* strains used are listed in Table 2.2

<i>S. equi</i> 4047 Strain	Comment	Source
WT	Wild type (WT) strain 4047, originally isolated in 1990 from a submandibular abscess of a New Forest pony	Culture collection of the Animal Health Trust, Newmarket, United Kingdom.
Δ PrtM	PrtM-deficient mutant strain (Δ <i>prtM</i> ₁₃₈₋₂₁₃), with a deletion of nucleotides 138 to 213. Em ^r	Animal Health Trust, United Kingdom, following the work of Hamilton <i>et al.</i> (2006).
Δ PrtM123/pVA838	Complemented mutant: PrtM-deficient mutant strain (Δ <i>prtM</i> ₁₃₈₋₂₁₃), transformed with pVA838 to which the N-terminal, the Central and the C-terminal domain genes of PrtM had been cloned; Cm ^r .	This study
Δ PrtM12/pVA838	Complemented mutant: PrtM-deficient mutant strain (Δ <i>prtM</i> ₁₃₈₋₂₁₃), transformed with pVA838 to which only the N-terminal and the Central domain genes of PrtM had been cloned; Cm ^r .	This study
Δ PrtM/pVA838	PrtM-deficient mutant strain (Δ <i>prtM</i> ₁₃₈₋₂₁₃) transformed with the plasmid pVA838. Cm ^r .	This study
WT 4047/pVA838- Cm ^r	<i>S. equi</i> WT 4047 transformed with the plasmid pVA838. Cm ^r	This study
WT 4047/pVA838- Em ^r	<i>S. equi</i> WT 4047 transformed with the plasmid pVA838. Em ^r	This study

Table 2.1 *Streptococcus equi* subspecies *equi* strains used in this research.

<i>E. coli</i> strain	Use	Characteristics	Source
One Shot TOP10	Cloning host	F ⁻ mcrA Δ (mrr-hsdRMS-mcrBC) Φ 80lacZ Δ M15 Δ lacX74 recA1 araD139 Δ (ara leu) 7697 galU galK rpsL (StrR) endA1 nupG	Invitrogen
BL21 (DE3)	Protein hyper expression host	F ⁻ <i>ompT hsdSB</i> (rB- mB-) <i>gal dcm</i> (DE3)	Invitrogen

Table 2.2 *E. coli* strains used in this research.

2.1.2 Cryogenic storage of bacterial stocks

Per 100 mL

100 % (v/v) glycerol

50.0 mL

50% (v/v) glycerol was aliquoted into 1 mL amounts in 1.5 mL microcentrifuge tubes, autoclaved and cooled to room temperature before use.

Bacterial stocks were prepared by mixing 0.5 mL of broth culture with 1.0 mL of sterile 50% (v/v) glycerol. These stocks were then stored at -20°C (medium-term storage) or -80 °C (long term storage).

2.1.3 Growth Media

Culture media (Table 2.3) were prepared using distilled water, and sterilised by autoclaving, unless otherwise stated. Liquid culture media were stored at room temperature, and solid media at 4°C. pH adjustments of solutions are stated and were performed using a pH meter (Appendix B2), and HCl or NaOH, unless otherwise stated.

THB and THA were chosen as culture media of choice for *S. equi* strains according to the methods of Hamilton *et al.* (2000); and Sutcliffe *et al.* (2000).

Media	Composition	Sterilisation
Todd-Hewith Broth (THB)	Todd-Hewith Broth base (Oxoid): 36.4 g per L.	Autoclaved at 121°C for 15 min
Todd-Hewith Agar (THA)	THB, Agar (bacteriological agar #1) 15 g per L.	Autoclaved at 121°C for 15 min
THB / 0.5 M Sucrose	THB, Sucrose 34.23 g per 200 mL	Autoclaved at 121°C for 15 min
THB / 0.5 M Sucrose / 20mM MgCl ₂ / 2 mM CaCl ₂ .	THB, 6.846 g, MgCl ₂ .6H ₂ O 0.2033 g, CaCl ₂ 0.294 g per 50 mL	Autoclaved at 121°C for 15 min
THB / 90 mM glycine	THB	Autoclaved at 121°C for 15 min, glycine filter sterilised using a 0.2 µm filter
THB / 90 mM glycine / 0.1% Tween-80	THB / 90 mM glycine, Tween-80 1 g per L.	Autoclaved at 121°C for 15 min, glycine filter sterilised using a 0.2 µm filter
Luria Bertiani (LB) broth - Low Salt	Tryptone 10 g, Yeast extract 5 g, Sodium Chloride 5 g - per L	Autoclaved at 121°C for 15 min
LB agar	Tryptone 10 g, Yeast extract 5 g, Sodium Chloride 5 g, agar 15 g - per L	Autoclaved at 121°C for 15 min
Foetal Calf Serum (FCS)	Commercially prepared (Appendix A5)	Commercially pre- sterilized stored at -20°C
Todd Hewith broth with Foetal Calf Serum (THB + FCS)	10% (v/v) FCS in THB (adapted from Hamilton <i>et al.</i> , 2006)	Used freshly prepared
5% Horse blood agar	40 g of blood agar base (Merck) was heated in 1litre of boiling water and autoclaved for 15 min at 121°C. This was left to cool to 50- 45°C. Finally, 5-6% sterile defibrinated horse blood was added and mixed carefully to avoid formation of bubbles. 15-20 mL volumes were poured onto petri dishes.	Plates were stored in the refrigerator at 4°C until ready for use.

Table 2.3 Growth media and supplements used in this research

2.1.4 Antibiotics

Compositions of the stock solutions of antibiotics used in this study are shown in Table 2.4. Appropriate volumes of the stock solutions were diluted in liquid media for antibiotics selection. Autoclaved media was cooled to less than 55°C before the addition of antibiotics. Final concentrations of antibiotic used are stated in the method section for specific assays.

Antibiotic	Stock Concentration	Solvent	Storage.
Ampicillin	10 mg/mL	18.2 MΩ/cm H ₂ O	-20°C
Chloramphenicol	34 mg/mL	100% Ethanol	-20°C
Erythromycin	1 mg/mL	18.2 MΩ/cm H ₂ O	-20°C
Gentamycin	500 mg/mL	18.2 MΩ/cm H ₂ O	-20°C
Kanamycin	10 mg/mL	18.2 MΩ/cm H ₂ O	-20°C
Norfloxacin	5 mg/mL	18.2 MΩ/cm H ₂ O	-20°C
Penicillin G	6 mg/mL	18.2 MΩ/cm H ₂ O	-20°C
Streptomycin	500 mg/mL	18.2 MΩ/cm H ₂ O	-20°C
Vancomycin	30 mg/mL	18.2 MΩ/cm H ₂ O	-20°C

Table 2.4 Stock concentrations of antibiotics used in this research.

2.2 Reagents, Buffers, Enzymes and Kits

The reagents and buffers that were used in this study are listed in tables 2.5, 2.6 and 2.7.

Reagent/ Buffer	Composition	Use
TAE Running Buffer	50 x stock : Tris HCl 242.0 g, 17.51 M Glacial acetic acid 57.1 mL, 0.5 M EDTA pH 8.0 100 mL - per L.	Diluted to 1 x by a 1:50 dilution for agarose gel electrophoresis.
Bromophenol Blue Loading Buffer	6 x sample loading buffer: Bromophenol Blue 0.025 g, glycerol 3.0 g - per 10 mL	Diluted to 1 x with sample prior to loading. For agarose gel electrophoresis
Xylene cyanol Loading Buffer	6 x sample loading buffer: Xylene cyanol 0.025 g, glycerol 3.0 g - per 10 mL	Diluted to 1 x with sample prior to loading. For agarose gel electrophoresis of DNA bands < 1Kb
Bioline Hyperladder 1 (size standard)	DNA ladder (10, 8, 6, 5, 4, 3, 2.5, 2, 1.5, 1.0, 0.8, 0.6, 0.4, and 0.2 kb) (Appendix A8)	Agarose gel electrophoresis
Ethidium Bromide	10 mg/mL	Diluted to 10 µg/ mL for agarose gel electrophoresis
TE buffer, pH 7.5	0.5 M Tris-HCL pH 7.5 20 mL, 0.5 M EDTA pH 8.0 2.0 mL - per L	Elution, storage and agarose gel electrophoresis of DNA
STET buffer, pH 8.0	Sucrose 80 g, Triton X-100 50 mL, 0.5 M EDTA pH 8.0 100 mL, Tris-HCL 6.06 g – per L	Crude plasmid preparation.

Table 2.5: Reagents and Buffers for DNA analysis

Reagent	Composition	Use
12% (w/v) Acrylamide resolving gel	40% (w/v) solution (37.5:1 acrylamide:bisacrylamide) 3.0 mL, Solution B (2M Tris-HCL pH 8.8 75 mL, 10% w/v SDS 4 mL per 100 mL) 2.5 mL, 18.2 MΩ/cm H ₂ O 4.5 mL, 10% w/v ammonium persulphate 50 μL, TEMED 10 μL	SDS-PAGE
13% (w/v) Acrylamide resolving gel	40% (w/v) solution (37.5:1 acrylamide:bisacrylamide) 3.25 mL, Solution B (2M Tris-HCL pH 8.8 75 mL, 10% w/v SDS 4 mL per 100 mL) 2.5 mL, 18.2 MΩ/cm H ₂ O 3.25 mL, 10% w/v ammonium persulphate 50 μL, TEMED 10 μL	SDS-PAGE
15% (w/v) Acrylamide resolving gel	40% (w/v) solution (37.5:1 acrylamide:bisacrylamide) 3.75 mL, Solution B (2M Tris-HCL pH 8.8 75 mL, 10% w/v SDS 4 mL per 100 mL) 2.5 mL, 18.2MΩ/cm H ₂ O 3.75 mL, 10% w/v ammonium persulphate 50 μL, TEMED 10 μL	SDS-PAGE
4% (w/v) Acrylamide stacking gel	40% (w/v) solution (37.5:1 acrylamide:bisacrylamide) 0.5 mL, Solution C (1M Tris-HCL pH 6.8 50 mL, 10% w/v SDS 4 mL per 100 mL) 1.0 mL, 18.2MΩ/cm H ₂ O 2.5 mL, 10% w/v ammonium persulphate 30 μL, TEMED 10 μL	SDS-PAGE
Running buffer, pH 8.8	10 x stock: Tris-HCl 30.3 g, Glycine 144 g, SDS 10 g – per L	Diluted 1:10 for SDS-PAGE and 2D-E
SDS-PAGE sample loading buffer	5 x stock: 2 M Tris-HCl pH 6.8 0.3 mL, 50% v/v glycerol 5 mL, 10% w/v SDS 2 mL, 14.4 mM β-mercaptoethanol 0.5 mL, 1% w/v bromophenol blue 1 mL - per 10 mL. Stored in a brown bottle at 4°C.	1 volume buffer to 4 volumes sample. For SDS-PAGE.
SDS - PAGE sample cracking buffer	2.4 g Urea, 7.6 mL SDS-PAGE sample loading buffer. Stored at 4°C.	For protein extraction for SDS-PAGE.
HMW Protein size standard	M.W. (KDa): 205, 116, 97, 84, 66, 55, 45, 36. Lyophilized standard. reconstituted with 18.2 MΩ/cm H ₂ O 200 μL and SDS-PAGE sample buffer (5 x) 25 μL, boiled for 3 min and stored at 4°C.	SDS-PAGE
LMW Protein size standard	M.W. (KDa): 66, 45, 36, 29, 24, 20. Lyophilized standard reconstituted with 18.2 MΩ/cm H ₂ O 200 μL and SDS-PAGE sample buffer (5 x) 25 μL, boiled for 3 min and stored at 4°C.	SDS-PAGE
Coomassie blue staining solution	Coomassie blue R-250 1 g, Glacial acetic acid 100 mL, Methanol 450 mL – per L. Room temperature storage.	SDS-PAGE
Coomassie blue gel destaining solution	Glacial acetic acid 100 mL, methanol 100 mL – per L. Room temperature storage.	SDS-PAGE
PBST	PBS, 0.05% (v/v) Tween-80. Stored at 4°C	Western Blotting
Transfer Buffer	Tris base 2.9 1g, Glycine 1.465 g, 18.2 MΩ/cm H ₂ O 400 mL, Methanol 100 mL. Room temperature storage.	Western Blotting
Start Buffer	Na ₂ HPO ₄ 2.84 g, NaCl 29.2 g Imidazole 0.68 g – per L, pH 7.4. Room temperature storage.	IMAC
Elution Buffer	Na ₂ HPO ₄ 2.84 g, NaCl 29.2 g Imidazole 34 g – per L, pH 7.4. Room temperature storage.	IMAC

Table 2.6: Reagents and Buffers for Protein Analysis.

Reagent	Composition	Use
Phosphate Buffered Saline (PBS)	NaCl 8 g, KCl 0.2 g, KH ₂ PO ₄ 0.2 g, Na ₂ HPO ₄ .12 H ₂ O 1.44 g	Proteomics
Lysis Solution	Urea 4.8 g, CHAPS 0.4 g – per 10 mL. Aliquoted into 1 mL quantities and stored at -20°C. 2 µL of IPG buffer (pH 2-10 or 4-7) added to every 100 µL before use	Proteomics
Rehydration Solution	Urea 4.8 g, CHAPS 0.4 g, 1% v/v bromophenol blue 20 µL – per 10 mL. Aliquoted into 0.7 mL quantities and stored at -20°C. 14 µL of IPG buffer (pH 3-10 or 4-7) and 1.4 mg DTT added to each tube before use	Proteomics. 2D-E
Equilibration stock solution	1.5 M Tris-HCl pH 8.8 10 mL, Urea 72 g, Glycerol 69 mL, SDS 2g, 1% (v/v) bromophenol blue 200 µL – per 200 mL. Stored in 20 mL aliquots at -2°C	Proteomics. 2D-E
Equilibration buffer with DTT	Equilibration stock solution 20 mL, DTT 0.2 g – per 20 mL	Proteomics. 2D-E
Equilibration buffer with IAA	Equilibration stock solution 20 mL, IAA 0.9 g – per 20 mL	Proteomics. 2D-E
14% (w/v) Resolving Gel	18.2MΩ/cm H ₂ O 58 mL, 1.5 M Tris-HCl pH 8.8 37.5 mL, 10% (w/v) SDS stock 1.5 mL, 40% (v/v) solution of 37.5:1 acrylamide:bisacrylamide 52.2 mL, 10% (w/v) APS 750 µL, TEMED 75 µL	Proteomics. 2D-E
Agarose sealing solution	Running Buffer (1x) 19 mL, Agarose 0.1g, 1% (w/v) bromophenol blue 40 µL.	Proteomics. 2D-E
2D gel fixing solution	Methanol 500 mL, Glacial acetic acid 120 mL per L	Proteomics. 2D-E
Colloidal Coomassie blue stock	Ammonium sulphate 100 g, Phosphoric acid 20 mL, Coomassie blue G-250 1 g – per L. Dissolve Coomassie blue G-250 in 20 mL of 18.2MΩ/cm H ₂ O and add to ammonium sulphate previously dissolved in phosphoric acid and 18.2MΩ/cm H ₂ O. Made up to 1 L. Stored at room temperature.	Proteomics. 2D-E
Colloidal Coomassie blue staining solution	Well mixed Colloidal Coomassie blue stock solution 4 parts, Methanol 1 part.	Proteomics. 2D-E
Trypsin stock (1 µg/µL). Promega and NEB.	Trypsin lyophilized powder 20 µg/ mL (100 µg), 50mM Glacial acetic acid (100 µL). Long term storage at -80°C, shot term storage at -20°C	Proteomics. Protein digestion.
Trypsin solution (20 µg/mL).	1 µg/µL Trypsin stock (2 µL), 50 mM NH ₄ HCO ₃ (98 µL)	Proteomics. Protein digestion.
LC-MS buffer A	LC-MS grade Water 95 mL, LC-MS grade Acetonitile 5 mL, Formic acid 0.1 mL - per 100 mL	Proteomics. LC-MS analysis
LC-MS buffer B	LC-MS grade Water 5 mL, LC-MS grade Acetonitile 95 mL, Formic acid 0.1 mL	Proteomics. LC-MS analysis
LC-MS Buffer C	50% (v/v) acetonitrile in LC-MS grade water	Proteomics. LC-MS analysis
BSA Standards	10 mg/mL stored at 20°C and diluted for use	Estimation of protein concentration
Bradford Reagent	Brilliant blue G in phosphoric acid and methanol (Sigma), stored at 4°C	For Bradford's assay

Table 2.7: Reagents and Buffers for Proteomics studies

Enzyme/Peptide	Co-constituent	Use	Supplier
KOD Hot Start DNA Polymerase – 1.0 U/μl	10 x PCR Buffer for KOD Hot Start DNA Polymerase (recipe not available), 25 mM MgSO ₄ , dNTP Mix (2 mM each)	PCR	Novagen
T4 DNA ligase (133 U/μL)	50 mM Tris-HCl pH 7.5, 10 mM MgCl ₂ , 10 mM DTT, 1 mM ATP, 25 μg/mL acetylated BSA	PCR	New England Biolabs
RibonucleaseA	10 μL of 10 mg/mL RNase to 1 mL of TE buffer		Sigma
Lysozyme (Hen egg white, 10 mg/mL)	Used unbuffered		New England Biolabs
<i>Nde</i> I	1 x Buffer 4 (20 mM Tris-acetate, 10 mM Magnesium acetate, 50 mM Potassium acetate, 1 mM DTT, pH 7.9)	Restriction	New England Biolabs
<i>Xho</i> I	1 x Buffer 2 (10 mM Tris-HCl, 10 mM MgCl ₂ , 50 mM NaCl, 1 mM DTT, pH 7.9)	Restriction	New England Biolabs
<i>Bam</i> H I	1 x Buffer E (6 mM Tris-HCl, 6 mM MgCl ₂ , 100 mM NaCl, 1 mM DTT, pH 7.5)	Restriction	Promega
<i>Sal</i> I	1 x Buffer D (6 mM Tris-HCl, 6 mM MgCl ₂ , 150 mM NaCl, 1 mM DTT, pH 7.9)	Restriction	Promega
α-chymotrypsin from bovine pancrease	20 mg/mL, diluted in a solution of 0.001 M HCl / 0.002 M CaCl ₂ ; once diluted stored at -20°C and used up within 1 week	Protease coupled enzyme assay	Sigma
Cyclophilin from calf thymus	1 mg/mL, diluted in a solution of 20 mM HEPES / 140 mM NaCl / 10% (v/v) glycerol; stored at -20°C	Protease coupled enzyme assay	Sigma
Peptide (Pep1)	Suc-Ala-Phe-Pro-Phe-pNa, M.W. 700.75 Da; diluted in 66% (v/v) DMSO	Protease coupled enzyme assay	Sigma
Peptide (Pep2)	Suc-Ala-Lys-Pro-Phe-pNa, M.W. 681.75 Da; diluted in 66% (v/v) DMSO	Protease coupled enzyme assay	Bachem
Peptide (Pep3)	Suc-Ala-Ala-Pro-Phe-pNa, M.W. 624.7 Da; diluted in 66% (v/v) DMSO	Protease coupled enzyme assay	Bachem

Table 2.8 Enzymes and Peptides used in this study

Kit	Use	Source
Prolex Steptococcal Grouping Latex Kit	Lancefield Streptococcal grouping	PRO-LAB DIAGNOSTICS
BCIP/NBT Substrate Kit (substrate for alkaline phosphatase)	Western Blotting	ZYMED Laboratories
DNeasy Blood and Tissue Kit®	Extraction and purification of genomic DNA	Qiagen
PCR purification kit	Purification of dsDNA from a PCR.	Qiagen
Miniprep kit (NZytech)	Extraction of pDNA from <i>E. coli</i>	Prozomix
NZYGelpure Kit	DNA gel purification	Prozomix
Hyaluronan Test Kit (Version 1.01)	Quantitation of hyaluronic acid capsule	Nzomics Biocatalysis

Table 2.9 Kits used in this study

2.3 Bioinformatics Tools

The programmes and websites used for bioinformatics analysis are discussed below.

2.3.1 Prediction of *S. equi* 4047 PrtM full gene, coding sequence, and protein sequence

The coding sequence for the gene of interest (the putative maturase lipoprotein - PrtM - Hamilton *et al.*, 2006) from *S. equi* 4047 was obtained via the Nucleotide database of the National Center for Biotechnology Information (NCBI) - <http://www.ncbi.nlm.nih.gov/nuccore>. The FASTA format of the sequence (http://www.ncbi.nlm.nih.gov/nuccore/NC_012471.1?report=fasta&from=676781&to=677782) shows a 1 kb region from base 676781 to 677782.

The protein sequence (accession number YP_002746044) of the gene was obtained via Protein database of NCBI (<http://www.ncbi.nlm.nih.gov/protein/>). The 'Translate tool' in ExPasy was also used to translate the gene sequence into amino acid sequence (<http://www.expasy.org/tools/dna.html>).

2.3.2 Signal Peptide prediction

The presence of signal peptides was predicted by using the SignalP 3.0 server (<http://www.cbs.dtu.dk/services/SignalP/>).

2.3.3 Gene Search and Conserved Domain/Sequence Similarity Search

A gene search via NCBI (<http://www.ncbi.nlm.nih.gov/gene>) was used to identify genes of interest and their protein sequences identified via NCBI (<http://www.ncbi.nlm.nih.gov/protein>) as well. After obtaining a protein sequence, protein sequences related by sequence similarity were determined by viewing related sequences under the related information link on the same page. The related sequences from different organisms obtained are automatically generated via Basic Local Alignment Search Tool (BLAST) of NCBI. Conserved domain search was carried out via NCBI conserved domain (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) search tool.

2.3.4 Multiple sequence alignment and domain prediction

The following softwares/websites were used to align similar sequences and to evaluate sequence homology with the sequence of *S. equi* 4047 PrtM.

- The ClustalW (www.ebi.ac.uk/Tools/clustalw , Larkin *et al.*, 2007.)

- M-Coffee (<http://tcoffee.crg.cat/apps/tcoffee/do:mcoffee>, Wallace *et al.*, 2006; and Moretti *et al.*, 2007),
- T-coffee version 9.01 (<http://tcoffee.crg.cat/apps/tcoffee/do:regular>, Notredame *et al.*, 2000 and Di Tommaso *et al.*, 2011), and
- uniprot (<http://www.uniprot.org/>, The UniProt Consortium, 2013).

After aligning the sequences they were copied into Word files for formatting (including colour coding the domains). The conserved and semi conserved residues of the sequence were determined, and finally the N-terminal, Central and C-terminal domains were predicted.

2.3.5 Reverse Translation of Protein Sequences

The Sequence Manipulation Suite (SMS) sequence analysis tool (http://www.bioinformatics.org/sms2/rev_trans.html) was used for the reverse translation of protein sequences.

2.3.6 Primer Design

Primers were designed via Eurofins MWG Operon's Oligo Design and Calculations tools (<http://www.eurofinsdna.com/products-services/oligonucleotides0/design-calculation-tools.html>).

The webcutter website (<http://rna.lundberg.gu.se/cutter2/>) was used to look for internal restriction sites.

2.3.7 Determination of Protein MW, pI and molar extinction coefficient.

The expert protein analysis system (Expasy) identification and characterisation tool (<http://web.expasy.org/protparam/>) was used to determine the molecular weight and pI of the proteins of interest.

2.3.8 Determination of protein three dimensional (3D) structures:

PrtM central domain protein sequence (underlined yellow in Figure 4.9a) was inputted into the PSIPRED Protein Sequence Analysis Workbench (<http://bioinf.cs.ucl.ac.uk/psipred/>). The PSIPRED v3.3, pGenTHREADER and BioSert v2.0 softwares, which are part of the PSIPRED protein sequence analysis workbench, were used for this analysis. PSIPRED v3.3 was selected to predict protein secondary structure. pGenTHREADER was selected for profile based fold recognition. BioSert v2.0 was selected for automated homology modelling. A valid MODELLER key (obtained via <http://salilab.org/modeller/registration.html>) was entered via the BioSert tab. Finally, the predict button was selected after which the protein 3D structure information generated by the PSIPRED protein sequence analysis workbench was emailed to a preapproved email address.

2.4 General Microbiology

2.4.1 Growth of bacteria on agar plates

An agar plate was surface dried by placing it open and face down at 65°C for 10-15 min. An inoculum was streaked onto the dried agar plate, which was subsequently incubated under the appropriate conditions.

S. equi strains used in this study were grown on Todd Hewitt agar (Table 2.3) plates (supplemented with the appropriate antibiotics if required) overnight (or as long as specified for individual experiments) at 37°C in a candle jar. *E. coli* strains were grown in LB agar (Table 2.3) at 37°C, supplemented with appropriate antibiotics as required. All agar cultures were stored at 4°C.

2.4.2 Growth of Bacteria in Broth

Overnight starter broth culture was prepared by inoculating a single colony of bacteria, or a 1:100 dilution of glycerol stock (thawed on ice), into a specified volume of the appropriate media (with antibiotics if required) followed by incubation at specified temperature and time, as stated for individual experiments.

S. equi broth cultures were grown in Todd Hewitt broth (Table 2.3) without agitation at 37°C. Colonies derived from each glycerol stock were stained (Gram staining section 2.4.3) to confirm the nature and purity of the culture.

It had previously been reported (Hamilton *et al*, 2006) that at optical density of approximately 0.3 (OD_{600 nm}) this density of bacteria corresponds to approximately 2 x

10^8 CFU/mL of *S. equi* 4047. Unless otherwise stated, biomass of *S. equi* 4047 was harvested at this optical density.

E. coli strains were grown in LB broth (Table 2.3) at the appropriate temperature and time, supplemented with appropriate antibiotics as required.

2.4.3 Gram Staining and Microscopy

An inoculum of the organism was heat fixed onto a clean glass slide. The heat fixed film was flooded with methyl violet for 30-40 sec. The stained slide was rinsed off with tap water and flooded with Gram's iodine for 30 - 40 sec. The slide was rinsed again with tap water and decolourised with acetone for 2 – 3 sec. After this, the smear was rinsed under running tap water, excess water poured off and counterstained with safranin for 15- 20 sec. The slide was rinsed with tap water and blotted dry with lint-free tissue. The slide was completely dried and examined using the oil immersion objective of a microscope. Gram-positive bacteria stained deep purple and Gram-negative bacteria stained pale pink.

2.4.4 Lancefield Streptococcal grouping of *S. equi* 4047 WT and Δ PrtM

Prolex Streptococcal Grouping Latex Kit (Table 2.9) was used to carry out Lancefield grouping of the *S. equi* 4047 WT and Δ PrtM strains for identity confirmation. Ready to use polyvalent antigens extracted from *Streptococcus* of Lancefield group B (provided in kit) were used as a positive control and *Staphylococcus aureus* colonies were used as a negative control.

According to the manufacturer's instructions, to one drop of Extraction Reagent 1 in an Eppendorf tube was added a few bacterial colonies from an agar plate and one drop of

Extraction reagent 2 and mixed together for 5-10 sec. Five drops of extraction reagent 3 were added to the tube and the contents mixed. One drop of blue latex suspension (group B) was dispensed onto separate circles on the test card provided in kit. One drop of bacterial extract from each tube was pipetted besides each drop of blue latex suspension and mixed. The card was rocked gently for one minute and observed for agglutination under normal lighting.

2.4.5 β -haemolysis test.

An inoculum from *S. equi* 4047 WT and Δ PrtM overnight starter cultures (each 20 mL THB inoculated with 100 μ l glycerol stock of WT or Δ PrtM and incubated at 37°C overnight) was inoculated onto 5% (v/v) horse blood agar plates (Table 2.3) and THA plates. The plates were incubated in candle jars at 37°C overnight. The 5% (v/v) horse blood agar plates were observed for clear zones of β -haemolysis.

2.4.6 Growth Curve

A starter culture (section 2.4.2) in 20 mL THB (in 25 mL universals, without antibiotics for *S. equi* 4047 WT and Δ PrtM, but with 5 μ g/mL chloramphenicol for complemented mutants) was incubated at 37°C overnight. Next day, a 1 in 20 dilution of the overnight starter culture in THB (with antibiotics if required) was done. 200 μ L of the diluted overnight starter culture was dispensed into each well of a 96-well plate and OD_{570 nm} read hourly over a 24 h period, via an automated ultra microplate reader (EL 808, appendix B7). Growth curve results were obtained from a minimum of three independent assays.

2.4.7 Sodium Chloride Stress test

1st passage cultures of *S. equi* 4047 WT or Δ PrtM were grown to an OD_{570 nm} of approximately 0.5 and 1.2 mL of culture was spun down for 10 min at 4000 x g at room temperature. The supernatant was discarded and the pellets in each tube were re-suspended in 1.2 mL of THB, sterile 18.2M Ω /cm H₂O, or a given concentration of NaCl (0.9%, 14.7% or 29.4% [w/v]). After incubation at 37°C overnight for 24 h and 48 h, serial dilutions (1 in 2, 1 in 10, 1 in 100 and 1 in 1000) of tubes containing sterile 18.2M Ω /cm H₂O, and NaCl) were plated onto THA plates to evaluate survival by colony count. For the serial dilutions, the tubes containing sterile 18.2M Ω /cm H₂O were diluted with only 18.2M Ω /cm H₂O; the tubes containing NaCl were diluted with the corresponding range of concentrations of NaCl solution. The tube containing only THB was also plated onto THA plate as control; to evaluate viability of the organism after centrifugation. THA plates were incubated in candle jars at 37°C overnight and colony counts of experimental plates recorded (a modification of the methods of Reffuveille *et al.* (2012a) and Reffiveille *et al.* (2012b). NaCl stress test results were obtained from a minimum of three independent assays.

2.4.8 Disc Diffusion Antibiotics Sensitivity Test

200 μ L of overnight starter culture of *S. equi* 4047 WT or Δ PrtM was spread onto THA plate. Each plate was seeded with discs each containing one of the following antibiotics: ampicillin 10 μ g, penicillin-G 6 μ g, gentamicin 500 μ g, streptomycin 500 μ g, norfloxacin 5 μ g and vancomycin 30 μ g (a modification of the methods of Reffuveille *et al.* (2012a) and Reffiveille *et al.* (2012b). 200 μ L of overnight starter culture of *S. equi* 4047 WT or Δ PrtM was also spread onto a plain THA plate. This was repeated for each strain in triplicate.

2.4.9 Broth Dilution Antibiotics Sensitivity Test

20 mL THB (in 25 mL universal, with varying dilutions of antibiotics) was inoculated with 200 μ L of overnight starter culture (of *S. equi* 4047 WT, Δ PrtM, or complemented mutants) and incubated overnight at 37°C. Next day, broth cultures were checked for turbidity, after which 200 μ L was spread onto agar plates (with varying dilutions of antibiotics) and incubated overnight at 37°C in a candle jar.

2.4.10 Rapid Detection of Hyaluronic acid capsule by Density Bouyancy Centrifugation

A modification of the method of DeAngelis and Weigel (1994) was applied. Overnight starter cultures (each of *S. equi* 4047 WT or Δ PrtM in THB) were diluted 1 in 20 in 20 mL THB (1 universal per strain) and incubated at 37°C to early log phase ($OD_{600\text{ nm}}$ 0.2). Another set of overnight starter cultures (each of *S. equi* 4047 WT or Δ PrtM in THB containing 34 μ g/mL hyaluronidase) were diluted 1 in 20 in 20 mL THB containing 50 μ g/mL hyaluronidase (1 universal per strain) and incubated at 37°C to early log phase ($OD_{600\text{ nm}}$ 0.2).

4 mL of each early log phase culture in a screw cap 15 mL centrifuge tube was carefully and slowly underlaid (using a 9' Pasteur pipette) with 2 mL of 65% or 50% (v/v) Percoll (buffered with 25mM sodium phosphate, pH 7.0). The 65% or 50% (v/v) Percoll layer was carefully and slowly underlaid (using a 9' Pasteur pipette) with 2 mL of 100% Percoll pad. The tubes were centrifuged at 1000 x g for 10 min. The locations of encapsulated cells at the top (yellow THB / 65% or 50% [v/v] Percoll) interface; and the acapsular or hyaluronidase treated cells at the lower (65% or 50% (v/v) Percoll / 100% (v/v) Percoll) interface were observed (Figure 2.0). Three replicas of this experiment were carried out.

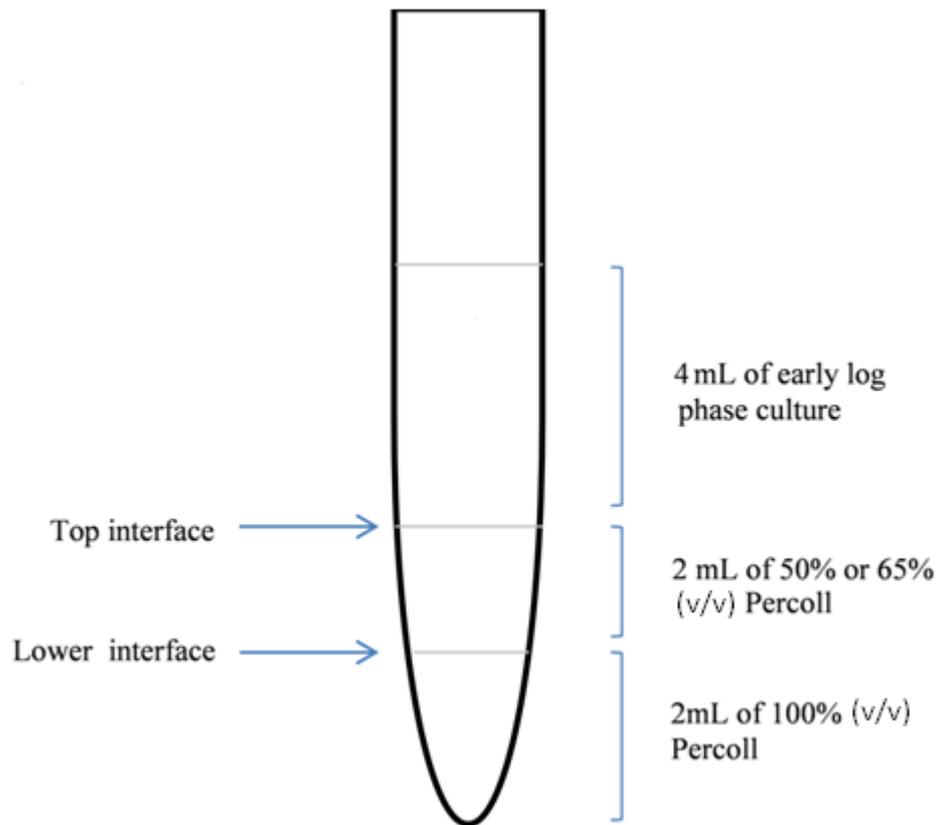


Figure 2.0: Rapid Bouyant Density Separation.

2.4.11 Colonial morphological assessment of hyaluronic acid capsule

An inoculum from an overnight starter culture of *S. equi* 4047 WT or Δ PrtM was plated onto THA (plus antibiotics for complemented mutants) plate which was incubated at 37°C for 24 – 48 h in a candle jar [a modification of the method of Prasad *et al.* (2010)]. The morphology of the colonies was noted.

2.4.12 Congo red/Safranin staining for assessment of hyaluronic acid capsule

An overnight starter culture of *S. equi* 4047 WT or Δ PrtM was diluted 1:20 and grown to OD_{600 nm} of 0.2. A smear (on glass slide) from a culture inoculum was air dried. The

smear was stained with safranin stain (a basic dye) for 2 min and the stain tipped off. This was followed by staining of the smears with Congo red (a negative dye) for 2 min. The smear was then washed slightly with water, air dried and examined under the oil immersion objective of a microscope. Safranin stained the cell surface red, congo red stained the background red, while capsules remained colourless.

2.4.13 Alcian Blue staining for assessment of hyaluronic acid capsule

An overnight starter culture of *S. equi* 4047 WT or Δ PrtM was diluted 1:20 and grown to OD_{600 nm} of 0.2. A smear (on glass slide) from a culture inoculum was air dried. The smear was stained for 1 min with a freshly prepared 1:9 dilution of alcian blue stain stock solution (1 g Alcian blue 8GX in 100 mL of 95% ethanol). After 1 min, the stain was washed off with water and counterstained with Ziehl-Neelsen carbol fuchsin, washed immediately with water and air dried. The slide was examined under the oil immersion objective of a microscope. Hyaluronic acid stained blue, while the cell stained pink-red. This was a modification of the method of McKinney (1953).

2.4.14 Crystal violet staining for assessment of hyaluronic acid capsule

The capsular staining method of Hiss (1905) modified by Anthony (1931) was modified and employed. An overnight starter culture of *S. equi* 4047 WT or Δ PrtM was diluted 1:20 and grown to OD_{600 nm} of 0.2. A smear (on glass slide) from an inoculum was air dried and then flooded with a solution of 1% (w/v) crystal violet for 2 min. The crystal violet was rinsed gently with a solution of 20% (w/v) copper sulfate. The slide was air dried and examined under the oil immersion objective of a microscope. Cells stained purple, while capsules stained faint blue or remained as a white halo.

2.4.15 Quantitative Hyaluronic acid capsule test

An overnight starter culture of *S. equi* 4047 WT or Δ PrtM was diluted 1:20 in THB and incubated at 37°C to OD_{600 nm} of 0.4. A volume of 0.5 mL culture (OD_{600 nm} of 0.4) was centrifuged and pellet resuspended in 500 μ L of sterile 18.2 M Ω /cm H₂O. Serial dilutions of the bacterial suspension were plated onto THA to confirm equivalent cfu per 1 mL. Chloroform was added to 400 μ L of the bacterial suspension in a 2 mL screw cap tube and shaken for 5 min in a Stuart Rotator (appendix B28). The mixture was centrifuged at 13,000 x *g* for 10 min. Hyaluronic acid in the aqueous phase was determined using a Hyaluronic Acid Test Kit (Version 1.01, Nzomics Biocatalysis). 2 mL of 18.2 M Ω /cm H₂O, 0.2 mL solution 1 (buffer), and 0.2 mL of test solution (extracted aqueous phase sample or control - hyaluronic acid from Sigma) were pipetted into a 3 mL quartz cuvette. A second cuvette (blank) contained 0.2 mL of 18.2 M Ω /cm H₂O instead of test solution. Each cuvette was covered and the solutions mixed and left to equilibrate for 3 min in a spectrophotometer set at 235 nm (room temperature). The starting absorbance (A1) was recorded and each cuvette swirled to homogenise the suspension. 0.2 mL of solution 2 (hyaluronate lyase enzyme) was added into each cuvette and mixed. The absorbance was read spectrophotometrically continuously for 4 min until it plateaued. The plateaued absorbance (A2) was recorded and the concentrations of hyaluronan in each sample calculated from the formula below:

$$[(A2 - A1) \text{ Sample} - (A2 - A1) \text{ Blank}] \times 0.2314 = \text{mg/mL of hyaluronan.}$$

2.5 General Biomolecular Methods

2.5.1 Plasmids

The plasmids used in this study are listed in Table 2.10

Plasmid	Characteristics	Application	Source
pET-28a	Kan ^r , T7, <i>lac</i> , <i>lacI</i> ^q	Expression vector	Novagen (appendix A17)
pVA838	9.2 kb, Em ^r , Cm ^r . <i>SalI</i> <i>SphI</i> , <i>BamHI</i> , <i>NruI</i> and <i>XbaI</i> cleavage sites	<i>E. coli</i> - <i>Streptococcus</i> shuttle vector	Macrina <i>et al.</i> , 1982. (appendix A18)
pVA838/PrtM123	pVA838 to which the N-terminal, the Central and the C-terminal domain genes of PrtM had been cloned; Cm ^r .	Production of complemented mutant Δ PrtM123/pVA838	This study
pVA838/PrtM12	pVA838 to which only the N-terminal and the Central domain genes of PrtM had been cloned; Cm ^r .	Production of complemented mutant Δ PrtM12/pVA838	This study
pET28a/PrtM	pET28a to which <i>S. equi</i> 4047 PrtM full gene had been ligated. Kan ^r	Transformation into <i>E. coli</i> BL21 for expression of Full length protein	This study
pET28a/central-PrtM	pET28a to which <i>S. equi</i> 4047 PrtM central domain gene had been ligated. Kan ^r	Transformation into <i>E. coli</i> BL21 for expression of central domain protein	This study

Table 2.10 Plasmids used in this research.

2.5.2 Extraction of Genomic DNA

1 mL of overnight broth culture was centrifuged (13,000 x g at room temperature) in Eppendorf tubes for 2 min. Supernatant was discarded and cell pellets centrifuged again for 40 sec to remove excess supernatant. Gram staining and Lancefield grouping were done to confirm the identity of isolates in biomass. All cell pellets of about the same

size were used immediately or stored in the freezer (-20°C) pending DNA extraction. Genomic DNA was extracted from each pellet using Qiagen DNeasy DNA Extraction Kit (Table 2.9), according to the manufacturer's instructions.

2.5.3 Oligonucleotide Primers

Oligonucleotide primers were synthesized by Eurofins MWG Operon (<http://www.eurofinsdna.com/products-services/>). The oligonucleotide primers used in this study are listed in Table 2.11

Oligonucleotide name	Sequence (Primer Length) 5' → 3'	Restriction Site	Annealing temperatures 1° and 2°	GC content	Use
SequiPrtMf	TTC CCA TTA TCC AGC ATG AG (20)	n/a	55.3 °C and 50.3°C	45%	PCR/ID of WT and Δ
SequiPrtMr	ACA ATT CAA AGC CCC ACT CA (20)	n/a	55.3 °C and 50.3°C	45%	PCR/ID of WT and Δ
SEQPrtmf	gatcgatccatATGTGTCAGTCTA CAAATGACAATACAAGTG (42)	<i>NdeI</i>	69.4 °C and 64.4°C	38.1%	PCR/cloning of full PrtM gene
SEQPrtmr	gatcgatcctcgagATATTTTTCTG ACTTAGATTTAGAAGATTGA C (46)	<i>XhoI</i>	70.3 °C and 64.4°C	37%	PCR/cloning of full PrtM gene
Prtmidcolif	tgccatagcatATGACTACTCAGG TCACTACTCTAGACAATG (42)	<i>NdeI</i>	71.4 °C and 64.5°C	42.9%	PCR/cloning of PrtM central domain
Prtmidcolir	tgccatagctcgagttaGGCTTTTTG GTTACCTTAACA (39)	<i>XhoI</i>	69.5 °C and 64.5°C	41%	PCR/cloning of PrtM central domain
Prtmequif	tgccataggatccGGTGGAAATGGT TGTTTTAGATGATG (39)	<i>BamH I</i>	71.6 °C	46.2%	PCR/ complement ed mutant production
Prtm123equir	tgccataggtcgacCTATTTTTCTGG ACTTAGATTTAGAAGATTGA C (46)	<i>Sal I</i>	70.3 °C and 65.3°C	37%	PCR/ complement ed mutant production
Prtm12equir	tgccataggtcgacctaggGCTTTTTG GTTACCTTAACA (39)	<i>Sal I</i>	70.5 and 65.5°C	43.6%	PCR/ complement ed mutant production

Table 2.11 Oligonucleotide Primers used in this study. Those nucleotides that do not anneal to the initial template are shown in lowercase. 1° indicates primary annealing temperature. 2° indicates secondary annealing temperature.

2.5.4 Polymerase Chain Reaction (PCR)

PCR was carried out using primers, DNA template and other components (dNTPs, KOD Hot Start polymerase, MgSO₄ Buffer) from Novagen (Appendix A9). The constitution of a typical PCR mix is shown in Table 2.12.

Order	Component	Volume
1	Sterile 18.2 MΩ/cm H ₂ O	35 μL
2	dNTPs (2 mM)	5 μL
3	Buffer (10 x)	5 μL
4	MgSO ₄ (25 mM)	2 μL
5	F primer (20 μM)	1 μL of 1 in 10 dilution
6	R primer (20 μM)	1 μL of 1 in 10 dilution
7	Template DNA	1 μL
8	KOD Hot Start polymerase (1 U/μl)	1 μL

Table 2.12 Typical PCR Mix

A PCR mix was prepared in the order shown in Table 2.12, to give a final reaction volume of 50 μl in labeled PCR (0.2 mL) tube. Each PCR reaction was run overnight under the thermal cycling conditions shown in Table 2.13.

PCR Stage	Temperature (°C)	Time	# of Cycles
Initial denaturing	95	2 min	1
Denaturing	95	15 sec	5
1° Annealing	X	30 sec	
Extension	72	1 min/kb	
Denaturing	95	15 sec	25
2° Annealing	Y	30 sec	
Extension	72	1 min/kb	
Final Extension	72	10 min	1
Hold	10	-	1

Table 2.13 PCR Stages and Conditions. X indicates primary annealing temperatures and Y indicates secondary annealing temperatures for the primers as shown in Table 2.11

When required, colony PCR was carried out by suspending a single bacterial colony in 100 μL of sterile 18.2 M Ω /cm H_2O and boiled for 10 min. 2 μL of this mixture was then used as a template for the PCR reaction.

To ascertain successful amplification, after PCR reaction, 5-10 μL aliquot was analysed by agarose gel electrophoresis.

2.5.5 Agarose Gel Electrophoresis (AGE)

A 1% (w/v) agarose gel was typically used and the volume prepared depended on the number of samples and/or the size of the electrophoresis tank. For a small tank, for example, 0.3 g agarose powder was added to 30 mL of 1 x TAE buffer and boiled to get a clear gel solution. The solution was allowed to cool to about 55°C before being carefully poured into the gel tray (containing well forming comb) in an electrophoresis tank. The gel was allowed to cool and set; after which the comb was removed.

For non-ethidium bromide stained gels, 5 μL of SYBR-safe (Appendix A7) was added to 30 mL of boiled solution at 55°C before being poured into gel tray. However, for ethidium bromide stained gels, staining was carried out after electrophoresis. Gels were stained for 10 min in 10 $\mu\text{g}/\text{mL}$ ethidium bromide solution.

2 μL of loading buffer ([0.25% (w/v) bromophenol blue; 30% (v/v) glycerol] or [0.25% (w/v) xylene cyanol; 30% (v/v) glycerol] depending on the expected size of DNA) was added to 10 μL DNA sample. This mixture was loaded into each well of the cast gel and 5 μL of Hyperladder 1 (Appendix A8) was loaded into one well as a standard marker. Samples were electrophoresed for 30-40 min at 120 mA (200 V). DNA bands were

visualised, images acquired and saved using a gel documentation system (Bio-Rad Gel Doc 2000 with Quantity One software).

PCR products were purified using the PCR clean-up protocol of the NZYGelpure kit (Table 2.9), according to manufacturer's instructions.

2.5.6 Gel Purification of DNA

DNA bands of interest were excised from agarose gel and purified using NZYGelpure Kit (Table 2.9), according to the manufacturer's instructions.

2.5.7 Quantification of DNA in agarose gel

DNA in a band in agarose gel was quantified via Quantity One software with the Bio-Rad Gel Doc 2000 imaging system.

2.5.8 Quantification of DNA in solution

DNA in solution was quantified using an automated NanoDrop uv/vis spectrophotometer (ND-1000, appendix B27) or manually via an ultra violet (UV) spectrophotometer.

For DNA quantitation via the UV spectrophotometer (Appendix B29), the instrument was zeroed with water (50-100 μL in a quartz cuvette) after which the diluted DNA sample (50-100 μL in a quartz cuvette) was scanned in the UV region between 200-300 nm. The optimum wavelength for absorbance of DNA was assumed to 260 nm, while the optimum wavelength to detect protein contamination was assumed to be 280 nm. ssDNA of 33 $\mu\text{g}/\text{mL}$, as well as dsDNA of 50 $\mu\text{g}/\text{mL}$ each give an $\text{OD}_{260 \text{ nm}}$ reading of

1.0. The concentration and purity of DNA in solution was therefore determined as described below:

$$[\text{dsDNA}] \mu\text{g}/\mu\text{L} = (A_{260 \text{ nm}}) \times 50 \times \text{dilution}/1000$$

$$\text{DNA purity ratio} = (A_{260 \text{ nm}})/(A_{280 \text{ nm}})$$

Pure DNA solutions would have an $(A_{260 \text{ nm}})/(A_{280 \text{ nm}})$ between 1.8 - 2.0

2.5.9 Restriction digest

Restriction endonuclease (New England Biolabs) digests were performed according to the manufacturer's instructions. Typically, each μg of DNA was cut with 5 U of enzymes at 37°C for >1.5 h, unless otherwise stated. When necessary, heat inactivation was carried out at 65°C for 10 min. BSA (at a final concentration of 0.1 mg/mL) and the appropriate buffer (at a final concentration of 1 x, Table 2.8) were added to the reaction mixture. In order to avoid interference with enzyme specificity, glycerol concentration in the final reaction did not exceed 10% (v/v). The products of digestion were subjected to agarose gel electrophoresis (section 2.5.5); the DNA fragment recovered and purified using NZYGelpure Kit (section 2.5.6).

Internal restriction recognition sites for DNA sequences and vectors were determined by the NEBcutter program (2012). A list of restriction endonucleases used to cut plasmids/vectors is given in Table 2.14. The restriction endonuclease used in this study and their corresponding reaction buffers are shown in Table 2.8.

Plasmid/Vector	Restiction endonuclease
pET-28a	<i>NdeI/XhoI</i> or <i>XbaI/XhoI</i>
pVA838	<i>BamHI/SalI</i>

Table 2.14 Restriction endonucleases used to cut plasmids/vectors

2.5.10 Ligation

Concentrations of DNA insert were determined on samples (after agarose gel electrophoresis) using the analysis tool of the Bio-Rad Gel Doc 2000 Quantity One software. For a typical ligation experiment, 100 ng of cut vector required 3-10 times more molecules of insert. Other components of the reaction include 400 U of T4 DNA Ligase (New England Biolabs), 1 μ L 10 x T4 DNA ligase Buffer (New England Biolabs). The ligation mixture was made up to a final volume of 10 μ L with sterile 18.2 M Ω /cm H₂O and incubated overnight at 16°C.

2.5.11 Preparation of chemically competent *E. coli* cells

The CaCl₂ method of Cohen *et al.* (1972) was applied for the preparation of chemically competent *E. coli* TOP10 or BL21 cells. A single bacterial colony was inoculated into 5 mL of LB broth and incubated at 37°C overnight. The overnight starter culture (1% v/v) was used to inoculate 50 mL of LB broth in a 250 mL conical flask and incubated at 37°C, shaking at 200rpm, to an OD_{600 nm} of 0.35, after which the culture was incubated on ice for 30 min. The culture was centrifuged at 2700 x g for 10 min at 4°C and the cell pellet resuspended, by gentle vortexing, in 15 mL of ice-cold solution of 80 mM MgCl₂ - 20 mM CaCl₂. After a further incubation on ice 10 min, the sample was centrifuged at 2700 x g for 10 min at 4°C. The cell pellet was resuspended (by gentle vortexing) in 1 mL of ice-cold 100 mM CaCl₂ and placed on ice for 1h. The cells were either used immediately or 50% (v/v) glycerol added to a final concentration of 15% (v/v) and stored in 50 μ L aliquots at -80°C.

2.5.12 Heat-Shock transformation of chemically competent *E. coli* (TOP10 or BL21) cells

DNA (10 μ L from a ligation reaction, or 1 μ L from a plasmid preparation) was added to 50 μ L of chemically competent *E. coli* cells, mixed gently with a pipette tip and incubated on ice for 20 min. The cells were heat shocked for 90sec at 42°C and incubated again on ice for 2 min. The cells were recovered aseptically by adding 200 μ L of room temperature NZY⁺ enrichment broth and incubated at 37°C for 1 h, shaking horizontally (for maximum aeration) at 200 rpm. The recovered cells were plated, aseptically onto LB agar plates containing appropriate antibiotics and incubated at 37°C for approximately 16-24 h.

2.5.13 Crude Preparation of pDNA for screening of successful transformation (STET DNA).

5 mL of LB broth containing the appropriate antibiotics was inoculated with a single colony of the transformant and the culture incubated overnight at 37°C, shaking at 200 rpm. 3 mL of the overnight bacterial culture was centrifuged in two 1.5 mL microcentrifuge tubes at 10,000 x g for 1 min. The pellet was pulsed and residual supernatant removed. Pellet was resuspended (by vortexing) in 150 μ L STET buffer (Table 2.5) and 10 μ L of freshly prepared 10 mg/mL hen egg white lysozyme added to lyse the cells. Sample was boiled for 40 sec and immediately after removal from the boiling water bath, centrifuged at 14,000 x g for 15 min. The viscous pellet was removed and discarded. 150 μ L of chilled isopropanol was added to the supernatant and mixed before incubation at -20°C for 1 h, to allow for precipitation of DNA. After 1 h incubation at -20°C, the sample was immediately centrifuged at 14,000 x g for 5 min and the supernatant discarded. After removal of residual supernatant by pulsing, the

pellet was washed in 500 μ L of ethanol and centrifuged at 14,000 x g for 3 min. The supernatant was discarded and the pellet pulsed to remove residual supernatant. The sample was desiccated for 10 min (to remove any latent ethanol) and resuspended in 30 μ L of TE buffer containing 100 μ g/mL of RNase.

2.5.14 Standard Preparation of pDNA (SPIN DNA)

An overnight culture containing insert (after section 2.5.12) was inoculated 1:100 into 5 mL or 100 mL LB broth containing the appropriate antibiotics and incubated overnight at 37°C, shaking at 200 rpm. The plasmid was purified using the NZYminiprep (5 mL culture) or NZYmidiprep (100 mL culture) according to the manufacturer's instructions. The pDNA was eluted into TE buffer, the plasmid concentration determined spectrophotometrically and the plasmid integrity determined by agarose gel electrophoresis (AGE).

2.5.15 pDNA sequencing

The standard preparation of pDNA (Section 2.5.13) was employed. However, the pDNA was eluted into sterile 18.2 M Ω /cm H₂O, the concentration determined using nanodrop spectrophotometer (Section 2.5.18) and the sample sent to GATC Biotech for sequencing.

2.5.16 Production of electrocompetent cells and electrotransformation of *S. equi* 4047 WT and Δ PrtM

A modification of the methods of Slater *et al.* (2003), Pajunen *et al.* (2005) and Hamilton *et al.* (2006) was employed in the preparation of competent *S. equi* cells. An overnight culture of *S. equi* 4047 Δ PrtM (grown in THB) or *S. equi* 4047 WT (grown in THB containing 30 μ g/mL hyaluronidase) was diluted 1:20 in 200 mL of same medium

and grown at 37°C to OD_{600 nm} between 0.12 -0.13. The cells were harvested by centrifugation (*S. equi* 4047 ΔPrtM at 4000 x g; and *S. equi* 4047 WT at 8000 x g) at 4°C for 10 min. Pellets were washed three times in 10 mL of ice-cold 0.5 M sucrose by centrifuging at same speed, temperature and time. After the last wash, the competent cells were resuspended in 1 mL of ice-cold 0.5 M sucrose / 10% (v/v) glycerol and 100 μL aliquots were used immediately for electrotransformation or stored frozen at -80°C. All tubes or storage vials used were always ice-cold prior to coming in contact with competent cells.

A modification of the methods of Wyckoff and Whitehead (1997) and Hamilton *et al.* (2006) was employed in the electrotransformation of *S. equi* competent cells. 1-5 μg of pDNA or recombinant-pDNA was added to 100 μL of competent cells in a pre-chilled 2 mm Bio-Rad (U.K.) electroporation cuvettes and left to stand on ice for 10 min. Electroporation was carried out in a Gene Pulser electroporator (Appendix 31) with pulse settings of 2.5 kV/cm, 200 Ω, and 25 μF. Pulse times ranged from 4 – 5 sec. After electroporation, 1 mL of ice cold THB / 0.5 M sucrose / 20 mM MgCl₂ / 2 mM CaCl₂ was added to the cells and left to stand on ice for 10 min. Cells were then incubated at 37°C for 2 h, after which they were put back on ice. Within 30 min, serial dilutions (in THB) were plated onto THA plates containing 5 μg/mL chloramphenicol, followed by incubation in a candle jar at 37°C for 24 - 96 h, until colonies were observed.

2.5.17 Bradford's assay

Protein determination by the Bradford (1976) method entailed the preparation of 10 dilutions of BSA in 500 μL aliquots, ranging from 1-10μg/mL. 500 μL of Bradford's solution (Table 2.7) was then added to each standard dilution and mixed. Protein concentration in each standard was measured in a glass cuvette at A_{595 nm} against a blank

containing 500 μL of 18.2 $\text{M}\Omega/\text{cm}$ H_2O mixed with 500 μL of Bradford's solution. After every measurement, the glass cuvette was washed with methanol. All measurements were completed within 40 min of addition of Bradford's solution, followed by the drawing of a standard curve (Figure 2.1). Dilutions of the protein to be determined were made in 500 μL volumes and measured after the addition of Bradford's solution in the same way as the standards. Diluted sample $A_{595 \text{ nm}}$ values that fell on the standard curve were then used to calculate protein concentration, using the equation below.

Protein concentration $\text{mg/mL} = (\text{Concentration obtained from the curve}) \times (\text{dilution of sample}) / 1000$

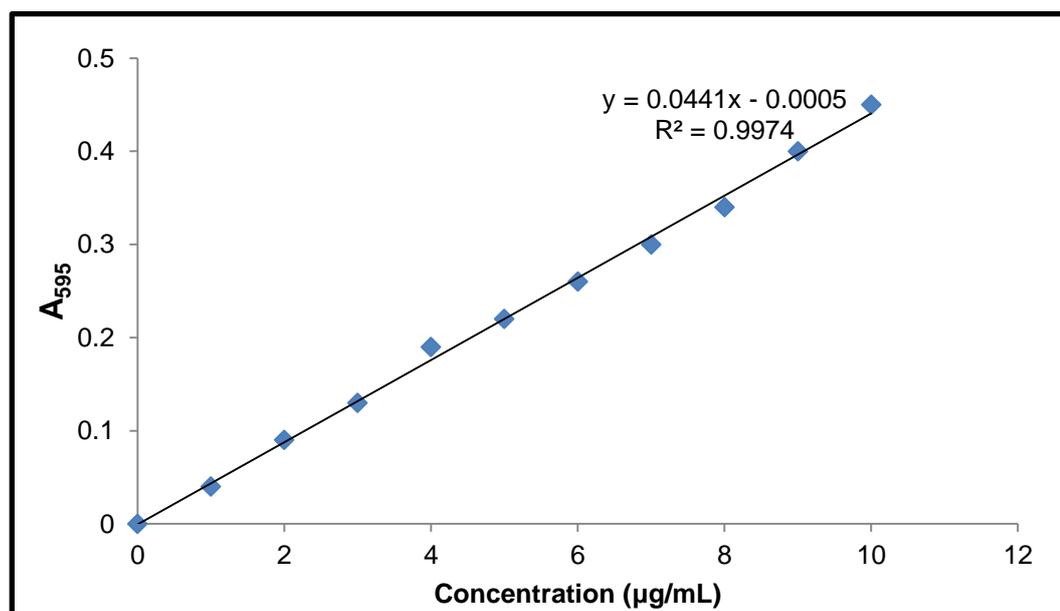


Figure 2.1: Standard curve for the quantification of protein by Bradford assay.

The curve was generated by the addition of 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, + 10 $\mu\text{g/mL}$ BSA in 500 μL 18.2 $\text{M}\Omega/\text{cm}$ H_2O to 500 μL Bradford's reagent. The $A_{595 \text{ nm}}$ of the solution was determined after 5 min incubation at room temperature.

2.5.18 Spectrophotometric determination of protein concentration

Determination of protein concentration using the UV spectrophotometer (Appendix B29) was achieved by taking A_{280nm} readings of diluted protein sample, with the diluent serving as the blank with which the instrument had initially been zeroed. The molar extinction coefficient of the protein was determined via the Expaty website (section 2.3.3) so as to enable the calculation of the concentration of protein in the sample using the following formula:

Protein concentration ($\mu\text{g/mL}$) = [(A280) x molecular weight of protein x dilution factor] / molar extinction coefficient of protein.

2.5.19 SDS-PAGE

SDS-PAGE was carried out according to the method of Laemmli (1970) using The BioRad Mini Protean III kit. Two clean glass plates of dimensions 10.1 X 7.2 cm and 10.1 X 8.2 cm (larger glass plate had a spacer ridge of 0.75 mm attached) were aligned in parallel, clamped and mounted onto the surface of a rubber gasket by applying vertical downward pressure. The components of the resolving gel were made up in a plastic container and pipetted into the space between the two plates, filling it up to about 2 cm from the top of the smallest plate. In order to prevent inhibition of polymerisation by oxygen, and also to produce a straight top, the surface of the gel was layered with 18.2 M Ω /cm H₂O. The gel was left to set for 20 min, after which that water was removed from the surface of the set gel and residual unset gel or water removed with the aid of blotting paper (without touching the gel surface with the blotting paper). The components of the stacking gel were mixed and pipetted on to the surface of the resolving gel, filling it up to the top of the small glass plate. Immediately, a 10-tooth comb (1.1 X 0.75 cm) was inserted into the gap between the plates and the gel was then

allowed to polymerise for 20 min. The combs were then gently removed to reveal the wells which were rinsed immediately with 18.2 MΩ/cm H₂O. The gels were placed in the electrophoresis module with the smaller glass plates facing inwards, towards the centre so as to create a reservoir in the central compartment. The module was then lowered vertically into the electrophoresis tank and 1 x SDS-PAGE running buffer carefully (avoiding creation of bubbles) poured into the reservoir. The buffer in the reservoir was allowed to flow into the wells and formed a layer on the top of the gel.

Samples were prepared by adding 5 μL of SDS-PAGE loading buffer to 20 μL of sample, mixed and boiled for 3 min. Size standards were similarly prepared, except that 8 μL of loading buffer was added to 4 μL of size standard. All samples and standards were then centrifuged at 14,000 x g for 1 min. With the aid of the Hamilton syringe, 20 μL of sample and 10 μL of standard were loaded into wells of the cast SDS-polyacrylamide gels. The pair of gels were electrophoresed at 120 mA, 200 V for 50-60 min, until the bromophenol blue dye had migrated off the bottom of the gel.

To visualize the protein bands after electrophoresis, gels were carefully removed from between the glass plates and stained in Coomassie blue stain for 10 min, destained overnight, washed in 18.2 MΩ/cm H₂O, photographed using the Bio-Rad gel doc system, while hard copies of the image were produced using a Mitsubishi Video Copy Processor with Mitsubishi thermal paper (Appendix B9).

2.5.20 Western Blotting

Western blotting can be used to indicate the presence of lipoproteins in either cell associated protein extract or secreted protein extract (Hamilton *et al.*, 2006). To evaluate immunogenicity, Western blotting was adapted from Hamilton *et al.* (2000).

SDS-PAGE of sample was carried out as described in section 2.5.15, except that the standard was the ready to use Bio-Rad Precision Plus Protein All Blue Standard (Appendix C8), unless otherwise stated. After SDS-PAGE, protein bands were electrophoretically transferred (Western blotting by Transblot apparatus, Appendix C13) onto nitrocellulose membranes (0.2 μ M, BioRad).

In order to view protein bands after transfer (a method adapted from Luque-Garcia *et al.*, 2006), blots were, optionally, reversibly stained with Ponceau-S solution (Appendix A1) and washed in several changes of PBS-Tween (Appendix C10).

After transfer, or after washing off Ponceau-S solution, non-specific binding sites on blot were blocked by incubating blot overnight in 20 mL 5% (w/v) skimmed milk in phosphate buffered saline containing 0.05% (w/v) Tween-80 (PBST, Table 2.6). The blocking solution was subsequently discarded and blots were incubated for 2-3 h in primary (1^o) antibody (Table 2.15) diluted in 5% (w/v) skimmed milk in PBST. After washing blots in five changes (5-10 min per wash) of PBST, blots were incubated for 2-3 h in secondary (2^o) antibody (Table 2.15) diluted in 5% (w/v) skimmed milk in PBST. Blots were washed again in five changes (5-10 min per wash) of PBST and developed in BCIP / NBT alkaline phosphatase substrate reagent (Appendix C12), according to the manufacturers instructions. Images of developed blots were acquired via the BioRad Gel doc system.

1° Antibody/Dilution	Corresponding 2° antibody/dilutions
Horse convalescent serum / 1:1000	α -horse / 1:20,000
Pony 5788 Pre or Post infection sera / 1:1000	α -horse / 1:20,000
Pony 5726 Pre or Post infection sera / 1:1000	α -horse / 1:20,000
α LppC / 1 :1000	α -rabbit /1:30,000
α PPMA / 1 :1000	α -rabbit /1:30,000
α HPr / 1 :2000	α -rabbit /1:30,000

Table 2.15: Antibodies and dilutions for Western blotting (adapted from Sutcliffe *et al.*, 2000).

2.5.21 Isolation of cell free extract (CFE) using cracking buffer

A 1:50 dilution in 100 mL THB of the overnight starter culture was incubated at 37°C to mid-late log phase and the 1 mL of the culture in a microcentrifuge tube subsequently centrifuged in at 13000 x g for 2 min at room temperature. The pellet was resuspended in a solution {containing 40 μ L of 18.2 M Ω /cm H₂O and 10 uL of SDS-PAGE sample cracking buffer (Table 2.6)} and boiled (in a water bath) for 10 min. After boiling, the cell debris was spun down at 13000 x g at room temperature for 2 min. The resulting supernatant (cell free protein extract) was collected into clean microcentrifuge tubes. CFE was used for SDS-PAGE and / or Western blotting.

2.5.22 Growth and harvesting of Cells/Supernatant for protein extraction

A 1:50 dilution in 100 mL THB of the overnight starter culture was incubated at 37°C to mid-log phase and the culture subsequently centrifuged at 4000 x g for 15 min at 4°C. The supernatant was processed immediately or stored in -20°C for extraction of secreted proteins. The cell pellet was centrifuged briefly at 4000 x g for 40 sec at 4°C and all excess supernatant removed. Pellet was resuspend and washed in two changed of 10 mL

of cold, sterile PBS at 4000 x g for 15 min at 4°C and supernatant decanted. Gram staining (section 2.4.3) and Lancefield grouping (section 2.4.4) were done to confirm the identity of isolates in biomass. All excess supernatant was discarded and washed cell pellet processed immediately or stored at -20 °C pending protein extraction for 2D-E.

2.5.23 Extraction of Cell-Associated Protein Extracts from Cell Pellets for 2D-E

Cell pellet (from section 2.5.22) was resuspended in 100 µl of reconstituted (ready to use) lysis solution (Table 2.7) to a total volume of approximately 200 µl, and lysed by sonication on ice at an amplitude of 14 microns, three times, in 10 bursts (10 sec) at 10 sec intervals. The protein mixture was harvested by centrifugation at 4000 x g at 4°C for 45 min and the supernatant saved.

2.5.24 Isolation of Secreted Proteins from Culture Supernatants

Culture supernatants (sections 2.5.22) were either freshly prepared or if retrieved from the freezer, thawed to room temperature. To enable precipitation of proteins, 1.5 mL of 100% (w/v) trichloroacetic acid was added to 25 mL of supernatant in a clean tube and kept on ice for 10 min. The mixture was centrifuged at 4000 x g at 4°C for 15 min and the supernatant discarded. The protein pellet was washed three times in 500 µl of ice-cold acetone, by centrifuging each time for 15 min at 4000 x g, 4°C. The acetone supernatant was discarded after each wash. The protein pellet was washed three times by dissolving it in 500 µl of ice-cold 18.2 MΩ/cm H₂O and centrifuged for 15 min at 4000 x g, 4°C. 200 µl of reconstituted (ready to use) lysis solution (Table 2.7) was added to each protein pellet and sonicated once at 14 microns in 10 bursts (10 sec) for complete suspension. Concentration of protein in sample was determined by Bradford's

assay (section 2.5.17.4). Any sample that was not used immediately was stored at -80°C until required.

2.5.25 Decontamination/Precipitation of Protein Extract

To each of the protein suspension (sections 2.5.23 and 2.5.24) in microcentrifuge tube, at room temperature, 800 µl (4 X sample volume) of cold (-20°C) acetone was added. Each tube was vortexed briefly and incubated for 1.5 h at -20° and then centrifuged for 10 min at 13,000 x g on a bench microcentrifuge. The supernatant was decanted and pellets washed with 4 volumes of ice-cold 80% (v/v) acetone by centrifuging for 10 min at 13,000 x g. The supernatant was decanted, each tube centrifuged briefly and residual supernatant removed with a pipette. The pellet was air dried for approximately 5 min at room temperature, being careful not to over dry. Cell pellet was resuspended (by sonication at 14 microns in 10 bursts, 10 sec, once) in 350 µl ready to use rehydration buffer (Table 2.7) for downstream application. Sample was used immediately or stored at -80°C, and thawed to room temperature when required.

2.5.26 Two Dimensional Gel Electrophoresis (2D-E)

Proteomics analysis (method of Zhang *et al.*, 2007) was carried out to identify and characterize proteins from *S. equi* 4047 WT, ΔPrtM and complemented mutants. 2D-E was carried out until at least six replica gels were obtained for the cell associated and secreted protein extracts.

Ready to use protein samples in rehydration buffer (section 2.5.25) were applied onto Immobiline DryStrips (18 cm, pH 3-10 or pH 4–7, Amersham Bioscience, appendix B16) for 16 h at room temperature. Isoelectric focusing (IEF) was done on a Multiphor II Isoelectric Focussing system (Amersham Bioscience) with running steps of 500 V for 1 min, 500–3500 V for 1.5 h and 3500 V for 7 h at 20°C. The strips were subsequently

incubated in equilibration buffer with DTT (Table 2.7) for 15 min at room temperature, followed by a second incubation in equilibration buffer with IAA (Table 2.7) for another 15 min.

The second dimension was carried out in either a 2 plate electrophoresis tank [14% w/v SDS-polyacrylamide gel using the Protean II XL 2-D cell (Bio-Rad, Appendix B11a) with running conditions of 40 mA per gel for 4 h at 10°C] or a 12 plate electrophoresis tank [12% w/v SDS-polyacrylamide gel using the Protean Plus Dodeca cell (Bio-Rad, Appendix B11b) with running conditions of 5 mA per gel for 1 h, 10 mA for 1 h and 12 mA for 16 h at 15°C]. Each gel was then fixed in 200 mL of 2D gel fixing solution (Table 2.7) for at least 1 h and stained in 200 mL of colloidal Coomassie blue solution (Table 2.7) for 16 h. Gels were destained in two changes of 18.2 MΩ/cm H₂O for at least 30 min per change, and gel images captured using a Bio-Rad GS-710 image densitometer (Appendix B21). Differential expression of proteins was analysed using PDQuest™ v 8.0 (Bio-Rad) or Progenesis SameSpots softwares.

2.5.27 Analysis of 2D-E Gels Using PDQuest™ v 8.0 Software

Gels were analysed, protein spots were detected and quantitated as described by Zhang *et al.*, (2007). However a higher version of the PDQuest™ v 8.0 (Bio-Rad) software was used. The gel background was subtracted, after which the intensity of the image pixel within each protein spot was quantified. Normalization of gels was based on the summed density in each gel of a matchset.

The spots from all the gels were individually assigned a spot number by the PDQuest software. A reference pattern was generated and a master gel image (Figure 3.25) with spot numbers was generated for each matchset. Each gel in the matchset was matched to the reference gel, the master gel image (Zhang *et al.*, 2007). Selection of differentially

expressed proteins was done according to Wil-coxon two-sample test ($p < 0.05$), such that protein spots were more intense (or less intense) on at least six gels in a matchset (Houtman *et al.*, 2003).

The master image contained all the spots that were found in all the gels in a matchset; and was very useful for correct identification, excision (cutting or spot picking), and tagging/labeling of the spots.

2.5.28 Analysis of 2D-E Gels Using Progenesis SameSpots v 4.5 Software

The analysis of 2D gels in the later part of this research was carried out using a fourth generation analysis software, Progenesis SameSpots (Nonlinear Dynamics, 2012). The objective of such fourth generation analysis software is to reduce the subjectivity of the image analysis (Silva *et al.*, 2010). The alignment step of the workflows utilized in previous generations of analysis software entailed tedious manual validation steps requiring significant user intervention to ensure correct spot detection and matching between gel images (Silva *et al.*, 2010). In the SameSpots work flow, the alignment step is performed before spot detection and manual review steps in order to prevent the user from introducing biased alignments between spots that are located in similar (but not identical) positions (Silva *et al.*, 2010). This also facilitates simultaneous spot detection on all gel images in an experiment, so that the resulting spot boundaries are identical on all gel images in the experiment (Silva *et al.*, 2010).

Gel images were imported into the programme for the Progenesis SameSpots Software. A representative gel image was selected and all the other gel images were aligned to the representative reference gel image. Unreliable spots, like those that were too small to be picked or those in damaged areas were removed by filtering them and deleting spots

that matched set criteria. To enable comparison of protein expression, matchsets were generated by defining which gel images represented a particular experimental condition. This was achieved by using the between-subject design in the experimental design setup screen of the Progenesis SameSpots Software.

Interesting and significantly changing spots were detected, using Anova p-value ≤ 0.05 and maximum fold change ≥ 2 (Nonlinear Dynamics, 2012). Finally, a picking list was generated and the spots to be picked were tagged.

2.5.29 In-gel Protein digestion

Protein spots in approximately 1 mm x 1 mm gel slices were cut from each Colloidal Coomassie blue stained spot on the 2D-E gels or a band from SDS-PAGE gel. Each 1 mm x 1 mm gel slice was transferred into clean, labeled 0.5 mL siliconized microcentrifuge tube and trypsinized immediately or preserved by the addition of 500 μ L of 18.2 M Ω /cm H₂O and stored at 4°C pending trypsinization. If the gel slice was stored, the 18.2 M Ω /cm H₂O was discarded just before further processing.

Colloidal Coomassie blue stain was washed out of each gel slice by the addition of freshly prepared 100 μ L of 100 mM NH₄HCO₃ and 60 μ L of LC-MS grade acetonitrile (ACN, LC-MS grade) was added to each siliconized microcentrifuge. The tube was shaken (200 rpm) for 30 min at 25°C and the supernatant discarded. This wash step was repeated three times until the blue colour was gone from the gel slice. Each gel slice was dehydrated by the addition of 60 μ L of ACN (LC-MS grade). The tube was then incubated at room temperature for 5 min and the liquid discarded. This process was repeated again for complete dehydration, after which the gel slices were dried in a centrifugal evaporator (Appendix B12) for 15 min at 25°C. Each dehydrated gel slice

was then incubated in 10 μ l of 20 μ g/ mL Promega trypsin solution (Table 2.7) in a water bath at 25°C for 1 h. After this incubation, approximately 30 μ l of 40 mM NH_4HCO_3 was added to each tube to cover the gel and the digestion was incubated at 37°C overnight.

The reaction was stopped by the addition of 30 μ l of 50% (v/v) ACN + 5% (v/v) formic acid solution, and shaken (200 rpm) for 30 min at 25°C. The supernatant was recovered and saved because it contained digested peptides. The remaining proteins in the gel slice were recovered by adding 30 μ l of 83% (v/v) ACN + 0.2% (v/v) formic acid solution, and shaken (200 rpm) for 30 min at 25°C. The supernatant was saved and pooled with the previous extract in a microcentrifuge tube.

One or two pin holes were bored on the lid of each microcentrifuge tube containing the pooled extract. The extract was then freeze dried for 24-48 h using the Alpha 1-2 LD plus freeze drier (Appendix B23). The freeze-dried extracts were either processed immediately for mass spectrometric analysis or stored at -80° for a few days pending further processing.

2.5.30 HPLC and Mass Spectrometry

Ion trap mass spectrometry can be used to identify peptides in the sub-mol range (Schweiger-Hufnagel *et al.*, 2001). Its multiple fragmentation stages give data with excellent information, thus allowing for highly reliable protein identification even from mixtures or poorly separated proteins; thus making ion trap mass spectrometry one of the leading mass spectrometric techniques in proteomics (Schweiger-Hufnagel *et al.*, 2001).

7 μ l of LC-MS buffer A (Table 2.7) was added to each freeze dried peptide extract (from section 2.5.27) and content mixed carefully with a clean pipette tip. 7 μ l of each solubilized sample was pipetted into one well of a 384-well microarray plate (Appendix B26). The solubilized peptides were analyzed on spherical high capacity traps (HCTultra™, Bruker Daltonics, appendix B25) using electrospray ionization (ESI) ion trap mass spectrometer coupled to the high performance liquid chromatography (HPLC) machine (UltiMate 3000™ - Dionex, Appendix B24). The mass spectrometer was controlled by the software EsquireControl™ (Bruker Daltonics). The samples' identities were electronically stored using the HyStar 3.2™ (Bruker Daltonics) software (a valuable software to help coordinate experiments when a liquid chromatography system is coupled with mass spectrometer, Bruker Daltonics, 2010). HyStar 3.2™ (Bruker Daltonics) functions by controlling the chromatography system instruments, which interface in diverting the solvent flow into the spectrometers or intermediate collectors (Bruker Daltonics, 2010).

Mass spectrometry of samples was carried out in ESI positive ion mode. Automated MS–MS mode was activated so that the mass spectrometer automatically detected and fragmented molecular ions whenever the MS ion signal intensity exceeded the previously fixed level (Schweiger-Hufnagel *et al.*, 2001). All across the LC peak, MS and MS–MS were performed alternately until the ion intensity was below the set intensity threshold again (Schweiger-Hufnagel *et al.*, 2001). A summary of the capillary LC-MS/MS peptide separation parameters is shown in Table 2.16

LC/MS Parameter	Range
LC system	UltiMate 3000™ - Dionex, Appendix B24
Column	polystyrene- divinylbenzene (PS-DVB) Monolithic Column 200 µm I.D. x 50 mm (60°C)
column	PS-DVB Monolithic, 200 µm i.d. x 5 mm
Loading solvent	LC-MS buffer A (Table 2.7)
Elution solvents	LC-MS buffer A (Table 2.7) LC-MS Buffer B (Table 2.7) LC-MS Buffer C (50% [v/v] acetonitrile in 18.2 MΩ/cm - Appendix F3)
Gradient	0-35%(v/v) ACN in 9 min, parallel LC
Samples	freeze dried peptide extract solubilized in 7 µl of LC-MS buffer A (section 2.5.18)
Injection	4 µL sample
Flow rate	3.003 µL/min
MS	HCTultra™ (Bruker Daltonics, Appendix B25) - electrospray ionization (ESI) ion trap mass spectrometer.
ESI-MS/MS	Positive ion mode, mass range 50-3000 m/z, cycle time 0.12 min
Max ion trap accumulate time	200.00 ms
Average scan	4
Number of precursors	3
Nebulizer of ionization source	10.0 psi
Dry gas of ionisation source	5.0 L/min
Dry temperature of ionization source	300°C

Table 2.16 Summary of the capillary LC-MS/MS peptide separation parameters

2.5.31 MS Data Analysis

The EsquireControl™ (Bruker Daltonics) has inbuilt procedure for MS/MS precursor ion selection and fragmentation; and can extract only the relevant information from the samples (Schweiger-Hufnagel *et al.*, 2001). The processing of the acquired MS and MS/MS data is triggered automatically by a script which includes the following steps:

- The EsquireControl™ (Bruker Daltonics) software performed chromatogram integration, mass annotation and charge deconvolution, after which the

deconvoluted and the non-deconvoluted data were combined and subsequently exported (Schweiger-Hufnagel *et al.*, 2001)

- This data was then exported to a protein database search engine on a local MASCOT (Matrix Science, 2009) server and the search result viewed and interpreted (Matrix Science, 2009). The MASCOT search employed MS/MS ion searches where confidence that a *protein*, (as opposed to a peptide), had been identified correctly came largely from getting multiple matches to peptides from the same protein (Matrix Science, 2009). This entailed identifying proteins based on raw MS/MS data from one or more peptides. The MASCOT (Matrix Science, 2009) search parameters for protein identification are shown in Table 2.17. The matched peptide and protein sequences were viewed in the ‘Protein hits’ results window.

Parameter	Identifier
Type of search	MS/MS ion search
Database	NCBIInr and/or SwissProt
Enzyme	Trypsin
Peptide Mass tolerance	±1.2 Da for the parent ion
Fragment Mass tolerance	±0.5 Da for the fragment ions
Maximum missed cleavages	2
Fixed modification	Carboxymethyl (C)
Variable modification	Oxidation (M)
Peptide charges	2+ and 3+
Mass values	monoisotopic
Protein mass	unrestricted
Instrument type	Default
Protein ID's definition	by at least 2 peptides, peptide ion score ≥ 14. Individual ion scores >60 indicate identity or extensive homology (p<0.05).

Table 2.17 Mascot search parameters for protein identification

Further evaluation of the proteins identified by Mass spectrometry was carried out using the criteria listed below:

- spot number
- protein identification - best match hit
- protein code for best match hit (i.e. the code SEQ_#### or Sez_####)
- comment as to whether expression is same in both strains, upregulated in wild type 4047 (= down regulated in mutant) or upregulated in mutant (= downregulated in wild type).

2.6 Overexpression and Purification of Recombinant Proteins

2.6.1 Small Scale Protein expression

To confirm protein expression and levels of expression, 5 mL LB broth (containing the appropriate antibiotics) in 25 mL universal was inoculated with a single bacterial colony and incubated at 37°C overnight, shaking at 200 rpm. Next day, the overnight starter culture was diluted 1:100 into two 50 mL LB broth (in 250 mL conical flask) containing appropriate antibiotics and incubated at 37°C, shaking at 200 rpm until OD_{600 nm} between 0.6 – 1. After incubation, 0.5 mL of 24 mg/mL IPTG was added to each flask. Both flasks were incubated overnight (one flask at 20°C and the other at 30°C) shaking at 200 rpm. Cells were harvested next day by centrifuging each culture at 4°C for 10 min at 4000 x g. Pellets were re-suspended in 5 mL START buffer (Table 2.6) and sonicated (at an amplitude of 14 microns, for a total of 1 min, with 10 sec interval between each 10 sec sonication) in 25 mL universal on ice. Samples were then centrifuged at 24,000 x g at 4°C for 20-30 min, the cell free extract (CFE) was harvested

and maintained on ice while the pellet was discarded. SDS-PAGE (section 2.5.15) of CFE was done to determine protein expression and levels.

2.6.2 Large scale protein expression

Two 5 mL LB broth (containing the appropriate antibiotics) cultures (in 25 mL universals) were each inoculated with a single bacterial colony and incubated at 37°C overnight, shaking at 200 rpm. Next day, the overnight starter culture was diluted 1:100 into 100 mL LB broth (in 1 L conical flask) containing appropriate antibiotics and incubated at 37°C, shaking at 200 rpm until $OD_{600\text{ nm}}$ between 0.6 – 1. Subsequently, 10 mL of 24 mg/mL IPTG was added to the flask which was incubated overnight at 30°C (optimum temperature of protein expression from section 2.6.1) shaking at 200 rpm. Next day, cells were harvested from 100 mL culture in four 500 mL plastic bottles which were centrifuged at 4°C for 10min at 4000 xg. Pellets were re-suspended in 5 mL Start buffer (Table 2.6) and sonicated (at an amplitude of 14 microns, for a total of 1 min, with 10 sec interval between each 10 sec sonication) in 25 mL universal on ice. Samples were then centrifuged at 24,000 x g at 4°C for 20-30 min, the CFE was harvested and maintained on ice while the pellet was discarded.

2.6.3 His-tagged Protein Purification

Recombinant proteins, under native conditions, containing a stretch of 6 histidine residues at the N-terminus (His₆ tag) were purified using immobilized metal affinity chromatography (IMAC) using a fast flow Ni column with Sepharose chelating resin. For high yield purification of recombinant protein, the automated FPLC system gradient elution technique was employed. The column was prepared by adding ~50 mL Sepharose chelating fast flow resin to a C series column (Pharmacia Biotech). The

column adapters were attached securely after which the column was connected to the FPLC system. 1 M NiSO₄ solution was passed through the resin until all the resin was a uniform colour. The column was subsequently washed by passing through ~ 200 mL of start buffer (Table 2.6). Once the column had been equilibrated, CFE (10-20 mL) was loaded onto the column, which was then washed with 100 mL of start buffer (Table 2.6) at a flow rate of 5 mL/min generated by the FPLC gradient pump. Over the next 50 min, the protein was eluted at a flow rate of 5 mL/min using a linear gradient of imidazole (elution buffer, Table 2.6) extending from 10 mM to 500 mM. An inline UV spectrophotometer connected to the FPLC system was used to determine the presence of the target protein by monitoring A_{280 nm}. Samples were collected in 5 mL volumes on a fraction collector.

To determine solubility of protein, two 1 mL aliquot from one tube (believed to contain protein fraction) were each mixed with 0.5 g of (NH₄)₂SO₄ and left to stand on ice for 1 h. Precipitate was resuspended in start buffer (Table 2.6) and 18.2 MΩ/cm H₂O.

To confirm purity, a 20 µL aliquot from each 5 mL fraction (in tubes thought to contain the target protein) was analysed by SDS-PAGE (section section 2.5.15) and the pure fractions pooled.

2.6.4 Dialysis

Dialysis of pooled full length recombinant protein fractions (section 2.6.3) was carried out using cellulose membrane tubing (M.W. 12,400, size 25 mm x 16 mm, Sigma) secured with dialysis tubing closure (gripping length 50 mm, Sigma). The dialysis tubing (always handled with gloves and cut into a length to accommodate solution to be dialysed) was boiled for about 15 min at 80°C in 18.2 MΩ/cm H₂O. The boiled dialysis

tube was then rinsed in 18.2 MΩ/cm H₂O and used immediately or stored for a maximum of one week in 18.2 MΩ/cm H₂O at 4°C. Recombinant protein (section 2.6.3) was dialysed at 4°C against 25 mM HEPES buffer (pH 7.4) for 1h, then in a second change of 25mM HEPES buffer (pH 7.4) for 6 h and finally in a third change of 25 mM HEPES buffer (pH 7.4) overnight. The dialysed recombinant protein was aliquoted in 1 mL volumes into microcentrifuge tubes (with pin holes on the cap). The concentration in one 1 mL microcentrifuge tube was determined by Bradford's assay (section 2.5.17). The remaining aliquots were frozen at -20°C and freeze dried for 1-2 days. The lyophilized recombinant protein was stored at -20°C and reconstituted in buffer or 18.2 MΩ/cm H₂O for assay. SDS-PAGE (section 2.5.18) was carried out using 18.2 MΩ/cm H₂O reconstituted lyophilized recombinant protein, to ascertain the integrity of the dialysed sample.

2.6.5 Concentration of IMAC purified proteins

Pooled recombinant protein (full length or central domain) fractions (section 2.6.3) were concentrated at 4°C, by centrifugation at 4000 x g in 10 kDa (full length recombinant protein) or 5 kDa (central domain recombinant protein) 6 mL cut-off concentrator units (Viva Science). After concentrating all the fractions containing the recombinant protein to about 200 μL, recombinant protein was further washed (desalted) three times in 5 mL of sterile 18.2 MΩ/cm H₂O. Each wash was done by centrifugation at 4,000 x g at 4°C, until about 200 μL of protein solution was left in the molecular weight concentrator tube. Purified and concentrated recombinant protein concentration was determined spectrophotometrically (section 2.5.17).

2.7 Crystallisation of Recombinant Protein

2.7.1 Manual hanging drop method

Crystals were grown by the vapour diffusion method. Crystallisation trays were set up in 24-multi well plates (Falcon ® Multiwell™ 24 well Becton Dickinson) into which 0.5 mL of crystallisation buffer (Appendix G1-7) was aliquoted into each well. Around the rim of each well was then greased with vacuum grease dispensed from a syringe. Silanised Cover slips (molecular dimension) were polished with a silk scarf prior to the addition of protein drops. 1 µl of protein (section 2.6.3 and 2.6.5) was added to 1 µl of crystallisation buffer (removed from the well) on a cover slip to form a 2 µl drop. The cover slip was inverted and sealed above the appropriate well, after which the plates were incubated at 22°C. This method was repeated for all crystallisation buffers (Appendix J1-7).

2.7.2 Microbatch - Automated 96-well plate - hanging drop method

Crystallisation trays (hanging drop method) were also set up using the microbatch method at York Structural Biology Laboratory (YSBL). The Hydra96 (Ribbins Scientific) dispensed the screens onto each well of the 96-well plate, after which the hanging drops were set up with Mosquito^R crystal screen robot (TTP LabTech). The screens set up by this method were PEG ion, Newcastle screen and Hampton screen 1 and 2 (Appendix J).

2.7.3 Mounting Crystals and Cryoprotectant Optimization conditions

The conditions in which crystals grew were then optimised to obtain crystals of good quality. This was achieved by varying the concentrations of buffer, salt and precipitant.

Once native crystals had grown, the same conditions were then used to set up crystallization trays of proteins from the central domain of the enzyme.

Samples were frozen in rayon-fibre loop and subjected to X-ray analysis. The point at which ice rings were not seen in the diffraction pattern indicated a suitable additive concentration to act as a cryoprotectant. Crystals were harvested in a rayon fibre loop, soaked briefly (few sec) in a given concentration of cryoprotectant (which was in the original screen) and flash frozen in liquid nitrogen.

2.7.4 Crystal screening

Each crystal was mounted onto a single-axis goniometer and screened at YSBL by X-ray (by Dr Edward Taylor) to determine diffraction quality before data collection. Crystal data was collected using a 345 mm Mar Research image-plate detector on a Rigaku rotating anode RU-200 X-ray generator, with a Cu target operating at 50 kV (100 mA) and focusing X-ray optics (MSC). The quality of a crystal was judged by its diffraction pattern.

2.8 Enzymology

Enzyme assays were carried out in triplicate. For kinetic experiments, the same aliquot of purified recombinant protein (method from section 2.6.3 and 2.6.5) was used. The concentration of the recombinant protein (full or central domain, prepared from sections 2.6.3 and 2.6.5) was determined spectrophotometrically (section 2.5.18). The standard protease-coupled PPIase assay employed to assay the activity of recombinant *S. equi* 4047 PrtM towards peptide substrates (Pep1, Pep2 and Pep3, Table 2.8) with a consensus sequence Suc-Ala-X-Pro-Phe-pNA (X = various amino acids that could precede the crucial proline residue and pNa = paranitroaniline) was a modification of the methods of Fischer *et al.* (1984), Kofron *et al.* (1991), Hani *et al.* (1999) and Alonzo *et al.* (2011).

2.8.1 Protease-coupled Peptidylprolyl Isomerase (PPIase) Assay

The PPIase assay was performed by mixing 10 μ L of recombinant protein (40-60 μ L recombinant protein diluted in 20 mM HEPES / 140 mM NaCl / 10% glycerol), cyclophilin (positive control, Table 2.8) or a solution of 20 mM HEPES / 140 mM NaCl / 10% glycerol (negative control) with 480 μ L of buffer (20 mM HEPES / 140 mM NaCl / 1 mM DTT, pH 7.4 or 8.0) in a microcentrifuge tube and allowing the recombinant protein to equilibrate on ice for 5 min. An empty cuvette was placed in the spectrophotometer (helios- α , Appendix B3) which was then zeroed at 390 nm. 10 μ L of ice-cold chymotrypsin (Table 2.8) was pipetted into the empty cuvette in the spectrophotometer. The 490 μ L mixture (10 μ L of recombinant protein, positive control or negative control plus 480 μ L of buffer) from the microcentrifuge tube was quickly added to and mixed with the chymotrypsin in cuvette in the spectrophotometer. Finally

and quickly, 500 μL of ice-cold buffer/tetrapeptide [tetrapeptide substrate (Table 2.8) diluted up to a final concentration of 25-70 μM in buffer (20 mM HEPES / 140 mM NaCl / 1 mM DTT, pH 7.4 or 8.0] solution was added to content in cuvette and mixed quickly by pipetting up and down 3 times. The final reaction volume was 1 mL and the final concentration of chymotrypsin in the reaction mixture was 0.2 mL/mL. The rate of the reaction (*cis-trans* isomerization) was measured by following the absorbance at 390 nm ($A_{390\text{ nm}}$), following colour formation (following cleavage of *pNa* from the trans form of the tetrapeptide substrate by protease, chymotrypsin), over time, for a maximum of 6 min. Spectrophotometric (helios- α , Appendix B3) readings were recorded automatically via the Vision 32 software (version 1.25 from Unicam Ltd) in attached computer system. Reported kinetic data are given as the mean value of triplicate measurements for every condition.

2.8.2 Phenylmethanesulfonyl flouride (PMSF) assay

10 μL purified full length (60 mg/mL) or central domain (40 mg/mL) protein was incubated with chymotrypsin (10 μL of 20 mg/mL) in 880 μL assay buffer (20 mM HEPES / 140 mM NaCl / 1 mM DTT, pH 7.4) for 20 sec, 2 min and 5 min at 0°C. The reaction was stopped by the addition of 100 μL of 10 mM PMSF and subsequent incubation for 5 min at 0°C. 10 μL purified full length (60 mg/mL) or central domain (40 mg/mL) protein incubated with PMSF-inactivated chymotrypsin (10 μL of 20 mg/mL), chymotrypsin with PMSF alone, chymotrypsin alone, recombinant protein with PMSF alone, and recombinant protein alone served as controls. The reactions were analyzed on a 15% (w/v) polyacrylamide gel (Table 2) and by SDS-PAGE (section 2.5.18).

2.8.3 Enzyme Kinetics

In order to determine the Steady-State Kinetic Parameters for PrtM, recombinant proteins were assayed at varied substrate concentrations as indicated in section 2.8.1.

k_{cat} ($/s^{-1}$) was calculated by using Beer-Lambert law: $A = \epsilon C d$

$d = \text{pathlength} = 1 \text{ cm}$

$C = \Delta A / \epsilon$

$C = \Delta A_{390 \text{ nm}} / \epsilon$

Where:

- $\Delta A_{390 \text{ nm}}$ = difference in absorbance (between catalyzed and uncatalysed reaction) per sec
- ϵ = extinction coefficient of para-nitroaniline (8270 / M / cm)
- C = moles of product (pNA) per sec, per L, per μg enzyme used
- $C / 1000 \times 10^6 / (\text{total enzyme added} - \text{mg}) = V_{\text{max}} \text{ U}/\mu\text{g}$
- $U = \mu\text{moles of product produced per min}$
- $V_{\text{max}} \times \text{MW of enzyme in grams} = k_{\text{cat}}$

The reaction volume was 1000 μL , therefore C was divided by 1000 to give moles of product per min per 1000 μL . This value was then multiplied by 10^6 to be converted into $\mu\text{moles of product produced per min per 1000 } \mu\text{L}$ and this was defined as U . U was then divided by the total amount of enzyme added to the assay (μg). This gave the value for V_{max} which is units of enzyme activity per sec per μg . V_{max} was then converted from $\mu\text{moles of product produced per sec per } \mu\text{g}$, to $\mu\text{moles of product produced per sec per } \mu\text{moles}$ by multiplying with the molecular weight of enzyme in μg to give the value for k_{cat} .

Absolute kinetic analysis was determined by assaying (2.8.1) 3-4 substrate concentrations (determined empirically) in triplicate. To ascertain if these data reflected true Michaelis-Menten kinetics, a Line-weaver-Burk plot was constructed and used to determine value of K_M (calculated by reciprocalising the X intercept in the Line-weaver-Burk plot).

Specificity constant ($/ M / s$) was determined by dividing k_{cat} by K_M .

3 Results of General Characterisation of *S. equi* 4047 WT and Δ PrtM

3.1 Introduction

One of the aims of this research was to characterize PrtM by evaluating its immunogenicity and conservation in *S. equi* 4047 WT and Δ PrtM. To achieve this, agar slant cultures [of *S. equi* 4047 WT which was originally isolated in 1990 from a submandibular abscess of a New Forest pony; and Δ PrtM (PrtM-deficient mutant strain - Δ *prtM*₁₃₈₋₂₁₃, with a deletion of nucleotides 138 to 213)] maintained in the culture collection of the Animal Health Trust, Newmarket, United Kingdom were evaluated.

The identities of the isolates (*S. equi* 4047 WT and Δ PrtM) were confirmed by carrying out initial screening: growth on THA plates (method in section 2.4.1 and result in section 3.2) Gram staining (method in section 2.4.3 and result in section 3.2), Lancefield grouping (method in section 2.4.4; result in section 3.2), β -haemolysis tests (method in section 2.4.5; result in section 3.2), genomic DNA extraction (sections 2.5.2 and 2.5.5; result in Figure 3.2-A) and PCR (primers SequiPrtMf and SequiPrtMr shown in Table 2.11 and method in section 2.5.4; result in Figure 3.2-B). After these experiments, an evaluation of the growth pattern of both *S. equi* 4047 WT and Δ PrtM strains via growth curve experiments (method in section 2.4.6; results in section 3.3) were carried out.

The strategic anchorage of lipoproteins to the outer leaflet of the membranes of Gram-positive bacteria suggests a role in interaction with the environment (Reffuveille *et al.*, 2012a). Among other important roles, lipoproteins are thought to play important roles in bacterial adaptive response to host environmental changes (Reffuveille *et al.*, 2012a).

With the initial results of this research in hand, it was therefore proper to evaluate the sensitivity of *S. equi* 4047 WT and Δ PrtM to some antibiotics (method in section 2.4.8; results in section 3.6) as well as to varying concentrations of NaCl (method in section 2.4.7; results in section 3.5); so as to find out how the two strains cope under environmental variation.

S. equi hyaluronic acid capsule may be involved in the pathogenesis of the organism (Anzai *et al.*, 1999; Harrington *et al.*, 2002; Timoney *et al.*, 2004). This information, coupled with the observation that it was more difficult to get the mid-log phase cells of the wild-type (*S. equi* 4047 WT) to pellet (at 4000 x g for 15 min) in 0.5 M sucrose, compared to the mutant strain (*S. equi* 4047 Δ PrtM), led to a series of investigations. Mid to late log phase cells were employed for hyaluronic acid screening because streptococcal hyaluronidase is known to destroy the hyaluronic acid capsule in later stages of growth (DeAngelis and Weigel, 1994). In the investigation of the hyaluronic acid capsule content of the wild-type and mutant strains in this study, the mid-log phase cells of both strains were stained by different methods (sections 2.4.12, 2.4.13, 2.4.14); followed by density gradient centrifugation (section 2.4.10). When qualitative tests (results in sections 3.7 and 3.8) showed that the mutant is encapsulated just like the wild-type, a quantitative assay (method in section 2.4.15 and results in section 3.9) was carried out to ascertain how much hyaluronic acid is produced by each strain.

Cell free extracts (CFE) obtained from cells (using cracking buffer - method in section 2.5.21) of *S. equi* 4047 WT and Δ PrtM were analysed by SDS-PAGE (method in section 2.5.19) and Western blots (methods in section 2.5.20; results in section 3.10) were used to compare the immunogenicity of both strains. In order to find out the differences and similarities between the proteomes of both strains, the proteomics

methods (sections 2.5.22 – 2.5.29) of Zhang *et al.* (2007) was carried used (results are shown in sections 3.12 – 3.14). However, before two-dimensional gel electrophoresis (2D-E), Western blots (method in section 2.5.20) of the cell and supernatant protein extracts (from sections 2.5.25) were double checked to confirm protein origin and immunogenicity –results are shown in section 3.11.

3.2 Results of Colonial morphology, Gram Staining, Lancefield grouping and β -haemolysis tests

Both *S. equi* 4047 WT and Δ PrtM (mutant) strains showed good growth of mucoid colonies on THB, with the WT showing slightly larger colonies than the mutant. Gram stained smear of all cultures and biomass (of *S. equi*) used for this research yielded Gram-positive cocci in chains. Lancefield grouping confirmed the identities of both the WT and mutant as being Lancefield group C Streptococci.

Figures 3.1B and 3.1D show the results of investigation of β -haemolysis in *S. equi* 4047 WT and Δ PrtM on 5% horse blood agar (right). These are representative of triplicate repeats. There was no apparent difference in the zones of β -haemolysis of both strains. Apart from β -haemolysis, the colonies of both strains also appeared mucoid on blood agar.

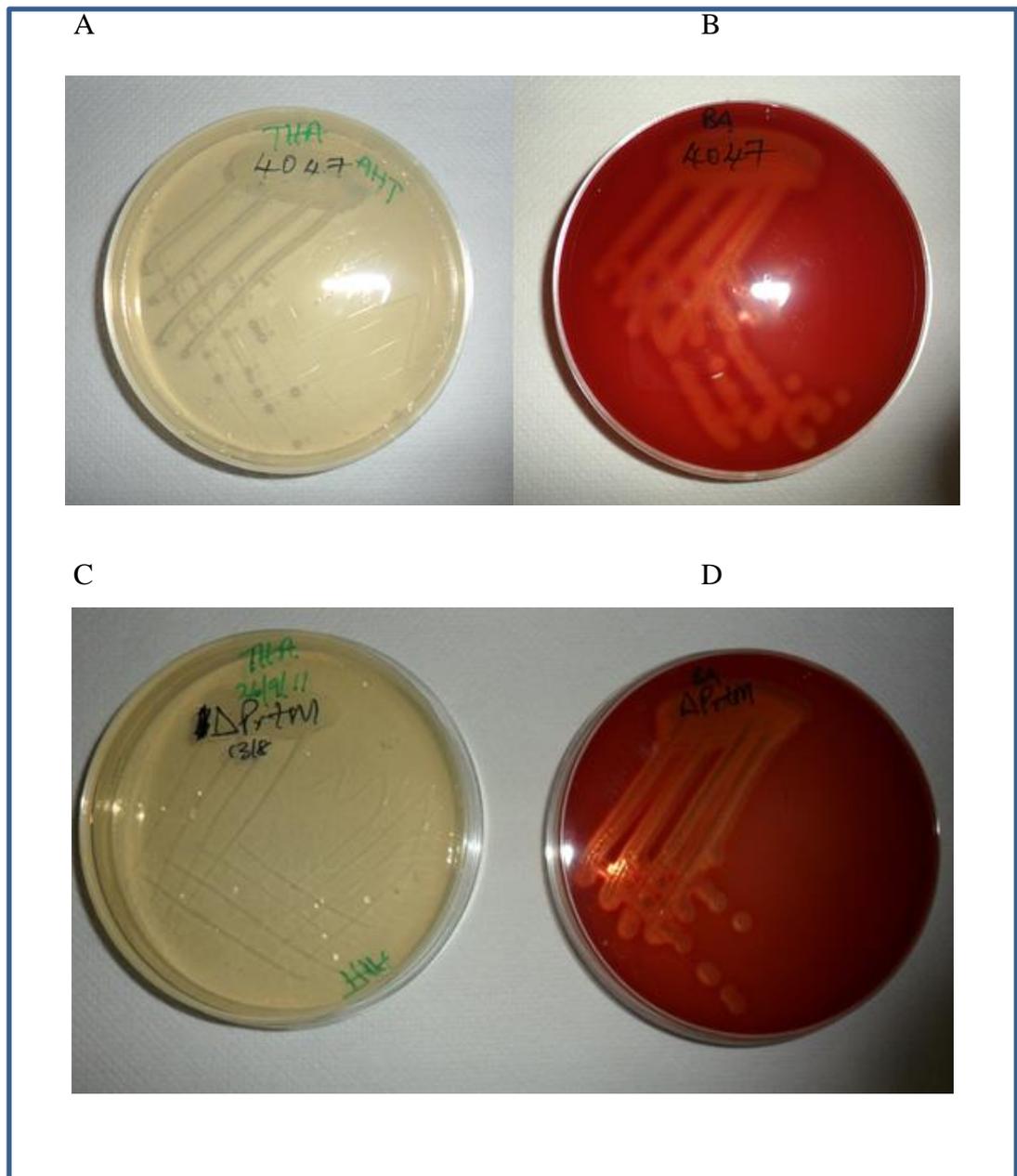


Figure 3.1: Result of Investigation of β -haemolysis in *S. equi* 4047 WT and Δ PrtM in 5% (v/v) horse blood agar (B=WT and D= Δ PrtM). TSA culture of *S. equi* 4047 WT (A) and Δ PrtM (C) show growth/viability of organism.

3.3 *S. equi* 4047 WT and Δ PrtM DNA Banding Patterns before and after PCR

S. equi 4047 WT and Δ PrtM genomic DNA (Figure 3.2 - A) both have higher molecular weight than the largest (10,000 bp) Hyperladder™ (Appendix 8) band.

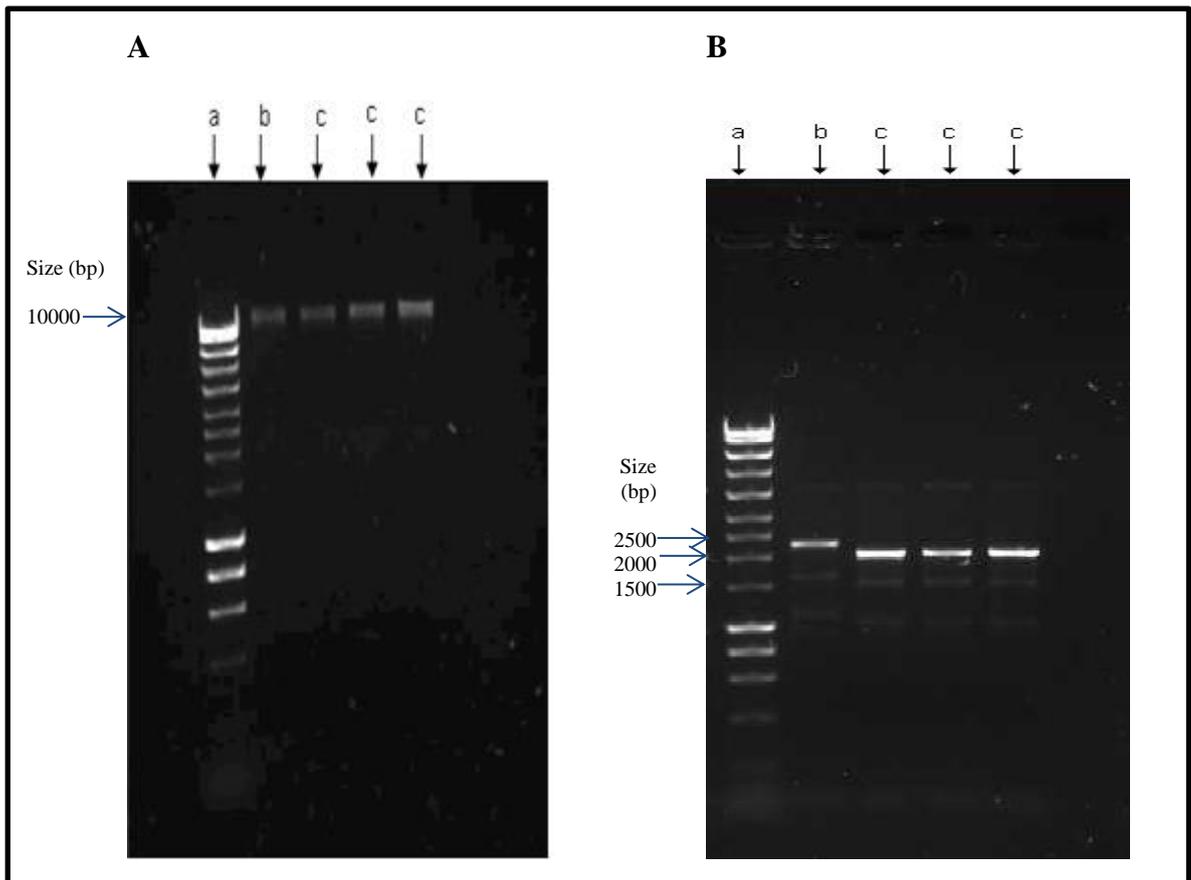


Figure 3.2: *S. equi* 4047 WT and ΔPrtM DNA Banding Pattern: Genomic DNA – Figure 3.2A. PCR of the PrtM gene locus shows that *S. equi* WT 4047 in band b of Figure 3.2B is larger than the PrtM gene locus on ΔPrtM in bands C of Figure 3.2B. Primers SequiPrtMf and SequiPrtMr shown in Table 2.11 and method in section 2.5.4. Post-PCR DNA - B. a = Hyperladder™ Standard (Appendix A8), b = *S. equi* 4047 WT and c = *S. equi* ΔPrtM.

From the results shown in Figure 3.2 (B), the amplified region of *S. equi* 4047 WT is larger than that of *S. equi* 4047 ΔPrtM. This result confirms that the mutant *S. equi* 4047 PrtM ($\Delta prtM_{138-213}$) used in this study has a deletion of nucleotides 138 to 213 as reported by Hamilton *et al.* (2006).

3.4 *S. equi* 4047 WT and ΔPrtM growth curves

The growth curves (Figure 3.3) of *S. equi* 4047 WT and ΔPrtM are shown in Figure 3.3.

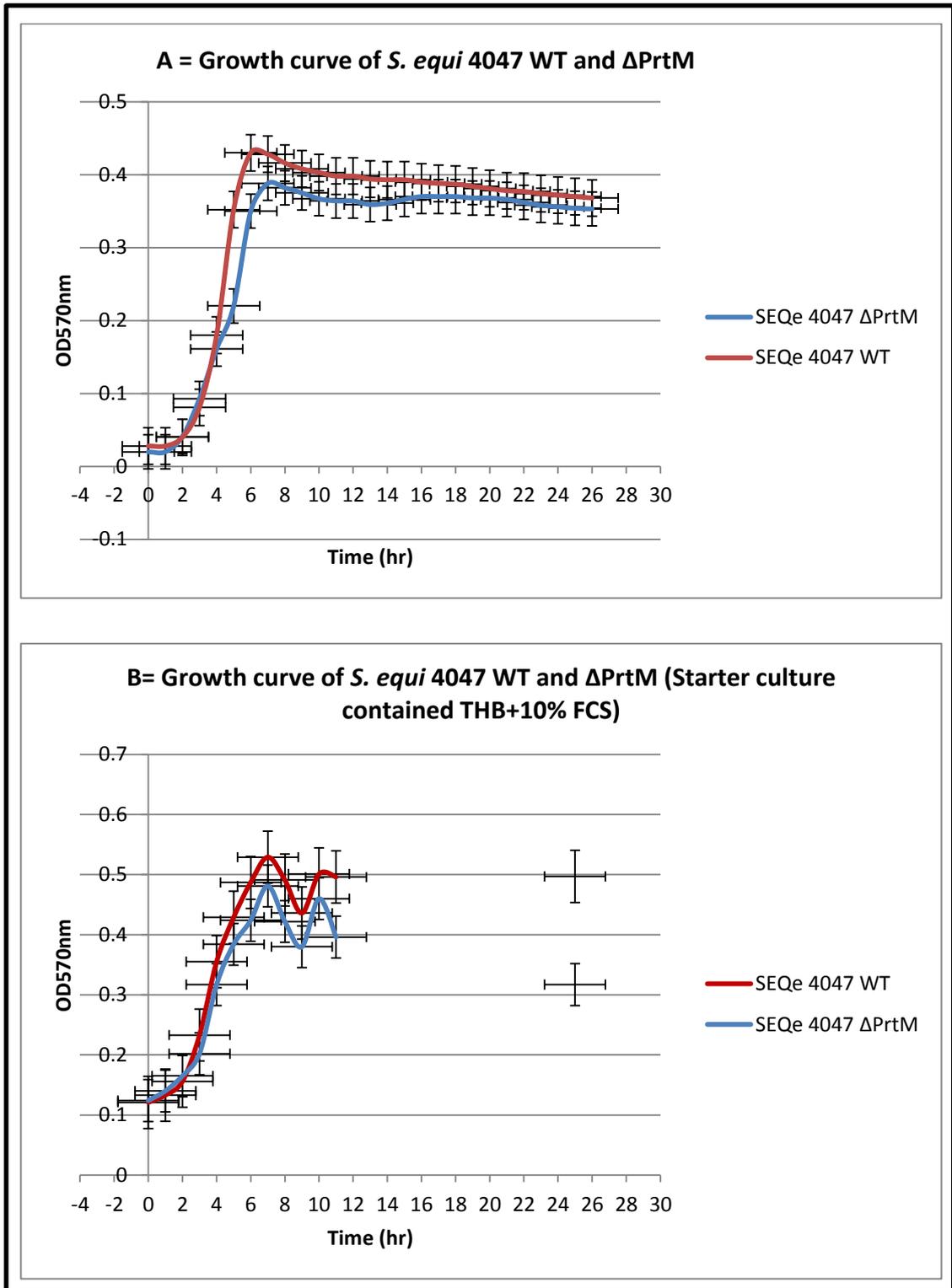


Figure 3.3 Growth Curve of *S. equi* 4047 WT and Mutant (Δ PrtM): A= in THB; B= in THB with starter culture containing THB+10% FCS. Actual cultures A and B did not contain any FCS. SEQe denotes *S. equi*

3.5 Sodium Chloride Stress Test

The averages of triplicate results of NaCl stress test (method in section 2.4.7) are shown in Table 3.1 below.

A						
<i>S. equi</i> 4047 Strain /Incubation time	H ₂ O “whole”	H ₂ O ½dilution -	H ₂ O 1/4dilution -	H ₂ O 1/10 dilution	H ₂ O 1/100 dilution	H ₂ O - 1/1000 dilution
WT / 24 h	Lawn	Lawn	Lawn	TNTC +++	TNTC +	75
ΔPrtM /24 h	TNTC +++	TNTC ++	TNTC+	TNTC	170	45
WT / 48 h	Lawn	TNTC ++	TNTC+	TNTC +	83	12
ΔPrtM /48 h	TNTC +	200	50	20	10	3
B						
<i>S. equi</i> 4047 Strain /Incubation time	0.9% (w/v) NaCl “whole”	0.9% (w/v) NaCl - ½ dilution	0.9% (w/v) NaCl - ¼ dilution	0.9% (w/v) NaCl 1/10 dilution	0.9% (w/v) NaCl 1/100 dilution	0.9% (w/v) NaCl 1/1000 dilution
WT / 24 h	Lawn	Lawn	Lawn	TNTC ++	TNTC +	110
ΔPrtM / 24 h	Lawn	TNTC +++	TNTC ++	TNTC +	250	74
WT / 48 h	Lawn	TNTC +++	TNTC ++	TNTC +	212	23
ΔPrtM / 48 h	TNTC +	160	60	38	19	9
C						
<i>S. equi</i> 4047 Strain /Incubation time	14.7% (w/v) NaCl “whole”	14.7% (w/v) NaCl - ½ dilution	14.7% (w/v) NaCl - ¼ dilution	14.7% (w/v) NaCl 1/10 dilution	14.7% (w/v) NaCl 1/100 dilution	14.7% (w/v) NaCl - 1/1000 dilution
WT / 24 h	TNTC +++	TNTC++	TNTC +	248	25	4
ΔPrtM / 24 h	NG	NG	NG	NG	NG	NG
WT / 48 h	218	125	80	10	3	1
ΔPrtM / 48 h	NG	NG	NG	NG	NG	NG
D						
<i>S. equi</i> 4047 Strain /Incubation time	29.4% (w/v) NaCl “whole”	29.4% (w/v) NaCl - ½ dilution	29.4% (w/v) NaCl - ¼ dilution	29.4% (w/v) NaCl - 1/10 dilution	29.4% (w/v) NaCl 1/100 dilution	29.4% (w/v) NaCl 1/1000 dilution
WT / 24 h	TNTC +	TNTC ±	TNTC +	103	4	1
ΔPrtM / 24 h	NG	NG	NG	NG	NG	NG
WT / 48 h	155	65	30	4	NG	NG
ΔPrtM / 48h	NG	NG	NG	NG	NG	NG

Table 3.1: NaCl Stress Test Result for *S. equi* 4047 WT and ΔPrtM. The average count of three replicate results is shown. Before plating on THA, that is after 24 h or 48 h incubation as described in section 2.4.7, the tubes containing sterile 18.2MΩ/cm H₂O (Table 3.1 A) were diluted with only 18.2MΩ/cm H₂O; the tubes containing NaCl (Tables 3.1 B, C and D) were diluted with the corresponding concentrations of NaCl solution. All broth cultures in tubes marked “whole” (second column of Table 3.1 A, B, C and D) were plated undiluted, onto THA, after 24h or 48h incubation. Serial dilutions were carried out to enable colony count. NG means no growth; TNTC means too numerous to count.

Due to the observation that only plates in the last column of Table 3.1 (A,B,C and D) showed consistency in countable colonies, their values were used to calculate percentage of total surviving organisms under NaCl stress over a 48h period. The percentage of surviving strains in 18.2MΩ/cm H₂O and varying concentrations of NaCl after 24 h and 48 h incubation are shown in Figure 3.4.

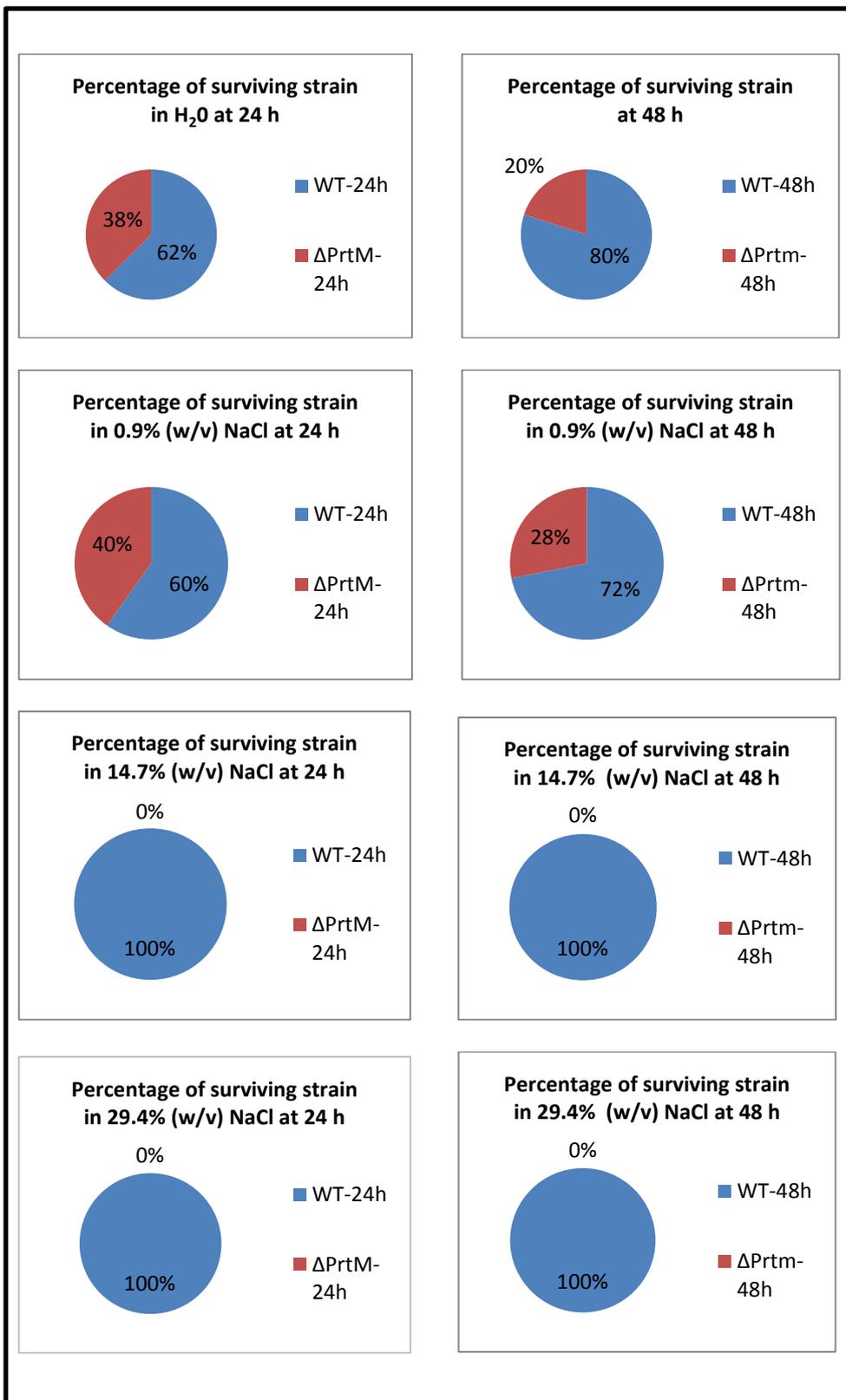


Figure 3.4: Percentage of surviving strain (*S. equi* 4047 WT and/or Δ PrtM) per NaCl stress test condition

3.6 Disc Diffusion Antibiotics Sensitivity Test

The sensitivity of *S. equi* 4047 WT and Δ PrtM to several antibiotics was assayed as described in section 2.4.8. The results are shown in Table 3.2, A and B. A comparison of antibiotic sensitivity and resistance levels of the WT and Δ PrtM strains of *S. equi* 4047 is shown in Figure 3.5.

A						
<i>S. equi</i> strain	Antibiotic concentration (μ g) / Zone of Inhibition (cm)					
WT 4047 - Triplicate Assays	PenicillinG (6 μ g)	Streptomycin (500 μ g)	Vancomycin (30 μ g)	Gentamycin (500 μ g)	Ampicillin (10 μ g)	Norfloxacin (5 μ g)
1	3.6 cm	1.9 cm	1.9 cm	2.2 cm	3.5 cm	1.2 cm
2	3.6 cm	1.9 cm	2.0 cm	2.4 cm	3.4 cm	1.2 cm
3	3.6 cm	2.2 cm	2.1 cm	2.2 cm	3.6 cm	1.1 cm
Average	3.6 cm	2.0 cm	2.0 cm	2.27 cm	3.5 cm	1.17 cm

B						
<i>S. equi</i> strain	Antibiotic concentration (μ g) / Zone of Inhibition (cm)					
Mutant (Δ PrtM) Triplicate Assays	PenicillinG (6 μ g)	Streptomycin (500 μ g)	Vancomycin (30 μ g)	Gentamycin (500 μ g)	Ampicillin (10 μ g)	Norfloxacin (5 μ g)
1	4.4 cm	3.2 cm	2.6 cm	3.4 cm	3.8 cm	1.8 cm
2	4.4 cm	3.0 cm	2.6 cm	3.2 cm	3.6 cm	2.0 cm
3	4.2 cm	2.6 cm	2.4 cm	3.0 cm	3.9 cm	1.8 cm
Average	4.33 cm	2.93 cm	2.53 cm	3.2 cm	3.77 cm	1.87 cm

Table 3.2: Antibiotic Sensitivity Pattern of *S. equi* 4047 WT (A) and Δ PrtM (B)

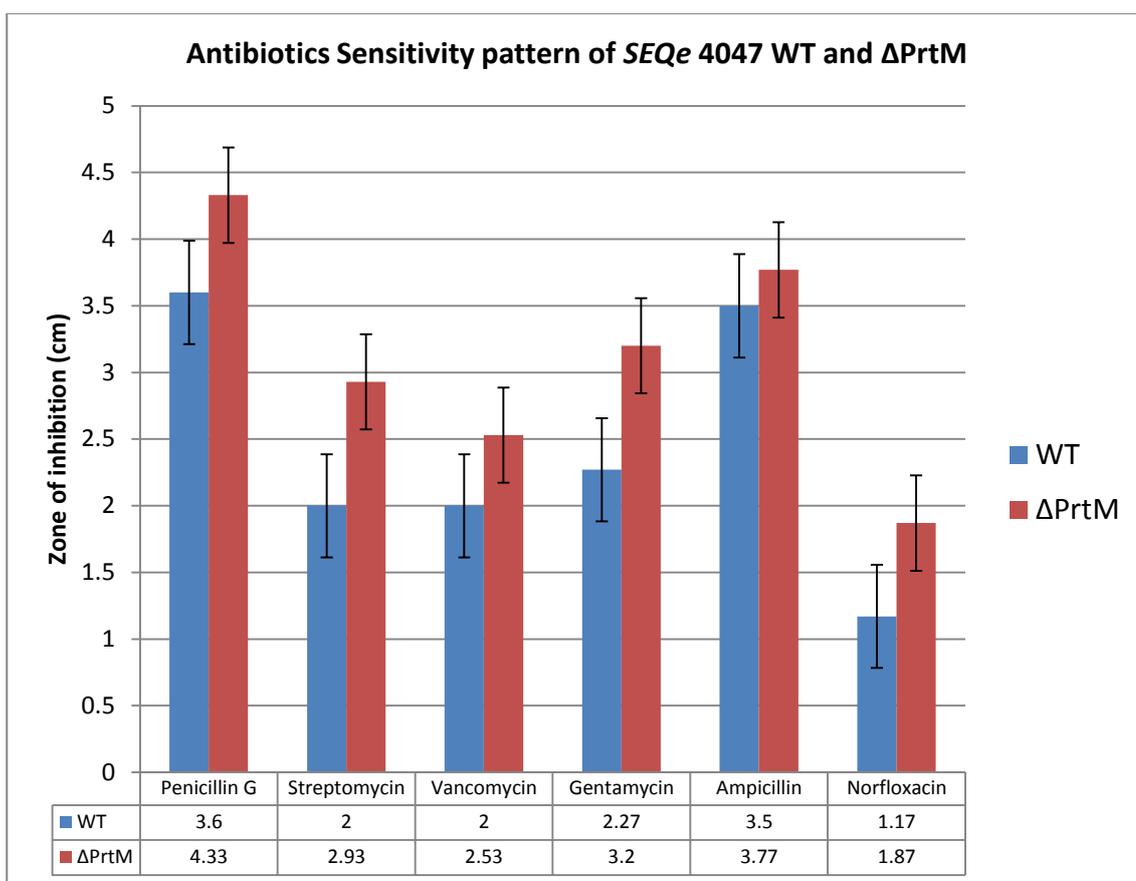


Figure 3.5 Comparison antibiotic sensitivity and resistance levels of the WT and ΔPrtM strains of *S. equi* 4047 – showing error bars with standard error. For all the antibiotics tested, areas of growth inhibition were significantly smaller (according to student T-test in appendix G) for the WT compared to the mutant. Mean values (on X-axis in cm) are shown.

3.7 Result of Hyaluronic Acid Capsule staining

An assessment of the hyaluronic acid capsule of *S. equi* 4047 WT and Δ PrtM was carried out by staining inoculums of each strain and analysing their colonial morphology as described in sections 2.4.11, 2.4.12, 2.4.13 and 2.4.14. The results are shown in Figure 3.6 below.

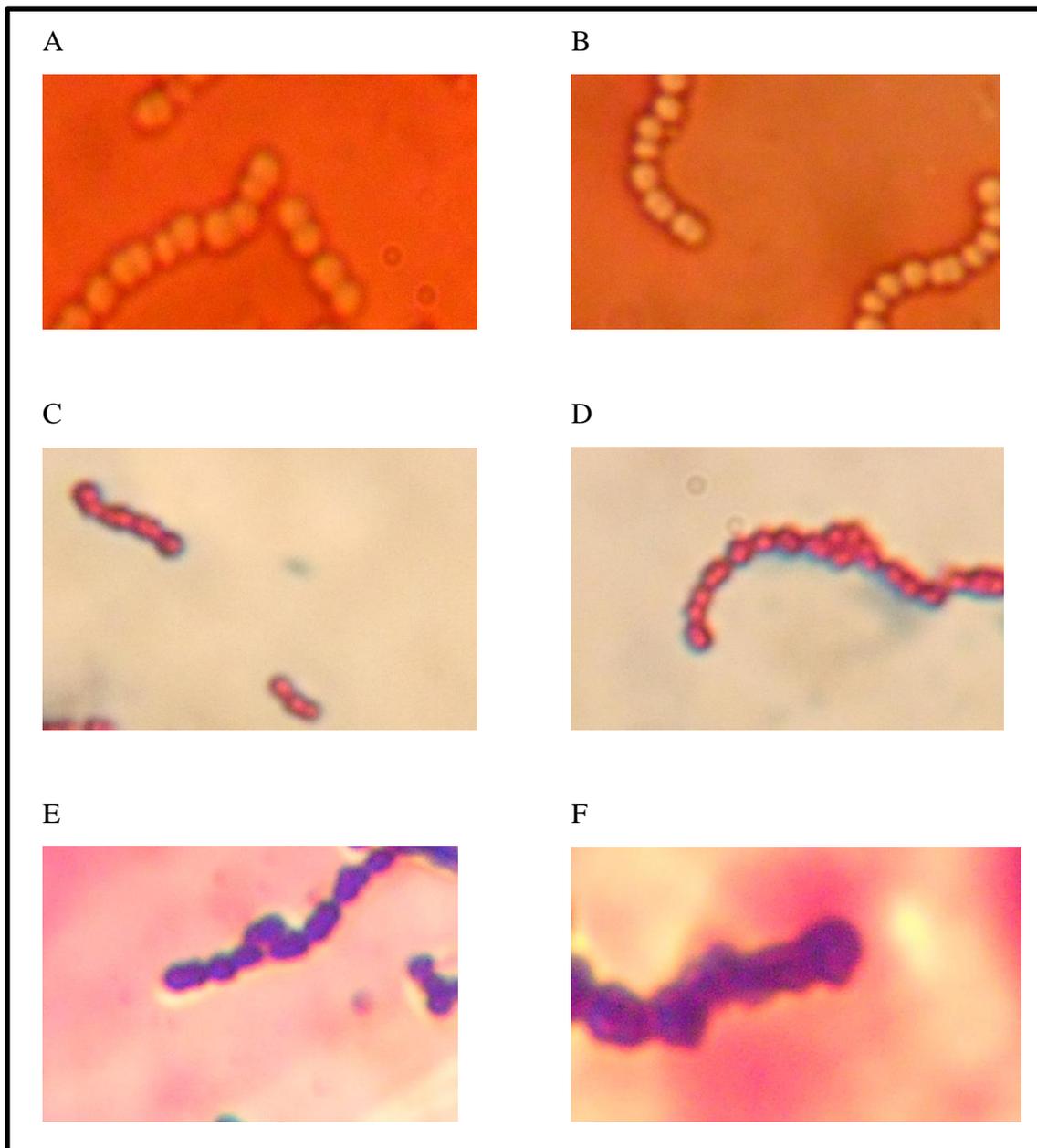


Figure 3.6: Images of capsular stained cells. The production of hyaluronic acid capsule by both strains is shown here by the results of capsular staining of *S. equi* isolates. A (*S. equi* 4047 WT) and B (*S. equi* Δ PrtM) = (safranin staining; method in section 2.4.12); C (*S. equi* 4047 WT) and D (*S. equi* 4047 Δ PrtM) = (Alcian blue staining; method in section 2.4.13); E (*S. equi* 4047 WT) and F (*S. equi* Δ PrtM) = (Crystal violet staining; method in section 2.4.14).

3.8 Hyaluronic acid capsules detected by Density Bouyancy Centrifugation

As a further confirmatory test for the presence of hyaluronic acid capsule in the mutant (*S. equi* Δ PrtM), density buoyancy centrifugation method (described in section 2.4.10) was used to evaluate both the wild type (*S. equi* WT) and mutant (*S. equi* Δ PrtM). The results of the assay are shown in Table 3.3.

<i>S. equi</i> 4047 strain	Hyaluronidase	Concentration of Percoll	Cells at Top interface	Cells at Lower interface
WT	-	50%	+	-
WT	+	50%	±	+
WT	-	65%	+	-
WT	+	65%	±	+
Δ PrtM	-	50%	+	-
Δ PrtM	+	50%	±	+
Δ PrtM	-	65%	+	-
Δ PrtM	+	65%	±	-

Table 3.3: Density buoyancy of *S. equi* 4047 WT and Δ PrtM. Hyaluronidase – (minus) means no hyaluronidase in culture media; hyaluronidase + (plus) means hyaluronidase was added to culture media. ± (plus/minus) means thin layer of a few cells at the interface. Method is described in section 2.4.10.

3.9 Concentrations of Hyaluronic Acid Produced by *S. equi* 4047 WT and Δ PrtM

The hyaluronic acid concentration per mL of culture was calculated as described in section 2.4.15. The results of triplicate assays are shown in Figure H1 (appendix H), while Figure 3.7 summarises the result in bar chart format.

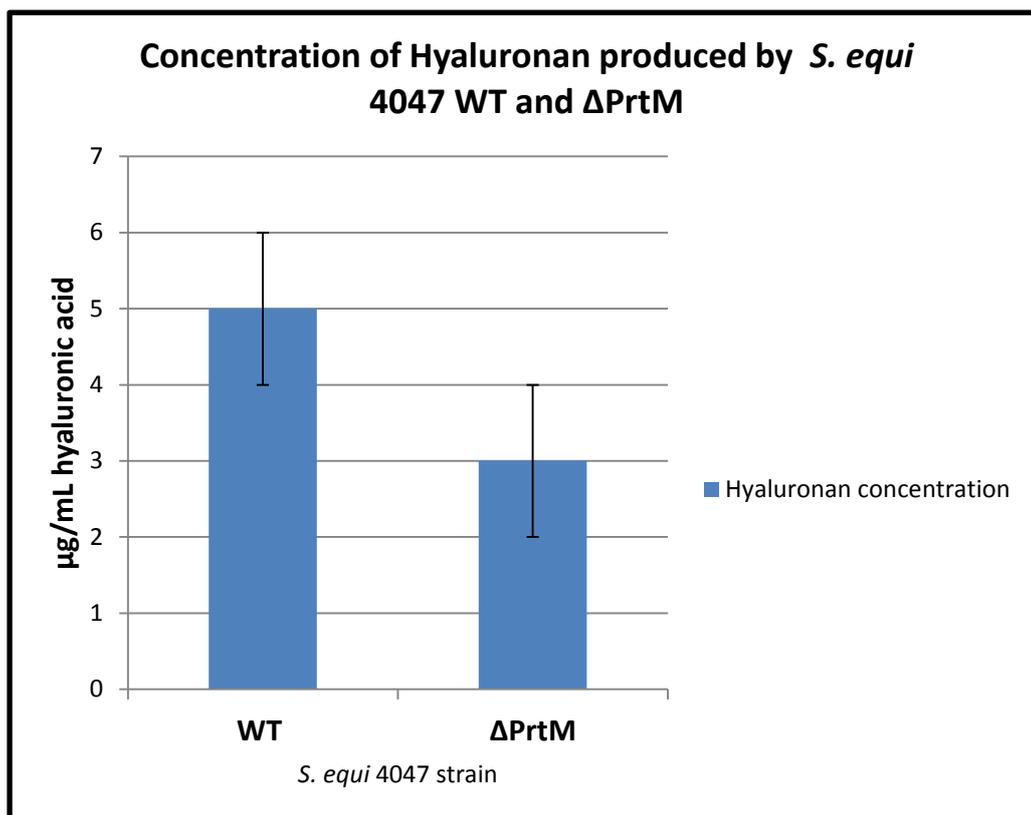


Figure 3.7: Hyaluronic acid production in *S. equi* 4047 WT and Δ PrtM. The WT strain produced 5 µg/mL and the mutant strain (Δ PrtM) produced 3 µg/mL hyaluronic acid in THB culture at an OD_{600nm} of 0.4.

3.10 Analysis of Cell Free Extract (CFE)

With knowledge of the growth pattern and general characteristics of the WT and mutant strains in hand (sections 3.1 to 3.9), and before Western blotting (method in section 2.5.20 and results in section 3.11), Coomassie blue R-250 (Table 2.6) was used to stain cell associated and secreted protein cell free extracts (method in section 2.5.21) on gel ran by SDS-PAGE (method in section 2.5.19). This enabled viewing and confirming the presence of extracted protein bands in gel before further assays were carried out. The result is shown in Figure 3.9, with protein bands in all four lanes (b, c, d and e).

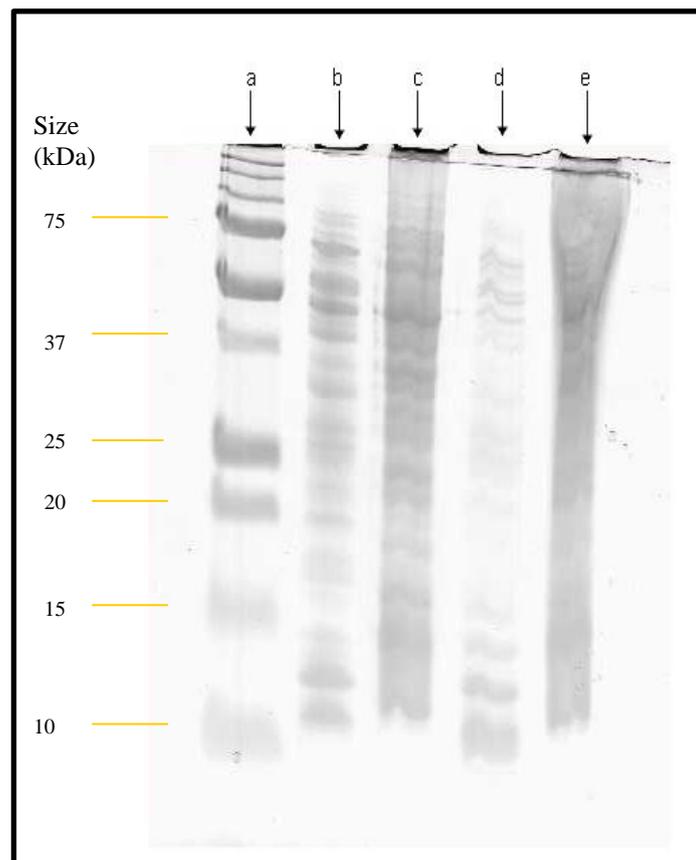


Figure 3.8: Protein Staining with Coomassie R-250. The presence of bands confirm the presence of protein in cell and secreted protein extracts (a) Protein Size Standard (appendix C8), (b): *S. equi* 4047 WT, (c) *S. equi* 4047 Δ PrtM, (d) *S. equi* 4047 WT, (e) *S. equi* 4047 Δ PrtM.

3.11 Results of Western Blots

Before cell associated (section 2.5.23) or secreted protein extracts (section 2.5.24) were used for 2D-E, Western blotting (method in section 2.5.20) was used to: double check and confirm the identities of both strains (*S. equi* 4047 WT and Δ PrtM); prove the presence of lipoproteins of *S. equi* origin, as well as evaluate immunogenicity of both strains.

Before proceeding to other steps of Western blotting, reversible Ponceau S staining (section 2.5.20) was carried out and used to analyse blots containing cell associated (section 2.5.23) and secreted protein extracts (section 2.5.24) of *S. equi* 4047 WT and Δ PrtM. This revealed and confirmed protein bands (Figure 3.9) from all extracts. Having confirmed the presence of protein bands on blots, Western blotting (section 2.5.20) was done using the following antibodies (Table 2.15): Horse Converlescent serum (results in Figure 3.10), α PPMA (result in Figure 3.11), α LppC (result in Figure 3.12), α HPr (result in Figure 3.13), as well as pre and post infection antisera from Ponies (results in Figure 3.14 and 3.15),

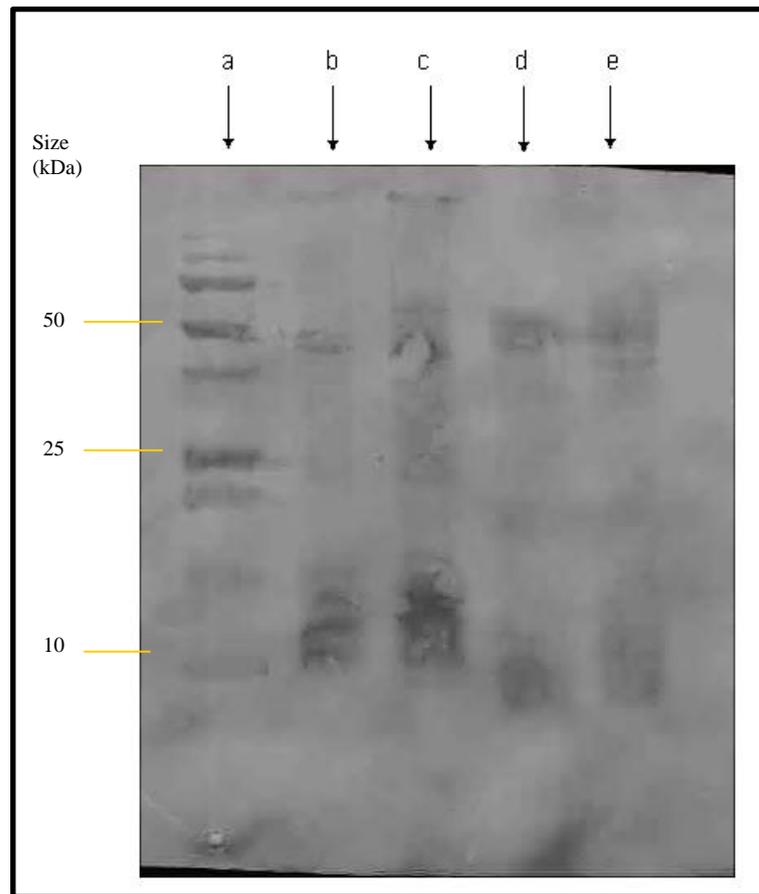


Figure 3.9: Reversible Staining with Ponceau S solution. The presence of protein bands on blots is confirmed here by the bands seen after reversible staining. (a) Protein Size Standard (Appendix C8), (b): Cell Associated Protein Extract of *S. equi* 4047 WT, (c) Cell Associated Protein Extract of *S. equi* 4047 Δ PrtM, (d) Secreted Protein Extract of *S. equi* 4047 WT, (e) Secreted Protein Extract of *S. equi* 4047 Δ PrtM

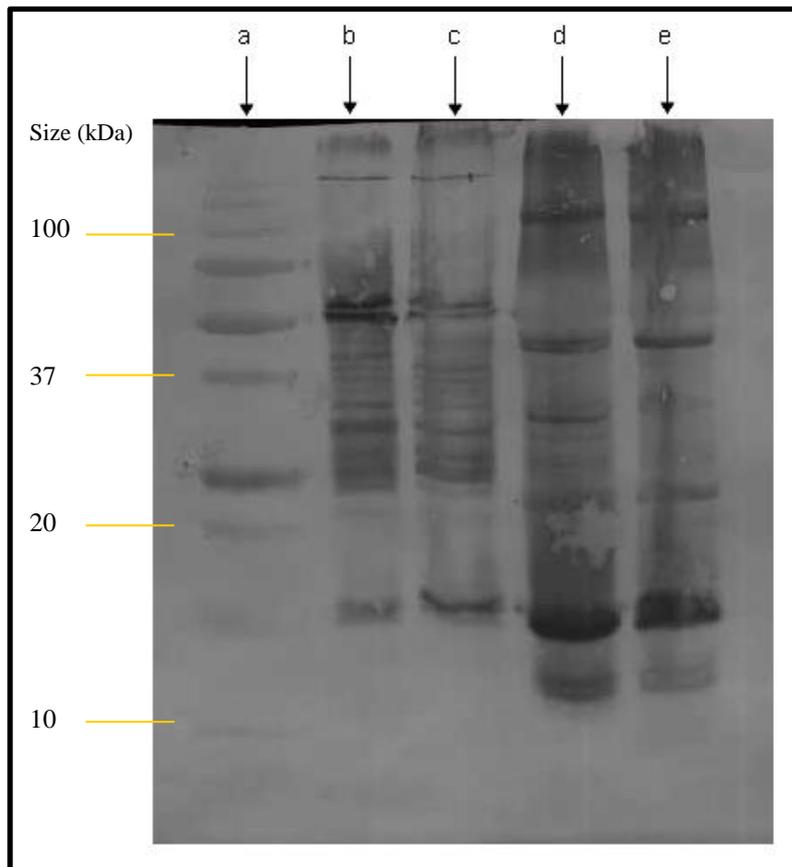


Figure 3.10: Western blot, Reactions of Cell-Associated and Secreted Protein Extracts from *S. equi* 4047 WT and *S. equi* 4047 Δ PrtM with Horse convalescent serum: (a) Protein Size Standard (appendix C8), (b): Cell Associated Protein Extract of *S. equi* 4047 WT, (c) Cell Associated Protein Extract of *S. equi* 4047 Δ PrtM (d) Secreted Protein Extract of *S. equi* 4047 WT, (e) Secreted Protein Extract of *S. equi* 4047 Δ PrtM. The Western blot results show that *S. equi* 4047 WT and Δ PrtM cross reacted with horse covalent serum.

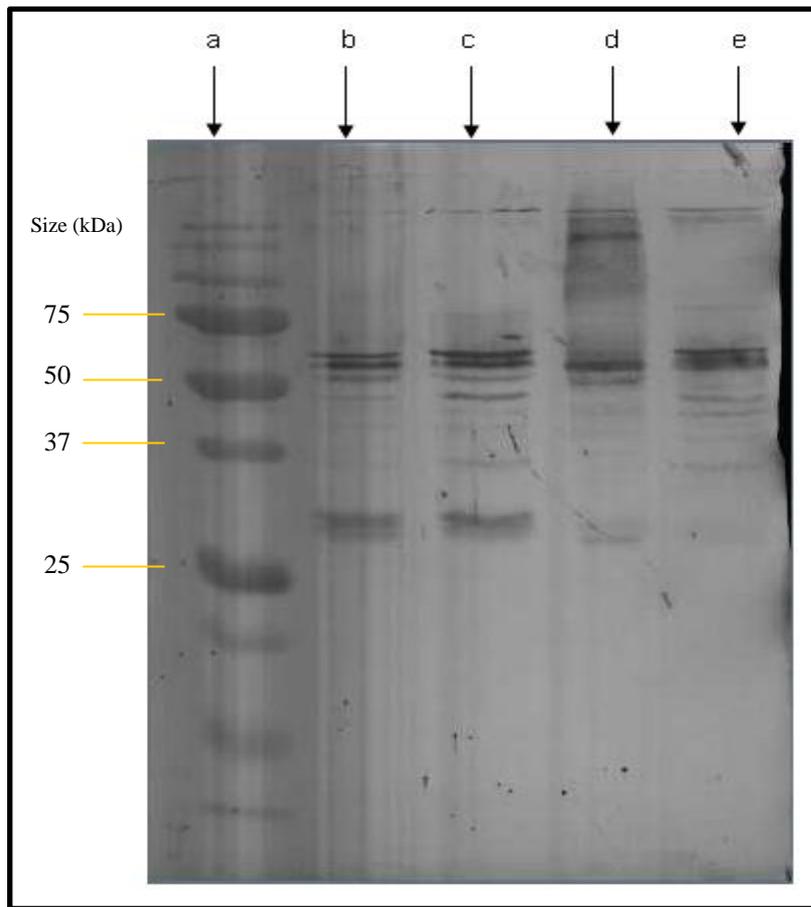


Figure 3.11: Reactions of Cell-Associated and Secreted Protein Extracts from *S. equi* 4047 WT and *S. equi* 4047 Δ PrtM with α PPMA: (a) Protein Size Standard (appendix C8), (b): Cell Associated Protein Extract of *S. equi* 4047 WT, (c) Cell Associated Protein Extract of *S. equi* 4047 Δ PrtM, (d) Secreted Protein Extract of *S. equi* 4047 WT, (e) Secreted Protein Extract of *S. equi* 4047 Δ PrtM, The cell associated and secreted protein extracts of both *S. equi* 4047 WT and *S. equi* 4047 Δ PrtM both cross reacted with the α PPMA antisera.

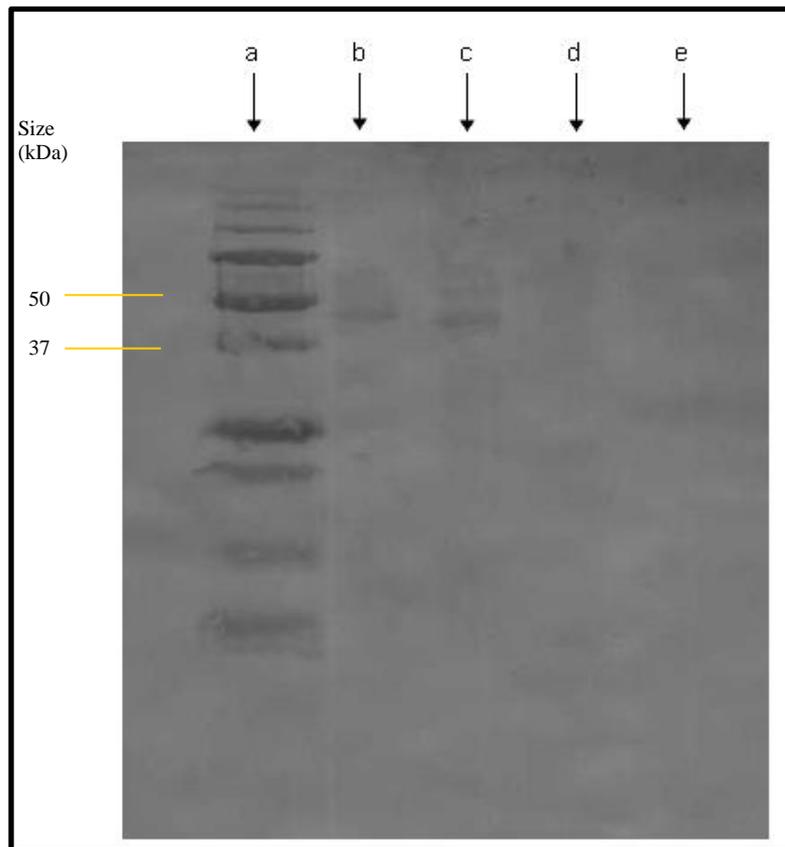


Figure 3.12: Reactions of Cell-Associated and Secreted Protein Extracts from *S. equi* 4047 WT and *S. equi* 4047 Δ PrtM with α LPPC antibody: (a) Protein Size Standard (appendix C8), (b): Cell Associated Protein Extract of *S. equi* 4047 WT, (c) Cell Associated Protein Extract of *S. equi* 4047 Δ PrtM, (d) Secreted Protein Extract of *S. equi* 4047 WT, (e) Secreted Protein Extract of *S. equi* 4047 Δ PrtM. Cell associated protein extracts of the WT and mutant both cross reacted with α LPPC Antibody. However, the secreted protein extract of both isolates did not show any cross reaction with this antisera.

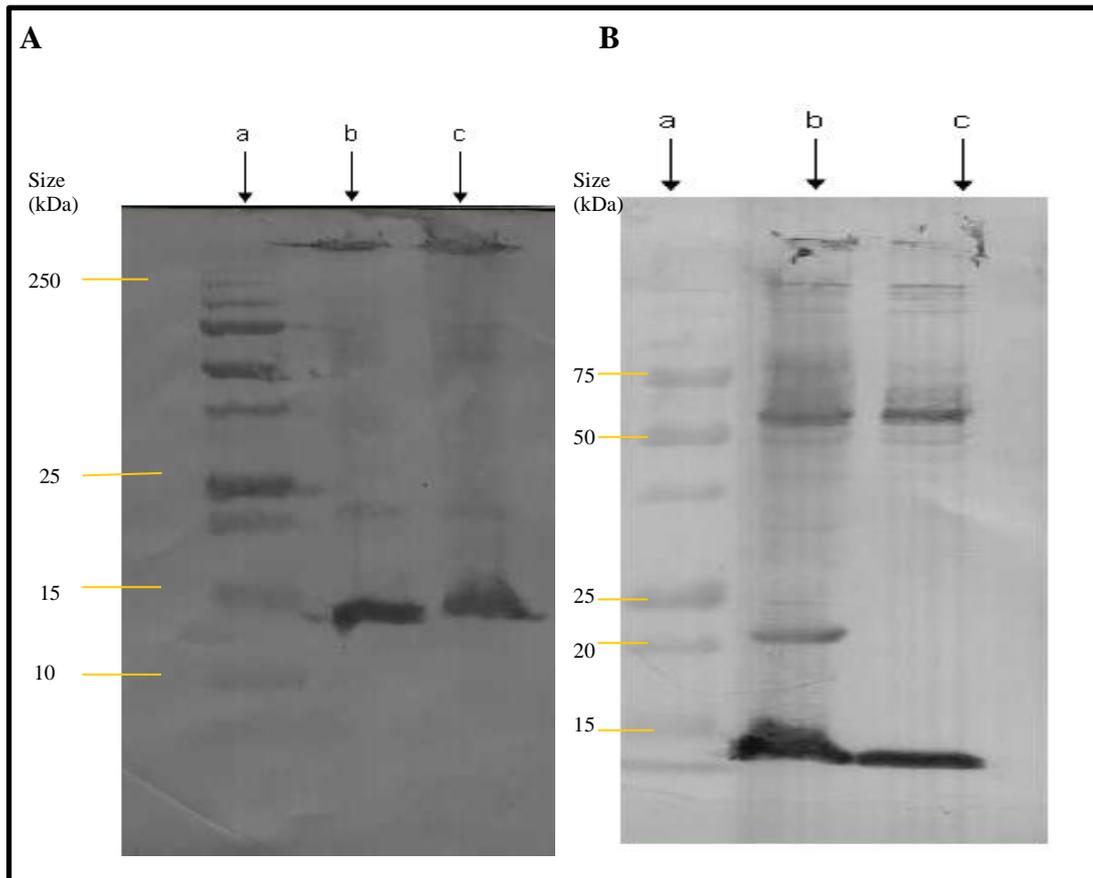


Figure 3.13: Reactions of *S. equi* 4047 WT and *S. equi* 4047 Δ PrtM with α HPr antibody: A = Cell associated protein extract; B= Secreted protein extract (a) Protein Size Standard (appendix C8), (b): *S. equi* 4047 WT, (c) *S. equi* 4047 Δ PrtM. The cell associated extracts of both *S. equi* 4047 WT and *S. equi* 4047 Δ PrtM cross reacted with α HPr antibody. However one band is missing from the reaction of secreted protein extract (B) of the mutant.

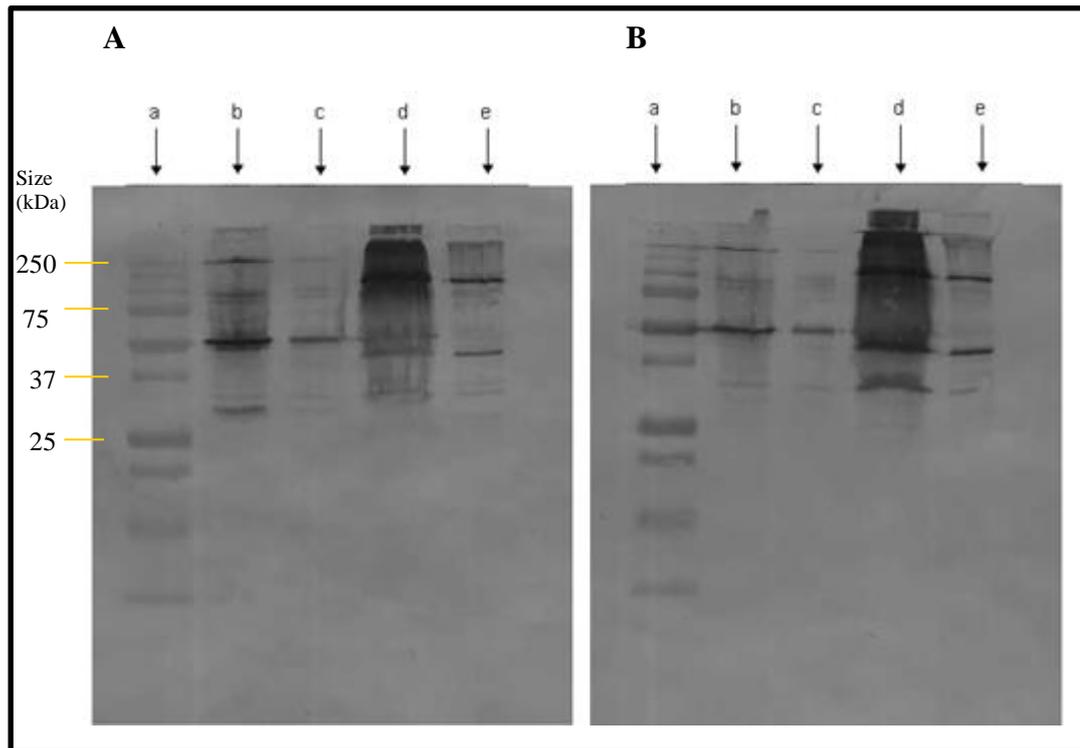


Figure 3.14: Reactions of Cell-Associated and Secreted Protein Extracts from *S. equi* 4047 WT and *S. equi* 4047 Δ PrtM with Pony 5788 antisera: A= Pre-Infection serum: (a) Protein Size Standard (appendix C8), (b): Cell Associated Protein Extract of *S. equi* 4047 WT, (c) Cell Associated Protein Extract of *S. equi* 4047 Δ PrtM, (d) Secreted Protein Extract of *S. equi* 4047 WT, (e) Secreted Protein Extract of *S. equi* 4047 Δ PrtM. B= Post-Infection serum: (a) Protein Size Standard (appendix C8), (b): Cell Associated Protein Extract of *S. equi* 4047 WT, (c) Cell Associated Protein Extract of *S. equi* 4047 Δ PrtM, (d) Secreted Protein Extract of *S. equi* 4047 WT, (e) Secreted Protein Extract of *S. equi* 4047 Δ PrtM. The reactions of both the cell associated and secreted protein extracts of both strains with pre and post infection serum from ponies confirmed the isolates as bonafide *S. equi* with lipoprotein activities.

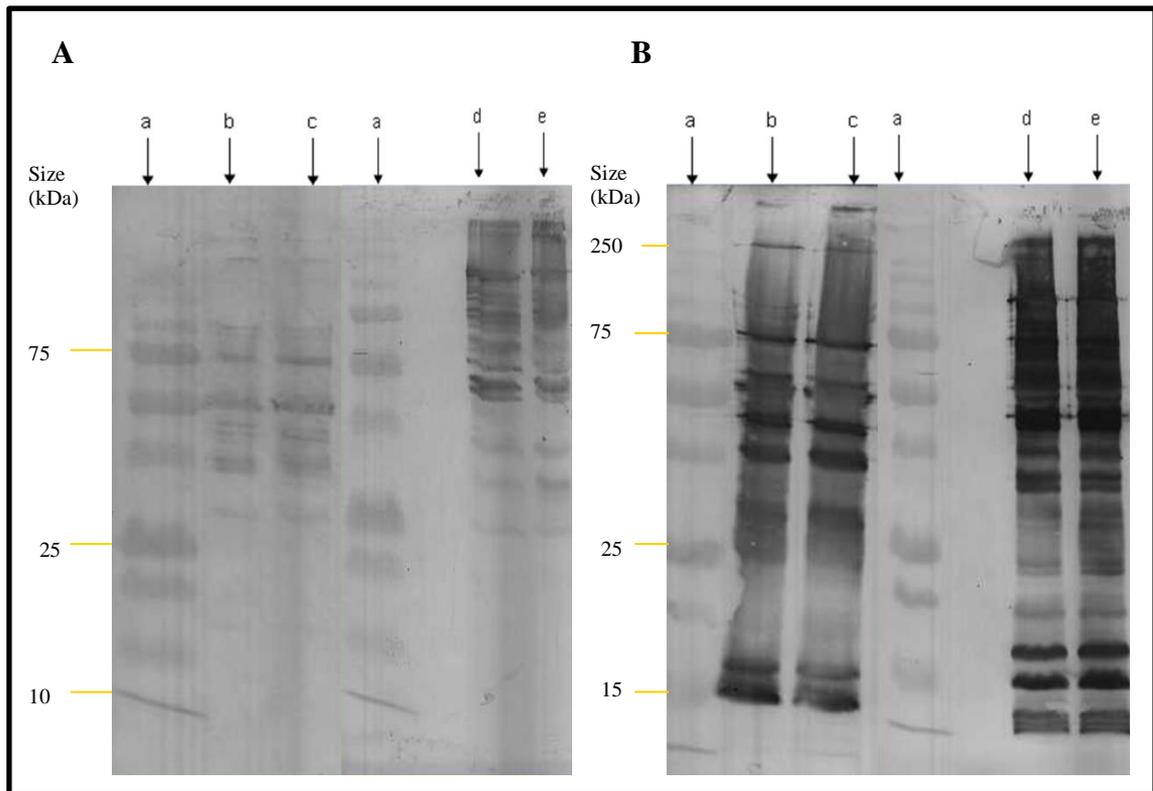


Figure 3.15: Reactions of Cell-Associated and Secreted Protein Extracts from *S. equi* 4047 WT and *S. equi* 4047 ΔPrtM Pony 5726 antisera: A= Pre-Infection serum: (a) Protein Size Standard (appendix C8), (b): Cell Associated Protein Extract of *S. equi* 4047 WT, (c) Cell Associated Protein Extract of *S. equi* 4047 ΔPrtM, (d) Secreted Protein Extract of *S. equi* 4047 WT, (e) Secreted Protein Extract of *S. equi* 4047 ΔPrtM. B = Post-Infection serum: (a) Protein Size Standard (appendix C8), (b): Cell Associated Protein Extract of *S. equi* 4047 WT, (c) Cell Associated Protein Extract of *S. equi* 4047 ΔPrtM, (d) Secreted Protein Extract of *S. equi* 4047 WT, (e) Secreted Protein Extract of *S. equi* 4047 ΔPrtM.

3.12 2D-E Gel images of *S. equi* 4047 WT and Δ PrtM Cell Associated and Secreted Protein extracts

After confirming by Western blotting that the protein extracts were of bonafide *S. equi* origin, 2D-E (method in section 2.5.26) was carried out with the intension of detecting differences in the proteome of *S. equi* 4047 WT and Δ PrtM. Initially, Immobiline Dry Strips at pH 3-10 were used for pilot study. The 2D-E gel images (Figures 3.16) of both the cell associated (obtained as described in sections 2.5.22 and 2.5.25) and secreted (obtained as described in sections 2.5.24 and 2.5.25) protein extracts of both strains revealed that most of the proteins are localized between pH 4-7. Hence the rest of the 2D-E was carried out in the pH 4-7 range (Figure 3.17).

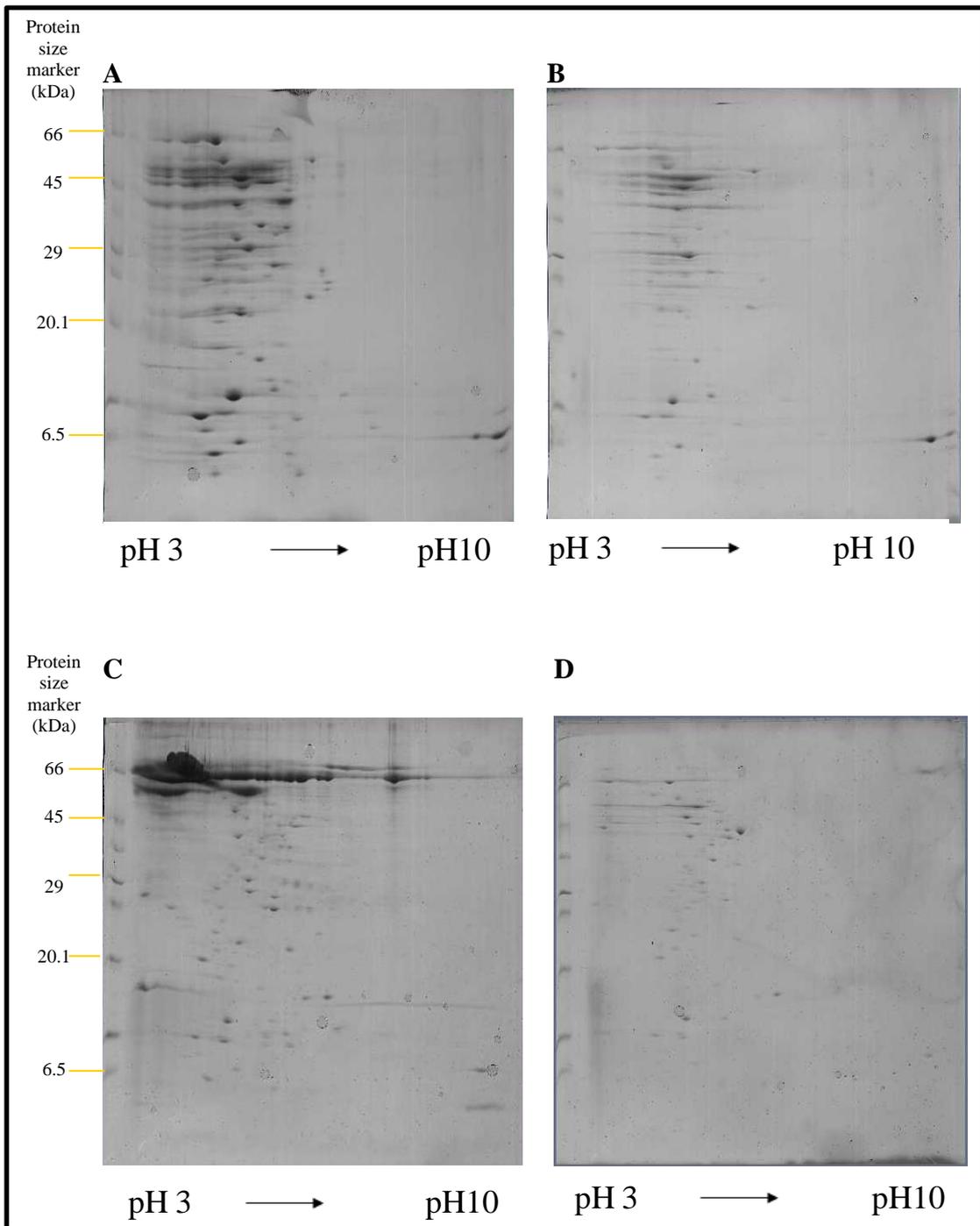


Figure 3.16: Representative 2D-E images of Protein Extract of *S. equi* 4047 WT and Δ PrtM at pH 3-10. A= Cell Associated Protein Extract of *S. equi* 4047 WT. B = Cell Associated Protein Extract of *S. equi* 4047 Δ PrtM. C = Secreted Protein Extract of *S. equi* 4047 WT. D = Secreted Protein Extract of *S. equi* 4047 Δ PrtM.

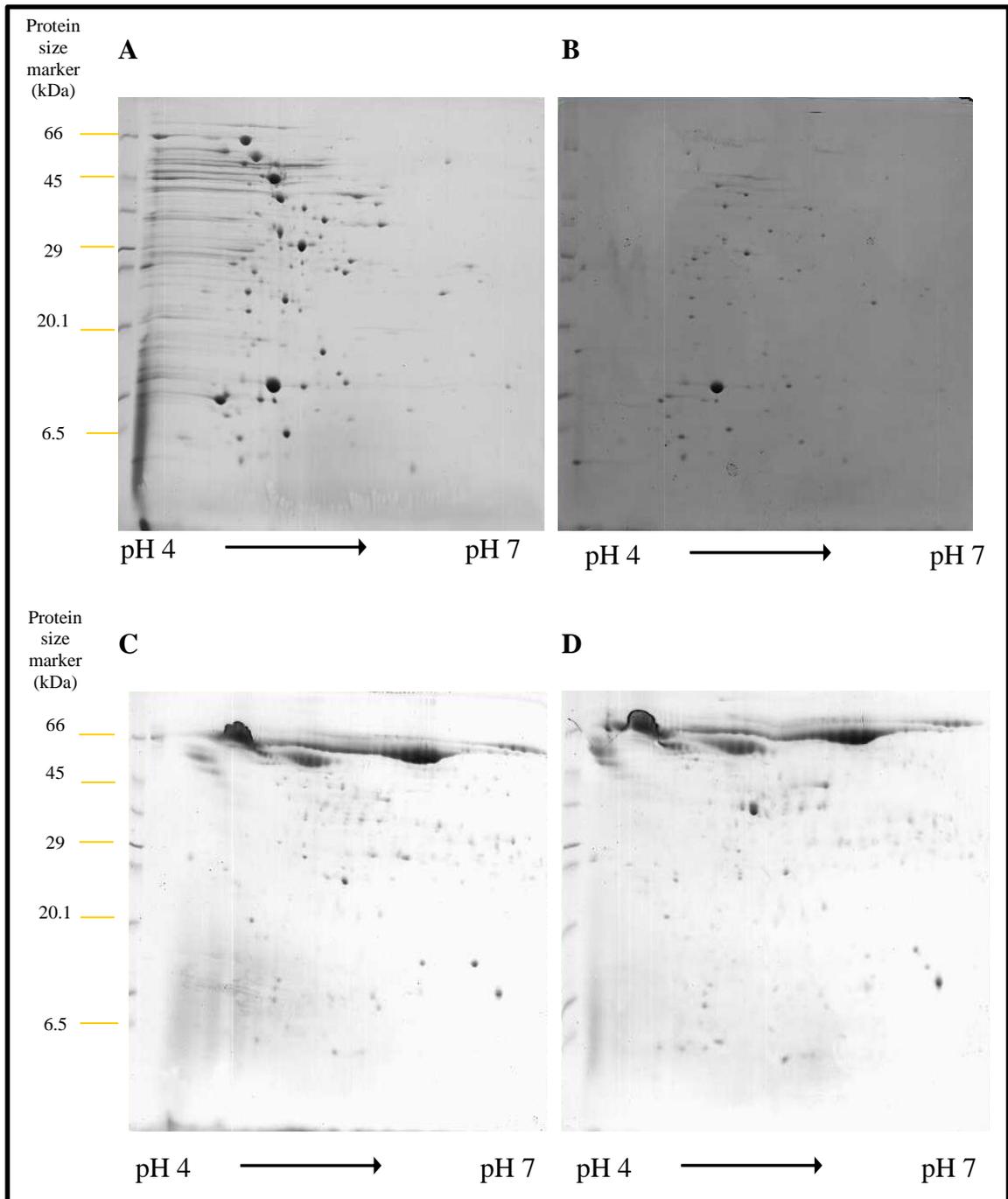


Figure 3.17: Representative 2D-E images of Protein Extract of *S. equi* 4047 WT and *S. equi* 4047 Δ PrtM at pH 4-7: A= Cell Associated Protein Extract of *S. equi* 4047 WT. B = Cell Associated Protein Extract of *S. equi* 4047 Δ PrtM. C = Secreted Protein Extract of *S. equi* 4047 WT. D = Secreted Protein Extract of *S. equi* 4047 Δ PrtM.

3.13 Results of Analysis of 2D-E Gels

The spots from all the gels were individually assigned a spot number by the PDQuest software. Finally, a master gel image (Figure 3.18, A and B) with spot numbers was generated for each matchset according to the manufacturer's (PDQuest) instructions (section 2.5.26.1). The master image contains all the spots that can be found in all the gels in a matchset; and was very useful for correct identification and excision (cutting or spot picking) of the spots.

The determination of presence and quantitative changes in protein expression was carried out using the PDQuest software (section 2.5.27); after which spots were picked and digested (method in section 2.5.29) for further analysis. HPLC/Mass spectrometry (method in section 2.5.30) of the digested proteins yielded the identities of proteins, after a MASCOT search (section 2.5.31). A list of the identities of all proteins that were expressed and identified from *S. equi* 4047 WT and *S. equi* 4047 Δ PrtM are shown in Appendix D; while Tables 3.4 to 3.12 show the spot numbers with a description of levels of expression.

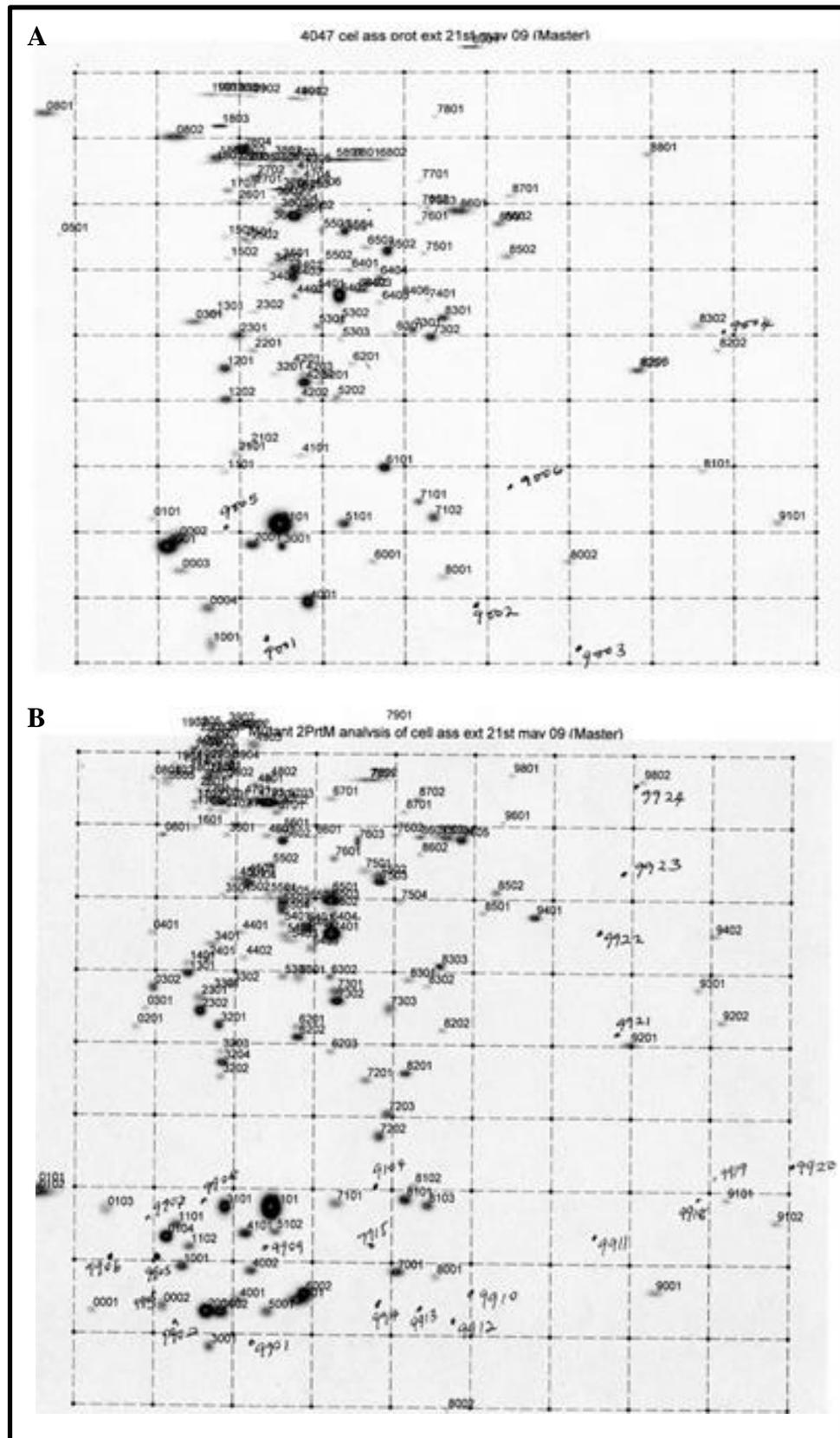


Figure 3.18: Representative master images with spot IDs: A = *S. equi* 4047 WT Cell Associated Proteins extract. B = *S. equi* 4047 Δ PrM Cell Associated Proteins

Significant protein spots from cell associated protein extracts of *S. equi* 4047 WT and *S. equi* 4047 Δ PrtM are given in Tables 3.4, 3.5, 3.6, 3.7 and 3.8. These include: common protein spots with equal levels of expression in both WT and mutant (Table 3.4), Common protein spots more highly expressed in the WT (Table 3.5), Common protein spots more highly expressed in the Mutant (Table 3.6), protein spots present in only the WT (Table 3.7) and protein spots present only in mutant (Table 3.8).

WT (4047)	=	Mutant (Δ PrtM)
Spot #3101: -50S ribosomal protein L21 [<i>Streptococcus pyogenes</i> M1 GAS] -30S ribosomal protein S11 [<i>Streptococcus pyogenes</i> M1 GAS] -50S ribosomal protein L14 [<i>Streptococcus pyogenes</i> M1 GAS] - heat-stable phosphocarrier protein, HPr [<i>Streptococcus mutans</i> , Ingbritt, Peptide, 86 aa] - hypothetical protein SAG1694 [<i>Streptococcus agalactiae</i> 2603V/R] - general stress protein [<i>Streptococcus equi subsp. zooepidemicus</i> MGCS10565]		Spot # 5101

Table 3.4: Common Spots with equal levels of expression in cell associated protein extract: expression in WT = expression in mutant.

WT (4047) >	Mutant (Δ PrtM)
Spot #7302: -apolipoprotein A-I precursor -RecName: Full=Apolipoprotein A-I; Short=Apo-AI; Short=ApoA-I; Flags: Precursor	Spot #8302: - apolipoprotein A-I precursor
Spot #4001: -30S ribosomal protein S6 [<i>Streptococcus equi subsp. zooepidemicus</i>] -30S ribosomal protein S6 [<i>Streptococcus equi subsp. zooepidemicus</i> MGCS10565] -hypothetical protein STRINF_01108 [<i>Streptococcus infantarius subsp. infantarius</i> ATCC BAA-102]	Spot #6002: -30S ribosomal protein S6 [<i>Streptococcus equi subsp. zooepidemicus</i>] -30S ribosomal protein S6 [<i>Streptococcus equi subsp. zooepidemicus</i> MGCS10565] -hypothetical protein STRINF_01108 [<i>Streptococcus infantarius subsp. infantarius</i> ATCC BAA-102]
4204: - hypothetical protein SEQ_1025 [<i>Streptococcus equi subsp. equi</i> 4047]	6202: - hypothetical protein Sez_0895 [<i>Streptococcus equi subsp. zooepidemicus</i> MGCS10565]

Table 3.5: Common Spots with differential level of expression in cell associated protein extract: expression in WT greater than expression in Mutant (Δ PrtM).

WT (4047) <	Mutant (Δ PrtM)
Spot #5303: - thioredoxin peroxidase [<i>Bacillus sp.</i> NRRL B-14911]	Spot #6302: - YkuQ [<i>Bacillus sp.</i> NRRL B-14911]

Table 3.6: Common Spots with differential level of expression in cell associated protein extract: *S. equi* 4047 WT expression less than *S. equi* 4047 Mutant (Δ PrtM) expression.

Spot ID#	Proteins
3201	Xaa-His dipeptidase [<i>Streptococcus equi subsp. zooepidemicus</i>] RecName: Full=NADP-dependent alcohol dehydrogenase Alcohol dehydrogenase zinc-binding domain protein [<i>Thermoanaerobacter italicus</i> Ab9] dipeptidase PepV [<i>Streptococcus pyogenes</i> M1 GAS]
2201	cyclophilin type peptidyl-prolyl cis-trans isomerase protein [<i>Streptococcus equi subsp. equi</i> 4047] co-chaperonin GroES [<i>Streptococcus equi subsp. zooepidemicus</i> MGCS10565]
5302	purine nucleoside phosphorylase [<i>Streptococcus equi subsp. zooepidemicus</i> MGCS10565]
6201	dTDP-4-Keto-6-Deoxyglucose-3 5-epimerase R mLC [<i>Streptococcus equi subsp. zooepidemicus</i> MGCS10565]
6001	hypothetical protein CaO19.10414 [<i>Candida albicans</i> SC5314]
3601	mannose-6-phosphate isomerase Pmi [<i>Streptococcus equi subsp. zooepidemicus</i> MGCS10565]
6501	6-phosphofructokinase [<i>Streptococcus equi subsp. zooepidemicus</i> MGCS10565]
5504	alcohol dehydrogenase [<i>Streptococcus equi subsp. zooepidemicus</i> MGCS10565] alcohol dehydrogenase [<i>Streptococcus equi subsp. zooepidemicus</i>]

Table 3.7: Spots present on only *S. equi* 4047 WT in cell associated protein extract.

Spot ID#	Proteins
0001	sugar phosphotransferase component II B [<i>Streptococcus equi subsp. zooepidemicus</i> MGCS10565]
9001	transcriptional regulator [<i>Bacillus sp.</i> NRRL B-14911]
8202	hypothetical protein GK3020 [<i>Geobacillus kaustophilus</i> HTA426]
8201	thioredoxin peroxidase [<i>Bacillus sp.</i> NRRL B-14911]
3203	transcription elongation factor GreA [<i>Streptococcus equi subsp. zooepidemicus</i> MGCS10565]
9401	ribosome recycling factor [<i>Streptococcus pyogenes</i> M1 GAS]

Table 3.8: Spots present on only *S. equi* 4047 Mutant (Δ PrtM in cell associated protein extract. NH means no hit; NRH means no relevant hit.

Significant protein spots from secreted protein extracts of *S. equi* 4047 WT and *S. equi* 4047 Δ PrtM are given in Tables 3.9, 3.10, 3.11, and 3.12. These include: common protein spots with equal levels of expression in both WT and mutant (Table 3.9), Common protein spots more highly expressed in the WT (Table 3.10), protein spots present in only the WT (Table 3.11) and protein spots present only in mutant (Table 3.12).

WT	=	Mutant (Δ PrtM)
Spot #9101: NH		Spot #7202: NH
Spot #0201: -esterase [<i>Streptococcus equi subsp. zooepidemicus</i> MGCS10565] -lipoprotein [<i>Streptococcus equi subsp. zooepidemicus</i>] -lipoprotein [<i>Streptococcus equi subsp. equi</i> 4047]		Spot #0401: -esterase [<i>Streptococcus equi subsp. zooepidemicus</i> MGCS10565] -RecName: Full=Hyaluronoglucosaminidase; Short=Hyaluronidase
Spot #7402: -RecName: Full=Hyaluronoglucosaminidase; Short=Hyaluronidase -fibronectin-binding protein [<i>Streptococcus equi</i>]		Spot #6503 -RecName: Full=Hyaluronoglucosaminidase; Short=Hyaluronidase

Table 3.9: Common Spots with equal level of expression in secreted protein extract: expression in WT = expression in Mutant. Note: NH means no hit; NRH means no relevant hit.

WT	>	Mutant (Δ <i>prtM</i> ₁₃₈₋₂₁₃)
Spot #5001: -unnamed protein product [<i>Manduca sexta</i>]		Spot #5103: -IgM heavy chain constant region, secretory form [<i>Bos taurus</i>]
Spot #8102: NRH		Spot #7201: NRH
Spot #9403: - exported protein [<i>Streptococcus equi subsp. equi</i> 4047]		Spot #7502: -esterase [<i>Streptococcus equi subsp. zooepidemicus</i> MGCS10565]

Table 3.10: Common Spots with differential level of expression in secreted protein extract: Expression in WT greater than expression in Mutant. NH means no hit; NRH means no relevant hit.

Spot ID #	Proteins
0005	-RecName: Full=NADP-dependent alcohol dehydrogenase - hypothetical protein AN7590.2 [<i>Aspergillus nidulans</i> FGSC A4] - unnamed protein product [<i>Escherichia coli</i> str. K-12 substr. MG1655] - L-xylulose reductase [<i>Neurospora crassa</i> OR74A] -Alcohol dehydrogenase zinc-binding domain protein [<i>Thermoanaerobacter italicus</i> Ab9]
1101	-unnamed protein product [<i>Escherichia coli</i> str. K-12 substr. MG1655]
8403	-ALB protein [<i>Bos taurus</i>] -serum albumin [<i>Bos indicus</i>] - albumin [<i>Felis catus</i>]
2201	-hypothetical protein Sez_0895 [<i>Streptococcus equi subsp. zooepidemicus</i> MGCS10565] -RecName: Full=Hyaluronoglucosaminidase; Short=Hyaluronidase
8501	-Mac family protein [<i>Streptococcus equi subsp. equi</i> 4047] -hypothetical protein CaO19.10414 [<i>Candida albicans</i> SC5314] -RecName: Full=NADP-dependent alcohol dehydrogenase
7301	-DNA/RNA non-specific endonuclease [<i>Streptococcus equi subsp. zooepidemicus</i>] -fibronectin-binding protein [<i>Streptococcus equi</i>] -serine proteinase inhibitor, clade A, member 1 precursor [<i>Bos taurus</i>]
9202	-fibronectin-binding protein [<i>Streptococcus equi</i>] - DNA/RNA non-specific endonuclease [<i>Streptococcus equi subsp. zooepidemicus</i>] -hypothetical protein TTHERM_00225940 [<i>Tetrahymena thermophila</i>] - RecName: Full=Hyaluronoglucosaminidase; Short=Hyaluronidase
2202	-hypothetical protein Sez_0895 [<i>Streptococcus equi subsp. zooepidemicus</i> MGCS10565] -RecName: Full=Hyaluronoglucosaminidase; Short=Hyaluronidase -fibronectin-binding protein [<i>Streptococcus equi</i>] - hyaluronoglucosaminidase [<i>Streptococcus pyogenes</i> MGAS10394]
1401	-lipoprotein [<i>Streptococcus equi subsp. zooepidemicus</i>] - lipoprotein [<i>Streptococcus equi subsp. equi</i> 4047] -hyaluronidase, phage associated [Streptococcus phage 370.1] -hyaluronoglucosaminidase [<i>Streptococcus pyogenes</i> MGAS10394] - hypothetical protein SPY_1154 [<i>Streptococcus pyogenes</i> M1 GAS]
2401	-extracellular protein [<i>Streptococcus equi subsp. zooepidemicus</i> MGCS10565]
1402	-extracellular protein [Streptococcus equi subsp. zooepidemicus MGCS10565] -hyaluronidase, phage associated [Streptococcus phage 370.1] -hyaluronoglucosaminidase [<i>Streptococcus pyogenes</i> MGAS10394] -unnamed protein product [<i>Escherichia coli</i> str. K-12 substr. MG1655] - RecName: Full=NADP-dependent alcohol dehydrogenase -Alcohol dehydrogenase zinc-binding domain protein [<i>Thermoanaerobacter italicus</i> Ab9]
8401	-Mac family protein [<i>Streptococcus equi subsp. equi</i> 4047]
2101	-hypothetical protein Sez_1583 [<i>Streptococcus equi subsp. zooepidemicus</i> MGCS10565] -RecName: Full=Hyaluronoglucosaminidase; Short=Hyaluronidase
1002	-co-chaperonin GroES [<i>Streptococcus equi subsp. zooepidemicus</i> MGCS10565] -hyaluronidase [Streptococcus phage P9]

Table 3.11: Spots present in secreted protein extract of only the WT

Spot ID #	Protein
1201	-RecName: Full=NADP-dependent alcohol dehydrogenase -YALIOB08052p [<i>Yarrowia lipolytica</i>] -hypothetical protein CaO19.10414 [<i>Candida albicans</i> SC5314] - Alcohol dehydrogenase zinc-binding domain protein [<i>Thermoanaerobacter italicus</i> Ab9] - hypothetical protein AN7590.2 [<i>Aspergillus nidulans</i> FGSC A4] - unnamed protein product [<i>Escherichia coli</i> str. K-12 substr. MG1655]
1103	-hypothetical protein Sez_1583 [<i>Streptococcus equi subsp. zooepidemicus</i> MGCS10565] -hyaluronidase, phage associated [<i>Streptococcus</i> phage 370.1] -hyaluronoglucosaminidase [<i>Streptococcus pyogenes</i> MGAS10394]
0101	-cold-shock protein, molecular chaperone, RNA-helicase co-factor [<i>Bacillus subtilis subsp. subtilis</i> str. 168]
0301	-A family sortase SrtA [<i>Streptococcus equi subsp. zooepidemicus</i> MGCS10565] -sortase SrtA [<i>Streptococcus equi subsp. zooepidemicus</i>] - RecName: Full=Hyaluronoglucosaminidase; Short=Hyaluronidase -
0304	-cold shock protein [<i>Bacillus sp.</i> NRRL B-14911]
1202	-oligopeptide ABC transporter periplasmic oligopeptide-binding protein OppA [<i>Streptococcus equi subsp. zooepidemicus</i> -MGCS10565] -YALIOB08052p [<i>Yarrowia lipolytica</i>] -RecName: Full=NADP-dependent alcohol dehydrogenase -Alcohol dehydrogenase zinc-binding domain protein [<i>Thermoanaerobacter italicus</i> Ab9] - hypothetical protein AN8113.2 [<i>Aspergillus nidulans</i> FGSC A4] -
2101	-30S ribosomal protein S6 [<i>Streptococcus uberis</i> 0140J] -cold shock protein [<i>Bacillus sp.</i> NRRL B-14911] -30S ribosomal protein S6 [<i>Streptococcus equi subsp. zooepidemicus</i> MGCS10565]
2301	-oligopeptide ABC transporter periplasmic oligopeptide-binding protein OppA [<i>Streptococcus equi subsp. zooepidemicus</i> MGCS10565]
5301	-thioredoxin peroxidase [<i>Bacillus sp.</i> NRRL B-14911]
4201	-50S ribosomal protein L10 [<i>Staphylococcus epidermidis</i> ATCC 12228]

Table 3.12: Spots present in secreted protein extract of only the Mutant.

3.14 Discussion

The results of statistical analysis (student T-test showing no significant difference $p < 0.05$ - in Appendix F) of growth curves (Figure 3.3) of *S. equi* 4047 WT and Δ PrtM are in accordance with previous work of Hamilton *et al.* (2006) who stated that the growth of the *S. equi* 4047 Δ PrtM in nutrient-rich broth was comparable to that of wild-type (*S. equi* 4047 WT). Not finding any significant difference between the growth rate of *S. equi* 4047 WT and Δ PrtM suggests that the deletion of the prtM gene from the

mutant, and consequently its (Δ PrtM) lack of PPIase activity (the PPIase activity of PrtM of *S. equi* was proven using recombinant PrtM from *S. equi* in a protease-coupled PPIase assay, results shown in chapter 6 of this study) does not affect growth under the conditions applied.

The Gram stain reaction and Lancefield grouping confirm the identities of both *S. equi* 4047 WT and Mutant (Δ PrtM) as being Gram-positive, Lancefield group C Streptococci.

The result of agarose gel electrophoresis of the genomic DNA (Figure 3.2-A) indicate that the genomic DNA extraction protocol (section 2.5.2) was successful. The amplification result (Figure 3.2-B) shows that *S. equi* 4047 WT has a higher molecular weight than mutant (Δ PrtM). Therefore, the wild type strain used in this study carries the PrtM gene (sequence 138-213), while the mutant (Δ PrtM) clearly lacks part of the PrtM gene (sequence 138-213) which is in agreement with the report of Hamilton *et al.* (2006). This PCR result also indicates that the growth conditions used in this experiment favour maturase expression.

Bacteria can survive under a wide range of environmental conditions, including osmotic variation (Visick and Clarke, 1995); however NaCl can cause damage to the folding of cell surface proteins (Ignatova and Gierasch, 2007). *S. equi* 4047 WT and Δ PrtM were subjected to growth in varying concentration of NaCl (results in Section 3.5: Table 3.1 and Figures 3.4). Both strains were able to survive better in 0.9% (w/v) NaCl (Figure 3.4) which is not surprising considering that this is the concentration of physiological saline which may have served to balance osmotic pressure for the bacterial cells. Considering both strains together, a 58% survival rate in 0.9% (w/v) NaCl was followed

by a 39% survival in water (Figure 3.4). Only 2% of the organism survived in 14.7% (w/v) NaCl (Figure 3.4) and out of this Figure, 100% were the wild type strain (Figure 3.4). The wild-type strain made up 100% of the 1% (Figure 3.4) of surviving cells in 29.4% (w/v) NaCl. These data suggest that *S. equi* 4047 is able to adapt to varying and high salt concentrations. However, compared to the mutant (Δ PrtM) which was only able to survive in H₂O and 0.9% (w/v) NaCl, the higher percentage of *S. equi* 4047 WT surviving in all conditions and at all the concentrations tested, indicate that the absence of the prtM gene in the mutant (Δ PrtM) greatly affects its ability to adapt to high NaCl concentrations. PPIases have been proven to be important for resistance to NaCl (Li *et al.*, 2005; Reffuveille *et al.*, 2012b). Having proven in this study (chapter 6) that PrtM recombinant protein of *S. equi* has PPIase activity, it can therefore be inferred that PPIase activity of *S. equi* maturase lipoprotein (PrtM) is involved in the bacterial adaptation to NaCl stress.

Triplicate repeats of antibiotics sensitivity tests (results in Table 3.2 and Figure 3.5) show that areas of growth inhibition were significantly smaller (student T-test in Appendix G) for the WT compared to the mutant (Δ PrtM). PPIases activity has been associated with cell wall synthesis and remodeling proteins. The role of the PPIase, PrsA, in *B. subtilis* viability has been associated with gross cell wall structural defects due to a loss of Penicillin Binding Protein (PBP) stability and/or activity upon PrsA depletion (Hyyrylainen *et al.*, 2010). In *L. monocytogenes*, the association of PBP activity with PrsA2 PPIase activity was established when a *prsA2* N+C construct (mutant lacking the entire central PPIase domain) was unable to complement increased bacterial sensitivity to Penicillin G exhibited by Δ *prsA2* mutants (Alonzo *et al.*, 2012). Compared to the wild type, the increased sensitivity of the mutant (Δ PrtM) to Penicillin G (Table 3.2 and Figure 3.5) indicates that just like *B. subtilis* PrsA and *L.*

monocytogenes PrsA2, in *S. equi* 4047 the PrtM PPIase activity is required, by the bacteria, for optimal resistance to β -lactam antibiotics. The fact that Δ PrtM exhibited higher sensitivity to all the antibiotics tested (Table 3.2 and Figure 3.5) is also indicative that *S. equi* PrtM PPIase activity is associated with cell wall synthesis and protein remodelling.

The production of mucoid colonies on THA and also on blood agar (Figure 3.1) by both strains, as well as the results of capsular staining (Figure 3.6), indicate that hyaluronic acid is produced by both *S. equi* 4047 WT and Δ PrtM. The hyaluronic acid synthase gene is located in the *has* operon which codes for the enzymes involved in the production of hyaluronic acid precursors (Blank *et al.*, 2008). In *S. equi*, the hyaluronic acid synthase genes are located in the *hasA/hasB/hasC* and *hasC/glmU/pgi* operons (Blank *et al.*, 2008).

Having ascertained from staining (results in Figure 3.6) that both strains produce hyaluronic acid, it was puzzling that it was impossible to concentrate all the cells of the wild-type suspended in 0.5 M sucrose, centrifuged at 4000 x g at 4°C for up to 30 min, whereas the mutant cells were pelleted after 15 min centrifugation under the same conditions. The rapid density buoyancy centrifugation assay (result in section 3.8, Table 3.3), which followed the capsular staining, also confirmed the fact that both strains produce hyaluronic acid. It is possible then that the wild type produces more hyaluronic acid than the mutant, hence the difficulty in completely pelleting the wild-type cells. The next option was therefore to find out how much hyaluronic acid was produced in broth culture, by each strain. DeAngelis and Weigel (1994) reported that a strain (NSA156) of *Streptococcus pyogenes* produced 7 μ g/ mL of hyaluronic acid and floated in 65% (v/v) Percoll. In this study, *S. equi* 4047 WT produced 5 μ g/ mL and *S. equi*

4047 Δ PrtM produced 3 μ g/ mL of hyaluronic acid in THB, at cell density OD_{600nm} at 0.4 (result in section 3.9, Figure 3.7); and both strains floated in 65% and 50% Percoll (Section 3.8, Table 3.3). The ratio of hyaluronic acid produced per mL of culture (Figure 3.7) is approximately 3:2 (60:40%) for the WT and Δ PrtM respectively. Is it possible then that PPIase activity of the PrtM of *S. equi* 4047 (as reported in chapter 6 of this study) is involved in speeding up the folding or remodeling of one or more of the enzymes involved in hyaluronic acid synthesis, hence *S. equi* 4047 WT produces more hyaluronic acid than the PrtM-deficient mutant strain? Such involvement is an area for future study.

Reversible Ponceau S staining of blots after transfer (method in sections 2.5.20 and result in Figure 3.9) was carried out in order to confirm the presence of protein bands in the cell associated and secreted protein extracts. The Western blot results (Figure 3.10) show that *S. equi* 4047 WT and Δ PrtM cross reacted with horse covalent serum. Both strains also cross reacted with pre and post infection sera of ponies (Figures 3.14 and 3.15). These reactions are consistent with bonafide *S. equi* isolates (Hamilton *et al.*, 2000; Hamilton *et al.*, 2006). The results were therefore valuable determining immunogenicity as well as in authenticating the identities of *S. equi* 4047 WT and Δ PrtM strains used for this research. In addition to this, the Western blot results were valuable for confirming the most favourable growth conditions for protein extraction for 2D-E.

Proteins which are involved in the pathogenesis of *S. pneumoniae* infections are viewed as interesting components for future conjugate or multicomponent protein vaccines (Overweg *et al.*, 2000a). The importance of a putative proteinase maturation protein A (PpmA) in pneumococcal pathogenesis was demonstrated in a mouse pneumonia model

and PpmA was shown to elicit species-specific opsonophagocytic antibodies which cross-reacted with various pneumococcal strains (Overweg *et al.*, 2000a). Since PpmA is the *S. pneumoniae* homologue of the PrtM (Hamilton *et al.*, 2006; and Overweg *et al.*, 2000a) it was interesting to find out the reaction of antibodies against PpmA with extracts from both *S. equi* 4047 WT and Δ PrtM. PpmA serum recognized a protein band with the correct molecular size (35 kDa) in whole-cell lysates of *S. pneumoniae* (Overweg *et al.*, 2000a). PpmA was therefore proven to be located at the surface of *S. pneumoniae*. In this research, the cell associated and secreted protein protein extract of both *S. equi* 4047 WT and Δ PrtM both cross reacted with the α PPMA antisera (Figure 3.11). The cross reaction of mutant (Δ PrtM) with α PPMA antisera suggests that PrtM is a good vaccine candidate.

Cell associated protein extracts of *S. equi* 4047 WT and Δ PrtM both cross reacted with α LPPC antibody (Figure 3.12) at the appropriate molecular weights, corresponding to previous studies by Hamilton *et al.* (2000) and Hamilton *et al.* (2006) where α LPPC antibody activity was detected in *S. equi* at approximately 31.9 kDa. However, the secreted protein extract of both isolates did not show any cross reaction with α LPPC antisera (Figure 3.12). This was not surprising because the *S. equi* LppC homologue was shown to be a lipoprotein located on the cell surface (Hamilton *et al.*, 2000).

The reactions of both the cell associated and secreted protein extracts of both strains (*S. equi* 4047 WT and Δ PrtM) with pre and post infection serum from ponies (Figures 3.14 and 3.15) confirmed the isolates as bonafide *S. equi* with lipoprotein activities. A good vaccine candidate should be able to induce antibodies that cross react with the target protein in all strains of the pathogen. The cross reaction of mutant (Δ PrtM) with serum

from all tested ponies' serum, as well as with horse covalent serum (Figure 3.10), therefore indicate that PrtM is a good vaccine candidate.

Bacteria are known to transport saccharides across the cell membrane and phosphorylate them before their release into the cytosol (Kundig *et al.*, 1964). Anaerobic and facultatively anaerobic bacteria have and use the phosphoenolpyruvate (PEP) phosphotransferase system (PTS) as their main carbohydrate transport system (Romano *et al.*, 1970; Saier, 1989). The PTS system is made up of enzyme I and a histidine-containing phosphocarrier protein (HPr) – as its two main energy-coupling components, and of enzymes II which are sugar-specific permeases (Herzberg *et al.*, 1992). HPr is a central part of the phosphoenolpyruvate sugar phosphate system by which Streptococci transport sugar as energy source (Hamilton, 1987, and Thompson, 1987 in Dubreuil *et al.*, 1996). HPr has been identified in *S. equi* (Sutcliffe *et al.*, 2000; and Dixon *et al.*, 2001), with HPr-1 (in which its N-terminal methionine been removed) being predominant (Sutcliffe *et al.*, 2000). This is a modification (as in HPr-1) that was previously associated with surface localization of streptococcal HPr proteins (Hamilton, 1987, and Thompson, 1987 in Dubreuil *et al.*, 1996). It is speculated by Dixon *et al.*, (2001) that surface-localised and probably released HPr-1 of *S. equi* may have roles to play in its (HPr-1) mitogenic activity which had previously been demonstrated in *S. pyogenes* by Gerlach *et al.*, (1992). Therefore, the reactions (Figures 3.13 A) of cell associated protein extracts of both *S. equi* 4047 WT and Δ PrtM with α HPr antibody is in accordance with previous studies. The post 2D-E/HPLC/MS finding of equal levels of expression of HPr (Table 3.4) in the cell associated protein extract of *S. equi* 4047 WT and Δ PrtM is therefore not surprising.

HPr of Gram-positive bacteria can be phosphorylated and dephosphorylated at the serine 46 residue by an ATP-dependent HPr kinase, to give HPr-P or HPr-(Ser-P) respectively (Asanuma and Hino, 2002). These two forms of HPr have been reported to give two distinct bands (HPr-P being in the upper band) on Western blots (Asanuma and Hino, 2002). In *B. subtilis*, HPr kinase activity is seen under conditions of good nutrient supply which are indicated by high ATP and fructose-1,6-bisphosphate concentrations; unlike phosphorylase activity which is triggered by high concentrations of inorganic phosphate, indicating the absence of good carbon sources (Halbedel *et al.*, 2004). In *S. mutans*, serine-phosphorylated HPr functions in concert with particular PTS permeases thereby prioritizing carbohydrate utilization through modulation of sugar transport activity and the transcription of catabolic operons (Zeng and Burne, 2010). The reaction of the secreted protein extract of only the WT but not the mutant (Δ PrtM) with anti-HPr (Figure 3.13-B), is suggestive that *S. equi* PrtM PPIase activity (Chapter 6) may be involved in the folding or remodeling of HPr kinase. This absence of the upper band (Figure 3.13-B) in the secreted protein extract of the PrtM deficient mutant also indicates that released HPr-1 of *S. equi* may have critical roles to play in HPr-1 mitogenic activity.

Proteomics approaches (methods of Zhang *et al.* 2007) were used to identify the cell associated and secreted proteins of importance in *S. equi* 4047 WT and Δ PrtM (methods in section 2.5). It was predicted that the inability of the mutant (Δ PrtM) to correctly fold the maturase substrates leads to differences in proteolytic processing of the substrate proteins and ultimately to differences in their migration on 2D-E gels using SDS-PAGE.

An evaluation of the HPLC/MS (methods in section 2.5.30 and 2.5.31) results of the post-2D-E gel trypsinized proteins spots of the cell associated protein extract (Table 3.5) indicate that SEQ_1025 (a hypothetical protein of *S. equi* with protein code [gi|225870399](#)) is common in both *S. equi* WT 4047 and Mutant ($\Delta prM_{138-213}$), is a best match hit; however it (the hypothetical protein) is upregulated (more highly expressed) in the *S. equi* 4047 WT. Another protein of interest identified (Table 3.5) was SEZ_0895 (hypothetical protein of *Streptococcus equi subsp. zooepidemicus* MGCS10565, code [gi|195978022](#)) and is also upregulated (more highly expressed) in the *S. equi* 4047 WT.

It is very interesting to note that from the secreted protein extract, multiple spots apparently contain the same proteins. For example both spot numbers WT9202 and WT7301 (Table 3.11) contain fibronectin binding protein (FNE) and SEQ0882 endonuclease.

IdeE (an IgG-endopeptidase and homologue of the leucocyte receptor Mac-1) was identified from a screen of clones reactive with convalescent serum from a gene library of *S. equi* CF32 (Timoney *et al.*, 2007). Antiphagocytic activity for equine neutrophils was proven to be neutralized by IdeE-specific antiserum (Timoney *et al.*, 2007). IdeE2 (Mac family protein) is also an endopeptidase but has greater activity towards horse IgG (Guss *et al.*, 2009). Interestingly, a MAC family protein (Table 3.11, spot number 8501) was identified in the secreted protein extract of only the WT (*S. equi* 4047 WT) but not in the mutant (*S. equi* 4047 $\Delta PrtM$).

The rotamase lipoprotein SlrA (streptococcal rotamase A a member of the cyclophilin family) and PpmA (proteinase maturation protein A which shares homologies to

PPIases of the parvulin family) have been identified in *Streptococcus pneumoniae* and are both involved in the colonization phase of infection and in the evasion of phagocytes (Reffuveille, 2012b). In this study, a PPIase of the cyclophilin type (Table 3.7, spot 2201) was identified in the cell associated protein extract of only the WT (*S. equi* 4047 WT) but not in the mutant (*S. equi* 4047 Δ PrtM). This suggests the absence of this particular surface located PPIase in the mutant (*S. equi* 4047 Δ PrtM).

Streptococcal fibronectin (Fn)-binding cell surface proteins are assumed to enhance the potential of the bacteria to cause disease (Lindmark *et al.*, 2001). Finding FNE and IdeE2, in the secreted protein extract of only *S. equi* 4047 WT and not the PrtM deficient-mutant (Δ PrtM) therefore suggests proteolytic degradation of misfolded secreted proteins; and very importantly as well, indicates that PrtM may not only be linked to the folding of one specific substrate but could be a multisubstrate foldase. This suggests PrtM involvement in *S. equi* antibiotics sensitivity reaction (Table 3.2 and Figure 3.5), in the adaptability of the organism to NaCl stress (Table 3.1, Figures 3.4 a), level of hyaluronic acid production (Figure 3.7), as well as in with HPr kinase folding and/or remodelling. All these buttress findings from this study that PrtM is a multisubstrate foldase.

The 2D-E results establish that the protein profile of *S. equi* 4047 WT has significant differences from that of the mutant (Δ PrtM). These results therefore confirm that, the absence of the maturase lipoprotein PrtM (as seen in Δ PrtM), results in readily detectable changes in *S. equi* proteome.

4.0 Bioinformatics

4.1 *S. equi* 4047 PrtM sequence and encoded protein sequence

The sequence (and encoded protein sequence) of the *S. equi* 4047 PrtM gene (Figures 4.1 and 4.2, respectively) were obtained as described in section 2.3.1.

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ATAGGTGGAATGGTTGTTTTAGATGATGTGTTTCAGGGGGGTGACATTGCTAAGCCGATCAAGGAGGTTTC
GGAGAGGACAGCGCACGATTTATCGTGGCCTTCAGGACTTATTTGCAGCAACTCTTGATAATCCTGGATT
GACAGCCAGCCTGCTGCCGCTTAGTGATGGCTTGCTGATGATTCGTAAAAATCAAGCAGATATTCGTTTG
TTAGATTAAGTTACGTTAAGAAATTGTGATATAATTACTGGTTAATGACAAAAAGGAGCACAAAATCA
ATGAAAAAATCAACTAAATTACTTGTGGTATCGTAACCCTAGCATCAGCAATGACCCTAGCAGCCTGT
CAGTCTACAAATGACAATACAAGTGTCATTACGATGAAGGGCGACACTATCAGTGTTAGTGATTTTTTACA
ATGAAACAAAAAATACAGAGATTTCTCAAAGAGCAATGCTAAACCTTGTGGTTAGTCGTGTTTTTGAGGA
CCAATACGGTAAAAAGGTTTCTAAGAAAAGAACGGAAGAAGCTTACAATAAATCAGCTGAGCAATACGGT
GCGTCATTCCTCTGCAGCCCTTGCAGCTCTGGCTTGACAACAGATAACCTACAAGCGTCAAATTCGCTCAG
CCATGCTGGTTGAATATGCTGTTAAAGAAGCAGCTAAAAAAGAGCTGACAGATGCTGATTACAAAAAAGC
CTATGAGTCATACACACCAGAAATGACTACTCAGGTCACTACTCTAGACAATGAAGAAACAGCAAAGGCT
GTTTTAGGTGAGGTTAAGGCTGAGGGTGTGACTTTGCTGCTATTGCTAAGGAAAAGACAACAGCAGCAG
ACAAGAGGTAGACTATAAGTTTGACTCAGGAGACACTAAGTTACCAGCAGATGTGATCAAGGCCGCCTC
AGGATTAAGAGGGTGATATTTCAGAGGTGGTTTTCTGTCTTAGATCCGGCTACTTATCAAACAAGTTC
TATATTGTTAAGGTAACCAAAAAAGCCGAAAAGGCTTCTGATTGGAAGAAATATAAGAAACGTCTAAAAG
AAATTGCTTTGGCTGAAAAGACACAAAACATTGATTTCCAAAATAAGGTCATTGCAAAGGCCTTAGACAA
GGCAAATGTTAAGATCAAAGACCAAGCATTGCTAATATCTTGGCACAGTATGCCAATACTGATAAAAAA
GCAAGCAAGGCGAACACAAGCAAGTCAGATCAGAAATCATCTTCAGACTCAAGCAAGGATAGTCAATCTT
CTAAATCTAAGTCAGAAAAATAG
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Figure 4.1: The *S. equi* 4047 PrtM-encoding 1283 nucleotide (nt) sequence: (>gi|225698891:676500-677782). The 281 nt upstream sequence contains promoter sequences (in red), a ribosome binding site (in blue) and a start codon (underlined). For details of primer sequences, refer to Table 2.11 in Chapter 2: Binding site of Prtm123equir highlighted yellow, binding site of Prtm12equir and Prtmidcolir highlighted lemon green. Binding site of Prtmidcolif highlighted purple. Binding site of Prtmequif highlighted grey. Binding site of SEQPrtmr underlined red. Binding site of SEQPrtmf underlined green. Central domain sequence underlined yellow.

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MKKSTKLLAGIVTLASAMTLAACQSTNDNTSVITMKGDTISVSDFYNETKNTEISQRAMLNLVV
SRVFEDQYGKKVSKKRTEEAYNKSAEQYGASFSALAQSGLTTDTYKRQIRSAMLVEYAVKEA
AKKELTDADYKKAYESYTPEMTTQVTTLDNEETAKAVLGEVKAEGADFAAIAKEKTTAADKK
VDYKFDSGDTKLPADVIKAASGLKEGDISEVVSVLDPATYQNKFYIVKVTKKAEKASDWKKYK
KRLKEIVLAEKTQNIDFQNKVIAKALDKANVKIKDQAFANILAQYANTDKKASKANTSKSDQKS
SSDSSKDSQSSKSKSEK
```

Figure 4.2: *S. equi* 4047 PrtM protein sequence with signal peptide (underlined).

4.2 *S. equi* 4047 PrtM signal peptide.

The predicted signal peptide of the *S. equi* 4047 PrtM domain, shown in Figure 4.3, was determined as described in section 2.3.2.

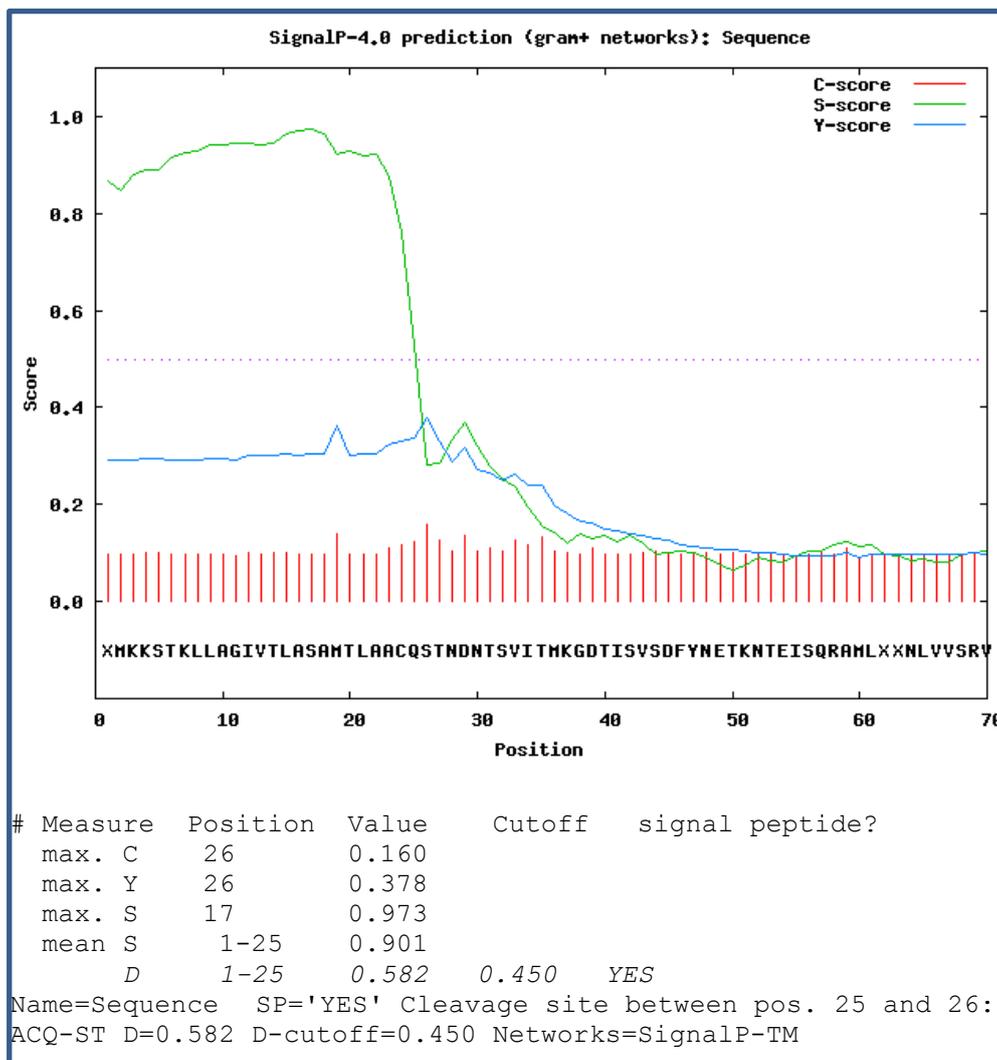


Figure 4.3: Predicted Signal peptide of *S. equi* 4047 PrtM via <http://www.cbs.dtu.dk/services/SignalP/>

4.3 *S. equi* 4047 PrtM Protein sequences, conserved domain and aligned sequences

The PrsA protein of *Bacillus subtilis* is an essential membrane-bound lipoprotein, proven to be a peptidyl-prolyl cis/trans isomerase (Vitikainen *et al.*, 2004). PrsA protein of *B. subtilis* is assumed to assist post-translocational folding of exported proteins and to stabilize them in the compartment between the cytoplasmic membrane and cell wall (Vitikainen *et al.*, 2004). PrsA has a stretch of 90 amino acids that are 45% identity to *E. coli* parvulin (Vitikainen *et al.*, 2004); *E. coli* PpiC being of the parvulin family (Rahfield *et al.*, 1994; Rudd *et al.*, 1995; Kuhlewein *et al.*, 2004). The PrsA protein of *B. subtilis* shares a high degree of sequence similarity across the entire length of its sequence with that of PrsA2 of *Listeria monocytogenes* (Alonzo *et al.*, 2011).

In order to predict the domain architecture of PrtM of *S. equi* 4047, its protein sequence (Figure 4.2) was matched (method in section 2.3.4) to the sequences of the following peptidyl-prolyl cis/trans isomerases (PPIases): PrsA2 of *L. monocytogenes*, PrsA of *B. subtilis* and PpiC of *E. coli*. PrtM was shown to align with the aforementioned PPIases (alignment results are shown in Figures 4.4, 4.5, 4.6 and 4.7). The sequence of the centrally packed conserved region of *S. equi* 4047 PrtM (Figure 4.8) was translated into protein sequence and the first reading frame was selected and used, in conjunction with a comparison of several sequence alignment results (Figures 4.4, 4.5, 4.6 and 4.7), in determining the sequence of the central domain (Figure 4.8). The central conserved domain is marked by a large number of weakly similar, strongly similar or identical amino acid residues (Figures 4.4, 4.5, 4.6 and 4.7).

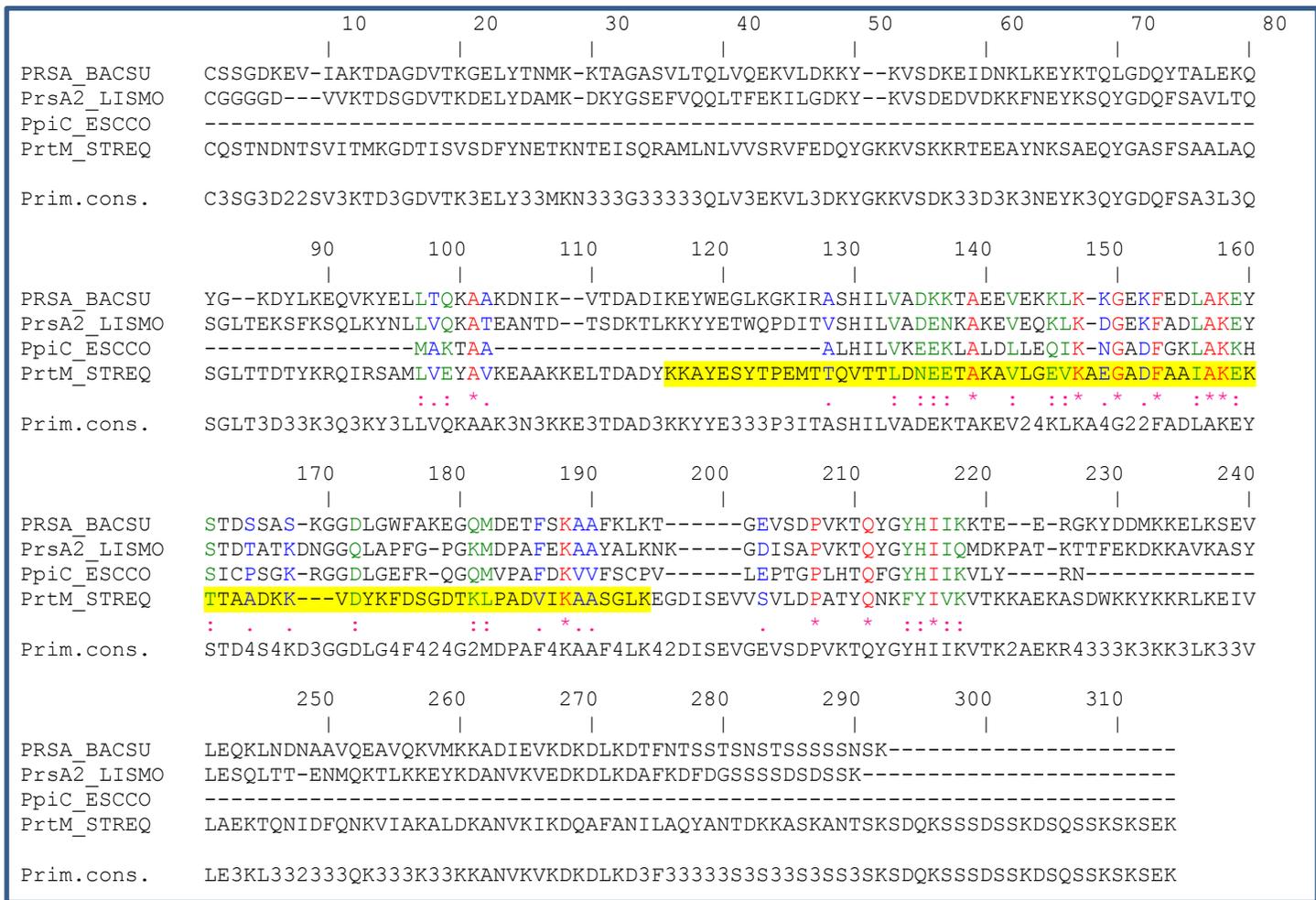


Figure 4.4: CLUSTALW alignment. Sequence highlighted in yellow = deleted in *S. equi* Mutant PrtM that was used for this research. Identical aa (*); strongly similar conserved aa substitutions (:) or weakly similar aa(.). Signal peptide sequences were excluded. Amino acids are denoted by single letters and letter colouration represents the physicochemical properties of the residues (Larkin et al., 2007). Red: small, hydrophobic, aromatic, but not Y. Blue: acidic. Magenta: Basic, excluding H. Green: Hydroxyl, sulfhydryl, amine, including G. Grey: others, including unusual amino/imino acids. The numbering of the residues is consistent with the numbering of the primary consensus (prim. cons.) Identical and conserved residues are concentrated in the central domain region. The number of amino acid possibilities at a relative position is represented by numbers within the prim. cons.

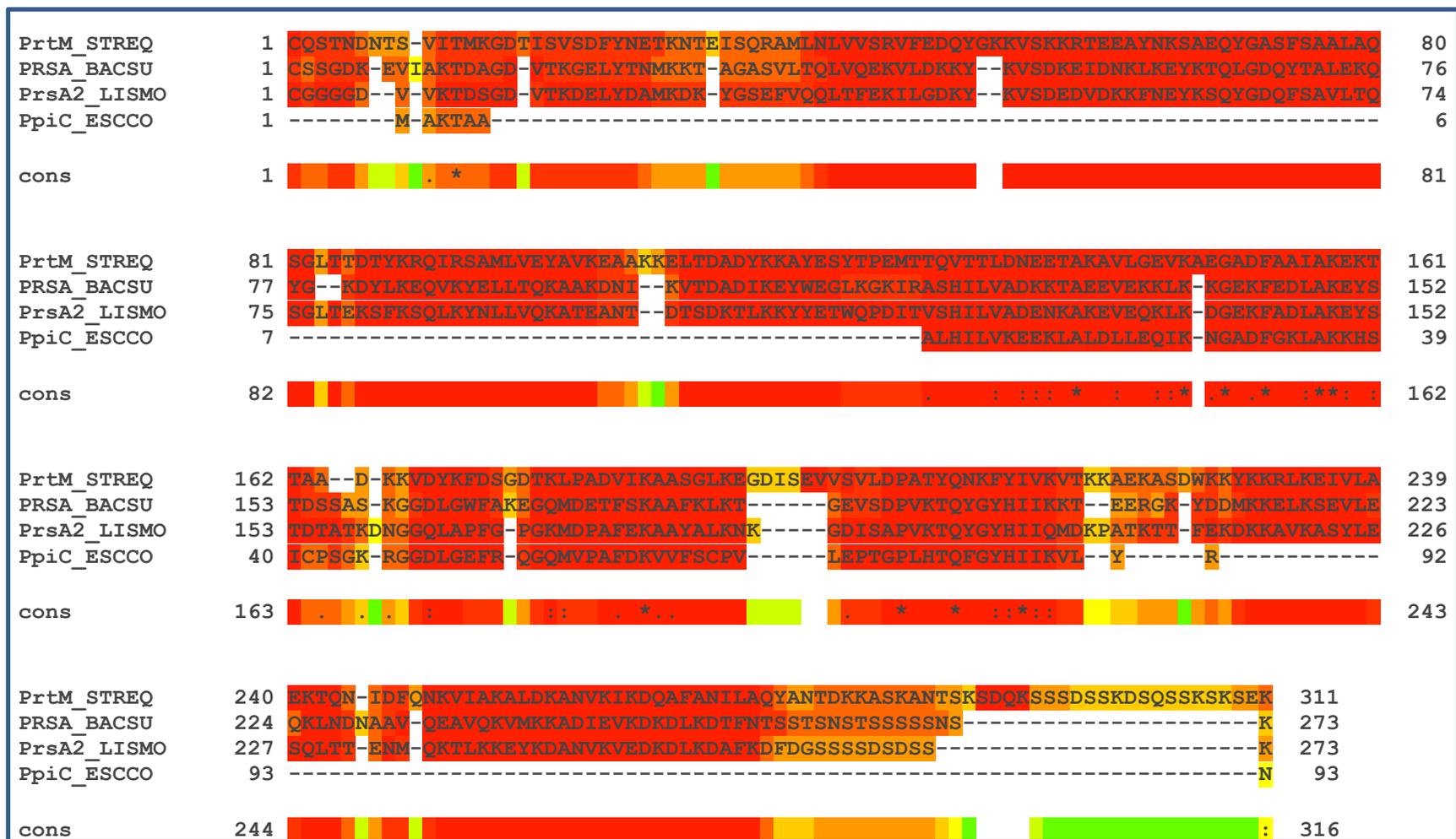


Figure 4.5: M-Coffee alignment (*Bacillus subtilis* PrsA, *Listeria monocytogenes* PrsA2, *Escherichia coli* PpiC, and *S. equi* 4047 PrtM). Identical aa (*); conserved aa substitutions (: or .). Signal peptide sequences were excluded. Identical and conserved residues are concentrated in the central domain region. The colour of individual residue indicates the level of agreement of its alignment with the multiple sequence alignments (Moretti *et al.*, 2007). Dark red: residues aligned in a similar fashion; blue: very low agreement; dark yellow, orange and red: reliably aligned (Moretti *et al.*, 2007). Cons: conserved residues.

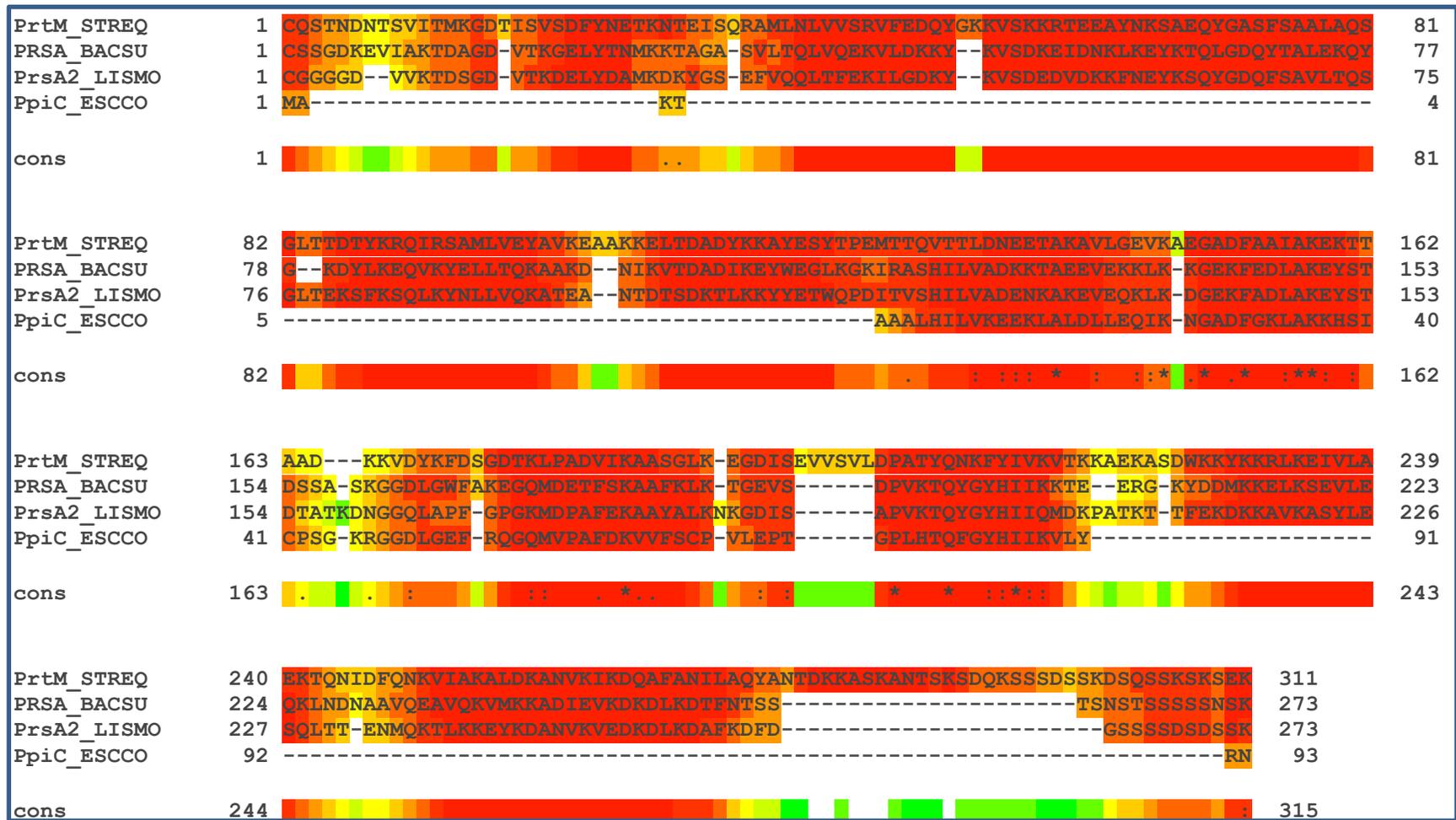


Figure 4.6: T-Coffee alignment (*Bacillus subtilis* PrsA, *Listeria monocytogenes* PrsA2, *Escherichia coli* PpiC, and *S. equi* 4047 PrtM). Identical aa (*); conserved aa substitutions (: or .). Signal peptide sequences were excluded. Identical and conserved residues are concentrated in the central domain region. The residue colour BAD AVG GOOD scheme represents the primary library support for alignment of the residue under consideration on a scale between 0:blue, poorly supported and 9:dark red, strongly supported (Di Tommaso *et al.*, 2011). Cons. means conserved residues.

1	-CSSGDKEVIAKTDAGDVTKGELYTNMK-KTAGASVLTQLVQEKVLDKKY--KVSDKEID	56	PRSA_BACSU
1	-CGGGGD--VVKTDSDGVTKDELYDAMK-DKYGSEFVQQLTFEKILGDKY--KVSDDEDVD	54	PrsA2_LISMO
1	-----	0	PpiC_ESCCO
1	CQSTNDNTSVITMKGDTISVSDFYNETKNTEISQRAMLNLVSRVFEDQYGKKVSKKRTE	60	PrtM_STREQ
57	NKLKEYKTQLGDQYTALEKQYG--KDYLKEQVKYELLTQKAAKDNIK--VTDADIKEYWE	112	PRSA_BACSU
55	KKFNEYKSQYGDQFSAVLTQSGLTEKSFKSQLKYNLLVQKATEANTD--TSDKTLKYYE	112	PrsA2_LISMO
1	-----	0	PpiC_ESCCO
61	EAYNKSAEQYGASFSAAALQSGLTDDTYKRQIRSAMLVEYAVKEAAKELTDADYKKAYE	120	PrtM_STREQ
	. : :: * : : * . * . * : ** : : . . :		
113	GLKGKIRASHILVADKKTAEVEVEKKLK-KGEKFDLAKEYSTDSSAS-KGGDLGWFAKEG	170	PRSA_BACSU
113	TWQPDITVSHILVADENKAKEVEQKLNK-DGEKFDLAKEYSTDATKDNGGQLAPFG-PG	170	PrsA2_LISMO
1	-MAKTAALHILVKEEKLALDLLEQIK-NGADFGKLAKKHSICPSGK-RGGDLGEFR-QG	56	PpiC_ESCCO
121	SYTPEMTTQVTTLDNEETAKAVLGEVKAEGADFAAIAKEKTTAADKK---VDYKFDSDGT	177	PrtM_STREQ
	:: . * .. . * * : ** : : .		
171	QMDETFSKAAFKLKT-----GEVSDPVKTQYGYHI IKKTE---ERGKYDDMKKELKSEV	221	PRSA_BACSU
171	KMDPAFEKAAYALKNK-----GDISAPVKTQYGYHI IQMDKP-ATKTTFEKDKKAVKASY	224	PrsA2_LISMO
57	QMVPAFDKVVFSCPV-----LEPTGPLHTQFGYHII-----KVLYRN-----	93	PpiC_ESCCO
178	KLPADVKAASGLKEGDISEVVSVLDPATYQNKFYIVKVTKKAEKASDWKKYKKRLKEIV	237	PrtM_STREQ
222	LEQKLNDNAAVQEAVQKVMKKADIEVKDKDLKDTFNSTSNSTSSSSSSNSK-----	273	PRSA_BACSU
225	LESQLTT-ENMQKTLKKEYKDANVKVEDKDLKDAFKDFGSSSSSDSDSSK-----	273	PrsA2_LISMO
94	-----	93	PpiC_ESCCO
238	LAEKTQNIDFQNKVIAKALDKANVKIKDQAFANILAQYANTDKKASKANTSKSDQKSSSD	297	PrtM_STREQ
274	-----	273	PRSA_BACSU
274	-----	273	PrsA2_LISMO
94	-----	93	PpiC_ESCCO
298	SSKDSQSSKSKSEK	311	PrtM_STREQ

Figure 4.7: UniProt alignment (*Bacillus subtilis* PrsA, *Listeria monocytogenes* PrsA2, *Escherichia coli* PpiC, and *S. equi* 4047 PrtM). Identical aa (*); conserved aa substitutions (: or .). Identical and conserved residues are concentrated in the central domain region. Signal peptide sequences were excluded.

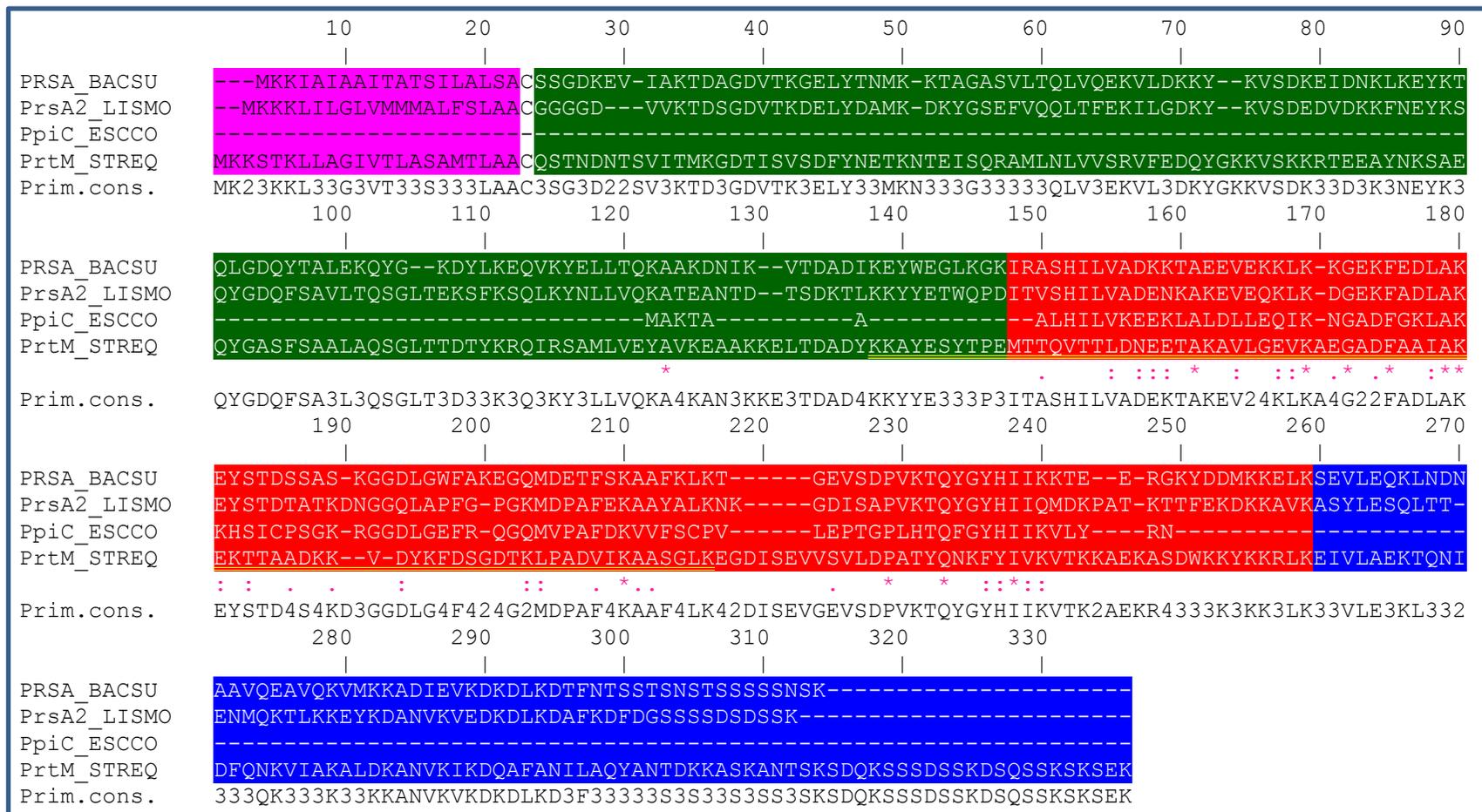


Figure 4.8: CLUSTALW amino acid sequence (*Bacillus subtilis* PrsA, *Listeria monocytogenes* PrsA2, *Escherichia coli* PpiC, and *S. equi* 4047 PrtM) alignment – with signal peptide. N-terminal signal peptide (pink), N-terminal domain (green), Central parvulin-type (PPIase) domain (red) and C-terminal domain (blue). Identical and conserved residues are concentrated in the central domain region. The region deleted in *S. equi* mutant PrtM that was used for this research is underlined yellow.

4.4: Predicted Domain Architecture

By analysing the information generated from Figures 4.4, 4.5, 4.6, 4.7 and 4.8; and by comparing the sequence alignment of PrtM to the domains of the other PPIases, the N-terminal, central and C-terminal domains of PrtM were predicted. The predicted domain architecture of PrtM of *S. equi* 4047 is shown in Figures 4.9a (protein sequence) and 4.9b (pictorial).

MKKSTKLLAGIVTLASAMTLAACQSTNDNTSVITMKGDTISVSDFYNETKNTEISQRAMLNLVVS RVFEDQYGKKVSKKRTEEAYNKSAEQYGASFSAALA
 QSLTTDTYKRQIRSAMLVEYAVKEAAKKELTDADY**KKAYESYTP****MTTOVTTL****NEETAKAVLGEVKAEGADFAAIAKEKT****TAAADKKVDYKFDSG**
DTKLPADV**IKAAASGL****KEGDISEVVS****VLDPATYQNKFYIVKVT****KKAEKASDWK****YKKRLKEIV****LAEKTQ****NIDFQNKVI****AKALD****KANVKIKD****QAFANILAQY**
ANTDKK**ASKANTS****KS****SDQK****SSSD****SKDS****QSSK****SKSEK**

Figure 4.9a: Predicted domain architecture of *S. equi* 4047 PrtM [N-terminal signal peptide (pink) – 22 amino acids; N-terminal domain (green) - 125 amino acids; Central parvulin (PPIase) domain (red) – 109 amino acids; and C-terminal domain (blue) – 77 amino acids]. In bold and underlined yellow is the sequence that was deleted (inframe internal deletion) in the *S. equi* 4047 mutant Δ PrtM that was used for this research.

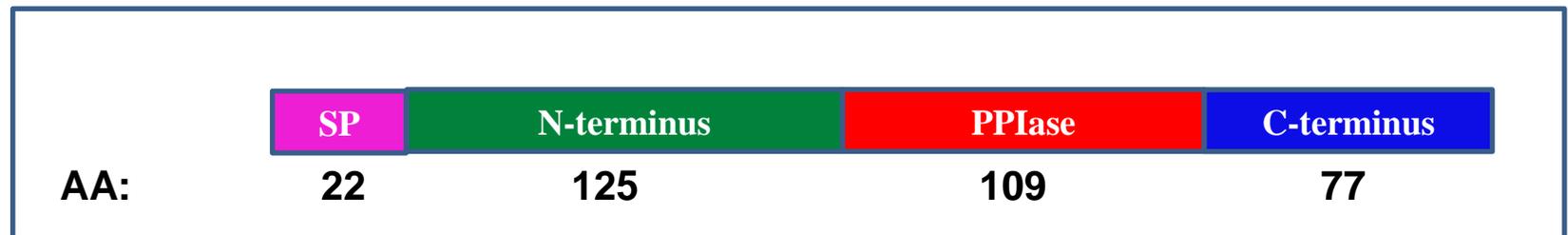


Figure 4.9b: Pictorial of Predicted domain architecture of *S. equi* 4047 PrtM [N-terminal signal peptide (pink) – 22 amino acids; N-terminal domain (green) - 125 amino acids; Central parvulin (PPIase) domain (red) – 109 amino acids; and C-terminal domain (blue) – 77 amino acids].

4.5 PrtM recombinant Protein characteristics

Figure 4.10 shows protein characteristics of the full-length his-tagged *S. equi* 4047 PrtM protein (without signal peptide). Figure 4.11 shows the protein characteristics of the his-tagged *S. equi* 4047 PrtM central domain. The molecular weight and pI of the his-tagged proteins of interest was determined as described in Section 2.3.7 and results shown in Figures 4.10 and 4.11.

10	20	30	40	50	60
HHHHHCQST	NDNTSVITMK	GDTISVSDFY	NETKNTEISQ	RAMLNLVVSR	VFEDQYGKKV
70	80	90	100	110	120
SKKRTEEAYN	KSAEQYGASF	SAALAQSGLT	TDTYKRQIRS	AMLVEYAVKE	AAKKELTDAD
130	140	150	160	170	180
YKKAYESYTP	EMTTQVTTLD	NEETAKAVLG	EVKAEGADFA	AIAKEKTAA	DKKVDYKFDS
190	200	210	220	230	240
GDTKLPADVI	KAASGLKEGD	ISEVVSVDLP	ATYQNKFYIV	KVTKKAEKAS	DWKKYKKRLK
250	260	270	280	290	300
EIVLAEKTQN	IDFQNKVIAK	ALDKANVKIK	DQAFANILAQ	YANTDKKASK	ANTSKSDQKS
310					
SSDSSKDSQS	SKSKSEK				
Number of amino acids: 317					
Molecular weight: 35183.4					
Theoretical pI: 9.07					
Amino acid composition: CSV format					
Ala (A)	38	12.0%			
Arg (R)	6	1.9%			
Asn (N)	14	4.4%			
Asp (D)	24	7.6%			
Cys (C)	1	0.3%			
Gln (Q)	14	4.4%			
Glu (E)	22	6.9%			
Gly (G)	9	2.8%			
His (H)	6	1.9%			
Ile (I)	13	4.1%			
Leu (L)	15	4.7%			
Lys (K)	48	15.1%			
Met (M)	4	1.3%			
Phe (F)	8	2.5%			
Pro (P)	3	0.9%			
Ser (S)	31	9.8%			
Thr (T)	25	7.9%			
Trp (W)	1	0.3%			
Tyr (Y)	14	4.4%			
Val (V)	21	6.6%			
Pyl (O)	0	0.0%			
Sec (U)	0	0.0%			
(B)	0	0.0%			
(Z)	0	0.0%			
(X)	0	0.0%			
Total number of negatively charged residues (Asp + Glu): 46					
Total number of positively charged residues (Arg + Lys): 54					
Atomic composition:					
Carbon	C	1537			
Hydrogen	H	2476			
Nitrogen	N	424			
Oxygen	O	508			
Sulfur	S	5			
Formula: C ₁₅₃₇ H ₂₄₇₆ N ₄₂₄ O ₅₀₈ S ₅					
Total number of atoms: 4950					
Extinction coefficients:					
Extinction coefficients are in units of M ⁻¹ cm ⁻¹ , at 280 nm measured in water.					
Ext. coefficient		26360			
Abs 0.1% (=1 g/l)		0.749			assuming all pairs of Cys residues form cystines
Ext. coefficient		26360			
Abs 0.1% (=1 g/l)		0.749			assuming all Cys residues are reduced

Figure 4.10 Protein characteristics of full-length his-tagged PrtM (without signal peptide).

10	20	30	40	50	60
HHHHHHSSGL VPRGSHMTTQ VTLLDNEETA KAVLGEVKAE GADFAAIAKE KTTAADKKVD					
	70	80	90	100	110
YKFDSDGDTKL PADVIKAASG LKEGDISEVV SVLDPATYQN KFYIVKVTKK A					
<hr/>					
Number of amino acids: 111					
Molecular weight: 11943.4					
Theoretical pI: 6.71					
Amino acid composition:					CSV format
Ala (A)	14		12.6%		
Arg (R)	1		0.9%		
Asn (N)	2		1.8%		
Asp (D)	9		8.1%		
Cys (C)	0		0.0%		
Gln (Q)	2		1.8%		
Glu (E)	7		6.3%		
Gly (G)	7		6.3%		
His (H)	7		6.3%		
Ile (I)	4		3.6%		
Leu (L)	6		5.4%		
Lys (K)	14		12.6%		
Met (M)	1		0.9%		
Phe (F)	3		2.7%		
Pro (P)	3		2.7%		
Ser (S)	7		6.3%		
Thr (T)	10		9.0%		
Trp (W)	0		0.0%		
Tyr (Y)	3		2.7%		
Val (V)	11		9.9%		
Pyl (O)	0		0.0%		
Sec (U)	0		0.0%		
(B)	0		0.0%		
(Z)	0		0.0%		
(X)	0		0.0%		
Total number of negatively charged residues (Asp + Glu): 16					
Total number of positively charged residues (Arg + Lys): 15					
Atomic composition:					
Carbon	C		527		
Hydrogen	H		842		
Nitrogen	N		146		
Oxygen	O		168		
Sulfur	S		1		
Formula: C ₅₂₇ H ₈₄₂ N ₁₄₆ O ₁₆₈ S ₁					
Total number of atoms: 1684					
Extinction coefficients:					
This protein does not contain any Trp residues. Experience shows that this could result in more than 10% error in the computed extinction coefficient.					
Extinction coefficients are in units of M ⁻¹ cm ⁻¹ , at 280 nm measured in water.					
Ext. coefficient		4470			
Abs 0.1% (=1 g/l)		0.374			

Figure 4.11 Protein characteristics of his-tagged central domain of *S. equi* 4047 PrtM.

4.6 Primers

All primers used for this study were designed as stated in section 2.3.6. These primers are listed in Table 2.11 and marked in Figure 4.1. In determining the primers for the *S. equi* 4047 PrtM full gene (results in Section 5), the forward primer was designed to upstream sequence containing native promoter and ribosome binding sites. The internal forward and reverse primers for amplifying the sequence encoding the PrtM central domain were designed with restriction sites to allow for cloning as described in Section 5. Forward and reverse primers for complemented mutants have restriction sites to enable PrtM to be cloned into plasmid pVA838 for complementation (results in Chapter 8). Internal forward and reverse primers have restriction sites to clone portions of PrtM into pVA828 for complementation. The reverse primers for cloning full PrtM gene into pET28a for recombinant gene expression in *E. coli* were designed with the same reverse primers used for cloning full PrtM gene into pVA838, to avoid designing another primer. Reverse primers had stop codon (TAG) included. For the expression of PrtM central domain recombinant protein in *E. coli*, internal forward primers were designed with restriction sites to enable cloning into pET28a.

4.7 Computer model of protein three dimensional (3D) structure:

The 3D structure of PrtM (central domain underlined yellow in Figure 4.9a) was obtained as described in Section 2.3.8.

The PSIPRED Protein Sequence Analysis Workbench (<http://bioinf.cs.ucl.ac.uk/psipred/>) generated the central domain 3D model (Figure 4.12) by selecting the best scoring hits using softwares PSI-BLAST, pGenTHREADER and HHPred against the current query protein and the protein database. pGenTHREADER software generated alignment scores against input sequences from the protein database. PSIPRED v3.3 generated a predicted protein secondary structure (Figure 4.13). All the information generated via The PSIPRED Protein Sequence Analysis Workbench (<http://bioinf.cs.ucl.ac.uk/psipred/>) can be viewed via this link: <http://bioinf.cs.ucl.ac.uk/psipred/result/22b4bc7c-3332-11e3-875b-00163e110593> (for *S. equi* PrtM central domain).

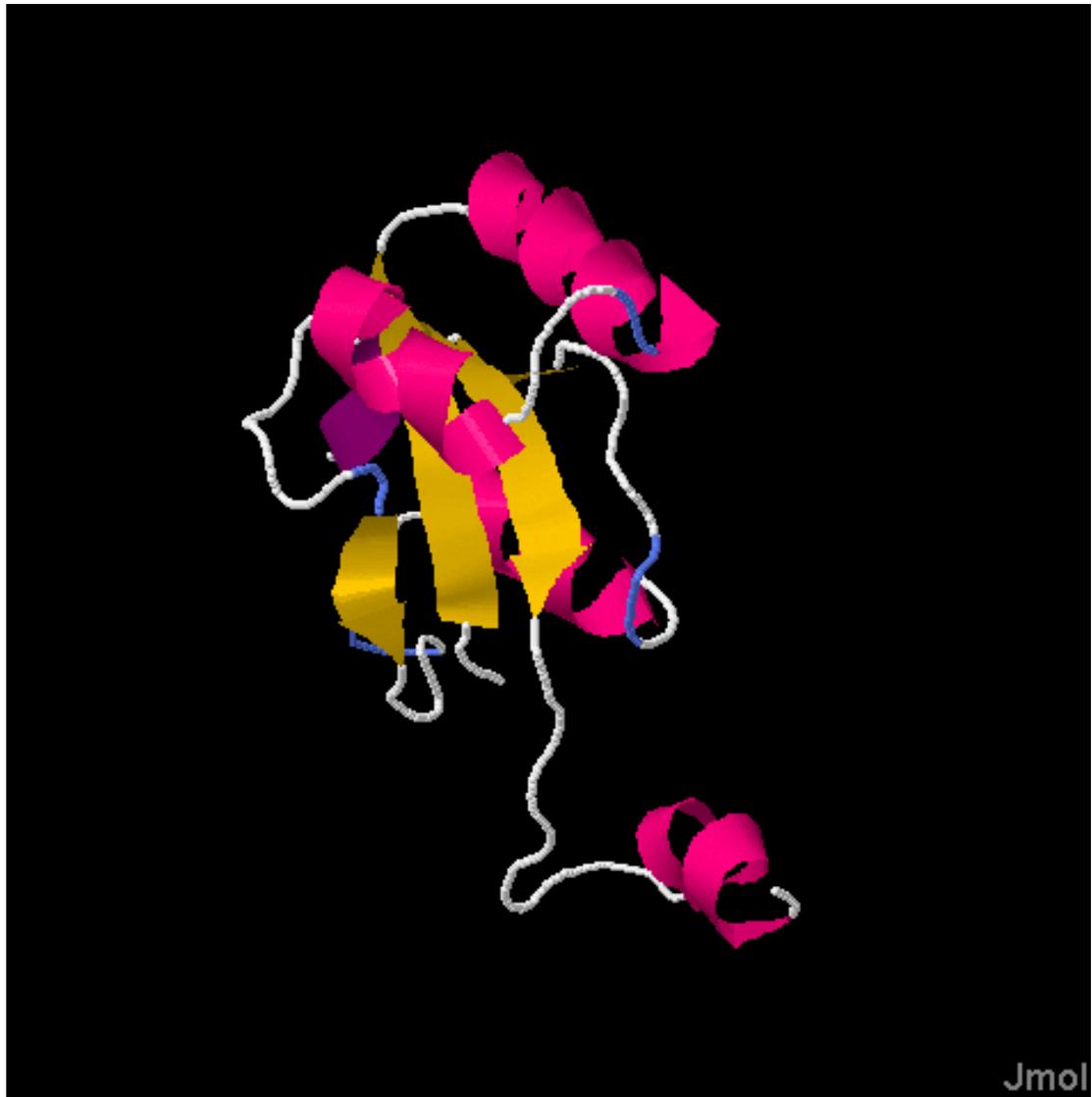


Figure 4.12: *S. equi* 4047 PrtM central domain 3D model. Helices are highlighted pink and sheets highlighted yellow.

4.8 Discussion

PrsA of *B. subtilis* shares a significant degree of amino acid similarity (45% identity, and 65% similarity) with *L. monocytogenes* PrsA2 (Alonzo *et al.*, 2011). Several alignment programs were used to get the most biologically correct alignment, by cross-examining the resulting alignments. ClustalW alignment yielded: 22.69% identity and 24.48 similarity for PrtM_STREQ and PRSA_BACSU; 29.17% identity and 21.73% similarity for PrtM_STREQ and PrsA2_LISMO; and 5.11% identity and 9.61% similarity for PrtM_STREQ and PpiC_ESCCO (see protein sequences in Figure 4.8) and T-coffee alignment, which is a consistency based alignment (Tommaso *et al.*, 2011), gave a main score of 85 (Figure 4.6) which is quite high considering the fact that a value of a 100 means full agreement between the considered alignment and its associated primary library (Tommaso *et al.*, 2011). The PPIase domains of PrtM of *S. equi* 4047, *E. coli* PpiC, *B. subtilis* PrsA and *L. monocytogenes* PrsA2 share identical putative active site residues (Figures 4.4, 4.5, 4.6, 4.7 and 4.8) with the identical and conserved residues being concentrated in the central domain region.

Multiple alignments, which are a necessary pre-requisite in phylogeny, remote homologue detection and structure prediction (Wallace *et al.*, 2006) were therefore valuable in elucidating the domain architecture of PrtM of *S. equi* 4047. This understanding was necessary for accurate design of primers used in Chapters 5 and 8 (see a list of primers used for this study in Table 2.11) and for the production of complemented mutants in chapter 8 of this study.

E. coli PpiC (PpiC_ESCCO in Figures 4.4 – 4.8) is the prototype of the parvulin protein family, and defines the minimal catalytic domain of the entire body of parvulin-like enzymes (Kuhlewein *et al.*, 2004). Figures 4.7 and 4.8 show that *E. coli* PpiC, *B. subtilis* PrsA and *L. monocytogenes* PrsA2 share a good number of conserved and identical amino acids with *S. equi* 4047 PrtM, especially in the central-parvulin domain. The central domain contains a lot of conserved amino acids, compared to the catalytic domain of other parvulins (Figures 4.8, 4.9a and 4.9b) suggesting that the central domain of the PrtM of *S. equi* 4047 is a parvulin domain.

Generally, parvulins are made up of one, rarely two parvulin boxes and additional N- and C-terminal domains (Kuhlewein *et al.*, 2004) with varying lengths and roles in substrate binding and/or chaperone-like catalysis of folding (Behrens *et al.*, 2001; Vitikainen *et al.*, 2004). The predicted modular organization of *S. equi* 4047 PrtM (Figures 4.9a and 4.9b) is similar to that of *B. subtilis* PrsA and *L. monocytogenes* PrsA2, with N- and C-terminal domains flanking a central domain which is predicted in this study to also be a PPIase domain. Protease-coupled PPIase assay results (Chapter 6 of this study) further confirm the initial prediction that the central domain of the PrtM of *S. equi* 4047 has PPIase activity.

Computer generated molecular model information (Figure 4.12 and 4.13) indicates that *S. equi* 4047 PrtM central domain is made up of four α -helices and four β -sheets.

Information generated from the protein parameters search (Section 4.5 - Figures 4.10 and 4.11) were valuable. For example, the knowledge of protein molecular weights generated

(35183.4 Da for his-tagged full-length protein and 11943.4 Da for his-tagged central domain protein in Figures 4.10 and 4.11 respectively) were valuable in comparing and confirming the sizes of the recombinant proteins (Chapter 5). The extinction coefficient (Figures 4.10 and 4.11) were valuable in calculating the concentrations (method in 2.5.18) of purified the recombinant proteins (generated in Chapter 5) which were used for enzyme assay (Chapter 6) and crystallography (Chapter 7).

5. Results of Production of PrtM (Full Length and Central Domain) recombinant Proteins of *S. equi* 4047 WT

Full length and central domain recombinant PrtM proteins from wild type (WT) *S. equi* 4047 were produced in *Escherichia coli* as discussed in this Chapter.

5.1 Introduction

The mode of action and the structure of PrtM of *S. equi* 4047 has never been studied. In order to evaluate the function and structure of the PrtM of *S. equi* 4047, and to ascertain whether individual domains within the predicted PrtM architecture (Chapter 4, Figure 4.9a and 4.9b) contribute to its PPIase activity, the full length and central domain genes of PrtM were cloned and transformed into expression host *E. coli* BL21 (results in Section 5.2). The best conditions for expressing the full length and central domain recombinant proteins were first optimized and confirmed on a small scale (results in Section 5.3), followed by large scale overexpression and then purification to homogeneity by IMAC (method in Sections 2.6.2 and 2.6.3 and results in Section 5.4) and desalted and concentrated by centrifugal filtration (method in Section 2.6.5) prior to characterization/function/structure analysis (in Sections 5.4, 5.5, 5.6; Chapters 6 and 7).

The purity of recombinant proteins was judged by SDS-PAGE (method in Section 2.5.19, result in Figure 5.5 and 5.6) and the identities of the recombinant proteins verified by peptide mass fingerprinting (results in Section 5.5).

5.2 Results of cloning

To produce full length and central domain recombinant proteins, the complete PrtM gene (Chapter 4, Figure 4.1) as well as the central domain (sequence underlined yellow in Figure 4.1, Chapter 4) were amplified by PCR (the sequences of primers SEQPrtmf and SEQPrtmr for full length encoding sequence, and Prtmidcolif and Prtmidcolir for the central domain encoding sequence are shown in Table 2.11, method of PCR in Section 2.5.4) from *S. equi* 4047 genomic DNA.

Following purification of PCR product, the PCR fragments (encoding full length and the central domain), as well as the pET28-a (diagram in Appendix A17) cloning/expression vector were digested (method of digestion in Section 2.5.9) with *NdeI/XhoI*. Images of AGE of double digest products are shown in Figure 5.2. The genes (encoding full length and central domain) were individually ligated (ligation method in Section 2.5.10) into pET28-a/*NdeI/XhoI* cloning vector and the ligation products were transformed into *E. coli* TOP10 (see method of TOP10 transformation in Section 2.5.12).

After transformation, plasmids were extracted from *E. coli* TOP 10 (see method of STET based extraction in Section 2.5.13). The purified plasmid was sequenced (method in section 2.5.15 and result in Appendix E) to confirm proper orientation of DNA insert and correct DNA sequence. The inserts (encoding full length and central domain) were digested out of the plasmid (images of AGE of digest products are shown in Figure 5.2) to confirm successful cloning. After this, recombinant pET28a, which had been re-extracted from *E. coli* TOP10 (using spin-column-based extraction, Figure 5.3, method in Section

2.5.15), were used to transform the expression host, *E coli* BL21 (DE3) (method of BL21 transformation in Section 2.5.12). All *E coli* (TOP10 or BL21) transformants were selectively isolated or grown in LB media containing $- 50 \mu\text{g} / \text{mL}$ kanamycin (Table 2.4). The pET28a cloning vector (Appendix A17) codes for an oligohistidine tag. Therefore, the expressed protein contained an oligohistidine tag at the N-terminus.

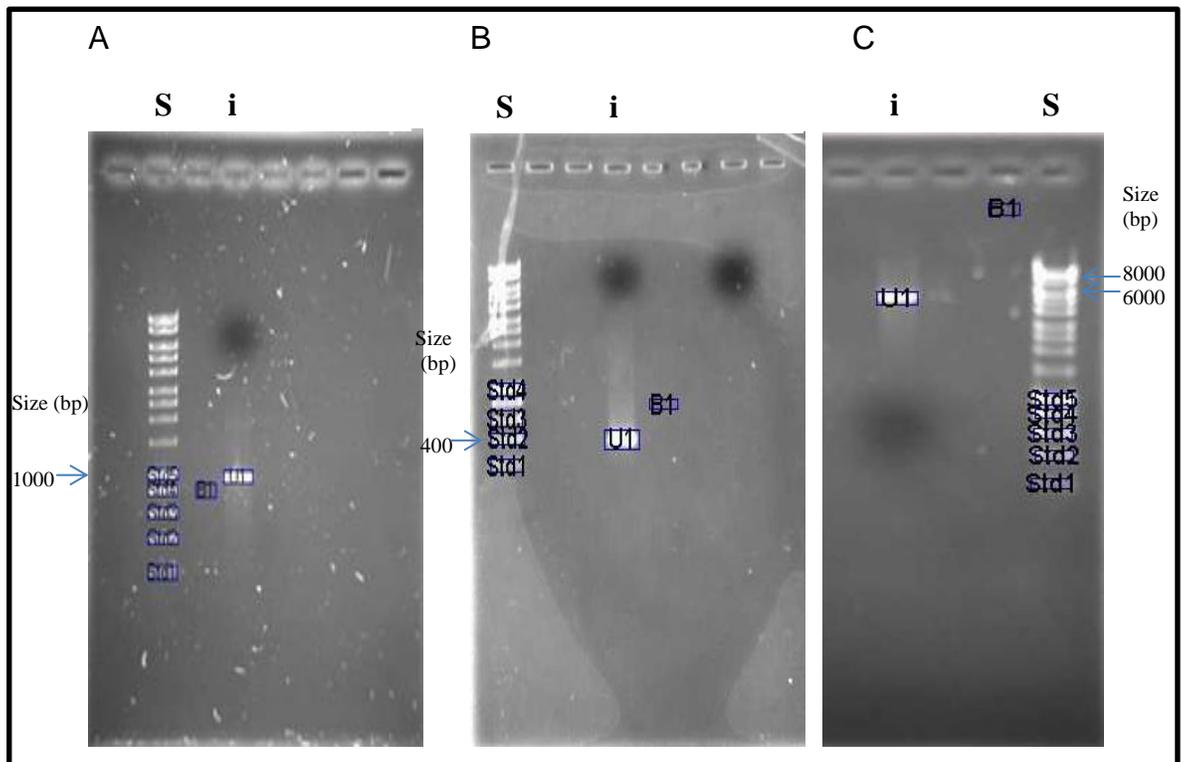


Figure 5.1: Concentration of digested ligation components, post gel purification. *S. equi* 4047 PrtM Full length A i = 53 ng/ μL . *S. equi* 4047 PrtM Central domain DNA B i = 37.3 ng/ μL . C i = purified pET28a/*NdeI/XhoI* double digest, 5296 bp = 93.13 ng/ μL . S = molecular weight DNA size standard (200 – 10,000 bp).

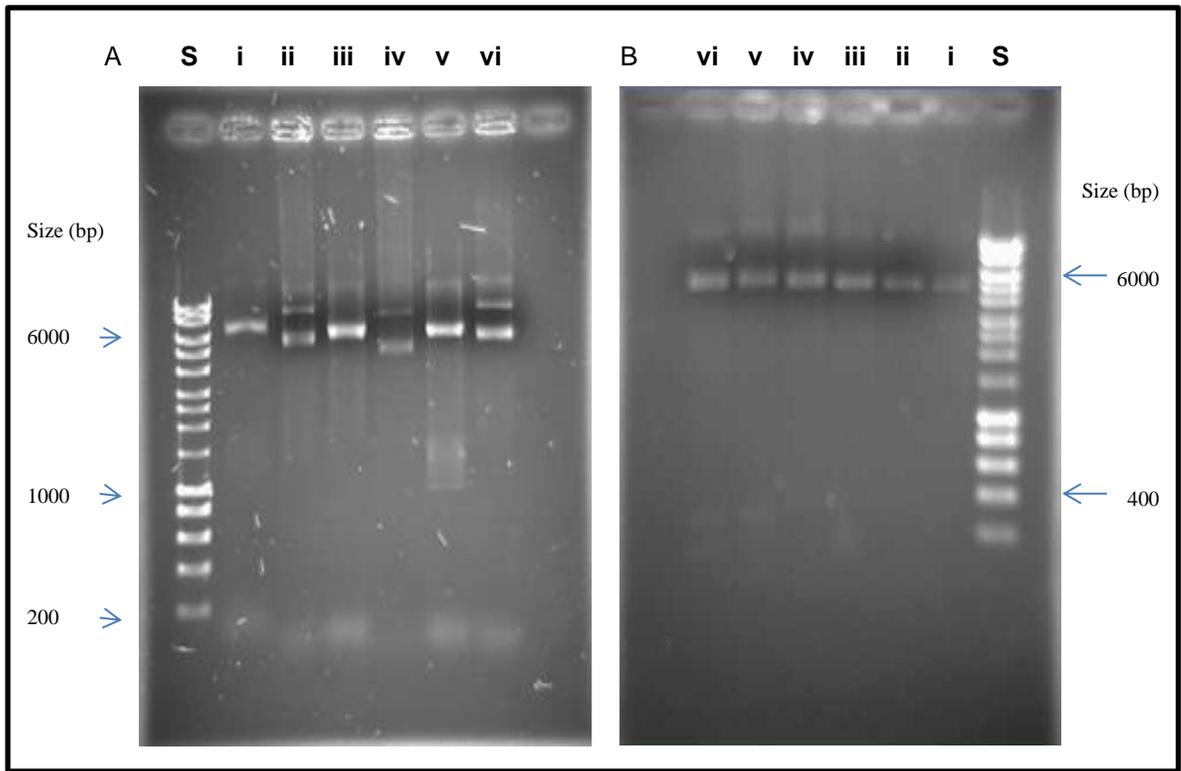


Figure 5.2 Double digestions of plasmids isolated from clones carrying the genes encoding PrtM Full Length and Central Domain. Successful cloning is confirmed here by the observed inserts. A = for Full length PrtM clone: *NdeI/XhoI* digested putative pET28a/full length PrtM clones. A v = Full length DNA insert = approximately 1000 bp in size. Ai, Aiii and Av = *XbaI/XhoI* digested plasmid DNA containing inserts; Aii, Aiv and Avi = *XbaI/XhoI* digested plasmid without insert. B = for PrtM Central Domain clone: *XbaI/XhoI* digested putative pET28a/central domain clones (268 bp band in lanes Bv and Bvi). S = molecular weight DNA size standard (200 – 10,000 bp).

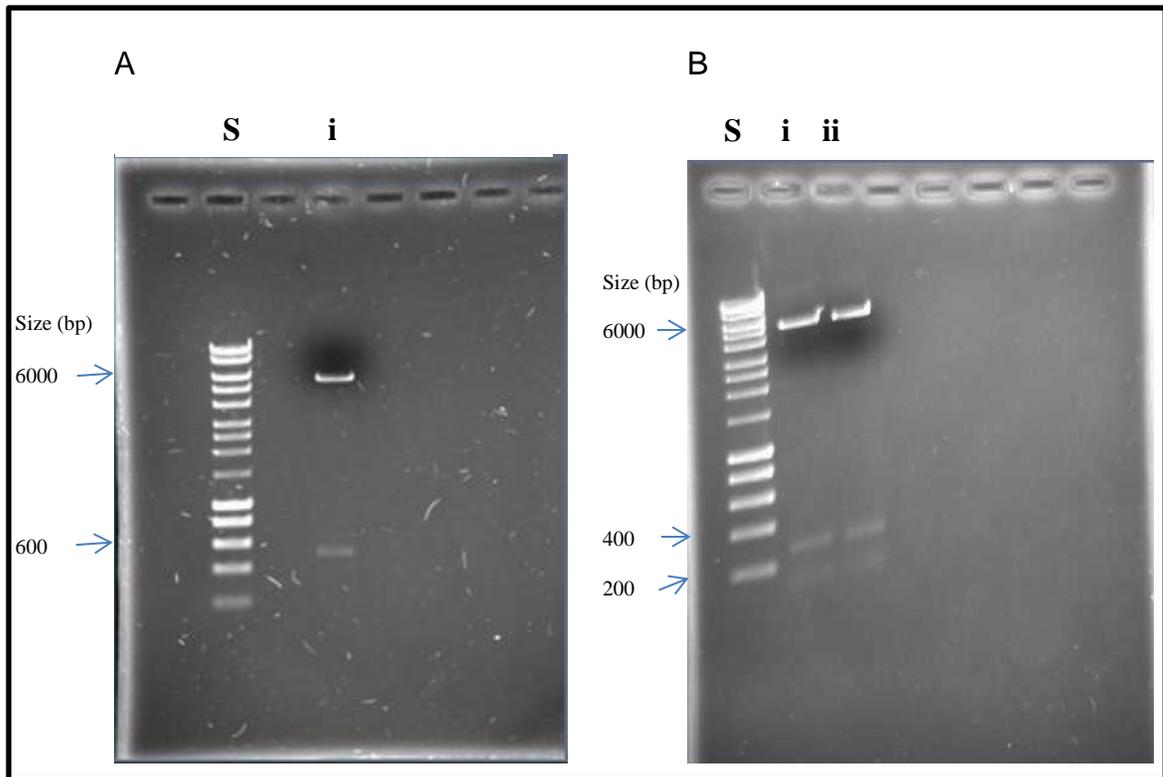


Figure 5.3 Spin-column-based extracted plasmid digestion. A i = *XbaI* digest of pET28a/full length PrtM DNA (bottom = 644 bp and top = 5588 bp fragments); B i and ii = *XbaI/XhoI* digest DNA of pET28a/PrtM central domain (top = 5269, middle = 268 and bottom = 118 bp). These results indicate success of cloning of both (full length and central domain) target inserts. S = molecular weight DNA size standard (200 – 10,000 bp).

5.3 Result of small scale protein expression

After cloning, the expression of recombinant proteins (full-length and central domain) in *E. coli* BL21 was optimized on a small scale as described in Section 2.6.1. Results of small scale expression are shown in SDS polyacrylamide gel in Figure 5.6A. The full length recombinant protein expressed very well cultured at 20°C and at 30°C induction temperatures (Figure 5.4 Aii and Aiii respectively); with slightly more soluble protein at 30°C. At both temperatures, the band size of about 36 kDa is in agreement with predicted

molecular weight (Chapter 4, Figure 4.10). For the central domain recombinant protein, CFE for samples Bii, and Biii (Figure 5.4B) were obtained as described in section 2.6.1. An initial SDS-polyacrylamide gel (SDS-PAG) using 13% (w/v) acrylamide showed bands of expressed recombinant protein in agreement with predicted molecular weight. The extracted CFE were then electrophoresed on 15% SDS-PAG (Figure 5.4B). Increasing the concentration of the SDS-PAG slowed down the migration of the central domain recombinant protein band (approximately 11.5 kDa) in the gel. The central domain recombinant protein (Figure 5.4 B) expressed very well at 20°C and 30°C induction temperatures.

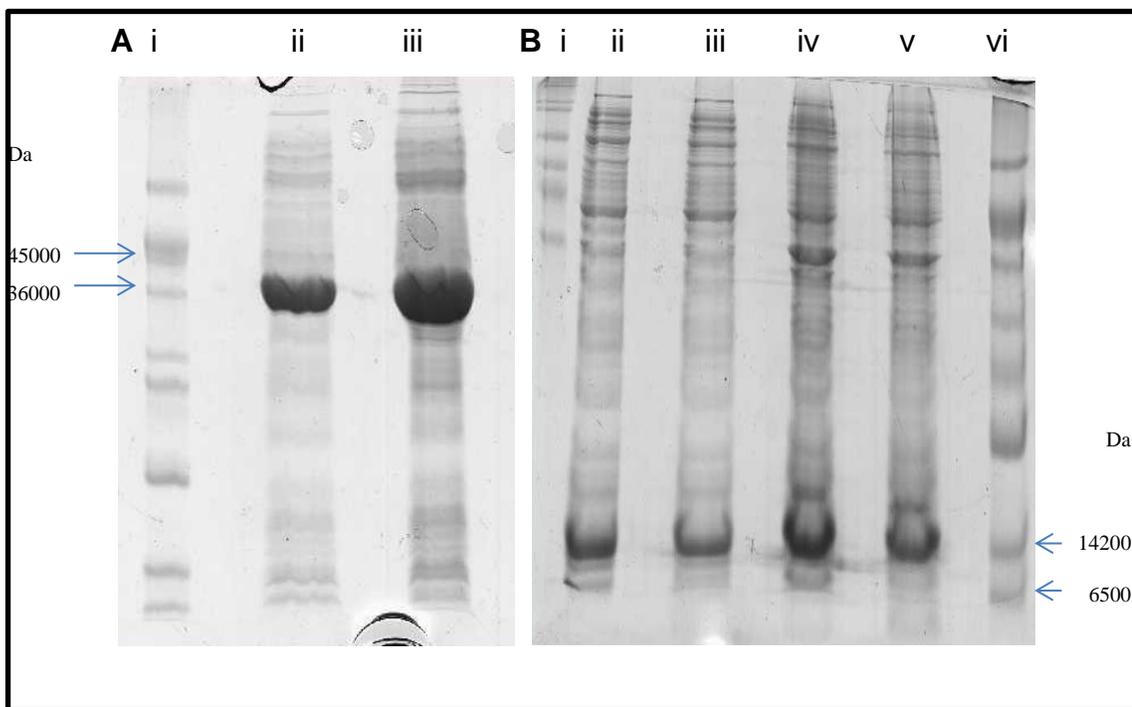


Figure 5.4 SDS-PAGE analysis of PrtM small scale protein expression. A = full length recombinant protein on a 13% (w/v) SDS polyacrylamide gel: Ai = LMW marker, Aii and Aiii were CFE obtained by method in section 2.6.1 and induced at 20°C and 30°C respectively. B = central domain recombinant protein on a 15% (w/v) SDS polyacrylamide gel: Bi = HMW marker, Bii and Biii were CFE obtained by method in section 2.6.1 and induced at 20°C and 30°C respectively. Biv and Bv were CFE obtained as described in section 2.5.21, and induced at 20°C and 30°C respectively. Bvi=LMW marker.

5.4 Result of large scale protein expression and purification

Following the success of small scale expression and optimization of expression conditions (results in Section 5.2), large scale expression of recombinant proteins was carried out using *E. coli* BL21 (DE3) (see method in Section 2.6.2) and CFE of *N*-terminally histidine tagged recombinant proteins purified by gradient elution IMAC with Ni Sepharose high performance resin (method in Section 2.6.3). After SDS-PAGE analysis of IMAC purification (results shown in Figure 5.5), the pure full length and central domain recombinant protein fractions were concentrated and washed using molecular weight concentrator (method in Section 2.6.5, and result in Figure 5.6).

Figure 5.7 shows the result of SDS-PAGE analysis of dialysed, freeze dried full length recombinant protein (method of dialysis in Section 2.6.4). Due to technical problems and the fact that no added advantage was observed, dialysis was discontinued and only molecular weight concentrator was used for washing and purification of proteins for further analysis.

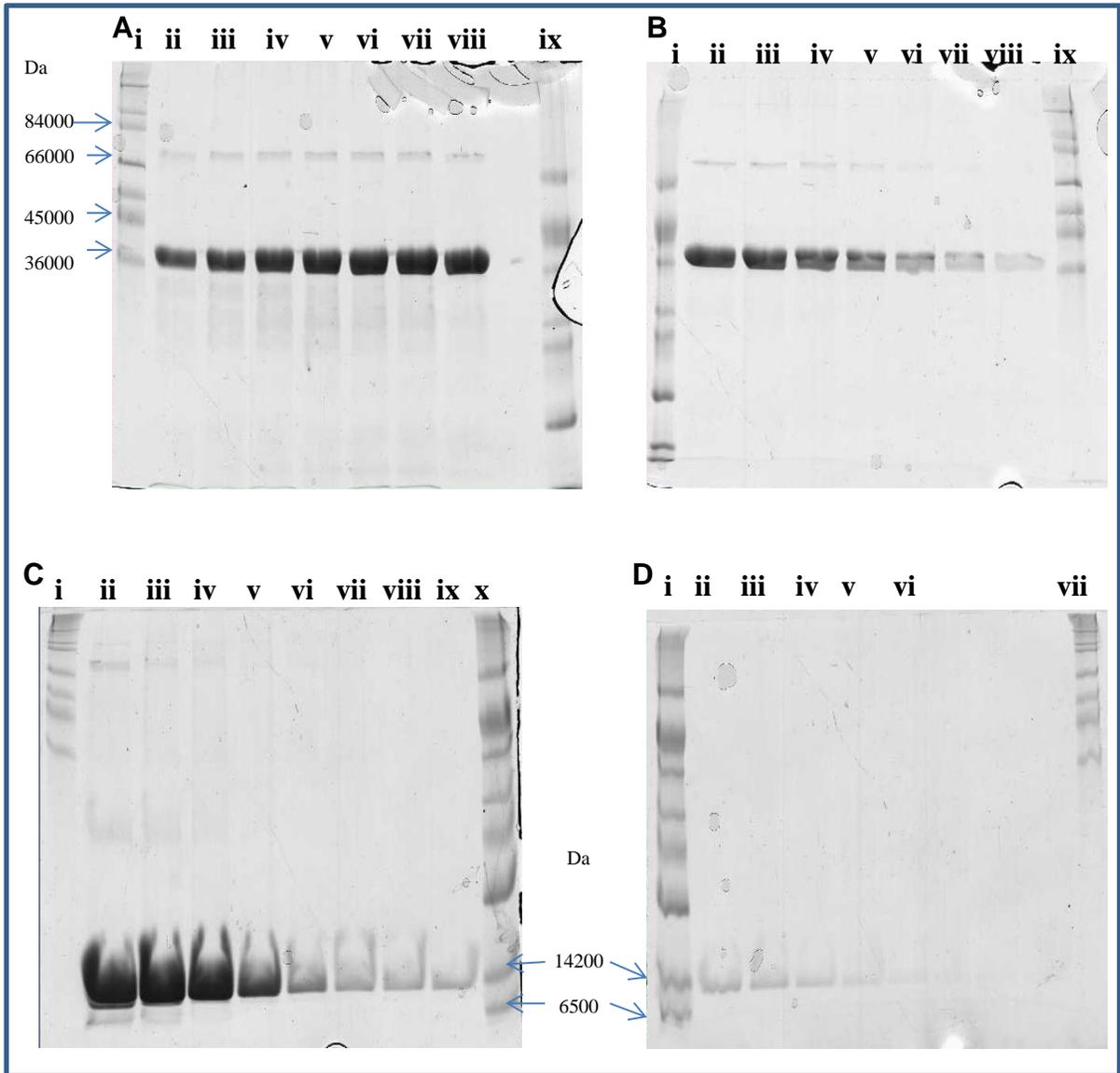


Figure 5.5 SDS-PAGE analysis of post-IMAC purified recombinant proteins. A ii – viii & B ii - viii = PrtM full length recombinant protein fractions. C ii to ix & D ii to vi = PrtM central domain recombinant protein fractions. A ix, B i, C x and D i = LMW marker. A i, B ix, C i and D vii = HMW marker.

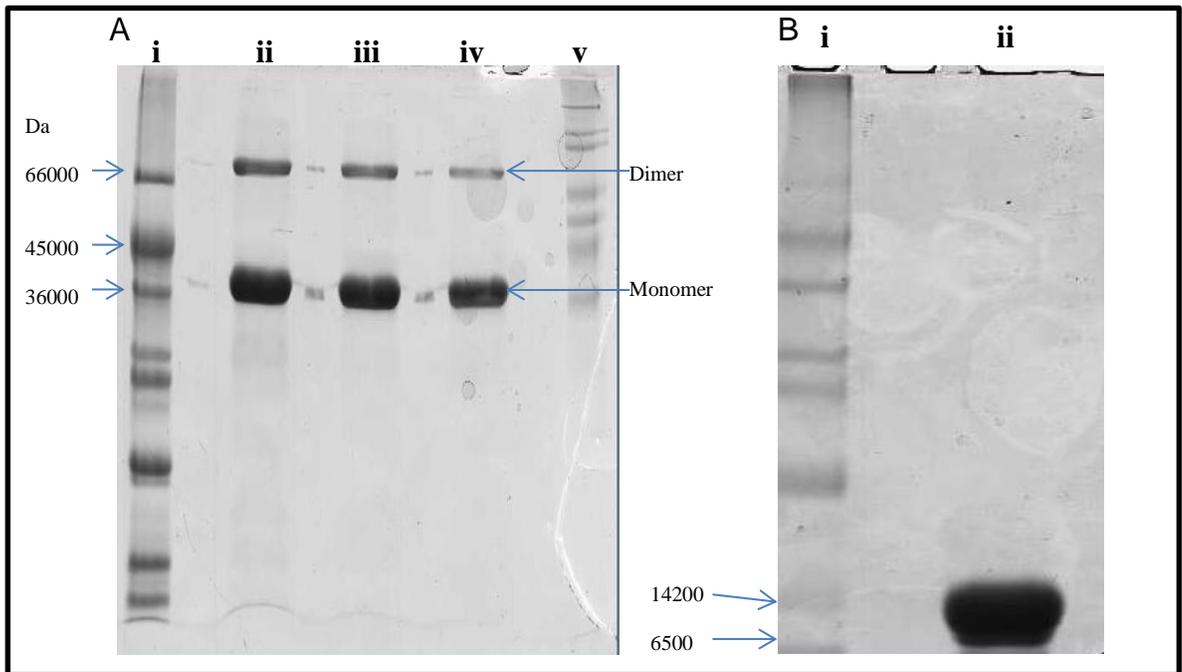


Figure 5.6 SDS-PAGE image of concentrated IMAC-purified recombinant protein: A ii, iii and iv = PrtM full length fractions; B ii = PrtM central domain fraction. The final concentration was determined spectrophotometrically (method in section 2.5.18). A i and B i = LMW marker, A v = HMW marker. The dimer (upper band in A ii, A iii, and A iv) gave a Mascot score of 1304, with 26 unique matches out of 64 and 56 % protein sequence coverage (Table E3, Appendix E). The monomer (lower band in A ii, A iii, and A iv) gave a Mascot score of 1475, with 36 unique matches out of 86 and 59 % protein sequence coverage (Table E3, Appendix E). The central domain recombinant protein (Figure 5.8 Bii) gave a mascot score of 312, with 6 unique matches out of 32 and 44% protein sequence coverage (Table E, Appendix E).

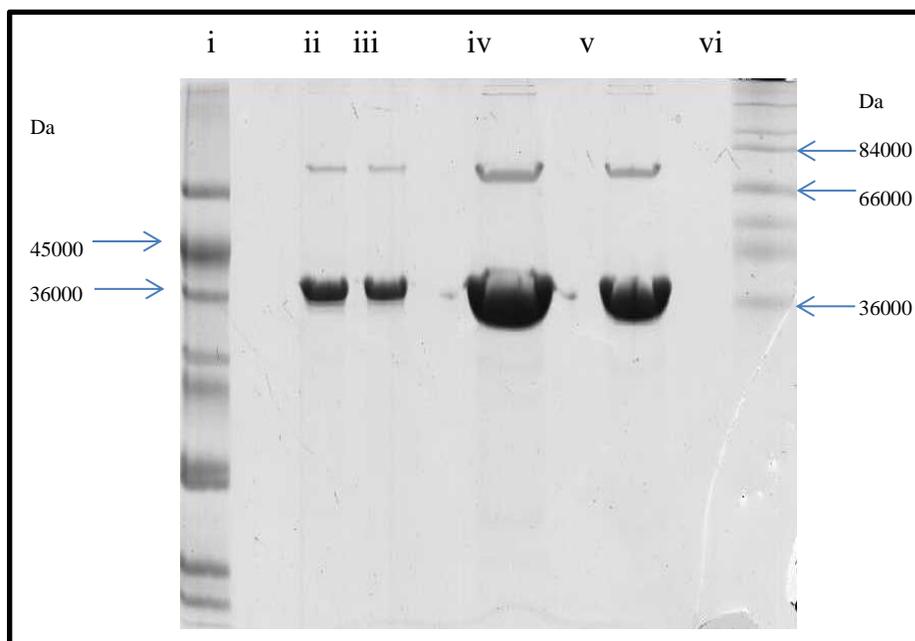


Figure 5.7 SDS-PAGE analysis of reconstituted dialysed full length recombinant protein. ii and iii were fractions from dialysed sample; iv and v were fractions which had been dialysed, freeze dried and resuspended in 18.2 M Ω /cm H₂O. i = LMW marker. vi = HMW marker.

5.5 Result of in-gel tryptic digest and identification of recombinant proteins by HPLC/MS

In order to confirm the identities of the concentrated full length and central domain recombinant proteins, bands from Figure 5.6 (A and B) were excised, trypsinized (method in Section 2.5.29) and identified by HPLC/MS (method in Section 2.5.30). Mascot spectrum analysis (method in Section 2.5.31) reports (links to reports are given below in Table 5.0) confirmed that the full length and central domain recombinant protein bands (Figure 5.6) were the correct proteins and were of *S. equi* 4047 origin.

Recombinant protein fraction	Mascot search result	
	Protein code	Protein name
Full Length - upper band - dimer	Protein hits : gi 225870097	foldase protein PrsA [<i>Streptococcus equi subsp. equi</i> 4047]
Full Length - lower band - monomer	Protein hits : gi 225870097	foldase protein PrsA [<i>Streptococcus equi subsp. equi</i> 4047]
Central domain	Protein hits : gi 225870097	foldase protein PrsA [<i>Streptococcus equi subsp. equi</i> 4047]

Table 5.0: Protein Codes from Mascot spectrum analysis following MS/MS of PrtM recombinant proteins.

5.6 Results of Western blots of recombinant proteins

Western blot analysis (method in Section 2.5.20) of the full length and central domain recombinant proteins revealed reactions of both fractions with α -PpmA antibody.

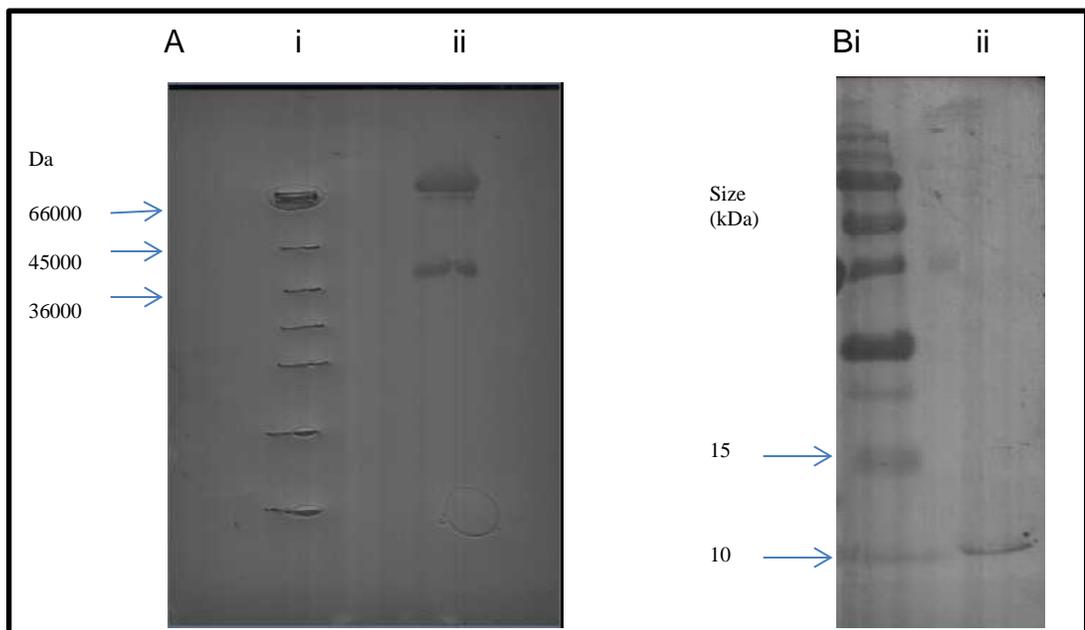


Figure 5.8 Image of Western blot of PrtM recombinant proteins with high titre α -PpmA. Ai = Sigma LMW marker, Aii = reaction of full length purified recombinant protein fraction; Bi = BioRad protein standard, B ii = reaction of central domain purified recombinant protein fraction. Sigma marker (Ai) which was not usually used for Western blots, was used because of unavailability of BioRad protein standard at the time.

5.7 Discussion

The purity of the recombinant protein was examined by SDS-PAGE and this showed that the purified full length recombinant protein (Figure 5.6A) had a molecular weight of about 36 kDa (monomeric unit) which is in agreement with the predicted molecular weight (Section 4, Figure 4.10). The molecular weight of the central domain recombinant protein fraction (Figure 5.6B) was about 11.5 kDa and this is also in agreement with the predicted molecular weight (Section 4, Figure 4.11). The MS and Mascot search results (Table 5.0) and the reactions of both the full length and central domain purified recombinant proteins with α PpmA antibody (Figure 5.8), all confirm that cloning and overexpression experiments were successful and that both protein fractions are of *S. equi* 4047 origin. With this outcome in hand, both purified recombinant protein fractions were used for enzyme assays (Chapter 6) and crystallography (Chapter 7).

Most bacteria have a rigid cell wall which maintains their cell shape and is responsible for resistance of internal turgor pressure (van den Ent *et al.*, 2006). The rigidity of the bacterial cell wall is due to a fine network of glycan strands which are linked together by short peptides forming a peptidoglycan layer - or murein sacculus (Bhavsar and Brown, 2006). Penicillin binding proteins (PBPs), which form and cross-link glycan strands through their glycosyl transferase and transpeptidase activities, are responsible for the assembly of the peptidoglycan layer (Popham and Young, 2003). It has been suggested that some of the proteins (like MreB, MreC and MreD - encoded by the *mreBCD* operon), which affect cell morphology might position the peptidoglycan synthesis machinery (Formstone *et al.*, 2008).

van den Ent *et al.*, (2006) showed that MreC is a dimeric protein whose structure may be important for its putative scaffolding function in the recruitment of PBPs and in organising cell wall synthesis. Consistently, the purified *S. equi* 4047 PrtM full length recombinant protein (Figure 5.6A) contained protein bands equivalent to the dimeric form of about 66 kDa as well as the monomeric form of about 36 kDa on SDS-PAGE. The formation of dimers and oligomers has also been reported for *B. subtilis* PrsA, which is a PPIase implicated in the folding and stability of PBPs (Hyyrylainen, 2010). *L. monocytogenes* PrsA2, a PPIase as well, also forms dimers in solution (Alonzo *et al.*, 2011). From the findings of this study, it is therefore very interesting to observe that the PrtM of *S. equi* 4047 forms dimers (Figure 5.6A), has been implicated to have a role in the folding of PBPs (Section 3.14) and exhibits PPIase activity (Chapter 6).

6.0 Results of Enzyme Assays of *S. equi* PrtM (Full Length and Central Domain) Proteins

6.1 Introduction

Peptidylprolyl *cis-trans* isomerases are found in all organisms and are important for the folding of newly synthesized proteins (Bang *et al.*, 2000; Gemmill *et al.*, 2005). PPIases are believed to be chaperones or folding isomerases which induce conformational changes in mature proteins, post-translationally altering their structure and intermolecular interactions, thereby affecting their activity (Rutherford and Zuker, 1994). Proline isomerization has been proposed as a potential rate-limiting step in *in vivo* protein folding (Brandts *et al.*, 1975; Lazar and Kolter, 1996).

In this study, the enzyme activity of *S. equi* 4047 PrtM was investigated as a follow up to bioinformatics analysis (Figures 4.9a, 4.9b, 4.12 and 4.13), to confirm that it (PrtM) is a true PPIase. To do this, the standard protease-coupled assay for PPIase activity (Section 2.8) was employed. The prolyl bond of the peptide substrates exist in an equilibrium of about 5-20% *cis*- and 80-95% *trans*-conformers (Hani *et al.*, 1999). Endopeptidases (like chymotrypsin, trypsin, or subtilisin) split off the C-terminal pNA residue (chromophore) only in the *trans* population of these proline-containing substrates such that in the presence of sufficient amounts of protease in the reaction mixture, the *trans* population is cleaved off rapidly, whereas the *cis* population remains intact (Kofron *et al.*, 1991; Kay 1996; and Hani *et al.*, 1999). After this first rapid (burst) phase, the slow isomerization reaction that

follows (second, slow phase) is accelerated by PPIases and results in the production of protease-cleavable *trans* substrate (Kofron *et al.*, 1991 and Kay 1996).

It had previously been demonstrated that PPIases exhibit a range of substrate specificities and that the PPIase assay is generally assessed by isomer-specific proteolysis using tetrapeptide derivatives as peptide substrates (Harrison and Stein, 1990; Hani *et al.*, 1999). Comparison of *S. aureus* PrsA with *B. subtilis* PrsA (both PPIases), indicates differences in the substrate preference. In this study three peptides (Pep1 - Suc-Ala-Phe-Pro-Phe-pNA; Pep2 - Suc-Ala-Lys-Pro-Phe-pNA; and Pep3 - Suc-Ala-Ala-Pro-Phe-pNA; Table 2.8) were used for the PPIase assay. Results (method in section 2.8.1 and results of analysis in Appendix I, Figures I1 and I2) revealed that Pep2 (having uncharged, polar and hydrophilic lysine before the proline residue) and Pep3 (having uncharged, non-polar alanine before the proline residue) were not substrates for PrtM. The enhanced rate of cleavage of varying concentrations of Pep1 in the presence of PrtM of *S. equi* 4047 (method in section 2.8.1 and results shown in Figures 6.1 – 6.4, and Appendix I3) confirms that PrtM of *S. equi* 4047 is a bonafide PPIase.

After the PPIase assay, the PMSF assay (method in Section 2.8.2, and results in Section 6.3) was carried out to find out if chymotrypsin (a protease) had any significant effect on the recombinant proteins. Although the central domain recombinant protein showed a faster rate of reaction with Pep1, than the full length protein (Figures 6.1 to 6.4), because of the observed significant cleavage of the central domain recombinant protein by

chymotrypsin (Figure 6.5B), the enzyme kinetics (method in Section 2.8.3) of only PrtM full length recombinant protein was subsequently calculated (results in Section 6.4).

PPIase activity of *S. equi* 4047 PrtM was expressed as k_{cat}/K_M ($\text{M}^{-1} \text{s}^{-1}$) – method in Section 2.8.3 and results in Section 6.4.

6.2 Protease coupled PPIase assay results

Results of the PPIase assay are shown in Figures 6.1 – 6.4.

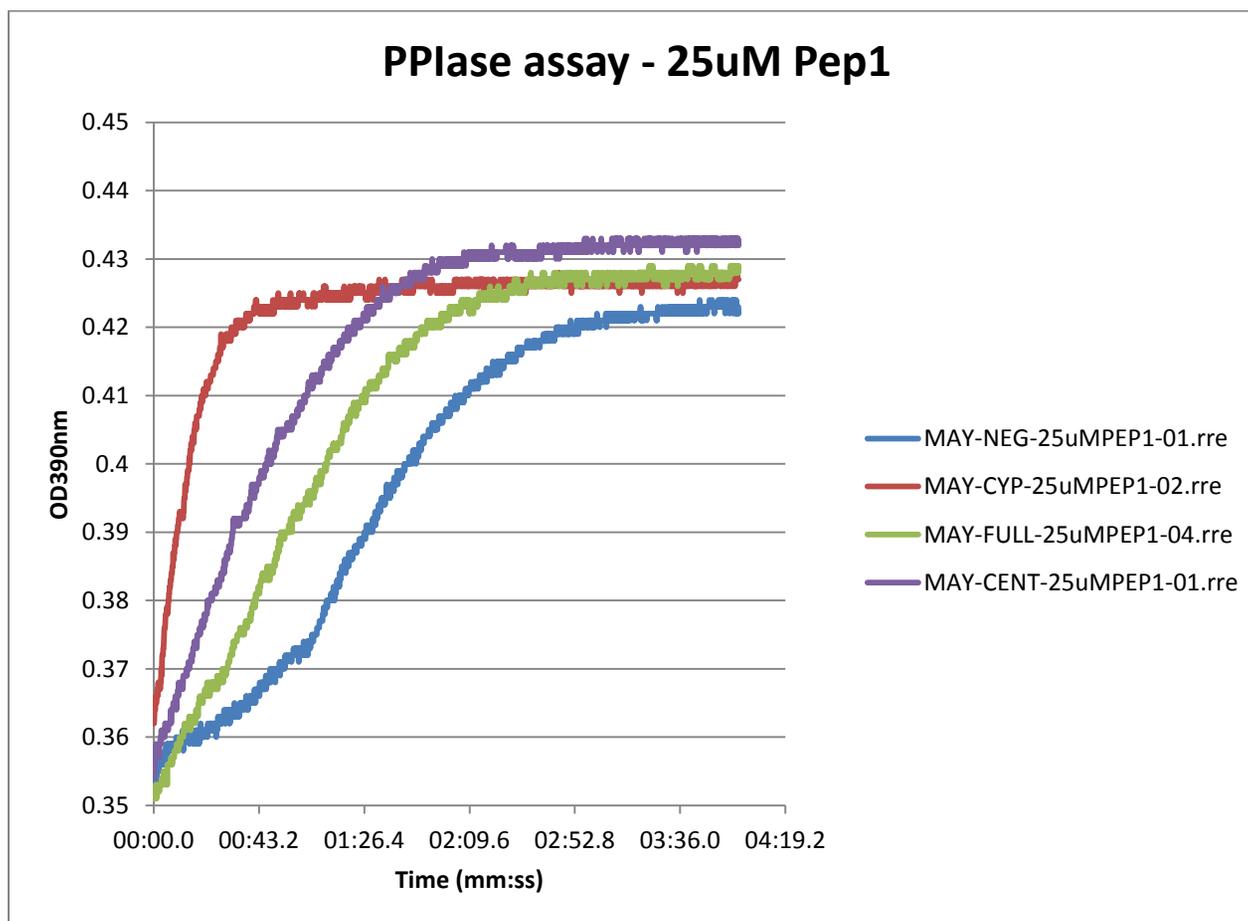


Figure 6.1: Protease coupled PPIase assay with 25 μ M Pep1. The enhanced rate of cleavage of Pep1 in the presence of 25 μ M of PrtM of *S. equi* 4047 is seen here. NEG = negative control, CYP = cyclophilin (positive control), FULL = full length recombinant protein. CENT = central domain recombinant protein.

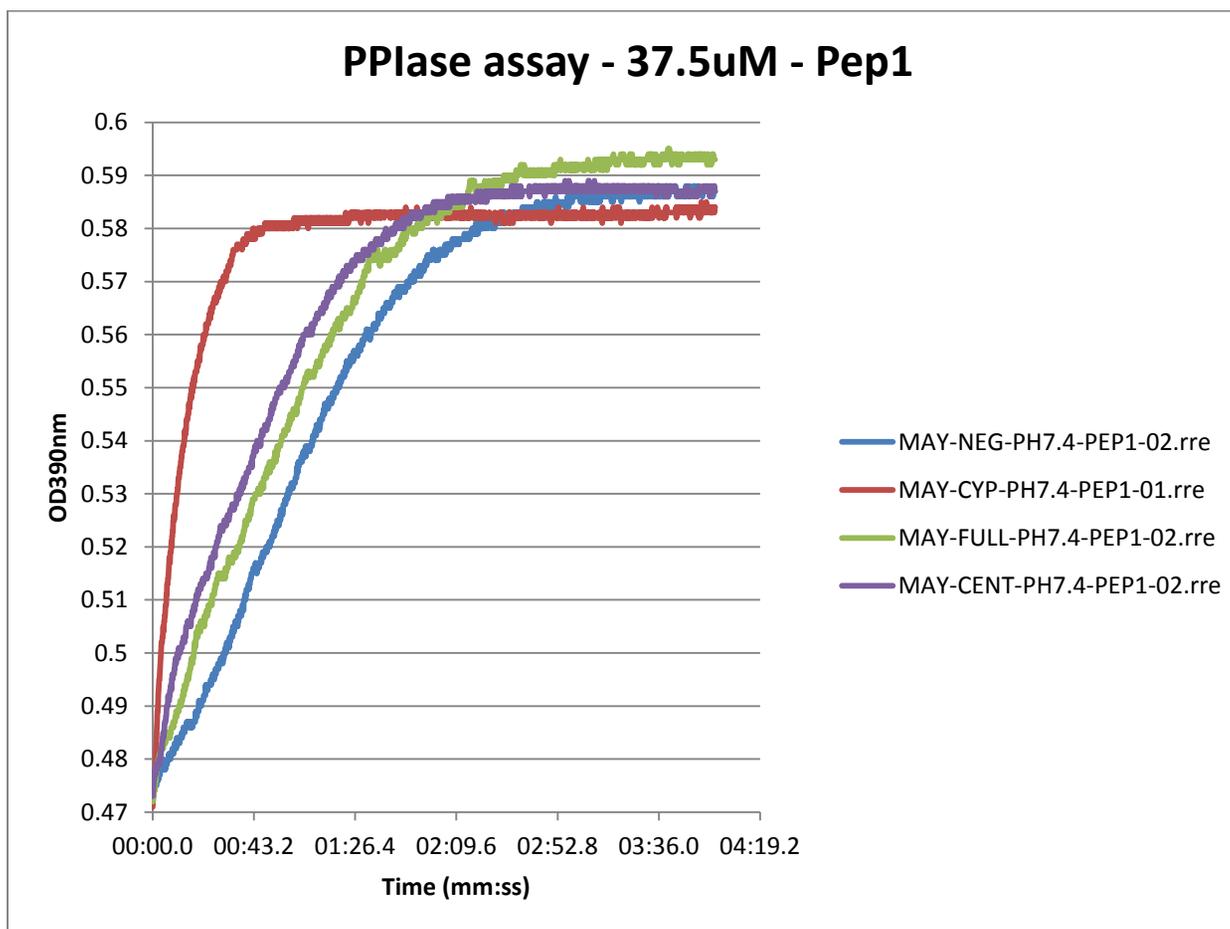


Figure 6.2: Protease coupled PPIase assay with 37.5 μ M Pep1. The enhanced rate of cleavage of Pep1 in the presence of 37.5 μ M of PrtM of *S. equi* 4047 is seen here. NEG = negative control, CYP = cyclophilin (positive control), FULL = full length recombinant protein. CENT = central domain recombinant protein.

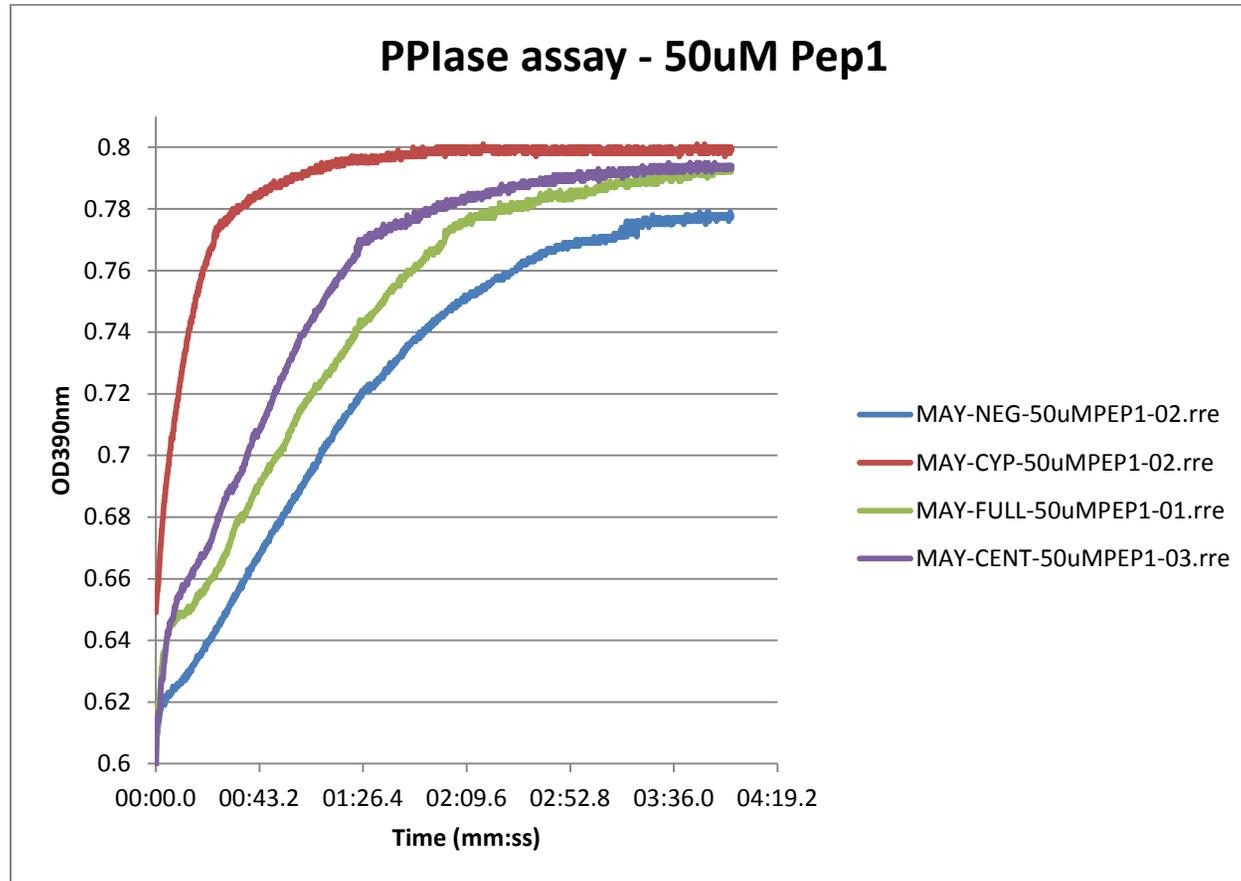


Figure 6.3: Protease coupled PPIase assay with 50 μ M Pep1. The enhanced rate of cleavage of Pep1 in the presence of 50 μ M of PrtM of *S. equi* 4047 is seen here. NEG = negative control, CYP = cyclophilin (positive control), FULL = full length recombinant protein. CENT = central domain recombinant protein.

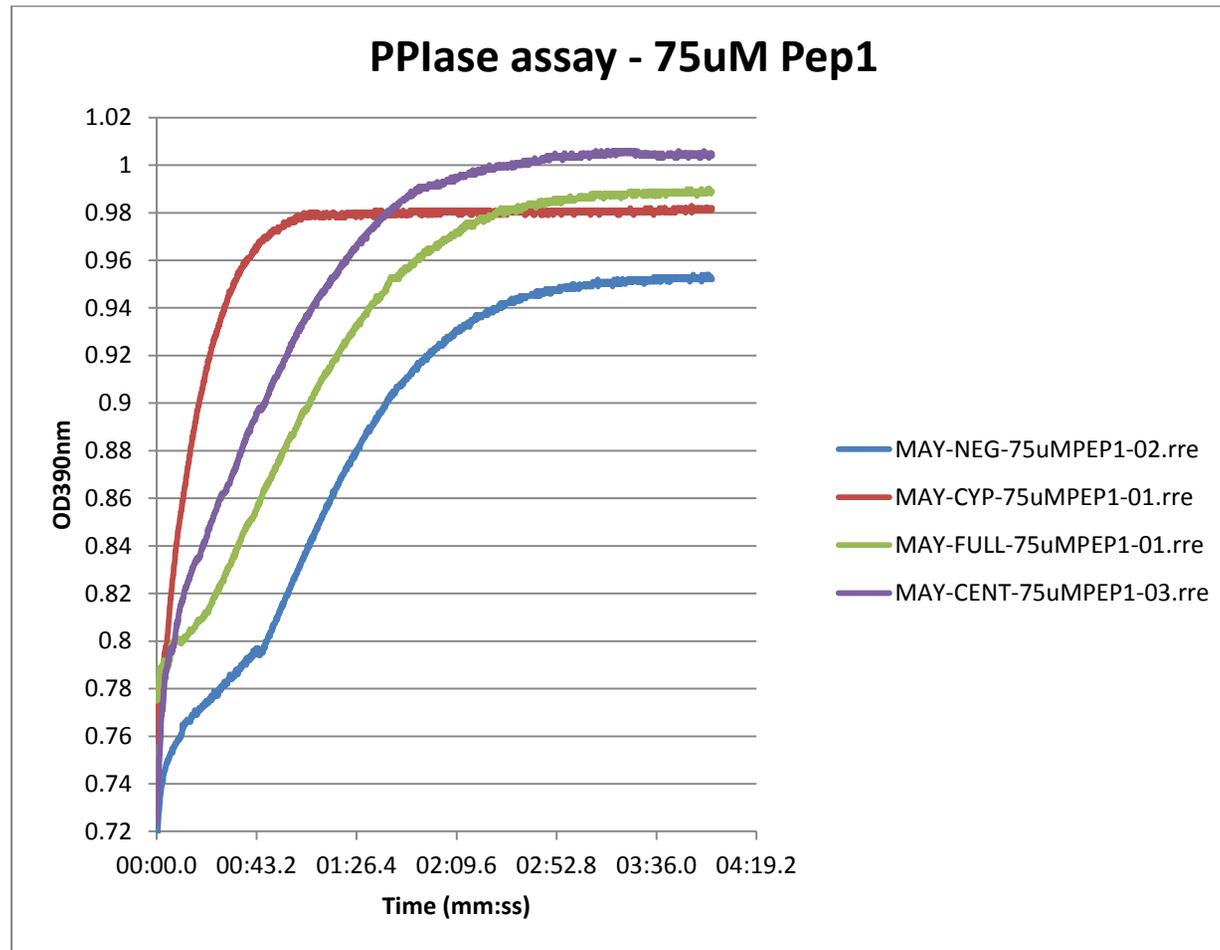


Figure 6.4: Protease coupled PPIase assay with 75 μ M Pep1. The enhanced rate of cleavage of Pep1 in the presence of 75 μ M of PrtM of *S. equi* 4047 is seen here. NEG = negative control, CYP = cyclophilin (positive control), FULL = full length recombinant protein. CENT = central domain recombinant protein.

6.3 Phenylmethanesulfonyl flouride (PMSF) assay result

The PMSF assay (method in Section 2.8.2) result is shown in figure 6.5 below.

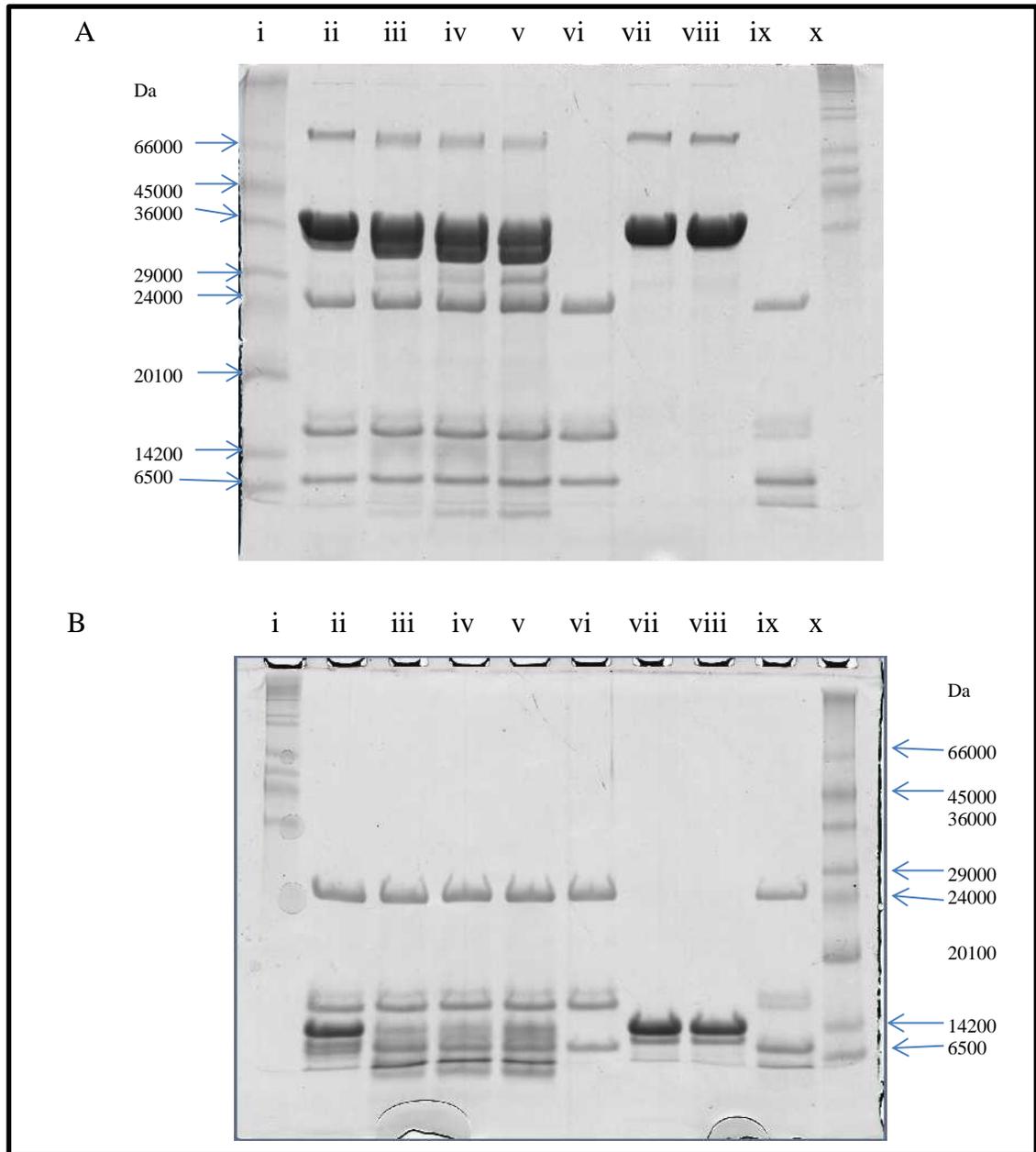


Figure 6.5 PMSF assay result: Method in section 2.8.2. A = SDS-PAGE analysis of the reaction of full length recombinant protein. B = SDS-PAGE analysis of the reaction of the central domain recombinant protein. High molecular weight size standard (i). PMSF inactivated chymotrypsin was incubated with the recombinant protein in assay buffer (ii). Before the protease reaction was stopped with PMSF, recombinant protein was incubated with chymotrypsin in assay buffer for 20 s (iii), 2 min (iv) and 5 min (v). Only chymotrypsin and PMSF in reaction buffer, no recombinant protein (vi). Recombinant protein and PMSF only (vii). Recombinant protein only (viii). Chymotrypsin only (ix).

6.4 Enzyme Kinetics

Triplicates assays for a range (25 μM , 37.5 μM , 50 μM , and 75 μM) of Pep1 were used for the PPIase assays (Figures 6.1 to 6.4). However, k_{cat} for the enzyme was calculated (see method in Section 2.8.3 and results in Figure 6.6) from average of triplicate PPIase assays of the full length recombinant protein with 37.5 μM Pep1 at pH 7.4.

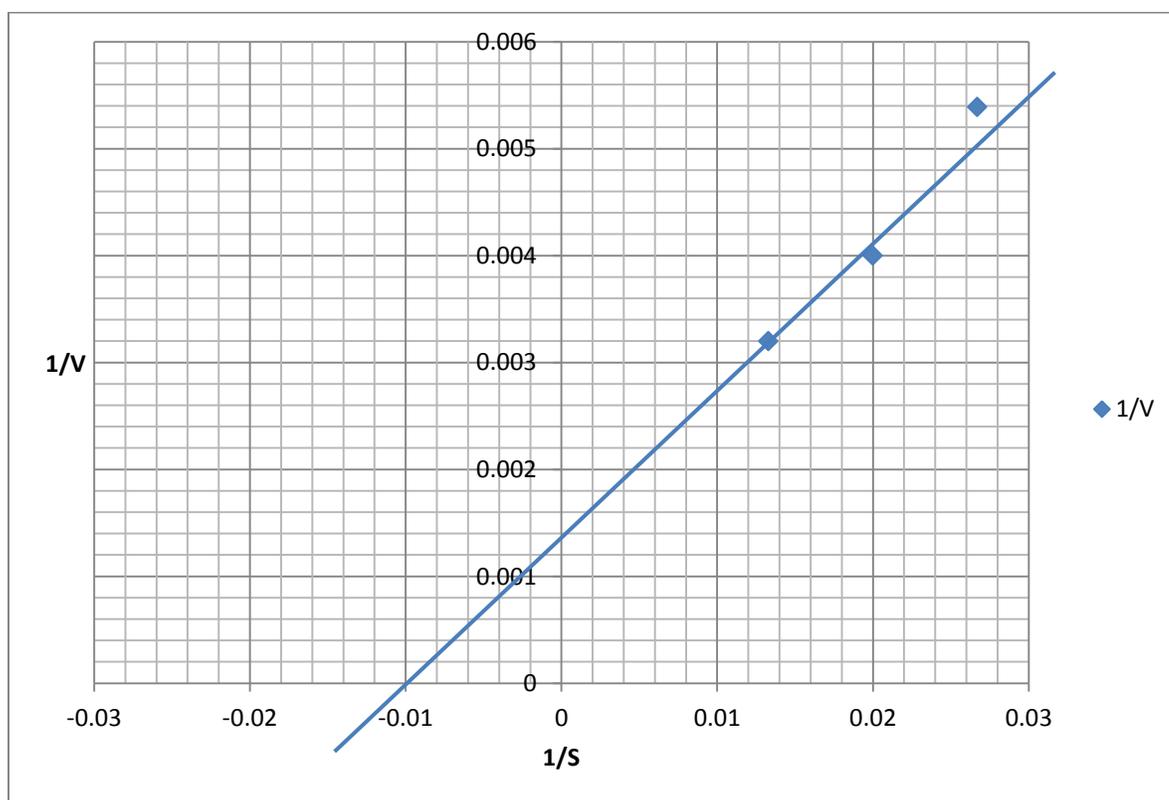


Figure 6.6 The First Order Rate Constant of PrtM of *S. equi* 4047: The k_{cat} for *S. equi* 4047 PrtM full length recombinant protein = 583.75 /s. K_{M} for *S. equi* 4047 PrtM full length recombinant protein = 100 μM . $k_{\text{cat}}/K_{\text{M}}$ for *S. equi* 4047 PrtM full length recombinant protein = $5.84 \times 10^6/\text{M/s}$.

6.5 Discussion

Parvulins (a class of PPIases) of eukaryotic cells, including Human Pin1, specifically recognize proline residues that are preceded by phosphorylated serine or threonine residues (Yaffe *et al.*, 1997; Hani *et al.*, 1999; Sun *et al.*, 2012). For other parvulins, their substrate recognition is independent of phosphorylation, they therefore have a wider substrate range (Lu *et al.*, 2007). *S. aureus* PrsA prefers a substrate having a negatively charged residue before the proline residue (preferred substrate Suc-AEPPF-*p*NA); while *B. subtilis* PrsA has the highest catalytic activity towards Suc-AKPF-*p*NA peptide (Heikkinen *et al.*, 2009). In this study, it has been proven that by the enhanced cleavage of the tetrapeptide (Pep1) by both the full length and central domain proteins in the protease coupled assay (Figures 6.1, 6.2, 6.3, 6.4 and 6.6), *S. equi* 4047 PrtM is a bonafide PPIase with preference for a substrate (Suc-Ala-Phe-Pro-Phe-*p*NA) with an uncharged, hydrophobic residue (phenylalanine) before the proline residue.

Interestingly, *B. subtilis* PrsA central domain has also been proven to have PPIase activity (Vitikainen *et al.*, 2004). In this study, the chymotrypsin, used in the PPIase assay to cleave the *trans* form of Phe-Pro, did not have any significant effect on PrtM full-length recombinant protein during the assay (Figure 6.5A). The central parvulin-domain recombinant protein was highly sensitive to chymotrypsin (Figure 6.5B), yet the small fraction that remained uncleaved by chymotrypsin still showed higher rate of PPIase reaction than the full length (Figures 6.1 to 6.4). This implies that the PPIase activity of the central parvulin domain of PrtM of *S. equi* 4047 is very high, much higher than the activity of the full length recombinant protein. The enhanced cleavage of the central domain

(Figure 6.5B) also implies that the N and C terminal domains are required for the stability of the enzyme (PrtM). The N and C terminal domains may help the PrtM enzyme in resisting proteolytic cleavage *in vivo*, therefore may have roles to play in enhancing the contribution of PrtM PPIase activity to *S. equi* 4047 virulence. In future, it will be worth finding out the effect of other endopeptidases (like trypsin and subtilisin) on PrtM full length and central domain recombinant proteins, and looking at the possibility of carrying out similar protease coupled PPIase assay with another endopeptidase.

Cyclophilin (which was used as a positive control) clearly displayed a higher reaction rate (Figures 6.1-6.4). This is consistent with findings of Vitikainen *et al.* (2004) where cyclophilin was found to have significantly up to 20-fold higher activity than *L. monocytogenes* PrsA2.

PPIase activity of parvulins can be very low and may be difficult to measure (Rouviere and Gross, 1996; Uchida *et al.*, 1999; Schmidpeter *et al.*, 2011; Alonzo *et al.*, 2012). This may be due to the observation that streptococcal parvulin homologues do not have a number of identical amino acids at essential positions of the functional parvulin domain (Drouault *et al.*, 2002). To date PPIase activity has not been detected in *S. pneumoniae* PpmA, a member of the parvulin family (Hermans *et al.*, 2006).

PPIase activity of *S. equi* 4047 PrtM was expressed as k_{cat}/K_M (/M/s). The specificity constant k_{cat}/K_M for the *cis* to *trans* interconversion of the -Phe-Pro- bond Suc-Ala-Phe-Pro-Phe-*p*Na is 5.84×10^6 /M/s (Section 6.4). Table 6.1 shows the specificity constant of

some PPIases determined by the protease coupled assay. The PPIase of PrtM of *S. equi* 4047 is shown at the end.

PPiase Family	Protein	k_{cat}/K_M (/M/s)	Substrate	Reference
Cyclophilin	<i>S. pneumoniae</i> S1rA	1.1×10^7	Suc-Ala-Ala-Pro-Phe- <i>pNa</i>	Hermans <i>et al.</i> , 2006
Cyclophilin	<i>S. pneumoniae</i> S1rA	4.0×10^6	Suc-Ala-Phe-Pro-Phe- <i>pNa</i>	Hermans <i>et al.</i> , 2006
FKBP	Human FKBP12	3.6×10^6	Suc-Ala-Leu-Pro-Phe- <i>pNa</i>	Bossard <i>et al.</i> , 1994
FKBP	<i>Plasmodium falciparum</i> PFKBP35	1.7×10^4	succinyl-Ala-Leu-Pro-Phe- <i>pNa</i>	Monaghan and Bell, 2005
Cyclophilin	Human cyclophilin A	3.2×10^7	Suc-Ala-Ala-Pro-Phe- <i>pNa</i>	Harrison and Stein, 1990
Cyclophilin	Human cyclophilin A	1.4×10^7	Suc-Ala-Phe-Pro-Phe- <i>pNa</i>	Harrison and Stein, 1990
FKBP	Bovine FKBP	0.66×10^6	Suc-Ala-Leu-Pro-Phe- <i>pNa</i>	Kofron <i>et al.</i> , 1991
Cyclophilin	<i>L. pneumophila</i> Cyp 18	4.6×10^6	Suc-Ala-Ala-Pro-Phe- <i>pNa</i>	Schmidt <i>et al.</i> , 1996
PTPA	Rabbit PTPA	1.1×10^6	Suc-Ala-Ala-Pro-Lys- <i>pNa</i>	Jordens <i>et al.</i> , 2006
Parvulin	<i>S. aureus</i> PrsA	3.3×10^5	Suc-Ala-Glu-Pro-Phe- <i>pNa</i>	Heikkinen <i>et al.</i> , 2009
Parvulin	<i>E. coli</i> Par10	1.35×10^7	Suc-Ala-Leu-Pro-Phe- <i>pNa</i>	Uchida <i>et al.</i> , 1999
Parvulin	<i>B. subtilis</i> PrsA	1.4×10^6	Suc-Ala-Lys-Pro-Phe- <i>pNa</i>	Vitikainen <i>et al.</i> , 2004
Parvulin	<i>Saccharomyces cerevisiae</i> Ptf1p	4.2×10^6	Suc-Ala-Glu-Pro-Phe- <i>pNa</i>	Hani <i>et al.</i> , 1999
Parvulin	Human Pin1	1.9×10^7	Ala-Ala-pSer-Pro-Arg- <i>pNa</i>	Yaffe <i>et al.</i> , 1997
Parvulin	<i>E. coli</i> SurA	3.4×10^4	Suc-Ala-Leu-Pro-Phe- <i>pNa</i>	Behrens <i>et al.</i> , 2001
Parvulin	<i>S. equi</i> 4047 PrtM	5.84×10^6	Suc-Ala-Phe-Pro-Phe- <i>pNa</i>	This study

Table 6.1: Specificity Constant of PPIases Determined by Protease Coupled Assay

The protease coupled assay is a simple inexpensive assay. However, one disadvantage of the protease-coupled assay is that it requires a high concentration of helper proteases to obtain the two-phase reaction (Hani *et al.*, 1999). Therefore, this assay can only be used for proteins that are relatively resistant to the protease in assay, for the duration of the experiment and these parameters should be determined for each PPIase in combination with the substrate and the protease (Jordens *et al.*, 2006).

Although the central domain recombinant protein showed significant activity (see Figures 6.1 – 6.4), its kinetic values were not calculated because of its high sensitivity to the protease (chymotrypsin) degradation (Figure 6.5B). The kinetic values for PrtM was therefore calculated only from values obtained from PPIase assay with the full length recombinant protein which just like the human eukaryotic parvulin homologue -hEPVH (Rulten *et al.*, 1999), was more stable to attack by chymotrypsin for the duration of the experiment (Figure 6.5A). However, unlike hEPVH, PrtM was considerably resistant to chymotrypsin attack at the concentrations used in this study.

Another limitation to the protease coupled assay is that only one direction of the reversible isomerisation is measured (*cis* → *trans*) and only about 10% of the *cis* content of the substrate is monitored, such that the signal-to-noise ratio can cause problems. This background can be reduced by dissolving the substrates in 0.48 M LiCl/trifluoroethanol (TFE), which shifts the *cis* content up to 70% (Kofron *et al.*, 1991). Cost and time constraints were limiting factors in applying other techniques to determine PPIase activity.

However, the protease coupled method employed in this study gave reliable PPIase activity results for the full length recombinant protein.

Since the central domain recombinant protein was highly sensitive toward chymotrypsin (Section 6.3), its PPIase activity and kinetic constants would be more accurately measured by other techniques, like a protease-free assay. One such assay would be an uncoupled protease-free PPIase assay, which uses the standard tetrapeptide substrates of the proteolytically coupled test system such that differences in the UV/vis absorption spectra of *cis* and *trans* conformations of Suc-Ala-Xaa-Pro-Phe-(Y-) anilide (Xaa = Ala, Leu, Phe; Y = 4-nitro, 2,4-difluoro) are exploited to monitor the time course of the *cis/trans* isomerization subsequent to a solvent jump from LiCl/trifluoroethanol into aqueous solution (Janowski *et al.*, 1997; Bang *et al.*, 2001).

Peptidylprolyl *cis-trans* isomerases facilitate the refolding of some denatured proteins in vitro (Schonbrunner *et al.*, 1991). PPIase activity of *S. equi* 4047 PrtM full length and central domain recombinant protein could also be measured by studying the refolding kinetics of denatured proteins like ribonuclease T1 (RNase T1). The refolding kinetics of RNase T1 and some other proline containing proteins has been monitored by electron microscopy, fluorescence or protolysis (Schonbrunner *et al.*, 1991; Budiman *et al.*, 2011; Golbik *et al.*, 2005; Davis *et al.*, 1989). The use of dynamic proton NMR spectroscopy in assaying PPIase activity (Jordens *et al.*, 2006) is another method worth exploring. The inhibitory effect of juglone on PrtM of *S. equi* 4047 is also worth evaluating in future.

Distinct functional roles in PPIase activity have been observed for the central, N- and C-terminal domains of *L. monocytogenes* PrsA2 (Alonzo *et al.*, 2012). The PrsA2 N+C mutant (lacking the central domain) was unable to catalyze *cis* → *trans* isomerization (Alonzo *et al.*, 2012). In this study, the full length (PrtM) recombinant protein was proven to have PPIase activity; and PrtM central domain has also shown activity. It is hereby inferred that a PrtM N+C mutant (lacking the central domain) will be unable to catalyze the *cis* → *trans* isomerization of Pep1 (Suc-Ala-Phe-Pro-Phe-pNA). However, this is subject to confirmation by future work.

7.0 Results of Crystallisation of *S. equi* PrtM (Full Length and Central Domain)

7.1 Introduction

Knowing the 3-D structure of the PrtM of *S. equi* 4047 would be beneficial for the development of highly specific drug or peptide analogues which interfere with its activity, and thereby provide alternative therapeutic strategies to vaccination. Crystallisation screens of the purified PrtM full length and central domain recombinant proteins were carried out as described in Sections 2.7.1 – 2.7.4. Initial crystallization screens were carried out using the Clear Strategy Screen (Molecular Dimensions: CSS1 -Appendix J1 and CSS2-Appendix J2), the Hampton Screen 1 and 2 (Hampton Research: Appendix J3a and J3b), the PEG Ion Screen (Hampton Research: Appendix J4), the Newcastle Screen (Appendix J6a-J6d) and the Index Screen (Index Screen: Appendix J7a and J7b).

PrtM microcrystals were obtained in 4-6 weeks from Newcastle Screen (condition number #93 which was made up of 0.8 M sodium formate, 10% PEG 8000, 10% {w/v} PEG 1000 and 0.1 M imidazole pH8.0) and from Hampton Screen 2 (condition #24 which was made up of 0.05 M caesium chloride, 0.1 M MES pH 6.6 and 30% Jeffamine M.600). These screens were optimized (see details of optimization conditions in Figures 7.0A and 7.1) by manual hanging drop method in 24-well XRL plates (Section 2.7.1). Microcrystals were also harvested from Hampton Screen 1&2 (set up in Grenier 96-well plates as described in Section 2.7.2) under conditions described in Figure 7.2.

Microcrystals were harvested (method in Section 2.7.3) in Northumbria University and transported in liquid nitrogen to University of York Structural Biology Laboratory (YSBL) where x-ray diffraction carried out as described in Section 2.7.4. Results of x-ray diffraction analysis of microcrystals by Dr Edward Taylor of YSBL are shown in section 7.3.

7.2 Crystals of *S. equi* PrtM (Full Length and Central Domain)

Images of crystals are shown in Figures 7.0, 7.1 and 7.2.

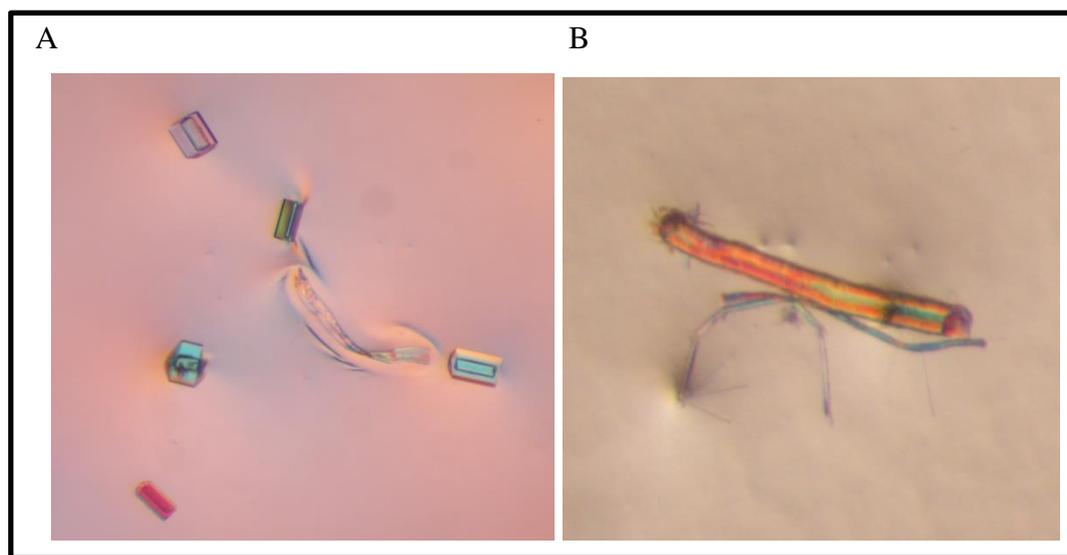


Figure 7.0: *S. equi* 4047 PrtM full length protein Crystals: A= crystals in optimized Newcastle Screen condition #93 (optimized to contain 0.8 M sodium formate, 20% PEG 8000, 20% {w/v} PEG 1000 and 0.2 M imidazole pH8.0). In addition to poor diffraction pattern in Figure 7.4B, crystals (such as in Figure 7.0A) were also seen in mother liquor, analysis of these salts crystals was discontinued. B = crystals in Hampton Screen 2 condition #24 (made up of 0.05 M caesium chloride, 0.1 M MES pH 6.6 and 30% Jeffamine M.600)

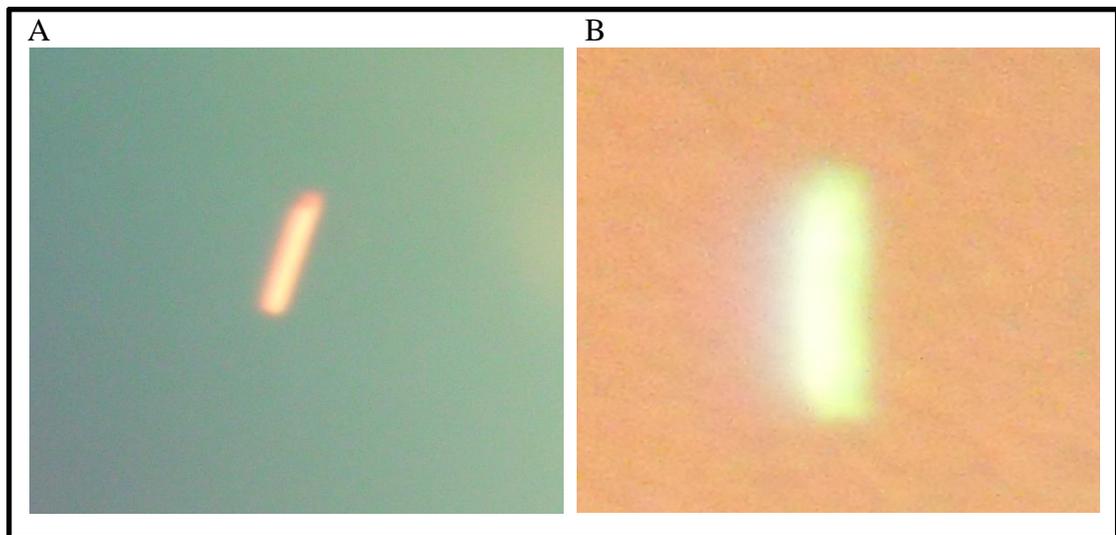


Figure 7.1: *S. equi* 4047 PrtM protein Crystals in Hampton Screen 2 condition #24 - optimized manually. A = Full length protein crystals. B = central domain protein crystals. Condition #24 of Hampton Screen 2 was optimized (to contain 0.01 M caesium chloride, 0.1 M MES pH6.6 and 20% Jeffamine M.600) for both crystals in Figures 7.1A and 7.1B.

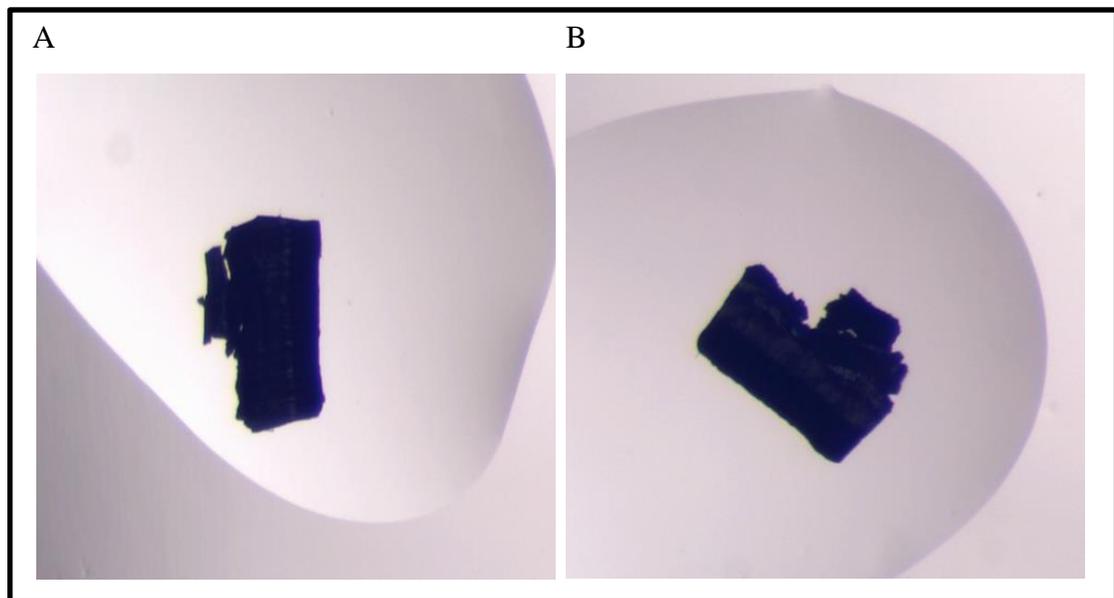


Figure 7.2 PrtM protein crystals in Hampton Screen 1&2: A = *S. equi* 4047 PrtM full protein in well E1 of 96-well plate (containing 0.1 M sodium acetate and 0.1 M imidazole pH 6.5). B= *S. equi* 4047 PrtM central domain protein crystals in well B1 of 96-well plate (containing 1.4 M sodium acetate and 0.1 M sodium cacodylate pH 6.5). Both trays were set up via mosquito. Crystal did not yield good diffraction patterns.

7.3 Diffraction Analysis of Microcrystals

A typical example of the image from good diffraction of a crystal is shown in Figure 7.3; while Figure 7.4 shows images of patterns obtained from diffraction analysis of crystals in this study.

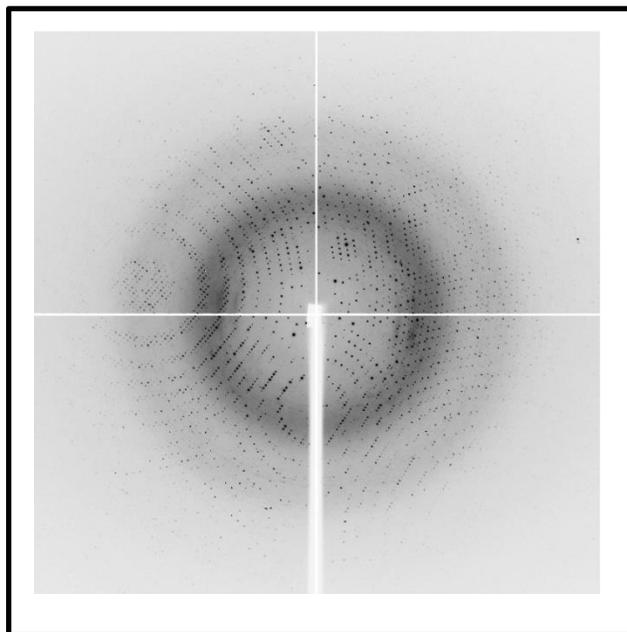


Figure 7.3: Sample x-ray diffraction pattern of a diffracting protein crystal (Courtesy Dr Edward Taylor, YSBL).

Diffraction analysis (method in Section 2.7.4) by Dr E. Taylor of YSBL revealed that some of the microcrystals obtained were either salts (microcrystal in Figure 7.0A, diffraction pattern in Figure 7.4B) or may have deteriorated during transportation and/or freezing (for example, microcrystal in Figure 7.1A, diffraction pattern in Figure 7.4A).

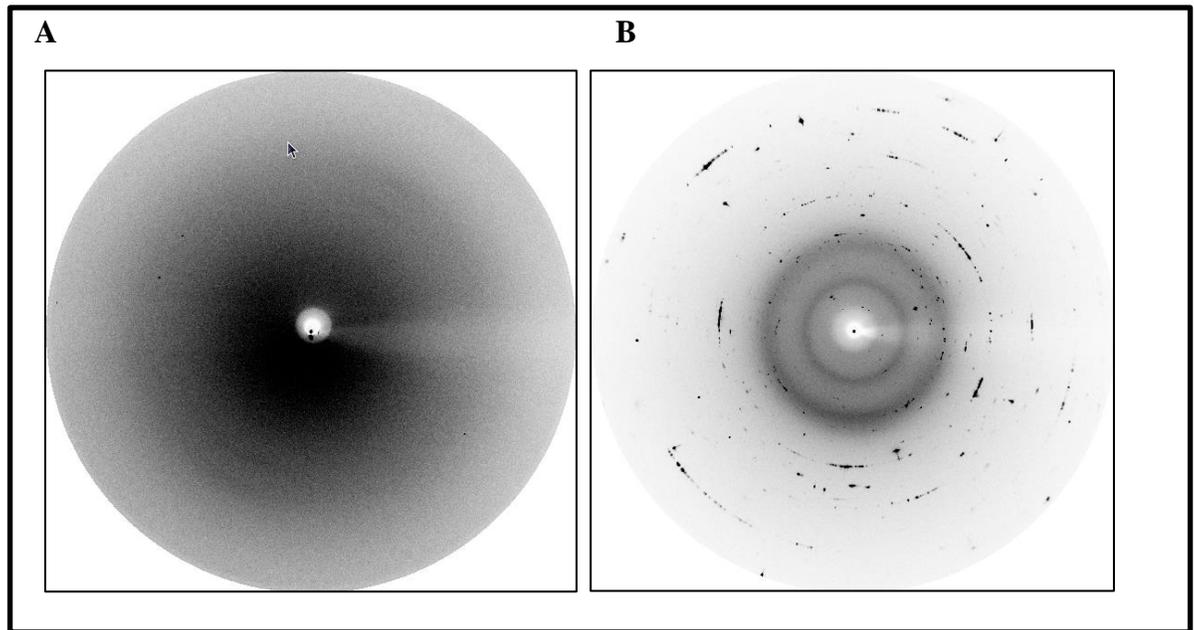


Figure 7.4 Images of non-diffracting/salt microcrystals: A = non-diffracting microcrystal image, derived from *S. equi* 4047 PrtM full length recombinant protein microcrystal in Figure 7.0B: microcrystal may have fragmented during freezing or transportation, hence non-diffraction pattern. B = crystal from of *S. equi* 4047 PrtM full length recombinant protein microcrystal in Figure 7.0A showing a typical diffraction pattern of a salt crystal.

Due to the fact that crystals were either salts or non-diffracting, it was pointless to send them for data collection at the Diamond Light Source synchrotron radiation facility (located at the Harwell Science and Innovation Campus in Oxfordshire) where the protein (PrtM) structure would have been solved.

7.4. Discussion

Parvulins are ubiquitous globular protein domains of about 100 residues that fold into a four-stranded antiparallel β -sheet core made up of four α -helices $\beta\alpha_3\beta\alpha_2$ (Fanghanel and Fischer, 2004). The structure of some parvulins can be found in the Protein Data Bank (Research Collaboratory for Structural Bioinformatics, 2012). The prototypical human Pin1 (hPin1) has its structure in the Protein Data Bank (Research Collaboratory for Structural Bioinformatics, 2012). That of *B. subtilis* PrsA is also in the Protein Data Bank (Research Collaboratory for Structural Bioinformatics, 2012). Parvulins differ in the length and the composition of the S1-H1 loop between the strand β_1 and the helix α_1 (Weininger *et al.*, 2010).

The preference, by Human Pin1-type parvulins, for substrates having a negatively charged residue (preferably a phosphorylated serine/threonine before the processed proline) is believed to be mediated by their loop which has a high number of positively charged residues (Ranganathan *et al.*, 1997). This type of loop is missing in Par14-type parvulins. In the SurA PPIase domain for example, the S1-H1 loop is made up of mainly hydrophobic residues (Bitto and McKay, 2002).

The structure of the *S. aureus* PrsA resembles that of hPin1; and just like hPin1, the *S. aureus* PrsA prefers a substrate (preferred substrate is Suc-AEPPF-*p*NA) with a negatively charged residue before the proline residue (Heikkinen *et al.*, 2009). However *B. subtilis* PrsA has the highest catalytic activity towards a substrate (preferred substrate Suc-AKPF-*p*NA peptide.) with positively charged residue before the proline residue. *E. coli* Par10 has

a preference for nonpolar residues before and after the proline (Schmidpeter *et al.*, 2011). Considering bioinformatics analysis (in which PrtM was predicted to have a central, N- and C- terminal domains just like some other parvulins - Chapter 4), and the fact that PrtM has been proven in this study (Chapter 6) to be a PPIase with a preference for a substrate (Suc-Ala-Phe-Pro-Phe-pNA) having an uncharged, bulky hydrophobic residue (phenylalanine) before and after the proline residue, it can be predicted that PrtM PPIase domain will have a typical parvulin structure ($\beta\alpha_3\beta\alpha_2$) with a four-stranded antiparallel β -sheet core and four α -helices. This prediction is further supported by the computer generated molecular model information (Figures 4.12 and 4.13) generated in Chapter 4.

The growth of a visually perfect crystal does not always mean that the structure of the protein will be solvable by x-ray crystallography, because there is limited correlation between a microscopically identified crystal and its diffraction quality (Owen and Garman, 2005). Difficulties were faced in attempts to solve the structure of the ParC55 breakage-reunion domain of topoisomerase IV from *Streptococcus pneumoniae* as most of the crystals were twinned, non-diffracting or exhibited a high mosaic spread. Also the crystals, which were grown under conditions that favoured diffraction, were difficult to flash-freeze without losing their ability to diffract (Sohi *et al.*, 2008). The best crystals obtained in this study were non-diffracting.

Since attempts to solve the structure of PrtM of *S. equi* 4047 by x-ray crystallography were unsuccessful in this study, some other techniques may be explored to solve the crystal structure of PrtM in future. PrtM has been proven (Chapter 5 of this study) to form dimers

in solution. *Bordetella pertussis* Par27, was the first parvulin protein identified as forming dimers in solution (Hodak *et al.*, 2008). In an attempt to solve the structure of *B. pertussis* Par27, its PPIase domains gave rise to diffuse scattering and could not be solved (Clantin *et al.*, 2010). Crystal structures were obtained only for its N- and C- terminal domains, therefore the structure of Par27 was characterized by applying other techniques (template-based modelling and small-angle scattering), in addition to X-ray crystallography (Clantin *et al.*, 2010).

NMR was used in solving the structure of human Par14 (Terada *et al.*, 2001) and *B. subtilis* PrsA (Tossavainen *et al.*, 2006). Solution-state NMR is worth exploring, in future attempts to solve the structure PrtM of *S. equi* 4047. However, solid-state NMR may be a better option because this technique offers the chance to solve the structures of proteins which are difficult to solve even at atomic resolution (Huber *et al.*, 2012).

8.0 Results of Production and characterization of Complemented mutants (Δ PrtM123/pVA838 and Δ PrtM12/pVA838).

8.1 Introduction

In a pony infection study, Hamilton *et al.* (2006) discovered that 4 out of 4 ponies infected with the *S. equi* 4047 WT (wild-type) exhibited pathological signs of strangles compared with 0 out of 5 ponies infected with Δ PrtM (Δ *prtM*₁₃₈₋₂₁₃, mutant with a deletion of nucleotides 138 to 213). In this study, the information generated so far confirm that PrtM is indeed a significant player in the virulence of *S. equi* 4047. The next task undertaken in this research was therefore to demonstrate the effect of complementing the mutant with plasmid (pVA838) to which the full (N, central and C terminal domains - Δ PrtM123/pVA838) or partial (N and central domains only - Δ PrtM12/pVA838) PrtM DNA sequence had been cloned. Demonstrating this effect *in vivo* is beyond the scope of this study. Therefore, complemented mutants (Δ PrtM123/pVA838 and Δ PrtM12/pVA838, Table 2.1) were produced, characterised and their proteomes compared (by comparative proteomic analysis as discussed in chapter 9) to those of controls (WT 4047/pVA838 and Δ PrtM/pVA838). Mukouhara *et al.* (2011) used the plasmid pVA838 to produce complemented mutants in their study of the contribution *S. mutans* lipoprotein PpiA to suppression of macrophage receptor with collagenous structure (MARCO) mediated phagocytosis by macrophages.

To produce the complemented mutants in this study, the full length PrtM gene (PrtM123, including the 281 nt upstream – Chapter 4, Figure 4.1) as well as the gene segment

containing the N-terminus and the central domain (PrtM12, including the 281 nt upstream – Chapter 4, Figure 4.1) were cloned into plasmid pVA838 (results in Section 8.2) to make recombinant plasmids. This was followed by electrotransformation of the mutant (Δ PrtM) with the recombinant plasmids (Section 8.3) to give the complemented mutants (Δ PrtM123/pVA838 and Δ PrtM12/pVA838). Subsequently, control strains (*S. equi* 4047 WT/pVA838 and Δ PrtM/pVA838) were produced (Section 8.4).

Gram stain reaction (result in Section 8.5) was carried out to confirm the identity of complemented mutants and controls. Before carrying out growth curve experiment (results in Section 8.7), antibiotics broth dilution assay (method in Section 2.4.9 and results in Section 8.6) was done to ascertain the most suitable antibiotics concentration for further assays. This was necessitated by the fact that unlike the complemented mutants and Δ PrtM123/pVA838 (control), *S. equi* 4047 WT/pVA838 struggled to grow in THB containing >10 μ g/mL of chloramphenicol. Including the wild type (*S. equi* 4047 WT) and mutant (Δ PrtM) in the assay (results in Section 8.6) was to confirm that growth of complemented mutants and controls was as a result of newly acquired recombinant plasmid.

A preliminary analysis of the protein profile of the complemented mutants and controls was achieved via SDS-PAGE analysis of protein extracts (results in Section 8.8). This was followed by immunogenic evaluation of proteins by Western blot analysis (results in Section 8.9). Comparison of the proteomes of the revertants and controls was carried out by 2D-E and HPLC/MS (results in Sections 8.10 - 8.13).

8.2 Result of construction of recombinant plasmids pVA838/PrtM123 and pVA838/PrtM12

The full length gene (encoding PrtM123 containing the N-, C-terminal and central domains and including the 281 nucleotides - upstream sequence containing native promoter and ribosome binding site –Chapter 4, Figure 4.1) was amplified (PCR method in Section 2.5.4 and results in Figure 8.0A) from *S. equi* 4047 genomic DNA (Section 3, Figure 3.2A) using primers Prtmequif and Prtm123equir (Section 2, Table 2.11). The gene section encoding PrtM12 (containing only the N-, terminal and central domains and including the 281 nucleotides - upstream sequence containing native promoter and ribosome binding site – Chapter 4, Figure 4.1) was amplified (PCR method in Section 2.5.4, and results in Figure 8.0B) from *S. equi* 4047 genomic DNA (Section 3, Figure 3.2A) using primers Prtmequif and Prtm12equir (Section 2, Table 2.11). The PCR products were purified (method in Section 2.5.6) and along with pVA838, digested (method in Section 2.5.9 and results in Figure 8.1). After double digests, PrtM123 and PrtM12 were separately ligated (method in Section 2.5.10) into pVA838 to yield recombinant plasmids pVA838/PrtM123 and pVA838/PrtM12, respectively.

E. coli (TOP10, Table 2.2) was transformed (method in Section 2.5.12) with the ligation. Transformants were selected on LBA (Table 2.3) plates containing 34 µg/ mL chloramphenicol (Table 2.4). Plasmid DNA (method in Section 2.5.13, and result in Figure 8.2) was extracted from overnight LB/chloramphenicol cultures to check for recombinants. Plasmid DNA (method in Section 2.5.14) from midi-plasmid preparations of several of

each successful recombinant was then digested (method in Section 2.5.9, results of AGE analysis in Figure 8.3) to confirm that the recombinant plasmids contain DNA inserts. Having confirmed that recombinant plasmids were successfully produced, the next step was electrotransformation (Section 8.3) to produce complemented mutants.

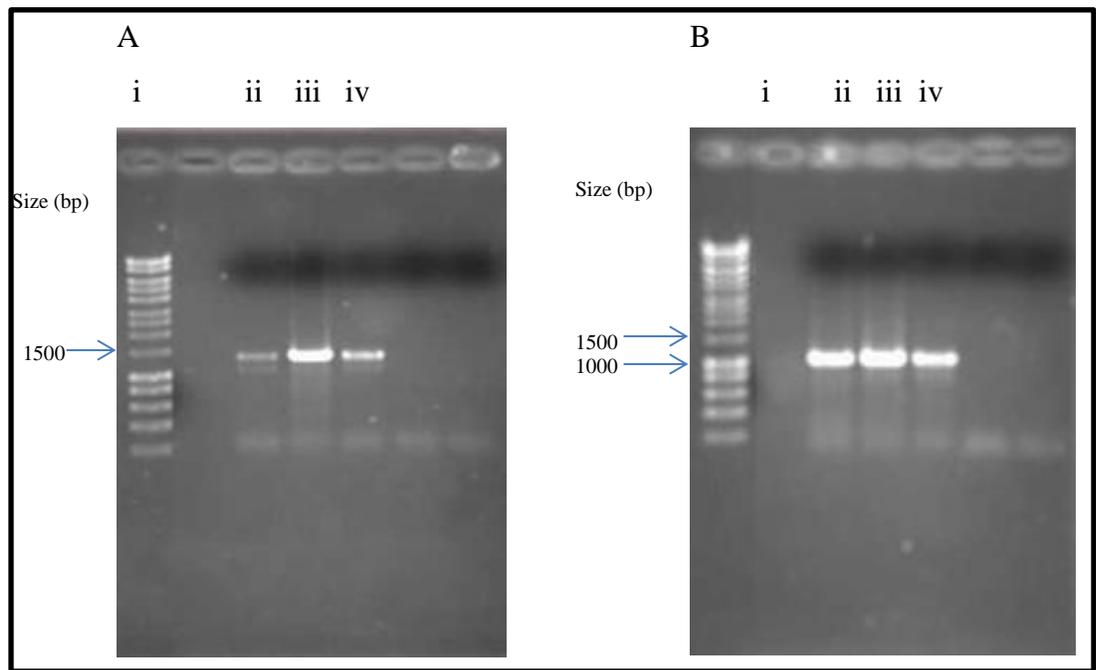


Figure 8.0: PCR products for production of complemented mutants: A= *S. equi* 4047 PrtM upstream sequences + full length PrtM gene (1277 bp lanes Aii-Aiv) and B = *S. equi* 4047 PrtM upstream sequences + N-terminal + Central Domain encoding sequences (1004 bp lanes Bii-Biv). Lane i = molecular weight DNA size standard (200 – 10,000 bp).

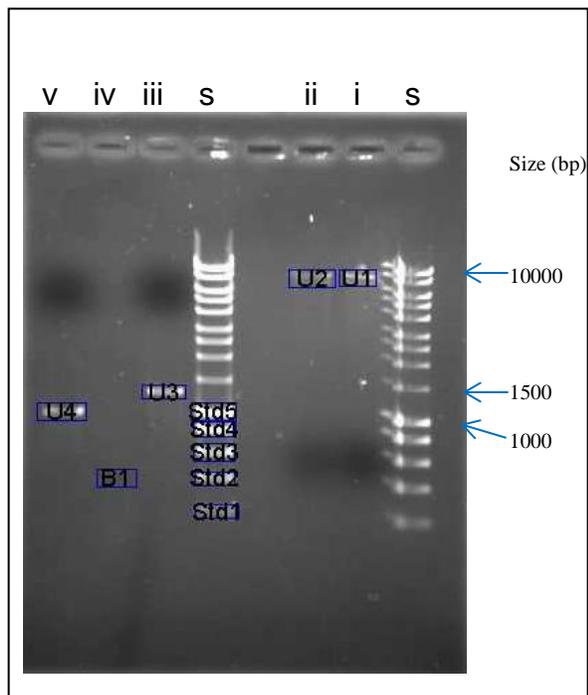


Figure 8.1 Concentrations of ligation components of agarose gel purified digested (*Bam*HI/*Sal*I) fragments (i = pVA838 U1= 10 μ g/uL), Δ PrtM123 gene (iii = U3 = 20.6 μ g/uL), Δ PrtM12 DNA sequence (v = U4 =21.1 μ g/uL. Lane s = molecular weight DNA size standard (200 – 10,000 bp).

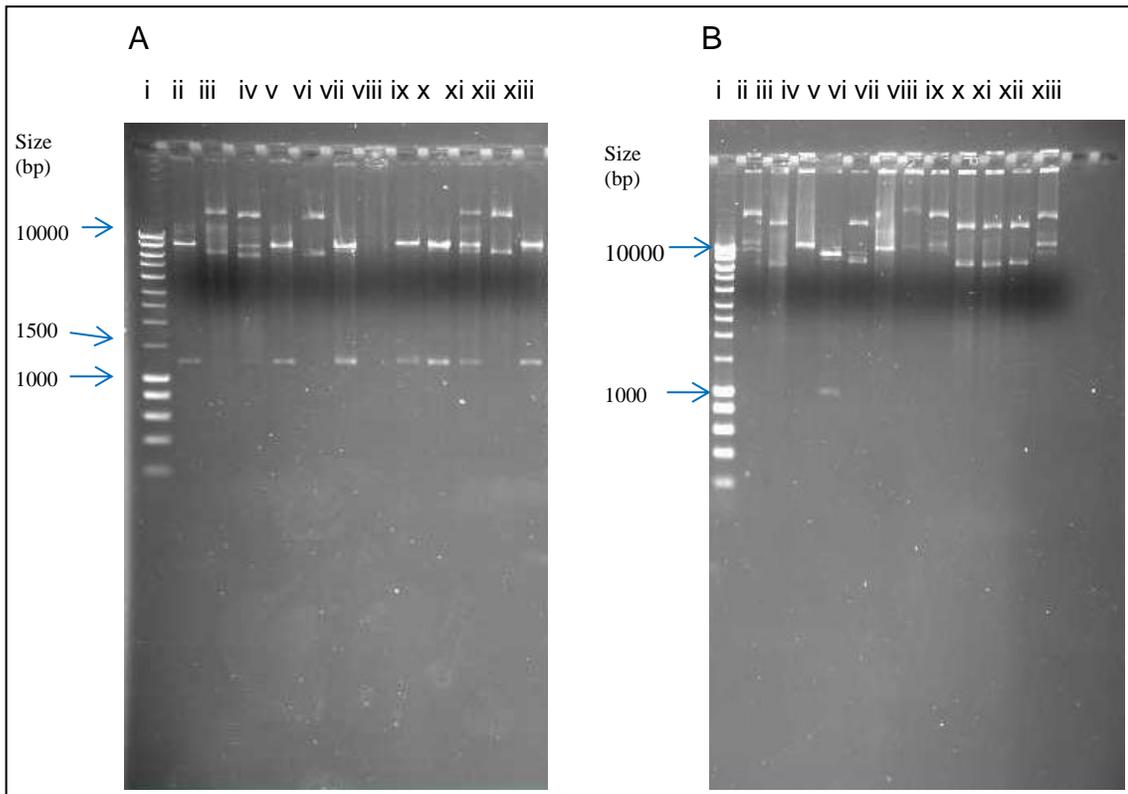


Figure 8.2 Result of screening of digestions (*Bam*HI/*Sal*I) of plasmid preparations from ligation transformants: A= pVA838/PrtM123 (with lanes ii, v, vii, ix, x, xi and xiii containing insert) left; and B= pVA838/PrtM12 (only lane v contains insert) right. Lane i = molecular weight DNA size standard (200 – 10,000 bp)

AGE images of midi-preparations of plasmid DNA from overnight cultures of *E. coli* TOP10 containing recombinant plasmids pVA838/PrtM123 and pVA838/PrtM12 are shown in Figure 8.3.

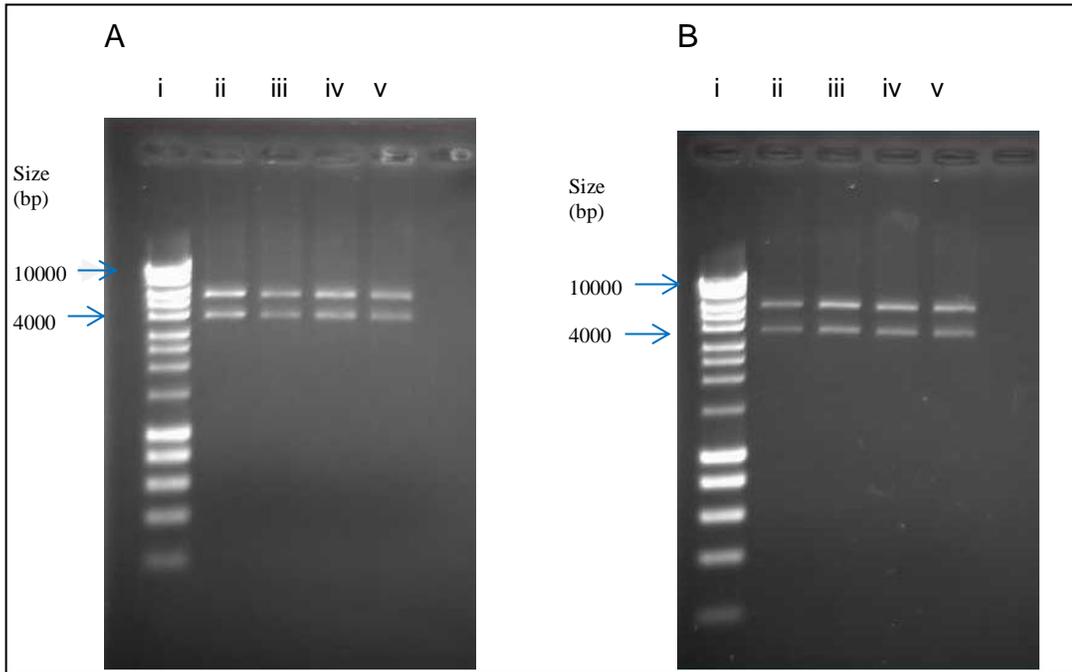


Figure 8.3 Image of diagnostic (*XbaI*) digest of plasmid DNA. *XbaI* cleaves pVA838 as well as PrtM123 and PrtM12 DNA. A = pVA838/PrtM123 (6477 bp and 4000 bp in lanes ii-v) left; and B = pVA838/PrtM12 (6364 bp and 3,900 bp in lanes ii - v) right. Lane i = molecular weight DNA size standard (200 – 10,000 bp).

Concentration of midi-preparation plasmid DNA pVA838/PrtM123 (above image left, estimated via nanodrop) = 2670.7 ng/ μ L.

Concentration of midi-preparation plasmid DNA pVA838/PrtM12 (above image, right, estimated via nanodrop) = 1390.1 ng/ μ L.

8.3 Results of production of complemented mutants

Complemented mutants $\Delta\text{PrtM123/pVA838}$ and $\Delta\text{PrtM12/pVA838}$ were produced using competent ΔPrtM cells (Section 2.5.16) and recombinant plasmids (pVA838/PrtM123 and pVA838/PrtM12 respectively, from Section 8.2). Electrotransformation was carried out as described in Chapter 2 (Section 2.5.16). $\Delta\text{PrtM123/pVA838}$ yielded 2.99×10^2 transformants/ μg of recombinant plasmid DNA (pVA838/PrtM123) after 24-48 h incubation. $\Delta\text{PrtM12/pVA838}$ yielded $3.6 \times 10^2/\mu\text{g}$ transformants/ μg of recombinant plasmid DNA (pVA838/PrtM12) after 24-48 h incubation. Single colonies of transformants were inoculated into THB containing 5 $\mu\text{g/mL}$ of chloramphenicol and incubated overnight at 37°C. Overnight broth cultures were stored in 1 mL aliquots as glycerol stocks (at a final concentration of 25% (v/v) glycerol) at -80°C.

8.4 Result of Production of controls - *S. equi* 4047 WT/pVA838 and $\Delta\text{PrtM /pVA838}$

S. equi 4047 WT/pVA838, that is the wild type (*S. equi* 4047 WT) strain containing the plasmid (pVA838) without any other insert, was produced as a control that was chloramphenicol resistant just like the complemented mutants. $\Delta\text{PrtM/pVA838}$, that is the mutant (*S. equi* 4047 ΔPrtM) strain containing the plasmid (pVA838) without any other insert, was also produced as a control that was chloramphenicol resistant just like the complemented mutants. Competent cells and electrotransformations were carried out (method in Section 2.5.16) using plasmid pVA838 (original concentration = 1317.7 ng/ μL). $\Delta\text{PrtM/pVA838}$ yielded 3.4×10^2 transformant/ μg of plasmid DNA (pVA838)

after 24-48 h incubation. *S. equi* 4047 WT/pVA838 yielded 0.24×10^2 transformants/ μg of plasmid DNA (pVA838) after 72-96 h incubation. Single colonies of transformants were inoculated into THB containing $5\mu\text{g/mL}$ chloramphenicol and incubated overnight at 37°C . Overnight broth cultures were stored in 1 mL aliquots as glycerol stocks (at a final concentration of 25% (v/v) glycerol) at -80°C .

8.5 Result of Gram stain of complemented mutants.

Gram stain (method in Section 2.4.3) of single colonies of transformants yielded Gram-positive streptococci for the complemented mutants and controls.

8.6 Result of Broth dilution antibiotics sensitivity

Jacks *et al.* (2003) have reported that the minimum concentration at which 90% of β -haemolytic streptococci (including *S. equi*) were inhibited by chloramphenicol was $\leq 4\mu\text{g/mL}$. Broth dilution sensitivity to chloramphenicol was carried out as described in section 2.4.9. After 24 – 48 h incubation, uncomplemented *S. equi* 4047 WT and uncomplemented mutant (ΔPrtM) were unable to survive (culture media remained clear after incubation) in THB cultures containing $5.0\mu\text{g/mL}$, $6.0\mu\text{g/mL}$ and $10.0\mu\text{g/mL}$ of chloramphenicol. However, all the complemented strains (WT 4047/pVA838, $\Delta\text{PrtM/pVA838}$, $\Delta\text{PrtM-123/pVA838}$ and $\Delta\text{PrtM-12/pVA838}$) were able to survive (survival indicated by turbid cultures after 24 – 48 h incubation) in broth cultures containing aforementioned ($5.0\mu\text{g/mL}$, $6.0\mu\text{g/mL}$ and $10.0\mu\text{g/mL}$) concentrations of

chloramphenicol. All strains (complemented and uncomplemented) survived and grew well (survival indicated by turbid cultures after 24 – 48 h incubation) in THB to which no chloramphenicol was added. These results indicate successful transformation with pVA838 plasmids into all complemented strains which were resistant to chloramphenicol at the concentrations used. These results were also valuable in ascertaining the chloramphenicol concentrations in which the complemented mutants could resist and thrive.

8.7 Result of Growth curve

Growth curve of three independent samples (a, b and c) of the complemented mutants and control was generated (results in Figure 8.4 and Appendix K) as described in Section 2.4.6, with the addition of 5 µg/mL chloramphenicol to the THB growth medium.

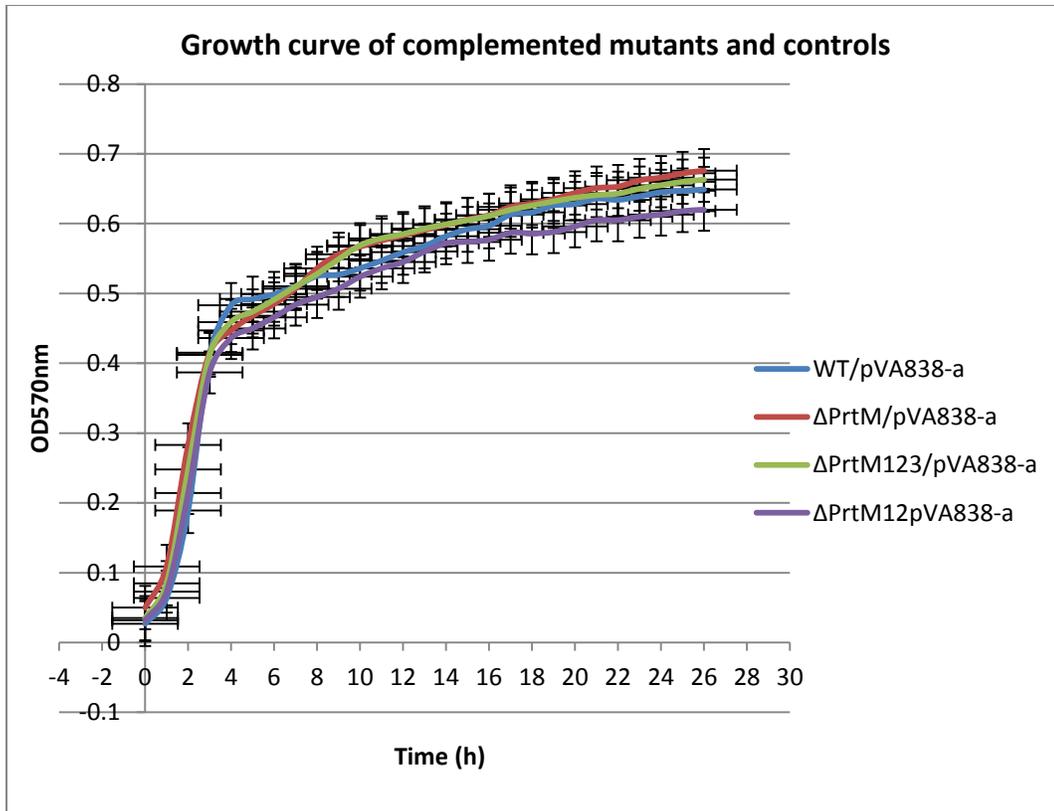


Figure 8.4: Growth curve of complemented mutants and controls: Error bars show no significant difference in growth rate up to late exponential phase.

8.8 SDS-PAGE of secreted protein extract and CFE of cell associated protein extract

Figure 8.5 shows image of SDS-PAGE of protein extracts (method of obtaining CFE and secreted protein extract discussed in Sections 2.5.21 and 2.5.24 respectively) of the complemented mutants and controls.

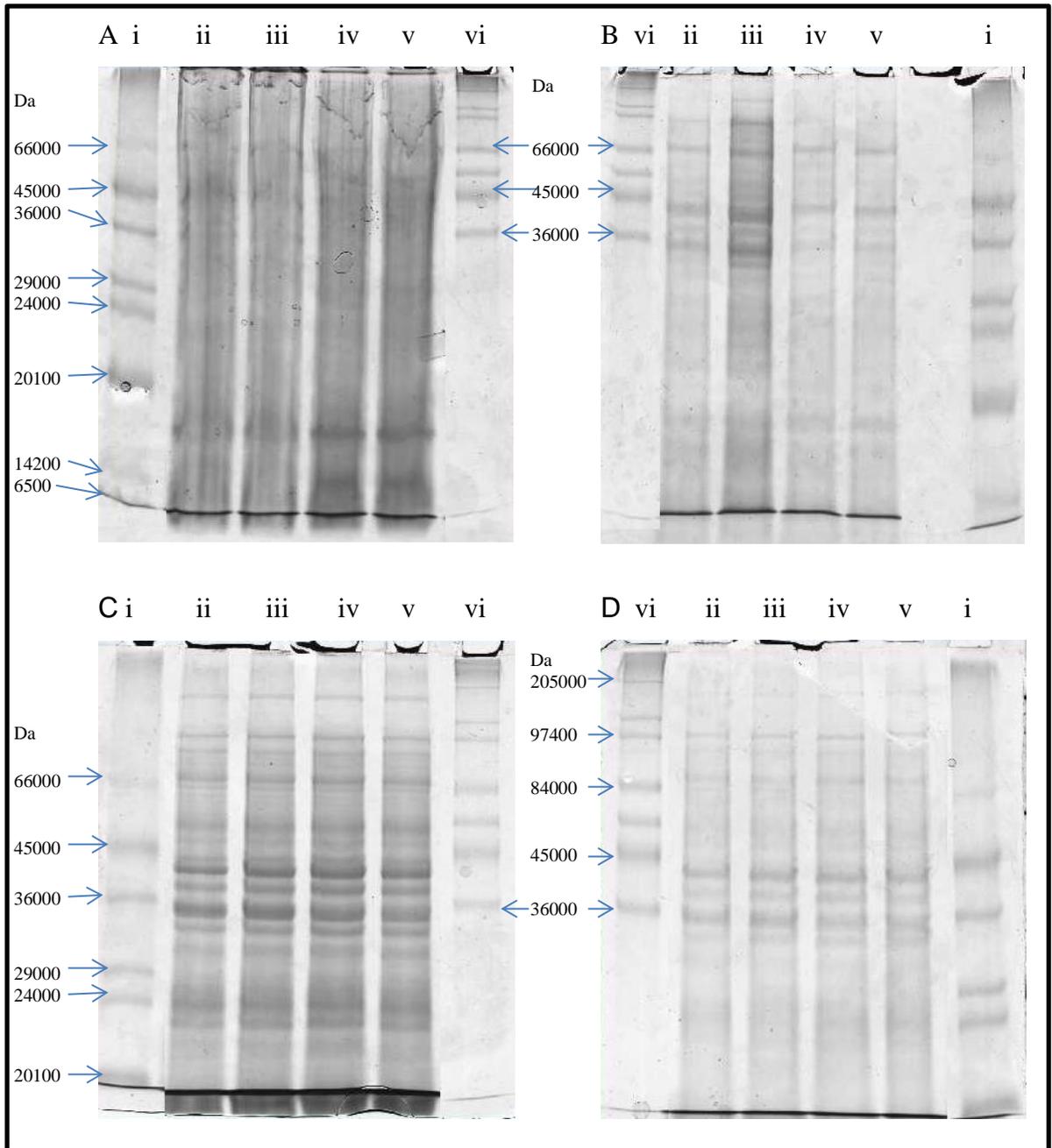


Figure 8.5: Results of SDS-PAGE of proteins from complemented mutants and controls. A&B = 15% (w/v) SDS-PAGE of CFE of cell associated protein extracts: from 20 mL cultures (A) and $\frac{1}{4}$ dilutions of CFE from 20 mL cultures (B). C & D = 12% (w/v) SDS-PAGE of secreted protein extract: From 20 mL cultures (C) and $\frac{1}{4}$ dilutions from 20 mL cultures (D). i = LMW, vi = HMW markers. ii = control *S. equi* 4047WT /pVA838, iii = Δ Prtm123/pVA838, iv = Δ Prtm12/pVA838, v = Δ Prtm/pVA838.

8.9 Western Blotting

Figure 8.7 shows the immunogenic (Western blot method in Section 2.5.20) reactions of cell associated and secreted protein extracts of the complemented mutants and controls with high titre α -PPMA.

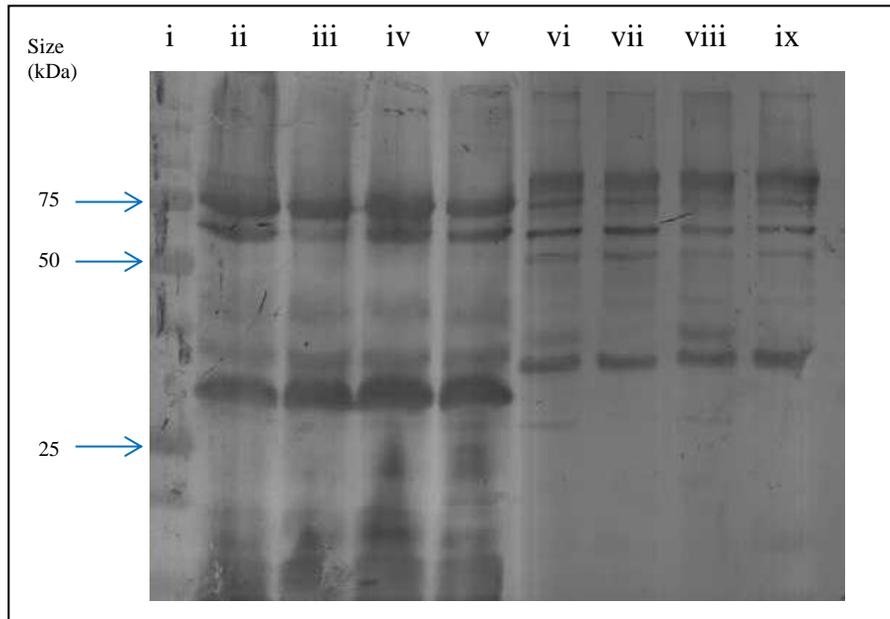


Figure 8.6: Western Blot with high titre α -Ppma: Cell associated protein extract (ii = control *S. equi* 4047 WT /pVA838, iii = Δ Prtm123/pVA838, iv = Δ Prtm12/pVA838, v = control Δ Prtm/pVA838), secreted protein extract (vi = control *S. equi* 4047 WT /pVA838, vii = Δ Prtm123/pVA838, viii = Δ Prtm12/pVA838, ix = Δ Prtm/pVA838). i = protein standard (BIO-RAD, Appendix A13).

8.10 2D-E of cell associated protein

Representative images of the proteomes of the cell associated protein extracts of the complemented mutants and controls obtained after 2D-E (method in Sections 2.5.23, 2.5.25 and 2.5.26) are shown in Figure 8.7.

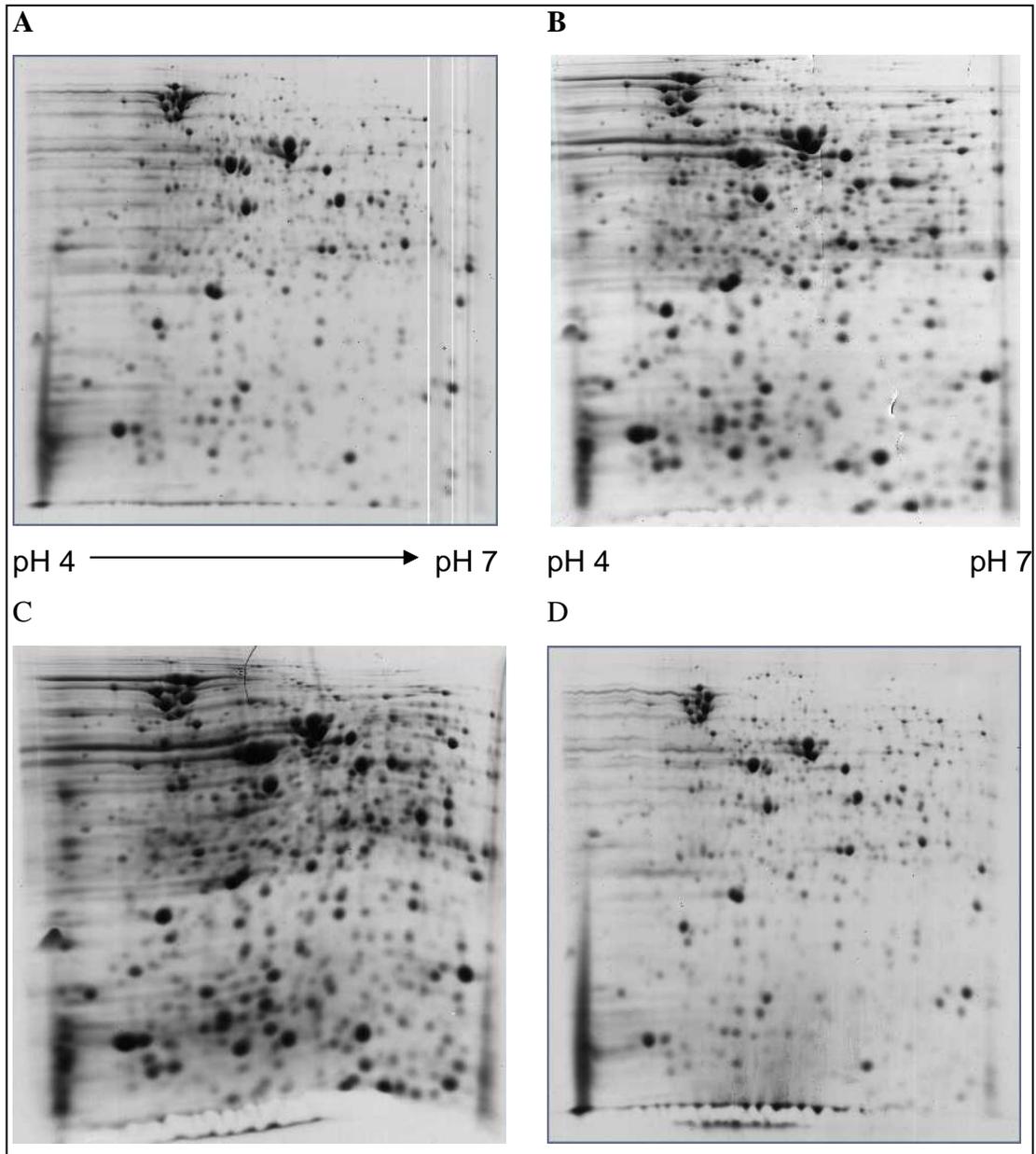


Figure 8.7 Representative Images of 2D-E gels of cell associated protein extract: A = control, *S. equi* WT 4047/pVA838; B = control *S. equi* Δ PrtM/pVA838; C = *S. equi* Δ PrtM123/pVA838; D = *S. equi* Δ PrtM12/pVA838.

8.11 2D-E of Secreted Protein extracts

Representative images of the proteomes of the secreted protein extracts of the complemented mutants and controls obtained after 2D-E (method in Sections 2.5.24, 2.5.25 and 2.5.26) are shown in Figure 8.8.

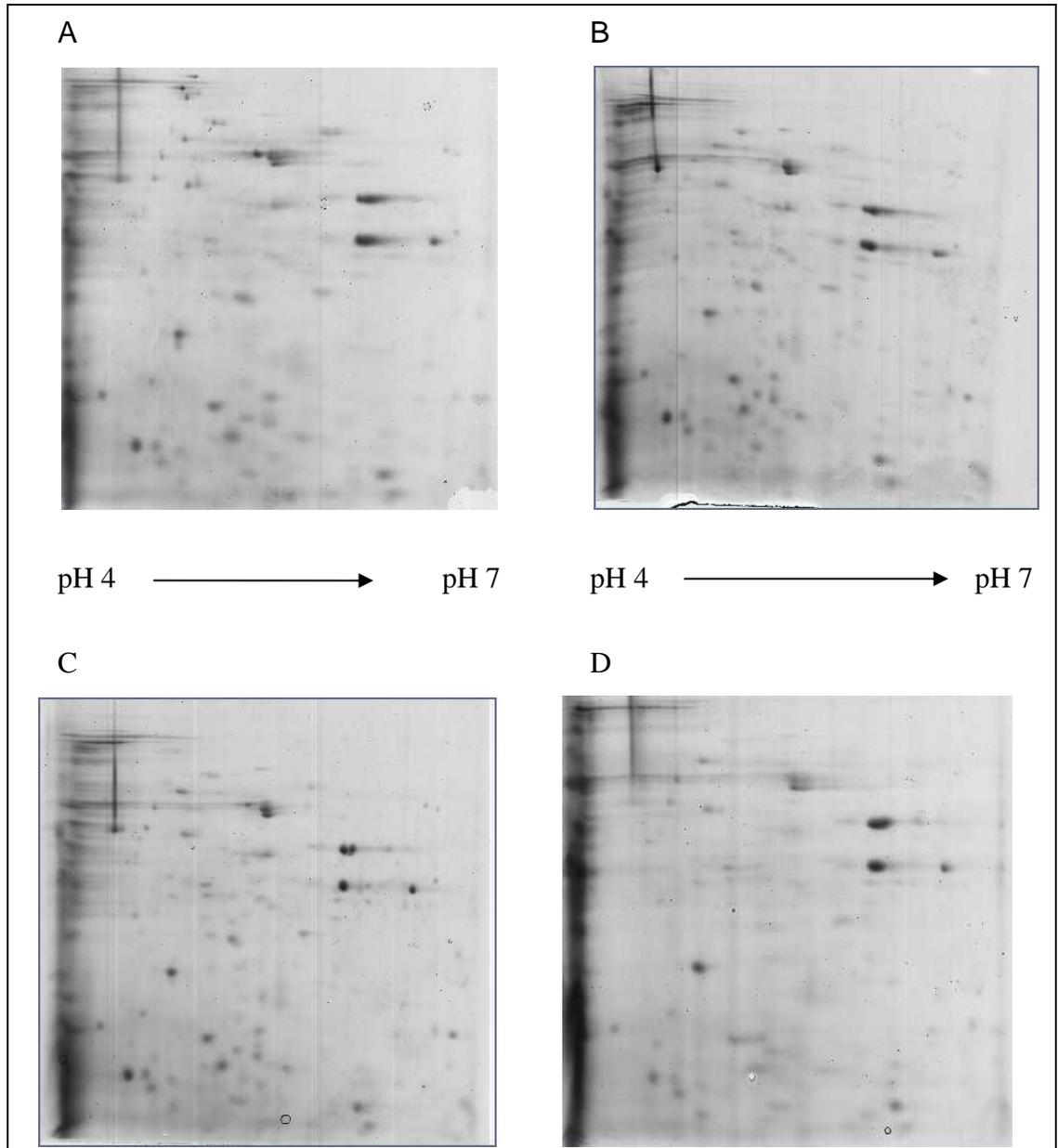


Figure 8.8: Representative images of 2D-E gels of secreted protein extract: A = control *S. equi* 4047 WT/pVA838, B = control *S. equi* 4047 Δ Prtm/pVA838; C = complemented mutant - Δ Prtm123/pVA838, D = complemented mutant - Δ Prtm12/pVA838.

8.12 Results of Analysis of 2D-gel images by Progenesis SameSpots software

The detection and quantification of spots was done using Progenesis SameSpots software (version 4.5, Nonlinear Dynamics, U.K.). Six Coomassie blue stained 2D-E gel images from each set were manually and automatically matched and aligned to six of a second set. The software (Progenesis SameSpots) automatically chose the most representative image from each matchset (Figures 8.9 and 8.10). Spots were prefiltered and manually checked, after which statistical analysis of variance criteria was applied ($p < 0.05$ and $\text{fold} > 2.0$). Spots showing significant and reproducible changes were automatically marked by the software (Appendix L).

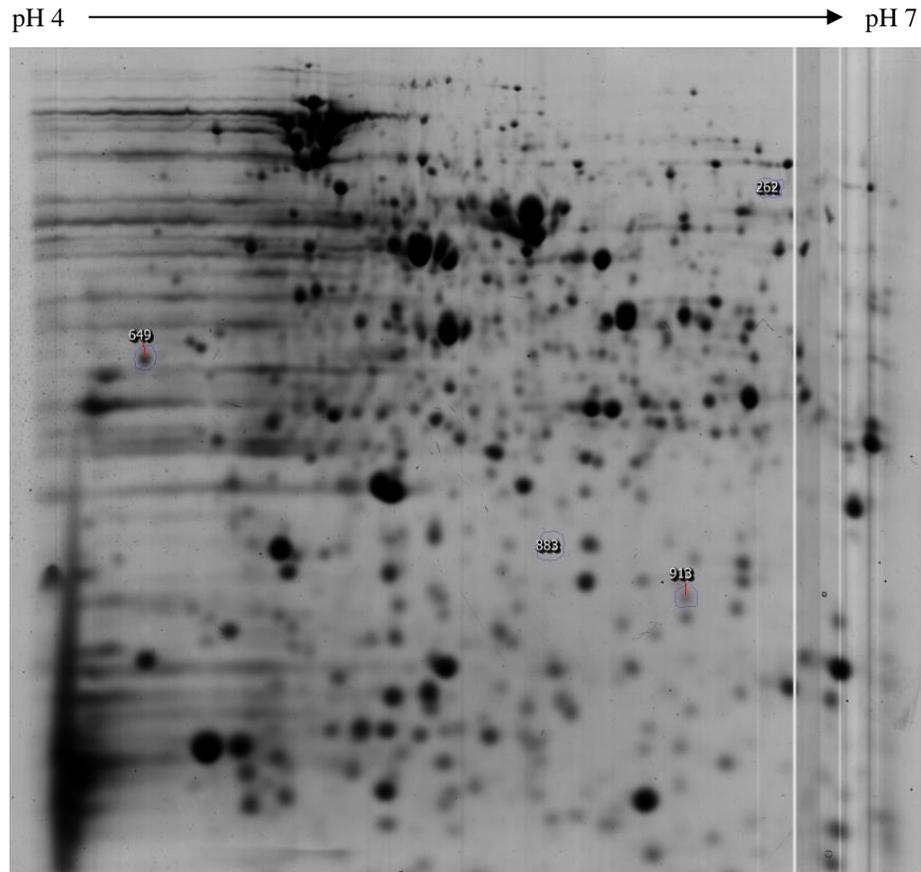


Figure 8.9: Matchset of *S. equi* 4047 WT/pVA838 versus Δ Prtm123/pVA838 – Cell associated protein extract. Shown here is the most representative image from the matchset of *S. equi* 4047 WT/pVA838 versus Δ Prtm123/pVA838 as determined by the software (Progenesis SameSpots). Circled spots with numbers indicate differentially expressed proteins. Expression levels were determined for each numbered spot as described in section 2.5.28.

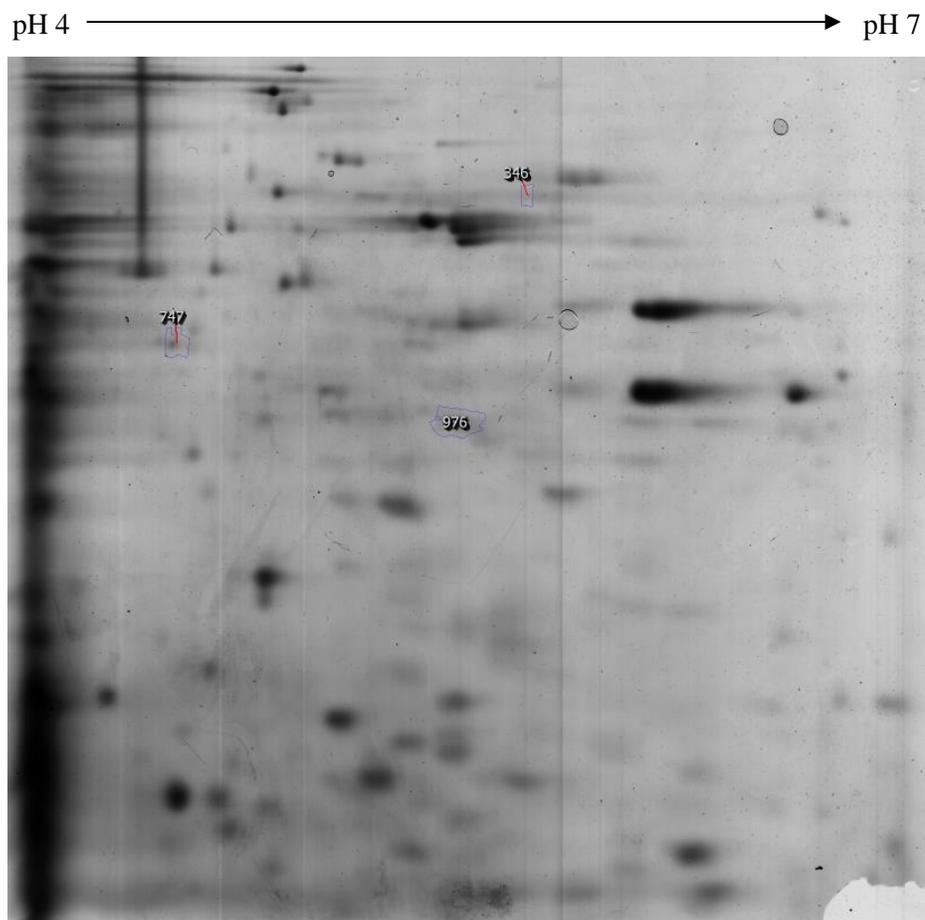


Figure 8.10: Matchset of *S. equi* 4047 WT/pVA838 versus Δ Prtm123/pVA838 – Secreted protein extract. Shown here is the most representative image from the matchset of *S. equi* 4047 WT/pVA838 versus Δ Prtm123/pVA838 as determined by the software (Progenesis SameSpots). Circled spots with numbers indicate differentially expressed proteins. Expression levels were determined for each numbered spot as described in section 2.5.28.

8.13 Results of LC/MS and Mascot Search

After analyzing the 2D-gels using Progenesis SameSpot software as described in Section 2.5.28, the significant spots from the gels of each matchset (mass spectrometric results tabulated in Tables 8.1 – 8.12) were picked, digested (method in Section 2.5.29) and then processed by HPLC/MS (method in Section 2.5.30). Analysis of the results of HPLC/MS by Mascot search (method in Section 2.5.31) gave the identities of proteins from some of the significant spots (Tables 8.1 – 8.12).

Spot ID	WT/ pVA838	Δ PrtM123/p VA838	Protein ID via Mascot search of LC-MS result
649	+	\pm	gi 154721499 translation elongation factor Tu [<i>Bacillus subtilis</i> subsp. <i>spizizenii</i> ATCC 6633]
262	\pm	+	gi 220930559 bifunctional acetaldehyde-CoA/alcohol dehydrogenase [<i>Clostridium cellulolyticum</i> H10]
913	+	-	NSH
883	-	+	NSH

Table 8.1: Mascot search results of spots from cell associated protein extract of *S. equi* 4047 WT/pVA838 vs Δ PrtM123/pVA838: Results of Mascot search following 2D-E, Progenesis SameSpot analysis and HPLC/MS/MS. The identities of some of the proteins from significant spots are shown. + means present, - means absent, \pm means present but at lower levels. NSH means no significant hit.

Spot ID	WT/ pVA838	Δ PrtM12/ pVA838	Protein ID via Mascot search of LC-MS result
661	+	-	gi 225870097 foldase protein PrsA [<i>Streptococcus equi</i> subsp. <i>equi</i> 4047]
662	+	-	gi 225870097 foldase protein PrsA [<i>Streptococcus equi</i> subsp. <i>equi</i> 4047]
485	+	-	gi 225870097 foldase protein PrsA [<i>Streptococcus equi</i> subsp. <i>equi</i> 4047]
663	+	\pm	NSH
688	\pm	+	NSH

Table 8.2: Mascot search results of spots from cell associated protein extract of *S. equi* 4047 WT/pVA838 vs Δ PrtM12/pVA838: Results of Mascot search following 2D-E, Progenesis SameSpot analysis and HPLC/MS/MS. The identities of some of the proteins from significant spots are shown. + means present, - means absent, \pm means present but at lower levels. NSH means no significant hit.

Spot ID	WT/ pVA838	ΔPrtM/ pVA838	Protein ID via Mascot search of LC-MS result
904	+	-	NSH
913	+	-	PYRH OCEIH Uridylate kinase OS= <i>Oceanobacillus iheyensis</i> (strain DSM 14371 / JCM 11309 / KCTC 3954 / HTE831) GN=pyrH PE=3 SV=1
934	+	-	NSH
906	+	-	SYH CLOA B Histidine--tRNA ligase OS= <i>Clostridium acetobutylicum</i> (strain ATCC 824 / DSM 792 / JCM 1419 / LMG 5710 / VKM B-1787) GN=hisS PE=3 SV=1
875	+	-	RL6 DESAP 50S ribosomal protein L6 OS= <i>Desulforudis audaxviator</i> (strain MP104C) GN=rp1F PE=3 SV=1
221	+	-	LEPA MO OTA Elongation factor 4 OS= <i>Moorella thermoacetica</i> (strain ATCC 39073) GN=lepA PE=3 SV=1
1075	-	+	NSH
883	-	+	FPG LACL C Formamidopyrimidine-DNA glycosylase OS= <i>Lactococcus lactis subsp. cremoris</i> GN=mutM PE=1 SV=3
891	-	+	NSH
148	+	±	NSH
966	+	±	RPOA O ENO B DNA-directed RNA polymerase subunit alpha OS= <i>Oenococcus oeni</i> (strain ATCC BAA-331 / PSU-1) GN=rpOA PE=3 SV=1
879	±	+	NSH

Table 8.3: Mascot search results of spots from cell associated protein extract of *S. equi* 4047 WT/pVA838 vs ΔPrtM/pVA838: Results of Mascot search following 2D-E, Progenesis SameSpot analysis and HPLC/MS/MS. The identities of some of the proteins from significant spots are shown. + means present, - means absent, ± means present but at lower level of expression. NSH means no significant hit.

Spot ID	Δ PrtM123 / pVA838	Δ PrtM12/ pVA838	Protein ID via Mascot search of LC-MS result
322	+	-	NSH
934	-	+	NSH
688	-	+	gi 307688041 outer membrane protein A [<i>Clostridium cellulovorans</i> 743B]
262	+	\pm	FPG LA Formamidopyrimidine-DNA glycosylase OS= <i>Lactococcus lactis</i> subsp. CLC <i>cremoris</i> GN=mutM PE=1 SV=3
672	+	\pm	NSH
900	+	\pm	MGSA EX Methylglyoxal synthase OS= <i>Exiguobacterium</i> sp. (strain ATCC BAA-1283 / ISA AT1b) GN=mgsA PE=3 SV=1

Table 8.4: Mascot search results of spots from cell associated protein extract of *S. equi* 4047 Δ PrtM123/pVA838 vs Δ PrtM12/pVA838: Results of Mascot search following 2D-E, Progenesis SameSpot analysis and HPLC/MS/MS. The identities of some of the proteins from significant spots are shown. + means present, - means absent, \pm means present but at lower level of expression. NSH means no significant hit.

Spot ID	Δ PrtM123 / pVA838	Δ PrtM/ pVA838	Protein ID via Mascot search of LC-MS result
556	+	-	NSH
874	+	-	2::gi 35187683 iron-containing superoxide dismutase [<i>Bacillus cereus</i>]
198	+	-	gi 35187683 iron-containing superoxide dismutase [<i>Bacillus cereus</i>]
933	+	\pm	NSH
1075	\pm	+	NSH
1078	\pm	+	RBSA BAC Ribose import ATP-binding protein RbsA OS= <i>Bacillus anthracis</i> GN=rbsA AN PE=3 SV=2
899	\pm	+	GLNH ABC transporter glutamine-binding protein glnH OS= <i>Bacillus subtilis</i> (strain BACSU 168) GN=glnH PE=2 SV=1

Table 8.5: Mascot search results of spots from cell associated protein extract of *S. equi* 4047 Δ PrtM123/pVA838 vs Δ PrtM/pVA838: Results of Mascot search following 2D-E, Progenesis SameSpot analysis and HPLC/MS/MS. The identities of some of the proteins from significant spots are shown. + means present, - means absent, \pm means present but at lower level of expression. NSH means no significant hit.

Spot ID	Δ PrtM12/ pVA838	Δ PrtM/ pVA838	Protein ID via Mascot search of LC-MS result
906	+	-	gi 398814302 hypothetical protein PMI05_01395 [<i>Brevibacillus sp.</i> BC25]
934	+	-	NSH
112	+	-	NSH
904	+	-	NSH
913	+	-	NSH
883	-	+	NSH
672	-	+	NSH
316	-	+	NSH
613	+	\pm	NSH
1025	+	\pm	IF2 GE Translation initiation factor IF-2 OS= <i>Geobacillus kaustophilus</i> (strain OKA HTA426) GN=infB PE=3 SV=1

Table 8.6: Mascot search results of spots from cell associated protein extract of *S. equi* 4047 Δ PrtM12/pVA838 vs Δ PrtM/pVA838: Results of Mascot search following 2D-E, Progenesis SameSpot analysis and HPLC/MS/MS. The identities of some of the proteins from significant spots are shown. + means present, - means absent, \pm means present but at lower level of expression. NSH means no significant hit.

Spot ID	WT/ pVA838	Δ PrtM123/ pVA838	Protein ID via Mascot search of LC-MS result
346	+	\pm	NSH
976	\pm	+	NSH
747	\pm	+	gi 154721499 translation elongation factor Tu [<i>Bacillus subtilis</i> subsp. <i>spizizenii</i> ATCC 6633]

Table 8.7: Mascot search results of spots from secreted protein extract of *S. equi* 4047 WT/pVA838 vs Δ PrtM123/pVA838: Results of Mascot search following 2D-E, Progenesis SameSpot analysis and HPLC/MS/MS. The identities of some of the proteins from significant spots are shown. + means present, - means absent, \pm means present but at lower level of expression. NSH means no significant hit.

Spot ID	WT/ pVA838	ΔPrtM12/ pVA838	Protein ID via Mascot search of LC-MS result
347	+	-	gi 1547214 translation elongation factor Tu [<i>Bacillus subtilis</i> subsp. <i>spizizenii</i> ATCC 6633]
1268	+	-	NSH
50	+	-	gi 60549198 60 kDa chaperonin [<i>Listeria innocua</i>]

Table 8.8: Mascot search of spots from secreted protein extract of *S. equi* 4047 WT/pVA838 vs ΔPrtM12/pVA838: Results of Mascot search following 2D-E, Progenesis SameSpot analysis and HPLC/MS/MS. The identities of some of the proteins from significant spots are shown. + means present, - means absent, ± means present but at lower level of expression. NSH means no significant hit.

Spot ID	WT/ pVA838	ΔPrtM/ pVA838	Protein ID via Mascot search of LC-MS result
355	+	-	gi 392461346 elongation factor Tu domain-containing protein, partial [<i>Pelosinus fermentans</i> A12]
346	+	-	gi 154721499 translation elongation factor Tu [<i>Bacillus subtilis</i> subsp. <i>spizizenii</i> ATCC 6633]
322	+	-	gi 154721499 translation elongation factor Tu [<i>Bacillus subtilis</i> subsp. <i>spizizenii</i> ATCC 6633]
1848	-	+	NSH
662	+	±	NSH
656	±	+	gi 307688041 outer membrane protein A [<i>Clostridium cellulovorans</i> 743B]
976	±	+	NSH
1518	±	+	NSH
1779	±	+	GUAA_LIS GMP synthase [glutamine-hydrolyzing] OS= <i>Listeria monocytogenes</i> serovar 1/2a MO (strain ATCC BAA-679 / EGD-e) GN=guaA PE=3 SV=1

Table 8.9: Mascot search results of spots from secreted protein extract of *S. equi* 4047 WT/pVA838 vs ΔPrtM/pVA838: Results of Mascot search following 2D-E, Progenesis SameSpot analysis and HPLC/MS/MS. The identities of some of the proteins from significant spots are shown. + means present, - means absent, ± means present but at lower level of expression. NSH means no significant hit.

Spot ID	Δ PrtM123 / pVA838	Δ PrtM12/ pVA838	Protein ID via Mascot search of LC-MS result
415	+	-	NSH
1460	+	-	NSH
1037	+	-	NSH
1891	+	-	NSH
904	+	\pm	gi 307688041 outer membrane protein A [<i>Clostridium cellulovorans</i> 743B]
1853	+	\pm	RL24_BACP2 50S ribosomal protein L24 OS= <i>Bacillus pumilus</i> (strain SAFR-032) GN=rplX PE=3 SV=1
355	\pm	+	NSH

Table 8.10: Mascot search results of spots from secreted protein extract of *S. equi* 4047 Δ PrtM123/pVA838 vs Δ PrtM12/pVA838. Results of Mascot search following 2D-E, Progenesis SameSpot analysis and HPLC/MS/MS. The identities of some of the proteins from significant spots are shown. + means present, - means absent, \pm means present but at lower level of expression. NSH means no significant hit.

Spot ID	Δ PrtM123 / pVA838	Δ PrtM/ pVA838	Protein ID via Mascot search of LC-MS result
1943	+	-	NSH
1932	+	-	NSH
1037	+	-	NSH
1460	+	-	NSH
415	+	-	NSH
322	+	-	gi 154721499 translation elongation factor Tu [<i>Bacillus subtilis</i> subsp. <i>spizizenii</i> ATCC 6633]
1848	-	+	NSH
1853	+	\pm	NSH
904	+	\pm	gi 307688041 outer membrane protein A [<i>Clostridium cellulovorans</i> 743B]
656	\pm	+	NSH
1918	\pm	+	NSH

Table 8.11: Mascot search results of spots from secreted protein extract of *S. equi* 4047 Δ PrtM123/pVA838 vs Δ PrtM/pVA838: Results of Mascot search following 2D-E, Progenesis SameSpot analysis and HPLC/MS/MS. The identities of some of the proteins from significant spots are shown. + means present, - means absent, \pm means present but at lower level of expression. NSH means no significant hit.

Spot ID	Δ PrtM12/ pVA838	Δ PrtM/ pVA838	Protein ID via Mascot search of LC-MS result
355	+	-	gi 392461346 elongation factor Tu domain-containing protein, partial [<i>Pelosinus fermentans</i> A12]
276	+	-	NSH
377	+	-	gi 6015080 RecName: Full=Elongation factor Tu; Short=EF-Tu
1918	-	+	NSH
351	+	\pm .	gi 154721499 translation elongation factor Tu [<i>Bacillus subtilis</i> subsp. <i>spizizenii</i> ATCC 6633]
656	\pm	+	NSH
976	\pm	+	ADDB ATP-dependent helicase/deoxyribonuclease subunit B OS= <i>Bacillus cereus</i> BACCN subsp. <i>cytotoxis</i> (strain NVH 391-98) GN=addB PE=3 SV=1

Table 8.12: Mascot search results of spots from secreted protein extract of *S. equi* 4047 Δ PrtM12/pVA838 vs Δ PrtM/pVA838: Results of Mascot search following 2D-E, Progenesis SameSpot analysis and HPLC/MS/MS. The identities of some of the proteins from significant spots are shown. + means present, - means absent, \pm means present but at lower level of expression. NSH means no significant hit.

8.14 Discussion

Complemented mutants (Δ PrtM123/pVA838 and Δ PrtM12/pVA838) were successfully produced as shown in Section 8.1-8.3. However, for meaningful comparison it was necessary to introduce the plasmid (pVA838) without any insert into *S. equi* 4047 WT and Δ PrtM. This way, the effect of the plasmid (pVA838) on the proteome of complemented mutants was nullified, so that only the effect of the introduction of the inserts (via the recombinant plasmids pVA838/PrtM123 and pVA838/PrtM12) was evaluated; and only proteins whose stability or localization were dependent on the full (N-terminal, Central and C-terminal domains) or partial (N-terminal and central domains) PrtM gene were isolated and evaluated from the cell as well as from the secreted proteins.

The observation of very little difference between the proteomes of the WT (WT/pVA838) and complemented mutant (Δ PrtM123/pVA838) containing the full PrtM gene as shown in Tables 8.1 and 8.7, indicates that the full PrtM gene restored the secreted and cell associated protein profiles back to the mutant (Δ PrtM). However, a significant number of cell associated proteins (up to four) were present in the WT/pVA838 but absent in the complemented mutant (Δ PrtM12/pVA838) containing the partial PrtM gene; notable was the presence of the foldase protein PrSA from *S. equi* 4047 in the WT/pVA838 and its absence in Δ PrtM12/pVA838 (Table 8.2). Up to three proteins were detected in the secreted protein extract of WT/pVA838 but absent in the complemented mutant (Δ PrtM12/pVA838) containing the partial PrtM gene; notable was a 60 kDa chaperonin (Table 8.8). Ma *et al.* (2006) implicated peptidyl-prolyl isomerase (*prsA*) in the

pathogenesis of the Gram-positive Group A *Streptococci*. *prsA* has also been found to be an essential gene that assists in the secretion, folding, and sequestration of a number of secreted proteins in *B. subtilis* (Vitikainen *et al.*, 2004; and Tossavainen *et al.*, 2006). In this study, PPIase activity was observed in the PrtM full length recombinant protein as well as in the PrtM central domain recombinant protein (Chapter 6). The results of this Chapter (8) therefore suggest that the full PrtM gene is essential for optimal PPIase activity *in vivo*.

The observation from a comparison of the proteomes of WT/pVA838 and Δ PrtM123/pVA838 suggest that the cell associated and secreted protein profiles of the mutant (*S. equi* 4047 Δ PrtM) can be largely restored by the introduction of the full PrtM gene. Also very importantly, the absence of significant proteins from the proteome of Δ PrtM12/pVA838, indicate that the C-terminal domain is very important and that the full PrtM gene is required for the stability and proper localization of some important proteins like PrsA from *S. equi* 4047 (in Table 8.2) and 60 kDa chaperonin (Table 8.8).

Although the impact of the deletion of the C terminal domain has been evaluated in this study, another area worth exploring in future is the impact a deletion of the N + C terminal domains of PrtM would have on *S. equi in vitro*. Also worth re-evaluating in future are the spots that yielded no significant hits on mascot search.

9.0 General Discussion and Future work

9.1 General Discussion

In spite of extensive efforts to prevent outbreaks, *S. equi* infection remains spread worldwide. Strangles continues to be a highly contagious and sometimes deadly equine disease and antibiotic use is not consensual since most treatment with antibiotics have proven to be ineffective after external signs of disease are noticed (Rodrigues *et al.*, 2012). Commercially available vaccines have not been successful against strangles (Harrington *et al.*, 2002; Waller and Jolley, 2007; Rodrigues *et al.*, 2012). The need to identify effective and safe therapeutic targets for the prevention and treatment of strangles therefore fuelled, and has been satisfied, by this research.

As discussed in chapter 1 (Section 1.7), although Hamilton *et al.* (2006) previously demonstrated that the putative lipoprotein maturase (PrtM) of *S. equi* 4047 is a major virulence factor, no functional role had been ascribed to it (PrtM). Hence this research set out to characterize PrtM and evaluate it as a potential therapeutic target.

It was proven in Chapter 3 (Section 3.5 and 3.14) of this study, that PrtM is involved in *S. equi* 4047 adaptation to NaCl stress. The finding that PrtM exhibits PPIase activity (Chapter 6) and the increased sensitivity of the mutant (Δ PrtM) to various antibiotics (Section 3.6) suggest that the PPIase activity of PrtM is associated with cell wall synthesis and protein remodelling. The findings of this study also suggest that PrtM may be involved in speeding up the folding or remodelling of one or more of the enzymes involved in

hyaluronic acid synthesis (Section 3.8). All these, coupled with the observation that the mutant (Δ PrtM) cross reacted with horse convalescent serum, *S. equi* pre-and post-infection sera, as well as with α PPMA antisera (Chapter 3) suggest that PrtM is a good vaccine candidate. Further support for the fact that PrtM is a good therapeutic target, was the reaction of the secreted protein extract of only the WT but not the mutant (Δ PrtM) with anti-HPr (Chapter 3, Figure 3.14-B), suggesting that *S. equi* PrtM may be involved in the folding or remodelling of HPr kinase and that released HPr-1 of *S. equi* may have critical roles to play in HPr-1 mitogenic activity.

Proteomics analysis of the WT, mutant - Δ PrtM (Chapter 3) and complemented mutants - Δ PrtM123/pVA838 and Δ PrtM12/pVA838 (Chapter 8) establish that the protein profile of *S. equi* 4047 WT has some differences from that of the mutant (Δ PrtM). These results confirm that, the absence of parts or all of the full PrtM gene result in readily detectable changes in the proteome of *S. equi* 4047. Finding FNE and IdeE2, in the secreted protein extract of only *S. equi* 4047 WT and not the PrtM deficient-mutant (Chapter 3); and finding the foldase protein PrSA from *S. equi* 4047 in the WT/pVA838 but not in Δ PrtM12/pVA838 (Chapter 8) suggest proteolytic degradation of misfolded secreted proteins and indicates that PrtM may not only be linked to the folding of one specific substrate but could be a multisubstrate foldase. Finding foldase protein PrSA from *S. equi* 4047 in the WT/pVA838 (Chapter 8) is consistent with the identification of the same protein from the dimeric full length recombinant protein of *S. equi* 4047 WT (Chapter 5).

In *B. subtilis*, the PrsA lipoprotein (a parvulin foldase) is essential for protein secretion and viability (Kontinen and Sarvas, 1993). Interestingly, the predicted modular organization of *S. equi* 4047 PrtM (Figures 4.9a and 4.9b in Chapter 4) is similar to that of *B. subtilis* PrsA and *L. monocytogenes* PrsA2, with N- and C- terminal domains flanking a central domain which is predicted in this study (Chapter 4) to also be a PPIase domain.

Most of the proteins that are translocated across the cytoplasmic membrane of Gram-positive bacteria are delivered to the membrane–cell wall interface in an essentially unfolded form (Sarvas *et al.*, 2004). To gain functional activity, most proteins must be folded into defined three-dimensional structures (Hartl *et al.*, 2011). After translocation, proteins rely on various folding factors (including PPIases and chaperones) to reach their native state (Foster *et al.*, 2011). Chaperones are able to assist proteins folding into their native conformations by inhibiting improper protein-protein interactions and altering the activation energy necessary for proper folding (Ellis and van der Vies, 1991).

As mentioned in chapter 1 (Section 1.5), PPIases are folding proteins that catalyze the rate limiting *cis/trans* isomerization of peptidylprolyl (Lazar and Kolter, 1996; Reffuveille *et al.*, 2012). Protein folding can be assisted by both molecular chaperones and foldases (Zhang *et al.*, 2013). It has been observed that some multidomain PPIases act as both PPIases and chaperones (Mok *et al.*, 2006; Zhang *et al.*, 2013). In addition to PPIase activity, prokaryotic parvulins also exhibit chaperone-like activities (He *et al.*, 2004). It is not surprising therefore, that in addition to PPIase activity that was observed in the full length and central domain recombinant proteins of *S. equi* 4047 WT (Chapter 7), a 60 kDa

chaperone was also detected in the *S. equi* 4047 WT (WT/pVA838 in Chapter 8), indicating the co-existence of PPIase and chaperone activities in PrtM. Finding a 60 kDa chaperone as well as the foldase protein PrSA from *S. equi* 4047 in the WT/pVA838 but not in Δ PrtM12/pVA838 (Chapter 8 of this study) is also suggestive that the C-terminal domain is essential for the PPIase-Chaperonin function of the PrtM of *S. equi* 4047. It is predicted that the central domain of *S. equi* is made up of parvulin-like four-stranded antiparallel β -sheet core and four α -helices $\beta\alpha_3\beta\alpha_2$ (Chapters 4 and 7). Due to the fact that PPIases play important roles in a wide range of physiological processes, the design of selective and potent inhibitors against PPIases (to potentially treat viral, parasitic, fungal and bacterial infections) has been widely researched as is quite promising (Dugave, 2006).

9.2 Future work.

As discussed in Section 3.14, the possible involvement of the PPIase activity of the PrtM of *S. equi* 4047 (Chapter 6) in speeding up the folding or remodeling of one or more of the enzymes involved in hyaluronic acid synthesis is worth exploring in future because *S. equi* 4047 WT produced more hyaluronic acid than the PrtM-deficient mutant strain (Chapter 3, Sections 3.9).

The reaction of the secreted protein extract of only the WT but not the mutant (Δ PrtM) with anti-HPr (Chapter 3, Sections 3.11 and 3.14), is suggestive that *S. equi* PrtM PPIase activity (Chapter 6) may be involved in the folding or remodeling of HPr kinase. This absence of the upper band (Chapter 3, Section 3.11, Figure 3.14-B) in the secreted protein extract of the PrtM deficient mutant also indicates that released HPr-1 of *S. equi* may have critical roles to play in HPr-1 mitogenic activity. Therefore, as extensively discussed in Section 3.14, the relationship of PrtM to HPr kinase remodeling and HPr-1 mitogenic activity is worth further evaluation.

The enzyme kinetics of the full length recombinant protein of *S. equi* 4047 was successfully calculated in this study (Chapter 6, Section 6.4). The central domain recombinant protein was highly sensitive toward chymotrypsin (Chapter 6, Section 6.3). It was therefore suggested (Chapter 6, Section 6.5) that other proteases (like trypsin or subtilisin) or a protease-free assay could be used to accurately measure the kinetic constants of the central domain recombinant protein fraction of *S. equi* 4047. In addition to

this, the benefit of evaluating PPIase activity via other techniques (like studying the refolding kinetics of denatured proteins like ribonuclease T1 and dynamic proton NMR spectroscopy) was also discussed in Section 6.5.

The inference (in Chapter 6, Section 6.5) that a PrtM N+C mutant (lacking the central domain) will be unable to catalyze the *cis* → *trans* isomerization of Pep1 (Suc-Ala-Phe-Pro-Phe-pNa) is subject to confirmation in future work. In addition to this, as discussed in Chapter 8 (Section 8.14), it would be valuable to evaluate the proteome of a complemented mutant lacking the N+C terminal domains; and also re-evaluate the spots that yielded no significant hits on mascot search.

As mentioned (Chapter 7, Section 7.4), the growth of a visually perfect crystal does not always mean that the protein's structure will be solved by x-ray crystallography, due to limited correlation between a microscopically beautiful crystal and its diffraction quality (Owen and Garman, 2005). Despite success is producing crystals under different conditions (Chapter 7, Section 7.2), attempts to solve the structure of PrtM of *S. equi* 4047 by x-ray crystallography were unsuccessful (Chapter 7, Sections 7.3 and 7.4). The knowledge of the structure and catalytic mechanism of PrsA-PPIase is necessary for successful design of efficient and selective enzyme inhibitors which could be used as antibacterial agents against Gram-positive bacteria (Heikkinen *et al.*, 2009). In addition to the proven PPIase activity of PrtM (Chapter 6), knowing its (PrtM) crystal structure would enhance the selection and design of inhibitors. It is predicted that the central PPIase domain of *S. equi* contains four-stranded antiparallel β -sheet core and four α -helices,

$\beta\alpha_3\beta\alpha_2$ (Chapter 4 and Chapter 7). Therefore, the possible use of other techniques (like template-based modelling and small-angle scattering in addition to X-ray crystallography and solid state NMR) in solving the structure of PrtM in future was suggested and discussed in Section 7.4.

9.3 Conclusion

The involvement of PrtM of *S. equi* 4047 in adaptation to NaCl stress (Section 3.5 and 3.14); its involvement in regulating sensitivity to antibiotics and thereby cell wall synthesis and protein remodeling (Section 3.6); its possible roles in speeding up the synthesis of hyaluronic acid (Section 3.8) and in the folding or remodeling of HPr Kinase (Section 3.11); its parvulin gene organisation elucidated by bioinformatics analysis (Chapter 4), the cross reactions of the mutant (Δ PrtM) with a number of antisera (Section 3.11), the observation that PrtM may be a multisubstrate foldase due to the detectable and significant differences in the proteomes of the WT, mutant (Chapter 3) and complemented mutants (Chapter 8); the dimeric protein formed by the full length recombinant protein fraction of *S. equi* 4047 WT (Chapter 5); and the PPIase-Chaperonine activities of PrtM (Chapters 6 and 8) all validate PrtM of *S. equi* 4047 as a viable and novel therapeutic candidate which pharmaceutical industries should extensively evaluate for the prevention and treatment of strangles.

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Zeng Lin and Burne A. Robert (2010). Seryl-phosphorylated HPr Regulates CcpA-Independent Carbon Catabolite Repression in Conjunction with PTS Permeases in *Streptococcus mutans*. **Molecular Microbiology**, **75 (5):1145-1158; March, 2010.**

Appendices

Appendix A

A1: Chemicals, Media, Enzymes, and Reagents

Acros

Adenine
p-aminobenzoate
Ammonia solution
Cysteine
3, 5-Dinitrosalicylic acid (DNSA)
Disodium phosphate
Guanine
Iodoacetamide
Monosodium phosphate
2,7 – naphthalene-disulfonic acid solution
Nicotinamide
Sodium hyaluronate
Uracil

Aldrich

Pantothenate

Bio-Rad Laboratories

Coomassie G250
Nitrocellulose membrane

British Drugs House (Merck)

Alcian Blue 8GX
Biotin
Folic acid

Fisher BioReagents

Acrylamide/Bisacrylamide 37.5:1, 40 % solution
Brilliant blue G 250
Coomassie Blue R-250
Lysozyme
Methanol
Phenol

Fisher Chemicals

Acetic acid, glacial
Acetone
Acetonitrile

Ammonium bicarbonate
Ammonium sulphate
Dimethyl sulfoxide
Ethanol
Formaldehyde
Formic acid
Glutaraldehyde
Phosphoric acid
Sodium carbonate
Sodium tetraborate
Trichloroacetic acid (TCA)
Trisodium citrate

Fluka Analytical Chemicals

Carbol-Fichsin solution according to Ziehl-Heelsen

Melford Laboratories Ltd

Agarose (High gel strength)
Dithiothreitol
Glycine
Sodium dodecylsulphate
Tris [Hydroxymethyl] aminomethane (Tris-HCl)

Oxoid

THA
THB

Promega

Trypsin Gold

Riedel-deHaen

Hydrochloric acid
Sulfuric acid

Sigma

Albumin
Ammonium sulphate
Angiotensin I
APS
Bovine serum albumin, Fraction V (BSA)
Bromophenol blue
Carbonic anhydrase
CHAPS
Citric acid
Dipotassium phosphate
Ethidium bromide

Ethylene diamine tetraacetic acid, disodium salt (EDTA)
Ferrous sulphate
 β -Galactosidase
Glucose
Glucuronic acid
L-glutamic dehydrogenase
Glyceraldehydes-3-phosphate dehydrogenase
Glycerol
Isopropanol (2- propanol)
 α - lactalbumin
Manganous sulphate
Magnesium chloride
 β -mercaptoethanol
Ovalbumin
Phosphorylase b
Ponceau S solution
Potassium Chloride
Potassium phosphate monobasic
Riboflavin
Silver nitrate
Sodium acetate
Sodium chloride
Sodium hydroxide
Sodium sulphite
Sucrose
TEMED
Thiamine
Trypsinogen
Urea

A2: Protein Size Markers

SigmaMarker™:

Proteins	M.W. (Da)	High	Low
Myosin, rabbit muscle	205000	X	
β -Galactosidase, <i>E. coli</i>	116000	X	
Phosphorylase b, rabbit muscle	97000	X	
Fructose-6-phosphate Kinase, rabbit muscle	84000	X	
Albumin, bovine serum	66000	X	X
Glutamic Dehydrogenase, bovine liver	55000	X	
Ovalbumin, chicken egg	45000	X	X
Glyceraldehydes-3-phosphate Dehydrogenase, rabbit muscle	36000	X	X
Carbonic Anhydrase, bovine erythrocytes	29000		X
Trypsinogen, bovine pancreas	24000		X
Trypsin Inhibitor, soybean	20000		X
α -Lactalbumin, bovine milk	14200		X
Aprotinin, bovine lung	6500		X

Table A1: Molecular weight distribution in Sigma Markers.

Lyophilized standards were stored at 2-8°. Lyophilized standards were reconstituted with 100 μ l of 18.2 M Ω /cm H₂O to give a final concentration of 2.0-3.5 mg/ mL. The reconstituted standard was aliquoted into 4 μ l amounts and stored at 20°C. Before use, 8 μ l of SDS-PAGE loading buffer (appendix D6) was mixed with the thawed standard and boiled for 3mins.

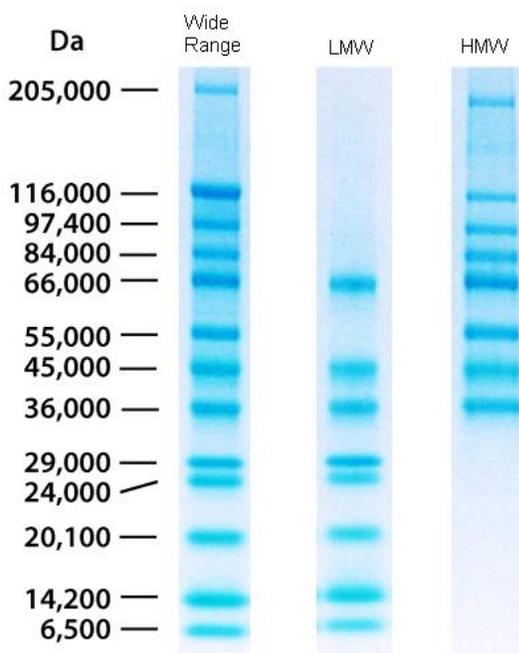


Figure A1: Molecular weight distribution in Sigma Markers (Wide range molecular weight, low molecular weight (LMW), and high molecular weight (HMW)). Adapted from: http://www.sigmaaldrich.com/catalog/ProductDetail.do?N4=M3913|SIGMA&N5=SEARCH_CONCAT_PNO|BRAND_KEY&F=SPEC&lang=en_US (assessed on 20th August, 2009).

A3 ProlexTM Streptococcal Grouping Latex Kit – PRO-LAB DIAGNOSTICS

The ProlexTM Streptococcal Grouping Latex Kit was used to serologically carry out Lancefield grouping of the S equi strains (wild type and mutant).

A3.1 ProlexTM Streptococcal Grouping Latex Kit Components

- Blue Latex Suspension Group A
- Blue Latex Suspension Group B
- Blue Latex Suspension Group C
- Blue Latex Suspension Group D
- Blue Latex Suspension Group F
- Blue Latex Suspension Group G
- Extraction Reagent 1
- Extraction Reagent 2
- Extraction Reagent 3
- Polyvalent Positive Control
- Mixing Sticks
- Latex Test Cards

A3.2 Streptococcal Grouping Test Protocol:

All components were brought to room temperature (22-28 °C) prior to use. One reaction well was labeled for each specimen and one drop of extraction reagent 1 was added to each well. Selected 2-4 colonies with a disposable loop and suspended them in the extraction reagent 1 until it became turbid. Added one drop of extraction reagent 2 to each well and mixed reaction by rocking the plate for 5-10 seconds. Added a few drops of extraction reagent 3 to each well and mixed the reaction again for 5 – 10 seconds. Dispensed one drop of each blue latex suspension onto separate circles on the tset card. With a Pasteur pipette, placed one drop of each extract beside each drop of latex suspension. Mixed the blue latex and extract with wooden sticks and rocked the card slowly for 60 seconds. Observed for agglutination.

A4 Foetal Calf Serum (FCS)

From EU approved origin
GIBCO 2012-10
Ref: 10106 -151
Lot #: 41F5674F
Invitrogen Life Technology

Stored aliquots of 10 mL X 10 at -20 °C.

A5 Nzomics-biocatalysis Hyaluronan Test Kit (Version 1.01)

A6 QIAGEN DNeasy DNA Extraction Kit:

Blood and Tissue Kit 50D (Catalogue # 69504)

A6.1 Enzymatic Lysis Buffer from kit (Appendix A6): 20mg/ mL lysozyme

- 1 20mM Tris.Cl, pH 8.0
- 2 2mM sodium EDTA
- 3 1.2% Triton[®] X-100
- 4 20mg/ mL lysozyme; add lysozyme just before use

Components 1, 2 and 3 were mixed prior to lysis. However, lysozyme was added only just before extraction was actually carried out. To get 20mg/ mL lysozyme:

Weigh 19.3mg lysozyme
 $19.3/20 = 0,965$

Therefore, added 965µl of premixed lysis buffer to 19.3mg lysozyme. This give 20mg/ mL lysozyme.

A.6.2 Buffers (as supplied in kit -appendix A6)

Buffer AL (216 mL)	216 mL Lysis Buffer
Buffer ATL (200 mL)	200 mL Tissue Lysis Buffer
Buffer AW1 (Concentrate, 242 mL)	242 mL Wash Buffer (1) Concentrate
Buffer AW2 (Concentrate, 324 mL)	324 mL Wash Buffer (2) Concentrate
Buffer AE (240 mL)	240 mL Elution Buff

A7: SYBR-safe DNA Gel Stain (Invitrogen)

10,000 X concentrate in DMSO

S33102, Lot # 410108

Invitrogen molecular Probes, Eugene, Oregon, U.S.A, 541 465 8300.

probes.invitrogen.com

A8: Hyperladder™ 1 (Bioline)

Lot # H1-105B

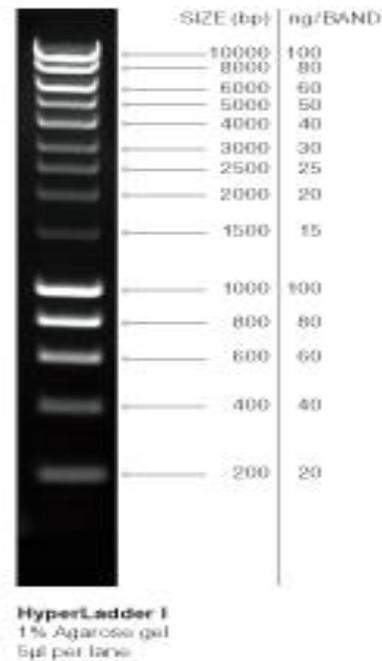


Figure A2: Bioline Hyperladder banding pattern 5µl on 1% agarose.

A9: PCR Components (Novagen)

dNTPs	2mM
KOD Hot Start polymerase	1 u/ μ
MgSO ₄	25mM
Buffer	10X

A11 Blood agar base (Merck, #VM402286 515; www.merck.de)

A12 NZYGelpure™ Kit (Prozomix)

<http://www.prozomix.com/products/view?product=814¤cy=33>

A13 Protein Standard: Bio-Rad Precision Plus Protein™ All Blue Standard: for Western blotting

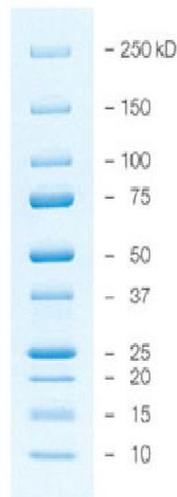


Figure A3: Bio-Rad Precision Plus Protein™ All Blue Standard Migration distance

A14: BCIP/NBT Substrate Kit (substrate for alkaline phosphatase) – ZYMED Laboratories

Kit Contents:

A- concentrated BCIP/buffer solution (10X), 100 mL

B- concentrated NBT solution (10X), 100 mL

store at 2-8°C.

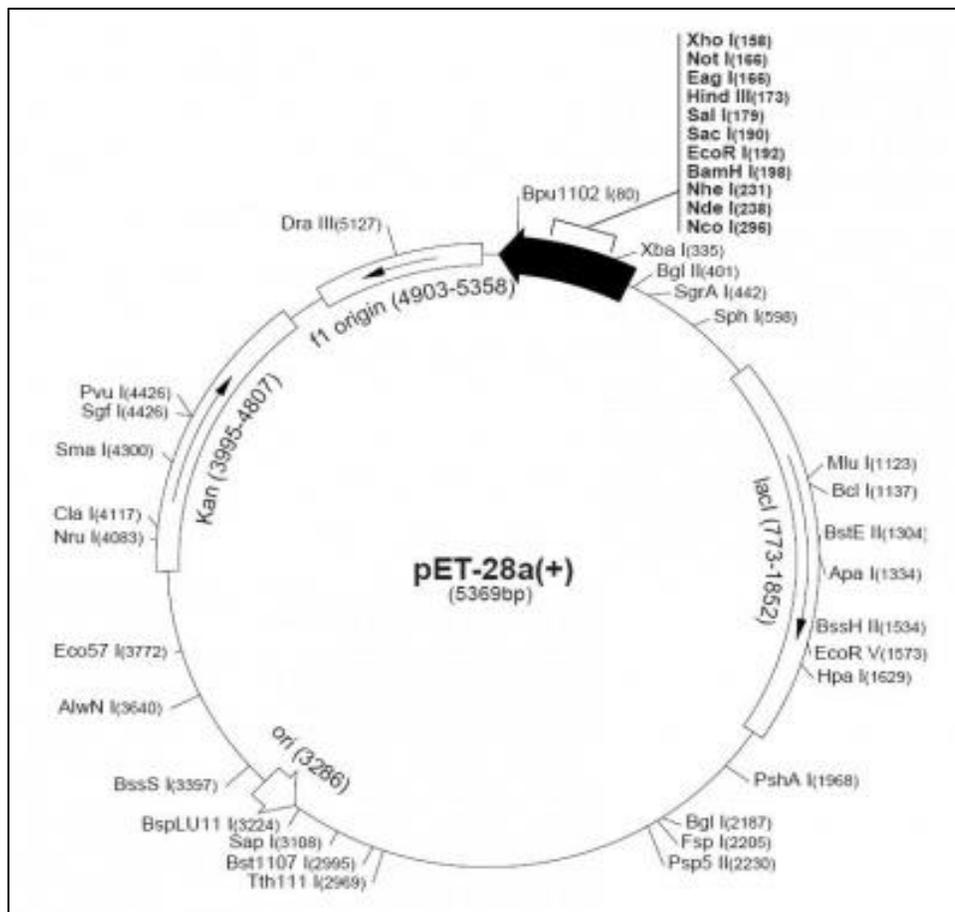
BCIP is an insoluble substrate for alkaline phosphatase which is good for staining blots. Added 1 mL of each reagent to 8 mL of distilled water immediately before use.

A15 IPG Buffer (GE Healthcare)

Selected and used an IPG buffer with the same pH interval as the immobiline DryStrip to be rehydrated.

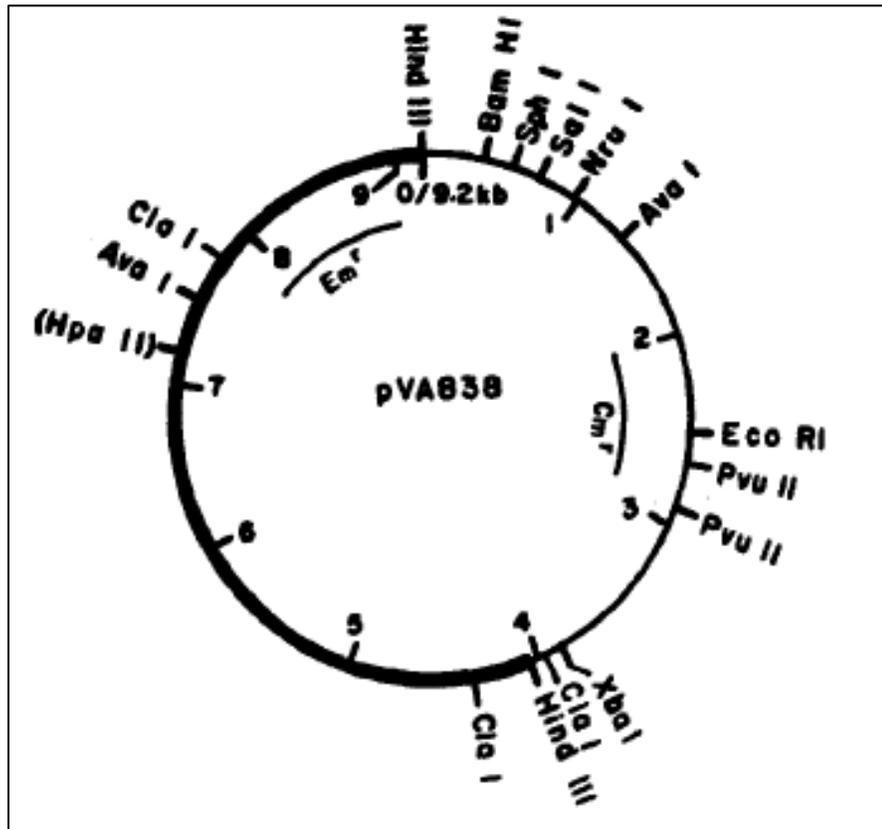
A16 Immobiline™ DryStrip Cover Fluid – GE Lifesciences

A17 Map of pET-28a vector (Novagen)



FigureA4: Map of pET-28a vector (Novagen)

A18 Map of pVA838 (Macrina *et al.*, 1983)



FigureA5: Map of pVA838 (Macrina *et al.*, 1983, p.146)

Appendix B

B1: Autoclaving

Autoclave sterilisation was achieved using a benchtop Prestige® Medical 2100 Classic autoclave at 121 °C, 32 lb/inch² pressure for 20 min.

B2: pH meter

All adjustments to the pH of solutions and media were achieved using a Jenway Ion Meter 3340 calibrated with buffers at pH 4.0, 7.0, and 9.2.

B3: UV-Visible Spectrophotometer (Spectronic Unicam Helios- α , Thermos Electron Corporation).

B4: Incubators

Growth of bacteria was performed in static Gallenkamp incubators

B5: Centrifugation

Centrifugation of volumes below 1.5 mL was achieved using a small Sigma 1-15 bench top micro-centrifuge. Volumes above 1.5 mL were centrifuged in a large Sigma 3K18C refrigerated bench top centrifuge, using appropriate rotors and inserts.

B6: Sonication

Cell lysis was carried out using a MSE Soniprep 150 ultra-sonication machine.

B7: Microtitre plate reader

Growth curve assays were measured from samples in 96-well plate at OD₅₇₀ using EL 808 Ultra Microplate Reader, Bio-Tek Instrument Inc.

B8: SDS-PAGE gel kit

Electrophoresis of proteins was carried out with Bio-Rad Mini-PROTEAN® 3 Cell kit powered by an E-C 570-90 E-C apparatus corporation power pack.

B9: Gel documentation

Visualisation of SDS-PAGE gels was done with Bio-Rad Gel Doc 2000 system or a Bio-Rad GS-710 densitometer system and Quantity One™ software. Hard copies of the gel picture were produced using a Mitsubishi Video Copy Processor (Model P91), with Mitsubishi thermal paper (K65HM-CE /High density type, 110 mm x 21 m).

B10: Immobiline DryStrip Kit

This kit is designed for running the first dimension of 2-D electrophoresis using precast Immobiline™ DryStrip gels.

B11a: Protean II XL 2-D cells and accessories for 2D-E: BioRad

B11b: Protean Plus Dodeca Cell and accessories for 2D-E: Biorad

B12: Centrifugal evaporator

Extraction of protein digests were dried using CHRIST centrifugal evaporator RVC 2-18

B13: Progenesis SameSpot™ software (a product of Nonlinear Dynamics)

B14: Nitrocellulose membrane (Bio-Rad)

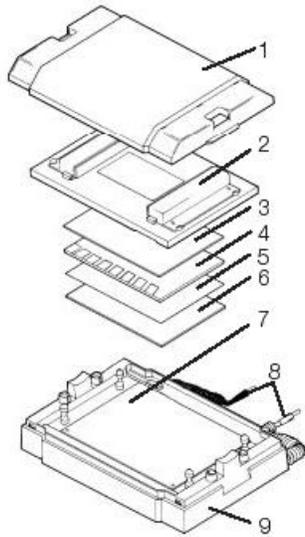
B15: 1D-Electrophoresis Apparatus (Mini-PROTEAN® 3 Cell Assembly) - BioRad

B16 (Immobiline DryStrip Gels - GE Healthcare)

B17 Immobiline™ DryStrip Reswelling Trays (GE Healthcare)

- B18 Multiphor™ II Electrophoresis System and Unit (GE Lifesciences)**
- B19 IEF Electrode Strips (paper): GE Lifesciences**
- B21 Bio-Rad GS-710 image densitometer**
- B22: Javac Laboratory High Vacuum pump (D00061 Wombat JL – 2.5 double stage)**
- B23: Freeze Drier (Alpha-12 LD plus Christ Freeze Drier)**
- B24 HPLC Machine (Ultimate 3000 - Dionex):**
- B25 Mass Spectrometer HCTultra - Bruker Daltonics. A High Capacity Trap (HCT) ultra spectrophotometer for ion trap analysis.**
- B26: 384-well microarray plate – Fisher Scientific**
- B27 NanoDrop Spectrophotometer (ND-1000)**
Supplied by LabTech International,
- B28 Stuart Rotator (SB2)**
bibby-scientific
- B29 UV/Visible spectrophotometer**
Pharmacia Biotech, Ultrospec 2000.

B30 Western blotting Semi-Dry Transfer Assembly



Trans-Blot SD Semi Dry Cell:

1. Safety lid
2. Cathode assembly with latches
3. 2 layers soaked filter papers
4. Gel
5. Nitrocellulose membrane
6. 2 layers soaked filter papers
7. Spring-loaded platinum anode platform, mounted on four guide posts
8. power cables
9. base

Figure B1: Bio-Rad Semi-Dry Trans-Blot Cell Assembly

B31 Electroporator – Gene Pulser (Bio-Rad, U.K.)

Appendix C: 2D-E gel images and Spot IDs by PDQuest Software

Appendix C1: Images of 2D-E gels (pH 4-7)

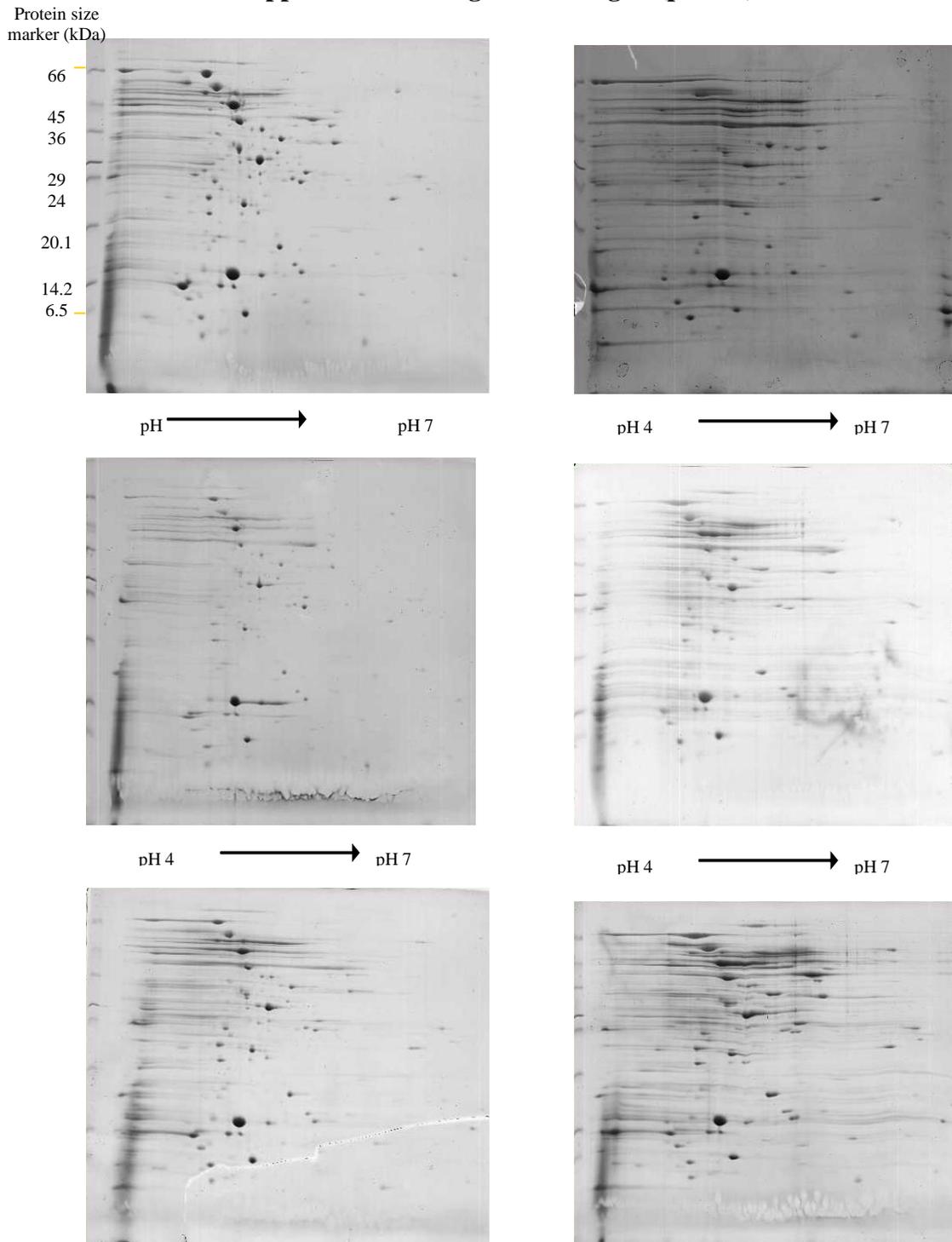


Figure C1: Images of 2D-E gels of Cell Associated Protein Extract of *S. equi* 4047 WT at pH 4 - 7.

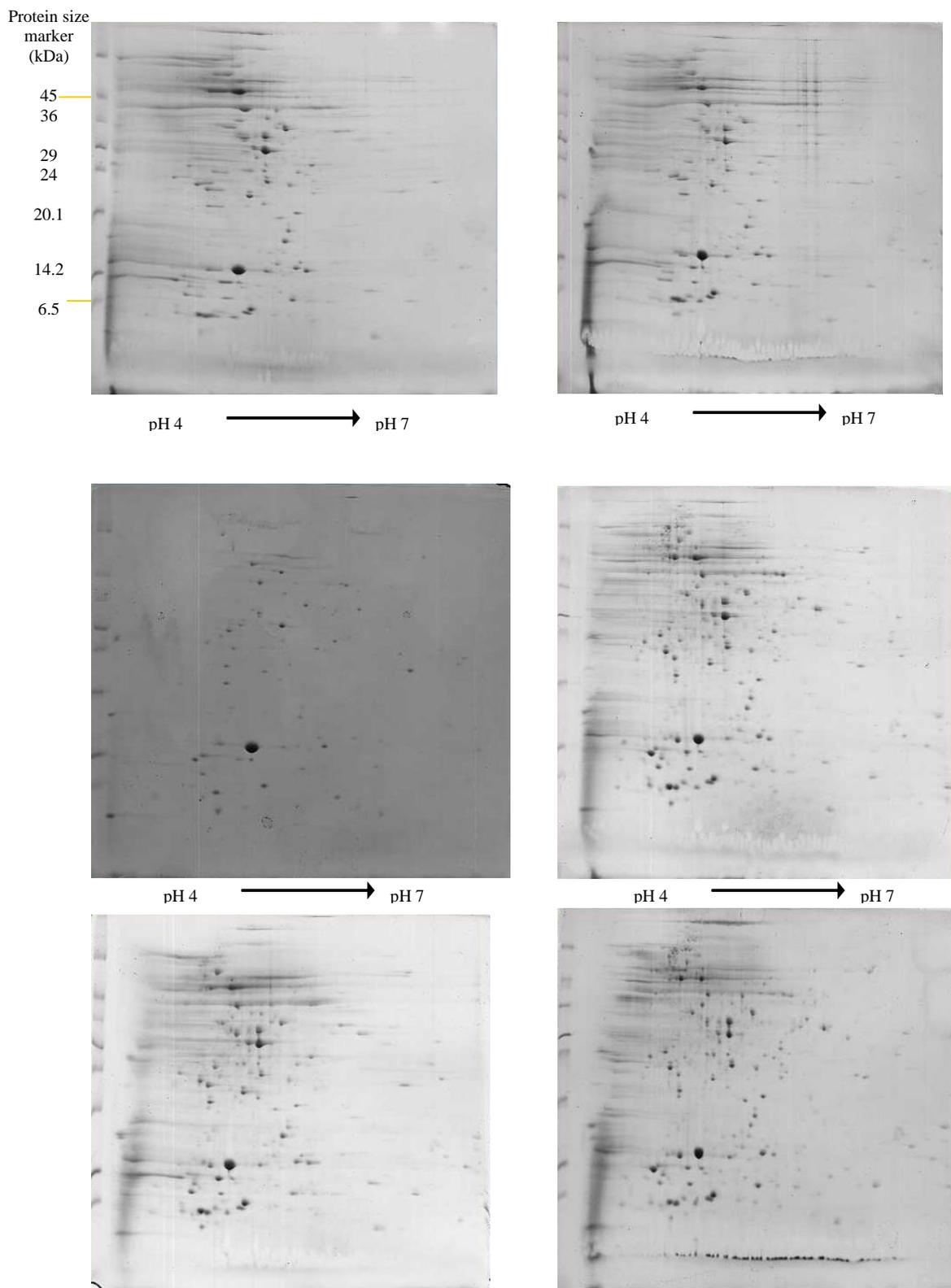


Figure C2: Gel Image of 2D-E of Cell Associated Protein Extract of *S. equi* 4047 Δ PrtM at pH 4-7

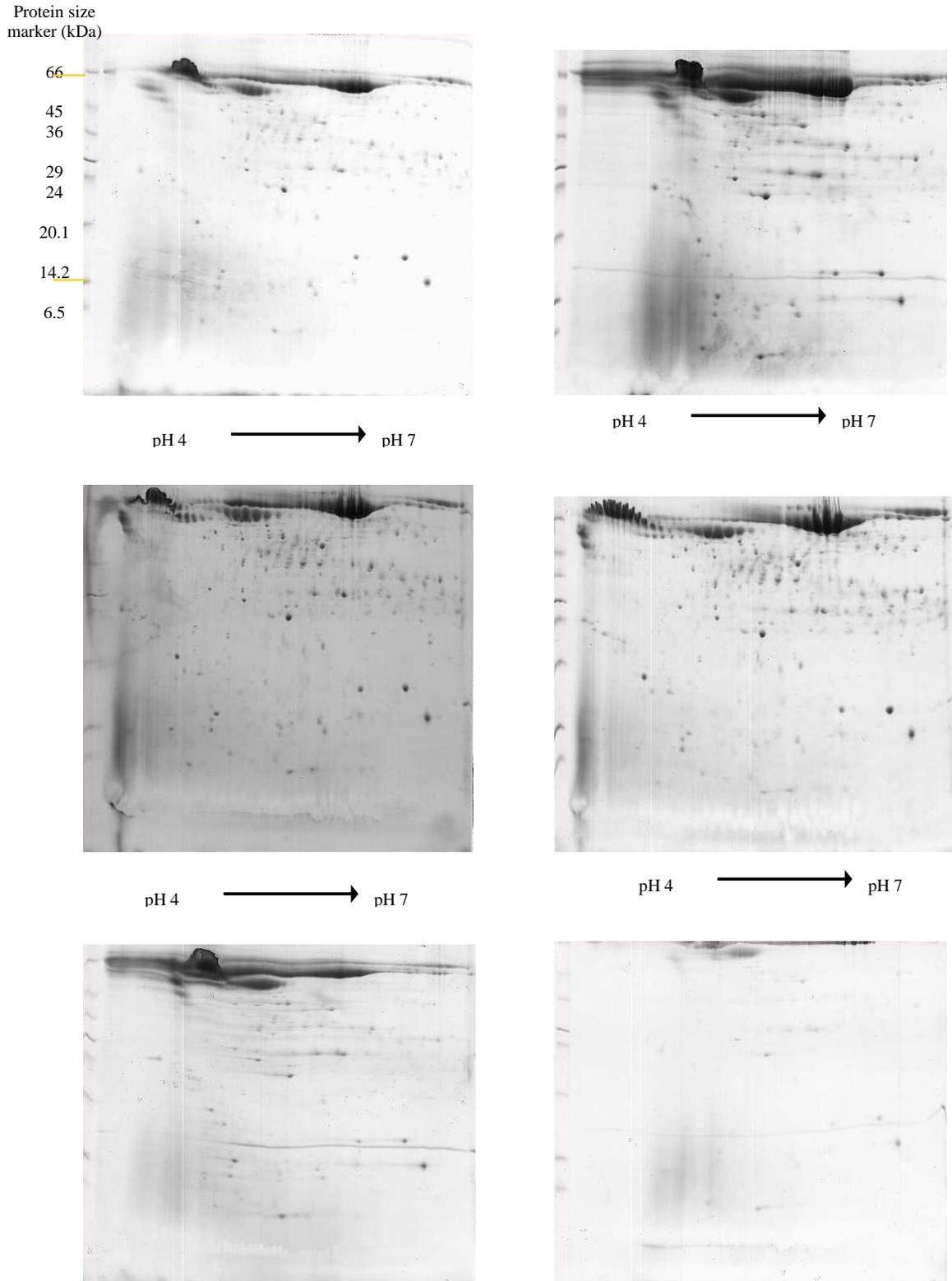


Figure C3 Gel Image from 2D-E of Secreted Protein Extract of *S. equi* 4047 WT at pH 4 – 7.

Protein
size marker
(kDa)

66

45

36

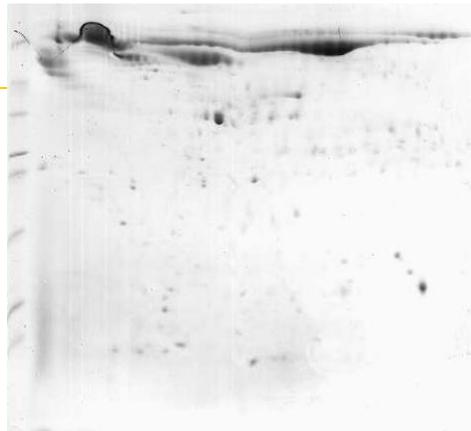
29

24

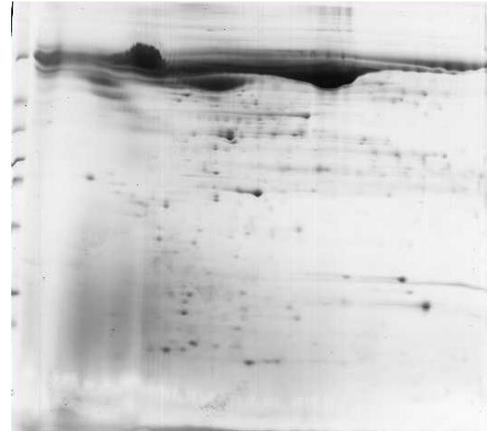
20.1

14.2

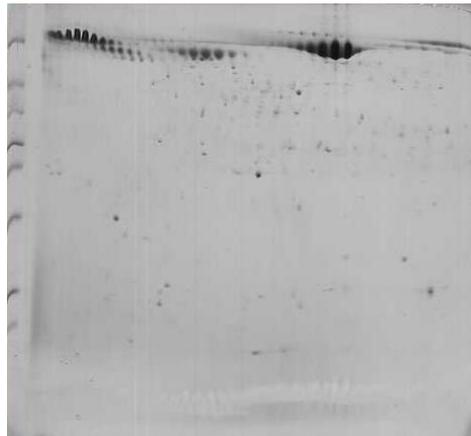
6.5



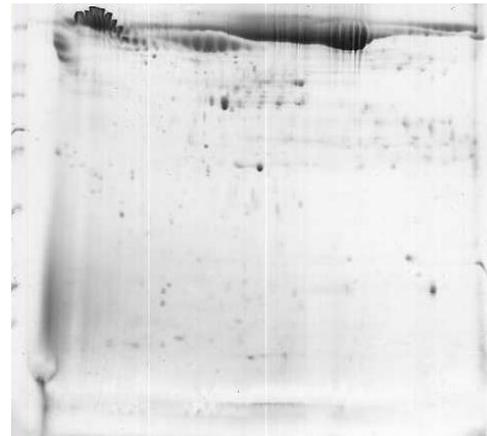
pH 4 → pH 7



pH 4 → pH 7



pH 4 → pH 7



pH 4 → pH 7

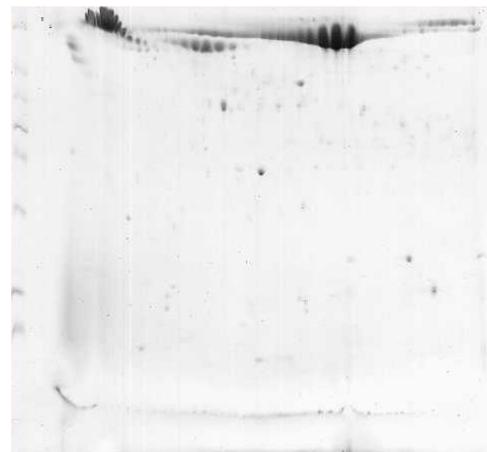
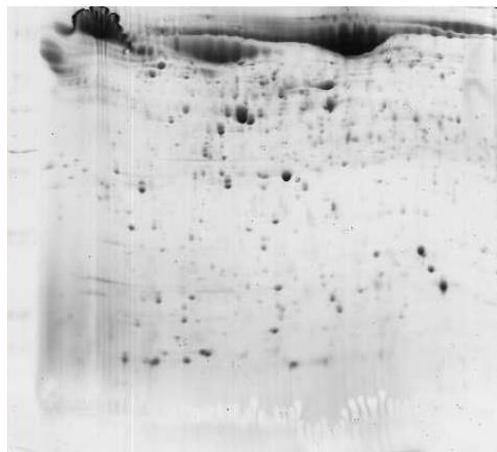


Figure C4 Gel Image from 2D-E of Secreted Protein Extract of *S. equi* 4047 Δ PrtM at pH 4-7

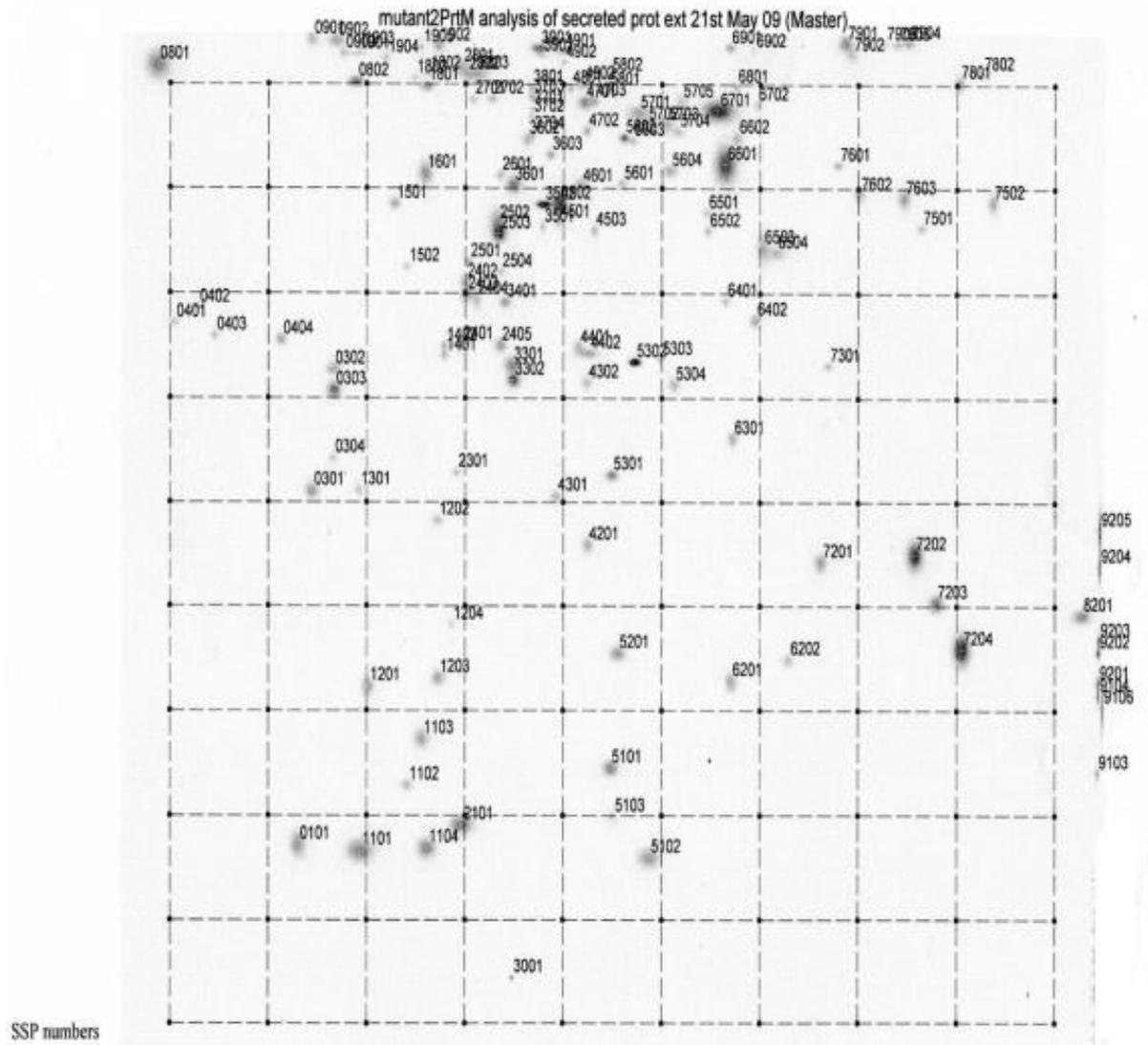


Figure C6: Master image with spot IDs for 2D-E gel images of *S. equi* 4047 Mutant (Δ PrtM) Secreted Protein Extract

Appendix D: Identities of all proteins after MS/MS

Table D1 List of Protein from *S. equi* 4047 WT and Mutant (Δ PrtM): Cell Associated Protein Extract

<u>Spot #</u>	<u>Spot #</u>	<u>Protein name</u>	<u>Protein code</u>	<u>Comment on expression</u>	<u>Comment on level of hit</u>	<u>Protein code for best match hit</u>
WT3101	PrtM5 101	50S ribosomal protein L21 [Streptococcus pyogenes M1 GAS]	gi15674860	same in WT and mutant		
WT3101	PrtM5 101	30S ribosomal protein S11 [Streptococcus pyogenes M1 GAS]	gi15674312	same in WT and mutant		
WT3101	PrtM5 101	50S ribosomal protein L14 [Streptococcus pyogenes M1 GAS]	gi15674297	same in WT and mutant		
WT3101	PrtM5 101	heat-stable phosphocarrier protein, HPr [Streptococcus mutans, Ingbritt, Peptide, 86 aa]	gi546175	same in WT and mutant		
WT3101	PrtM5 101	hypothetical protein SAG1694 [Streptococcus agalactiae 2603V/R]	gi22537834	same in WT and mutant		
WT3101	PrtM5 101	general stress protein [Streptococcus equi subsp. zooepidemicus MGCS10565]	gi195977498	same in WT and mutant	First choice hit is <i>S. equi</i>	
WT7302	PrtM8 302	apolipoprotein A-I precursor RecName: Full=Apolipoprotein A-I;	gi162678	up in mutant		
WT7302	PrtM8 302	Short=Apo-AI; Short=ApoA-I; Flags: Precursor	gi3915607	up in WT/down in mutant		
WT4001	PrtM6 002	30S ribosomal protein S6 [Streptococcus equi subsp. zooepidemicus]	gi225869197	up in WT/down in mutant	First choice hit is <i>S. equi</i>	
WT4001	PrtM6 002	30S ribosomal protein S6 [Streptococcus equi subsp. zooepidemicus MGCS10565]	gi195977487	up in WT/down in mutant	lower choice hit is <i>S. equi</i>	
WT4001	PrtM6 002	hypothetical protein STRINF_01108 [Streptococcus infantarius subsp. infantarius ATCC BAA-102]	gi171779260	up in WT/down in mutant		
WT4204	PrtM6 202	hypothetical protein SEQ_1025 [Streptococcus equi subsp. equi 4047]	gi225870399	up in WT/down in mutant	Best match hit	SEQ_1025
WT4204	PrtM6 202	hypothetical protein Sez_0895 [Streptococcus equi subsp. zooepidemicus MGCS10565]	gi195978022	up in WT/down in mutant	lower choice hit is <i>S. equi</i>	Sez_0895
WT5303	PrtM6 302	thioredoxin peroxidase [Bacillus sp. NRRL B-14911]	gi89100074	up in mutant/down in WT		
WT5303	PrtM6 302	YkuQ [Bacillus sp. NRRL B-14911]	gi89099375	up in mutant/down in WT		
WT3201		Xaa-His dipeptidase [Streptococcus equi subsp. zooepidemicus]	gi225868376	only in WT	First choice hit is <i>S. equi</i>	
WT3201		RecName: Full=NADP-dependent alcohol dehydrogenase	gi113443	only in WT		
WT3201		Alcohol dehydrogenase zinc-binding domain protein [Thermoanaerobacter italicus Ab9]	gi255255024	only in WT		
WT3201		dipeptidase PepV [Streptococcus pyogenes M1 GAS]	gi15675062	only in WT		
WT3201		beta-glucosidase protein [Rhizobium etli CFN 42]	gi86359230	only in WT		
WT3201		hypothetical protein CaO19.10414	gi68480910	only in WT		

	[Candida albicans SC5314]			
	phosphopyruvate hydratase			
WT2201	[Streptococcus equi subsp. zoepidemicus MGCS10565] RecName: Full=Enolase; AltName: Full=2-phosphoglycerate dehydratase; AltName: Full=2-phospho-D-glycerate hydro-lyase	gi 195977904	only in WT	First choice hit is S. equi
WT2201		gi 57015278	only in WT	
WT2201	enolase [Streptococcus sobrinus]	gi 28866546	only in WT	
WT2201	phosphopyruvate hydratase			
WT2201	[Streptococcus pneumoniae TIGR4]	gi 15900994	only in WT	
WT2201	phosphopyruvate hydratase			
WT2201	[Streptococcus mutans UA159]	gi 24379669	only in WT	
WT2201	hypothetical protein STRINF_01784			
WT2201	[Streptococcus infantarius subsp. infantarius ATCC BAA-102]	gi 171779997	only in WT	
WT2201	enolase [Enterococcus faecalis V583]	gi 29376483	only in WT	
WT2201	phosphopyruvate hydratase			
WT2201	[Streptococcus gordonii str. Challis substr. CH1]	gi 157151177	only in WT	
WT2201	enolase [Bacillus coagulans 36D1]	gi 229543256	only in WT	
WT2201	cyclophilin type peptidyl-prolyl cis-trans isomerase protein			
WT2201	[Streptococcus equi subsp. equi 4047]	gi 225871058	only in WT	First choice hit is S. equi
WT2201	Phosphopyruvate hydratase			
WT2201	[Desulfuromonas acetoxidans DSM 684]	gi 95929414	only in WT	
WT2201	enolase [Pediococcus pentosaceus ATCC 25745]	gi 116492264	only in WT	
WT2201	enolase [Lactobacillus salivarius UCC118]	gi 90962138	only in WT	
WT2201	co-chaperonin GroES [Streptococcus equi subsp. zoepidemicus MGCS10565]	gi 195977292	only in WT	lower choice hit is S. equi
WT2201	enolase [Heliobacterium modesticaldum Ice1]	gi 167629687	only in WT	
WT5302	purine nucleoside phosphorylase			
WT6201	[Streptococcus equi subsp. zoepidemicus MGCS10565]	gi 195978281	only in WT	lower choice hit is S. equi
WT6001	dTDP-4-Keto-6-Deoxyglucose-3 5-epimerase R mLC [Streptococcus equi subsp. zoepidemicus MGCS10565]	gi 195978346	only in WT	lower choice hit is S. equi
WT3601	hypothetical protein CaO19.10414 [Candida albicans SC5314]	gi 68480910	only in WT	
WT3601	mannose-6-phosphate isomerase Pmi			
WT3601	[Streptococcus equi subsp. zoepidemicus MGCS10565]	gi 195977511	only in WT	First choice hit is S. equi
WT3601	RecName: Full=NADP-dependent alcohol dehydrogenase	gi 113443	only in WT	
WT3601	Alcohol dehydrogenase zinc-binding domain protein [Thermoanaerobacter italicus Ab9]	gi 255255024	only in WT	
WT3601	hypothetical protein AN7590.2			
WT3601	[Aspergillus nidulans FGSC A4]	gi 67901206	only in WT	
WT6501	L-xylulose reductase [Neurospora crassa OR74A]	gi 85091614	only in WT	
WT6501	6-phosphofructokinase [Streptococcus equi subsp. zoepidemicus MGCS10565]	gi 195978008	only in WT	First choice hit is S. equi
WT6501	6-phosphofructokinase [Clostridium perfringens str. 13]	gi 18309343	only in WT	
WT6501	6-phosphofructokinase [Idiomarina baltica OS145]	gi 85711053	only in WT	
WT6501	6-phosphofructokinase [Deinococcus geothermalis DSM 11300]	gi 94985737	only in WT	
WT6501	6-phosphofructokinase [Enterococcus faecalis V583]	gi 29375624	only in WT	
WT5504	alcohol dehydrogenase [Streptococcus	gi 195977198	only in WT	Best match

		equi subsp. zooepidemicus MGCS10565]			hit
WT5504		alcohol dehydrogenase [Streptococcus equi subsp. zooepidemicus]	gi 225867661	only in WT	lower choice hit is S. equi
	PrtM001	sugar phosphotransferase component II B [Streptococcus equi subsp. zooepidemicus MGCS10565]	gi 195977278	only in mutant	lower choice hit is S. equi
	PrtM9001	transcriptional regulator [Bacillus sp. NRRL B-14911]	gi 89101180	only in mutant	
	PrtM8202	hypothetical protein GK3020 [Geobacillus kaustophilus HTA426]	gi 56421555	only in mutant	
	PrtM8201	thioredoxin peroxidase [Bacillus sp. NRRL B-14911]	gi 89100074	only in mutant	
	PrtM3203	transcription elongation factor GreA [Streptococcus equi subsp. zooepidemicus MGCS10565]	gi 195978755	only in mutant	First choice hit is S. equi
	PrtM9401	ribosome recycling factor [Streptococcus pyogenes M1 GAS]	gi 15674582	only in mutant	

Table D2 List of Proteins from *S. equi* 4047 WT and Mutant (Δ PrtM): Secreted Protein Extract

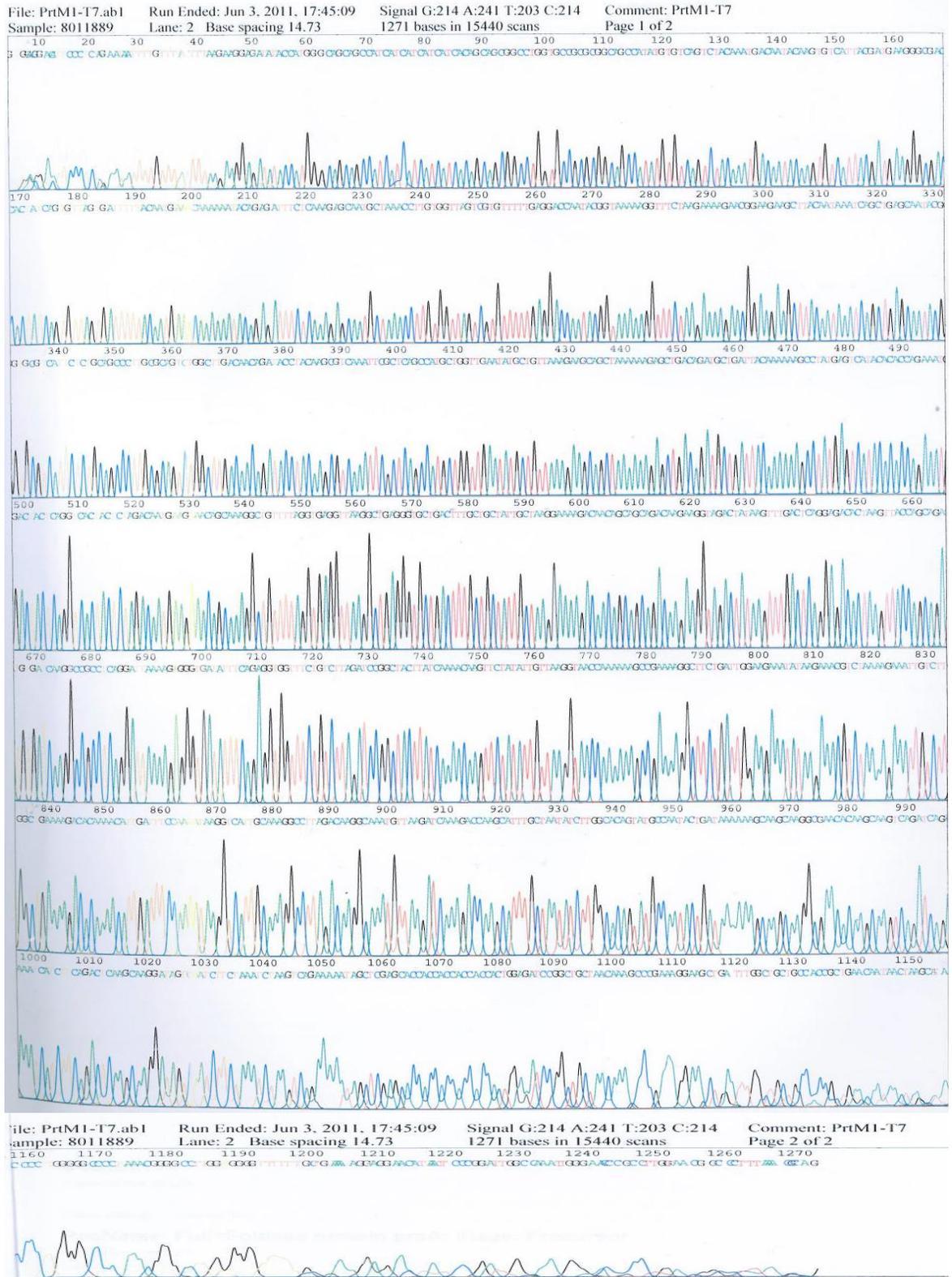
<u>Spot #</u>	<u>Spot #</u>	<u>Protein name</u>	<u>Protein code</u>	<u>Comment on expression</u>	<u>Comment on level of hit</u>	<u>Protein code for best match hit</u>
WT0201	PrtM0401	-esterase [Streptococcus equi subsp. zooepidemicus MGCS10565]	gi 195977587	Same in both WT and mutant	First choice hit is S. equi	
WT0201	PrtM0401	-lipoprotein [Streptococcus equi subsp. zooepidemicus]	gi 225867979	Same in both WT and mutant	lower choice hit is S. equi	
WT0201	PrtM0401	-lipoprotein [Streptococcus equi subsp. equi 4047]	gi 225871106	Same in both WT and mutant	lower choice hit is S. equi	
WT0201	PrtM0401	-hypothetical protein CaO19.10414 [Candida albicans SC5314]	gi 68480910	Same in both WT and mutant		
WT0201	PrtM0401	-RecName: Full=NADP-dependent alcohol dehydrogenase -RecName:	gi 113443	Same in both WT and mutant		
WT7402	PrtM6503	Full=Hyaluronoglucosaminidase; Short=Hyaluronidase	gi 3913881	same in both WT and mutant		
WT7402	PrtM6503	-fibronectin-binding protein [Streptococcus equi]	gi 1617432	same in both WT and mutant	lower choice hit is S. equi	
WT5001	PrtM5103	unnamed protein product [Manduca sexta]	gi 9725	up in WT/down in mutant		
WT5001	PrtM5103	IgM heavy chain constant region, secretory form [Bos taurus]	gi 28592070	up in WT/down in mutant	First choice hit is S. equi	
WT5001	PrtM5103	-IgM heavy chain constant region [Bos taurus]	gi 2232299	up in WT/down in mutant		
WT9403	PrtM7502	exported protein [Streptococcus equi subsp. equi 4047]	gi 225869943	up in WT/down in mutant	First choice hit is S. equi	
WT9403	PrtM7502	: -esterase [Streptococcus equi subsp. zooepidemicus MGCS10565]	gi 195977587	up in WT/down in mutant	First choice hit is S. equi	
WT9403	PrtM7502	-6-phosphofructokinase [Bacillus subtilis subsp. subtilis str. 168]	gi 16079971	up in WT/down in mutant		
WT9403	PrtM7502	-ALB protein [Bos taurus]	gi 74267962	up in WT/down in		

WT9403	PrtM7502	-6-phosphofructokinase [Bacillus sp. SG-1]	gi 149181139	mutant up in WT/down in mutant	
WT9403	PrtM7502	-6-phosphofructokinase [Bacillus sp. NRRL B-14911]	gi 89100372	up in WT/down in mutant	
WT3301	Prtm2504	-peptidyl-prolyl cis-trans isomerase cyclophilin-type [Streptococcus equi subsp. zooepidemicus MGCS10565]	gi 195978568	up in WT/down in mutant	First choice hit is S. equi
WT3301	Prtm2504	-REDICTED: similar to keratin 1 isoform 7 [Macaca mulatta]	gi 109096823	up in WT/down in mutant	
WT3301	Prtm2504	-serine proteinase inhibitor, clade A, member 1 precursor [Bos taurus]	gi 27806941	up in WT/down in mutant	
WT3301	Prtm2504	hypothetical protein CaO19.10414 [Candida albicans SC5314]	gi 68480910	up in WT/down in mutant	
WT3301	Prtm2504	-esterase [Streptococcus equi subsp. zooepidemicus MGCS10565]	gi 195977587	up in WT/down in mutant	lower choice hit is S. equi
WT0005		-RecName: Full=NADP-dependent alcohol dehydrogenase	gi 113443	only in WT	
WT0005		- hypothetical protein AN7590.2 [Aspergillus nidulans FGSC A4]	gi 67901206	only in WT	
WT0005		- unnamed protein product [Escherichia coli str. K-12 substr. MG1655]	gi 466691	only in WT	
WT0005		- L-xylulose reductase [Neurospora crassa OR74A]	gi 85091614	only in WT	
WT0005		-Alcohol dehydrogenase zinc-binding domain protein [Thermoanaerobacter italicus Ab9]	gi 255255024	only in WT	
WT1101		-unnamed protein product [Escherichia coli str. K-12 substr. MG1655]	gi 466691	only in WT	
WT8403		-ALB protein [Bos taurus]	gi 74267962	only in WT	
WT8403		-serum albumin [Bos indicus]	gi 76445989	only in WT	
WT8403		- albumin [Felis catus]	gi 30962111	only in WT	
WT2201		-PREDICTED: similar to Keratin, type I cytoskeletal 14 (Cytokeratin-14) (CK-14) (Keratin-14) (K14) [Pan troglodytes]	gi 114667176	only in WT	
WT2201		-hypothetical protein Sez_0895 [Streptococcus equi subsp. zooepidemicus MGCS10565]	gi 195978022	only in WT	lower choice hit is S. equi Sez_0895
WT2201		-RecName: Full=Hyaluronoglucosaminidase; Short=Hyaluronidase	gi 3913881	only in WT	
WT8501		-Mac family protein [Streptococcus equi subsp. equi 4047]	gi 225870319	only in WT	First choice hit is S. equi
WT8501		-hypothetical protein CaO19.10414 [Candida albicans SC5314]	gi 68480910	only in WT	
WT8501		-RecName: Full=NADP-dependent alcohol dehydrogenase	gi 113443	only in WT	
WT7301		-DNA/RNA non-specific endonuclease [Streptococcus equi subsp. zooepidemicus]	gi 225868777	only in WT	First choice hit is S. equi lower choice hit is S. equi
WT7301		-fibronectin-binding protein [Streptococcus equi]	gi 1617432	only in WT	
WT7301		-serine proteinase inhibitor, clade A, member 1 precursor [Bos taurus]	gi 27806941	only in WT	
WT9202		-fibronectin-binding protein [Streptococcus equi]	gi 1617432	only in WT	First choice hit is S. equi

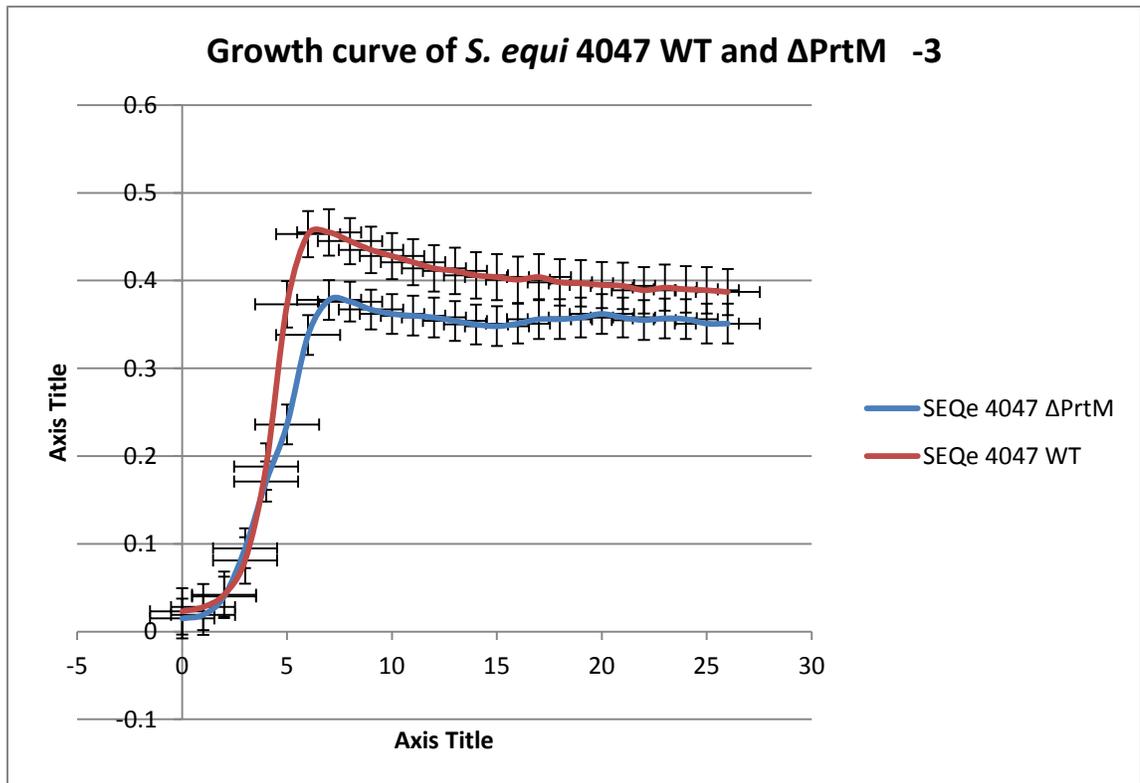
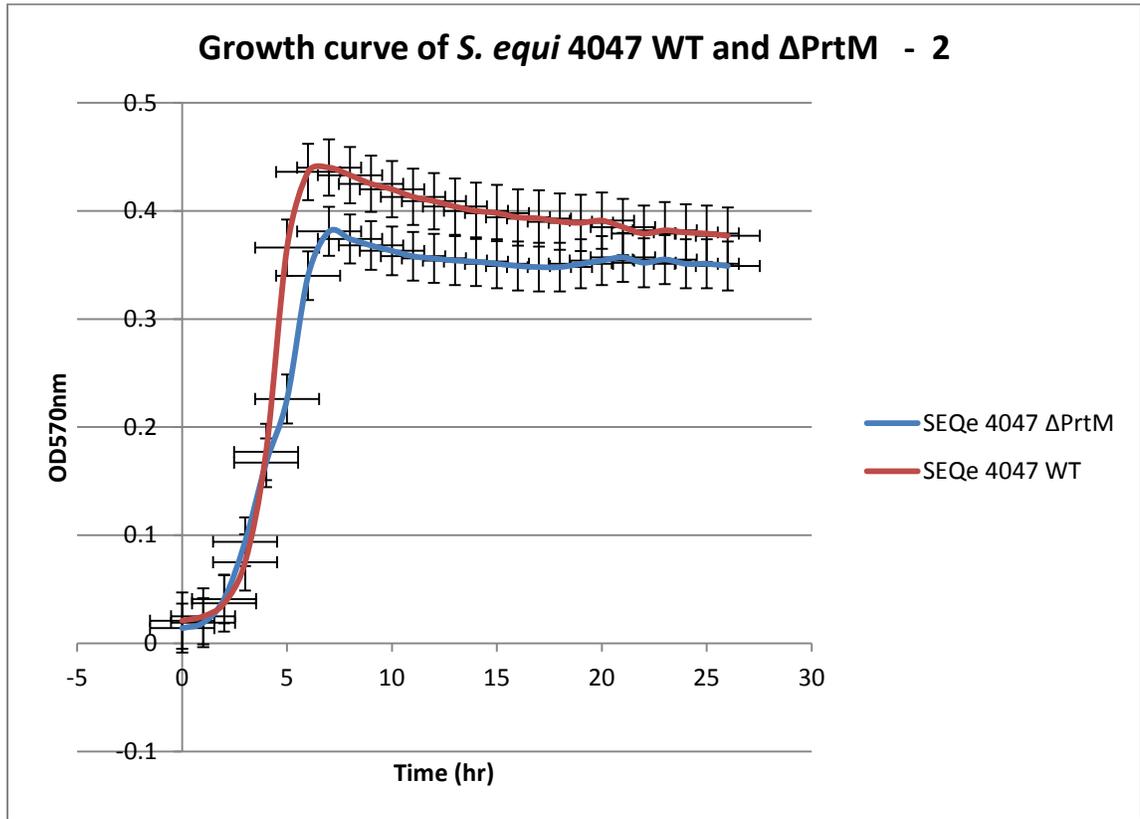
WT9202	- DNA/RNA non-specific endonuclease [Streptococcus equi subsp. zooepidemicus]	gi 225868777	only in WT	lower choice hit is S. equi	
WT9202	-hypothetical protein TTHERM_00225940 [Tetrahymena thermophila]	gi 146164853	only in WT		
WT9202	- RecName: Full=Hyaluronoglucosaminidase; Short=Hyaluronidase	gi 3913881	only in WT		
WT2202	-hypothetical protein Sez_0895 [Streptococcus equi subsp. zooepidemicus MGCS10565]	gi 195978022	only in WT	Best match hit	Sez_0895
WT2202	-RecName: Full=Hyaluronoglucosaminidase; Short=Hyaluronidase	gi 3913881	only in WT		
WT2202	-fibronectin-binding protein [Streptococcus equi]	gi 1617432	only in WT	lower choice hit is S. equi	
WT2202	- hyaluronoglucosaminidase [Streptococcus pyogenes MGAS10394]	gi 50914896	only in WT		
WT1401	-lipoprotein [Streptococcus equi subsp. zooepidemicus]	gi 225867979	only in WT	First choice hit is S. equi	
WT1401	- lipoprotein [Streptococcus equi subsp. equi 4047]	gi 225871106	only in WT	lower choice hit is S. equi	
WT1401	-hyaluronidase, phage associated [Streptococcus phage 370.1]	gi 15674762	only in WT		
WT1401	-hyaluronoglucosaminidase [Streptococcus pyogenes MGAS10394]	gi 50914896	only in WT		
WT1401	- hypothetical protein SPy_1154 [Streptococcus pyogenes M1 GAS]	gi 15675130	only in WT		
WT2401	-extracellular protein [Streptococcus equi subsp. zooepidemicus MGCS10565]	gi 195978139	only in WT	First choice hit is S. equi	
WT2401	- PREDICTED: similar to keratin 1 isoform 7 [Macaca mulatta]	gi 109096823	only in WT		
WT1402	-extracellular protein [Streptococcus equi subsp. zooepidemicus MGCS10565]	gi 195978139	only in WT	First choice hit is S. equi	
WT1402	-hyaluronidase, phage associated [Streptococcus phage 370.1]	gi 15674762	only in WT	lower choice hit is S. equi	
WT1402	-hyaluronoglucosaminidase [Streptococcus pyogenes MGAS10394]	gi 50914896	only in WT		
WT1402	-unnamed protein product [Escherichia coli str. K-12 substr. MG1655]	gi 466691	only in WT		
WT1402	- RecName: Full=NADP-dependent alcohol dehydrogenase	gi 113443	only in WT		
WT1402	-Alcohol dehydrogenase zinc-binding domain protein [Thermoanaerobacter italicus Ab9]	gi 255255024	only in WT		
WT8401	-Mac family protein [Streptococcus equi subsp. equi 4047]	gi 225870319	only in WT	First choice hit is S. equi	
WT2101	-hypothetical protein Sez_1583 [Streptococcus equi subsp. zooepidemicus MGCS10565]	gi 195978685	only in WT	Best match hit is S. equi	Sez_1583
WT2101	-RecName: Full=Hyaluronoglucosaminidase; Short=Hyaluronidase	gi 3913881	only in WT		
WT1002	-co-chaperonin GroES [Streptococcus equi subsp. zooepidemicus MGCS10565]	gi 195977292	only in WT	First choice hit is S. equi	
WT1002	-PREDICTED: similar to keratin 1 isoform 7 [Macaca mulatta]	gi 109096823	only in WT		

WT1002	-hyaluronidase [Streptococcus phage P9	gi157311178	only in WT		
PrtM1201	-RecName: Full=NADP-dependent alcohol dehydrogenase	gi113443	only in mutant		
PrtM1201	-YALIOB08052p [Yarrowia lipolytica]	gi50546208	only in mutant		
PrtM1201	-hypothetical protein CaO19.10414 [Candida albicans SC5314]	gi68480910	only in mutant		
PrtM1201	- Alcohol dehydrogenase zinc-binding domain protein [Thermoanaerobacter italicus Ab9]	gi255255024	only in mutant		
PrtM1201	- hypothetical protein AN7590.2 [Aspergillus nidulans FGSC A4]	gi67901206	only in mutant		
PrtM1201	- unnamed protein product [Escherichia coli str. K-12 substr. MG1655]	gi466691	only in mutant		
PrtM1103	-hypothetical protein Sez_1583 [Streptococcus equi subsp. zooepidemicus MGCS10565]	gi195978685		Best match hit	Sez_1583
PrtM1103	-hyaluronidase, phage associated [Streptococcus phage 370.1]	gi15674762	only in mutant		
PrtM1103	-hyaluronoglucosaminidase [Streptococcus pyogenes MGAS10394]	gi50914896	only in mutant		
PrtM0101	-cold-shock protein, molecular chaperone, RNA-helicase co-factor [Bacillus subtilis subsp. subtilis str. 168	gi16079252	only in mutant		
PrtM0301	-A family sortase SrtA [Streptococcus equi subsp. zooepidemicus MGCS10565]	gi195978143	only in mutant	First choice hit is S. equi	
PrtM0301	-sortase SrtA [Streptococcus equi subsp. zooepidemicus]	gi225868523	only in mutant	lower choice hit is S. equi	
PrtM0301	- RecName: Full=Hyaluronoglucosaminidase; Short=Hyaluronidase	gi3913881	only in mutant		
PrtM0304	-cold shock protein [Bacillus sp. NRRL B-14911	gi89098567	only in mutant		
PrtM1202	-oligopeptide ABC transporter periplasmic oligopeptide-binding protein OppA [Streptococcus equi subsp. zooepidemicus -MGCS10565]	gi195978795	only in mutant		
PrtM1202	-YALIOB08052p [Yarrowia lipolytica]	gi50546208	only in mutant		
PrtM1202	-RecName: Full=NADP-dependent alcohol dehydrogenase	gi113443	only in mutant		
PrtM1202	-Alcohol dehydrogenase zinc-binding domain protein [Thermoanaerobacter italicus Ab9]	gi255255024	only in mutant		
PrtM1202	- hypothetical protein AN8113.2 [Aspergillus nidulans FGSC A4]	gi67902252	only in mutant		
PrtM2101	-30S ribosomal protein S6 [Streptococcus uberis 0140J]	gi222153666	only in mutant		
PrtM2101	-cold shock protein [Bacillus sp. NRRL B-14911]	gi89098567	only in mutant		
PrtM2101	-30S ribosomal protein S6 [Streptococcus equi subsp. zooepidemicus MGCS10565]	gi195977487	only in mutant	lower choice hit is S. equi	
PrtM2301	-oligopeptide ABC transporter periplasmic oligopeptide-binding protein OppA [Streptococcus equi subsp. zooepidemicus MGCS10565]	gi195978795	only in mutant		
PrtM5301	-thioredoxin peroxidase [Bacillus sp. NRRL B-14911	gi89100074	only in mutant		
PrtM4201	-50S ribosomal protein L10 [Staphylococcus epidermidis ATCC 12228	gi27467221	only in mutant		

Appendix E: DNA Sequence of Plasmid plus PrtM DNA insert without the 281 nucleotide upstream sequences:



Appendix F: Growth Curve and student T-test of growth curve ODs



Student T-test of growth curve ODs

Time (hrs)	<i>S. equi</i> 4047 ΔPrtM	<i>S. equi</i> 4047 WT	Time (hrs)	<i>S. equi</i> 4047 ΔPrtM	<i>S. equi</i> 4047 WT
4	0.161	0.18	14	0.361	0.393
4	0.167	0.177	14	0.353	0.4
4	0.171	0.188	14	0.35	0.406
Student T-test=	0.015117		Student T-test=	0.011677	
Time (hrs)	<i>S. equi</i> 4047 ΔPrtM	<i>S. equi</i> 4047 WT	Time (hrs)	<i>S. equi</i> 4047 ΔPrtM	<i>S. equi</i> 4047 WT
6	0.35	0.43	16	0.37	0.39
6	0.34	0.436	16	0.349	0.394
6	0.338	0.453	16	0.351	0.401
Student T-test=	0.005351		Student T-test=	0.026953	
Time (hrs)	<i>S. equi</i> 4047 ΔPrtM	<i>S. equi</i> 4047 WT	Time (hrs)	<i>S. equi</i> 4047 ΔPrtM	<i>S. equi</i> 4047 WT
8	0.382	0.416	18	0.37	0.387
8	0.374	0.433	18	0.348	0.39
8	0.376	0.445	18	0.356	0.398
Student T-test=	0.017601		Student T-test=	0.028079	
Time (hrs)	<i>S. equi</i> 4047 ΔPrtM	<i>S. equi</i> 4047 WT	Time (hrs)	<i>S. equi</i> 4047 ΔPrtM	<i>S. equi</i> 4047 WT
10	0.367	0.403	20	0.368	0.381
10	0.363	0.42	20	0.354	0.391
10	0.362	0.428	20	0.362	0.395
Student T-test=	0.013495		Student T-test=	0.032526	
Time (hrs)	<i>S. equi</i> 4047 ΔPrtM	<i>S. equi</i> 4047 WT	Time (hrs)	<i>S. equi</i> 4047 ΔPrtM	<i>S. equi</i> 4047 WT
12	0.364	0.398	22	0.362	0.377
12	0.356	0.409	22	0.352	0.379
12	0.358	0.414	22	0.355	0.389
Student T-test=	0.010125		Student T-test=	0.022381	

Appendix G: Student T-test of antibiotics sensitivity result

Antibiotic	PenicillinG		Streptomycin	
<i>S. equi</i> 4047 strain	WT	Δ PrtM	WT	Δ PrtM
1st replica -antibiotic concentration in cm	3.6	4.4	1.9	3.2
2nd replica -antibiotic concentration in cm	3.6	4.4	1.9	3
3rd replica -antibiotic concentration in cm	3.6	4.2	2.2	2.6
Student T-test	0.00408170		0.037931147	
Antibiotic	Vancomycin		Gentamycin	
<i>S. equi</i> 4047 strain	WT	Δ PrtM	WT	Δ PrtM
1st replica -antibiotic concentration in cm	1.9	2.6	2.2	3.4
2nd replica -antibiotic concentration in cm	2	2.6	2.4	3.2
3rd replica -antibiotic concentration in cm	2.1	2.4	2.2	3
Student T-test	0.023606933		0.009901971	
Antibiotic	Ampicillin		Norfloxacin	
<i>S. equi</i> 4047 strain	WT	Δ PrtM	WT	Δ PrtM
1st replica -antibiotic concentration in cm	3.5	3.8	1.2	1.8
2nd replica -antibiotic concentration in cm	3.4	3.6	1.2	2
3rd replica -antibiotic concentration in cm	3.6	3.9	1.1	1.8
Student T-test	0.007634036		0.003367042	

Appendix H: Hyaluronic acid quantitation result

The kit manufacturer's (Nozomis Biocatalysis) formular that was:

$$[(A2-A1)Sample - (A2-A1)Blank] \times 0.2314 = \text{mg/ mL of hyaluronan.}$$

The results of triplicate hyaluronic acid quantitation assays are summarized in figure H1 (appendix H)

Blank	A1 (OD_{235 nm})	A2 (OD_{235 nm})	A2-A1
BLANK1	0.617	0.623	0.006
BLANK2	0.632	0.638	0.006
BLANK3	0.598	0.603	0.005
Average of Blank A2 - A1 =			0.00567
S. equi 4047			
strain	A1 (OD_{235 nm})	A2 (OD_{235 nm})	A2-A1
WT	0.795	0.808	0.013
WT	0.755	0.768	0.013
WT	0.726	0.749	0.023
Average of WT A2-A1 =			0.01633
WT average (A2 - A1) - Blank Average			0.01067
0.01067 x 0.2314 =			0.002469038 mg/ mL
Since 0.5 mL of culture was used, multiplied result by 2			
5.0 x 10 ⁻³ mg/ mL = 5 µg/ mL hyaluronan in WT culture			
S. equi 4047			
strain	A1 (OD_{235 nm})	A2 (OD_{235 nm})	A2-A1
ΔPrtM	0.761	0.769	0.008
ΔPrtM	0.757	0.768	0.011
ΔPrtM	0.75	0.768	0.018
Average of ΔPrtM A2 - A1 =			0.01233
ΔPrtM Average (A2-A1) -Blank average			0.00667
0.00667 x 0.2314 =			0.001543438 mg/ mL
Since 0.5 mL of culture was used, multiplied result by 2			
3.0 x 10 ⁻³ mg/ mL = 3 µg/ mL hyaluronan in ΔPrtM culture			

Figure H1: Summary of Hyaluronic acid quantitation result

Appendix I – Analysis of Protease coupled PPIase assays

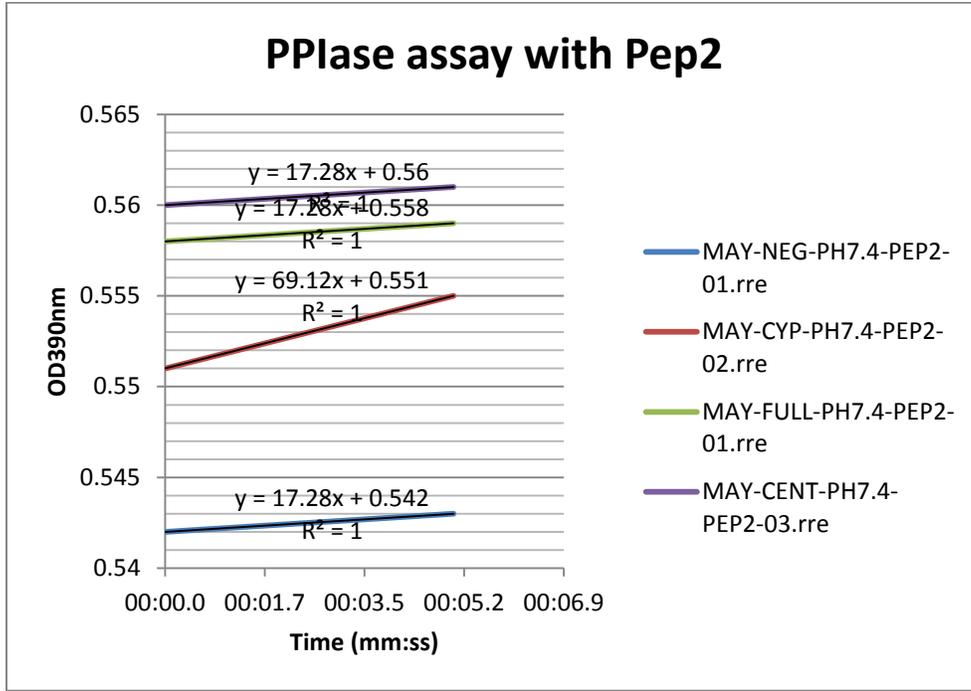


Figure I1: Analysis of reactions of Pep2: Full length (FULL) and central domain (CENT) recombinant protein fractions.

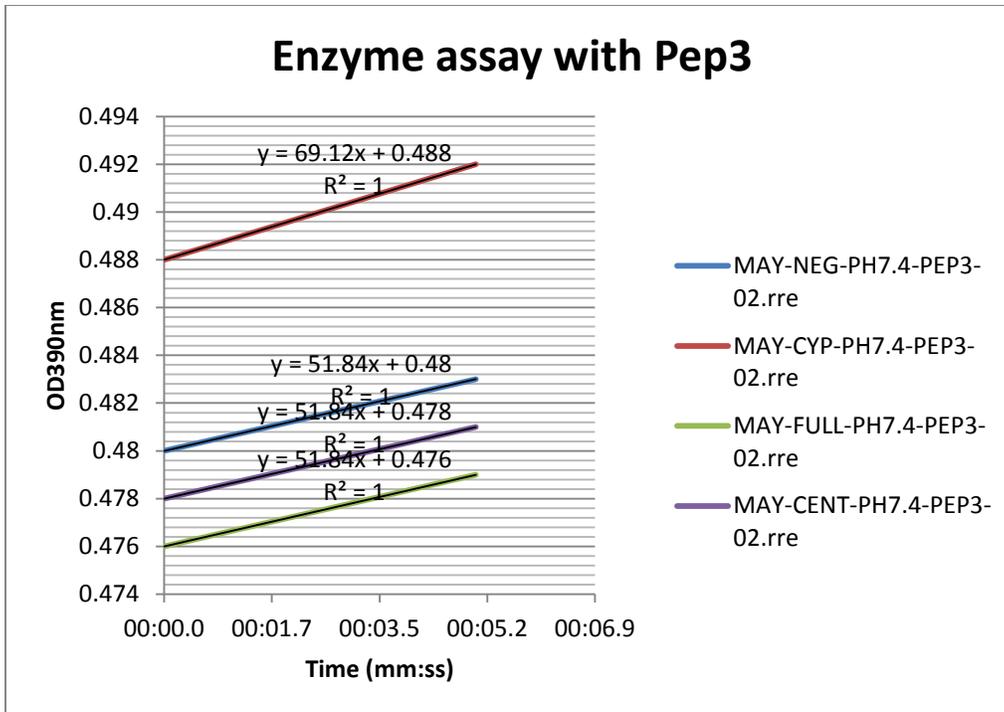


Figure I2: Analysis of reactions of Pep3: Full length (FULL) and central domain (CENT) recombinant protein fractions.

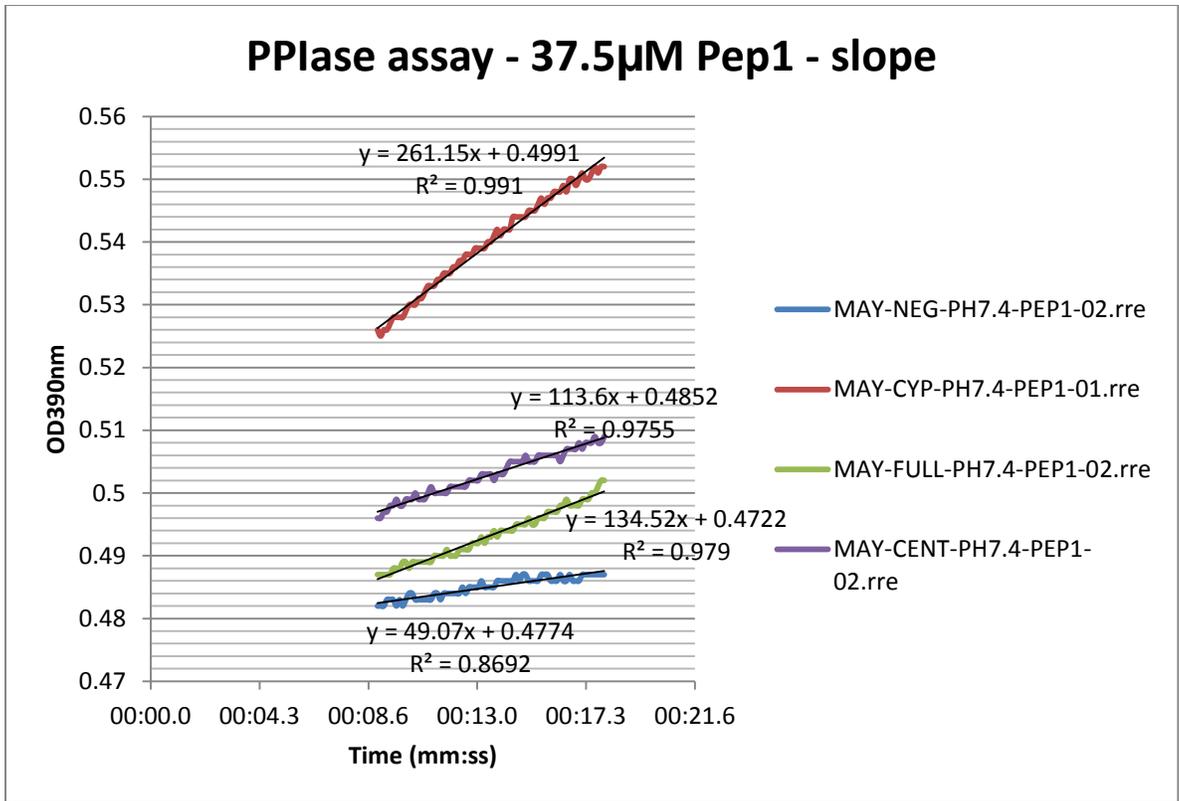


Figure I3: Analysis of reactions of Pep1: Full length (FULL) and central domain (CENT) recombinant protein fractions.

Appendix J

Protein crystallisation screens

Tables J1, J2, J3, J4, J5, J6 and J7 show the solutions used to screen for crystal development. Those screens in tables HI and HII are named Clear Strategy Screen (CSS) I and II respectively from Molecular Dimensions. The following abbreviations describe precipitants: polyethylene glycol (PEG), monomethylether (MME), Jeffamine (O-(2-Aminopropyl)-O'-(2-methoxy-ethyl) polypropylene glycol 500).

J1: CSS 1

Tube Number	Salt	Precipitant
1	0.3M Sodium Acetate	25% (w/v) PEG 2K _{mme}
2	0.2M Lithium Sulphate	25% (w/v) PEG 2K _{mme}
3	0.2M Magnesium Chloride	25% (w/v) PEG 2K _{mme}
4	0.2M Potassium Bromide	25% (w/v) PEG 2K _{mme}
5	0.2M Potassium Thiocyanate	25% (w/v) PEG 2K _{mme}
6	0.8M Sodium Formate	25% (w/v) PEG 2K _{mme}
7	0.3M Sodium Acetate	15% (w/v) PEG 4K
8	0.2M Lithium Sulphate	15% (w/v) PEG 4K
9	0.2M Magnesium Chloride	15% (w/v) PEG 4K
10	0.2M Potassium Bromide	15% (w/v) PEG 4K
11	0.2M Potassium Thiocyanate	15% (w/v) PEG 4K
12	0.8M Sodium Formate	15% (w/v) PEG 4K
13	0.3M Sodium Acetate	10% (w/v) PEG 8K, 10% (w/v) PEG 1K
14	0.2M Lithium Sulphate	10% (w/v) PEG 8K, 10% (w/v) PEG 1K
15	0.2M Magnesium Chloride	10% (w/v) PEG 8K, 10% (w/v) PEG 1K
16	0.2M Potassium Bromide	10% (w/v) PEG 8K, 10% (w/v) PEG 1K
17	0.2M Potassium Thiocyanate	10% (w/v) PEG 8K, 10% (w/v) PEG 1K
18	0.8M Sodium Formate	10% (w/v) PEG 8K, 10% (w/v) PEG 1K
19	0.3M Sodium Acetate	8% (w/v) PEG 20K, 8% (w/v) PEG 550 _{mme}
20	0.2M Lithium Sulphate	8% (w/v) PEG 20K, 8% (w/v) PEG 550 _{mme}
21	0.2M Magnesium Chloride	8% (w/v) PEG 20K, 8% (w/v) PEG 550 _{mme}
22	0.2M Potassium Bromide	8% (w/v) PEG 20K, 8% (w/v) PEG 550 _{mme}
23	0.2M Potassium Thiocyanate	8% (w/v) PEG 20K, 8% (w/v) PEG 550 _{mme}
24	0.8M Sodium Formate	8% (w/v) PEG 20K, 8% (w/v) PEG 550 _{mme}

Table J1: CSS I components.

J2: CSS II

Tube Number	Salt	Precipitant
1	1.5M Ammonium Sulphate	
2	0.8M Lithium Sulphate	
3	2M Sodium Formate	
4	0.5M Potassium dihydrogen Phosphate	
5	0.2M Calcium Acetate	25% (w/v) PEG 2K _{mme}
6	0.2M Calcium Acetate	15% (w/v) PEG 4K
7	2.7M Ammonium Sulphate	
8	1.8M Lithium Sulphate	
9	4M Sodium Formate	
10	1M Potassium dihydrogen Phosphate	
11	0.2M Calcium Acetate	10% (w/v) PEG 8K, 10% (w/v) PEG 1K
12	0.2M Calcium Acetate	8% (w/v) PEG 20K, 8% (w/v) PEG 550 _{mme}
13		40% (v/v) MPD
14		40% (v/v) 2,3-Butane-diol
15	5mM Cadmium Chloride	20% (w/v) PEG 4K
16	0.15M Potassium Thiocyanate	20% (w/v) PEG 550 _{mme}
17	0.15M Potassium Thiocyanate	20% (w/v) PEG 600
18	0.15M Potassium Thiocyanate	20% (w/v) PEG 1500
19		35% (v/v) Isopropanol
20		30% (v/v) Jeffamine 600M pH 6.5
21	5mM Nickel Chloride	20% (w/v) PEG 4K
22	0.15M Potassium Thiocyanate	18% (w/v) PEG 3350
23	0.15M Potassium Thiocyanate	18% (w/v) PEG 5K _{mme}
24	0.15M Potassium Thiocyanate	15% (w/v) PEG 6K

Table JII: CSS II components.**J3a: Hampton screen I**

Tube Number	Salt	Buffer	Precipitant
1	0.02M Calcium Chloride dihydrate	0.1M Sodium Acetate trihydrate pH 4.6	30% (v/v) 2-Methyl-2, 4-pentenediol
2			0.4M Potassium Sodium Tartrate tetrahydrate
3			0.4M Ammonium dihydrogen Phosphate
4		0.1M Tris Hydrochloride pH 8.5	2M Ammonium Sulphate
5	0.2M tri-Sodium Citrate dihydrate	0.1M HEPES- Sodium pH 7.5	30% (v/v) 2-Methyl-2, 4-pentenediol
6	0.2M Magnesium Chloride hexahydrate	0.1M Tris Hydrochloride pH 8.5	30% (w/v) PEG 4000
7		0.1M Sodium Cacodylate pH 6.5	1.4M Sodium Acetate trihydrate
8	0.2M tri-Sodium Citrate dihydrate	0.1M Sodium Cacodylate pH 6.5	30% (v/v) iso-Propanol
9	0.2M Ammonium Acetate	0.1M tri-Sodium Citrate dihydrate pH 5.6	30% (w/v) PEG 4000
10	0.2M Ammonium Acetate	0.1M Sodium Acetate trihydrate pH 4.6	30% (w/v) PEG 4000
11		0.1M tri-Sodium Citrate dihydrate pH 5.6	1M Ammonium dihydrogen Phosphate
12	0.2M Magnesium Chloride hexahydrate	0.1M HEPES- Sodium pH 7.5	30% (v/v) iso-Propanol
13	0.2M tri-Sodium Citrate dihydrate	0.1M Tris Hydrochloride pH 8.5	30% (v/v) PEG 400

14	0.2M Calcium Chloride dihydrate	0.1M HEPES- Sodium pH 7.5	28% (v/v) PEG 400
15	0.2M Ammonium Sulphate	0.1M Sodium Cacodylate pH 6.5	30% (w/v) PEG 8000
16		0.1M HEPES- Sodium pH 7.5	1.5M Lithium Sulphate monohydrate
17	0.2M Lithium Sulphate monohydrate	0.1M Tris Hydrochloride pH 8.5	30% (w/v) PEG 4000
18	0.2M Magnesium Acetate tetrahydrate	0.1M Sodium Cacodylate pH 6.5	20% (w/v) PEG 8000
19	0.2M Ammonium Acetate	0.1M Tris Hydrochloride pH 8.5	30% (v/v) iso-Propanol
20	0.2M Ammonium Sulphate	0.1M Sodium Acetate trihydrate pH 4.6	25% (w/v) PEG 4000
21	0.2M Magnesium Acetate tetrahydrate	0.1M Sodium Cacodylate pH 6.5	30% (v/v) 2-Methyl-2, 4-pentanediol
22	0.2M Sodium Acetate trihydrate	0.1M Tris Hydrochloride pH 8.5	30% (w/v) PEG 4000
23	0.2M Magnesium Chloride hexahydrate	0.1M HEPES- Sodium pH 7.5	30% (v/v) PEG 400
24	0.2M Calcium Chloride dihydrate	0.1M Sodium Acetate trihydrate pH 4.6	20% (v/v) iso-Propanol
25		0.1M Imidazole pH 6.5	1M Sodium Acetate trihydrate
26	0.2M Ammonium Acetate	0.1M tri-Sodium Citrate dihydrate pH 5.6	30% (v/v) 2-Methyl-2, 4-pentanediol
27	0.2M tri-Sodium Citrate dehydrate	0.1M HEPES- Sodium pH 7.5	20% (v/v) iso-Propanol
28	0.2M Sodium Acetate trihydrate	0.1M Sodium Cacodylate pH 6.5	30% (w/v) PEG 8000
29		0.1M HEPES- Sodium pH 7.5	0.8M Potassium Sodium Tartrate tetrahydrate
30	0.2M Ammonium Sulphate		30% (w/v) PEG 8000
31	0.2M Ammonium Sulphate		30% (w/v) PEG 4000
32			2M Ammonium Sulphate
33			4M Sodium Formate
34		0.1M Sodium Acetate trihydrate pH 4.6	2M Sodium Formate
35		0.1M HEPES- Sodium pH 7.5	0.8M Sodium dihydrogen Phosphate, 0.8M Potassium dihydrogen Phosphate
36		0.1M Tris Hydrochloride pH 8.5	8% (w/v) PEG 8000
37		0.1M Sodium Acetate trihydrate pH 4.6	8% (w/v) PEG 4000
38		0.1M HEPES- Sodium pH 7.5	1.4M tri-Sodium Citrate dihydrate
39		0.1M HEPES- Sodium pH 7.5	2% (v/v) PEG 400, 2M Ammonium Sulphate
40		0.1M tri-Sodium Citrate dihydrate pH 5.6	20% (v/v) iso-Propanol, 20% (w/v) PEG 4000
41		0.1M HEPES- Sodium pH 7.5	10% (v/v) iso-Propanol, 20% (w/v) PEG 4000
42	0.05M Potassium dihydrogen Phosphate		20% (w/v) PEG 8000
43			30% (w/v) PEG 1500
44			0.2M Magnesium Formate
45	0.2M Zinc Acetate dihydrate	0.1M Sodium Cacodylate pH 6.5	18% (w/v) PEG 8000
46	0.2M Calcium Acetate	0.1M Sodium Cacodylate	18% (w/v) PEG 8000

	hydrate	pH 6.5	
47		0.1M Sodium Acetate trihydrate pH 4.6	2M Ammonium Sulphate
48		0.1M Tris Hydrochloride pH 8.5	2M Ammonium dihydrogen Phosphate

Table J3a: Hampton screen I components.

J3B: Hampton screen II

Tube Number	Salt	Buffer	Precipitant
1	2M Sodium Chloride		10% (w/v) PEG 6000
2	0.5M Sodium Chloride; 0.1M Magnesium Chloride hexahydrate		0.01M Hexadecyltrimethylammonium Bromide
3			25% (v/v) Ethylene Glycol
4			35% (v/v) Dioxane
5	2M Ammonium Sulphate		5% (v/v) iso-Propanol
6			1M Imidazole pH 7.0
7			10% (w/v) PEG 1000; 10% (w/v) PEG 8000
8	1.5M Sodium Chloride		10% (v/v) Ethanol
9		0.1M Sodium Acetate trihydrate pH 4.6	2M Sodium Chloride
10	0.2M Sodium Chloride	0.1M Sodium Acetate trihydrate pH 4.6	30% (v/v) MPD
11	0.01M Cobaltous Chloride hexahydrate	0.1M Sodium Acetate trihydrate pH 4.6	1M 1,6 Hexanediol
12	0.1M Cadmium Chloride dihydrate	0.1M Sodium Acetate trihydrate pH 4.6	30% (v/v) PEG 400
13	0.2M Ammonium Sulphate	0.1M Sodium Acetate trihydrate pH 4.6	30% (w/v) PEG 2000mme
14	0.2M Potassium Sodium Tartrate tetrahydrate	0.1M tri-Sodium Citrate dihydrate pH 5.6	2M Ammonium Sulphate
15	0.5M Ammonium Sulphate	0.1M tri-Sodium Citrate dihydrate pH 5.6	1M Lithium Sulphate monohydrate
16	0.5M Sodium Chloride	0.1M tri-Sodium Citrate dihydrate pH 5.6	2% (w/v) Ethylene Imine Polymer
17		0.1M tri-Sodium Citrate dihydrate pH 5.6	35% (v/v) tert-Butanol
18	0.01M Ferric Chloride hexahydrate	0.1M tri-Sodium Citrate dihydrate pH 5.6	10% (v/v) Jeffamine M-600
19		0.1M tri-Sodium Citrate dihydrate pH 5.6	2.5M 1,6 Hexanediol
20		0.1M MES pH 6.5	1.6M Magnesium Sulphate heptahydrate
21	0.1M Sodium dihydrogen Phosphate; 0.1M Potassium dihydrogen Phosphate	0.1M MES pH 6.5	2M Sodium Chloride
22		0.1M MES pH 6.5	12% (w/v) PEG 20000
23	1.6M Ammonium Sulphate	0.1M MES pH 6.5	10% (v/v) Dioxane
24	0.05M Caesium Chloride	0.1M MES pH 6.5	30% (v/v) Jeffamine M-600

25	0.01M Cobaltous Chloride hexahydrate	0.1M MES pH 6.5	1.8M Ammonium Sulphate
26	0.2M Ammonium Sulphate	0.1M MES pH 6.5	30% (w/v) PEG 5000mme
27	0.01M Zinc Sulphate heptahydrate	0.1M MES pH 6.5	25% (v/v) PEG 550mme
28			1.6M tri-Sodium Citrate dihydrate pH 6.5
29	0.5M Ammonium Sulphate	0.1M HEPES pH 7.5	30% (v/v) MPD
30		0.1M HEPES pH 7.5	10% (w/v) PEG 6000; 5% (v/v) MPD
31		0.1M HEPES pH 7.5	20% (v/v) Jeffamine M-600
32	0.1M Sodium Chloride	0.1M HEPES pH 7.5	1.6M Ammonium Sulphate
33		0.1M HEPES pH 7.5	2M Ammonium Formate
34	0.05M Cadmium Sulphate hydrate	0.1M HEPES pH 7.5	1M Sodium Acetate trihydrate
35		0.1M HEPES pH 7.5	70% (v/v) MPD
36		0.1M HEPES pH 7.5	4.3M Sodium Chloride
37		0.1M HEPES pH 7.5	10% (w/v) PEG 8000; 8% (v/v) Ethylene Glycol
38		0.1M HEPES pH 7.5	20% (w/v) PEG 10000
39	0.2M Magnesium Chloride hexahydrate	0.1M Tris pH 8.5	3.4M 1,6 Hexanediol
40		0.1M Tris pH 8.5	25% (v/v) tert-Butanol
41	0.01M Nickel (II) Chloride hexahydrate	0.1M Tris pH 8.5	1M Lithium Sulphate monohydrate
42	1.5M Ammonium Sulphate	0.1M Tris pH 8.5	12% (v/v) Glycerol anhydrous
43	0.2M Ammonium dihydrogen Phosphate	0.1M Tris pH 8.5	50% (v/v) MPD
44		0.1M Tris pH 8.5	20% (v/v) Ethanol
45	0.01M Nickel (II) Chloride hexahydrate	0.1M Tris pH 8.5	20% (w/v) PEG 2000mme
46	0.1M Sodium Chloride	0.1M Bicine pH 9.0	20% (w/v) PEG 550mme
47		0.1M Bicine pH 9.0	2M Magnesium Chloride hexahydrate
48		0.1M Bicine pH 9.0	2% (v/v) Dioxane; 20% (w/v) PEG 2000mme 10% (w/v) PEG 20000

Table J3b: Hampton screen II components

J4: PEG ion screen

Tube Number	Salt	Precipitant
1	0.2M Sodium Fluoride	20% (w/v) PEG 3350
2	0.2M Potassium Fluoride	20% (w/v) PEG 3350
3	0.2M Ammonium Fluoride	20% (w/v) PEG 3350
4	0.2M Lithium Chloride anhydrous	20% (w/v) PEG 3350
5	0.2M Magnesium Chloride hexahydrate	20% (w/v) PEG 3350
6	0.2M Sodium Chloride	20% (w/v) PEG 3350
7	0.2M Calcium Chloride dihydrate	20% (w/v) PEG 3350
8	0.2M Potassium Chloride	20% (w/v) PEG 3350
9	0.2M Ammonium Chloride	20% (w/v) PEG 3350
10	0.2M Sodium Iodide	20% (w/v) PEG 3350
11	0.2M Potassium Iodide	20% (w/v) PEG 3350
12	0.2M Ammonium Iodide	20% (w/v) PEG 3350
13	0.2M Sodium Thiocyanate	20% (w/v) PEG 3350
14	0.2M Potassium Thiocyanate	20% (w/v) PEG 3350
15	0.2M Lithium Nitrate	20% (w/v) PEG 3350
16	0.2M Magnesium Nitrate hexahydrate	20% (w/v) PEG 3350

17	0.2M Sodium Nitrate	20% (w/v) PEG 3350
18	0.2M Potassium Nitrate	20% (w/v) PEG 3350
19	0.2M Ammonium Nitrate	20% (w/v) PEG 3350
20	0.2M Magnesium Formate	20% (w/v) PEG 3350
21	0.2M Sodium Formate	20% (w/v) PEG 3350
22	0.2M Potassium Formate	20% (w/v) PEG 3350
23	0.2M Ammonium Formate	20% (w/v) PEG 3350
24	0.2M Lithium Acetate dihydrate	20% (w/v) PEG 3350
25	0.2M Magnesium Acetate tetrahydrate	20% (w/v) PEG 3350
26	0.2M Zinc Acetate dihydrate	20% (w/v) PEG 3350
27	0.2M Sodium Acetate trihydrate	20% (w/v) PEG 3350
28	0.2M Calcium Acetate hydrate	20% (w/v) PEG 3350
29	0.2M Potassium Acetate	20% (w/v) PEG 3350
30	0.2M Ammonium Acetate	20% (w/v) PEG 3350
31	0.2M Lithium Sulphate monohydrate	20% (w/v) PEG 3350
32	0.2M Magnesium Sulphate heptahydrate	20% (w/v) PEG 3350
33	0.2M Sodium Sulphate decahydrate	20% (w/v) PEG 3350
34	0.2M Potassium Sulphate	20% (w/v) PEG 3350
35	0.2M Ammonium Sulphate	20% (w/v) PEG 3350
36	0.2M di-Sodium tartrate dihydrate	20% (w/v) PEG 3350
37	0.2M Potassium Sodium Tartrate tetrahydrate	20% (w/v) PEG 3350
38	0.2M di-Ammonium Tartrate	20% (w/v) PEG 3350
39	0.2M Sodium dihydrogen Phosphate monohydrate	20% (w/v) PEG 3350
40	0.2M di-Sodium hydrogen Phosphate dihydrate	20% (w/v) PEG 3350
41	0.2M Potassium dihydrogen Phosphate	20% (w/v) PEG 3350
42	0.2M di-Potassium hydrogen Phosphate	20% (w/v) PEG 3350
43	0.2M Ammonium dihydrogen Phosphate	20% (w/v) PEG 3350
44	0.2M di-Ammonium hydrogen Phosphate	20% (w/v) PEG 3350
45	0.2M tri-Lithium Citrate tetrahydrate	20% (w/v) PEG 3350
46	0.2M tri-Sodium Citrate dihydrate	20% (w/v) PEG 3350
47	0.2M tri-Potassium Citrate monohydrate	20% (w/v) PEG 3350
48	0.2M di-Ammonium hydrogen Citrate	20% (w/v) PEG 3350

Table J4: PEG ion screen components.

J6a Newcastle screen

1	50% (w/v) PEG 400	0.2 M Lithium Sulphate	0.1 M Sodium acetate pH 5.1	
2	20% (w/v) PEG 3000	0.1 M Sodium citrate pH 5.5		
3	20% (w/v) PEG 3350	0.2 M Diammonium hydrogen citrate pH 5.0		
4	30% (v/v) MPD	0.08 M Calcium chloride	0.1 M Sodium acetate pH 4.6	
5	20% (w/v) PEG 3350	0.2 M Magnesium formate pH 5.9		
6	20% (w/v) PEG 1000	0.2 M lithium sulphate	0.25 M Sodiumcitrate pH 4.2	0.25 M sodium dihydrogen phosphate pH 5.2
7	20% (w/v) PEG 8000	0.1M CHES pH 9.5		
8	20% (w/v) PEG 3350	0.2 M Ammonium formate pH6.6		
9	20% (w/v) PEG 3350	0.2 M Ammonium chloride pH 6.3		
10	20% (w/v) PEG 3350	0.2 M Potassium formate pH 7.3		
11	50% (v/v) MPD	0.2 M Ammonium dihydrogen phosphate	0.1M Tris pH 8.5	

12	20% (w/v) PEG 3350	0.2 M Potassium nitrate pH 6.9		
13	0.8 M Ammonium sulphate	0.1M Citric acid pH 4.0		
14	20% (w/v) PEG 3350	0.2 M Sodium thiocyanate pH 6.9		
15	20% (w/v) PEG 6000	0.1M Bicine pH 9.0		
16	10% (w/v) PEG 8000	8% (v/v) Ethylene glycol	0.1 M HEPES pH 7.5	
17	40% (v/v) MPD	5% (w/v) PEG 8000	0.1M Sodium cacodylate pH 7.0	
18	40% (v/v) Ethanol	5% (w/v) PEG 1000	0.25 M Sodium citrate pH 5.2	0.25 M Sodium dihydrogen phosphate pH 5.2
19	8% (w/v) PEG 4000	0.1M Sodium acetate pH 4.6		
20	10% (w/v) PEG 8000	0.2 M Magnesium chloride	0.1 M Tris pH 7.0	
21	20% (w/v) PEG 6000	0.1M Citric acid pH 5.0		
22	50% (w/v) PEG 200	0.2 M Magnesium chloride	0.1 M Sodium cacodylate pH 6.6	
23	1.6 M Sodium citrate pH 6.5			
24	20% (w/v) PEG 3350	0.2 M Potassium citrate pH 8.3		

J6b Newcastle screen

25	30% (v/v) MPD	0.02 M Calcium chloride	0.1M Sodium acetate pH 4.6	
26	20% (w/v) PEG 8000	0.2 M Sodium chloride	0.25 M Sodium citrate pH 4.2	0.25 M Sodium dihydrogen phosphate pH 4.2
27	20% (w/v) PEG 6000	1.0 M Lithium chloride	0.1 M Citric acid pH 4.0	
28	20% (w/v) PEG 3350	0.2 M Ammonium nitrate pH 6.3		
29	10% (w/v) PEG 6000	0.1M HEPES pH 7.0		
30	0.8 M Ammonium dihydrogen phosphate	0.8 M Potassium dihydrogen phosphate	0.1M HEPES pH 7.5	
31	40% (w/v) PEG 300	0.25 M Sodium citrate pH 5.2	0.25 M Sodium dihydrogen phosphate pH 5.2	
32	10% (w/v) PEG 3000	0.2 M Zinc acetate	0.1 M Sodium acetate pH 4.5	
33	20% (v/v) Ethanol	0.1 M Tris pH 8.5		
34	25% (v/v) 1-2-Propanediol	0.1M Sodium potassium phosphate pH 6.8	10% (v/v) glycerol	
35	10% (w/v) PEG 20000	2% (v/v) Dioxane	0.1M Bicine pH 9.0	
36	2.0 M Ammonium sulphate	0.1M Sodium acetate pH 4.6		
37	10% (w/v) PEG 1000	10% (w/v) PEG 8000		
38	24% (w/v) PEG 1000	20% (v/v) Glycerol		
39	30% (v/v) PEG 400	0.2 M Magnesium chloride	0.1M HEPES pH 7.5	

40	50% (w/v) PEG 200	0.2 M Sodium chloride	0.1 M Sodium potassium phosphate pH 7.2	
41	30% (w/v) PEG 8000	0.2 M Lithium sulphate	0.1M Sodium acetate pH 4.5	
42	70% (v/v) MPD	0.2 M Magnesium chloride	0.1 M HEPES pH 7.5	
43	20% (w/v) PEG 8000	0.1M Tris pH 8.5		
44	40% (v/v) PEG 400	0.2 M Lithium sulphate	0.1 M Tris pH 8.4	
45	40% (v/v) MPD	0.1M Tris pH 8.0		
46	25.5% (w/v) PEG 4000	0.17 M Ammonium sulphate	15% (v/v) Glycerol	
47	40% (w/v) PEG 300	0.2 M Calcium acetate	0.1M Sodium cacodylate pH 7.0	
48	14% (v/v) Isopropanol	0.14 M Calcium chloride	0.07M Sodium acetate pH 4.6	30% (v/v) glycerol

J6c Newcastle screen

49	16% (w/v)PEG 8000	0.04 M Potassium dihydrogen phosphate	20% (v/v) Glycerol	
50	1.0 M Sodium citrate	0.1 M sodium cacodylate pH 6.5		
51	2.0 M Ammonium sulphate	0.2 M Sodium chloride	0.1 M Sodium cacodylate pH 6.5	
52	10% (v/v) Isopropanol	0.2 M Sodium chloride	0.1 M HEPES pH 7.5	
53	1.26 M Ammonium sulphate	0.2 M Lithium sulphate	0.1M Tris pH 8.5	
54	40% (v/v) MPD	0.1M CAPS pH 10.1		
55	20% (w/v)PEG 3000	0.2 M Zinc acetate	0.1M Imidazole pH 8.0	
56	10% (v/v) Isopropanol	0.2 M Zinc acetate	0.1M Sodium cacodylate pH 6.5	
57	1.0M Diammonium hydrogen phosphate	0.1 M Sodium acetate pH 4.5		
58	1.6 M Magnesium sulphate	0.1M MES pH 6.5		
59	10% (w/v)PEG 6000	0.1 M Bicine pH 9.0		
60	14.4% (w/v)PEG 8000	0.16 M Calcium acetate	0.08 M Sodium cacodylate pH 6.5	20% (v/v) glycerol
61	10% (w/v)PEG 8000	0.1 M Imidazol pH 8.0		
62	30% (v/v) Jeffamine	0.05 M Caesiumchloride	0.1 M MES pH 6.5	
63	3.2 M Ammonium sulphate	0.1 M Citric acid pH 5.0		
64	20% (v/v) MPD	0.1 M Tris pH 8.0		
65	20% (v/v) Jeffamine	0.1M HEPES pH 6.5		
66	50% (v/v) Ethylene glycol	0.2 M Magnesium chloride	0.1 M Tris pH 8.5	
67	10% (v/v) MPD	0.1 M Bicine pH 9.0		
68	0.2 M Ammonium sulphate	0.1 M Sodium acetate pH 4.6	30% (w/v) PEG MME 2000	
69	0.2 M Ammonium sulphate	0.1 M MES pH 6.5	30% (w/v) PEG MME 5000	
70	0.01 M Zinc sulphate	0.1 M MES pH 6.5	25% (w/v) PEG MME 550	
71	0.01 M Nickel chloride	0.1 M Tris pH 8.5	20% (w/v) PEG MME 2000	
72	0.1 M Sodium chloride	0.1 M Bicine pH 9.0	20% (w/v) PEG MME 550	

J6d Newcastle Screen

73	0.005 M Magnesium chloride	0.05 M HEPES pH 7.0	25% (w/v) PEG MME 550	
74	0.1 M Potassium chloride	0.015 M Magnesium chloride	0.05 M Tris pH 7.5	10% PEG MME 550
75	20% (v/v)1-4- Butandiol	0.1 M MES pH 6.0	0.2 M Lithium sulphate	
76	1 M Sodium potassium tartrate	0.1 M Imidazole pH 8.0	0.2 M Sodium chloride	
77	20% (v/v)1-4-Butandiol		0.1M Sodium acetate pH 4.5	
78	1M Sodium potassium tartrate	0.1 M CHES pH 9.5	0.2 M Lithium sulphate	
79	35% (v/v) Propanol	0.1 M Sodium cacodylate pH6.5		
80	35% (v/v) Propanol	0.1 M Tris pH 8.5		
81	3.5 M Sodium formate			
82	0.8M Succinic acid pH 7.0			
83	2.1 M Malic acid pH 7.0			
84	2.4 M Sodium malonate pH 7.0			
85	0.2 M Potassium chloride	0.05 M HEPES pH 7.5	35% (v/v) Pentaerythritol propoxylate	
86	0.005 M Ammonium sulphate	0.05 M Tris pH 6.5	30% (v/v) Pentaerythritol ethoxylate	
87	0.2 M Potassium bromide	25% (w/v) PEG MME 2000	0.1 M HEPES pH 7.5	
88	0.2M Potassium bromide	8% (w/v)PEG 20000	8% (w/v) PEG MME 550	0.1 M Tris pH 8.5
89	1.0 M Potassium dihydrogen phosphate	0.1 M Sodium citrate pH 4.6		
90	0.5 M Potassium dihydrogen phosphate	0.1M HEPES pH 7.0		
91	20% (w/v)PEG 4000	0.005 M Cadmium chloride	0.1 M Tris pH 8.0	
92	20% (w/v)PEG 4000	0.005 M Nickel chloride	0.1 M MED pH 6.5	
93	0.8 M Sodium formate	10% (w/v) PEG 8000	10% PEG 1000	0.1 M imidazol pH 8.0
94	15% (w/v)PEG 4000	0.005 M Cadmium sulphate	0.1 M Sodium cacodylate pH 6.5	
95	20% (w/v) PEG 600	0.005 M Cobalt chloride	0.1 M HEPES pH 7.5	
96	2 M Ammonium sulphate	10% (v/v) Jeffamine	0.1 M Tris pH 8.0	

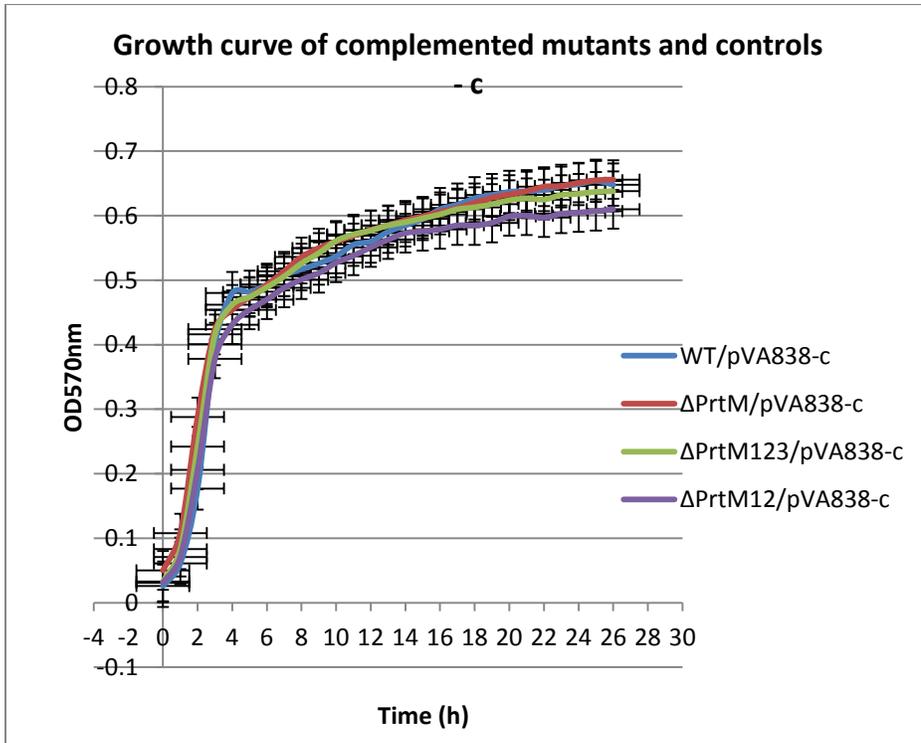
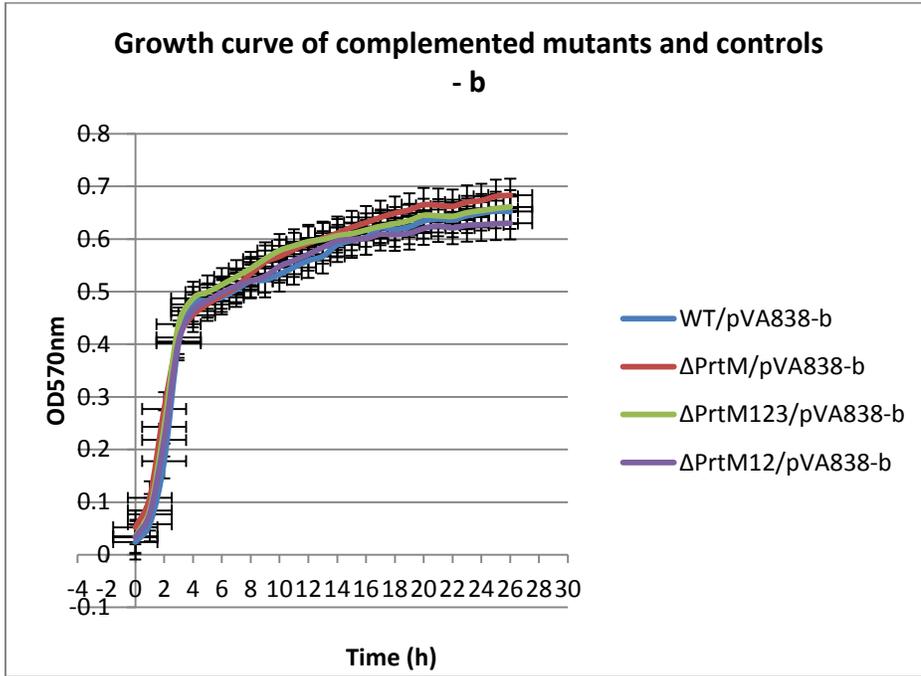
J7a Index Screen

1. 0.1 M Citric Acid pH 3.5, 2.0 M Ammonium Sulfate
2. 0.1 M Na Acetate pH 4.5, 2.0 M Ammonium Sulfate
3. 0.1 M Bis-Tris pH 5.5, 2.0 M Ammonium Sulfate
4. 0.1 M Bis-Tris pH 6.5, 2.0 M Ammonium Sulfate
5. 0.1 M HEPES pH 7.5, 2.0 M Ammonium Sulfate
6. 0.1 M Tris pH 8.5, 2.0 M Ammonium Sulfate
7. 0.1 M Citric Acid pH 3.5, 3.0 M Sodium Chloride
8. 0.1 M Na Acetate pH 4.5, 3.0 M Sodium Chloride
9. 0.1 M Bis-Tris pH 5.5, 3.0 M Sodium Chloride
10. 0.1 M Bis-Tris pH 6.5, 3.0 M Sodium Chloride
11. 0.1 M HEPES pH 7.5, 3.0 M Sodium Chloride
12. 0.1 M Tris pH 8.5, 3.0 M Sodium Chloride
13. 0.1 M Bis-Tris pH 5.5, 0.5 M Magnesium Formate
14. 0.1 M Bis-Tris pH 6.5, 0.5 M Magnesium Formate
15. 0.1 M HEPES pH 7.5, 0.3 M Magnesium Formate
16. 0.1 M Tris pH 8.5, 0.3 M Magnesium Formate
17. 1.4 M Sodium/Potassium Phosphate pH 5.6
18. 1.4 M Sodium Potassium Phosphate pH 6.9
19. 1.4 M Sodium Potassium Phosphate pH 8.2
20. 0.1 M HEPES pH 7.5, 1.4 M Sodium Citrate
21. 1.8 M tri-Ammonium Citrate pH 7.0
22. 0.8 M Succinic Acid pH 7.0
23. 2.1 M DL-Malic Acid pH 7.0
24. 2.8 M Sodium Acetate pH 7.0
25. 3.5 M Sodium Formate pH 7.0
26. 1.1 M di-Ammonium Tartrate pH 7.0
27. 2.4 M Sodium Malonate pH 7.0
28. 35% v/v Tacsimate pH 7.0
29. 60% v/v Tacsimate pH 7.0
30. 0.1 M Sodium Chloride, 0.1 M Bis-Tris pH 6.5, 1.5 M Ammonium Sulfate
31. 0.8 M K/Na Tartrate, 0.1 M Tris pH 8.5, 0.5% PEG MME 5000
32. 1.0 M Ammonium Sulfate, 0.1 M Bis-Tris pH 5.5, 1% w/v PEG 3350
33. 1.1 M Na Malonate pH 7.0, 0.1 M HEPES pH 7.0, 0.5% v/v Jeffamine ED-2001 pH 7.0
34. 1.0 M Succinic Acid pH 7.0, 0.1 M HEPES pH 7.0, 1% w/v PEG MME 2000
35. 1.0 M Ammonium Sulfate, 0.1 M HEPES pH 7.0, 0.5% w/v PEG 8000
36. 15% v/v Tacsimate pH 7.0, 0.1 M HEPES pH 7.0, 2% w/v PEG 3350
37. 25% w/v PEG 1500
38. 0.1 M HEPES pH 7.0, 30% v/v Jeffamine M-600 pH 7.0
39. 0.1 M HEPES pH 7.0, 30% v/v Jeffamine ED-2001 pH 7.0
40. 0.1 M Citric Acid pH 3.5, 25% w/v PEG 3350
41. 0.1 M Na Acetate pH 4.5, 25% w/v PEG 3350
42. 0.1 M Bis-Tris pH 5.5, 25% w/v PEG 3350
43. 0.1 M Bis-Tris pH 6.5, 25% w/v PEG 3350
44. 0.1 M HEPES pH 7.5, 25% w/v PEG 3350
45. 0.1 M Tris pH 8.5, 25% w/v PEG 3350
46. 0.1 M Bis-Tris pH 6.5, 20% w/v PEG MME 5000
47. 0.1 M Bis-Tris pH 6.5, 28% w/v PEG MME 2000
48. 0.2 M Ca Chloride, 0.1 M Bis-Tris pH 5.5, 45% v/v MPD
49. 0.2 M Ca Chloride, 0.1 M Bis-Tris pH 6.5, 45% v/v MPD
50. 0.2 M Ammonium Acetate, 0.1 M Bis-Tris pH 5.5, 45% v/v MPD
51. 0.2 M Ammonium Acetate, 0.1 M Bis-Tris pH 6.5, 45% v/v MPD
52. 0.2 M Ammonium Acetate, 0.1 M HEPES pH 7.5, 45% v/v MPD
53. 0.2 M Ammonium Acetate, 0.1 M Tris pH 8.5, 45% v/v MPD

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54. 0.05 M Ca Chloride, 0.1 M Bis-Tris pH 6.5, 30% v/v PEG MME 550
55. 0.05 M Mg Chloride, 0.1 M HEPES pH 7.5, 30% v/v PEG MME 550
56. 0.2 M K Chloride, 0.05 M HEPES pH 7.5, 35% v/v Pentaerythritol Propoxylate (5/4 PO/OH)
57. 0.05 M Ammonium Sulfate, 0.05 M Bis-Tris pH 6.5, 30% v/v Pentaerythritol Ethoxylate (15/4 EO/OH)
58. 0.05 M Bis-Tris pH 6.5, 45% v/v PPG P 400
59. 0.02 M Mg Chloride, 0.1 M HEPES pH 7.5, 22% w/v Polyacrylic Acid 5100
60. 0.01 M Co Chloride, 0.1 M Tris pH 8.5, 20% w/v Polyvinylpyrrolidone K15
61. 0.2 M Proline, 0.1 M HEPES pH 7.5, 10% w/v PEG 3350
62. 0.2 M Trimethylamine N-oxide, 0.1 M Tris pH 8.5, 20% PEG MME 2000
63. 0.1 M HEPES pH 7.0, 5% v/v Tacsimate pH 7.0, 10% w/v PEG MME 5000
64. 0.005 M Co Chloride, 0.005 M Ni Chloride, 0.005 M Cd Chloride, Mg Chloride, 0.1 M HEPES pH 7.5, 12% w/v Polyethylene Glycol 3350
65. 0.1 M Ammonium Acetate, 0.1 M Bis-Tris pH 5.5, 17% w/v PEG 10,000
66. 0.2 M Ammonium Sulfate, 0.1 M Bis-Tris pH 5.5, 25% w/v PEG 3350
67. 0.2 M Ammonium Sulfate, 0.1 M Bis-Tris pH 6.5, 25% w/v PEG 3350
68. 0.2 M Ammonium Sulfate, 0.1 M HEPES pH 7.5, 25% w/v PEG 3350
69. 0.2 M Ammonium Sulfate, 0.1 M Tris pH 8.5, 25% w/v PEG 3350
70. 0.2 M Na Chloride, 0.1 M Bis-Tris pH 5.5, 25% w/v PEG 3350
71. 0.2 M Na Chloride, 0.1 M Bis-Tris pH 6.5, 25% w/v PEG 3350
72. 0.2 M Na Chloride, 0.1 M HEPES pH 7.5, 25% w/v PEG 3350
73. 0.2 M Na Chloride, 0.1 M Tris pH 8.5, 25% w/v PEG 3350
74. 0.2 M Li Sulfate, 0.1 M Bis-Tris pH 5.5, 25% w/v PEG 3350
75. 0.2 M Li Sulfate, 0.1 M Bis-Tris pH 6.5, 25% w/v PEG 3350
76. 0.2 M Li Sulfate, 0.1 M HEPES pH 7.5, 25% w/v PEG 3350
77. 0.2 M Li Sulfate, 0.1 M Tris pH 8.5, 25% w/v PEG 3350
78. 0.2 M Ammonium Acetate, 0.1 M Bis-Tris pH 5.5, 25% w/v PEG 3350
79. 0.2 M Ammonium Acetate, 0.1 M Bis-Tris pH 6.5, 25% w/v PEG 3350
80. 0.2 M Ammonium Acetate, 0.1 M HEPES pH 7.5, 25% w/v PEG 3350
81. 0.2 M Ammonium Acetate, 0.1 M Tris pH 8.5, 25% w/v PEG 3350
82. 0.2 M Mg Chloride, 0.1 M Bis-Tris pH 5.5, 25% w/v PEG 3350
83. 0.2 M Mg Chloride, 0.1 M Bis-Tris pH 6.5, 25% w/v PEG 3350
84. 0.2 M Mg Chloride, 0.1 M HEPES pH 7.5, 25% w/v PEG 3350
85. 0.2 M Mg Chloride, 0.1 M Tris Hydrochloride pH 8.5, 25% w/v PEG 3350
86. 0.2 M K/Na Tartrate, 20% w/v PEG 3350
87. 0.2 M Na Malonate pH 7.0, 20% w/v PEG 3350
88. 0.2 M tri-Ammonium Citrate pH 7.0, 20% w/v PEG 3350
89. 0.1 M Succinic Acid pH 7.0, 15% w/v PEG 3350
90. 0.2 M Na Formate, 20% w/v PEG 3350
91. 0.15 M DL-Malic Acid pH 7.0, 20% w/v PEG 3350
92. 0.1 M Mg Formate, 15% w/v PEG 3350
93. 0.05 M Zinc Acetate, 20% w/v PEG 3350
94. 0.2 M Na Citrate, 20% w/v PEG 3350
95. 0.1 M K Thiocyanate, 30% w/v PEG MME 2000
96. 0.15 M K Bromide, 30% w/v PEG MME 2000

Appendix K : Growth curve of complemented mutants and controls



Appendix L

Progenesis SameSpots Analysis of 2D-E images of Complemented mutants and Control.

pH 4 \longrightarrow pH 7

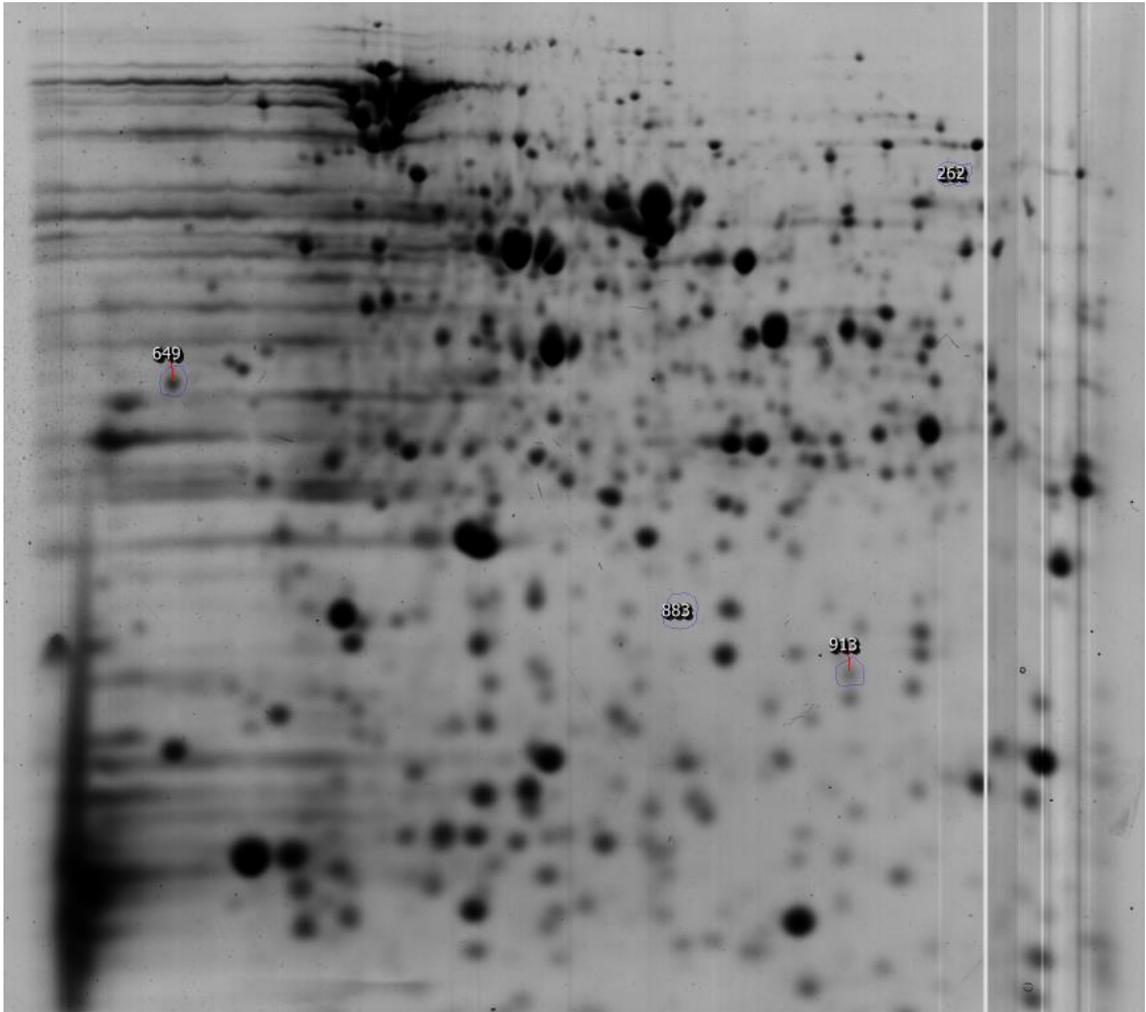


Figure L1: Progenesis Comparison of *S. equi* 4047 WT/pVA838 versus Δ Prtm123/pVA838 – Cell Associated Protein Extract

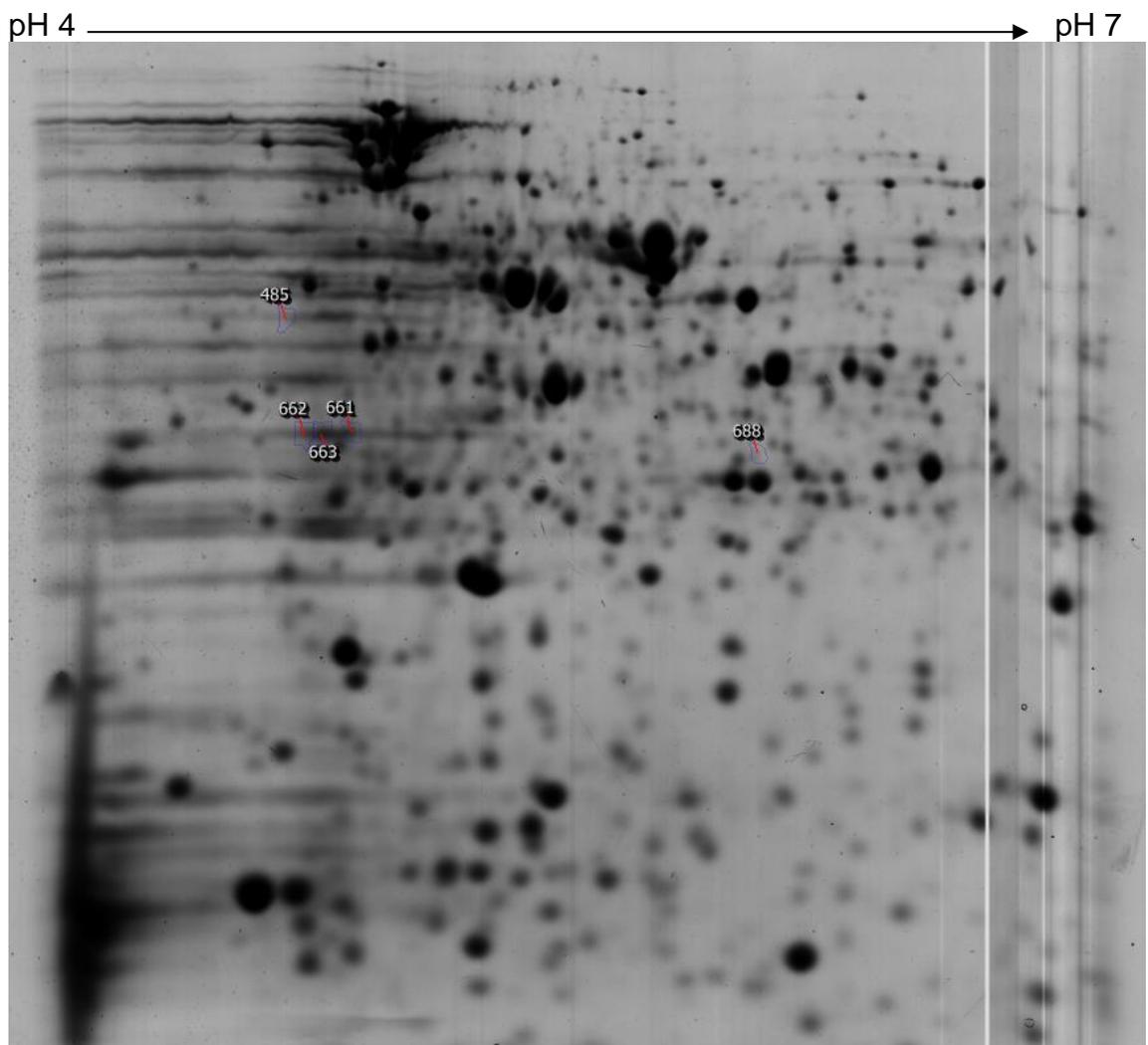


Figure L2: Progenesis Comparison of *S. equi* 4047 WT/pVA838 versus Δ Prtm12/pVA838 – Cell Associated Protein Extract

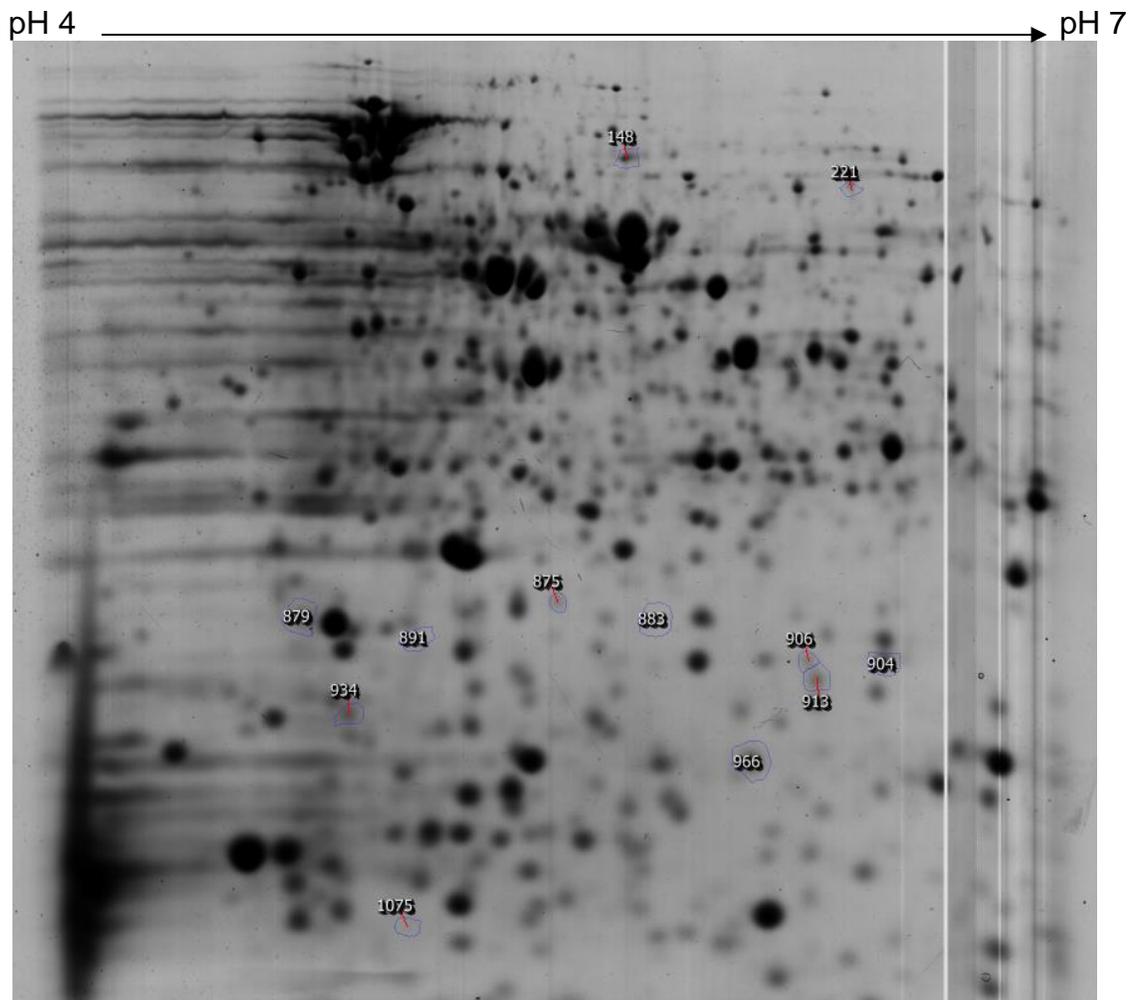


Figure L3: Progenesis Comparison of *S. equi* 4047 WT/pVA838 versus Δ Prtm/pVA838 – Cell Associated Protein Extract

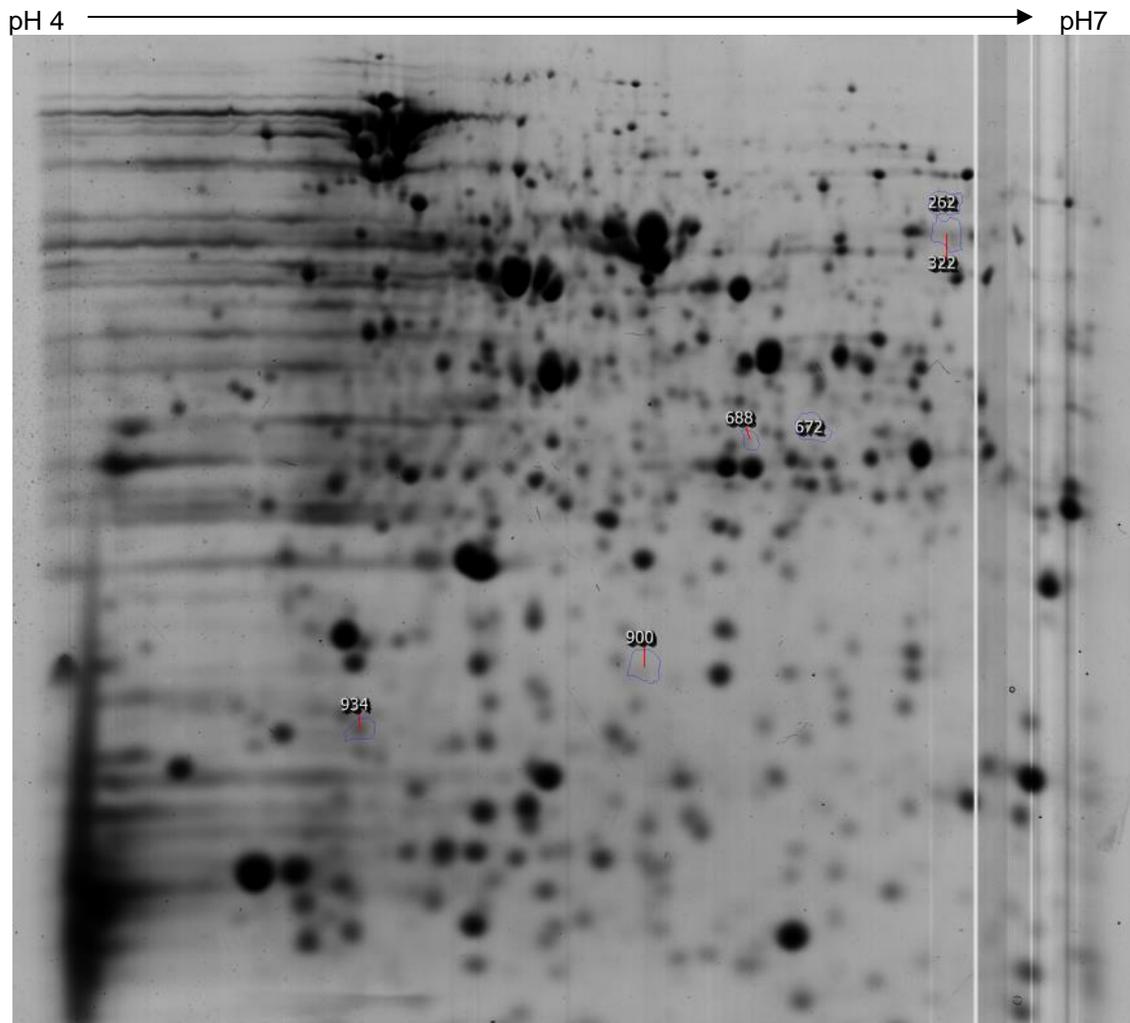


Figure L4: Progenesis Comparison of *S. equi* 4047 Δ Prtm123/pVA838 versus Δ Prtm12/pVA838 – Cell Associated Protein Extract

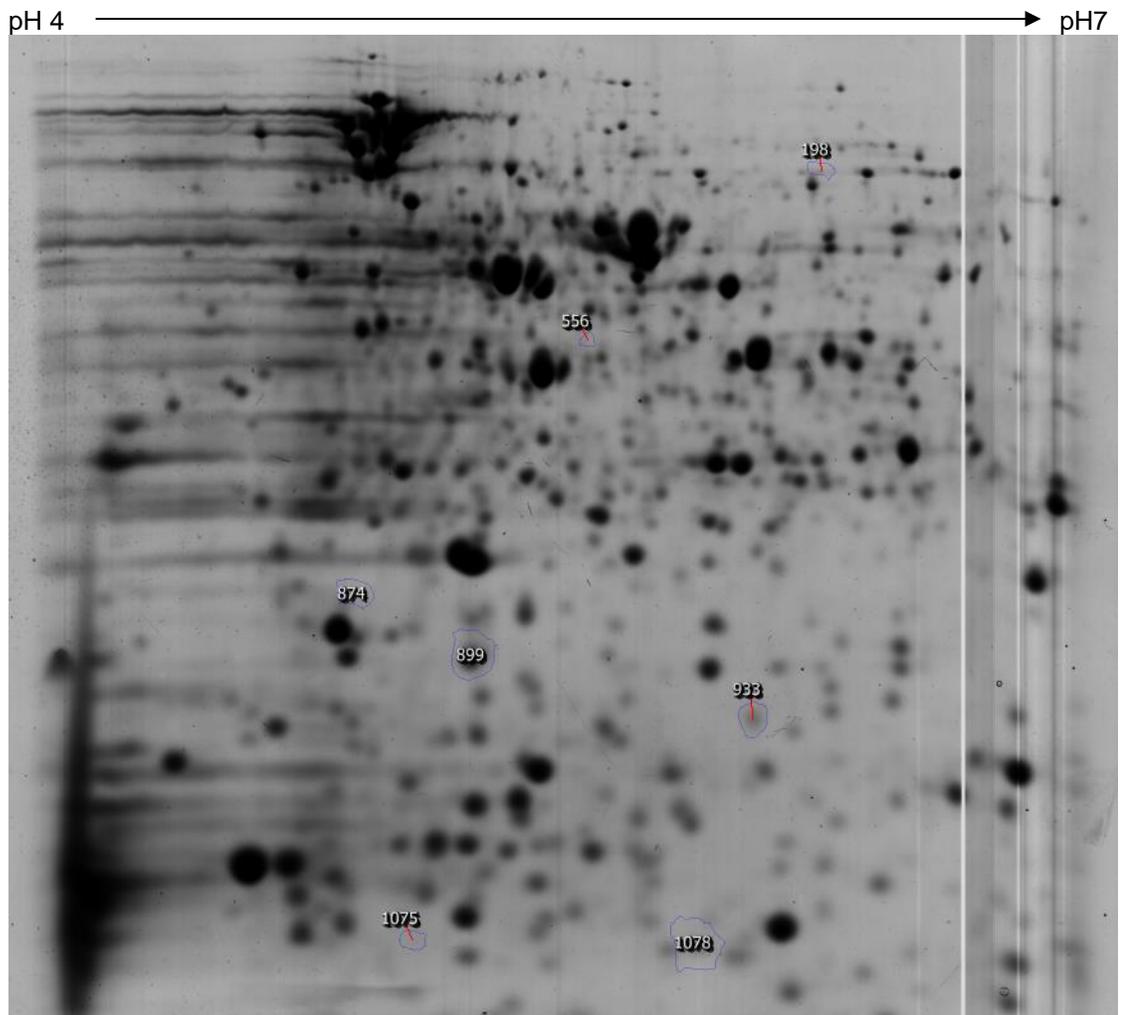


Figure L5: Progenesis Comparison of *S. equi* 4047 Δ Prtm123/pVA838 versus Δ Prtm/pVA838 – Cell Associated Protein Extract

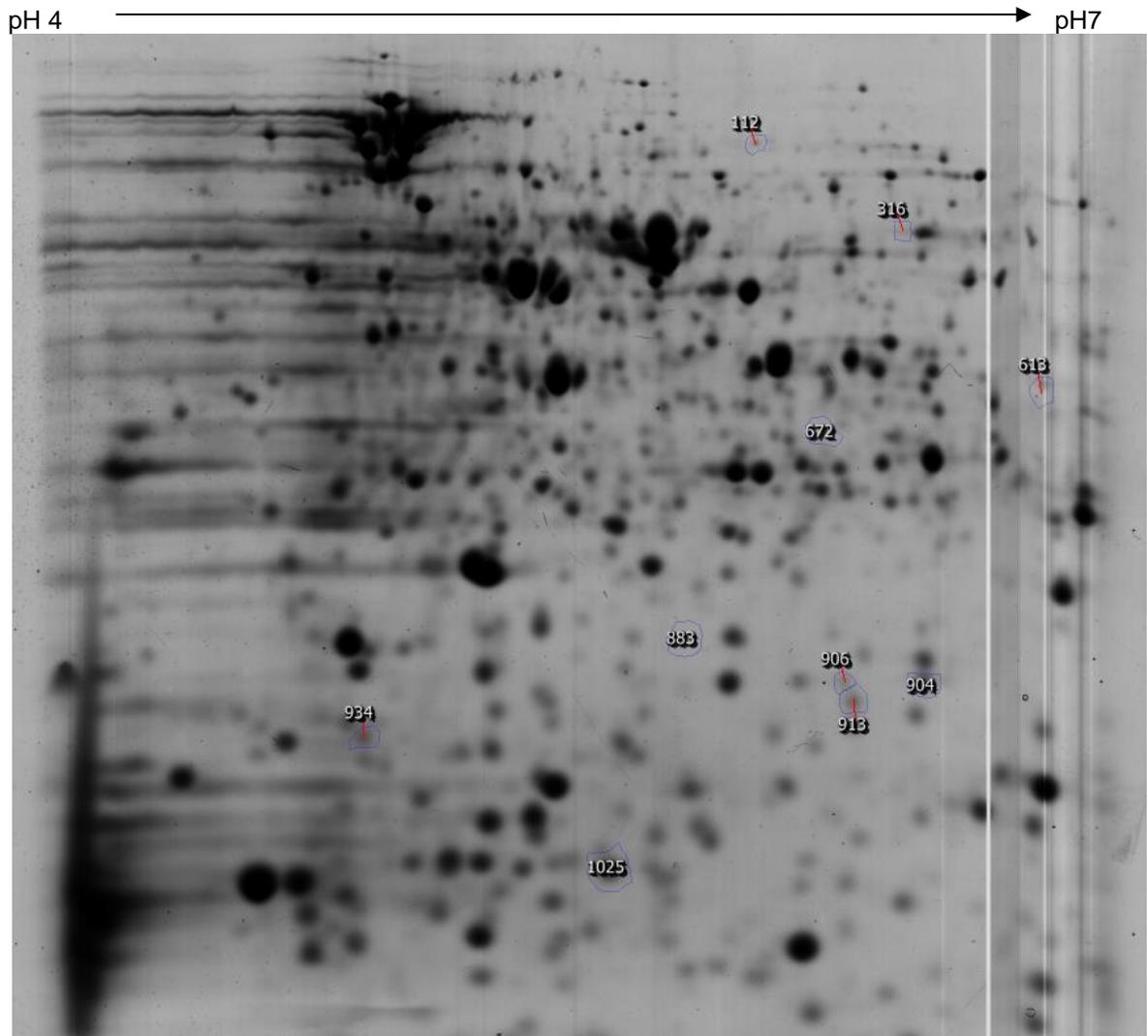


Figure L6: Progenesis Comparison of *S. equi* 4047 Δ Prtm/pVA838 versus Δ Prtm12/pVA838 – Cell Associated Protein Extract

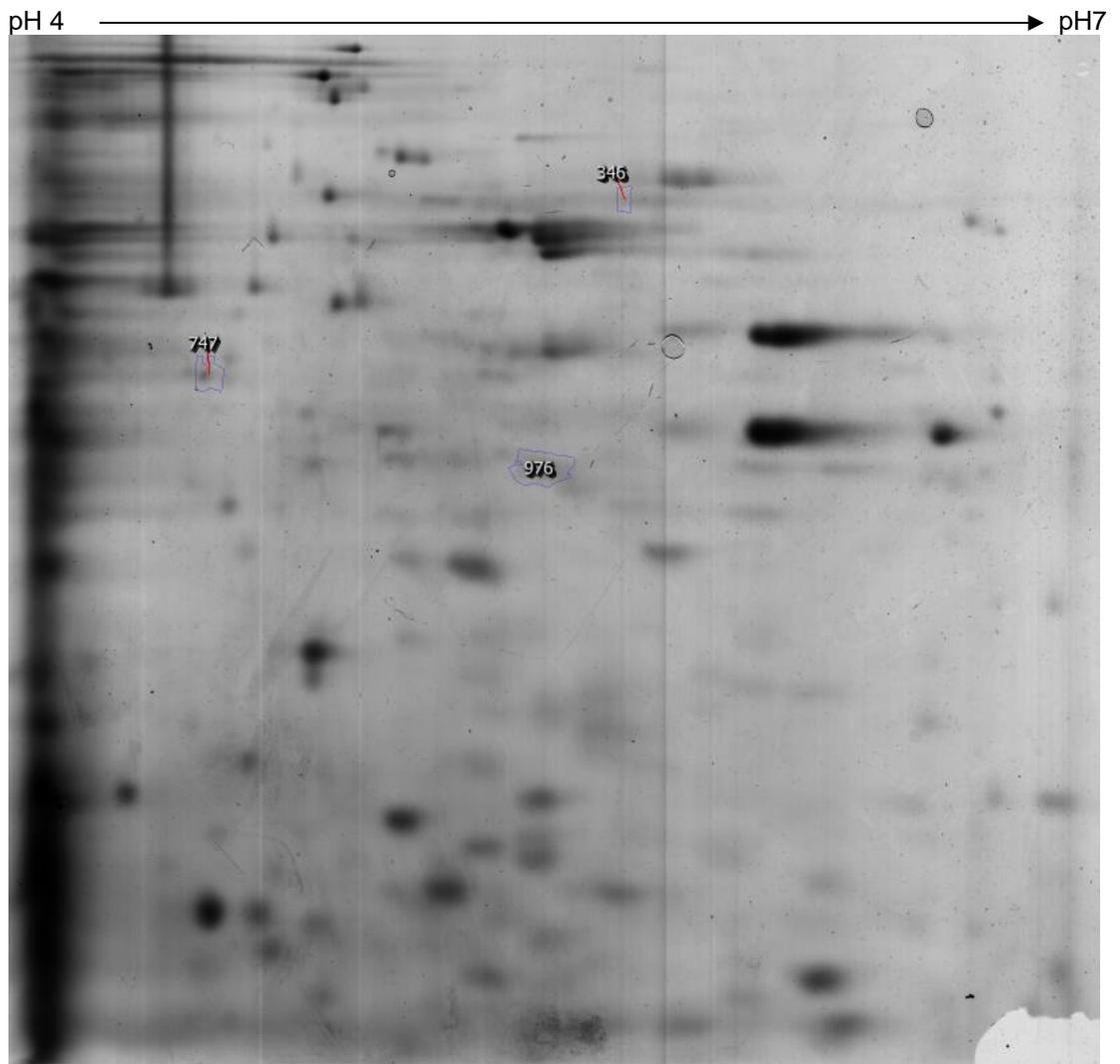


Figure L7: Progenesis Comparison of *S. equi* 4047 WT/pVA838 versus Δ Prtm123/pVA838 –Secreted Protein Extract

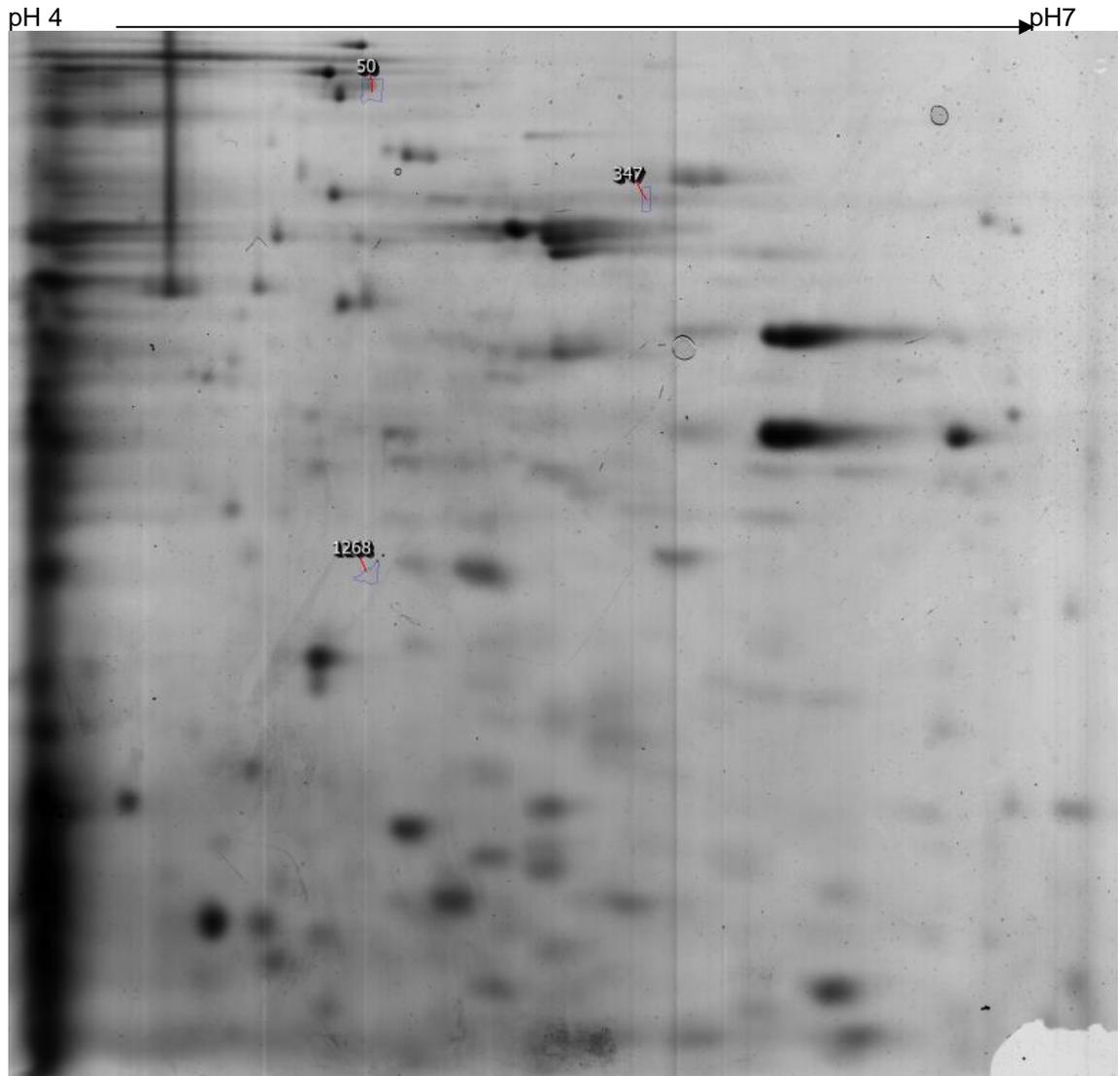


Figure L8: Progenesis Comparison of *S. equi* 4047 WT/pVA838 versus Δ Prtm12/pVA838 –Secreted Protein Extract

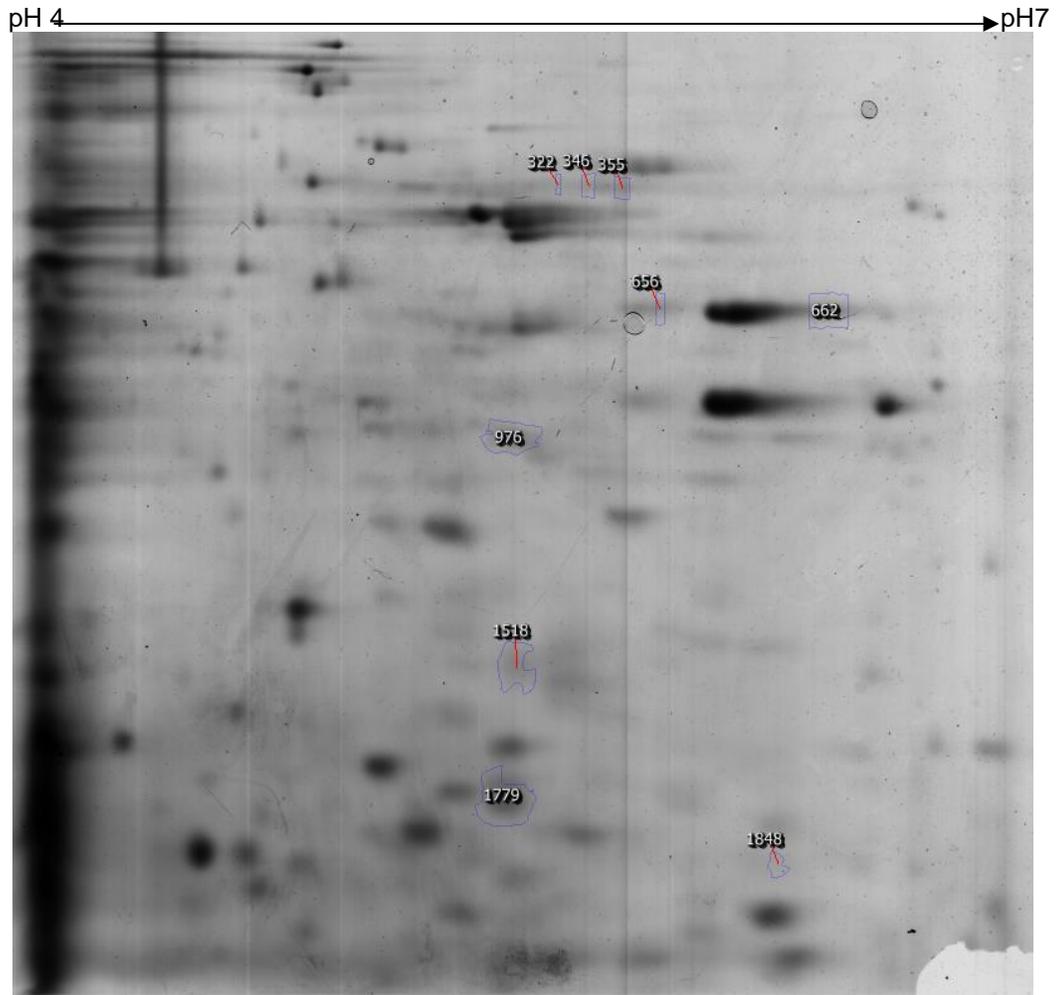


Figure L9: Progenesis Comparison of *S. equi* 4047 WT/pVA838 versus Δ Prtm/pVA838 – Secreted Protein Extract

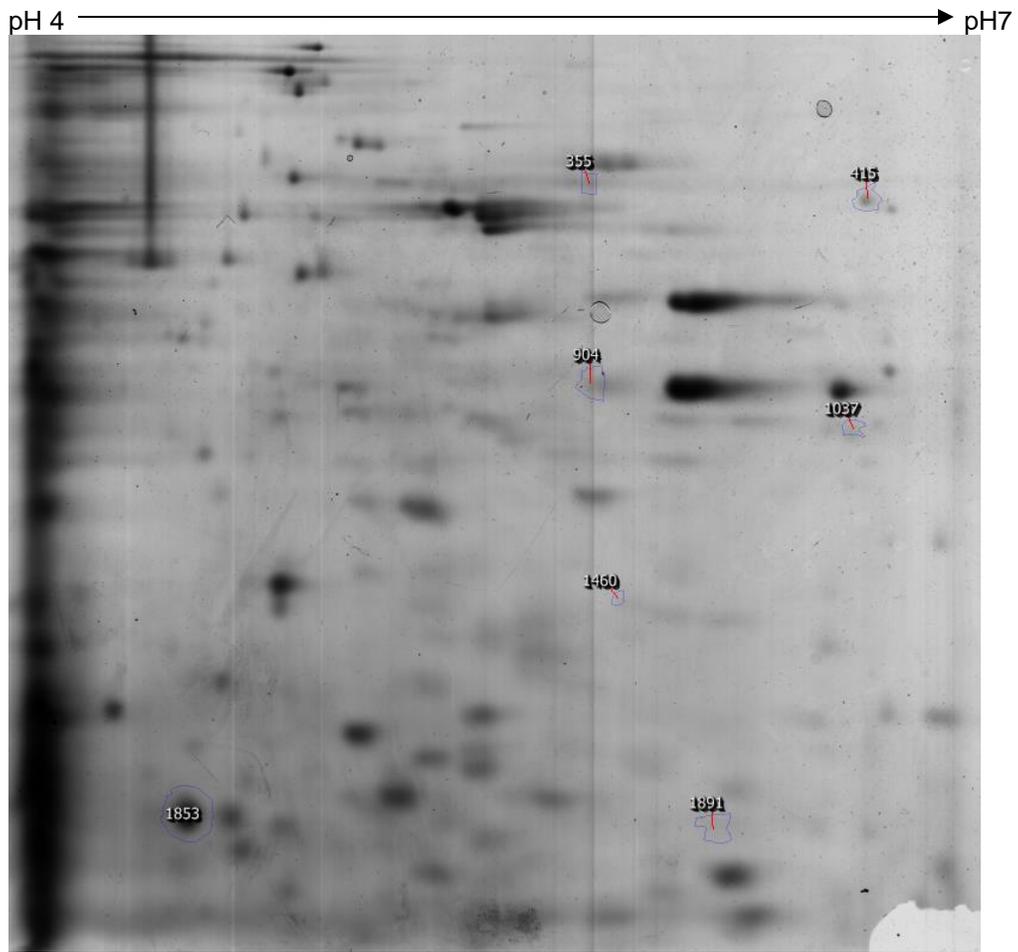


Figure L10: Progenesis Comparison of *S. equi* 4047 Δ Prtm123/pVA838 versus Δ Prtm12/pVA838 –Secreted Protein Extract

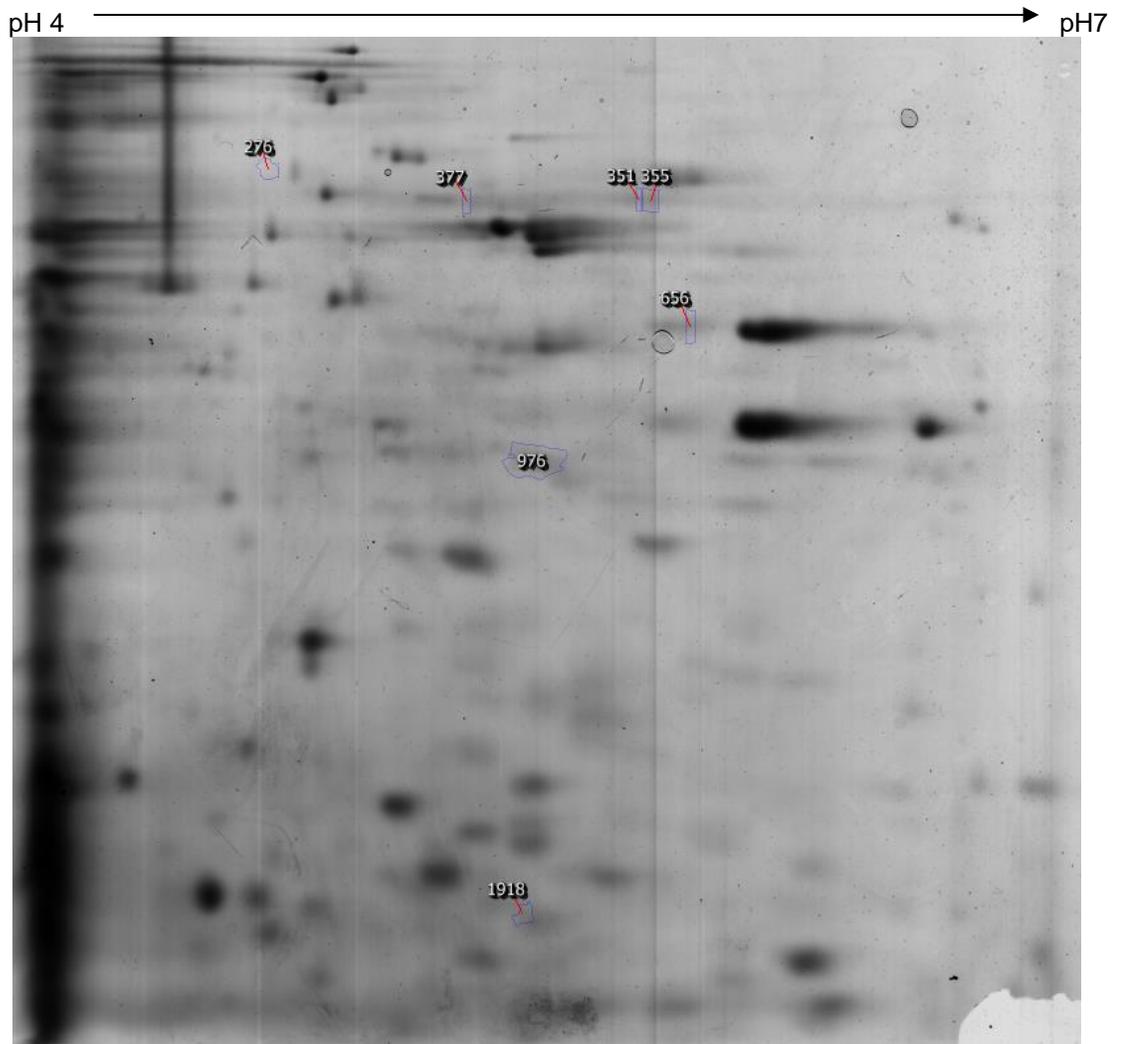


Figure L11: Progenesis Comparison of *S. equi* 4047 Δ Prtm12/pVA838 versus Δ Prtm/pVA838 – Secreted Protein Extract

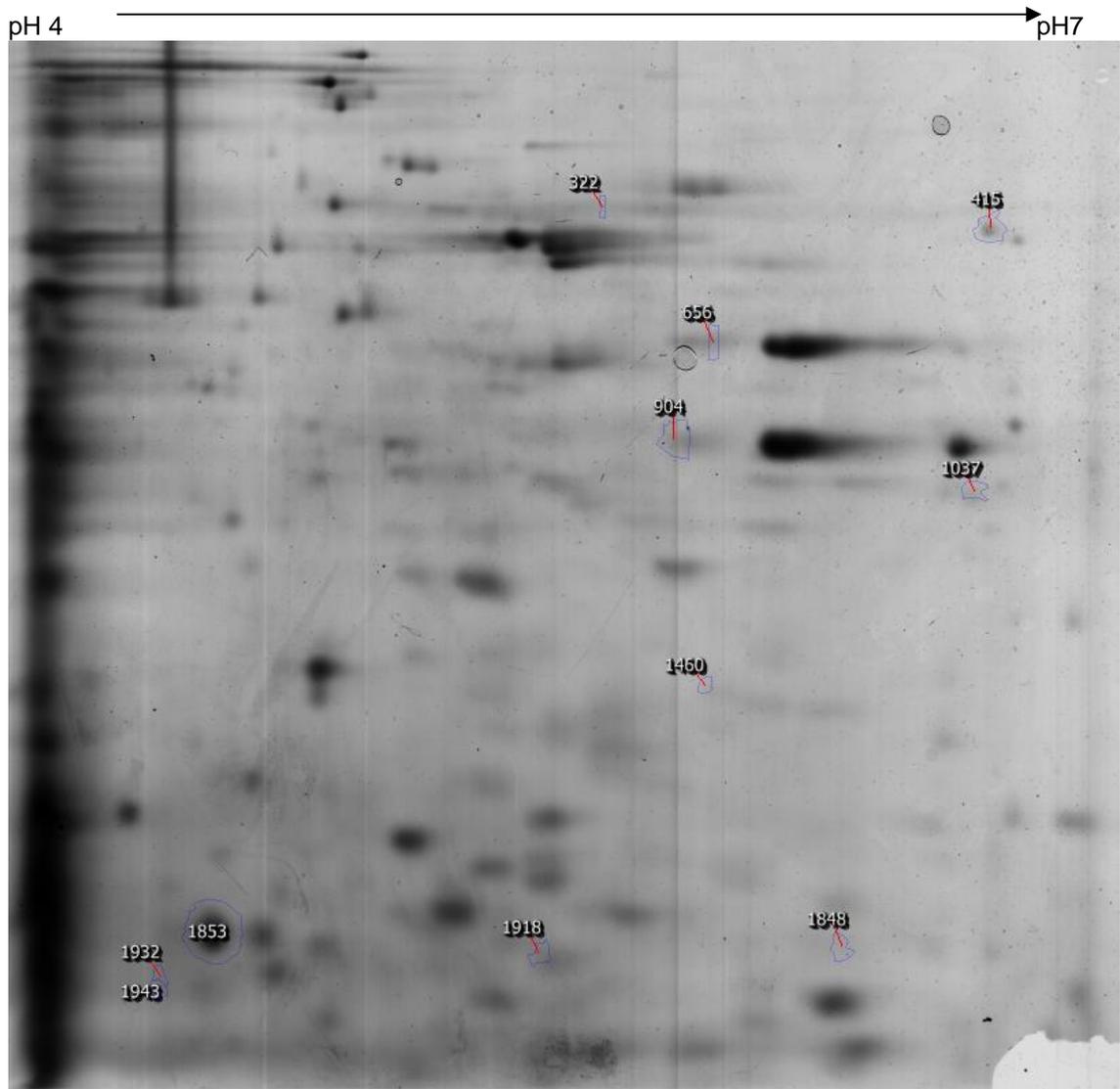


Figure L12: Progenesis Comparison of *S. equi* 4047 Δ Prtm/pVA838 versus Δ Prtm123/pVA838 –Secreted Protein Extract.

