Comparative outer membrane proteomic analyses of bovine and ovine isolates of *Mannheimia haemolytica* and *Mannheimia glucosida*

JONATHAN DAVID ARTHUR HOUNSOME

Institute of Infection, Immunity and Inflammation
College of Medical, Veterinary and Life Sciences
University of Glasgow

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Summary

In the present study, recombinant proteins representing the transmembrane domain of *M. haemolytica* outer membrane protein A (OmpA) from a bovine serotype A1 isolate (rOmpA1) and an ovine serotype A2 isolate (rOmpA2) were over-expressed, purified and used to generate anti-rOmpA1 and anti-rOmpA2 antibodies, respectively. An examination of the binding specificities of these antibodies to *M. haemolytica* isolates representing different OmpA subclasses revealed that cross-absorbed anti-rOmpA1 antibodies recognised OmpA1-type proteins but not OmpA2-type proteins; conversely, cross-absorbed anti-rOmpA2 antibodies recognised OmpA2-type proteins but not OmpA1 type proteins. This demonstrated that OmpA1 and OmpA2 are surface-exposed and could potentially bind to different receptors in cattle and sheep. The outer membrane subproteomes of seven *M. haemolytica* isolates and one *M. glucosida* isolate were also characterised and compared after growth in complex growth medium in order to identify OMPs with putative roles in host-specificity and virulence. First, a simple bioinformatic workflow (E-Komon *et al.*, 2011b) was used to confidently predict 93 unique OMPs encoded among the genomes of a bovine serotype A1 *M. haemolytica* isolate and two serotype A2 isolates (one bovine and one ovine). Secondly, a combination of gel-based and gel-free proteomic approaches employing MALDI-TOF-TOF and LC-ESI-QqTOF mass spectrometry identified 55 unique OMPs among the outer membrane fractions of seven *M. haemolytica* isolates and one *M. glucosida* isolate (of which 50 were predicted by the bioinformatic approach). A role in host-specific adaptation could not be established for any of the identified OMPs, however, the study represents the most comprehensive analysis of *M. haemolytica* and *M. glucosida* outer membrane subproteomes to date. In order to identify putative virulence-associated OMPs, the outer membrane subproteomes of the same representative isolates were also characterised after *in vitro* growth under conditions that were designed to mimic the *in vivo* host respiratory tract microenvironment. These conditions included growth in iron-restricted medium, serum-supplemented tissue culture media and growth on solid-surface agar (in the absence or presence of Congo red). This approach allowed the identification of 13 additional OMPs that were not identified after growth in complex medium alone.

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Dedication

This four year study is dedicated to my parents, Sandy and David Hounsome.

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Presentations/Publications

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2. Hounsome, J. D. A., Burchmore, R. J. S. & Davies, R. L. Comparative analyses of the outer membrane subproteomes of bovine and ovine *Mannheimia haemolytica* isolates grown under iron-replete and iron-restricted conditions. Poster presentation at the 8th International *Pasteurellaceae* Conference, 24th-27th August 2011, Helsingør, Denmark.

3. Hounsome, J. D. A., Baillie, S., Riboldi-Tunnicliffe, A., Isaacs, N. W., Burchmore, R. J. S. & Davies, R. L. Purification and crystallisation of outer membrane protein A (OmpA) of *Mannheimia haemolytica*. Poster presentation at the 7th International *Pasteurellaceae* Conference, 12th-15th October 2008, Sorrento, Italy.

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Declaration

I hereby declare that the work presented in this thesis is my own, except where otherwise cited or acknowledged. No part of this thesis has been presented for any other degree. The research for this thesis was performed between September 2007 and September 2011.

Jonathan David Arthur Hounsome

Abbreviations

1-D	One-dimensional
2-DE	Two-dimensional electrophoresis
A	Adenine
ABC	ATP-binding cassette
ACN	Acetonitrile
ATP	Adenosine triphosphate
BAM	ß-barrel assembly machinery
BHI	Brain heart infusion
BHIA	Brain heart infusion agar
BHIB	Brain heart infusion broth
BSA	Bovine serum albumin
˚C	Degrees Celsius
C	Cytosine
cfu	Colony forming unit(s)
CID	Collision-induced dissociation
CO_2	Carbon dioxide
CPS	Capsular polysaccharide
Da	Dalton(s)
dH_2O	Distilled water
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
emPAI	Exponentially modified protein abundance index
ESI	Electrospray ionisation
ET	Electrophoretic type

et al.	*et alios* (and others)
FCS	Foetal calf serum
FT	Fourier transform
× *g*	A unit of force equal to the force exerted by gravity
g	Gram(s)
G	Guanine
GRAVY	Grand average of hydropathicity
h	Hour(s)
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGT	Horizontal gene transfer
Ig	Immunoglubulin
IM	Inner membrane
IPTG	Isopropyl-ß-D-thiogalactopyranoside
K	Kelvin
kDa	Kilodalton(s)
kV	Kilovolt(s)
LB	Luria-bertani
LC	Liquid chromatography
LDAO	*N*,*N*-Dimethyldodecylamine *N*-oxide
Lol	Lipoprotein localisation machinery
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
M	Molar
m/*z*	Mass-to-charge
M199	Medium 199
MALDI	Matrix assisted laser desorption ionisation
MFP	Membrane fusion protein

µ	Microgram
mg	Milligram
$MgSO_4$	Magnesium sulphate
µl	Microlitre(s)
min	Minute(s)
ml	Millilitre(s)
mm	Millimetre(s)
MLEE	Multilocus enzyme electrophoresis
mM	Millimolar
MOWSE	Molecular weight search
MS	Mass spectrometry
NaCl	Sodium chloride
NCBI	National Centre for Biotechnology Information
nm	Nanometre(s)
NCS	Newborn calf serum
NPG	nitrophenyl-β-D-galactopyraniside
$OD_{X\ nm}$	Optical density at wavelength X nm
OM	Outer membrane
OMP	Outer membrane protein
PBP	Periplasmic binding protein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDB	Protein Data Bank
pH	A measure of the acidity of a solution
pmol	Picomole(s)
POTRA	Polypeptide transport-associated
Q/g	Quadrupole
r	Recombinant

RNA	Ribonucleic acid
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute
rRNA	Ribosomal RNA
RTX	Repeats-in-toxin
s	Second(s)
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SS	Sheep serum
T	Thymidine
TCA	Trichloroacetic acid
TFA	Trifluoroacetic acid
TOF	Time-of-flight
TPS	Two-partner secretion
Tris	2-amino-2-(hydroxymethyl)1,3-propandiol
TTBS	0.05% Tween 20 in TBS
UV	Ultraviolet
V	Volt(s)
v/v	Volume per volume
VIS	Visible
w/v	Weight per volume

1. INTRODUCTION

1.1 The organisms

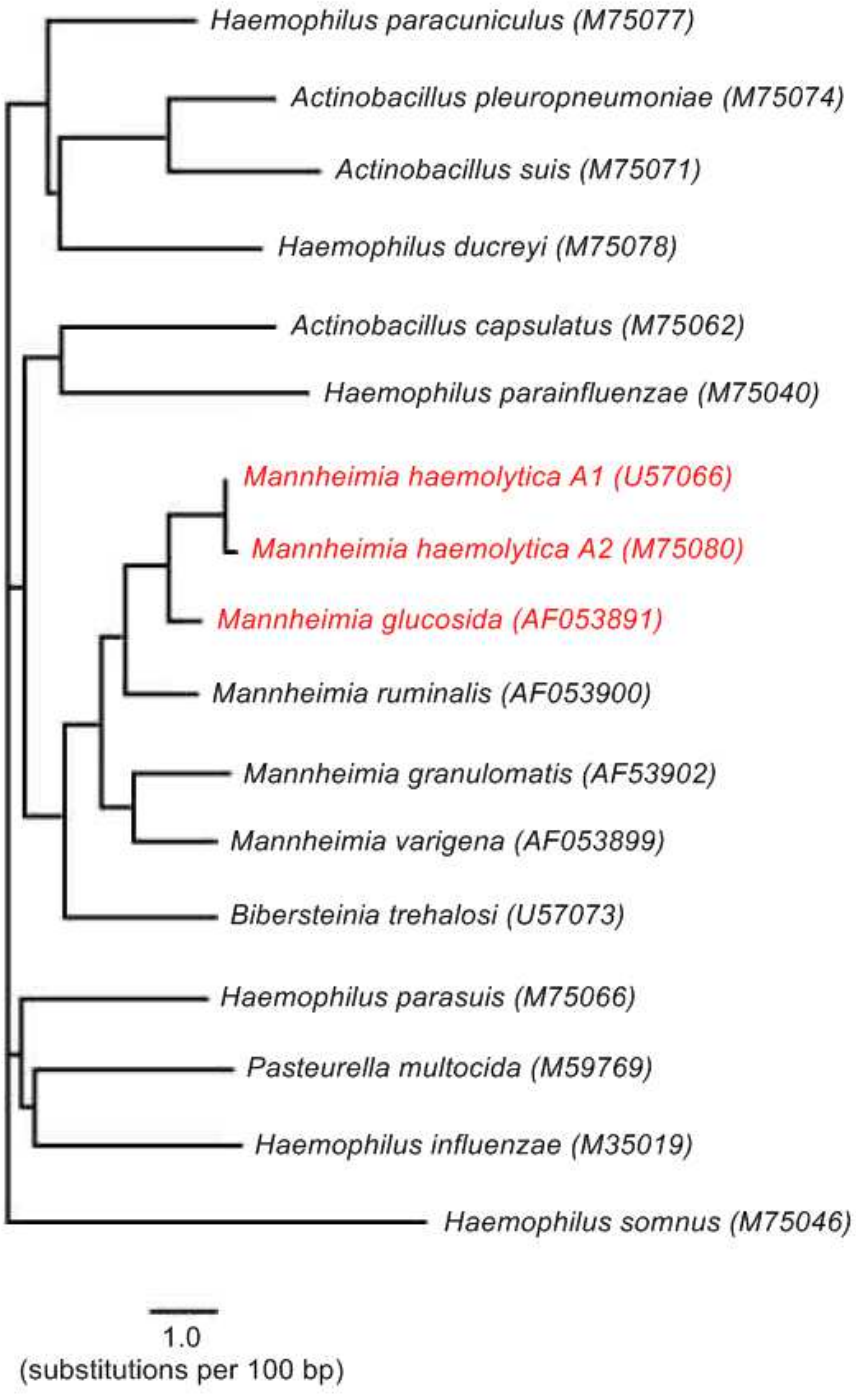
1.1.1 History and taxonomy of *M. haemolytica* and *M. glucosida*

The causative agent of a disease which caused pneumonic pasteurellosis in calves was originally described as *Bacterium bipolare multocidum* in 1885 (Kitt, 1885). It was later renamed *Bacillus boviseptica* in 1896, and then further subdivided into separate groups that cause either bovine fibrinous pneumonia (*Pasteurella boviseptica*) or haemorrhagic septicaemia (now *Pasteurella multocida*) (Jones, 1921). Newsom & Cross studied this group in detail and suggested the name *Pasteurella haemolytica* for the bacterium that causes pneumonia in calves (Newsome and Cross, 1932). This species was subdivided into biotypes A and T, according to their ability to ferment L-arabinose and trehalose, respectively (Smith, 1961, Smith, 1959). Capsular serotyping of *P. haemolytica* has established seventeen different serotypes to date (Biberstein *et al.*, 1960, Biberstein and Gills, 1962, Angen *et al.*, 1999a). *P. haemolytica* was later removed from the genus *Pasteurella* based on DNA-DNA hybridization (Mutters *et al.*, 1985). The trehalose fermenters were also later reclassified as a separate species, *Pasteurella trehalosi* (now *Bibersteinia trehalosi*), based on evidence from numerical taxonomic analyses and DNA-DNA hybridization (Sneath and Stevens, 1990, Biberstein and Francis, 1968). Twelve of the biotype A organisms (serotypes 1, 2, 5-9, 12-14, 16 and 17) have been reclassified as *Mannheimia haemolytica* and the A11 serotype has been renamed as a separate species, *Mannheimia glucosida* (Angen *et al.*, 1999a).

M. haemolytica is no longer a *Pasteurella sensu stricto*, but now occupies a place in the *Mannheimia* genus on a branch of a phylogenetic tree within the gamma subdivision of the Proteobacteria, whose closest relatives are *B. trehalosi*, *Actinobacillus capsulatus*, and *Haemophilus parainfluenzae* (Highlander, 2001) (Fig. 1.1).

Figure 1.1. Phylogenetic tree of members of the *Pasteurellaceae* produced from 16S rRNA sequences. GenBank Accession numbers for the sequences used are shown in parentheses.

Phylogenetic tree adapted from Highlander *et al*. (2001).



1.1.2 Morphological and biochemical characteristics of *M. haemolytica* and *M. glucosida*

1.1.2.1 Cell morphology

M. haemolytica and *M. glucosida* are small, non-motile, encapsulated Gram-negative coccobacillus exhibiting slight pleomorphism and occasional bipolar staining (Adlam, 1989). Fimbriae have been described on the surface of serotype A1 *M. haemolytica* isolates grown in culture (Potter *et al.*, 1988, Morck *et al.*, 1987) and isolated from infected cattle (Morck *et al.*, 1989), but have not been identified on other *M. haemolytica* serotypes or on *M. glucosida*.

1.1.2.2 Cultural morphology

Colonies of *M. haemolytica* and *M. glucosida* are smooth and greyish in colour and are 1-2 mm in diameter after 24 h growth on blood agar (Angen *et al.*, 1999a). Most strains of *M. haemolytica* and all strains of *M. glucosida* are haemolytic on bovine blood agar (Angen *et al.*, 1999a).

1.1.2.3 Biochemical characteristics

The biochemical characteristics of *M. haemolytica* and *M. glucosida* have been previously described (Angen *et al.*, 1999a, Mutters *et al.*, 1989) and are summarised in Table 1.1. The two species have common characteristics for haemolysis and the fermentation of trehalose, D-sorbitol, D-xylose, maltose, dextrin, indole and D-melibiose, but can be differentiated by the NPG test.

1.2 The diseases

1.2.1 Pneumonic pasteurellosis in cattle (shipping fever)

M. haemolytica is the principal etiological agent of bovine pneumonic pasteurellosis, a disease which is often referred to as shipping fever (also transit fever, bovine respiratory disease, dust pneumonia, bronchial pneumonia, fibrinous pleuropneumonia and bovine epizootic pneumonia). Various combinations of environmental stresses can contribute to the pathogenesis of the disease, including shipment, weaning, overcrowding and inclement weather (Singh *et al.*, 2011). Several viruses that are ubiquitous in the cattle population are also associated with the disease, including bovine parainfluenzae virus 3,

Table 1.1. Biochemical characteristics of *M. haemolytica* and *M. glucosida*.

Property	*M. haemolytica*[a]	*M. glucosida*[a]
Haemolysis	+	+
Orthinine decarboxylase	−	+/−
Trehalose	−	−
L-Arabinose	−	+/−
D-Sorbitol	+	+
D-Xylose	+	+
Maltose	+	+
Dextrin	+	+
Glucosides	−	+/−
Gentiobiose	−	+/−
NPG (β-glucosidase)	−	+
Meso-Inositol	+/−	+
ONPF (α-fucosidase)	+	+/−
ONPX (β-xylosidase)	+/−	+/−
ONPG (β-galactosidase)	+/−	+
Indole	−	−
D-Melibiose	−	−

[a]See references Angen *et al.* (1999a) and Mutters *et al*., 1989.
'+' = positive; '−' = negative; '+/−' = positive or negative

bovine respiratory syncytial virus, bovine herpesvirus 1, bovine viral diarrhoeae virus, *Mycoplasma* species, and other bacteria (Singh *et al.*, 2011). The processes by which environmental stresses or viral infections, either alone or in combination with each other, predispose cattle to shipping fever are not fully understood. In healthy cattle, *M. haemolytica* are carried in the nasopharynx and tonsils in low number and are not readily detected by nasal swab cultures (Frank, 1989). Under the context of immune suppression, caused by environmental stress or viral infection, specific serotypes of *M. haemolytica* rapidly multiply in the upper respiratory tract. Serotype A1 isolates are most commonly associated with cases of bovine disease (although serotype A6 isolates are responsible for an increasing proportion of disease cases), whereas serotype A2 isolates are frequently recovered from asymptomatic animals. The larger bacterial numbers result in aerosolised droplets containing bacteria accessing the trachea and lungs and causing pneumonia. Death can occur in as little as 2 to 3 days, or the infection can proliferate and cause chronic lung damage. The early clinical symptoms of the disease are fever, cough, depression, anorexia, dyspnoea, and nasal and eye discharge (Frank, 1989).

1.2.2 Pneumonic pasteurellosis in sheep

Pasteurellosis is one of the most common infectious bacterial diseases of sheep (Gilmour and Gilmour, 1989). *M. haemolytica* is responsible for causing pneumonia in all ages of sheep and septicaemia in young lambs (Gilmour and Gilmour, 1989). All *M. haemolytica* serotypes are recovered from sheep. In ovine pneumonic pasteurellosis disease cases, serotype A2 is most frequently recovered, followed by serotypes A7 and A9, whereas serotype A1 isolates are frequently recovered from asymptomatic animals. Similar to bovine disease cases, predisposing factors to ovine pneumonic pasteurellosis include environmental stresses and viral infection. *M. glucosida* is an opportunistic pathogen of sheep that is not normally associated with disease conditions and probably represents part of the resident microflora in the upper respiratory tract (Biberstein and Gills, 1962, Angen *et al.*, 1999a).

1.3 The Gram-negative cell envelope

The cell envelope of Gram-negative bacteria is comprised of two membranes, the inner membrane and the outer membrane, separated by the periplasm. The inner membrane is at the boundary between the cytoplasm and the periplasm, whereas the outer membrane is at the boundary between the periplasm from the external environment. Although these two membranes are both lipid bilayers which contain proteins, their structure and composition are dramatically different as a result of their different functions and the dissimilar environments that they contact (Ruiz *et al.*, 2006) (Fig 1.2).

1.3.1 Inner membrane

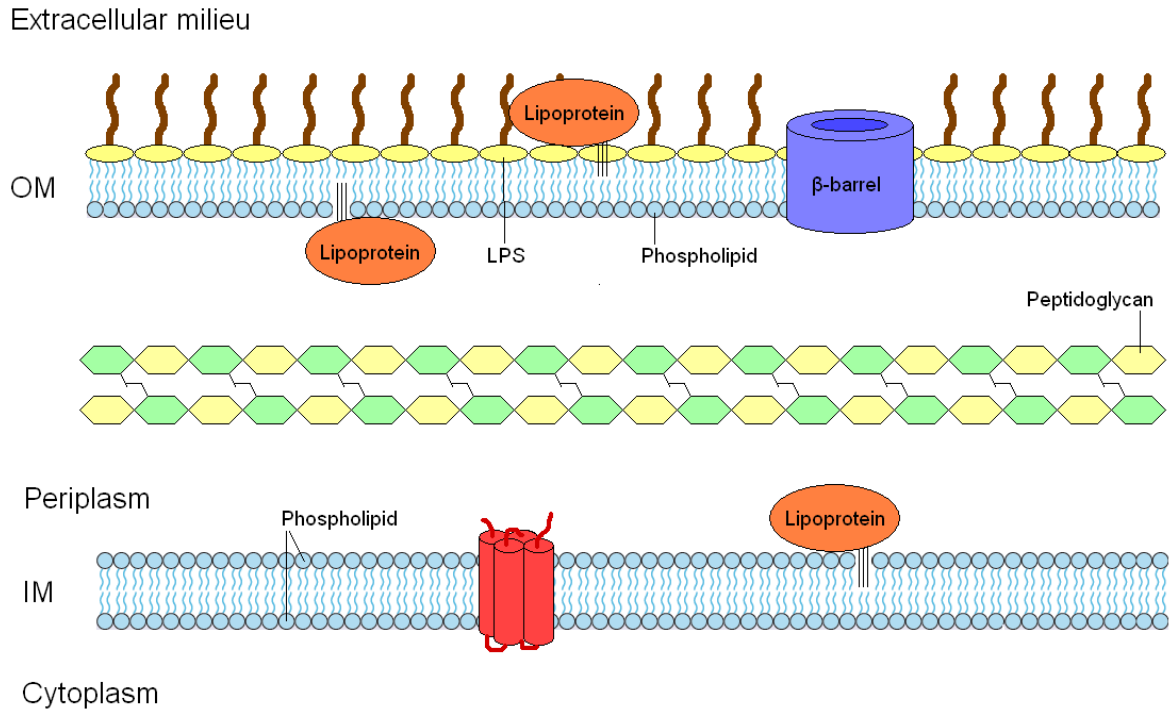
The inner membrane is composed of a symmetrical phospholipid bilayer interspersed with proteins. Although inner membrane phospholipid content varies between different species of bacteria, in *E. coli* 70-80% is phosphatidylethanolamine, 15-20% is phosphatidylglycerol and 5% or less is cardiolipin (Kanemasa *et al.*, 1967, Yamagami *et al.*, 1970). These proteins can be of two types, integral proteins and lipoproteins. Integral inner membrane proteins span the inner membrane with α-helical transmembrane domains, whereas inner membrane lipoproteins are anchored to the periplasmic side of the membrane by lipid modifications at the N-terminus of the prolipoprotein. Inner membrane proteins are involved in important cellular processes including energy production and conservation in the respiratory chain, signal transduction, cell division and transport across the membrane.

1.3.2 Periplasm

The periplasm is a highly viscous compartment that contains soluble proteins and a thin peptidoglycan layer. Proteins in this compartment are involved in processes such as protein folding, transport and degradation, outer membrane biogenesis, and sensing and responding to environmental stimuli. It is an oxidising environment and contains enzymes which catalyse disulphide bond formation, a crucial process for the folding and stability of proteins secreted through the cell envelope (Nakamoto and Bardwell, 2004). The peptidoglycan layer serves as an extracytoplasmic cytoskeleton that contributes to the rigidity of the cell envelope, maintains cell shape and prevents cell lysis.

Figure 1.2. Schematic diagram of the Gram-negative cell envelope.

The cell envelope of Gram-negative bacteria is comprised of an inner membrane (IM), the periplasm and the outer membrane (OM). The inner membrane is a symmetrical phospholipid bilayer containing integral membrane proteins that have α-helical transmembrane domains. The peptidoglycan separates the inner and outer membrane and contains a thin layer of peptidoglycan. The outer membrane is an asymmetrical lipid bilayer containing phospholipids on the inner leaflet and LPS on the outer leaflet. The outer membrane also contains integral proteins that mainly adopt ß-barrel conformations. Both membranes contain lipoproteins that anchored to the periplasmic side. The outer membrane also contains lipoproteins which are exposed on the outer leaflet. Figure adapted from Ruiz *et al*. (2006).



1.3.3 Outer membrane

In contrast to the inner membrane, the outer membrane is asymmetric, with the inner leaflet composed of phospholipids and the outer leaflet composed mainly of LPS. The phospholipid composition is slightly different to that of the inner membrane, as the outer membrane is enriched with saturated fatty acids and phosphatidylethanolamine (Lugtenberg and Peters, 1976, White *et al.*, 1972). The LPS functions as an effective permeability barrier due to its low fluidity and strong lateral interactions between molecules (Nikaido, 2003). Like the inner membrane, the outer membrane also contains both integral OMPs and lipoproteins; however, in contrast to the α-helical integral proteins of the inner membrane, integral OMPs span the outer membrane predominantly with amphipathic antiparallel ß-strands which adopt a ß-barrel conformation that allows many OMPs to serve as channels (Koebnik *et al.*, 2000, Schulz, 2002). Similar ß-barrel proteins are also found in the membranes of chloroplasts (Schleiff *et al.*, 2003) and mitochondria (Casadio *et al.*, 2002). Outer membrane lipoproteins can be anchored to either the periplasmic or extracellular face of the outer membrane (Tokuda and Matsuyama, 2004). About 50% of the outer membrane mass consists of integral OMPs and lipoproteins (Koebnik *et al.*, 2000).

1.3.3.1 Getting things in: OMPs involved in nutrient uptake

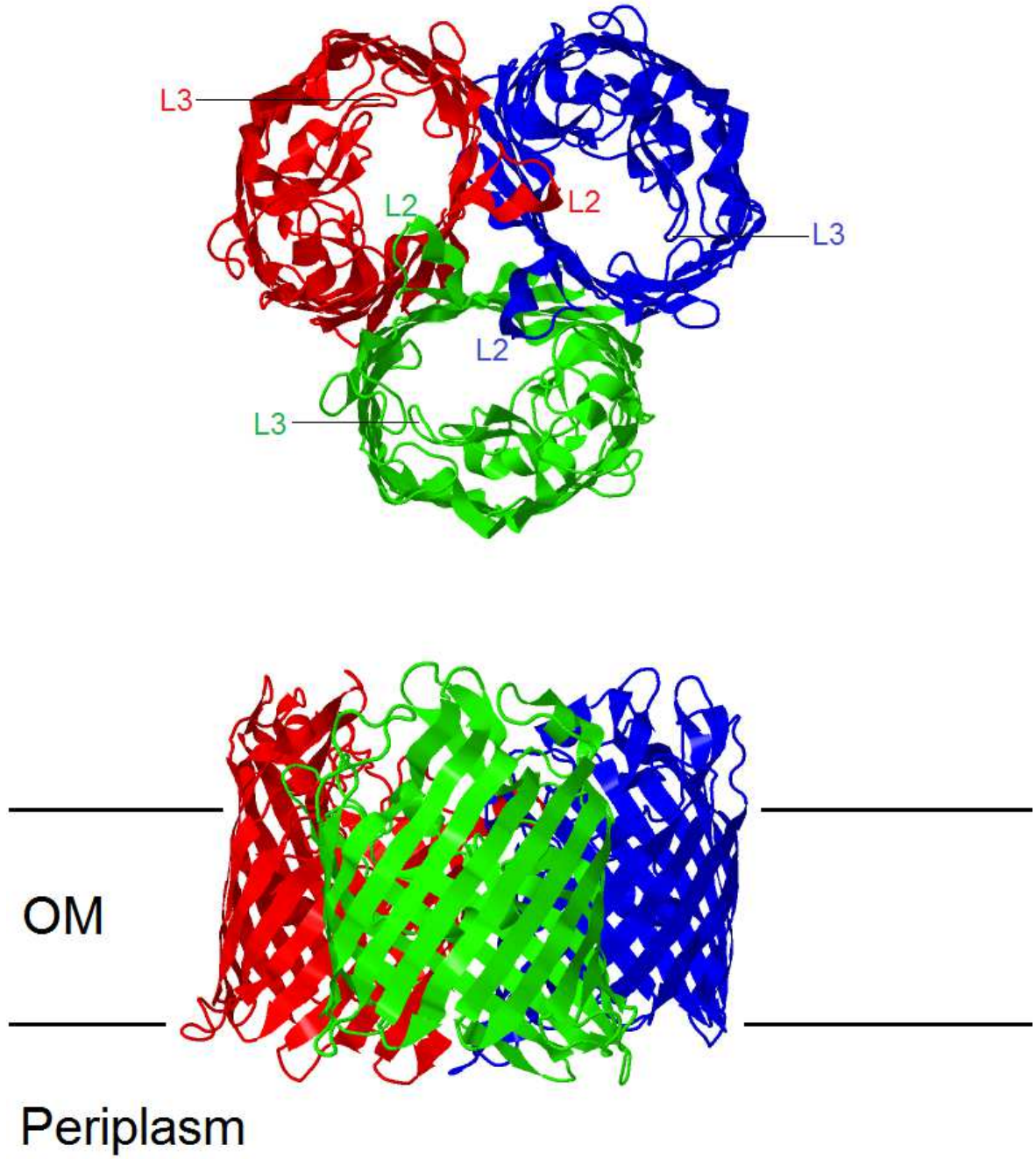
The outer membrane of Gram-negative bacteria is a very effective permeability barrier that also poses a challenge with respect to nutrient uptake. For this reason, Gram-negative bacteria possess a range of OMPs for either passive or active uptake of essential nutrients from the extracellular milieu.

1.3.3.1.1 General porins

The general porins form aqueous channels that allow diffusion of small hydrophilic molecules (< 600 Da) into the cell and show no particular substrate specificity (with the exception of preferences for cations or anions) (Nikaido, 2003). They are generally between 30 and 50 kDa in size (Nikaido, 2003). The first porin crystal structures were of the *Rhodobacter capsulatus* porin (Weiss *et al.*, 1991) and the *E. coli* general porins PhoE and OmpF (Cowan *et al.*, 1992) (Fig. 1.3). These revealed that the porins were present as homotrimers in the

Figure 1.3. The crystal structure of the *E. coli* OmpF porin homotrimer.

Extracellular loops 2 (L2) and 3 (L3) are indicated for each subunit monomer. OmpF structure was adapted from Cowan *et al*. (1992); PDB reference 10PF.



outer membrane and that each monomer was a ß-barrel containing 16 antiparallel ß-strands. The size of the eyelet region of OmpF was measured at 0.7 by 1.1 nm (Cowan *et al.*, 1992). In porins, the third extracellular loop (L3) folds back into the barrel, forming a constriction zone and contributing significantly to the permeability properties of the pore (i.e. exclusion limit and ion selectivity). The second extreacellular loop (L2) bends over the wall of the neighbouring barrel subunit, playing a significant role in trimer stabilisation (Nikaido, 2003). Porins are able to transition between open or closed states depending on the transmembrane potential across the outer membrane (Koebnik *et al.*, 2000).

1.3.3.1.2 Substrate-specific porins

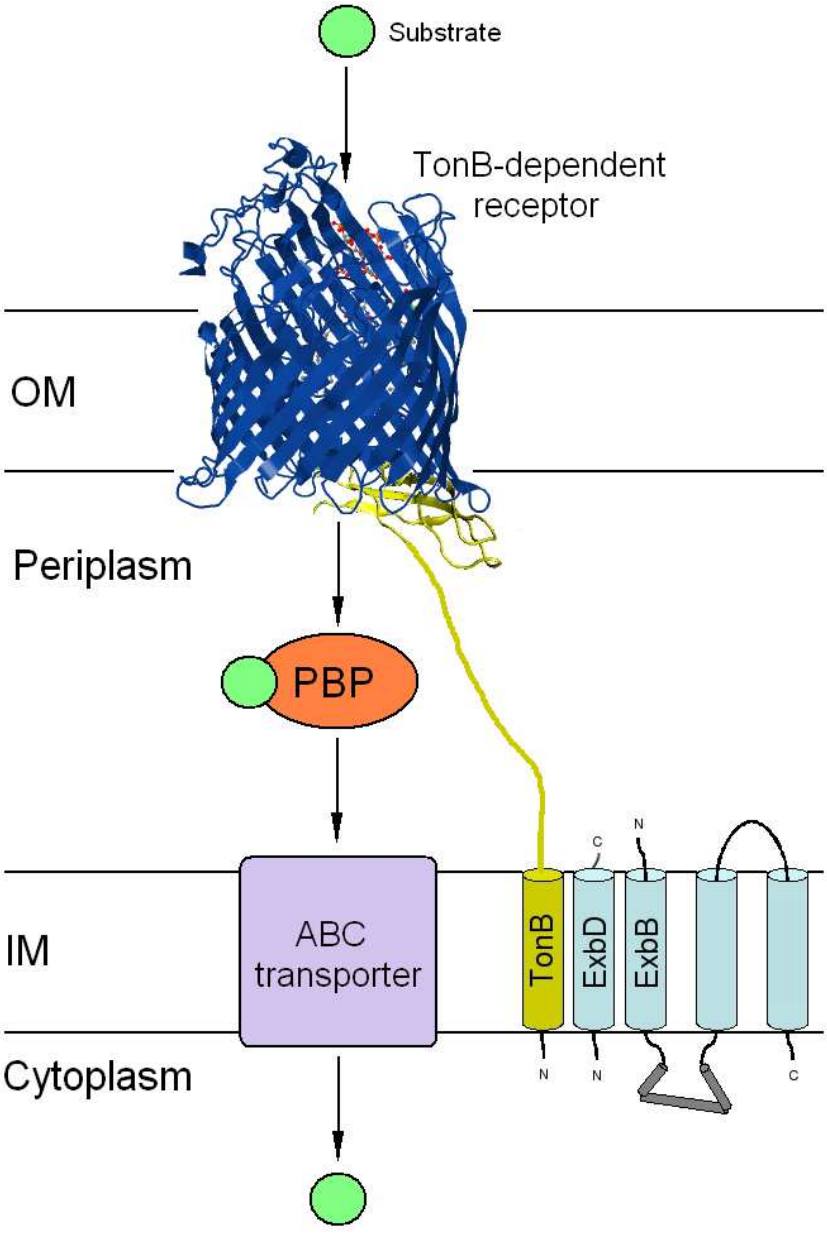
Besides the general diffusion porins described above, the outer membrane also contains porins that are substrate-specific. The best-studied example is the *E. coli* maltoporin LamB, a specific transporter of maltooligosaccharides of various sizes. Like the general porins, LamB is present as a homotrimer in the outer membrane and each monomer is a 47 kDa ß-barrel containing 18 antiparallel ß-strands (Schirmer *et al.*, 1995). The L1, L3 and L6 loops fold back inside the barrel, forming a channel (0.5 to 0.6 nm in diameter) that is more constricted than the general porins (Schirmer *et al.*, 1995). The length of the channel interior contains a row of aromatic amino acid residues (greasy slide) that are lined up by polar residues (polar track), creating a maltooligosaccharide-specific translocation pathway that facilitates passage through the channel (Dutzler *et al.*, 1996). Similar structural characteristics are also seen in the *Salmonella typhimurium* sucrose-specific porin ScrY (Forst *et al.*, 1998).

1.3.3.1.3 TonB-dependent receptors

TonB-dependent receptors bind and actively transport specific substrates across the outer membrane which are either poorly permeable through porins (> 600 Da) or are present at very low concentrations and require energised transport for their translocation. These substrates include host iron-binding proteins, siderophores, haemophores, vitamin B12, nickel chelates and carbohydrates (Schauer *et al.*, 2008). TonB-dependent receptors contain a C-terminal integral membrane ß-barrel comprised of 22 antiparallel ß-strands (Ferguson et al., 1998, Locher et al., 1998, Buchanan et al., 1999) (Fig. 1.4) that form a pore

Figure 1.4. Schematic diagram of TonB-dependent receptor transport.

A substrate binds to the TonB-dependent receptor with high-affinity and is transported across the outer membrane. In the periplasm, the substrate binds to a periplasmic-binding protein (PBP) that transports it across the periplasm to the inner membrane. The substrate is then transported across the inner membrane into the cytoplasm by an ATP-binding cassette (ABC) transporter. TonB-dependent receptor structure was adapted from Ferguson *et al.* (1998); PDB reference 2FCP.



which is larger (3.5 by 4.7 nm) than that of the porins. The N-terminus forms a plug domain which occludes the pore and prohibits the passage of substrates. Substrate binding occurs at sites on the extracellular side of the plug domain and on the walls and extracellular loops of the ß-barrel. Transport of the bound substrate through the ß-barrel is an energy-dependent process; however, the outer membrane is not capable of producing energy due to the absence of either ATP-hydrolysing enzymes or a proton gradient. The required energy is obtained from the proton motive force of the inner membrane and transduced to the outer membrane via three inner membrane proteins: TonB, ExbB and ExbD. TonB is anchored to the inner membrane by the ExbB-ExbD transmembrane complex and interacts with an eight amino acid sequence in the receptor plug domain called the 'TonB box'. The exact mechanism of energy transduction is not understood. However, it is thought that upon substrate binding and subsequent energy transduction conformational changes in TonB result in a conformational change in the plug domain that allows transport of the substrate through the receptor. Once in the periplasm, the substrate is transported to the inner membrane by a periplasmic binding protein. At the inner membrane, the substrate is transported into the cytoplasm by an ATP-binding cassette (ABC) protein.

1.3.3.2 Getting things out: OMPs involved in secretion

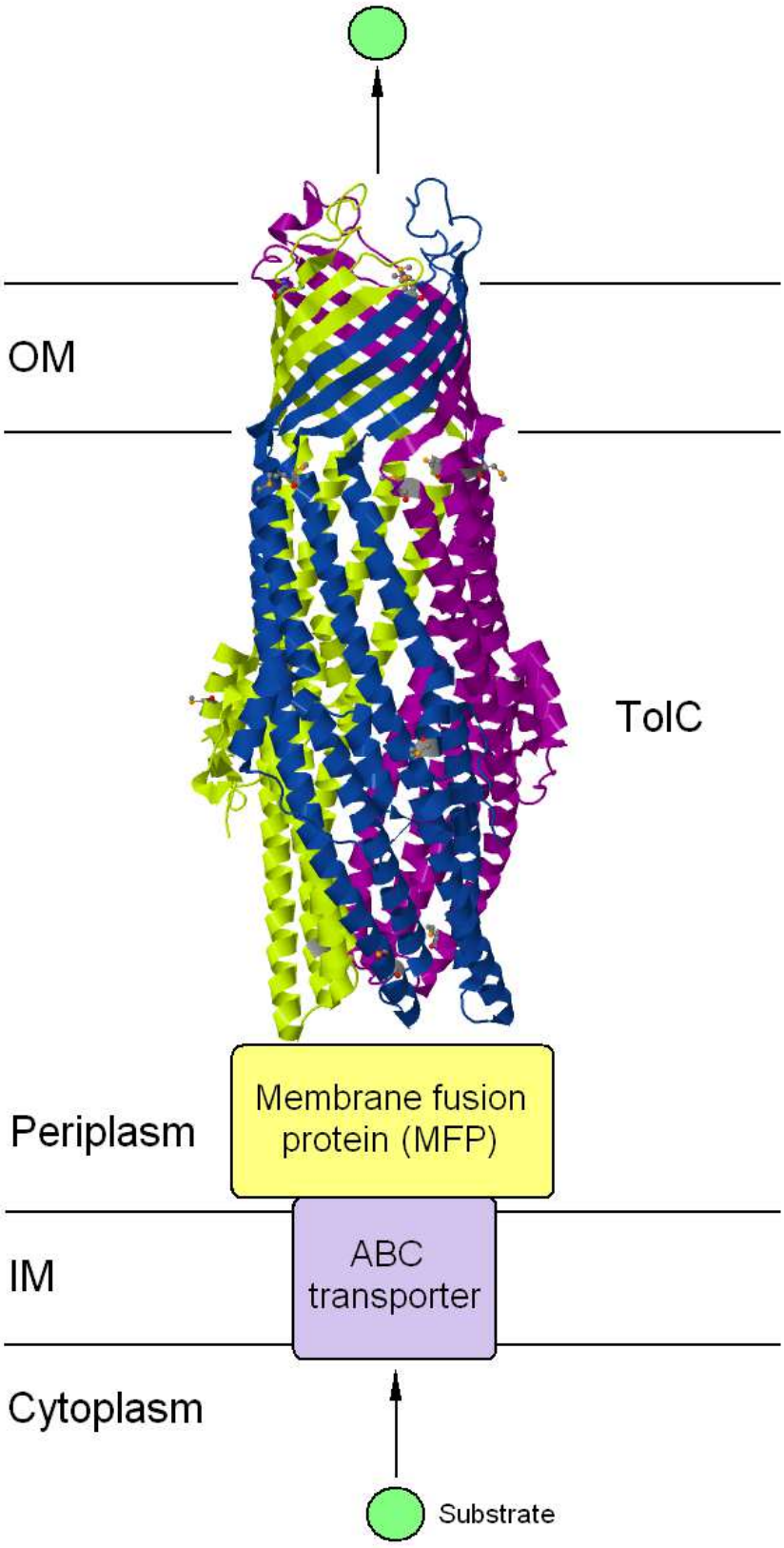
Gram-negative bacteria possess a wide array of secretion systems for exporting proteins and other substances out through the cell envelope. A range of different OMPs are utilised in these systems which act in conjunction with other proteins in the inner membrane and periplasm.

1.3.3.2.1 Type I secretion (TolC export channel)

Type I secretion systems consist of three proteins: a pore-forming OMP (TolC) and two inner membrane proteins, a membrane fusion protein (MFP) and an ABC transporter protein (Fig. 1.5). The ABC transporter recognises a specific secretion signal motif at the C-terminal end of the protein which is to be secreted. Subsequent binding of the MFP protein to TolC allows secretion of the protein into the extracellular milieu. Hence, secretion occurs across the inner and outer membrane in one continuous step, without the formation of periplasmic intermediates. TolC is an outer membrane channel comprised of

Figure 1.5. Schematic diagram of the type 1 secretion pathway, including the crystal structure of the TolC protein.

TolC structure adapted from Koronakis *et al*. (2000); PDB reference 1EK9.



three TolC monomers that each contribute four ß-strands to the formation of a ß-barrel containing 12 antiparallel ß-strands (Koronakis *et al.*, 2000). The channel also possesses an α-helical barrel that extends from the integral outer membrane ß-barrel into the periplasm (Koronakis *et al.*, 2000). Efflux of antibacterial drugs and other small inhibitory molecules may also occur via an interaction between TolC, an MFP, and an inner membrane translocase which can be either an ABC protein, or a proton antiporter of either the resistance nodulation division or major facilitator superfamily classes (Koronakis *et al.*, 2004).

The *E. coli* alpha-haemolysin (HlyA) was the first RTX toxin described to be secreted via a type I secretion system (Gentschev *et al.*, 2002); however, another well characterised example is the secretion pathway of *M. haemolytica* LktA (described in 1.4.1.1).

1.3.3.2.2 Type II and type III secretion *(secretin channels)*

Outer membrane proteins of the secretin family are present in type II (Brok *et al.*, 1999, Chami *et al.*, 2005) and type III (Marlovits *et al.*, 2004, Burghout *et al.*, 2004, Hodgkinson *et al.*, 2009) secretion systems. They are also present in type IV pilus biogenesis systems (Martin *et al.*, 1993, Collins *et al.*, 2001) and filamentous phage secretion systems (Opalka *et al.*, 2003). Secretins typically form highly stable oligomers of 12-14 subunits in the outer membrane.

In type II secretion, secreted proteins are first translocated across the inner membrane by either the Sec (Pugsley *et al.*, 1991) or Tat (Voulhoux *et al.*, 2001) translocons. They are subsequently transported out of the cell by a complex of 12-15 proteins which span the cell envelope (Pugsley, 1993, Sandkvist, 2001). The prototypical type II outer membrane secretin is the PulD protein of *Klebsiella oxytoca* which is required for the secretion of pullulanase (Chami *et al.*, 2005).

Type III secretion systems are used by many pathogenic bacteria to directly inject effector proteins into eukaryotic cells and are hence often referred to as ‘injectisomes’. At least 20 different proteins are utilised in this system (Worrall *et al.*, 2011) of which several are genetically and structurally related to the bacterial flagellum (Blocker *et al.*, 2003). Proteins enter the injectisome directly

from the cytoplasm in a process that is Sec- and Tat- independent. The prototypical type III outer membrane secretin is the YscC protein of pathogenic *Yersinia* species (Burghout *et al.*, 2004). A putative type III secretion system has been identified in the sequenced bovine and ovine serotype A2 *M. haemolytica* genomes, but not the bovine serotype A1 *M. haemolytica* genome, that shows low to moderate homology to type III secretion components of E. coli O157 (Lawrence *et al.*, 2010b). Unlike typical type III secretion systems, the M. haemolytica genes encoding this putative system are not arranged in an operon or separate plasmid. The presence of this system may represent a new method of bacterial effector protein delivery into host cells for *M. haemolytica*.

1.3.3.2.3 Type IV secretion (double membrane spanning channel)

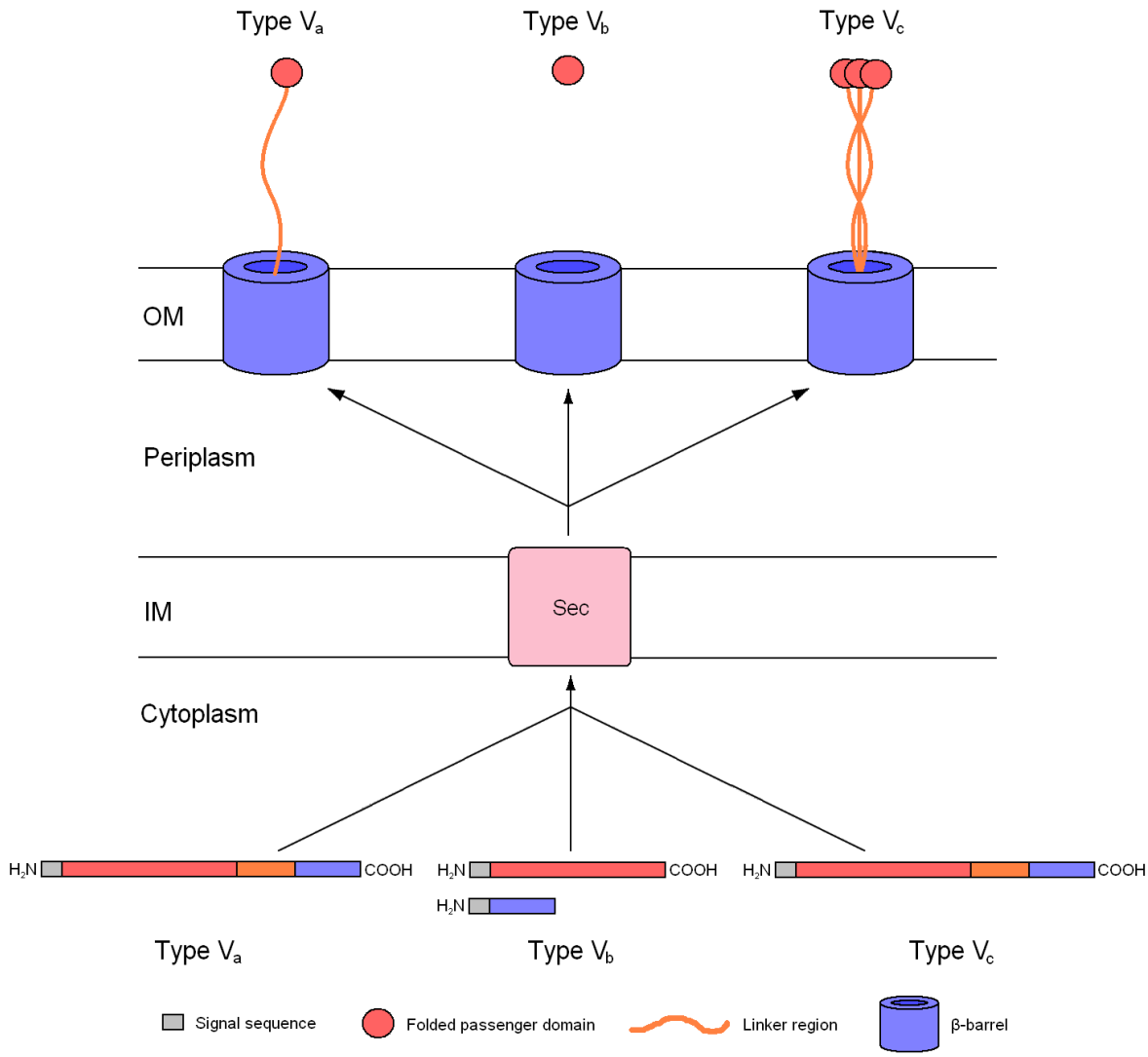
The type IV secretion system is used by a variety of Gram-negative bacteria (and Gram-positive bacteria) to mediate the transfer of effector proteins into host cells or for the conjugation of plasmid DNA and transposons into other cells (Christie *et al.*, 2005). In *Agrobacterium tumefaciens*, the core complex of the type IV secretion system is composed of three proteins, VirB7, VirB9 and VirB10, that are each present in 14 copies and form a 1.1-megadalton two chambered structure that spans both the inner and outer membrane (Chandran *et al.*, 2009, Fronzes *et al.*, 2009). The outer membrane chamber is the largest secretion channel to be found associated with the outer membrane to date (Chandran *et al.*, 2009). It also contains a hydrophobic double α-helical transmembrane domain that is the only other example, alongside Wza, of α-helical insertions in the outer membrane (Chandran *et al.*, 2009).

1.3.3.2.4 Type V secretion (autotransporter pathway)

Gram-negative bacteria possess a range of OMPs that contain the apparatus for the transport of their secreted domains across the outer membrane and are hence names autotransporters. These proteins are members of the type V secretion pathway and can be grouped into three distinct groups (Fig. 1.6) which reflect similarities in their primary structures and modes of biogenesis: classical autotransporters (type V_a secretion), two-partner secretion systems (type V_b

Figure 1.6. Schematic diagram of the different groups of type V (autotransporter) secretion systems.

Figure was adapted from Henderson *et al*. (2004).



secretion), and trimeric autotransporters (type V_c secretion) (Henderson *et al.*, 2004).

Classical autotransporters (type V_a). The primary structure of classical autotransporters contains three distinct domains: the N-terminal signal sequence, the passenger domain and the C-terminal translocation unit (Henderson *et al.*, 2004). The signal sequence targets the protein to the inner membrane for Sec-dependent translocation into the periplasm. The passenger domain contains the effector function of the protein. The translocation unit consists of a short α-helical linker region and a ß-core that forms a ß-barrel with 12 antiparallel ß-strands after insertion into the outer membrane (Oomen *et al.*, 2004, Barnard *et al.*, 2007, van den Berg, 2010, Zhai *et al.*, 2011), which facilitates translocation of the passenger domain from the periplasm to the bacterial surface. Once exposed on the bacterial surface, the passenger domain can either remain intact as a large protein extending into the extracellular milieu or it can be proteolytically cleaved. Cleavage results in the passenger domain being either released into the extracellular milieu or remaining non-covalently associated with the ß-barrel domain. Proteolytic cleavage can occur by either autoproteolysis or by another membrane-bound protease. Examples of *M. haemolytica* proteins with homology to classical autotransporters are Ssa, NanH and Iga1-like proteases (described in 1.4.3.4, 1.4.3.5 and 1.4.3.6, respectively)

Two-partner secretion (type V_b). Whereas in the autotransporter pathway the secreted protein is translated as a single polypeptide, in the two-partner secretion (TPS) pathway the passenger domain and the pore-forming ß-barrel of the secreted protein are translated as two separate proteins, termed TpsA and TpsB proteins, respectively (Henderson *et al.*, 2004). The genes encoding these proteins are generally arranged in a *tpsA-tpsB* operon. The TpsA protein contains an N-terminal signal sequence which targets the protein for Sec-dependent transport into the periplasm. It also contains an N-proximal TPS domain which interacts specifically with the TpsB protein prior to translocation across the outer membrane. The crystal structure of one TpsB protein, FhaC of *Bordetella pertussis*, has been solved and contain 16 ß-strands (Clantin *et al.*, 2007). They may also be involved in the processing of the TpsA protein into its active form.

The prototypical TPS system is that of the *B. pertussis* filamentous haemagglutinin protein, FhaB, which is secreted through the pore forming ß-barrel FhaC (Locht *et al.*, 1992, Willems *et al.*, 1994). It has been reported that another *B. pertussis* autotransporter, SphB1, is involved in the surface maturation of FhaB (Coutte *et al.*, 2001). Genes encoding proteins that are orthologous to the FhaB and FhaC proteins of *B. pertussis* are present in *M. haemolytica* genomes (Gioia *et al.*, 2006, Lawrence *et al.*, 2010a) (described in 1.4.1.3).

Trimeric autotransporters (type V_c). Trimeric autotransporters contains six distinct domains: (i) an N-terminal signal sequence, (ii) head, (iii) neck, (iv) stalk, (v) linker region, and (vi) a C-terminal region consisting of four ß-strands (Hoiczyk *et al.*, 2000, Henderson *et al.*, 2004). The head, neck and stalk comprise the passenger domain and the linker and C-terminal region comprise the translocator domain. Trimerisation of C-terminal subunits creates a ß-barrel with 12 ß-strands that confers an overall lollipop-like structure to the protein displayed on the cell surface (Hoiczyk *et al.*, 2000). The prototypical trimeric autotransporter is the YadA protein of *Yersinia pestis* (Nummelin *et al.*, 2004). Several trimeric autotransporters have been described in *M. haemolytica* (described in 1.4.3.7).

1.3.3.3 OMPs involved in outer membrane biogenesis and maintenance

In addition to controlling the passage of substances into and out of the cell, several OMPs are also involved in the biogenesis and maintenance of the outer membrane.

1.3.3.3.1 The BAM complex (integral OMP assembly)

Nascent integral OMPs are synthesised in the cytoplasm and possess an N-terminal extension, the signal peptide, which is required to deliver the preproteins to the Sec translocon located in the inner membrane. Translocation of the nascent OMP across the Sec apparatus is dependent on the proton motive force (Driessen and Nouwen, 2008). Upon entering the periplasm, the signal peptide is removed by a signal peptidase enzyme and the nascent OMP associates with one or more periplasmic chaperones (Ruiz *et al.*, 2006). In *E. coli*, the periplasmic chaperones DegP, SurA and Skp mainly facilitate delivery to

the outer membrane (Knowles *et al.*, 2009). It has been proposed that SurA is primarily responsible for the assembly of most nascent OMPs, whereas Skp And DegP rescue those that have missed the normal assembly route (Sklar *et al.*, 2007). Unlike the actively driven Sec translocon, the outer membrane contains the passive ß-barrel assembly machinery (BAM) complex (Fig. 1.7). The BAM complex is required for the correct folding and insertion into the outer membrane of nearly all integral OMPs identified to date. In *E. coli*, the BAM complex is comprised of five proteins: BamA (formerly known as YaeT, or Omp85/D15 in *N. meningitidis*), an integral OMP, and four accessory lipoproteins, BamB (YfgL), BamC (NlpB), BamD (YfiO) and BamE (SmpA), which are localised to the inner leaflet of the outer membrane. BamA is universally present in all Gram-negative bacteria and contains two major domains: a set of five POTRA (polypeptide transport-associated) domains which extend into the periplasm and an integral ß-barrel inserted into the outer membrane (Sanchez-Pulido *et al.*, 2003). It is thought that the POTRA domains have a role in binding unfolded OMPs (Sanchez-Pulido *et al.*, 2003, Robert *et al.*, 2006). The BamA protein recognises a specific C-terminal motif in unfolded OMPs that is required for efficient assembly into the outer membrane *in vivo* (Robert *et al.*, 2006, Struyve *et al.*, 1991). The exact functions of the accessory outer membrane lipoproteins are unknown, but they have been demonstrated to have roles in OMP biogenesis as their depletion results in OMP assembly defects (Werner and Misra, 2005, Wu *et al.*, 2005, Doerrler and Raetz, 2005, Malinverni *et al.*, 2006).

1.3.3.3.2 The LolABCDE complex (lipoprotein assembly)

Prelipoproteins are synthesised in the cytoplasm and possess an N-terminal signal peptide containing a consensus lipobox sequence close to the signal peptide cleavage site (Hayashi and Wu, 1990, Inouye *et al.*, 1977). The prelipoprotein is then translocated to the periplasmic face of the inner membrane by the Sec translocon and processed into its mature form. This is achieved by the attachment of a diacylglycerol moiety to the sidechain of a conserved cysteine residue in the lipobox (Sankaran and Wu, 1994). Mature lipoproteins are localised to the periplasmic face of the inner or outer membrane by the lipoprotein localisation (Lol) machinery, which consists of an inner membrane transmembrane protein complex (LolCDE), a periplasmic chaperone (LolA) and an outer membrane lipoprotein receptor (LolB) (Fig 1.8)

Figure 1.7. Schematic diagram of integral OMP biogenesis by the BAM complex in *E. coli*.

Nascent unfolded OMPs are translocated across the inner membrane by the Sec translocon and delivered to outer membrane BAM complex by either SurA or Skp/DegP chaperones. The BAM complex inserts the protein into the outer membrane and folds it into its ß-barrel conformation. Figure was adapted from Knowles *et al*. (2009).

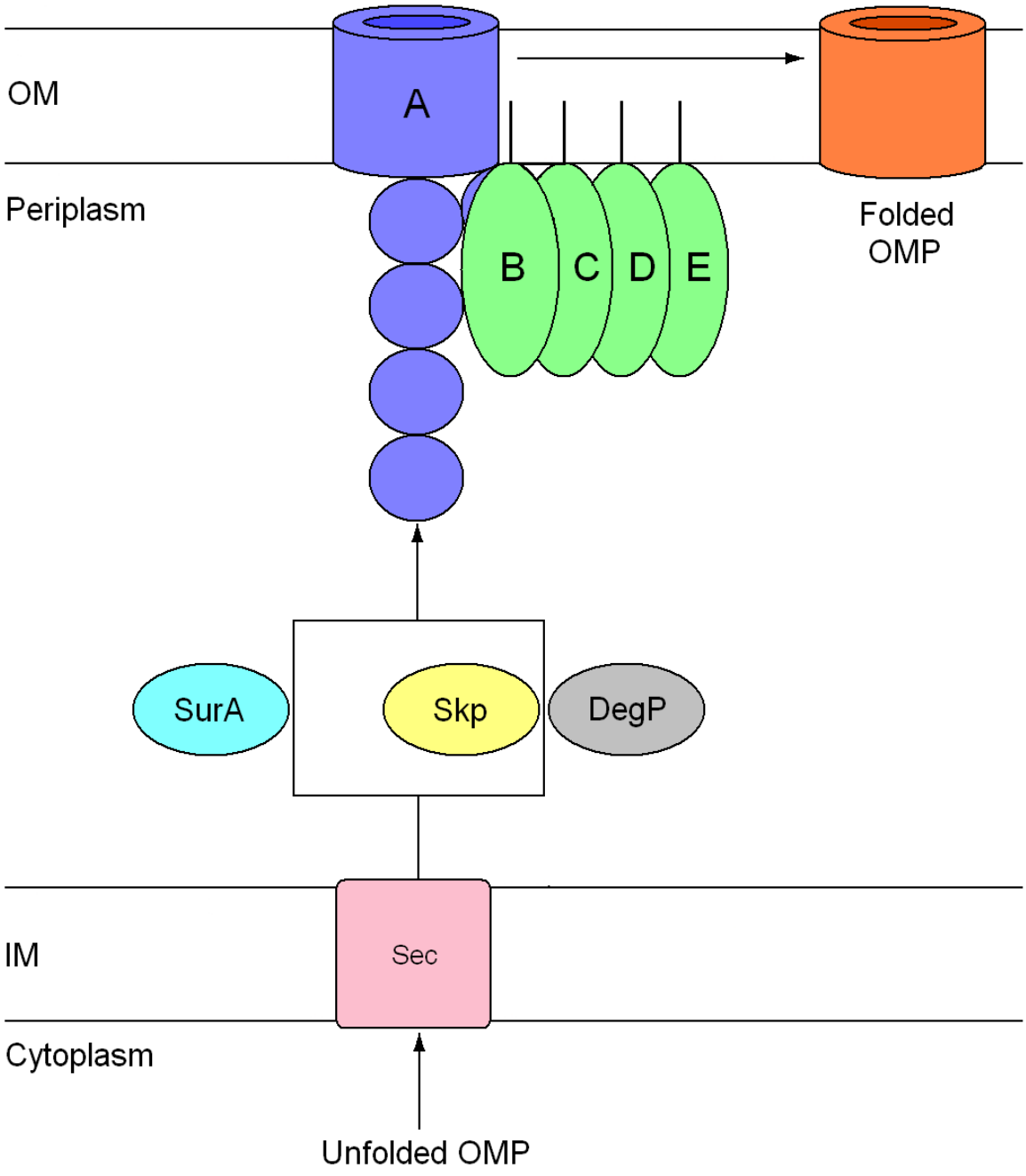
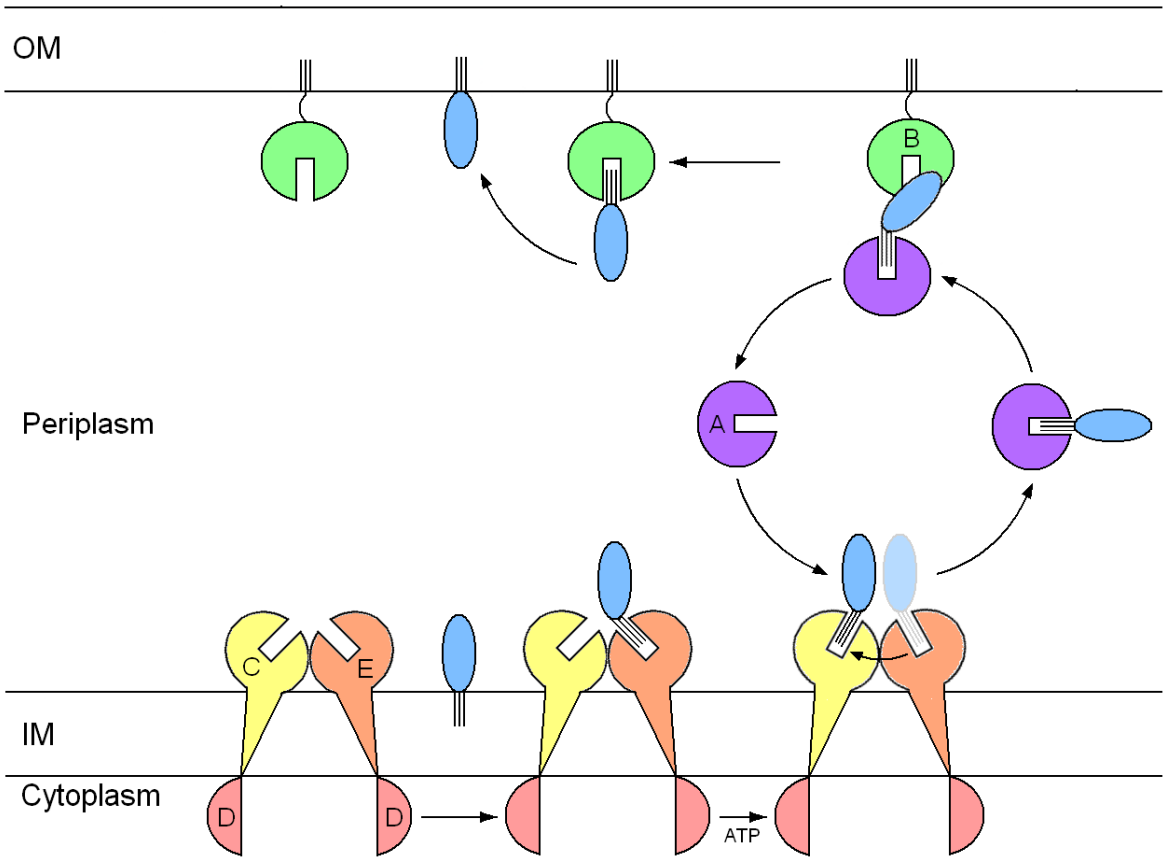


Figure 1.8. Schematic diagram of outer membrane lipoprotein biogenesis by the Lol machinery in *E. coli*.

A lipoprotein destined for the outer membrane binds to LolE and is then subsequently transferred to LolC. The lipoprotein is accepted by LolA in an ATP-dependent manner. The LolA-lipoprotein complex traverses the periplasm and reaches the outer membrane. The lipoprotein is transferred from LolA to LolB and is subsequently inserted into the outer membrane. Figure adapted from Okuda and Tokuda (2009).



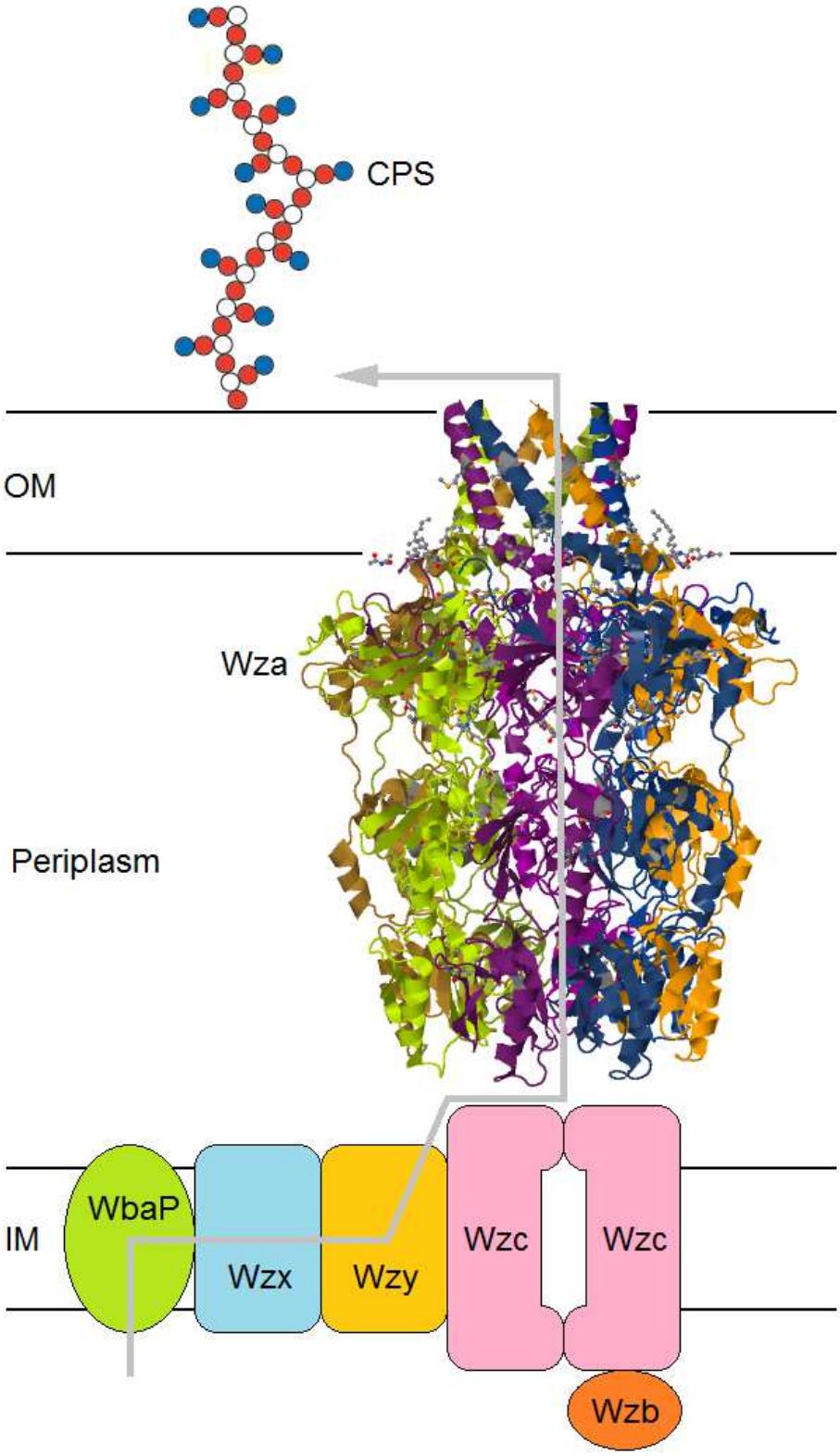
(Tokuda, 2009, Okuda and Tokuda, 2009). Lipoproteins which are targeted to the outer membrane are received by the LolCDE complex and subsequently released to LolA. The LolA-lipoprotein complex traverses the periplasm, whereby the lipoprotein is accepted by LolB and incorporated into the outer membrane. The determining factor which is responsible for a lipoprotein being directed to the outer membrane by the Lol apparatus or retained at the inner membrane was initially believed to be the identity of the amino acid adjacent to the conserved N-terminal cysteine residue, known as the +2 rule (Yamaguchi *et al.*, 1988). An aspartate residue at this position acts as a LolCDE avoidance signal, leading to retention of the lipoprotein at the inner membrane. Substitution of this aspartate residue with a different amino acid results in Lol-mediated translocation to the outer membrane. The +2 rule is not universal (Seydel *et al.*, 1999), and amino acid residues at positions +3 and +4 have been demonstrated to be involved in targeting lipoproteins to the outer membrane in other Gram-negative species (Narita and Tokuda, 2007, Silva-Herzog *et al.*, 2008). Lipoproteins are also present on the outer leaflet of the outer membrane in several Gram-negative bacteria, although the mechanism by which they are translocated through the membrane remains unknown (Kovacs-Simon *et al.*, 2011).

1.3.3.3.3 Wza (CPS transport)

The *E. coli*, repeat polysaccharide polymer units are assembled in the cytoplasm by a reaction initiated by WbaP, and are subsequently flipped across the inner membrane by Wzx (Collins and Derrick, 2007). Polymerisation occurs at the periplasm and is dependent on the integral membrane protein Wzy, a putative polymerase (Collins and Derrick, 2007). Polymerisation by Wzy requires the activity of the tetrameric Wzc protein. Wzb is a protein tyrosine phosphate enzyme that enables the cycling phosphorylation of Wzc, a process that is crucial for export (Collins and Derrick, 2007). Wzc interacts with Wza to form a complex that spans the periplasm. Wza is an the outer membrane lipoprotein that forms ring-shaped multimeric complexes (Drummelsmith and Whitfield, 2000). The crystal structure of Wza revealed an octomer containing a unique α-helical transmembrane barrel structure (Dong *et al.*, 2006) (Fig. 1.9), representing a new structural paradigm for OMPs (Collins and Derrick, 2007).

Figure 1.9. Synthesis and export of CPS export in *E.* coli, showing the crystal structure of Wza octomer.

Figure adapted from Dong *et al*. (2006). See text for details. Wza structure adapted from Dong *et al*. (2006). PDB ref. 2J58.



The capsule biosynthetic locus of serotype A1 *M. haemolytica* has been characterised and comprises twelve genes that are grouped into three regions based on their functions: capsule transport, capsule biosynthesis and phospholipid substitution (Lo *et al.*, 2001).

1.3.3.3.4 LptD and LptE (LPS transport)

Biosynthesis of core-lipid A occurs at the inner leaflet of the inner membrane (Osborn *et al.*, 1972) and is flipped across the inner membrane by the ABC transporter MsbA (Doerrler *et al.*, 2001, Doerrler and Raetz, 2002). O-antigen repeat units are synthesised in the cytoplasm, flipped across to the periplasm, and ligated to core-lipid A to form mature LPS. Transport of mature LPS to the outer membrane requires the LPS transport (Lpt) machinery, a protein complex comprised of seven different proteins which spans the entire cell envelope (Sperandeo *et al.*, 2009) (Fig. 1.10). The integral inner membrane proteins LptG and LptF, along with the cytoplasmic ABC protein LptB, most likely provide energy to the complex through ATP hydrolysis (Sperandeo *et al.*, 2009). The integral protein LptD and the outer lipoprotein LptE form a complex in the outer membrane (Wu *et al.*, 2006). It has recently been shown in *E. coli* that LptE is required at the outer membrane for the correct insertion of LptD (Chimalakonda *et al.*, 2011). LptA, a periplasmic protein with a fibrillar structure (Suits *et al.*, 2008), is expected to connect the inner membrane to and the LptD/LptE outer membrane components, and to chaperone LPS across the periplasm (Sperandeo *et al.*, 2009). The function of LptC is currently unknown, although it is thought to be a docking site to the inner membrane for LptA (Sperandeo *et al.*, 2009).

1.4 Virulence factors of *M. haemolytica*

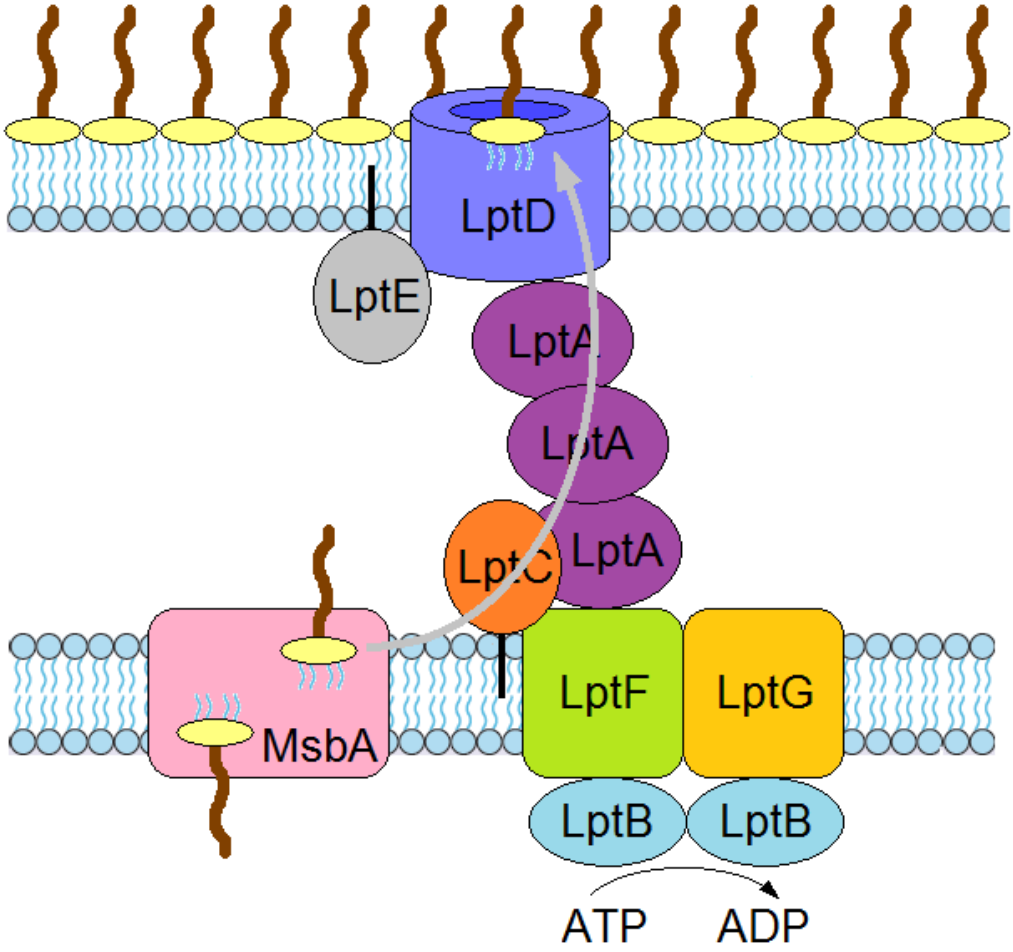
1.4.1 Secreted proteins

1.4.1.1 Leukotoxin (LktA)

The *M. haemolytica* LktA protein is a member of the RTX (repeats-in-toxin) pore-forming cytotoxins (Linhartova *et al.*, 2010) that includes the *Escherichia coli* alpha-haemolysin (Strathdee and Lo, 1987) and toxins of other *Pasteurellaceae* (Kraig *et al.*, 1990, Frey *et al.*, 1993).

Figure 1.10 Transport of LPS from the inner membrane to the outer membrane via MsbA and the Lpt transport system

Figure adapted from Sperandeo *et al.* (2009). See text for details.



LktA is produced by all *M. haemolytica* serotypes and in nearly all isolates examined to date (Burrows *et al.*, 1993, Saadati *et al.*, 1997). LktA is species-specific and has leukotoxic activity only against ruminant lymphocytes (Shewen and Wilkie, 1982, Kaehler *et al.*, 1980, Clinkenbeard and Upton, 1991). It also has haemolytic activity (Murphy *et al.*, 1995). Cytolysis is dependent on the interaction of LktA with the lymphocyte-function associated antigen 1 (LFA-1) molecule of target cells (Li *et al.*, 1999, Jeyaseelan *et al.*, 2000). At high concentrations, LktA creates pores in the cell membrane of target cells that results in swelling and lysis (Clinkenbeard *et al.*, 1989). At sub-lytic concentrations, LktA activates neutrophils (Czuprynski *et al.*, 1991), induces release of proinflammatory cytokines (Yoo *et al.*, 1995b) and respiratory burst products (Maheswaran *et al.*, 1992), and causes cytoskeletal changes resulting in apoptosis (Stevens and Czuprynski, 1996, Sun *et al.*, 1999). LktA has also recently been demonstrated to target leukocyte mitochondria (Kisiela *et al.*, 2010). LktA is a significant *M. haemolytica* virulence factor and is considered to be largely responsible for widespread tissue damage caused during infection (Highlander, 2001).

LktA is encoded in the *lktCABD* operon, where *lktA* encodes the inactive prototoxin, *lktC* is required for posttranslational activation of the prototoxin prior to secretion, *lktB* encodes an inner membrane ABC transporter, and *lktD* encodes a membrane fusion protein. Like other RTX proteins, LktA is secreted via a type I secretion mechanism directly from the cytoplasm into the extracellular space through a continuous channel created by LktB, LktD and TolC (Linhartova *et al.*, 2010). The *lktCABD* operon has a highly complex mosaic structure resulting from extensive inter- and intra-species horizontal DNA transfer and assortative recombination (Davies *et al.*, 2002, Davies *et al.*, 2001).

1.4.1.2 Glycoprotease (Gcp)

The glycoprotease protein, Gcp, is a zinc metalloglycoprotease that cleaves O-sialoglycoproteins from host cells (Abdullah *et al.*, 1992, Sutherland *et al.*, 1992, Otulakowski *et al.*, 1983). All serotypes of *M. haemolytica* possess the *gcp* gene and have glycoprotease activity whereas *M. glucosida* possesses the *gcp* gene but does not display glycoprotease activity (Abdullah *et al.*, 1990, Lee *et al.*, 1994). The protease is thought to enhance adhesion of *M. haemolytica* to host cells and

is also capable of inducing platelet activation, adhesion, and aggregation, leading to their deposition in the lung alveoli (Nyarko *et al.*, 1998).

1.4.1.3 Filamentous haemagglutinin (FhaB)

M. haemolytica encodes a putative filamentous haemagglutinin protein (FhaB) which shares significant amino acid similarity to the FhaB protein produced by the human respiratory pathogen *Bordetella pertussis* and other organisms (Gioia *et al.*, 2006). The *B. pertussis* FhaB protein is a 220 kDa protein found both on the bacterial cell surface and in the extracellular milieu. It is transported across the outer membrane via FhaC, a pore-forming OMP encoded by a gene which lies immediately downstream of FhaB (Locht *et al.*, 1992, Willems *et al.*, 1994). An *fhaC* orthologue is also present adjacent to *fhaB* in *M. haemolytica*, suggesting that *M. haemolytica* FhaB is secreted via a similar mechanism (Gioia *et al.*, 2006). In *B. pertussis* FhaB is a well characterised adhesin (Smith *et al.*, 2001), but the function of FhaB in *M. haemolytica* is currently unknown. In *Bordetella bronchiseptica*, a respiratory pathogen of pigs, FhaB is required for the efficient establishment of colonisation in the lower respiratory tract (Cotter *et al.*, 1998) and *fhaB* mutants are unable to cause disease (Nicholson *et al.*, 2009). An FhaB homologue in the bovine respiratory pathogen *Histophilus somni* was also suggested to have an important role in biofilm formation (Sandal *et al.*, 2009). In *Actinobacillus pleuropneumoniae*, the gene encoding an FhaB homologue was transcribed at much higher numbers in isolates recovered from diseased pig lungs compared to an isolate grown in complex growth medium (Deslandes *et al.*, 2010). If the *M. haemolytica* FhaB protein does function as an adhesin then it must have a different binding physiology to *B. pertussis* FhaB, as it lacks an integrin-binding RGD motif (Gioia *et al.*, 2006). It does, however, contain a bacterial intein-like (BIL) domain (Amitai *et al.*, 2003) at the carboxyl terminus which does not feature in *B. pertussis* FhaB (Gioia *et al.*, 2006). These domains are involved in post-translational autoproteolysis, hence the possibility of allowing variability in receptor recognition and receptor release. Since autoproteolysis would change the structure of FhaB, it may also interfere with host immune recognition and help evade the host immune response. To date, no expressed FhaB protein been identified in *M. haemolytica*.

1.4.2 Cell wall carbohydrates and pili

1.4.2.1 Capsular polysaccharide (CPS)

To date, twelve different capsular serotypes have been identified in *M. haemolytica* (A1, A2, A5-A9, A12-A14, A16 and A17) and one in *M. glucosida* (A11) (Angen *et al.*, 1999b). The serotype A1 capsule has been implicated in diverse functions including adherence to alveolar surfaces (Brogden *et al.*, 1989), resistance to complement-mediated serum killing (Chae *et al.*, 1990), resistance to phagocytosis by bovine neutrophils and alveolar macrophages (Chae *et al.*, 1990, Czuprynski *et al.*, 1989), and facilitating microcolony formation in the bovine pneumonic lung (Morck *et al.*, 1989, Morck *et al.*, 1988).

1.4.2.2 Lipopolysaccharide (LPS)

Lipopolysaccharide (LPS) is the major glycolipid molecule present in the cell envelope of Gram-negative bacteria and makes up between 12 and 25% of the dried cell weight of *M. haemolytica* (Keiss *et al.*, 1964). The basic structure of LPS is composed of three well-defined regions: (i) lipid A, composed of sugars and fatty acids and anchors LPS to the outer membrane, (ii) the oligosaccharide core, composed of approximately 10 monosaccharides and connects the lipid A and O-antigen regions and, (iii) the O-specific antigen, consisting of repeating units containing between one to seven monosaccharides (Hitchcock *et al.*, 1986). It is common for Gram-negative bacteria to possess LPS that does not contain O-antigen side chains. The two different LPS types, with and without O-antigen side chains, are commonly described as 'smooth-type' and 'rough-type' LPS, respectively, according to bacterial colony morphology (Hitchcock *et al.*, 1986). Enteric bacteria generally possess smooth-type LPS whereas, in contrast, bacteria that reside on respiratory and genital mucosa have evolved a unique set of surface glycolipids called lipooligosaccharides (LOSs) (Preston *et al.*, 1996). The LOS molecules are analogous to LPS but their O-antigen side chains are limited to around ten saccharide units. *M. haemolytica* LPS is unusual in that both smooth- and rough-types occur independently in different isolates (Ali *et al.*, 1992, Davies and Donachie, 1996, Lacroix *et al.*, 1993, Davies *et al.*, 1991). Interestingly, only a single O-antigen type is present in isolates possessing smooth-type LPS, suggesting that the gene encoding this part of the LPS molecule may have been recently obtained by horizontal transfer (Davies and

Donachie, 1996). It is possible that the structure of *M. haemolytica* LPS is actually closer to that of LOS, but this has not yet been demonstrated.

LPS is an important *M. haemolytica* virulence factor in serotype A1 isolates, causing damage to pulmonary endothelial cells (Paulsen *et al.*, 1989) and inducing alveolar macrophages to release proinflammatory cytokines (Yoo *et al.*, 1995a, Lafleur *et al.*, 1998) and reactive intermediates (Yoo *et al.*, 1996). It also forms aggregates with and enhances the effects of the leukotoxin LktA (Lafleur *et al.*, 2001, Li and Clinkenbeard, 1999).

1.4.2.3 Pili

The genomes of *M. haemolytica* contain genes that encode the type IV pili locus *pilABCD* (Gioia *et al.*, 2006, Lawrence *et al.*, 2010a). Type IV pili are known to be involved in several bacterial processes including adhesion, DNA uptake and twitching motility (Proft and Baker, 2009). The amino acid sequence of PilC is identical in the bovine serotype A1 and serotype A2 genomes, whereas in the ovine serotype A2 isolate there is a 29 amino acid deletion at the amino terminus (Lawrence *et al.*, 2010b). In *Neisseria gonorrhoeae*, PilC is localised to pili tips and acts as an adhesin which binds to human epithelial cells (Rudel *et al.*, 1995). It has been suggested that the amino-terminal deletion in the ovine serotype A2 PilC may serve as a modification for host species-specific binding to ovine epithelial cells (Lawrence *et al.*, 2010b).

1.4.3 Outer membrane proteins (OMPs)

1.4.3.1 Outer membrane protein A (OmpA)

The OmpA protein is a highly conserved, integral OMP that is universally present in Gram-negative bacteria and has been implicated in a diverse range of functions in different species (Smith *et al.*, 2007). It comprises an N-terminal transmembrane ß-barrel domain embedded in the outer membrane and a C-terminal globular domain which extends into the periplasm and interacts with the underlying peptidoglycan (Demot and Vanderleyden, 1994). The N-terminal domain consists of eight membrane-spanning anti-parallel ß-sheets and four relatively long, mobile, hydrophilic external loops (Pautsch and Schulz, 1998).

Molecular mass heterogeneity of OmpA has been observed among bovine and ovine *M. haemolytica* isolates that correlates with the host of origin (Davies and Donachie, 1996). Subsequent comparative nucleotide sequence analysis of the *ompA* gene from 31 *M. haemolytica* isolates revealed the presence of hypervariable domains within the four surface-exposed loops (Davies and Lee, 2004). The amino acid sequences of these domains are very different in bovine and ovine isolates but are highly conserved among isolates recovered from the same host species. The *ompA* gene can be categorised into four distinct alleles, I to IV. The class I (*ompA1*) alleles are associated exclusively with bovine *M. haemolytica* isolates, whereas the class II to IV (*ompA2* to *ompA4*) alleles occur only in ovine isolates of *M. haemolytica* (Davies and Lee, 2004). Significantly, the *ompA1*- and *ompA2*-type alleles are associated with the major pathogenic lineages of bovine [electrophoretic type 1 (ET 1)] and ovine (ETs 21 and 22) isolates, respectively (Davies *et al.*, 1997). Based on the distribution of the OmpA1 and OmpA2 proteins among bovine and ovine isolates, respectively, and the localisation of the amino acid variation to the tips of the four loops, it was hypothesised that OmpA plays an important role in adherence and is involved in host specificity. The exact role of *M. haemolytica* OmpA is unknown, although studies from bovine serotype A1 *M. haemolytica* isolates have demonstrated binding of OmpA to bovine bronchial epithelial cells (Kisiela and Czuprynski, 2009) and fibronectin (Lo and Sorensen, 2007), indicating a role in host adhesion. It has been demonstrated in several other Gram-negative bacteria, including *H. infuenzae*, *P. multocida*, *and E. coli*, that OmpA plays an important role in adherence and invasion of host cells (Bookwalter *et al.*, 2008, Dabo *et al.*, 2003, Hill *et al.*, 2001, Prasadarao *et al.*, 1996, Reddy *et al.*, 1996).

1.4.3.2 Iron-acquisition OMPs

An essential prerequisite for successful colonisation of mammalian hosts by bacteria is the ability to adapt to a microenvironment containing virtually no free iron. In host tissues, nearly all iron is sequestered by high-affinity iron-binding proteins such as transferrin, lactoferrin, ferritin and haemoglobin. High-affinity host haem-binding proteins such as haemopexin and haptoglobin also contribute to the sequestering of free haem. Bacteria which colonise mucosal surfaces have therefore evolved a number of iron acquisition systems including the secretion of siderophores (small molecules with high affinity for iron) and

outer membrane receptors specific for iron-containing host proteins (Wandersman and Delepelaire, 2004, Wooldridge and Williams, 1993). In many bacteria, expression of iron acquisition proteins is under the control of a transcriptional repressor called the ferric uptake regulator (Fur) (Andrews *et al.*, 2003). *M. haemolytica* also possesses a fur gene, although its function may be atypical as its transcription is not repressed by iron (Gioia and Highlander, 2007).

1.4.3.2.1 Transferrin binding receptor (TbpA and TbpB)

The *M. haemolytica* transferrin receptor, comprised of TbpA and TbpB, is responsible for acquiring iron that has been sequestered by host transferrin (Ogunnariwo *et al.*, 1997). TbpA is a TonB-dependent integral OMP that forms a pore through which iron passes after its release from transferrin. TbpB is a putatively surface-exposed outer membrane lipoprotein that is thought to increase the efficiency of iron uptake from transferrin (Moraes *et al.*, 2009). TbpA and TbpB have molecular masses of approximately 107 and 63 kDa, respectively, and together form a receptor that is highly specific for ruminant transferrin (Ogunnariwo and Schryvers, 1990, Yu *et al.*, 1992). The genes encoding the two proteins are arranged in an operon arrangement of *tbpB-tbpA*. In a recent study, comparative nucleotide sequence analysis revealed that the *tbpBA* operon has a complex mosaic structure and a common gene pool exists for *tbpBA* in *M. haemolytica* and the closely related species *M. glucosida* and *B. trehalosi* (Lee and Davies, 2011).

1.4.3.2.2 Haem/haemoglobin receptors

Transcription of genes encoding two putative haemoglobin receptors was strongly upregulated in a bovine serotype A1 *M. haemolytica* isolate under iron-restricted conditions and in infected lung tissue (Roehrig *et al.*, 2007). Furthermore, transcription of genes encoding a putative haemin [ferric (Fe^{3+}) haem] receptor complex was also upregulated under iron-restriction (Roehrig *et al.*, 2007). It has been suggested that LktA could play an important role in the liberation of haemin and haemoglobin from host erythrocytes, thereby providing substrates for these receptors (Roehrig *et al.*, 2007).

1.4.3.2.3 Haem-haemopexin acquisition system (HxuCBA)

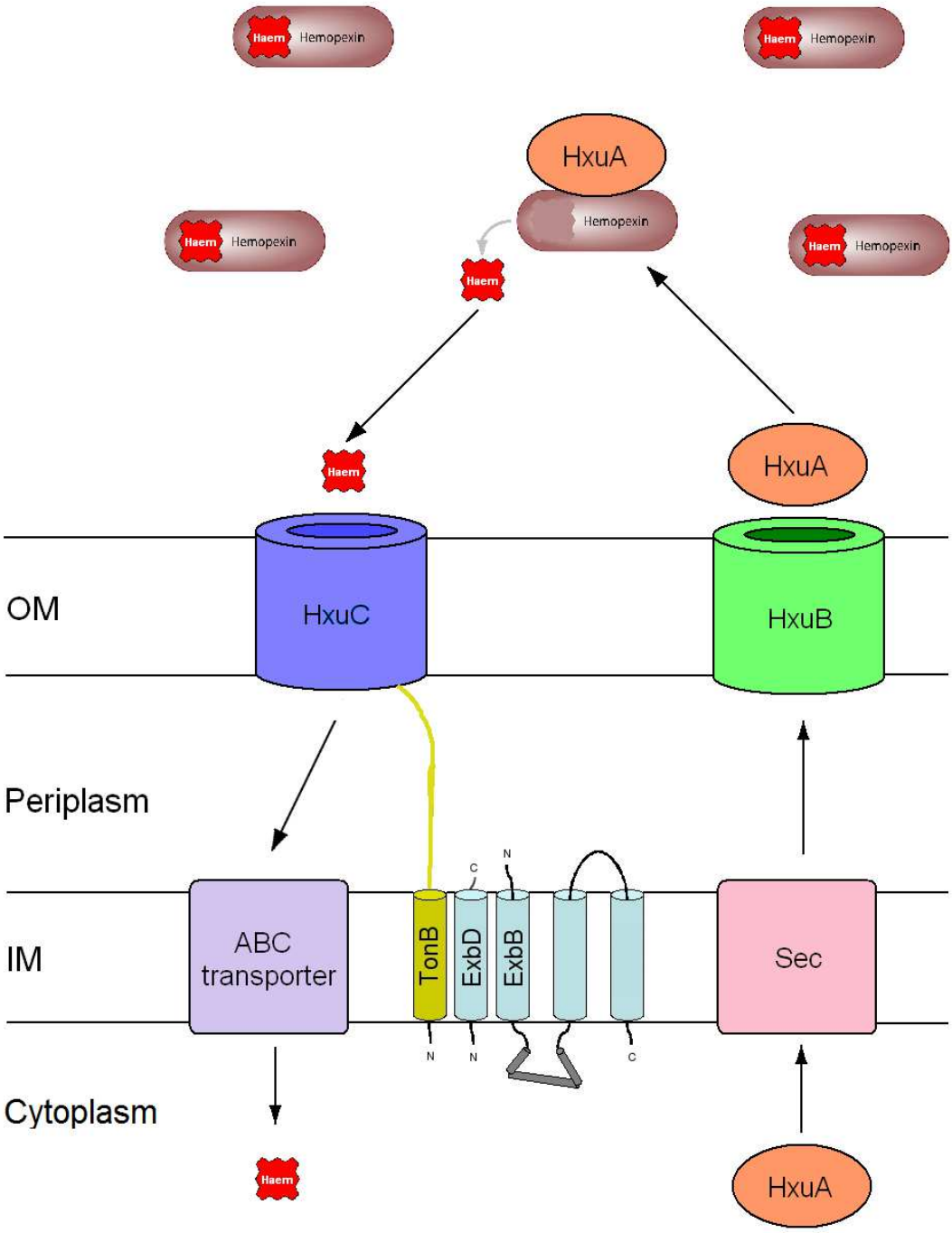
The bovine and ovine serotype A2 *M. haemolytica* genomes encode the haem-haemopexin acquisition operon *hxuCBA* (Lawrence *et al.*, 2010a). In the bovine serotype A1 genome only the *hxuA* gene has been described (Gioia *et al.*, 2006). The HxuCBA system was first discovered in *Haemophilus influenzae* (Hanson *et al.*, 1992) and is an important virulence determinant in this organism (Morton *et al.*, 2007) and in *Haemophilus parasuis* (Melnikow *et al.*, 2005) (Fig. 1.11). The HxuC protein is an outer membrane TonB-dependent receptor (Cope *et al.*, 2001, Cope *et al.*, 1995). The HxuB and HxuA proteins comprise a two-partner secretion (TPS) system, whereby HxuA is transported across the outer membrane by HxuB, a specific pore-forming OMP. HxuA binds to the host haem-haemopexin complex, and facilitates haem delivery to the cell surface via HxuC and an inner membrane ABC transporter (Cope *et al.*, 1998, Cope *et al.*, 1994). Recent evidence suggests that HxuA might not actually function as a haemophore in *H. influenzae*, but instead releases haem upon interaction with the haem-haemopexin complex (Fournier *et al.*, 2011). Furthermore, the primary activity of HxuA may actually be to sequester haemopexin in its inactive form, thereby decreasing the high-affinity binding of haem by the serum (Fournier *et al.*, 2011). None of the HxuCBA components have been identified at the transcriptomic or protein level in *M. haemolytica*.

1.4.3.2.4 Siderophore uptake systems

M. haemolytica does not produce siderophores, although genes encoding several proteins that are similar to siderophore uptake receptors of other Gram-negative bacteria have been identified. One of these proteins has significant homology to the *N. gonorrhoeae* FrpB protein that has been associated with ferric uptake from transferrin (Beucher and Sparling, 1995, Dyer *et al.*, 1988) and, more recently, as a receptor for the ferric siderophore enterobactin (Carson *et al.*, 1999). *N. gonorrhoeae* does not synthesise siderophores but can utilise those produced by other bacteria (xenosiderophores) such as *E. coli* aerobactin (West and Sparling, 1987) and enterobactin (Carson *et al.*, 1999, Rutz *et al.*, 1991). This suggests the possibility that the FrpB orthologue of *M. haemolytica* may also utilise xenosiderophores in a similar manner to *N. gonorrhoeae*. Indeed, some bacterial isolates of the bovine nasopharyngeal flora have been reported to

Figure 1.11. Schematic diagram of the *H. influenzae* HxuCBA haem-haemopexin uptake system.

HxuA is synthesised in the cytoplasm and translocated to the periplasm by the Sec translocon. HxuA is exported across the outer membrane into the host environment by HxuB. HxuA binds to the haem-haemopexin complex, releasing haem and allowing it to be imported back through the outer membrane via the TonB-dependent receptor HxuC, and then subsequently transported across the inner membrane by an ABC transporter.



stimulate growth of *M. haemolytica* and other closely-related Gram-negative bacteria (Corbeil *et al.*, 1985), possibly as a result of this mechanism.

Transcription of the *frpB* gene was upregulated in a bovine serotype A1 *M. haemolytica* isolate (Roehrig *et al.*, 2007) and in *A. pleuropneumoniae* (Klitgaard *et al.*, 2010) under iron-restricted conditions. In *A. pleuropneumoniae*, FrpB has been demonstrated to be both immunoreactive (Liao *et al.*, 2009) and an essential virulence determinant, as *frpB* deletion mutants are unable to colonise the host or cause clinical disease symptoms upon experimental infection (Buettner *et al.*, 2009).

Transcription of genes encoding proteins with homology to some of the proteins in the *A. pleuropneumoniae* ferrichrome uptake system FhuABCD (Mikael *et al.*, 2002) were upregulated in a bovine serotype A1 isolate under iron restricted conditions (Roehrig *et al.*, 2007). Although *fhuBCD* genes were all upregulated, the putative outer membrane receptor gene *fhuA* was not. In contrast to FrpB, the FhuA protein is not an essential virulence determinant in *A. pleuropneumoniae* (Baltes *et al.*, 2003).

1.4.3.3 Lipoproteins (PlpABC, PlpD and PlpE)

Several outer membrane-associated lipoproteins have been identified in *M. haemolytica*. Three contiguous genes arranged in an operon, *plpABC*, are present in all *M. haemolytica* and *M. glucosida* isolates that encode three 28-30 kDa lipoproteins (Cooney and Lo, 1993, Murphy and Whitworth, 1993). These proteins are highly immunogenic (Dabo *et al.*, 1994), and a *M. haemolytica* mutant lacking this operon was demonstrated to have increased susceptibility to complement-mediated killing and reduced survival *in vivo* (Murphy *et al.*, 1998). PlpA has been demonstrated to be surface-exposed and capable of binding to bovine bronchial epithelial cells *in vitro* (Kisiela and Czuprynski, 2009). It has also been identified in an immunoproteomic study of the OMPs of a bovine serotype A1 isolate (Ayalew *et al.*, 2010).

Another gene, *plpD*, is located on a different part of the chromosome to *plpABC* and encodes a 31 kDa lipoprotein (Nardini *et al.*, 1998). Amino acid sequence analysis indicates that PlpD is lipid modified at the N-terminus, but contains a C-terminal region that is similar to that of OmpA (Nardini *et al.*, 1998). This

protein is recognised by sera from calves immunised with a culture supernatant vaccine (Nardini *et al.*, 1998) but its exact function is yet to be determined.

The gene *plpE* is also located on a different part of the chromosome to *plpABC* and *plpD* and encodes a 45 kDa outer membrane lipoprotein that is present in all serotypes of *M. haemolytica* but not in *M. glucosida* (Pandher *et al.*, 1998). It has been demonstrated that anti-PlpE antibodies promote complement-mediated killing of bovine serotype A1 isolates (Nardini *et al.*, 1998, Ayalew *et al.*, 2004). The addition of recombinant PlpE to a commercial vaccine also conferred enhanced resistance against experimental challenge in cattle compared to the commercial vaccine alone (Confer *et al.*, 2003). The exact function of PlpE is also yet to be determined.

1.4.3.4 Serotype-specific antigen (Ssa)

The Ssa protein was originally designated as Ssa1 (serotype-specific antigen-1) as it was identified in a bovine serotype A1 *M. haemolytica* isolate (Gonzalezrayos *et al.*, 1986, Lo *et al.*, 1991). The *ssa* gene has since been discovered in all serotypes examined, with the exception of A8, but is absent from *M. glucosida* (Gonzalez *et al.*, 1991). Normally only *M. haemolytica* isolates of serotype A2 are identified in the upper respiratory tract of healthy cattle, and it was hypothesised that the Ssa protein might function as a specific adhesin in serotype A1 isolates which facilitates the selective colonisation of these isolates in the diseased bovine lung (Gonzalez *et al.*, 1995). Ssa was later categorised as a classical autotransporter and a subtilisin-like serine protease (Siezen and Leunissen, 1997) which shares significant homology to similar proteins in other species including AasP of *A. pleuropneumoniae* (Ali *et al.*, 2008), NalP of *Neisseria meningitidis* (Turner *et al.*, 2002) and SphB1 of *B. pertussis* (Coutte *et al.*, 2001), all of which act as surface maturation proteases of other autotransporters. A similar function has not yet been described in *M. haemolytica*.

1.4.3.5 Neuraminidase (NanH)

A neuraminidase protein (NanH) was detected and active in all capsular serotypes of *M. haemolytica*, but not *M. glucosida* (Straus *et al.*, 1993). In *M. haemolytica*, NanH has been suggested to facilitate the colonisation of mucosal

surfaces (Straus *et al.*, 1998), most likely through the modification of sialic acid molecules on host cells. Its primary amino acid sequence is similar to that of a classical autotransporter.

1.4.3.6 Immunglobulin proteases

Three genes encoding proteins that are orthologous to the Iga1 proteases of Gram-negative species including *H. influenzae*, *N. meningitidis* and *N. gonorrhoeae*, have been identified in *M. haemolytica* genomes (Gioia *et al.*, 2006, Lawrence *et al.*, 2010a). The *M. haemolytica* Iga1 orthologues contain Iga1 domains and conserved active serine protease sites that are similar to the active sites of *H. influenzae* Iga1 protease (Poulsen *et al.*, 1992). These serine protease sites cleave the hinge region of host IgA antibodies, thereby eliminating their agglutinating ability and facilitating host colonisation (St Geme, 2000). While IgA protease activity has not been detected in the supernatants of *M. haemolytica* (Abdullah *et al.*, 1992), IgG-specific protease activity was detected in partially purified culture supernatants (Lee and Shewen, 1996). IgG is also the primary secretory antibody found in the lower respiratory tract of cattle (Duncan *et al.*, 1972), whereas IgA is the predominant antibody in the upper respiratory tract (Wilkie and Markham, 1981). It is therefore possible that at least one of the Iga1 orthologues identified in *M. haemolytica* actually cleaves IgG instead of IgA. The Iga1 protease of *N. gonorrhoeae* was the first classical autotransporter protein to be studied in detail (Pohlner *et al.*, 1987). In neisserial species, the C-terminal ß-barrel domain of Iga1 integrates into the outer membrane and forms a specific pore for the translocation of the passenger domain (containing the serine protease site) from the periplasm to the extracellular space (Klauser *et al.*, 1993, Pohlner *et al.*, 1987). Once exposed on the bacterial surface the protein is able to undergo autoproteolysis (Vitovski and Sayers, 2007), thereby facilitating IgA cleavage at both the cell surface and in the extracellular milieu. The autotransporter protein NalP has also been reported to have a role in the modulation and processing of Iga1 in *N. meningitidis* at the cell surface (van Ulsen *et al.*, 2003). To date, no Iga1-like proteases have been identified at the protein level in any *M. haemolytica* isolates.

1.4.3.7 Trimeric autotransporter adhesins

Two putative trimeric autotransporter proteins have been identified in *M. haemolytica* genomes (Gioia *et al.*, 2006, Lawrence *et al.*, 2010a). One has similarity to the Hsf proteins of *A. pleuropneumoniae* and *P. multocida*, and the other has similarity to the Hia protein of *H. influenzae* and Hia orthologues of *A. pleuropneumoniae* and *P. multocida*. Roles in adherence are common to most of the trimeric autotransporters identified to date (Cotter *et al.*, 2005). Indeed, roles in adherence to epithelial cells have been associated with Hsf in *A. pleuropneumoniae* (Auger *et al.*, 2009) and *H. influenzae* (Hallstrom *et al.*, 2006), and also Hia in *H. influenzae* (Barenkamp and StGeme, 1996). Another gene locus, designated *ahs*, was also identified in a serotype A1 isolate and encodes a collagen-binding trimeric autotransporter purported to be involved in host adhesion (results obtained using recombinant Ahs) (Lo *et al.*, 2006, Daigneault and Lo, 2009). Despite the presence of genes encoding these proteins in *M. haemolytica* they have not yet been identified at the protein level in any isolate.

1.5 Phenotypic and genetic variation of *M. haemolytica* and *M. glucosida*

1.5.1 Classification based on phenotypic relationships

1.5.1.1 Capsular serotyping

The serotypes of *M. haemolytica* isolates are determined by performing the indirect haemagglutination assay (IHA) using sera raised against reference capsular polysaccharide types (Fraser *et al.*, 1983). To date, twelve different capsular serotypes have been identified in *M. haemolytica* (A1, A2, A5-A9, A12-A14, A16 and A17) and one in *M. glucosida* (A11) (Angen *et al.*, 1999b); however, untypeable isolates of both organisms are frequently isolated (Donachie *et al.*, 1984, Gilmour and Gilmour, 1989). Capsular serotyping of other *M. haemolytica*-like isolates that are phenotypically and genetically distinct from *M. haemolytica* have been shown to possess A6, A9 and A16 capsular serotypes, indicating that serotyping is of limited use in the differentiation of *M. haemolytica* isolates (Angen *et al.*, 1999b).

The various capsule structures of *M. haemolytica* have been previously characterised and are summarised in Table 1.2. The structure of the serotype A1 polysaccharide is highly similar to the widely distributed 'enterobacterial common antigen' (Adlam *et al.*, 1984). The polymer of serotype A2 polysaccharide is colaminic acid and is identical to the capsular polysaccharides of *N. meningitidis* B and *E. coli* K1 (Adlam *et al.*, 1987). Serotype A7 polysaccharide is similar to the phosphate-containing polysaccharides of *N. meningitidis* serogroup L and *H. influenzae* type F (Adlam *et al.*, 1986). It has been suggested that the production of similar or identical capsular structures among pathogenic bacteria from different genera represents a common defence mechanism developed through convergent evolution (Adlam, 1989).

1.5.1.2 LPS profiles

The LPS profiles of *M. haemolytica* and *M. glucosida* were analysed by SDS-PAGE (Davies and Donachie, 1996, Ali *et al.*, 1992, Lacroix *et al.*, 1993). Eight LPS types were recognised in *M. haemolytica* and one in *M. glucosida* (Davies and Donachie, 1996). In *M. haemolytica* isolates there were four different types of core-oligosaccharide (1-4), each either associated or not associated (A or B) with a single O-antigen type. There was a clear association between LPS profiles and capsular serotypes: serotypes A1, A5, A6, A9 and A12 with LPS type 1A, serotypes A2, A8, A14 and A16 with LPS types 1B and 3B, and serotypes A7 and A13 with LPS type 4A (Davies and Donachie, 1996). *M. glucosida* isolates were represented by a single LPS type, designated type 4C, which possessed a core oligosaccharide region similar to LPS type 4 but with a distinct O-antigen type different from that in *M. haemolytica* isolates (Davies and Donachie, 1996).

1.5.1.3 OMP profiles

The OMP profiles of *M. haemolytica* and *M. glucosida* isolates were analysed by SDS-PAGE (Davies and Donachie, 1996). Twenty different OMP profiles were recognised in *M. haemolytica* isolates and two in *M. glucosida* isolates. There was a clear association between the OMP profile types and capsular serotypes. Based on the variation in OMP profiles, the capsular serotypes of *M. haemolytica* could be sub-divided into three main groups: serotypes A1, A5, A6, A8, A9 and A12 with OMP type 1, serotypes A2, A14 and A16 with OMP type 2, and serotypes A7 and A13 with OMP type 3. The OMP profiles of *M. glucosida* were similar to

Table 1.2. Capsule structure of serotypes A1, A2, and A7 of *M. haemolytica*.

Serotype	Capsule structure	Similar capsule structure in other pathogens
A1	→3)-*O*-(2-acetamido-2-deoxy-4-*O*-acetyl-*β*-D-mannopyranosyluronic acid)-(1→4)-*O*-(2-acetamido-2-deoxy-*β*-D-mannopyranose)-(1→	Enterobacterial common antigen
A2	→2)-α-D-*N*-acetylneuraminic acid-(8→ (and a dextran polymer)	*N. meningitidis* serogroup B *E. coli* K1
A7	→3)-*β*-2-acetamido-2-deoxygalactopyranose-(1→3)-α-2-acetamido-2-deoxy-6-*O*-acetylglucopyranose-(1-phosphate→	*N. meningitidis* serogroup L *H. influenzae* type F

those of A7 and A13 and were also considered to be of OMP type 3. Furthermore, there were associations between specific OMP profiles and host of origin (i.e. cattle or sheep). For example, serotype A1 and A6 isolates were divided into two distinct subgroups, OMP types, 1.1 and 1.2, which were associated with cattle and sheep, respectively. A similar distinction could also be made for serotype A2 isolates (OMP types 2.1 and 2.2). The finding that bovine and ovine isolates of these three serotypes could be clearly differentiated based on their OMP profiles suggest that OMPs may contribute towards host-specificity.

1.5.2 Classification based on genetic relationships

1.5.2.1 Nucleotide sequence analysis of the 16S rRNA gene

Comparative nucleotide sequence analysis of the 16S rRNA gene demonstrated that *M. haemolytica* and *M. glucosida* isolates represent distinct phylogenetic lineages (Davies *et al.*, 1996, Angen *et al.*, 1999a). However, due to the highly conserved nature of the 16S rRNA only two different alleles were identified in all serotypes of *M. haemolytica*, demonstrating the limited use of this approach for differentiating isolates within a species (Davies *et al.*, 1996, Angen *et al.*, 1999a). Isolates of serotypes A1, A5 to A9, A12 to A14, and A16 had identical 16S rRNA nucleotide sequences and differed from the serotype A2 16S rRNA gene at only two nucleotide positions.

1.5.2.2 Multilocus enzyme electrophoresis (MLEE)

Multilocus enzyme electrophoresis (MLEE) is a technique which can be used to examine genetic diversity and structure in natural populations of bacteria, providing a genetic framework for the analysis of variation in phenotypic characteristics (Selander *et al.*, 1986). Bacterial isolates are characterised by measuring variations in the electrophoretic mobility of several housekeeping proteins which can be directly equated to allelic variation at the corresponding gene loci (Selander *et al.*, 1986). MLEE was used to analyse the genetic diversity and population structure of *M. haemolytica* and *M. glucosida* isolates and subsequently correlate variation in capsular serotypes, LPS types and OMP types with the underlying genetic framework (Davies *et al.*, 1997). *M. haemolytica* isolates were classified into 22 electrophoretic types (ETs) based on allelic variation at 18 housekeeping gene loci. The serotype A1 and A6 isolates of ET1

were responsible for 75% of bovine disease cases, marking two virulent, cattle-specific clonal groups. Similarly, the serotype A2 isolates of ET 21 and 22 were responsible for 40% of ovine disease cases, marking a virulent, cattle-specific clonal group. Bovine serotype A1 and A6 isolates and bovine serotype A2 isolates were phylogenetically distinct from ovine isolates of the same serotypes, suggesting that different subpopulations of these serotypes are associated with disease in the two animals. Furthermore, the MLEE data also confirmed previous observations (Davies and Donachie, 1996) that bovine and ovine isolates of the A1, A2 and A6 capsular serotypes could be consistently differentiated based on their OMP profiles, indicating a role in host-specificity.

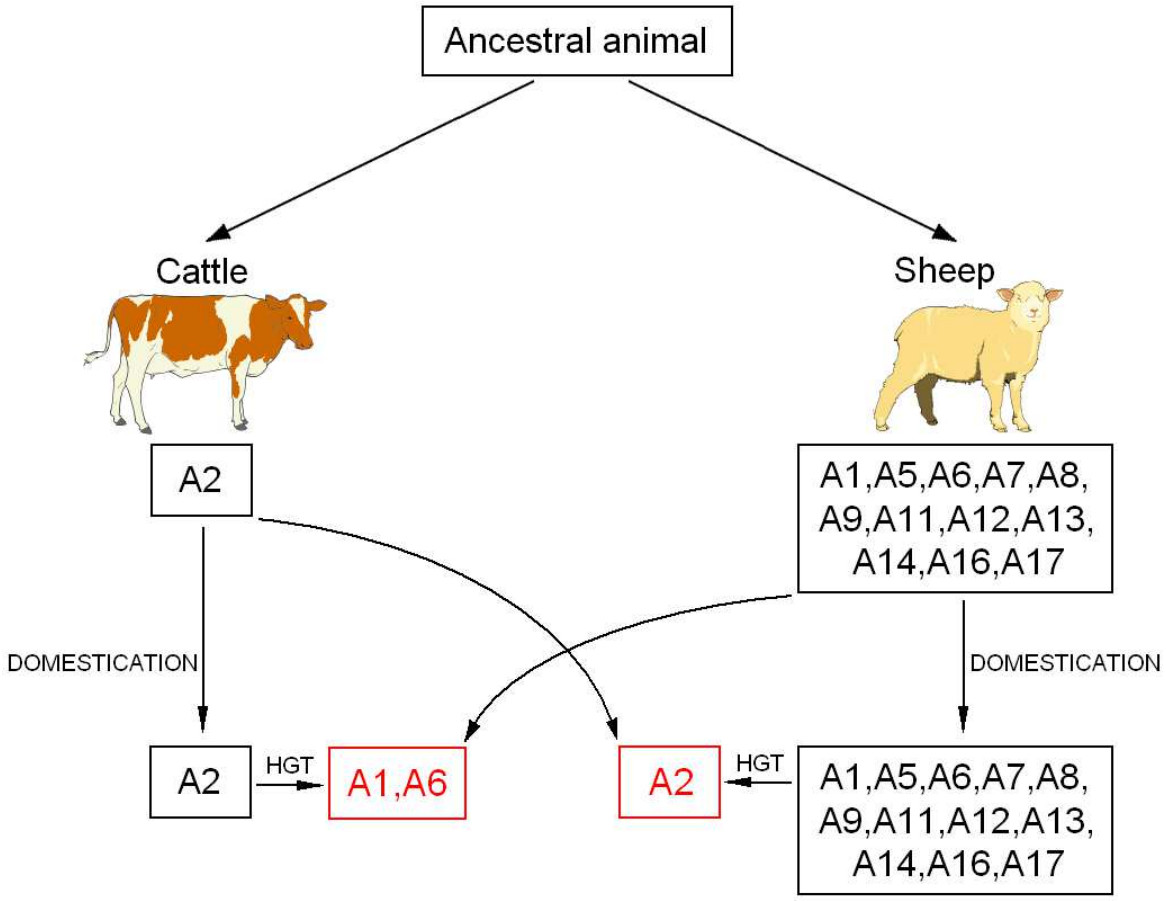
The MLEE data was consistent with previous findings of 16S rRNA sequence comparison (Davies *et al.*, 1996) that *M. glucosida* represents a heterogeneous group of bacteria that are phylogenetically distinct from *M. haemolytica* (Davies *et al.*, 1997).

1.5.3 Evolution and host-switching in *M. haemolytica*

Comparative nucleotide sequence analysis has shown that horizontal DNA transfer and assortative (entire gene) recombination have contributed significantly to the evolution of the leukotoxin operon (Davies *et al.*, 2001, Davies *et al.*, 2002), the *ompA* gene (Davies and Lee, 2004) and the *tbpBA* operon (Lee and Davies, 2011). In these studies, the association of identical or nearly identical alleles and gene segments with divergent phylogenetic lineages of *M. haemolytica* collectively provide evidence to support the hypothesis that host switching of strains from cattle to sheep and vice versa have contributed to the emergence of new strains. In this hypothesis (Fig. 1.12) the serotype A1 and A6 strains, which evolved independently in sheep, were transmitted to cattle around the time of domestication of these animals (around 10,000 years ago). Similarly, the A2 strains, which evolved independently in cattle, were transmitted to sheep. Exchange of genetic material from the native, non-pathogenic strains to the newly-transmitted strains allowed for their adaptation to their new host environment, giving rise to the pathogenic serotype A1 and A6 strains in cattle and the pathogenic serotype A2 strains in sheep.

Figure 1.12. The hypothesised route of *M. haemolytica* host switching.

Transmission of serotype A2 strains from cattle to sheep occurred around the time of animal domestication. Subsequent horizontal gene transfer (HGT) of genetic material from the native ovine strains gave rise to the pathogenic ovine serotype A2 strains (red). Similarly, transmission of serotype A1 and A6 strains from sheep to cattle and subsequent genetic exchange gave rise to the pathogenic bovine serotype A1 and A6 strains (red).



1.6 Bioinformatic tools for discriminating proteins located in different subcellular compartments

Once a bacterial protein has been synthesised in the cytoplasm it can either remain there or be targeted to a different subcellular location. Several of the sorting signals that are responsible for targeting a nascent polypeptide to a particular location have been well characterised, including the N-terminal signal peptide for targeting proteins outside of the cytoplasm. Many proteins also have secondary structural signatures which indicate their most probable location, such as the transmembrane α-helical secondary structures that are found in Gram-negative integral inner membrane proteins and the ß-barrel secondary structures present in Gram-negative integral OMPs. These signals and structural signatures are generally encoded in the amino acid sequence of the protein itself and can therefore be identified computationally using bioinformatics approaches. The availability of publicly available sequence information has expanded rapidly over recent years and bioinformatic prediction of such sequence features has become an important part of microbiological research. Proteins which are surface-exposed and potential vaccine targets can be rapidly identified bioinformatically from sequence data rather than experimental data, a process referred to as reverse vaccinology (Rappuoli, 2000, Vivona *et al.*, 2008). It is also useful to know the subcellular compartment that a protein is likely to be located in when designing protein isolation experiments and also for identifying contaminants in proteomic analyses.

1.6.1 Prediction tools for discrimination of OMPs

Outer membrane proteins have several unique sorting signals and structural signatures that allow them to be differentiated from proteins located in other subcellular compartments by bioinformatic predictors. These predictors can be categorised into three groups: ß-barrel predictors, outer membrane lipoprotein predictors and subcellular localisation predictors.

1.6.1.1 ß-barrel predictors

In general, two main types of structural signatures are present in Gram-negative integral membrane proteins, namely α-helices and ß-barrels, which are associated with the inner and outer membranes, respectively. The prediction of

integral inner membrane proteins is relatively easy, as the membrane-spanning regions consist of easily identifiable α-helical stretches consisting of 15 to 25 mostly non-polar amino acids (Santoni *et al.*, 2000). In contrast, predicting integral OMPs is more difficult, mainly due to the membrane-spanning ß-strands being much shorter and having highly variable properties (Koebnik *et al.*, 2000). In general, the amino acids of transmembrane ß-strands alternate between polar and non-polar, with polar residues facing the lipid bilayer and protein interfaces and non-polar residues facing the inside of the ß-barrel. However, some non-polar residues may also point inside the barrel, interrupting the regular alternation between polar and non-polar residues and making transmembrane ß-strands more difficult to identify (Schulz, 2000). Transmembrane ß-strands are also often flanked by a layer of aromatic amino acid residues (the 'aromatic girdle') which are believed to contribute towards providing stability in the outer membrane (Yau *et al.*, 1998). The majority of integral ß-barrel OMPs also contain a C-terminal signature sequence, in which there is a phenylalanine (or tryptophan) residue at the C-terminal position, and hydrophilic residues at positions 3, 5, 7 and 9 from the C-terminus (Struyve *et al.*, 1991). This sequence is required for translocation across the periplasm and correct insertion into the outer membrane by the BAM complex (Robert *et al.*, 2006).

Several bioinformatic methods have been developed for the prediction of integral ß-barrels from protein amino acid sequences. In these methods, the amino acid sequence is first encoded into a feature vector and then secondly entered into a classification model to produce the prediction. Several web-based resources are available for the prediction of ß-barrels, including TMB-Hunt (Garrow *et al.*, 2005), TMBETADISC-RBF (Ou *et al.*, 2008), MCMBB (Bagos *et al.*, 2004) and BOMP (Berven *et al.*, 2004).

1.6.1.2 Lipoprotein predictors

Proteins that are targeted for secretion via the Sec translocon contain an N-terminal signal sequence that is required for translocation across the inner membrane. Once in the periplasm, lipoprotein signal sequences are cleaved by an enzyme called signal peptidase II (SPaseII), whereas signal sequences of other proteins are cleaved by signal peptidase I (SPaseI). The key to bioinformatically predicting lipoproteins from a set of membrane proteins lies in the ability to

differentiate between these two signal sequences. In both lipoproteins and non-lipoproteins, signal sequences can be divided into three regions: an n-region, an h-region, and a c-region (Juncker *et al.*, 2003). Both protein types have a similar n-region, which is characterised by the presence of positive amino acids lysine and/or arginine. The h-region consists of hydrophobic amino acids and is shorter in lipoproteins than in non-lipoproteins. The c-region contains the signal peptidase recognition site and is necessary for cleavage. In lipoproteins, the c-region contains an apolar consensus sequence of four amino acids around the cleavage site known as the lipobox. The most conserved amino acids in the lipobox are a leucine at position -3 from the cleavage site, alanine at position -2 and glycine or alanine at position -1, although other substitutions have been demonstrated. A cysteine residue at position +1 is essential: LA(G,A)↓C (Vonheijne, 1989). In non-lipoproteins, the same region is polar and has small, uncharged residues at positions -3 and -1 (Vonheijne, 1989). Outer membrane lipoproteins can be further differentiated from inner membrane lipoproteins by examining the amino acid at position +2 from the cleavage site. In general, lipoproteins containing an aspartate residue at this position are retained at the inner membrane whereas those containing any other residue are translocated to the outer membrane (Yamaguchi *et al.*, 1988).

Web-based resources are available for the prediction of lipoproteins including DOLOP (Babu and Sankaran, 2002), LIPO (Berven *et al.*, 2006) and LipoP (Juncker *et al.*, 2003).

1.6.1.3 Subcellular localisation predictors

Subcellular localisation predictors are able to predict the localisation site of a protein from several different options. For Gram-negative bacterial proteins, these sites are the cytoplasm, inner membrane, periplasm, outer membrane and extracellular. Subcellular localisation predictors utilise many of the criteria described above for the differentiation of OMPs. The main advantage of using these predictors in conjunction with ß-barrel and lipoprotein predictors is that they can predict if a protein is likely to have more than one localisation site.

Several web-based Gram-negative subcellular localisation predictors are available including PSORTb (Gardy *et al.*, 2003, Gardy *et al.*, 2005), Proteome

Analyst (Szafron *et al.*, 2004), CELLO (Yu *et al.*, 2006) and SOSUI-GramN (Imai *et al.*, 2008).

1.7 Mass spectrometry (MS)-based outer membrane proteomics

Mass spectrometry (MS) is the method of choice for the identification of proteins in simple or complex samples. Proteomics in general deals with the determination of complete protein sets belonging to either a whole cell (a proteome) or a particular subcellular compartment (a subproteome). MS-based proteomics has only been made possible by the availability of gene and genome sequence databases, modern computing and large advances in MS technology. Outer membrane proteomics deals specifically with the identification of all integral OMPs and outer membrane lipoproteins (the outer membrane subproteome) within the outer membrane of a given Gram-negative bacterium. In order to undertake comprehensive studies of the outer membrane subproteome, the outer membrane must first be isolated from other cellular components and purified in order to minimise contamination with non-OMPs. This can be achieved through subcellular fractionation based on the specific properties of cellular components from different compartments. Complementary MS-based proteomic approaches can then be utilised to allow comprehensive identification of OMPs within a purified outer membrane sample.

1.7.1 Outer membrane isolation

This section will summarise three of the most popular techniques for the isolation of Gram-negative outer membranes: selective detergent solubilisation, spheroplasting and sucrose density gradient centrifugation.

1.7.1.1 Selective detergent solubilisation

This technique involves using an appropriate detergent to selectively solubilise the inner membrane, leaving the outer membrane largely intact so it can be pelleted and isolated. Before this can occur the cell envelope must first be separated from the components of the cytoplasm. Cells are broken in order to release their cytoplasmic contents, usually through sonication or French press. The cell lysate is centrifuged to pellet any unbroken cells which are

subsequently removed. The remaining supernatant is then subjected to ultracentrifugation to pellet the cell envelopes. One of the earliest detergents used for selective inner membrane solubilisation was the non-ionic detergent Triton X-100, which selectively solubilised the inner membrane of *E. coli* in the presence of Mg^{2+} ions (Schnaitman, 1971). The mild ionic detergent *N*-laurolysarcosine (Sarkosyl) was subsequently shown to selectively solubilise the inner membrane of *E. coli* in the absence of Mg^{2+} (Filip *et al.*, 1973), indicating that the selective solubilisation ability of Triton X-100 could be explained, at least in part, on the basis that Mg^{2+} cations stabilise the outer membrane. It is thought that divalent cations stabilise the outer membrane by interacting with the hydrophobic moiety of LPS (Schindler and Osborn, 1979). Sarkosyl has been demonstrated to selectively solubilise *M. haemolytica* inner membrane proteins more completely than Triton X-100 (Squire *et al.*, 1984) and yield more OMPs (Morton *et al.*, 1996). Furthermore, a recent study using *Campylobacter jejuni* compared a total of nine different OMP purification protocols and found that selective inner membrane solubilisation using Sarkosyl produced the purest and most complete outer membrane preparations and was the most reproducible in terms of consistent identification of OMPs (Hobb *et al.*, 2009). Sarkosyl is therefore a very popular choice for the isolation of OMPs in *M. haemolytica* (Davies and Donachie, 1996, McCluskey *et al.*, 1994, Davies *et al.*, 1994a, Davies *et al.*, 1992, Kisiela and Czuprynski, 2009, Gatewood *et al.*, 1994, Ayalew *et al.*, 2010) and several other Gram-negative organisms including *P. multocida* (Davies *et al.*, 2003b, Davies *et al.*, 2003a, Davies *et al.*, 2004), *A. pleuropneumoniae* (Chung *et al.*, 2007), *H. influenzae* (Kaur *et al.*, 2003), *C. jejuni* (Hobb *et al.*, 2009), *Shewanella oneidensis* (Brown *et al.*, 2010), *Flavobacterium columnare* (Liu *et al.*, 2008), *Coxiella burnetii* (Papadioti *et al.*, 2011), *Helicobacter pylori* (Baik *et al.*, 2004) and *Tannerella forsythia* (Veith *et al.*, 2009).

The mechanism of action of Sarkosyl is not fully understood, but it has been shown to be ineffective at dispersing LPS from the both *E. coli* and *Brucella abortus* cell envelopes which may also be the basis for its inability to solubilise the outer membrane (Moriyon and Berman, 1982). The length of its alkyl chain also appears to be a critical factor. Sarkosyl (which has an alkyl chain of 11 carbons) and Zwittergents 308 and 310 (alkyl chains of 8 and 10 carbons, respectively) are not effective in dispersing LPS in either *B. abortus* or *E. coli*,

whereas Zwittergents 312 and 316 (alkyl chains of 12 and 16 carbons, respectively) were very efficient at dispersing LPS in both bacteria (Moriyon and Berman, 1982). This suggests that a minimal alkyl chain length of 12 carbons is necessary for dispersing LPS and, therefore, that only detergents with 11 or fewer carbons are appropriate for the selective solubilisation of the inner membrane.

1.7.1.2 Spheroplasting

Spheroplasting involves breaking the outer membrane using lysozyme and EDTA, which peels away from the cell while the cytoplasmic membrane remains intact (i.e. the spheroplast) and is pelleted by centrifugation (Birdsell and Cota-Robles, 1967, Hill and Sillence, 1997, Osborn and Munson, 1974, Witholt *et al.*, 1976, Costerto.Jw *et al.*, 1967). One of the limitations of this technique is that the outer membrane may not become completely separated from the inner membrane, allowing the possibility that some OMPs will not be present in the outer membrane fraction (Hobb *et al.*, 2009).

1.7.1.3 Sucrose density gradient centrifugation

For sucrose density gradient centrifugation, a sucrose gradient is firstly created in a centrifuge tube whereby lower concentrations of sucrose are gently overlayed onto higher concentrations. Cell envelope samples are then loaded onto the top of the gradient and centrifuged. The membrane components move through the gradient until they reach the point at which their density matches that of the surrounding sucrose. The outer and inner membrane fractions are observed as separate bands within the gradient due to their different densities and can be subsequently isolated (Osborn *et al.*, 1972). Additional wash steps using chaotropic agents such as sodium bromide and sodium carbonate can be further used to remove contaminants (Schluesener *et al.*, 2005, Molloy *et al.*, 2000). Sucrose density gradient centrifugation is more laborious than detergent-based techniques and is generally not an appropriate technique for preparing several samples at once.

1.7.2 MS principles and instrumentation

Before the different strategies of MS-based OMP identification are discussed, it is first necessary to give an overview of the principles and instrumentation of MS. A

mass spectrometer consisting of three main components: an ion source that transfers peptides (from enzyme-digested proteins) into the gaseous phase, a mass analyser that measures the mass-to-charge (*m*/*z*) ratio of the ionised peptides, and a detector that records the number of ions at each *m*/*z* value. Protein identifications from MS data are made by peptide mass fingerprinting. By coupling two or more mass analysers together with a collision cell, peptides can be further fragmented to generate MS/MS spectra and produce protein identifications with greater sensitivity.

1.7.2.1 Ionisation techniques

In order for peptides to be analysed by MS it must first be vaporised and ionised. The success of MS in the life sciences is largely due to the introduction of electrospray ionisation (ESI) (Yamashita and Fenn, 1984a, Yamashita and Fenn, 1984b, Fenn *et al.*, 1989, Lane, 2005) (Fig. 1.13A) and matrix-assisted laser desorption ionisation (MALDI) (Karas and Hillenkamp, 1988) techniques, which allow large, polar, thermally labile proteins to enter into the gaseous phase (Lane, 2005) (Fig. 1.13B).

Peptides to be ionised by ESI are first dissolved in a solvent. This solution flows through a small capillary tube to which a high voltage is applied, resulting in the release of a fine mist of charged droplets. As the droplets migrate to the counter electrode they pass through a heating element, allowing the solvent to evaporate leaving behind multiply-charged gas-phase ions.

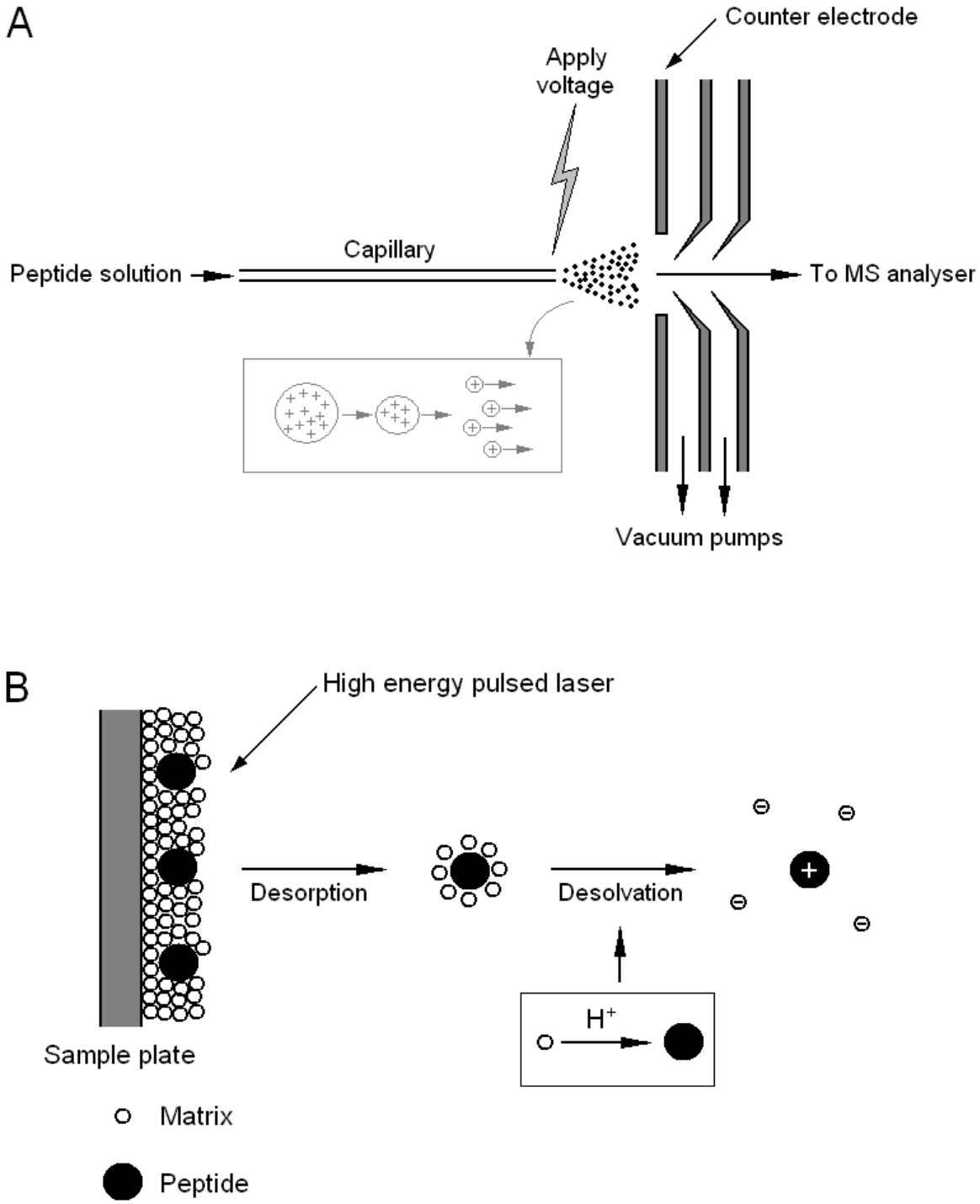
MALDI sublimates and ionises samples out of a dry, crystalline matrix using laser pulses. Samples are co-crystallised with an excess of a matrix material that strongly absorbs light from a laser. The matrix expands into the gas phase as it absorbs light from the laser, taking with it intact peptides.

1.7.2.2 Mass analysers

The mass analyser measures the *m*/*z* of ionised peptides to generate information-rich ion mass spectra from these peptides. There are four main types of mass analyser used in proteomics research: time-of-flight (TOF), quadrupole, ion trap and Fourier transform ion cyclotron (FT-MS) analysers. These can be either stand alone or, in some cases, put together in tandem to take advantage of the different strengths of each.

Figure 1.13. Ionisation and peptide sample introduction processes: (A) matrix-assisted laser desorption/ionisation (MALDI) and (B) electrospray ionisation (ESI).

Figure was adapted from Lane *et al*. (2005).

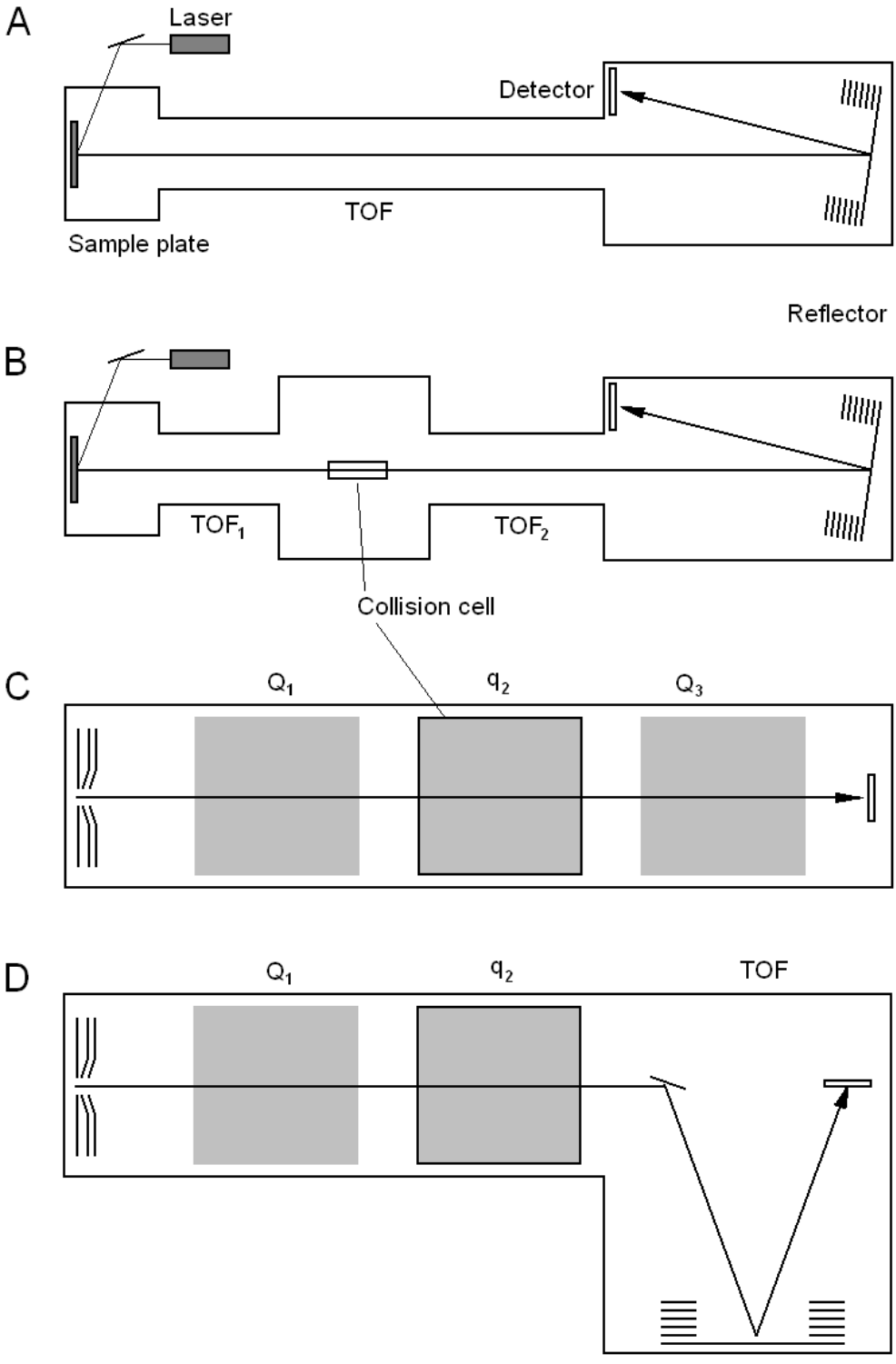


MALDI is usually combined with TOF analysers (MALDI-TOF) (Aebersold and Mann, 2003), which determine *m*/*z* ratios by measuring the time that peptide ions take to move through a tube of specified length that is under vacuum (Fig. 1.14A). MS/MS analysis can be achieved by placing a collision cell either between two TOF analysers (MALDI-TOF-TOF) (Medzihradszky *et al.*, 2000) (Fig 1.14B) or between a quadrupole mass filter (described below) and a TOF analyser (MALDI-Q-TOF) (Loboda *et al.*, 2000). Ions of a particular *m*/*z* are selected in the first analyser, fragmented in the collision cell by collision-induced dissociation (CID) and the fragments read by the second analyser. MALDI-MS has traditionally been used to analyse relatively simple peptide mixtures, although MALDI-MS/MS can now facilitate the analysis of more complex samples.

ESI is most commonly coupled to triple quadrupoles for the generation of MS/MS spectra (Aebersold and Mann, 2003). A quadrupole consists of four parallel rods equally spaced around a central axis. One set of opposing rods have a direct current, the other an alternating current, producing opposing positive and negative fields. Peptide ions are introduced in a continuous beam along the central axis between the poles, and are filtered according to their *m*/*z* ratios. The two positive rods create a high-pass mass filter that allows ions above a critical *m*/*z* ratio to pass through the centre of the quadrupole. The two negative rods create a low-pass mass filter that allows ions below a critical *m*/*z* ratio to pass through the centre of the quadrupole. The two overlapping mass filter regions create a 'band pass' area of mutual stability which allows ions of a certain *m*/*z* ratio to pass through. Ions outside of this area of mutual stability are not able to pass through the quadrupole and run into the rods. The *m*/*z* ratio of ions that are allowed to pass through the quadrupole is proportional to the voltage applied to the rods. For MS/MS analysis, three quadrupoles are combined whereby ions of a particular *m*/*z* are selected in the first, fragmented in the second by CID and the fragments read by third (Fig. 1.14C). The third quadrupole can also be substituted with a TOF analyser (ESI–QqTOF) to perform MS/MS analysis (Fig. 1.14D). ESI-MS systems are normally integrated with liquid chromatography (LC) technology for efficient separation of peptides prior to analysis.

Figure 1.14. Examples of mass spectrometers used in proteome research: (A) reflector time-of-flight (TOF), (B) time-of flight/time-of-flight (TOF-TOF), (C) triple quadrupole, (D) quadrupole time-of-flight (QqTOF).

Figure was adapted from Aebersold and Mann (2003).



1.7.2.3 Peptide mass fingerprinting

Prior to analysis by MS, a protein must be digested into peptides using an enzyme of high specificity (such as trypsin). A highly specific enzyme ensures that the peptides are not too short (as almost any three or four residue peptide will be found in several database entries) and, if being analysed by MALDI-MS, that they do not fall into the low mass region (below ~500 Da) that is obscured by the presence of matrix peaks. After analysis by MS, a list of experimental peptide masses is generated that represents the peptide mass fingerprint of that particular protein. The protein is then identified by matching the experimental peptide masses to the calculated enzyme-digested peptide masses of protein entries on a database. Several parameters can be set prior to this database comparison to improve search performance. The digestion enzyme may not cleave the protein at all of the expected amino acid sites, therefore the number of missed cleavage sites can be set (usually only one or two) to allow for this. The range of experimental peptide mass values that are compared to the database can be set so that they are large enough to offer good discrimination, but not so large that they are likely to be peptides with missed cleavage sites. A peptide mass tolerance value can also be set to allow for small differences between experimental and theoretical peptide masses.

Confidence in protein identification may come from having independent supporting evidence. For example, if the protein originated from a protein band at approximately 60 kDa on a one-dimension (1-D) SDS-PAGE separation of bacterial proteins, then the anticipated result of a peptide mass fingerprint is a 60 kDa bacterial protein. If the protein with the closest peptide matches meets this expectation then the search can be deemed successful. If it is a 150 kDa protein from a different species then the search is likely to have failed. In addition to this approach, many MS search programmes provide a score for each protein match which reflects the significance of that match. The popular Mascot search engine (Perkins *et al.*, 1999) incorporates a probability based implementation of the molecular weight search (MOWSE) scoring algorithm (Pappin *et al.*, 1993). In Mascot, the significance level is normally set at 5%, so that if the score for a particular match exceeds this level there is less than a 1 in 20 chance that the observed match is a random event.

One of the main drawbacks to peptide mass fingerprinting is caused by uncertainty in the mass of intact proteins. A protein will often undergo post-translational modification and processing after expression, resulting in experimental masses that are unlikely to be the same as that of the entry in the protein database. A peptide mass fingerprint can therefore only provide the statistically most probable identification, although this is still a much more advanced method than simply counting peptide mass matches. Peptide mass fingerprinting is also mostly suited to the identification of individual proteins, since mixtures of proteins create multiple peptide mass fingerprints and make protein identification more difficult.

1.7.2.4 MS/MS ion search

An MS/MS ions search has a higher level of specificity compared to that achieved by peptide mass fingerprinting because, in addition to peptide mass, MS/MS provides information about peptide sequence. However, this information only provides detail of amino acid composition and is not easily translatable into full and unambiguous peptide sequences. The Mascot search engine again can be used to perform probability-based scoring using MS/MS data. Alternative software such as Sequest (Eng *et al.*, 1994) and MS-TAG (Mann and Wilm, 1994) are also available for MS/MS data analyses that use cross-correlation and peptide sequence tag approaches, respectively. Data from MS/MS is particularly useful when multiple proteins are present within a sample. Furthermore, relative abundance of different proteins can be estimated by the Exponentially Modified Protein Abundance Index (emPAI), which offers relative quantification of proteins in a mixture based on protein coverage by the peptide matches in a database search result.

1.7.3 Membrane protein identification

There are two main approaches to the identification of membrane proteins using MS. The first is a combination of protein separation by 1-D SDS-PAGE or 2-DE and either MS or MS/MS (the gel-based approach). The second approach combines limited protein purification followed by MS/MS (the gel-free approach).

1.7.3.1 Gel-based approach

The gel-based approach to identifying proteins by MS involves the separation of a complex mixture of proteins by either 1-D SDS-PAGE or 2-DE followed by subsequent in-gel trypsin digestion and peptide mass fingerprinting by MALDI-TOF. MALDI-TOF-TOF can also be used to perform MS/MS, which is more appropriate for analysis where more than one protein is expected to be present in a gel slice/spot.

One-dimensional SDS-PAGE is particularly useful for the separation of membrane proteins as they are readily solubilised upon heating in SDS sample buffer. The technique is also relatively quick and simple to perform, making it suitable for comparative analysis of several samples at the same time. The main disadvantage of 1-D SDS-PAGE is that proteins are separated only by molecular mass, which can result in inadequate quantification and resolution if proteins of similar molecular masses are present in the sample. However, relative quantification of different proteins within a protein band can be estimated using emPAI.

Two-dimensional electrophoresis couples isoelectric focusing in the first dimension followed by SDS-PAGE in the second dimension to allow the separation of complex mixtures of proteins by isoelectric point, molecular mass, solubility and relative abundance. However, there are limitations to the use of this technique for the separation of membrane proteins. Many membrane proteins are not solubilised in the non-detergent isoelectric focusing sample buffer due their hydrophobic nature, and those that are solubilised often precipitate at their isoelectric point (Wu and Yates, 2003). Isoelectric focusing with immobilised pH gradients also results in severe protein losses on 2-D gels as these membrane proteins do not enter the second dimension of separation (Pasquali *et al.*, 1997, Adessi *et al.*, 1997). More general disadvantages of 2-DE include the large amount of sample handling that is involved, poor reproducibility, and the extra time and labour that is required to perform the technique. Due to these limitations, 1-D SDS-PAGE is considered by some to be a more favourable strategy than 2-DE for the separation, resolution and identification of membrane proteins (Gorg *et al.*, 2004).

1.7.3.2 Gel-free approach

The gel-free proteomic approaches provides a powerful alternative to gel-based approaches. It involves using LC-ESI-MS/MS to identify proteins in complex mixtures (Hunt *et al.*, 1992). Proteins are trypsin-digested (without prior gel-based separation) and the peptide fragments separated by one or more steps of LC. The peptides are then ionised by ESI and subsequently enter the mass spectrometer, where a mass spectrum of the peptides eluting at this time point is taken. A prioritised list of peptides is generated by a computer and these are subsequently fragmented by energetic collision with gas. The resulting MS/MS spectrum is recorded and used to make protein identifications.

As with gel-based proteomic approaches the solubility of membrane proteins is also an issue for gel-free approaches. However, a number of methods have been developed to overcome this issue including the use of detergents (Han *et al.*, 2001), organic solvents (Blonder *et al.*, 2002, Goshe *et al.*, 2003) and organic acids (Washburn *et al.*, 2001). The main advantage of the gel-free approach is that it provides comprehensive coverage of all proteins within a complex protein sample without the need for prior gel-based separation. It also allows for the identification of low abundance proteins which may not be adequately resolved and identified by gel-based approaches.

1.8 Objectives of research

The objectives of this thesis were threefold. The first objective was to investigate the surface-exposure and immune specificity of OmpA among bovine and ovine *M. haemolytica* isolates. This work builds upon a previous study (Davies and Lee, 2004) which demonstrated that the amino acid sequences within hypervariable loop domains of *M. haemolytica* OmpA were very different in bovine and ovine isolates but were highly conserved among isolates recovered from the same host species. As a result of this it was hypothesised that the OmpA1 and OmpA2 proteins of bovine and ovine isolates, respectively, play an important role in adherence and are involved in host-specificity. To achieve this objective, recombinant OmpA1 (rOmpA1) and OmpA2 (rOmpA2) proteins were over-expressed, purified and used to generate anti-OmpA1 and anti-OmpA2 antibodies, respectively. These antibodies were then used to explore OmpA

surface-exposure and epitope specificity using electron microscopy and immunofluorescence techniques. Attempts were also made to crystallise rOmpA1.

The second objective was to characterise and compare the outer membrane subproteomes of seven *M. haemolytica* isolates and one *M. glucosida* isolate (that were carefully selected to represent different host origins, disease statuses, capsular serotypes, OMP-types and phylogenetic lineages) after growth in complex medium in order to identify OMPs with putative roles in host-specificity and virulence under standard growth conditions. Previous work has demonstrated that bovine serotype A1 and A6 isolates and bovine serotype A2 *M. haemolytica* isolates are genetically distinct from ovine *M. haemolytica* isolates of the same serotypes, suggesting that different subpopulations of these serotypes are associated with disease in the two animals (Davies *et al.*, 1997). Bovine and ovine isolates of these three serotypes can also be differentiated based on their OMP profiles (Davies and Donachie, 1996), suggesting that certain OMPs are likely to be involved in host specificity and virulence. Firstly, the entire repertoire of encoded OMPs in the genomes of three publicly available *M. haemolytica* genomes was predicted using a simple bioinformatic framework. Qualitative comparisons and amino acid sequence comparisons were performed on the OMPs predicted among the genomes to identify those with potential roles in host adaptation. Secondly, complementary proteomics approaches were used to identify these predicted OMPs in the outer membranes of the representative isolates after they had been grown in complex growth medium. This comparative approach provided a comprehensive overview of *M. haemolytica* and *M. glucosida* subproteomes and insights into the roles of OMPs in host-specificity, virulence and mechanisms of pathogenesis.

The third objective was to identify OMPs that were differentially expressed in the representative isolates after growth under *in vitro* conditions which were designed to mimic the *in vivo* host environment. These conditions included iron-restriction, serum-supplementation and solid-surface growth (in the absence or presence of Congo red dye). This approach allowed the identification of several potential virulence-associated proteins, many of which were not previously identified after growth in complex medium alone. Furthermore, an examination

of the extracellular subproteome of the representative isolates was undertaken after growth in iron-restricted and iron-replete media.

2. OUTER MEMBRANE PROTEIN A (OMPA) OF BOVINE AND OVINE ISOLATES OF *M. HAEMOLYTICA* IS SURFACE-EXPOSED AND CONTAINS HOST-SPECIES SPECIFIC EPITOPES

2.1 Introduction

The Gram-negative bacterium *M. haemolytica* is a commensal of cattle, sheep, and other ruminants, but also causes bovine and ovine pneumonic pasteurellosis; these infections are responsible for considerable economic losses to the livestock industries (Frank, 1989, Gilmour and Gilmour, 1989). Twelve different capsular serotypes of *M. haemolytica* have been identified to date, but A1 and A2 are the most prevalent (Highlander, 2001) and strains of these serotypes are responsible for the majority of pneumonia cases worldwide in cattle and sheep, respectively. *M. haemolytica* consists of genetically distinct subpopulations that are differentially adapted to, and elicit disease in, either cattle or sheep (Davies *et al.*, 1997, Davies and Donachie, 1996). The molecular basis of host-adaptation and host-specificity in *M. haemolytica* is not understood, but it is likely that OMPs play important roles in these processes. The publication of the genome sequence of a bovine serotype A1 *M. haemolytica* isolate (Gioia *et al.*, 2006) and, more recently, of the genome sequences of bovine and ovine serotype A2 isolates (Lawrence *et al.*, 2010a), have revealed the presence of genes that encode various OMPs. Many of these proteins serve as adhesins that are involved in host receptor-specific binding (Daigneault and Lo, 2009) or as iron transport proteins (Roehrig *et al.*, 2007).

There is growing evidence to suggest that the OmpA protein of *M. haemolytica* functions as an adhesin (Lo and Sorensen, 2007, Kisiela and Czuprynski, 2009). OmpA is a highly conserved, integral, OMP of Gram-negative bacteria that has been implicated in a diverse range of functions in different species [reviewed in (Smith *et al.*, 2007)]. It comprises an N-terminal transmembrane ß-barrel domain embedded in the outer membrane and a C-terminal globular domain which extends into the periplasm to interact with the underlying peptidoglycan

(Demot and Vanderleyden, 1994). The N-terminal domain consists of eight membrane-traversing anti-parallel ß-sheets and four relatively long, mobile, hydrophilic external loops (Pautsch and Schulz, 1998). In previous studies, molecular mass heterogeneity of OmpA was observed among bovine and ovine *M. haemolytica* isolates that correlated with the host of origin (Davies and Donachie, 1996). Subsequently, comparative nucleotide sequence analysis of the *ompA* gene from 31 *M. haemolytica* isolates revealed the presence of hypervariable domains within the four surface-exposed loops (Davies and Lee, 2004). The amino acid sequences of these domains are very different in bovine and ovine isolates but are highly conserved among isolates recovered from the same host species (Davies and Lee, 2004). The *ompA* gene can be categorised into four distinct allelic classes, I to IV. The class I (*ompA1*) alleles are associated almost exclusively with bovine *M. haemolytica* isolates, whereas the class II to IV (*ompA2* to *ompA4*) alleles occur only in ovine *M. haemolytica* isolates (Davies and Lee, 2004). Significantly, the *ompA1*- and *ompA2*-type alleles are associated with the major pathogenic lineages of bovine (ET 1) and ovine (ETs 21 and 22) isolates, respectively (16). Based on the distribution of the OmpA1 and OmpA2 proteins among bovine and ovine isolates, respectively, and the localisation of the amino acid variation to the tips of the four loops, it was hypothesised that OmpA acts as a ligand, plays an important role in adherence and is involved in host-specificity (Davies and Lee, 2004). Subsequently, it was demonstrated that OmpA is involved in the binding of serotype A1 *M. haemolytica* to bovine bronchial epithelial cells (Kisiela and Czuprynski, 2009) and that fibronectin is a potential host receptor molecule in cattle (Lo and Sorensen, 2007).

The cell envelope of *M. haemolytica* is surrounded by a layer of capsular polysaccharide (CPS) (Adlam *et al.*, 1984, Lo *et al.*, 2001) which has been implicated in a number of functions including the adherence of the bacterium to alveolar surfaces (Brogden *et al.*, 1989, Whiteley *et al.*, 1990), inhibition of complement-mediated serum killing (Chae *et al.*, 1990), and inhibition of the phagocytic and bactericidal activities of neutrophils (Czuprynski *et al.*, 1989, Walker *et al.*, 1985). Visibly thicker capsules have been observed in *M. haemolytica* during *in vitro* early log phase growth compared to stationary phase growth in both capsular serotype A1 (Corstvet *et al.*, 1982) and A2 (Sutherland

et al., 1990) isolates. Crucially, polysaccharide capsules have been shown to inhibit outer membrane adhesin function in a range of capsular types in different bacterial species (Schembri *et al.*, 2004, Virji *et al.*, 1995, Shifrin *et al.*, 2008, Favre-Bonte *et al.*, 1999). Indeed, an acapsular serotype A1 *M. haemolytica* mutant was shown to have greater fibronectin-binding activity than the capsular parental strain, suggesting a shielding role of the capsule. In other species, CPS may be downregulated upon contact with host cells (Deghmane *et al.*, 2002, Corcionivoschi *et al.*, 2009, Auger *et al.*, 2009) or as a consequence of phase variable expression (Krinos *et al.*, 2001, deVries *et al.*, 1996, Bacon *et al.*, 2001), thus allowing transient exposure of outer membrane adhesins. The shielding of OMPs, including OmpA, by CPS is likely to have important implications for the function of surface proteins but has yet to be investigated in *M. haemolytica*.

The objectives of this study were twofold. First, we wished to determine if the *M. haemolytica* OmpA protein is surface-exposed or whether it is masked by the polysaccharide capsule under various growth conditions. Second, we wanted to demonstrate whether the bovine OmpA1 and ovine OmpA2 proteins are antigenically distinct, i.e. whether antibodies raised against each of these proteins interact in a strain-specific manner. This would provide clues as to whether these proteins are likely to interact with host-cell receptors in a host-specific manner. To achieve these aims, recombinant OmpA1 (rOmpA1) and OmpA2 (rOmpA2) proteins were over-expressed, purified and used to generate anti-OmpA1 and anti-OmpA2 antibodies, respectively. These antibodies were used to explore OmpA surface-exposure and epitope specificity using electron microscopy and immunofluorescence techniques. In addition, attempts were made to determine the crystal structure of purified rOmpA1 using crystallisation screening methods and X-ray diffraction.

2.2 Materials and methods

2.2.1 Bacterial strains and growth conditions

The *M. haemolytica* isolates used in this study are shown in Table 2.1. *E. coli* strains DH5α and Rosetta 2 (DE3) pLysS were obtained from Invitrogen and Novagen, respectively. The *M. haemolytica* isolates were stored at -80°C in 50%

Table 2.1. Properties of bovine and ovine *M. haemolytica* isolates.

Isolate	ET[a]	Serotype	Host Species	Clinical Status	Site of Origin	*ompA* allele	Immunogold-labelling		Immunofluorescence	
							anti-rOmpA1	anti-rOmpA2	anti-rOmpA1	anti-rOmpA2
PH2	1	A1	Bovine	Pneumonia	Lung	*ompA1.1*	+	−	+	−
PH30	1	A1	Bovine	Healthy	Nasopharynx	*ompA1.1*	+	−	+	+/−
PH376	1	A6	Bovine	Pneumonia	Lung	*ompA1.1*	+	−	+	−
PH540	2	A1	Bovine	-	Nasopharynx	*ompA1.2*	+	−	+	−
PH202	21	A2	Bovine	Healthy	Nasopharynx	*ompA1.3*	+	−	+	+/−
PH470	21	A2	Bovine	Pneumonia	Lung	*ompA1.3*	+	−	+	−
PH494	16	A2	Ovine	Pneumonia	Lung	*ompA1.4*	+	−	+	−
PH550	17	A2	Bovine	Healthy	Nasopharynx	*ompA1.5*	+	−	+	+/−
PH8	6	A1	Ovine	Pneumonia	Lung	*ompA2.1*	−	+	+/−	+
PH284	8	A6	Ovine	Pneumonia	Lung	*ompA2.1*	−	+	+/−	+
PH66	10	A14	Ovine	-	Lung	*ompA2.1*	−	+	+/−	+
PH56	5	A8	Ovine	Pneumonia	Lung	*ompA2.2*	−	+	+/−	+
PH278	21	A2	Ovine	Pneumonia	Lung	*ompA2.3*	−	+	−	+
PH292	22	A2	Ovine	Pneumonia	Lung	*ompA2.3*	−	+	−	+
PH196	18	A2	Bovine	Healthy	Nasopharynx	*ompA3.1*	−	+/−	−	+/−
PH296	12	A7	Ovine	Pneumonia	Lung	*ompA4.1*	−	+/−	−	+/−
PH484	14	A7	Ovine	Pneumonia	Lung	*ompA4.1*	−	+/−	−	+/−
PH588	15	A13	Ovine	Pneumonia	Lung	*ompA4.2*	−	+/−	−	+/−

[a]ET = electrophoretic type (Davies *et al.*, 1997)

(v/v) glycerol in brain heart infusion (BHI) broth and were subcultured on BHI agar containing 5% (v/v) defibrinated sheep's blood (blood agar) overnight at 37°C. Liquid starter cultures were prepared by inoculating a few colonies into 10 ml volumes of BHI broth and incubating overnight at 37°C with shaking at 120 rpm. *E. coli* strain DH5α was grown in Luria-Bertani (LB) broth containing 54 µg/ml carbenicillin. *E. coli* Rosetta 2 (DE3) pLysS was grown in LB broth containing 54 µg/ml carbenicillin and 34 µg/ml chloramphenicol. Both *E. coli* strains were grown at 37°C with shaking at 120 rpm.

2.2.2 Capsule staining

The polysaccharide capsule of *M. haemolytica* was demonstrated using the Maneval method (Corstvet *et al.*, 1982).

2.2.3 Preparation of chromosomal DNAs

Bacterial cells from 1 ml of overnight culture were harvested by centrifugation for 1 min at 13,000 × *g* and washed once with 1 ml of sterile distilled H_2O (dH_2O). DNAs were prepared using an InstaGene Matrix kit (Bio-Rad) according to the manufacturer's instructions and were stored at -20°C.

2.2.4 PCR amplification and plasmid construction

The *M. haemolytica ompA* gene fragments corresponding to the transmembrane domain of OmpA1 (amino acid positions 19 to 217) and OmpA2 (amino acid positions 19 to 211) were amplified by PCR from total genomic DNA from *M. haemolytica* isolates PH2 and PH278, respectively, using the following forward and reverse primers: 5'-AAGTTCTGTTTCAGGGCCCGCAAGCTAACACTTTCTACGCAG G-3' and 5'-ATGGTCTAGAAAGCTTTAACCTTGACCGAAACGGTATG-3'. PCR products containing the *ompA1* or *ompA2* gene fragments were amplified from chromosomal DNA in 50 µl reaction mixes using Platinum Pfx DNA Polymerase (Invitrogen) with 2 mM $MgSO_4$ and 50 pmol of each forward and reverse primer. PCRs were carried out in a GeneAmp PCR System 9700 (Applied Biosystems) thermal cycler using the following amplification parameters: initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 15 s, annealing at 65°C for 30 s, and extension at 68°C for 1 min 30 s. The production of PCR amplicons of the expected size was confirmed by electrophoresis in a 1% (w/v) agarose gel and visualisation with SybrSafe (Invitrogen). The PCR

amplicons were purified with a QIAquick PCR Purification Kit (Qiagen) and eluted in 30 µl of sterile dH_2O. Yields were assessed by agarose gel electrophoresis and the DNA stored at -20°C. The PCR products were cloned into separate pOPINF vectors (Oxford Protein Production Facility) (Berrow *et al.*, 2007) according to the manufacturer's instructions. The resulting plasmids, designated *ompA1*/His-pOPINF and *ompA2*/His-pOPINF, were verified by DNA sequencing. Five microlitres of *ompA1*/His-pOPINF or *ompA2*/His-pOPINF were added to a tube containing 50 µl *E. coli* DH5α cells (as supplied by the manufacturer) and incubated on ice for 30 min. The mixtures were heated in a water bath at 42°C for 30 s and returned to ice for 2 min. Four hundred and fifty microlitres of GS96 broth (QbioGene) were added to each tube, the contents mixed thoroughly and incubated at 37°C for 1 h. Transformant colonies were selected after plating onto LB agar containing 54 µg/ml carbenicillin and incubating overnight at 37°C. An individual colony was used to inoculate 50 ml of LB containing 54 µg/ml carbenicillin and grown overnight at 37°C with shaking at 170 rpm. Five millilitres of the overnight culture were centrifuged at 5,000 × *g* for 15 min at 4°C to pellet the cells. Plasmids were recovered using a QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions and eluted in 50 µl of sterile dH_2O. Plasmid size was confirmed by electrophoresis on a 1% (w/v) agarose gel and visualisation with SybrSafe (Invitrogen). The DNA was stored at -20°C.

2.2.5 Expression of rOmpA.

Two microlitres of *ompA1*/His-pOPINF or *ompA2*/His-pOPINF plasmids recovered from *E. coli* DH5α transformant cultures were added to a tube containing 100 µl *E. coli* Rosetta 2 (DE3) pLysS cells (Novagen) as supplied by the manufacturer and incubated on ice for 30 min. The bacterial cells were heated in a water bath at 42°C for 30 s and returned to ice for 2 min. Four hundred and fifty microlitres of GS96 broth were added to each tube, the contents mixed thoroughly and incubated at 37°C for 1 h. Transformant colonies were selected after plating onto LB agar containing 54 µg/ml carbenicillin and 34 µg/ml chloramphenicol and incubating overnight at 37°C. An individual colony was used to inoculate 15 ml LB and grown overnight at 37°C with shaking at 120 rpm. One hundred and fifty microlitres of overnight culture were inoculated into 30 ml LB broth containing 54 µg/ml carbenicillin and 34 µg/ml chloramphenicol and incubated

at 37°C with shaking at 170 rpm until an OD_{600nm} of 0.7-1.0 was achieved. Thirty microlitres of 1M isopropyl-ß-D-thiogalactopyranoside (IPTG) was added and the culture incubated at 25°C with shaking at 170 rpm for a further 18 h. One and a half millilitres of overnight culture were centrifuged for 1 min at 13,000 × *g* and the pellet resuspended in 100 µl of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. The sample was heated at 100°C for 5 min and analysed by SDS-PAGE to check for the presence of recombinant protein. A second colony from the *E. coli* Rosetta 2 (DE3) pLysS transformant plate was used to inoculate 15 ml LB broth and grown overnight at 37°C with shaking at 120 rpm. Two millilitres of overnight culture were used to inoculate each of eight 2-litre Ehrlenmeyer flasks containing 1 litre of LB broth supplemented with 54 µg/ml carbenicillin and 34 µg/ml chloramphenicol and incubated at 37°C with shaking at 170 rpm until an OD_{600nm} of 0.7-1.0 was achieved. One millilitre of 1M IPTG was added and the culture incubated at 25°C with shaking at 170 rpm for a further 18 h.

2.2.6 Purification of recombinant OmpA proteins (rOmpA1 and rOmpA2)

The protein purification protocol of Zhu *et al.* (Zhu *et al.*, 2007) was followed, with modifications. Bacterial cells from each 1 litre of culture were harvested by centrifugation at 10,000 × *g* for 20 min at 4°C, resuspended in 25 ml of buffer A (25 mM Tris, 200 mM NaCl [pH 8.0]) and stored at -20°C. The cells from 2 litres of culture medium were disrupted by sonication (six cycles of 30 s on, 30 s off) and the lysates were centrifuged at 4,700 × *g* for 30 min at 4°C to pellet the inclusion bodies. These were resuspended in 1% Triton X-100 in buffer A and centrifuged at 4,700 × *g* for 30 min at 4°C. The inclusion bodies were solubilised overnight at 4°C in 50 ml of 6 M urea. The solubilised protein was added drop-wise to 500 ml of 50 mM HEPES, 300 mM NaCl, 3% N,N-dimethyldodecylamine-N-oxide (LDAO), 5 mM dithiothreitol (DTT) (pH 8.0) at 4°C and stirred slowly at 4°C for 72 h and dialysed against buffer B (50 mM Tris, 300 mM NaCl, 0.1% LDAO [pH 7.6]). The protein solution was loaded onto a 5 ml HiTrap (GE Healthcare) column equilibrated with buffer B. Bound recombinant protein was eluted from the column using an imidazole gradient (0 - 300 mM imidazole in buffer B) and collected in 5 ml fractions. Protein concentrations were quantified using a Perkin Elmer Lambda 40 UV/VIS Spectrophotometer. Ten milligrams of eluted protein were loaded onto a Superdex G-200 gel filtration column (Pharmacia

Biotech) equilibrated with buffer B and collected in 5 ml fractions after running at 0.25 ml/min overnight. Concentrations of rOmpA in the collected fractions were determined by the modified Lowry procedure (Markwell *et al.*, 1978).

2.2.7 Circular dichroism spectroscopy

Circular dichroism spectroscopy (Kelly *et al.*, 2005) was performed to examine the secondary structure of refolded PH2 rOmpA1 and PH278 rOmpA2. Mean residue ellipticity was measured between 190 and 240 nm on a Jasco J-810 Spectropolarimeter (Jasco, UK). Percentage estimates of secondary structure content (α-helix, ß-strand, turn, unordered) were calculated using the modified CONTINLL algorithm (Provencher and Glockner, 1981, Vanstokkum *et al.*, 1990) provided by the online server DICHROWEB (Lobley *et al.*, 2002, Whitmore and Wallace, 2008).

2.2.8 Anti-OmpA antibody preparation

Purified rOmpA1 and rOmpA2 were sent to Eurogentec (Belgium; http://www.eurogentec.com/eu-home.html) and used to raise antibodies in rabbits using their 87-day Classic polyclonal antibody protocol. This protocol involves the injection of 0.2 mg of protein into each of two rabbits at days 0, 14, 28 and 56. Serum bleeds were obtained before the first injection (pre-immune) and 38, 66, and 87 (final bleed) days after the initial injection

2.2.9 Serum cross-absorption

Two hundred and fifty millilitres of overnight cultures of PH2 and PH278 were centrifuged at 10,000 × *g* for 20 min at 4°C and the cells washed three times in 50 ml phosphate buffered saline (PBS). The bacterial cells were resuspended in 5 ml PBS and 1 ml aliquoted into each of five microfuge tubes. Each microfuge tube was centrifuged (13,000 × *g*, 3 min) to pellet the cells and the supernatants removed. Cells from one tube were resuspended in 1 ml of heterologous final bleed serum and incubated at 37°C for 30 min on an orbital shaker. The cells were pelleted by centrifugation and the supernatant used to resuspend the cells in a second microfuge tube. These were incubated at 37°C for 30 min on an orbital shaker. This process was repeated for the remaining three tubes. The cross-absorbed sera were diluted 1:5 with dH_2O, filter sterilised and stored at -80°C.

2.2.10 Isolation of OMPs

Briefly, OMPs of *M. haemolytica* isolates PH2 and PH278 were prepared as described by Davies *et al*. (1992). Bacterial growth was stopped by chilling the culture medium in ice-cold water for 5 min. Bacterial cells were harvested by centrifuging the culture medium at 13,000 × *g* for 20 min at 4°C. The pellet was washed in 50 ml of 20 mM Tris/HCl (pH 7.2) and centrifuged at 12,000 × *g* for 20 min at 4°C. The pellet was resuspended in 7 ml of 20 mM Tris/HCl (pH 7.2) and sonicated, on ice, for 5 min using a Soniprep sonicator (12 microns amplitude). The sonicated samples were brought to a total volume of 10 ml with additional 20 mM Tris/HCl (pH 7.2) and centrifuged at 11,000 × *g* for 30 min at 4° C to remove unbroken cells. The supernatants were centrifuged at 84,000 × *g* at 1 h for 4°C in a Sorvall ultracentrifuge to pellet the cell envelopes. The gelatinous pellets were vigourously resuspended in 0.5 % sodium *N*-lauroylsarcosine (Sarkosyl; Sigma) for 20 min at room temperature to solubilise the cytoplasmic membranes and then centrifuged at 84,000 × *g* for 1 h at 4°C to pellet the OMs. The gelatinous OMs were resuspended in 20 mM Tris/HCl (pH 7.2) and centrifuged at 84,000 × *g* for 1 h at 4°C. The final pellets were resuspended in approximately 1 ml of 20 mM-Tris/HCl (pH 7.2). Fifty microlitre aliquots of these suspensions were transferred to separate tubes and their protein concentrations determined by the modified Lowry procedure. One hundred microlitre aliquots of the OM suspensions were adjusted to 2 mg/ml with 20 mM Tris/HCl (pH 7.2) and stored at -80°C.

2.2.11 SDS-PAGE

OMPs were separated by 1-D SDS-PAGE in a 12% polyacrylamide gel using the SDS continuous system (Laemmli, 1970) and the Hoefer SE600 electrophoresis equipment as previously described (Davies *et al.*, 1992).

2.2.12 Western blotting

Briefly, Western-blotting was performed essentially as described by (Davies *et al.*, 1994b). SDS-PAGE gels were equilibrated in transfer buffer (25mM Tris, 192mM glycine [pH 8.3]) for 30 min and the proteins were transferred to nitrocellulose membranes overnight at 30 V. The nitrocellulose membranes were washed twice in TBS (20 mM Tris, 50 mM NaCl [pH 7.5]) for 5 min, blocked with

3% gelatin in TBS for 1 h and washed twice in TTBS (0.05% Tween 20 in TBS) for 5 min each. The membranes were incubated with final bleed anti-rOmpA antibody diluted 1:100 in antibody buffer (1% gelatin in TTBS) for 1 h at room temperature and washed twice in TTBS for 5 min each. This was followed by incubation with secondary horse radish peroxidase-conjugated anti-rabbit antibody diluted 1:1000 in antibody buffer for 1 h at room temperature, two 5 min washes in TTBS and one 5 min wash in TBS. The membranes were developed in a substrate solution containing 0.05% (w/v) 4-chloro-napthol (dissolved in 20 ml of ice-cold methanol) and 0.05% (v/v) hydrogen peroxide in 100 ml of TBS. Development was stopped by washing the membranes in dH_2O.

2.2.13 Immunogold electron microscopy

Bacterial cells from 25 ml volumes of 6 or 8 h BHI broth cultures, or 6 h BHI broth cultures containing 100 µM 2,2'-dipyridyl, were harvested by centrifugation at 3,500 × *g* for 5 min and washed once with PBS. Alternatively, overnight cultures of bacteria were scraped off blood agar plates and washed once in PBS. The washed cells were resuspended in PBS to an OD_{600nm} of 0.4 (equivalent to 1.0×10^8 cfu/ml). One milliliter of this suspension was centrifuged at 13,000 × *g* for 1 min and the pellet resuspended in 1 ml 4% paraformaldehyde in PBS (pH 7.2) for 30 min at room temperature. The cells were washed twice in 50 mM glycine in PBS and resuspended in 0.5 ml 0.2% bovine serum albumin (BSA) in PBS for 30 min. The cells were centrifuged and resuspended in 100 µl primary anti-rOmpA antibody (1:10 dilution) in 0.2% BSA in PBS, incubated for 1 h, washed three times in 0.2% BSA in PBS and finally resuspended in 200 µl of 0.2% BSA in PBS. Twenty microlitres of this suspension were dropped onto a freshly prepared Poly L-Lysine-coated Formvar carbon-coated nickel grid, allowed to stand for 15 min and the excess fluid removed. The grids were floated face-down on 50 µl of secondary goat anti-rabbit IgG antibody conjugated to 10 nm gold particles (1:20 dilution) in 0.2% BSA in PBS and incubated for 1 h at room temperature. The grids were washed once in 0.2% BSA in PBS, three times in PBS (1 min each), and once in 1% glutaraldehyde in PBS (pH 7.4) for 5 min. The grids were finally washed three times with filtered de-ionized water (1 min each), allowed to air dry, and the bacteria visualised using a Zeiss 912 AB energy filtering transmission electron microscope operating under standard conditions at 80 kV.

2.2.14 Immunofluorescence staining

Bacteria were grown overnight on blood agar, resuspended in 5 ml PBS and adjusted to an OD_{600nm} of 0.5. The bacterial cells were mixed 1:1 with 8% paraformaldehyde in PBS (2×) for ten min. Five microlitres of bacterial suspension were added to each well of a multiwell microscope slide (Hendley-Essex, UK), allowed to air dry, and incubated with 20 µl of primary anti-rOmpA antibody (1:100 dilution) in 0.1% BSA in PBS for 60 min at room temperature. The slides were washed three times in 0.1% BSA in PBS and incubated with 20 µl of Alexa Fluor 488 (Invitrogen) goat anti-rabbit IgG antibody (15 µg/ml in 0.1% BSA in PBS) for 30 min. The slides were washed three times in 0.1% BSA in PBS and allowed to air dry. One or two drops of fluorescence mounting medium (Dako, Sweden) were added to each slide, covered with a cover slip, and sealed with clear nail varnish. Bacteria were visualised with a Zeiss Axioskop fluorescence microscope.

2.2.15 Crystallisation and X-ray analysis of rOmpA1

Buffer B containing purified and refolded rOmpA1 was replaced with 0.6% tetraethylene glycol monooctyl ether (C_8E_4) in dH_2O using a PD-10 buffer exchange column (GE Healthcare) and the eluted rOmpA1 protein concentrated to 40 mg/ml. Crystallisation screens of rOmpA1 in 0.6% C_8E_4 were set up by mixing 1 µl protein solution and 1 µl reservoir solution in MemGold and MemSys crystallisation screening trays (Molecular Dimensions) using the sitting-drop vapour diffusion method. Full details of screening conditions are shown in Appendices 7.3 and 7.4. Crystals were transferred to reservoir solution containing a cryoprotectant, mounted on a LithoLoop (Molecular Dimensions, UK), and placed directly in a cold nitrogen-gas stream at 100 K. X-ray diffraction images were collected using the MAR345dtb image plate detector system (MAR Research, Germany).

2.3 Results

2.3.1 Recombinant OmpA proteins are comprised predominantly of β-sheets and exhibit heat-modifiability after heat treatment in the presence of SDS.

Heat-modifiability is a characteristic property of the OmpA family of proteins (Reithmeier and Bragg, 1974, Nakamura and Mizushima, 1976, Beher *et al.*, 1980). The native form of the complete OmpA protein unfolds in the presence of SDS only after heat treatment at 100°C which exposes additional SDS-binding sites (Ohnishi *et al.*, 1998, Dornmair *et al.*, 1990). When analysed by SDS-PAGE, OmpA migrates through the gel according to its structural compactness with the native protein migrating faster than the fully denatured polypeptide (Dornmair *et al.*, 1990, Kleinschmidt *et al.*, 1999). Recombinant OmpA proteins from the *M. haemolytica* bovine isolate PH2 (rOmpA1) and ovine isolate PH278 (rOmpA2) were successfully expressed, purified and refolded. Mass spectrometric analysis of gel-extracted protein bands confirmed that native and heat-modified proteins (described below) represented *M. haemolytica* OmpA (Supplementary Table S2.1). The rOmpA1 and rOmpA2 proteins were heated at a range of temperatures between 50 and 100°C for 5 min prior to SDS-PAGE and both proteins exhibited heat-modifiability when analysed by SDS-PAGE, although this occurred at different temperatures (Fig. 2.1A). The native and heat-modified proteins migrated at approximately 24 and 22.5 kDa, respectively; however, PH2 rOmpA1 unfolded at 70°C whereas PH278 rOmpA2 unfolded at 100°C (Fig. 2.1A, arrows). In contrast, the native and heat-modified forms of the complete OmpA protein recovered from the outer membrane of *M. haemolytica* isolates PH2 and PH278 migrated at approximately 32 and 38 kDa, respectively, on SDS-polyacrylamide gels (Fig. 2.1B). In this case, the full-length OmpA protein of both isolates underwent heat modification at 100°C (Fig. 2.1B, arrows). The finding that rOmpA1 unfolds more readily at a lower temperature than rOmpA2 indicates a less stable structure for rOmpA1. Circular dichroism spectroscopy analysis predicted that the secondary structures of both recombinant proteins comprised predominantly ß-sheets and turns, indicating that they have folded into a ß-barrel conformation similar to native *M. haemolytica* OmpA (Fig. 2.2). However, PH2 rOmpA1 has a lower ß-sheet composition than PH278 rOmpA2 (33.6% and

Figure 2.1. Heat-modifiability of recombinant and full-length OmpA proteins of *M. haemolytica* isolates PH2 and PH278.

Panels A and B represent purified recombinant proteins rOmpA1 (PH2) and rOmpA2 (PH278) and Sarkosyl-extracted OMPs of bovine isolate PH2 (OmpA1) and ovine isolate PH278 (OmpA2), respectively. Samples were heated for 5 min at 50, 60, 70, 80, 90 and 100°C prior to separation by SDS-PAGE. Unmodified low-molecular-mass and heat-modified high-molecular-mass forms of rOmpA (A) and full-length OmpA (B) of isolates PH2 and PH278 are indicated by thick and thin arrows, respectively.

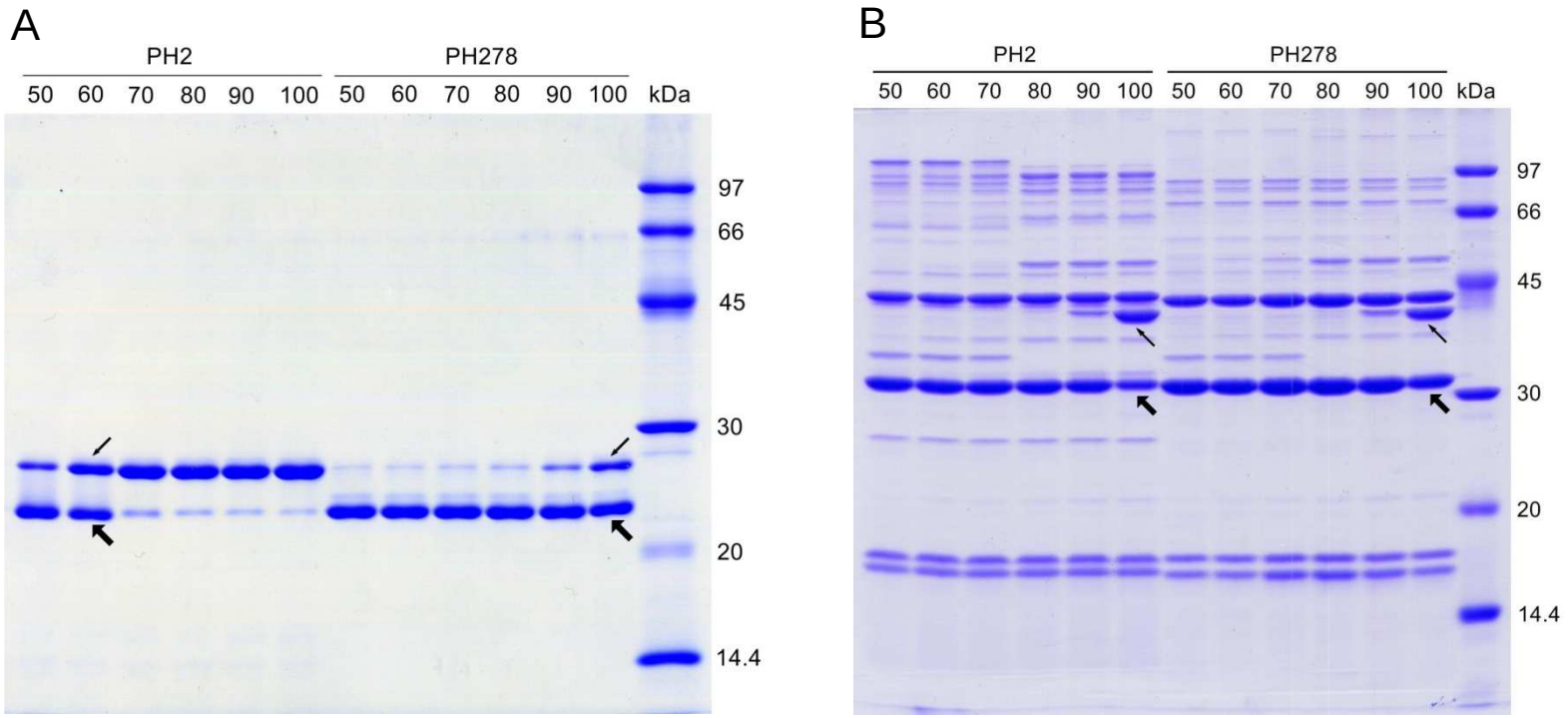
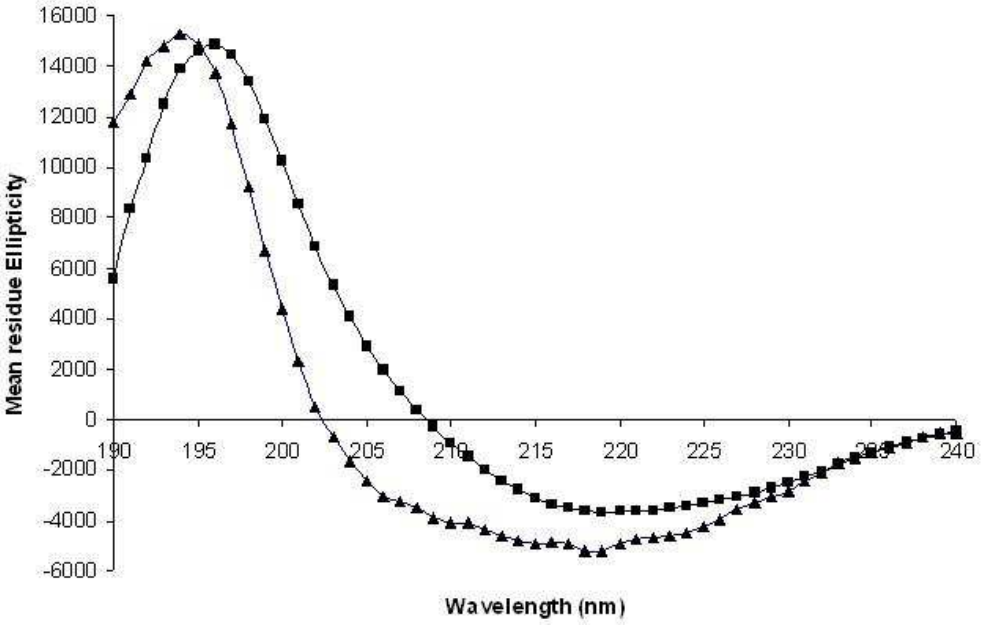


Figure 2.2. Circular dichroism spectroscopy analysis of purified rOmpA proteins.

Mean residue ellipticity was measured between 190 and 240 nm for PH2 rOmpA1 (▲) and PH278 rOmpA2 (■). Percentage estimates of secondary structure content (α-helix, ß-strand, turn, unordered) for each recombinant protein were calculated using the modified CONTINLL algorithm (Provencher and Glockner, 1981, Vanstokkum *et al.*, 1990) provided by the online server DICHROWEB (Lobley *et al.*, 2002, Whitmore and Wallace, 2008).



Protein	α-helix	β-strand	Turn	Unordered	Total
rOmpA1	0.109	0.336	0.228	0.327	1
rOmpA2	0.08	0.406	0.213	0.301	1

40.6%, respectively), which is consistent with the heat-modifiability results indicating a less stable structure for PH2 rOmpA1.

2.3.2 Anti-rOmpA1 and anti-rOmpA2 antibodies exhibit immune-specificity for the homologous proteins.

The binding specificities of the anti-rOmpA1 and anti-rOmpA2 antibodies to Sarkosyl-extracted OMPs and rOmpA from the bovine isolate PH2 and the ovine isolate PH278 were assessed by Western blotting (Fig. 2.3). Unabsorbed anti-rOmpA1 antibodies bound to the full-length OmpA and rOmpA proteins of both PH2 [Fig. 2.3A(ii)] and PH278 [Fig. 2.3B(ii)]. Similarly, unabsorbed anti-rOmpA2 antibodies bound to the full-length OmpA and rOmpA proteins of both PH2 [Fig. 2.3A(iii)] and PH278 [Fig. 2.3B(iii)]. Significantly, no other *M. haemolytica* proteins were recognised by either antibody in the complete Sarkosyl-extracted OMP samples. After cross-absorption with the heterologous isolate PH278, anti-rOmpA1 antibodies bound to PH2 OmpA and rOmpA1 [Fig. 2.3A(iv)] but not PH278 OmpA and rOmpA2 [Fig. 2.3B(iv)]. Similarly, anti-rOmpA2 antibodies cross-absorbed with the heterologous isolate PH2 bound to PH278 OmpA and rOmpA2 [Fig. 2.3B(v)] but not PH2 OmpA and rOmpA1 [Fig. 2.3A(v)]. However, the intensity of staining with the cross-absorbed antisera was noticeably weaker than for the unabsorbed antisera. These results indicate that the cross-absorbed anti-rOmpA1 and -rOmpA2 antibodies bind specifically to OmpA1 and OmpA2, respectively.

2.3.3 *M. haemolytica* OmpA is surface-exposed and recognised by anti-rOmpA antibodies *in vitro* in a strain-specific manner.

The ability of anti-rOmpA antibodies to recognise OmpA on the surface of *M. haemolytica* was assessed by immunogold labelling (Fig. 2.4) and immunofluorescent staining (Fig. 2.5). Immunogold labelling demonstrated that the OmpA proteins of *M. haemolytica* isolates PH2 and PH278 are both recognised by unabsorbed anti-rOmpA1 [Fig. 2.4A and B(i)] and anti-rOmpA2 [Fig. 2.4A and B(ii)] antibodies. However, cross-absorbed anti-rOmpA1 antibodies recognised OmpA of isolate PH2 [Fig. 2.4A(iii)] but not of isolate PH278 [Fig. 2.4B(iii)]; conversely, cross-absorbed anti-rOmpA2 antibodies recognised OmpA of isolate PH278 [Fig. 2.4B(iv)] but not of isolate PH2 [Fig. 2.4A(iv)]. Antibody binding was evenly distributed over the surface of the bacterial cells and was

Figure 2.3. Western-blot analysis of anti-rOmpA antibody binding to full-length OmpA and rOmpA of *M. haemolytica* isolates PH2 and PH278.

Panels A and B represent protein samples from bovine isolate PH2 and ovine isolate PH278, respectively. Lanes 1 and 2 represent Sarkosyl-extracted OMPs and purified rOmpA, respectively. The protein samples were separated by SDS-PAGE and either stained with Coomassie blue (i) or transferred to nitrocellulose (ii to v). The blotted proteins were probed with anti-rOmpA1 antibodies (ii), anti-rOmpA2 antibodies (iii), anti-rOmpA1 antibodies after cross-absorption with isolate PH278 (iv) and anti-rOmpA2 antibodies after cross-absorption with isolate PH2 (v). This was followed by incubation with secondary goat horse radish peroxidase-conjugated anti-rabbit antibody. Molecular mass markers (in kDa) are shown on the right.

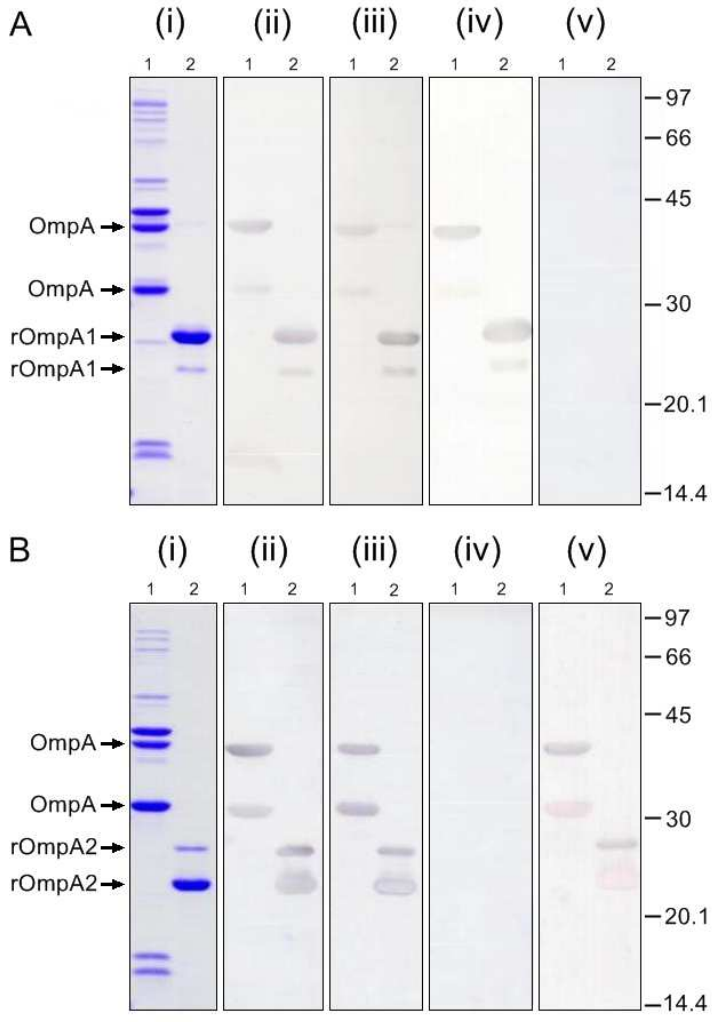


Figure 2.4. Immunogold labelling of the OmpA protein of *M. haemolytica* isolates PH2 and PH278 using anti-rOmpA antibodies.

Panels A and B represent bovine isolate PH2 and ovine isolate PH278, respectively. Isolates were incubated with anti-rOmpA1 antibodies (i), anti-rOmpA2 antibodies (ii), anti-rOmpA1 antibodies after cross-absorption with isolate PH278 (iii), and anti-rOmpA2 antibodies after cross-absorption with isolate PH2 (iv). This was followed by incubation with secondary goat anti-rabbit IgG antibody conjugated with 10 nm gold particles. Isolates were visualised by electron microscopy. Magnification was × 10,000.

Immunogold labelling images were produced by Dr. Mojtaba Noofeli.

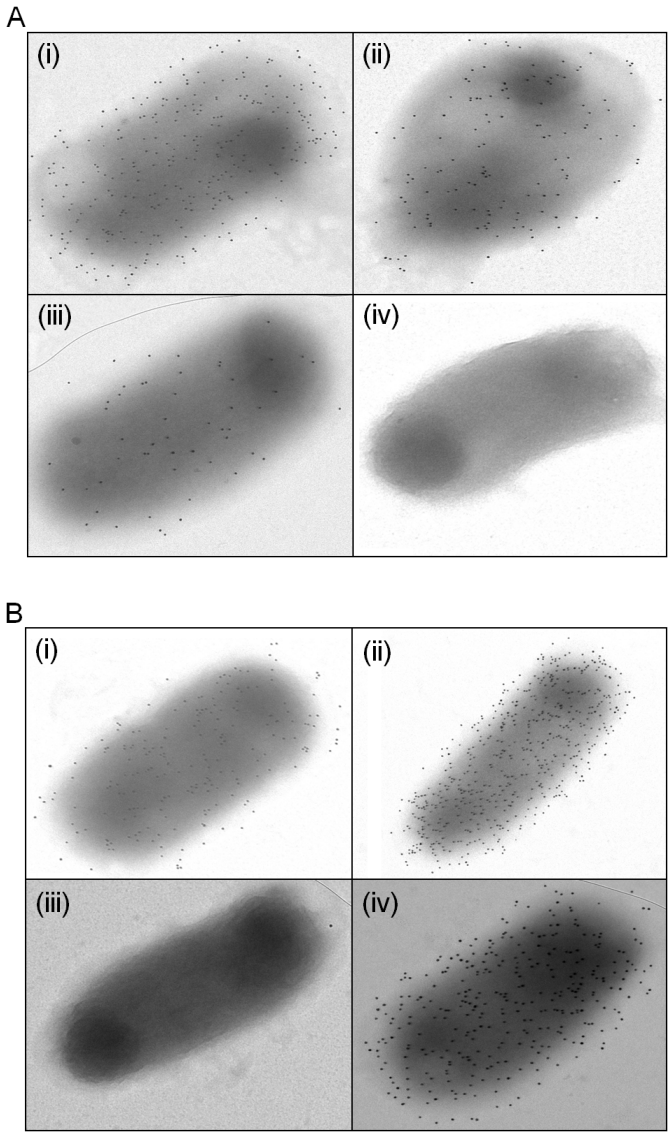
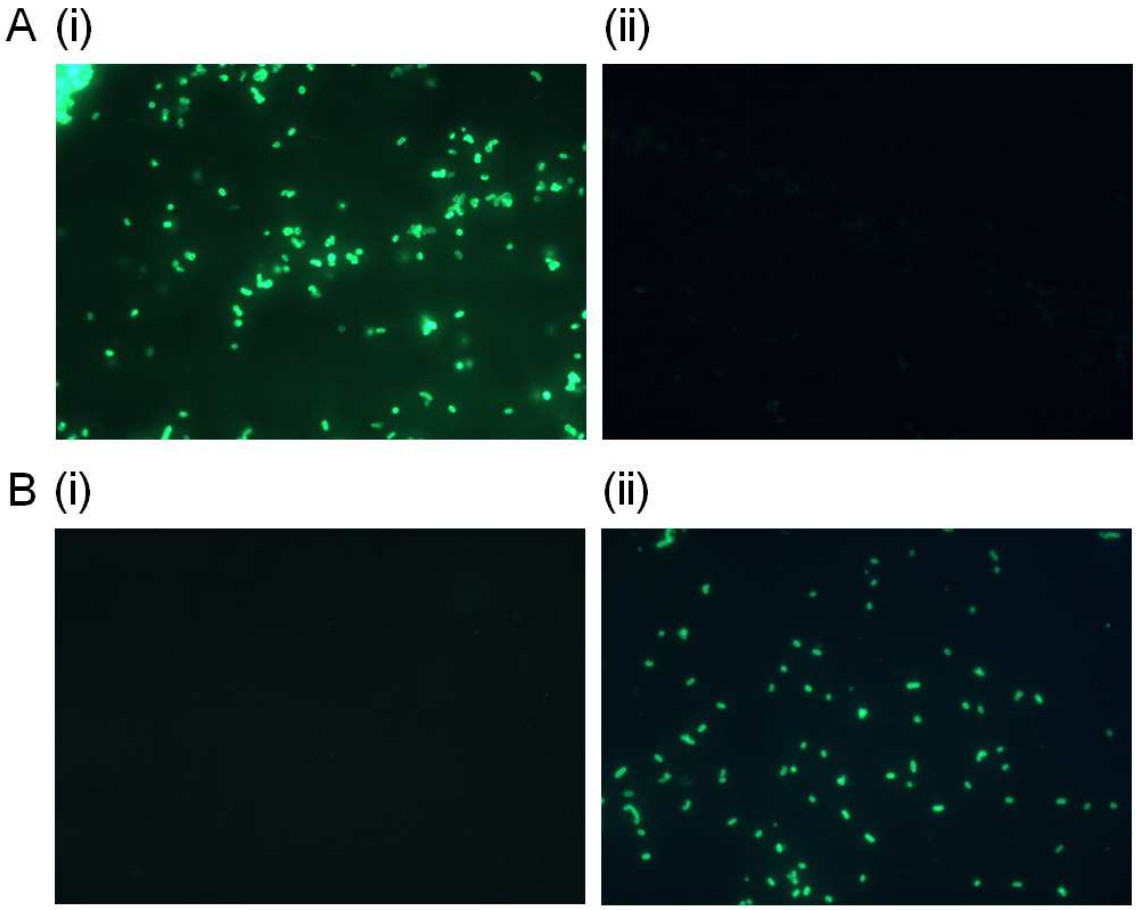


Figure 2.5. Immunofluorescent staining of *M. haemolytica* isolates PH2 and PH278 using cross-absorbed anti-rOmpA1 and anti-rOmpA2 antibodies.

Panels A and B represent bovine isolate PH2 and ovine isolate PH278, respectively. Isolates were incubated with anti-rOmpA1 antibodies after cross-absorption with isolate PH278 (i) or anti-rOmpA2 antibodies after cross-absorption with isolate PH2 (ii). These were then incubated with Alexa Fluor 488 (Invitrogen) goat anti-rabbit IgG antibody. Bound antibodies were visualised by immunofluorescence microscopy.

Immunofluorescent staining images were produced by Miss Susan Baillie.



not concentrated in any particular area. In addition, the density of labelling of isolate PH278 by the cross-absorbed anti-rOmpA2 antibody [Fig. 2.4B (iv)] was greater than that of PH2 by the cross-absorbed anti-rOmpA1 antibody [Fig. 2.4A (iii)] suggesting greater exposure of the OmpA2 protein in isolate PH278. Immunofluorescent staining confirmed that cross-absorbed anti-rOmpA1 antibodies recognised OmpA in isolate PH2 [Fig. 2.5A(i)] but not in isolate PH278 [Fig. 2.5B(i)], and that cross-absorbed anti-rOmpA2 antibodies recognised OmpA in isolate PH278 [Fig. 2.5B(ii)] but not in isolate PH2 [Fig. 2.5A(ii)].

* Immungold labelling and immunofluorescent staining work was carried out by Dr. Mojtaba Noofeli and Miss Susan Baillie, respectively, in this section and also in sections 2.3.4 and 2.3.5.

2.3.4 OmpA is surface-exposed at different stages of the growth cycle and after growth in different media.

Isolates PH2 and PH278 were grown for 6 h (logarithmic phase) and 18 h (stationary phase) in BHI broth and cells incubated with cross-absorbed anti-rOmpA1 or anti-rOmpA2 antibodies. No differences were observed in the degree of antibody binding between bacteria grown for 6 and 18 h (results not shown). These results demonstrated that there was no appreciable difference in OmpA surface-exposure between logarithmic and stationary-phase cells. Isolates PH2 and PH278 were also grown for 18 h on blood agar or in iron-restricted (100 μM 2,2'-dipyridyl) BHI broth and cells incubated with cross-absorbed anti-rOmpA1 or anti-rOmpA2 antibodies. Again, no differences were observed in antibody binding between bacteria grown in iron-restricted BHI broth or on blood agar plates compared to bacteria grown in BHI broth (results not shown). Capsulation of bacteria grown under these various conditions was also examined by Maneval staining (results not shown); there were no major differences between isolates grown under different conditions with the exception that 18 hour stationary phase cultures contained cells that were less consistent in overall size and shape. Taken together, these results demonstrated that OmpA is not masked by the polysaccharide capsule and that the degree of surface-exposure remains relatively unchanged when bacteria are grown under different growth conditions. For subsequent experiments bacterial cells were grown in BHI broth for 6 h. *Maneval staining work was carried out by Dr. Mojtaba Noofeli.

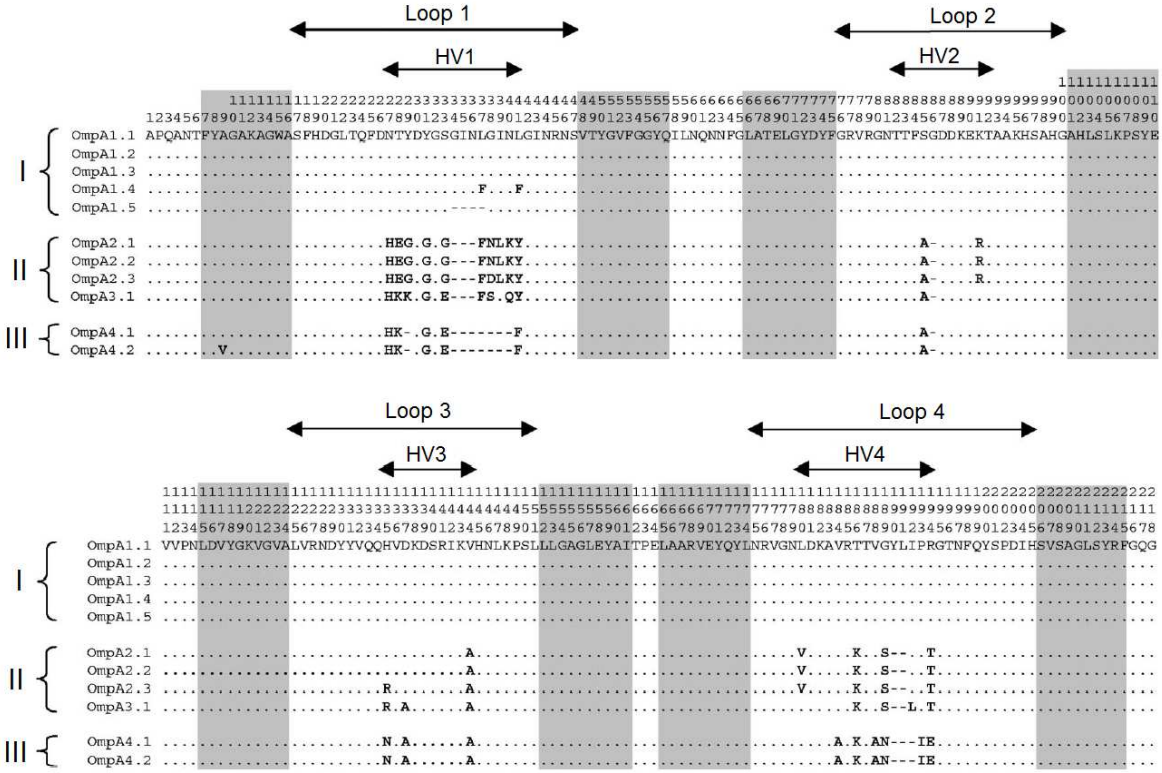
2.3.5 Binding specificities of cross-absorbed anti-rOmpA1 and anti-rOmpA2 antibodies to *M. haemolytica* isolates representing different OmpA subclasses.

Eleven distinct *ompA* alleles have previously been identified among 31 *M. haemolytica* isolates; these alleles were assigned to four subclasses, *ompA1* to *ompA4* (Davies and Lee, 2004). Individual alleles within each subclass were designated *ompA1.1*, *ompA1.2*, etc. The binding specificities of cross-absorbed anti-rOmpA1 and anti-rOmpA2 antibodies to 18 *M. haemolytica* isolates representing all eleven *ompA* alleles were determined by immunogold labelling and immunofluorescent staining (Table 2.1). Five *ompA1*-type alleles, representing the class I lineage, have been identified in *M. haemolytica* and these are associated almost exclusively with bovine isolates (Davies and Lee, 2004). The single exception, ovine isolate PH494, possesses a bovine-like LktA2-type leukotoxin (Davies *et al.*, 2001) in addition to a bovine-like OmpA1-type protein, and most likely represents a strain of bovine origin that has recently transferred to sheep. Anti-rOmpA1 antibodies were raised against bovine isolate PH2 OmpA, which possesses the *ompA1.1* allele. At the amino acid level, OmpA1.1 is identical to OmpA1.2 and OmpA1.3; OmpA1.4 differs from OmpA1.1 at two amino acid positions in HV1 and OmpA1.5 differs from OmpA1.1 in having four amino acid deletions in HV1 (Figure 2.6). Cross-absorbed anti-rOmpA1 antibodies bound to all eight OmpA1-type *M. haemolytica* isolates when examined by both immunogold labelling and immunofluorescent staining (Table 2.1). Cross-absorbed anti-rOmpA2 antibodies showed a negative binding response to all OmpA1-type isolates when examined by immunogold labelling, although weak fluorescence was detected in three isolates when examined by immunofluorescent staining (Table 2.1).

Three *ompA2*-type alleles, representing the class II lineage, have been identified in *M. haemolytica* and these are associated exclusively with ovine isolates (Davies and Lee, 2004). Anti-rOmpA2 antibodies were raised against ovine isolate PH278 OmpA, which possesses the *ompA2.3* allele. OmpA2.1 and OmpA2.2 are identical and differ from OmpA2.3 at one amino acid position in HV1 and one in HV3 (Figure 2.6). Cross-absorbed anti-rOmpA2 antibodies showed positive binding to all six OmpA2-type *M. haemolytica* isolates when examined by both immunogold labelling and immunofluorescent staining (Table 2.1). There was a

Figure 2.6 Distribution of variable amino acids in the transmembrane domains of the 11 OmpA proteins of *M. haemolytica*.

The major allele classes are represented by Roman numerals I to IV. Amino acid positions are designated above the sequences. Amino acids that match those of the first sequence are represented by dots. Gaps are indicated by dashes. The hypervariable regions within surface-exposed loops 1 to 4 are designated by HV1 to HV4. Membrane-spanning ß-strands are shaded.



negative binding response to all OmpA2-type isolates with cross-absorbed anti-rOmpA1 antibodies when examined by immunogold labelling, although weak fluorescence was detected in four isolates by immunofluorescent staining (Table 2.1).

The *ompA3.1* allele, also belonging to the class II lineage, is associated with the bovine isolate PH196. The OmpA3.1 protein was recognised by cross-absorbed anti-rOmpA2 (weakly) but not by anti-rOmpA1 antibodies when examined by immunogold labelling and immunofluorescent staining. Two *ompA4*-type alleles, representing the class III lineage, have been identified in *M. haemolytica* and these are associated with ovine serotype A7 and A13 isolates. The OmpA4-type proteins (OmpA4.1 and OmpA4.2) were recognised by cross-absorbed anti-rOmpA2 (weakly) but not by anti-rOmpA1 antibodies when examined by immunogold labelling and immunofluorescent staining.

2.3.6 Crystallisation of rOmpA1

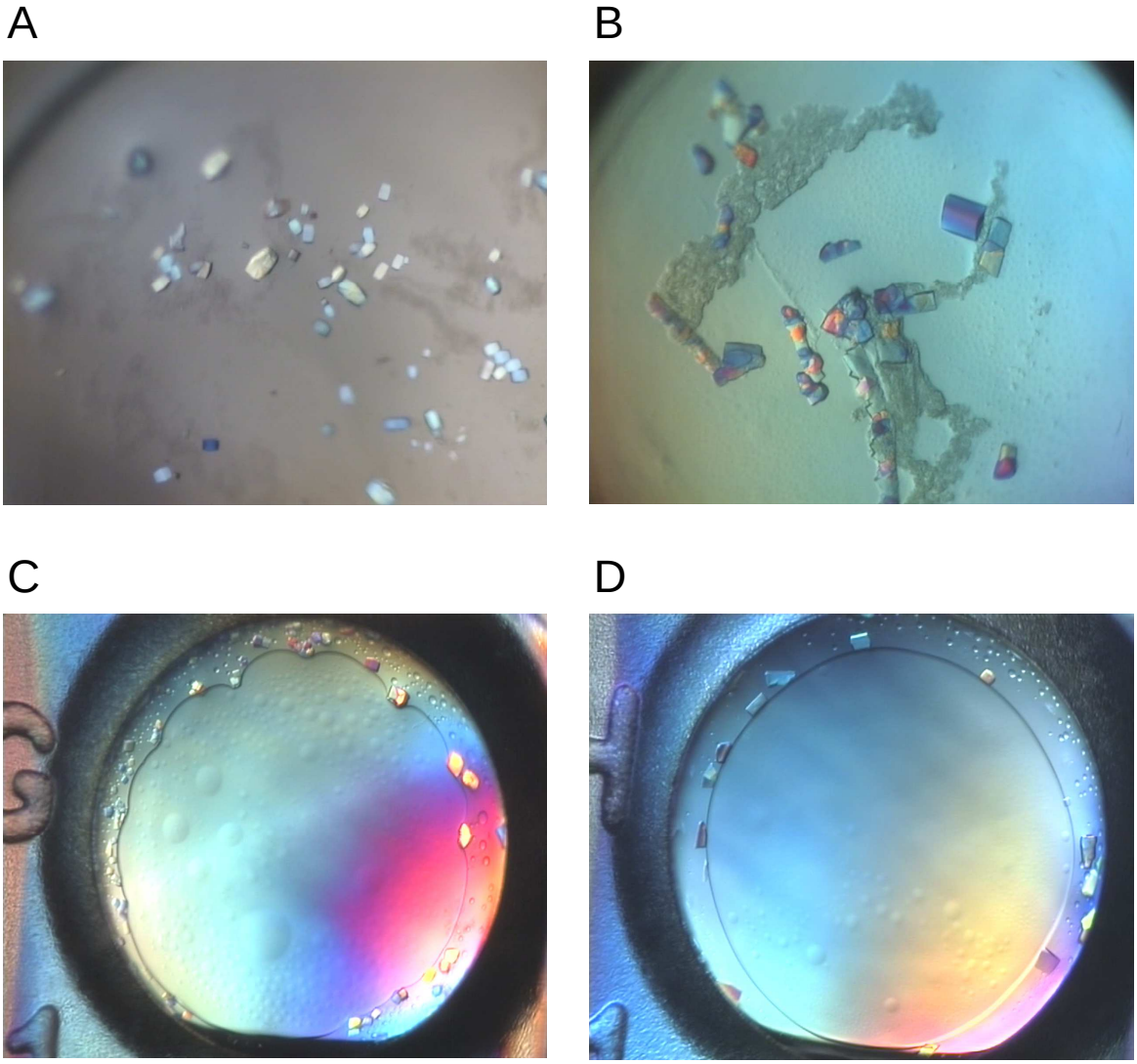
Crystals of rOmpA1 in 0.6% C_8E_4 detergent were obtained within 3 months of incubation in a MemSys crystallisation screening tray (Fig. 2.7). No crystals were obtained using the MemGold crystallisation screening tray. X-ray diffraction experiments were performed on rOmpA1 crystals from each screening condition shown in Fig. 2.7. None of the rOmpA1 crystals produced X-ray diffraction data, indicating that further optimisation of screening conditions will be necessary to improve crystal quality.

2.4 Discussion

In the present study, it has been clearly demonstrated that the OmpA protein of *M. haemolytica* is surface-exposed and able to bind anti-rOmpA antibodies *in vitro*. This was initially demonstrated in the bovine and ovine isolates PH2 and PH278 (Figs. 2.4 and 2.5), but was subsequently confirmed in a wide range of *M. haemolytica* isolates (Table 2.1). The degree of surface-exposure of OmpA in PH2 and PH278 was not affected by the stage of growth (i.e., logarithmic versus stationary phase) or the growth medium (i.e., blood agar and iron-replete or iron-restricted broth). Since a well-developed capsule was observed by Maneval staining in these isolates under different growth conditions, these findings strongly suggest that the capsule does not mask the OmpA protein since antibody

Figure 2.7. Crystallisation of rOmpA1 using a MemSys screening tray.

Panels A to D represent crystals that were obtained within three months of incubation in (A) 0.1 M MOPS, pH 7.0, 30% v/v PEG 400; (B) 0.1 M MOPS, pH 7.0, 12% PEG w/v 4000; (C) 0.1 M Na HEPES, pH 7.5, 12% w/v PEG 4000 and (D) 0.1 M sodium chloride, 0.1 M Na HEPES, pH 7.5, 12% w/v PEG 4000.



binding seemed unaffected. However, in a previous study the OmpA protein of an acapsular mutant was shown to have higher fibronectin-binding activity than the capsular parental strain (Lo and Sorensen, 2007), suggesting that the capsule may partially mask the protein.

The loops of the *M. haemolytica* OmpA protein range from 25 to 31 amino acids in length (Davies and Lee, 2004) and are estimated to extend less than 10 nm from the bacterial surface. However, the capsule of the parental strain of the acapsular mutant described above has been observed by electron microscopy to extend almost 200 nm from the bacterial surface (McKerral and Lo, 2002). It is therefore puzzling as to how the loops of OmpA are recognised by antibodies and, presumably, by host cell molecules through a capsule of this thickness. The *M. haemolytica* capsule is structurally fragile and it is possible that *in vitro* manipulation of bacterial cells causes sloughing of the capsule in some areas which exposes the tips of the loops and allows antibody binding. However, uniform immunogold labelling clearly demonstrated that OmpA is evenly distributed over the bacterial cell surface. The loops of the OmpA protein of *M. haemolytica* are longer than those of the OmpA protein of *E. coli* (Pautsch and Schulz, 1998), but not as long as the loops of other OMPs such as neisserial opacity (Opa) proteins which can extend to over fifty amino acids in length (de Jonge *et al.*, 2003). There are a number of reasons why OMPs might evolve loops of different lengths. The neisserial Opa proteins are involved in binding to host receptors and longer loops could be seen as advantageous for increasing the distance range to which they can bind host molecules. It is also reasonable to hypothesise that OMPs involved in host molecule binding might evolve loops which are long enough to traverse the capsule. However, this is contradicted by findings that long loops are still present in unencapsulated species such as *N. gonorrhoeae* and *H. influenzae*. Longer loops have also been correlated with virulence in neisserial species, where commensal organisms have shorter hypervariable domains (and thus shorter loops) than pathogenic species (Malorny *et al.*, 1998). There is evidence to suggest that in a number of bacterial species the capsule can be downregulated upon contact with host cells (Deghmane *et al.*, 2002, Corcionivoschi *et al.*, 2009, Auger *et al.*, 2009) and as a consequence of phase variable expression (Krinos *et al.*, 2001, deVries *et al.*, 1996, Bacon *et al.*, 2001), thus allowing transient exposure of OMP adhesins. Also, blebbing of

outer membrane vesicles has been observed in several Gram-negative bacteria [reviewed in (Kulp and Kuehn, 2010)], allowing OMPs to traverse the capsule and reach the extracellular environment. These phenomena have not been demonstrated in *M. haemolytica*, although they would provide appropriate mechanisms for the transient exposure of OmpA and other adhesin molecules for host-molecule binding.

A wider examination of the binding specificities of the cross-absorbed antibodies to *M. haemolytica* isolates representing different *ompA* subclasses demonstrated that anti-rOmpA1 antibodies recognise OmpA1-type (class I) proteins present in bovine isolates, but not OmpA2-type (class II) proteins that occur in ovine isolates. Conversely, cross-absorbed anti-rOmpA2 antibodies recognise OmpA2- but not OmpA1-type proteins. The ability of cross-absorbed anti-rOmpA antibodies to discriminate between OmpA proteins of the OmpA1 and OmpA2 subclasses is due to the greater degree of amino acid sequence variation (within the hypervariable domains) between each of the two subclasses compared to the variation within each subclass (Davies and Lee, 2004). There are only two variable amino acid sites within the OmpA1-type proteins; similarly, there are only two variable amino acid sites within the OmpA2-type proteins. However, there are 18 variable amino acid sites within the transmembrane domains of OmpA1 and OmpA2, all of which occur within the hypervariable regions (i.e., HV1 to HV4) located at the distal ends of the four external loops. The greater degree of variation between the two OmpA subclasses, compared to within each subclass, is sufficient to allow the cross-absorbed antibodies to discriminate between the bovine OmpA1- and ovine OmpA2-type subclasses. Overall, these results clearly demonstrate that the OmpA1 and OmpA2 proteins are sufficiently different in structure and epitope specificity that they are potentially capable of recognising host receptor molecules of differing specificities in cattle and sheep.

Previous studies have demonstrated that OmpA functions as a ligand, is involved in binding to specific host cell receptor molecules, and plays an important role in adherence and colonisation in a number of Gram-negative bacteria (Prasadarao *et al.*, 1996, Bookwalter *et al.*, 2008, Dabo *et al.*, 2003, Hill *et al.*, 2001, Reddy *et al.*, 1996, Millman *et al.*, 2001, Prasadarao, 2002, Torres and Kaper, 2003). In particular, it has been shown that the OmpA protein of bovine serotype A1 isolates of *M. haemolytica* binds to fibronectin (Lo and Sorensen,

2007) and to bovine bronchial epithelial cells (Kisiela and Czuprynski, 2009). However, a potential role of OmpA as an adhesin has yet to be investigated in ovine isolates of *M. haemolytica*. It has been previously shown that the OmpA1 and OmpA2 proteins are associated exclusively with bovine and ovine isolates, respectively, which led to the hypothesis that they are involved in binding to host-specific receptors in the upper respiratory tracts of these animals (Davies and Lee, 2004). This, in turn, might partially account for the different host-specificities of strains carrying the OmpA1 and OmpA2 protein types. Importantly, the present work has extended this previous study (Davies and Lee, 2004) by demonstrating that the binding specificities of the OmpA1 and OmpA2 proteins are very different from each other but are conserved within bovine and ovine *M. haemolytica* isolates representing different capsular serotypes and evolutionary lineages; there is no association between allele/protein type and clinical status (Table 2.1). From the findings of the present study, it is reasonable to conclude that OmpA1 and OmpA2 are capable of binding to different receptors within cattle and sheep, respectively. Although bovine fibronectin has been demonstrated to be a potential receptor for the OmpA protein of a bovine serotype A1 strain (Lo and Sorensen, 2007), it is interesting that the OmpA (P5) protein of the related species *H. influenzae* targets a different molecule, namely the carcinoembryonic antigen (CEA) family of cell adhesion molecules. Thus, it is reasonable to speculate that different molecular variants or regions of fibronectin, or even different molecules, are targeted in cattle and sheep. The production of antibodies against OmpA1 and OmpA2, and the demonstration that they are highly strain and protein specific, will allow further studies to be performed designed to investigate the role of OmpA in the adherence and colonisation of *M. haemolytica* in cattle and sheep.

Commercial vaccines have been produced and are important for the prevention of respiratory disease in both cattle and sheep (Bowland and Shewen, 2000, Hjerpe, 1990). Several studies have demonstrated the importance of *M. haemolytica* OMPs as surface antigens and their potential as vaccine components (Morton *et al.*, 1995, Confer *et al.*, 1995, Mosier *et al.*, 1989, Pandher *et al.*, 1999, Ayalew *et al.*, 2010). Antibodies against the immunogenic and surface-exposed lipoprotein PlpE contribute towards host defence (Pandher *et al.*, 1998) and the addition of recombinant PlpE to commercial vaccines significantly

enhances protection against experimental challenge (Confer *et al.*, 2003, Confer *et al.*, 2006). The incorporation of iron-regulated proteins into a vaccine was shown to enhance protection against experimental pasteurellosis in lambs (Gilmour *et al.*, 1991). An edible vaccine that expresses a fragment of the outer membrane antigen GS60 has also been developed (Lee *et al.*, 2008). Immune sera from cattle vaccinated with live or killed *M. haemolytica* cells (Mahasreshti *et al.*, 1997), and immune sera from naturally infected cattle (Zeng *et al.*, 1999), contain antibodies that recognise OmpA. In addition, in the closely related species *P. multocida*, OmpA is the major protein that cattle produce antibodies to after experimental challenge (Prado *et al.*, 2005). Clearly, OmpA is an important immunogen that should be considered for inclusion in *M. haemolytica* subunit vaccines. However, a critical property of any vaccine candidate is a high degree of amino acid conservation throughout bacterial populations. Hypervariability of surface-exposed loop regions is a common occurrence within OMPs of many bacterial species and poses a serious problem for vaccine design (Webb and Cripps, 1998, Duim *et al.*, 1997, Bolduc *et al.*, 2000, Martin *et al.*, 2000). It was previously demonstrated that four distinct OmpA classes occur within bovine and ovine *M. haemolytica* strains; OmpA1 and OmpA2 are associated with those strains that are responsible for the majority of disease in cattle and sheep, respectively, and have very different hypervariable domains within their surface-exposed loops (Davies and Lee, 2004). In the present study, it has been shown that antibodies specific for the surface-exposed loops of OmpA1 will not recognise the same regions of OmpA2 and *vice versa*. Therefore, an animal exposed to OmpA1 will generate anti-OmpA antibodies that are unlikely to confer protection against *M. haemolytica* isolates possessing OmpA2 and *vice versa* (although some cross-reacting antibodies may be present). These findings clearly have important implications with regard to vaccine development and, in particular, to the inclusion of OmpA in subunit vaccines that are designed to provide universal protection against heterologous *M. haemolytica* isolates. Essentially, an OmpA-based *M. haemolytica* subunit vaccine would need to include OmpA from more than one class to provide universal protection against heterologous strains.

3. COMPARATIVE OUTER MEMBRANE PROTEOMIC ANALYSES OF BOVINE AND OVINE ISOLATES OF *M. HAEMOLYTICA* AND *M. GLUCOSIDA* GROWN IN COMPLEX MEDIUM

3.1 Introduction

The Gram-negative bacterium *M. haemolytica* is the etiological agent of bovine and ovine pneumonic pasteurellosis, infections that are responsible for considerable economic losses to the livestock industries (Highlander, 2001). Twelve different capsular serotypes of *M. haemolytica* have been identified to date. Serotype A1 isolates are most commonly associated with cases of bovine pneumonic pasteurellosis, although serotype A6 isolates are responsible for an increasing proportion of disease cases. Serotype A2 isolates are often recovered from the nasopharynxes of healthy cattle but seldom cause disease. In contrast, serotype A2 isolates are responsible for the majority of disease cases of ovine pneumonic pasteurellosis. A wide range of other serotypes are also associated with sheep, including serotypes A1 and A6, although these are recovered much less frequently than serotype A2 isolates. *M. glucosida* was previously classified as the A11 serotype of *M. haemolytica* and comprises a heterogeneous group of organisms with low virulence that are mainly opportunistic pathogens of sheep (Angen *et al.*, 1999a, Davies *et al.*, 1997). Bovine serotype A1 and A6 isolates and bovine serotype A2 *M. haemolytica* isolates are genetically distinct from ovine *M. haemolytica* isolates of the same serotypes, suggesting that different subpopulations of these serotypes are associated with disease in the two animals (Davies *et al.*, 1997). Bovine and ovine isolates of these three serotypes can also be differentiated based on their OMP profiles (Davies and Donachie, 1996) and nucleotide sequence variation of the ompA gene (Davies and Lee, 2004). These observations suggest that certain OMPs are likely to be involved in host specificity and virulence. However, the mechanisms by which OMPs facilitate host-specific infection and disease in *M. haemolytica* are poorly understood.

The outer membrane is an asymmetrical lipid bilayer comprising an inner leaflet of phospholipid and an outer leaflet of mainly LPS. It serves as a selective

barrier for the passage of nutrients and other materials into and out of the cell and is at the interface between bacterium and host. Two types of proteins are also present in the outer membrane: integral OMPs and lipoproteins. Integral OMPs span the outer membrane with amphipathic antiparallel ß-strands that adopt a barrel-like conformation. Outer membrane lipoproteins are anchored to the outer membrane by N-terminal lipid modifications made at the inner membrane. The majority of proteins that are destined to be located in the outer membrane contain specific protein modifications, sorting signals and structural signatures that can be differentiated using bioinformatic prediction tools (Gromiha, 2005, Gromiha and Suwa, 2006, Jackups *et al.*, 2006, Juncker *et al.*, 2003). These predictors have been used to identify and quantify OMPs of several Gram-negative bacteria (Diaz-Mejia *et al.*, 2009, Berven *et al.*, 2006, Boyce *et al.*, 2006, Huntley *et al.*, 2007, Viratyosin *et al.*, 2008). A simple bioinformatic prediction framework encompassing three categories of bioinformatic predictors (and 10 prediction programmes) was recently developed to predict the total number of OMPs encoded in two *P. multocida* genomes (E-Komon *et al.*, 2011b). Complementary proteomic analyses can also be implemented to identify these putative OMPs in outer membrane fractions isolated from the bacterial cell envelope. Different proteomic methods have been used to characterise the outer membrane subproteome of several Gram-negative bacteria (Liu *et al.*, 2008, Molloy *et al.*, 2000, Cordwell *et al.*, 2008, Veith *et al.*, 2009). including the related species *P. multocida* (Boyce *et al.*, 2006, E-Komon *et al.*, 2011a) and *A. pleuropneumoniae* (Chung *et al.*, 2007).

In *M. haemolytica*, much effort has been given to comparing OMP expression between different isolates (Davies and Donachie, 1996, Davies *et al.*, 1992, McCluskey *et al.*, 1994, Morton *et al.*, 1996), characterising individual OMPs (Davies and Lee, 2004, Nardini *et al.*, 1998, Pandher and Murphy, 1996, Cooney and Lo, 1993, Lo *et al.*, 1991), and identifying those of immunological importance (Pandher *et al.*, 1999, McVicker and Tabatabai, 2002). In a recent study an immunoproteomic approach was used to identify potential vaccine candidate antigens in a bovine serotype A1 isolate (Ayalew *et al.*, 2010). A number of studies have also investigated the roles of specific OMPs in *M. haemolytica* adherence to host tissue and cells (Kisiela and Czuprynski, 2009, Daigneault and Lo, 2009, Lo and Sorensen, 2007, De la Mora *et al.*, 2006).

However, a comprehensive analysis of the *M. haemolytica* outer membrane subproteome has yet to be performed.

The objectives of this study were twofold. First, the entire repertoire of OMPs encoded in the genomes of a bovine serotype A1 *M. haemolytica* isolate (Gioia *et al.*, 2006) and two serotype A2 isolates (one bovine and one ovine) (Lawrence *et al.*, 2010a) were predicted using the same bioinformatic prediction approach developed for *P. multocida* (E-Komon *et al.*, 2011b). Comparative amino acid sequence analyses were also performed to identify predicted OMPs that could be involved in host-specific adaptation. Second, the OMPs present in the outer membrane fractions of seven *M. haemolytica* isolates which were carefully selected to represent different host species, disease statuses, capsular serotypes, OMP-types and phylogenetic lineages (Davies *et al.*, 1997, Davies and Donachie, 1996) were identified and compared using a combination of gel-based and gel-free proteomic approaches. The outer membrane fraction of a single *M. glucosida* isolate was also investigated for comparison. This is the first study to provide a comparative analysis of the outer membrane subproteomes of multiple isolates of *M. haemolytica*. This comparative approach will provide insights into the roles of OMPs in host-specificity in bovine and ovine hosts and identity putative virulence determinants and mechanisms of pathogenesis.

3.2 Materials and methods

3.2.1 Bioinformatic prediction of genome-encoded OMPs

The publicly available genomes of a bovine serotype A1 *M. haemolytica* isolate (GenBank ID: AASA00000000), an ovine serotype A2 isolate (GenBank ID: ACZX00000000) and a bovine serotype A2 isolate (GenBank ID: ACZY00000000) were used for all bioinformatic analyses. All *M. haemolytica* protein sequences (2695, 2682 and 2552 open reading frames from the bovine serotype A1, ovine serotype A2 and bovine serotype A2 genomes, respectively) were retrieved from NCBI. Each genome was scrutinised by bioinformatic approaches according to the workflow described by E-Komon *et al.* (2011b) to predict proteins which localise to the outer membrane. Each genome was analysed by three categories of bioinformatic prediction software, using a total of ten prediction tools. Subcellular localisation predictors included Proteome Analyst (Szafron *et al.*,

2004), PSORTb (Gardy *et al.*, 2005), CELLO (Yu *et al.*, 2006) and SOSUI-GramN (Imai *et al.*, 2008); ß-barrel predictors included TMB-Hunt (Garrow *et al.*, 2005), TMBETADISC-RBF (Ou *et al.*, 2008), MCMBB (Bagos *et al.*, 2004) and BOMP (Berven *et al.*, 2004); and outer membrane lipoprotein predictors included LipoP (Juncker *et al.*, 2003) and LIPO (Berven *et al.*, 2006). A consensus prediction framework was followed whereby proteins that were predicted to (a) be localised to the outer membrane by at least two subcellular localisation predictors, (b) have a ß-barrel conformation by at least three ß-barrel predictors or (c) be outer membrane lipoproteins by at least one lipoprotein predictor, were considered to be putative OMPs. In several instances, two predicted proteins within a genome were determined to constitute a single functional protein and were therefore grouped as such. A list of putative OMPs within each genome was produced by integrating the results from each of the predictor categories. A final integrated list of putative OMPs from all three genomes was produced by performing BLAST searches to determine whether each putative OMP was present in one, two or all three genomes. When a putative OMP was present in only one or two genomes, a protein BLAST search was performed against all annotated proteins to determine whether it was actually present or not in these other genome(s). Similarly, nucleotide BLAST searches were performed against whole genome shotgun contigs (ctgs) in these other genomes to determine whether a gene encoding an OMP was present but had not been annotated. Proteins with close homology at the amino acid level in more than one genome were assumed to have similar functions and were assigned the same protein name. The putative OMPs were further scrutinised using additional domain, homology and literature searches to assign likely functions and to predict their subcellular localisations with greater confidence. Based on this further information, each putative OMP was assigned to one of three localisation categories: (1) confidently predicted OMPs, (2) putative OMPs without confidently predicted subcellular location, and (3) false positives. Physiochemical properties including molecular mass, theoretical pI, aliphatic index, grand average of hydropathicity (GRAVY) score, number of ß-strands and helices, of putative OMPs were predicted by ProtParam (Gasteiger *et al.*, 2005), TMHMM (Krogh *et al.*, 2001), and TMBETA-NET (Jackups *et al.*, 2006).

3.2.2 Bacterial isolates and growth conditions

The eight bacterial isolates used in the present study are shown in Table 3.1. These included seven representative *M. haemolytica* isolates recovered from cattle (two) and sheep (five) and one *M. glucosida* isolate. These isolates were selected to represent the major clonal groups and electrophoretic types (ETs) that were previously identified by multilocus enzyme electrophoresis (MLEE) (Davies *et al.*, 1997) (Fig. 3.1) and comparative analyses of capsular serotypes, OMP profile types and disease statuses (McCluskey *et al.*, 1994, Davies and Donachie, 1996) (Table 3.1). Isolates were stored at -80°C in 50% (v/v) glycerol in brain heart infusion (BHI) broth and were subcultured on BHI agar containing 5% (v/v) defibrinated sheep's blood overnight at 37°C. For preparation of outer membrane fractions, liquid starter cultures were prepared by inoculating a few colonies into 15 ml volumes of BHI broth and incubating overnight at 37°C with shaking at 120 rpm. Eight hundred microlitres of overnight culture were used to inoculate a 2-litre Ehrlenmeyer flask containing 400 ml of BHI broth and incubated at 37°C with shaking at 120 rpm until an OD_{600nm} of 0.8-0.9 was achieved.

3.2.3 Preparation of OMPs.

Outer membrane proteins were prepared by Sarkosyl extraction as previously described in section 2.2.10.

3.2.4 Gel-based proteomic analysis

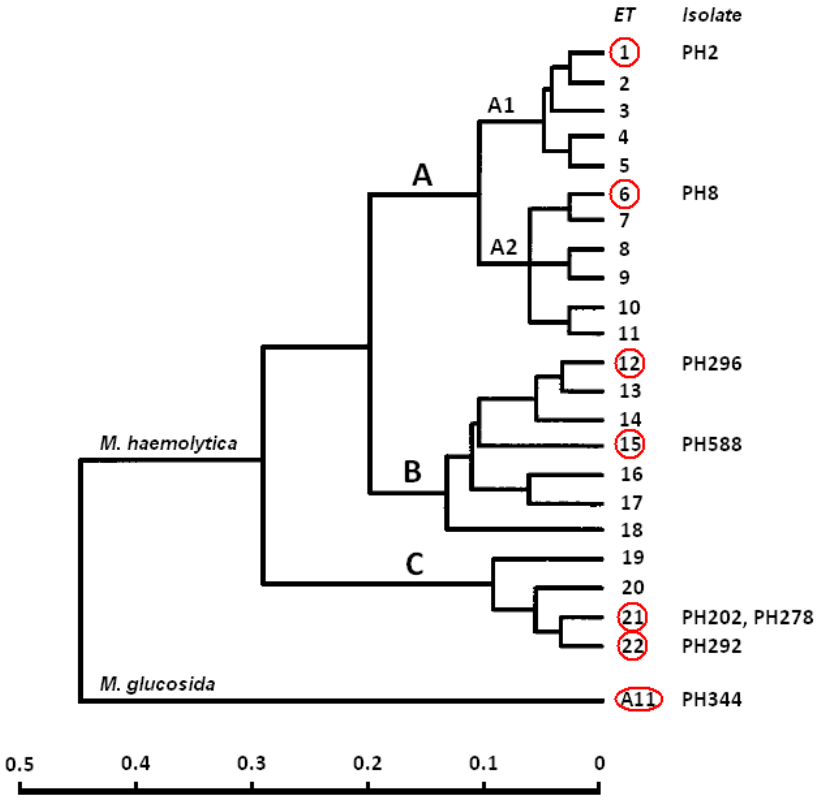
Twenty micrograms of each OMP preparation were separated by 1-D SDS-PAGE in a 12% linear polyacrylamide gel using the SDS discontinuous system (Laemmli, 1970) and the Hoefer SE600 electrophoresis equipment as previously described (Davies *et al.*, 1992, McCluskey *et al.*, 1994, Davies and Donachie, 1996, Davies *et al.*, 1994a). Proteins were visualised by staining with Coomassie brilliant blue. A total of 444 gel pieces, including both gel bands and gel fractions without protein bands from all isolates were manually excised and each gel piece placed into separate wells of 96-well plates. Automated in-gel trypsin digests were completed using an Ettan Spot Handling Workstation (Amersham Biosciences) according to the procedure described by Bridges *et al.* (Bridges *et al.*, 2008). Briefly, gel pieces were washed three times in 100 µl of 50 mM ammonium

Table 3.1. Properties of seven representative *M. haemolytica* isolates and one *M. glucosida* (PH344) isolate.

Isolate	Animal host	Electrophoretic type[a]	Capsular serotype	OMP type	Disease status
M. haemolytica					
PH2	Bovine	1	A1	1.1.1	Pneumonia
PH8	Ovine	6	A1	1.2.1	Pneumonia
PH202	Bovine	21	A2	2.1.2	Healthy
PH278	Ovine	21	A2	2.2.2	Pneumonia
PH292	Ovine	22	A2	2.2.1	Pneumonia
PH296	Ovine	12	A7	3.1.1	Pneumonia
PH588	Ovine	15	A13	3.3.2	Pneumonia
M. glucosida					
PH344	Ovine	N/A	A11	3.2.2	Septicaemia

Figure 3.1. Evolutionary relationships of seven representative *M. haemolytica* isolates and one *M. glucosida* (PH344) isolate, based on MLEE analysis.

Figure adapted from Davies *et al*. (1997).



bicarbonate, 50% v/v methanol and then twice in 100 µl of 75% v/v acetonitrile (ACN) before drying. Gel pieces were rehydrated with trypsin solution [20 µg trypsin/ml (Promega) in 20 mM ammonium bicarbonate] and incubated at 37°C for 4 h. Peptides were extracted by washing the gel pieces twice in 100 µl of 50% v/v ACN, 0.1% v/v trifluoroacetic acid (TFA), before being transferred in solution to a new 96-well plate and dried. Dried peptide samples were stored at -20°C until analysed by MALDI-TOF-TOF MS and/or LC-ESI-QqTOF MS.

3.2.5 Gel-free proteomic analysis

Outer membrane fractions were directly digested with trypsin without prior separation by 1-D SDS-PAGE using the methanol-aided trypsin digestion protocol as previously described by Bridges *et al.* (Bridges *et al.*, 2008). Briefly, twenty microlitres of 4 mg/ml protein was resuspended in 44 µl of 50 mM ammonium bicarbonate and placed in a sonicator bath for 20 min (with regular vortexing), before being incubated at 60°C for 20 min. Samples were placed on ice for 3 min before adding 60 µl of methanol and incubating for a further 5 min in the sonicator bath with regular vortexing. Sixteen microlitres of 200 µg/ml trypsin (Promega) in 25mM ammonium bicarbonate was added followed by 60 µl of methanol. After vortexing briefly samples were incubated at 37 °C for 12-16 h. The digested samples were dried down in an Eppendorf SpeedVac and stored at -20°C until analysis by mass spectrometry.

3.2.6 MALDI-TOF-TOF MS and data analysis

One microlitre of peptide solution was mixed with an equivalent volume of matrix solution (10 mg cyano-4-hydroxycinnamic acid in 500 µl 50% ACN, 0.1% TFA) on a MALDI-TOF target plate. Peptides were analysed using an Applied Biosystems 4700 Proteomics Analyzer. MS/MS was performed on the ten most intense precursor ions in each peptide sample. GPS Explorer Software (Applied Biosciences) was used to automate submission of collected data to MASCOT for searching the NCBI Eubacteria protein database with methionine oxidation selected as a variable modification, peptide and MS/MS tolerances of 1.2 and 0.5 Da, respectively, with one missed cleavage allowed. Only proteins identified with a significant MOWSE score ($p \leq 0.05$) were accepted. Each peptide sample was analysed three times. Any unidentified peptide samples were further analysed by LC-ESI-QqTOF MS.

3.2.7 LC-ESI-QqTOF MS and data analysis

The peptide samples obtained by gel-free trypsin-digestion were analysed by ESI-MS on a QSTAR XL Hybrid LC/MS/MS System according to the parameters described in Bridges *et al*. (Bridges *et al.*, 2008). Briefly, MASCOT Daemon Software (Matrix Science) was used to automate submission of collected data to MASCOT for searching the NCBI Eubacteria protein database with methionine oxidation selected as a variable modification, peptide and MS/MS tolerances of 1.2 and 0.4 Da, respectively, with one missed cleavage allowed. Only proteins identified with a significant MOWSE score ($p \leq 0.05$) were accepted.

3.3 Results

3.3.1 Bioinformatic prediction of OMPs in three *M. haemolytica* genomes.

Ten different bioinformatic tools were used to predict putative OMPs encoded in the fully-sequenced genomes of three *M. haemolytica* isolates (bovine serotype A1, ovine serotype A2 and bovine serotype A2) following the bioinformatic workflow of E-Komon *et al*. (2011b). These tools were categorised into three groups: subcellular localisation predictors (PA, PSORTb, CELLO, SOSUI-GramN), ß-barrel protein predictors (TMB-Hunt, TMBETADISC-RBF, BOMP, MCMBB), and outer membrane lipoprotein predictors (LIPO and LipoP). One hundred and sixty-four, 174 and 163 unique protein annotations (Supplementary Tables S3.1, S3.2 and S3.3) were predicted to be putative OMPs in the bovine serotype A1, ovine serotype A2 and bovine serotype A2 genomes, respectively, after following a consensus prediction framework and subsequently integrating the results within each genome. In some instances, two predicted annotations within a genome were deemed to comprise a single protein and were subsequently paired together to represent one protein. This reduced the number of putative OMPs to 164, 168 and 154 in the bovine serotype A1, ovine serotype A2 and bovine serotype A2 genomes, respectively. BLAST searches were performed on putative OMP amino acid sequences to determine if each was present in one, two or all three genomes. In some instances a protein was predicted to be a putative OMP in one or two genomes, but was not predicted in the other genome(s) despite its presence in that genome(s). This most often occurred when a protein annotation in one proteome had been annotated so that an important signal motif, such as

an N-terminal signal sequence, had been excluded from the annotation and hence not recognised by the bioinformatic predictors. Similarly, nucleotide BLAST searches were also performed against whole-genome shotgun contigs in each genome to determine if OMP-encoding genes were present that had not been annotated. Proteins which fell into these categories were included in the predicted proteins in their respective genomes. This amended the numbers of putative OMPs to 196, 202 and 188 in the bovine serotype A1, ovine serotype A2 and bovine serotype A2 genomes, respectively. A total of 217 unique putative OMPs were predicted across the three genomes. Of these proteins, 102 (47%), 67 (31%) and 114 (53%) were predicted by subcellular localisation, ß-barrel and outer membrane lipoprotein predictor categories, respectively. The 217 putative OMPs were further scrutinised using additional domain, homology and literature searches to predict the subcellular location of each protein with greater confidence and to remove false positives (Fig. 3.2). In this way, 93 (42.9%) proteins were confidently predicted to localise at the outer membrane (Table 3.2). Seventy proteins could not be predicted with great confidence to be localised to a particular subcellular compartment and were likely to include false positives as well as some true OMPs (Supplementary Table S3.4). Furthermore, 54 proteins were predicted to be localised to the cytoplasm, inner membrane, periplasm, or extracellular compartment and were therefore determined to be false-positives (Supplementary Table S3.5). Of the 93 confidently predicted OMPs, 63 (68%) were predicted by subcellular localisation predictors, 44 (47%) by ß-barrel predictors, and 39 (42%) by outer membrane lipoprotein predictors (Fig. 3.3A). Eighty-nine proteins were present in the bovine serotype A1 genome, 92 in the ovine serotype A2 genomes and 92 in the bovine serotype A2 genome (Fig. 3.3B), with 88 being present among all three genomes. Five OMPs were present in either one or two genomes (Table 3.3). Ninety-two OMPs were present among both bovine and ovine genomes, of which four were exclusively present in both the bovine and ovine serotype A2 genomes One OMP (PulD), was exclusively present in only the bovine serotype A1 genome. There were no OMPs present exclusively in either of the serotype A2 genomes. The finding that there were more OMPs present in both the bovine and ovine serotype A2 genomes (four) than between the bovine serotype A1 genome and either the bovine serotype A2 genome (zero) or the ovine serotype A2 genome (zero) is consistent with the hypothesis of serotype A2 host switching from cattle

Figure 3.2. Subcellular locations of 217 putative OMPs predicted by 10 bioinformatic prediction tools across three *M. haemolytica* genomes after further domain, homology and literature searches had been carried out on each protein.

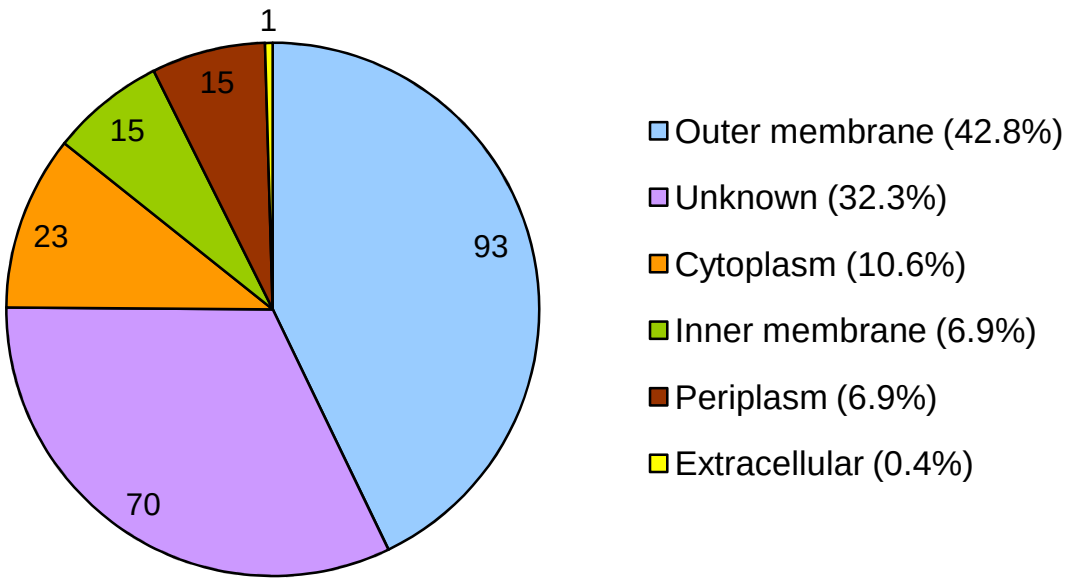


Table 3.2. Functional classifications of 93 confidently predicted OMPs encoded by three *M. haemolytica* genomes.

Name	Bovine A1[a]	Ovine A2[a]	Bovine A2[a]	Protein function	Sub[b]	TM[b]	Lipo[b]	Isol[c]
1. Outer membrane biogenesis and integrity								
OmpA	MHA_1054	COI_1980	COK_0402	Outer membrane integrity	+	+/−	−	8
Omp85	MHA_0691	COI_1174	COK_1967	Correct OMP folding and assembly	+	+	−	8
Imp/LptD	MHA_0291	COI_2627	COK_0922	LPS assembly	+	+	−	8
Pal	MHA_0263	COI_2595	COK_1835	Cell envelope integrity/peptidoglycan anchor	+	−	+	8
VacJ	MHA_2837	COI_1320	COK_1388	Phospholipid homeostasis	−	−	+	7
LppB/NlpD	MHA_1804	COI_0885	COK_0647	Cell wall formation and maintenance	+	+	+	4
RlpB/LptE	MHA_0669	COI_1194	COK_1987	LPS assembly	−	−	+	3
MltC	MHA_0242	COI_0470	COK_0285	Peptidoglycan maintenance and processing	+	−	+	3
ComL	MHA_1560	COI_0129	COK_2068	Outer membrane biogenesis/DNA transport	+	−	+	3
MltA	MHA_1133	COI_1670	COK_0590	Peptidoglycan maintenance and processing	+	+	+	2
LolB	MHA_2544	COI_0062	COK_2183	Chaperone & lipoprotein transport activity	+	−	+	
NlpE	MHA_1616	COI_0339	COK_2132	Cell envelope stress response/copper homeostasis regulator	−	−	+	
SmpA	MHA_1340	COI_0370	COK_0445	Cell envelope integrity and β-barrel protein assembly	−	−	+	
WzzB	MHA_0727	COI_1084	COK_0353	Lipopolysaccharide biosynthesis	+	−	−	
MltB	ctg173_107	COI_2519	COK_0949	Peptidoglycan maintenance and processing	−	−	+	

[a] Proteins annotations are shaded in grey if they were not predicted by bioinformatic software but have a homologue that was predicted in another genome.

Proteins annotations which were predicted by bioinformatic software are not shaded. Absence of protein homologue is shown by '−'

[b] Subcellular localisation, transmembrane β-barrel and lipoprotein prediction result; '+' = predicted in all genomes; '−' = not predicted in any genome;

+/−' = predicted in one/two genomes (see Supplementary tables 1-3 for specific result)

[c] The number of representative isolates that each protein has been identified in (see Table 3 and Figure 4)

Table 3.2. (continued)

Name	Bovine A1	Ovine A2	Bovine A2	Protein function	Sub	TM	Lipo	Isol
2. Transport and receptor								
TolC	MHA_1410	COI_0732	COK_0022	Protein secretion/transporter activity	+	+	+	8
OmpP1/FadL	MHA_0639	COI_1221	COK_2014	Hydrophobic compound transport	+	+	−	8
Wza	MHA_0527	COI_1313	COK_0141	Capsular polysaccharide transport	+	+	+	8
OmpP2-like	MHA_1793	COI_0051	COK_2166	Porin/ion transport activity	+	+	−	8
FrpB	MHA_2109	COI_0085	COK_0218	Receptor and transporter activities	+	+	−	8
TbpA	MHA_0196	COI_2333	COK_1753	Transferrin receptor & transport	+	+	−	7
OmpP4	MHA_2158	COI_0840	COK_2233	Heme acquisition/factor V utilisation	+	−	+	6
OmpP2	MHA_0735	ctg112	COK_1380	Porin/ion transport activity	+	+	−	4
FhaC	MHA_0867	COI_0226	COK_0335	Secretion of filamentous haemagglutinin (FhaB)	+	+	−	3
OmpW	MHA_2399	COI_1652/3	COK_1583/4	Small hydrophobic molecule transport	+	−	−	2
TonB-dependent receptor	MHA_0860	COI_1565	COK_2304	Receptor and transporter activities	+	+	−	2
HmbR1	MHA_2261	COI_2258	COK_1624	Haemoglobin receptor	+	+	−	2
HxuB	MHA_1005	COI_1367	COK_1209	Heme-hemopexin acquisition	+	+	−	2
TbpB	MHA_0197	COI_2332	COK_1752	Transferrin receptor & transport	+	+/−	+	1
HxuC	ctg59	COI_1368	COK_1207/8	Heme-hemopexin acquisition	+	+	−	1
TonB-dependent receptor	MHA_1346	COI_1921	ctg265	Receptor and transporter activities	+	+	−	1
LamB	MHA_0232	COI_2297	COK_1718	Maltoporin transport	+	+	−	
ComE	MHA_0164	COI_2362	COK_1782	DNA transport	+	−	−	
PulD	MHA_2514	−	−	Secretin	+	−	−	
HxuA	MHA_1004	COI_1366	COK_1210	Heme-haemopexin acquisition (Subcellular location: OM/E)	−	−	+	
FhuA	ctg68	COI_1905	COK_2465	Ferric hydroxamate receptor	+	+	−	

Table 3.2. (continued)

Name	Bovine A1	Ovine A2	Bovine A2	Protein function	Sub	TM	Lipo	Isol
FhuE	MHA_1541	COI_0658	COK_1513	Ferric hydroxamate receptor	+	+	−	
HmbR2	ctg86	COI_1762/3	COK_2539/40	Haemoglobin receptor	+	+	−	
Haemin receptor	ctg61	COI_2252/3	COK_1629/30	Haemin receptor	+	+	−	
Haemin-uptake lipoprotein	MHA_2255	COI_2251	COK_1631	Haemin-uptake lipoprotein	−	−	+	
TonB-dependent receptor	ctg58	COI_0092	COK_0224	Receptor and transporter activities	+	−	−	
TonB-dependent receptor	ctg85	COI_0006	COK_0109	Receptor and transporter activities	+	−	−	
TonB-dependent receptor	ctg58	COI_0090	COK_0222	Receptor and transporter activities	−	+	−	
3. Adherence								
YadA-like	MHA_0302	COI_2651	COK_0897/8	Adherence	+	+	−	2
Hsf	MHA_2701	COI_2393	COK_1437	Adherence	+	+/−	−	
Hia	MHA_1367	COI_1943/5	COK_2433/5	Adherence	+	−	−	
Ahs	ctg73_110_124_88	COI_1315	COK_1394	Adherence	+	−	−	
FhaB_1	MHA_0866	ctg15	COK_0334	Adherence (Subcellular location: OM/E)	+	+	−	
FhaB_2	ctg89_27_137	COI_1569	COK_2300	Adherence (Subcellular location: OM/E)	−	+	−	
4. Enzymatic activity								
Ssa	MHA_2492	COI_0850	COK_2411	Serine protease	+	+	−	8
Iga1_2	MHA_0563	COI_2430	COK_0634	Cleavage of host mucosal antibody	−	+	−	6
Iga1_3	MHA_1965	COI_1820	COK_2480	Cleavage of host mucosal antibody	+/−	+	−	3
NanH	MHA_1532	COI_0667	COK_1504	Terminal sialic acid hydrolysis from glycoconjugates	+	+	−	4
Iga1_1	MHA_2800	COI_2438	COK_1350	Cleavage of host mucosal antibody	+	+	−	
IgA1_4	−	COI_0585/6	COK_1280/81	Cleavage of host mucosal antibody	+	+	−	
GlpQ	MHA_2244	COI_1297	COK_0157	Glycerophosphodiester hydrolysis	+	−	−	

Table 3.2. (continued)

Name	Bovine A1	Ovine A2	Bovine A2	Protein function	Sub	TM	Lipo	Isol
NlpC/P60	MHA_1650	COI_0987	COK_0966	Cell wall hydrolysis during cell growth and division	+	−	+	
NlpD-like_1	MHA_1467	COI_0383	COK_1077	Metalloendopeptidase activity	+	−	−	
NlpD-like_2	MHA_0321	COI_0246	COK_0129	Metalloendopeptidase activity	+	−	−	
AmiC	MHA_0064	COI_1886	COK_0789	Cell envelope degradation	+	−	−	
5. Others								
Bor/Iss_2	MHA_0387	COI_1469	COK_1002	Survival in animal host	−	−	+	7
Bor/Iss_1	MHA_0386	COI_1470	COK_1001	Survival in animal host	−	−	+	1
fHbp_1	MHA_0965	COI_1330	COK_2564/5	Binding to complement factor H	−	−	+	4
fHbp_2	MHA_1406	COI_0728	COK_0018	Binding to complement factor H	−	−	+	
N1pI	MHA_0837	COI_2516	COK_0946	Cell division	−	−	+	
PilF	MHA_2059	COI_1784	COK_2516	Pilus assembly/stability	−	−	+	
EcnA	−	COI_0131	COK_2070	Toxin/antidote protein (cell apoptosis)	−	−	+	
6. Unknown								
PlpA	MHA_1433	COI_0758	COK_0049	Unknown	−	−	+	8
Possible OMP	MHA_0486	COI_0902	COK_2254	Unknown	+	+	−	8
Rickettsia-like surface ant.	−	COI_1456	COK_1118	Unknown	−	−	+	8
Lpp38	MHA_2152	COI_0845	COK_2238	Unknown	−	−	+	7
OMP18/16	MHA_2237	COI_2485	COK_1303	Unknown	+	−	−	7
YajG	−	COI_0336	COK_2129	Unknown	−	−	+	6
YtfM	MHA_0323	COI_0250	COK_0133	Unknown	+	+	−	6
Lpp/PCP/SlyB	MHA_0760	COI_1053	COK_0435	Unknown	−	−	+	6
HlpB	MHA_2282	COI_2279	COK_1699	Unknown	−	−	+	5

Table 3.2. (continued)

Name	Bovine A1	Ovine A2	Bovine A2	Protein function	Sub	TM	Lipo	Isol
PlpE	MHA_1514	COI_1139	COK_0733	Unknown	−	−	+	5
Possible OMP	MHA_0718	COI_2021	COK_0360	Unknown	+	−	−	5
PlpD	MHA_1464	COI_0386	COK_1074	Unknown	+	−	+/−	3
PlpC	MHA_1435	COI_0760	COK_0051	Unknown	−	−	+	3
LppC	MHA_2734	COI_0540	COK_2366/7	Unknown	+	+	+	2
Possible OMP	MHA_0964	COI_1329	COK_2563	Unknown	+	+	−	1
Possible OMP	MHA_2054	COI_1788	COK_2512	Unknown	−	+	−	1
Possible OMP	MHA_2761	COI_0565	COK_2341	Unknown	+	+	−	
Possible OMP	MHA_1407	COI_0729	COK_0019	Unknown	+	+	−	
Possible OMP	MHA_0862	COI_1567	COK_2302	Unknown	+	−	−	
Skp/Omp26	MHA_0690	COI_1175	COK_1968	Unknown	+	−	−	
OapB	MHA_2703	COI_1631	COK_1107	Unknown	−	−	+	
PlpB	MHA_1434	COI_0759	COK_0050	Unknown	−	−	+	
Autotransporter	ctg108	COI_1968/9	COK_0413/4	Unknown	+	+	−	
Autotransporter	MHA_0080	COI_1870	COK_0773	Unknown	+	+	−	
Lipoprotein	MHA_1803	COI_0886	COK_0646	Unknown	−	−	+	
Lipoprotein	MHA_1802	COI_0887	COK_0645	Unknown	−	−	+	

Figure 3.3. Distribution of 93 confidently predicted OMPs based on (A) the three categories of bioinformatic predictors (subcellular localisation, β-barrel and OM lipoprotein predictors) and (B) the three *M. haemolytica* genomes that were analysed (bovine A1, bovine A2, ovine A2).

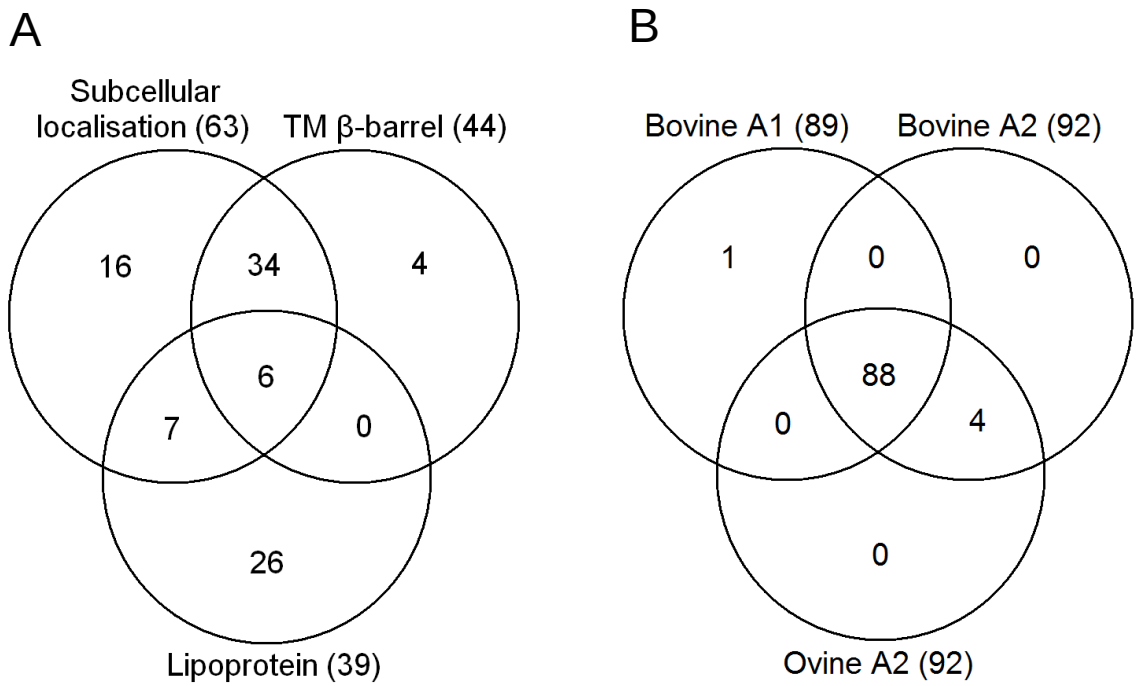


Table 3.3. Distribution of five confidently predicted OMPs present in only one or two *M. haemolytica* genomes.

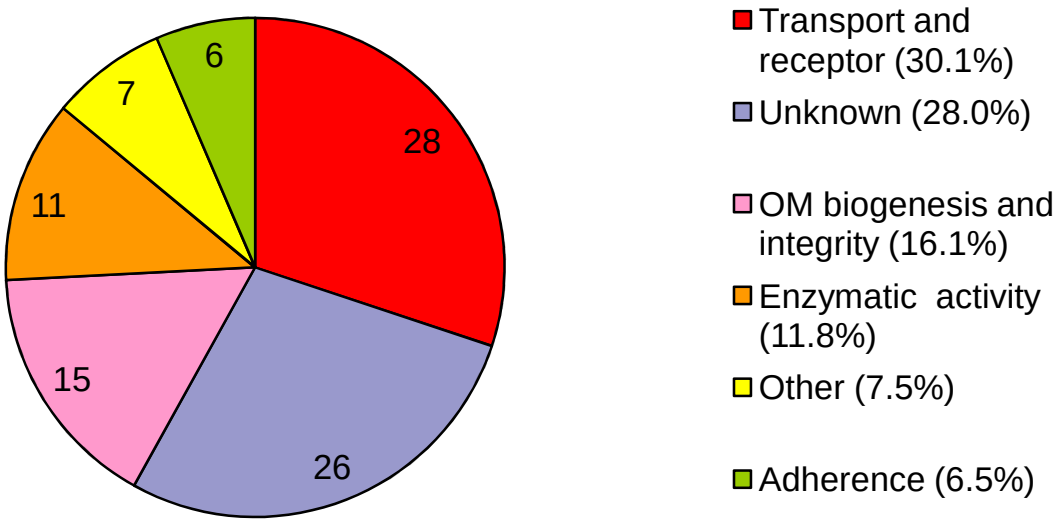
Confidently predicted OMP	Bovine A1	Bovine A2	Ovine A2
Iga1_4	−	+	+
EcnA	−	+	+
Rickettsia-like surface antigen	−	+	+
YajG	−	+	+
PulD	+	−	−

to sheep. The two serotype A2 genomes are expected to have similar gene content because of their common ancestral origin.

3.3.2 Functional classifications of confidently predicted OMPs.

The 93 confidently predicted OMPs could be assigned to six broad functional categories based on information gathered from the additional searches (Fig. 3.4; Table 3.2). Fifteen confidently predicted OMPs were involved in outer membrane biogenesis and integrity, all of which were present in the three genomes (OmpA, Omp85, Imp/LptD, Pal, VacJ, LppB/NlpD, RlpB/LptE, MltC, ComL, MltA, LolB, NlpE, SmpA, WzzB and MltB). Twenty-eight confidently predicted OMPs were involved in transport and receptor activities, of which 27 were present in all three genomes (TolC, OmpP1/FadL, Wza, OmpP2-like, FrpB, TbpA, OmpP4, OmpP2, FhaC, OmpW, TonB-dependent receptor MHA_0860/COI_1565/ COK_2304, HmbR1, HxuB, TbpB, HxuC, TonB-dependent receptor MHA_1346/COI_1921, LamB, ComE, HxuA, FhuE, HmbR2, haemin receptor, haemin-uptake lipoprotein, TonB-dependent receptor COI_0092/COK_0224, TonB-dependent receptor COI_0006/COK_0109 and TonB-dependent receptor COI_0090/COK_0222). The PulD protein was present only in the bovine serotype A1 genome. Notably, this was the only protein out of the 93 confidently predicted OMPs to be present (exclusively) in only one of the three genomes (Fig. 3.3B). Six confidently predicted OMPs were involved in adherence (YadA-like, Hsf, Hia, Ahs, FhaB_1 and FhaB_2), all of which were present in the three genomes. Eleven confidently predicted OMPs had enzymatic activity, of which 10 were present in all three genomes (Ssa, Iga1_2, Iga1_3, NanH, Iga1_1, GlpQ, NlpC/P60, two NlpD-like proteins, and AmiC). A fourth Iga1-like protein (Iga1_4) was predicted in both the bovine and ovine serotype A2 genomes but was notably absent from the bovine serotype A1 genome. Seven confidently predicted OMPs had functions which did not fall into any of the categories above. These included two proteins, Bor/Iss_1 and Bor/Iss_2, which promote bacterial resistance to serum complement killing and virulence (Barondess and Beckwith, 1995), two homologues of the neisserial factor H-binding protein (fHbp_1 and fHbp_2) (Welsch and Ram, 2008) and a pilus assembly protein (PilF). The remaining twenty-six confidently predicted OMPs were of unknown function; 24 of these were present in all three genomes.

Figure 3.4. Functional classifications of 93 confidently predicted OMPs present across three *M. haemolytica* genomes after domain, homology and literature searches were carried out on each protein.



3.3.3 Amino acid sequence variation in confidently predicted OMPs between different *M. haemolytica* genomes.

In order to identify OMPs that might be involved in host-specific adaptation, amino acid sequence comparisons were performed on the 93 confidently predicted OMPs in the three different *M. haemolytica* genomes. The OMPs present among the bovine and ovine serotype A2 genomes (Fig 3.5A) showed a higher degree of amino acid identity to each other than to the OMPs present among either of these two genomes and the bovine serotype A1 genome (Fig. 3.5B). Ninety-two confidently predicted OMPs were present in both the bovine and ovine serotype A2 genomes, of which 83 (92%) had an amino acid identity of 98% and above (Fig. 3.5A). The remaining nine OMPs (PlpE, Ssa, TbpB, OmpA, LppC, fHbp_2, Pal, Ahs and Hia) had amino acid identities ranging from 60 to 97% (below 98%), suggesting possible diversification that might be related to host adaptation (Fig. 3.5A; Table 3.4). However, only three of these proteins, OmpA, PlpE, and Ahs, had a higher amino acid identity when the bovine serotype A1 and A2 genomes were compared (Fig. 3.5B; Table 3.4). OmpA was 100% identical in the bovine serotype A1 and bovine serotype A2 genomes but was 95% identical when the genomes were compared to the ovine serotype A2 genome. PlpE was 91% identical in the bovine serotype A1 and A2 genomes but only 60% identical when these genomes were compared to the ovine serotype A2 genome, suggesting possible horizontal gene transfer. Ahs was 100% identical in the bovine serotype A1 and bovine serotype A2 genomes but was 97% identical when these genomes were compared to the ovine serotype A2 genome. Of the 88 confidently predicted OMPs that were present in both the bovine serotype A1 and A2 genomes, 67 (76%) had an amino acid identity of 98% and above (Fig. 3.5B). The remaining 21 OMPs (TbpB, TonB-dependent receptor COI_0090/COK_0222, fHbp_2, Hsf, OmpW, Ssa, FrpB, YadA-like, PlpE, OmpP2-like, TbpA, Pal, autotransporter COI_1968-9/COK_0413-4, possible OMP MHA_0862/COI_1567/COK_2302, LppC, fHbp_1, Hia, Wza, FhuA, FhaC and LolB) had amino acid identities ranging from 57 to 97% (below 98%) (Fig. 3.5B; Table 3.4). With the exception of PlpE, it is possible that these proteins have little or no role in host adaptation, and may be able to function in cattle and sheep equally well. The fact that there was a higher degree of amino acid conservation in OMPs present between the bovine and ovine serotype A2 genomes than between the bovine serotype A1 genome and bovine serotype A2 genome is

Figure 3.5. Amino acid sequence identity of 93 confidently predicted OMPs among (A) bovine and ovine serotype A2 genomes and (B) bovine serotype A1 and bovine serotype A2 genomes.

Bars are coloured based on the functional classifications of each OMP: OM biogenesis and maintenance (pink), transport and receptor (red), adherence (green), enzymatic activity (orange), other (yellow), unknown (blue).

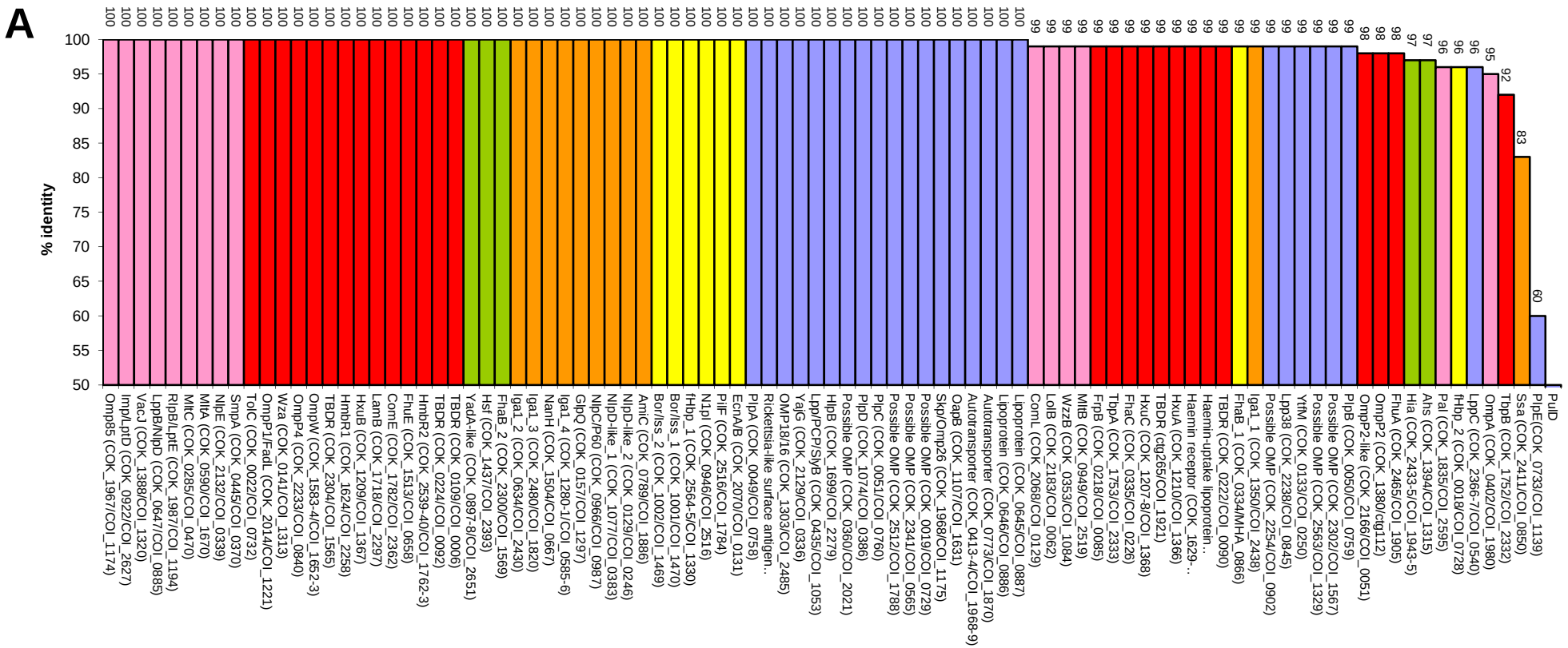


Figure 3.5 (continued)

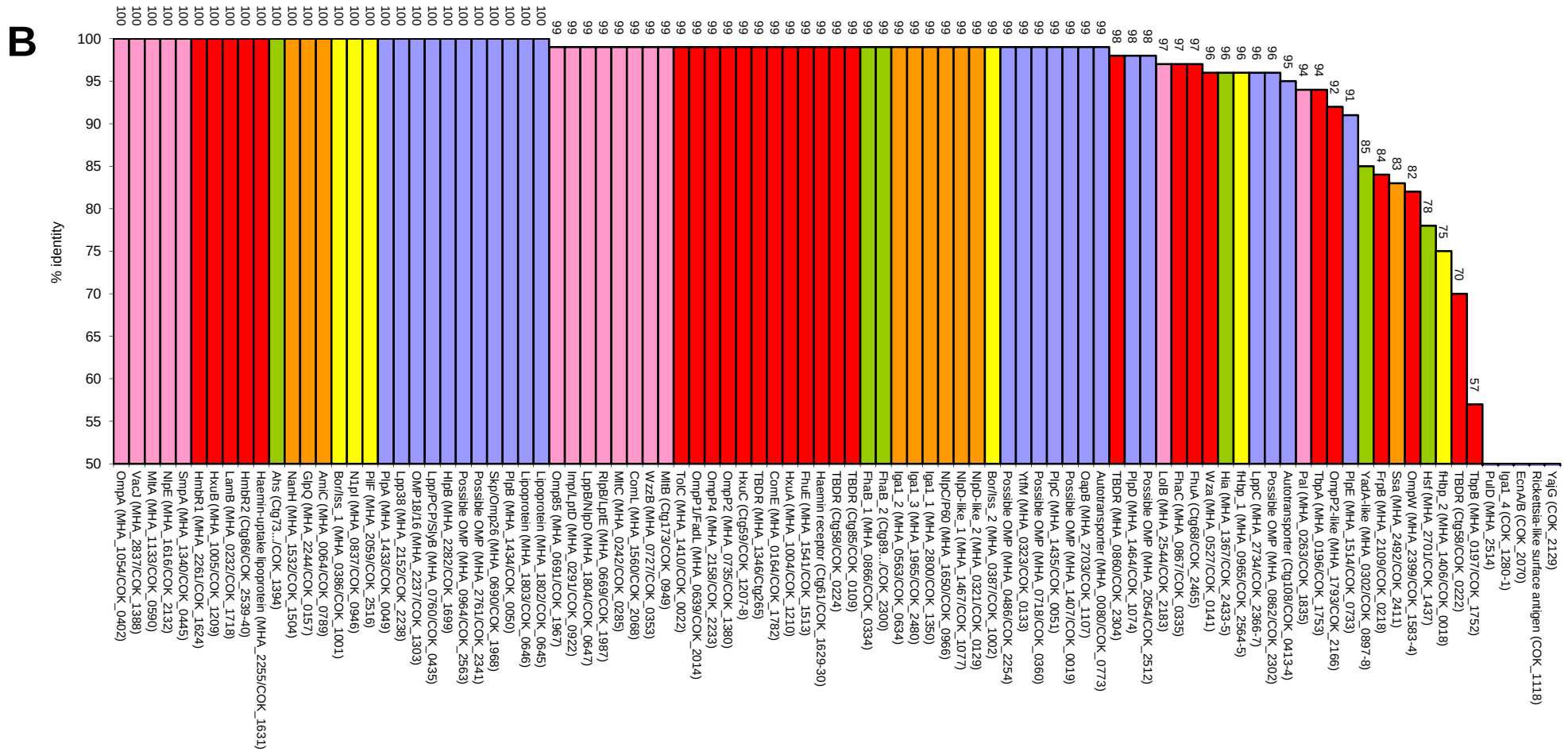


Table 3.4. Confidently predicted OMPs with amino acid sequence identity below 98% between the bovine serotype A2 and ovine serotype A2 genomes and/or between the bovine serotype A1 and A2 genomes.

Confidently predicted OMP	Bov. A2 vs. Ov. A2[a]	Bov. A1 vs. Bov. A2[a]
PlpE	60	91
Ssa	83	83
TbpB	92	57
OmpA	95	100
LppC	96	96
fHbp_2	96	75
Pal	96	94
Ahs	97	100
Hia	97	96
OmpP2-like	98	92
FhuA	98	97
TonB-dependent receptor ctg58/COI_0090/COK_0222	99	70
FrpB	99	84
TbpA	99	94
Possible OMP MHA_0862/COI_1567/COK_2302	99	96
FhaC	99	97
LolB	99	97
Wza	100	96
Autotransporter ctg108/COI_1968-9/COK_0413-4	100	95
Hsf	100	78
OmpW	100	82
YadA-like	100	85
fHbp_1	100	96

Proteins with higher percentage amino acid sequence identity between the bovine serotype A1 and A2 genomes than between the bovine serotype A2 and ovine serotype A2 genomes are shaded in grey.
[a]Percentage amino acid sequence identity.

further evidence to support the hypothesis of serotype A2 host switching from cattle to sheep.

3.3.4 Identification of *M. haemolytica* OMPs by gel-based and gel-free approaches.

The outer membrane fractions of seven *M. haemolytica* isolates and one *M. glucosida* isolate were extracted using the Sarkosyl extraction technique and analysed using gel-based and gel-free approaches. A 1-D SDS-polyacrylamide gel showing the OMP profiles of the representative isolates is shown in Fig. 3.6.

A total of 98 different proteins were identified using a combination of gel-based and gel-free approaches, of which 55 were classified as OMPs (Table 3.5). Fifty of these OMPs were confidently predicted to be OMPs using the bioinformatic prediction approach (Table 3.2). However, five proteins, LemA, HbpA, OapA, RlpA and CsgG were not predicted by the bioinformatic approach but were indicated by literature searches to be OMPs. Henceforth, the 50 confidently predicted OMPs and the five proteins described above will collectively be referred to together as OMPs.

Fifty-seven different proteins were identified using the gel-based approach. Forty-four of these were OMPs (Table 3.5; Figure 3.6), of which 21 were predicted to be ß-barrel proteins, 11 were predicted to be outer membrane lipoproteins, 6 were predicted to be both ß-barrel proteins and outer membrane lipoproteins and six were not predicted to be either (Fig. 3.7). Eighty-seven different proteins were identified using the gel-free approach. Forty-seven of these were OMPs (Table 3.5), of which 18 were predicted to be ß-barrel proteins, 18 were predicted to be outer membrane lipoproteins, five were predicted to be both ß-barrel proteins and outer membrane lipoproteins and six were not predicted to be either (Fig 3.7). A total of thirty-six OMPs were identified by both gel-based and gel-free methods. The gel-based approach exclusively identified eight OMPs that were not identified by the gel-free approach: Iga1_2, possible OMP MHA_0718/COI_2021/COK_0360, LppB/NlpD, HmbR1, HxuB, TonB-dependent receptor MHA_1346/COI_1921, possible OMP MHA_2054/COI_1788/COK_2512 and CsgG. Of these proteins, five were predicted to be ß-barrel proteins, one was predicted to be both a ß-barrel protein and an outer membrane lipoprotein and two were not predicted to be either (Fig. 3.7).

Figure 3.6. One-dimensional SDS-PAGE separation of outer membrane fractions from seven *M. haemolytica* isolates and one *M. glucosida* isolate.

Numbered proteins were identified by proteomic analysis (Table 3.5). Twenty micrograms of protein were loaded into each lane. Protein bands were stained with Coomassie brilliant blue.

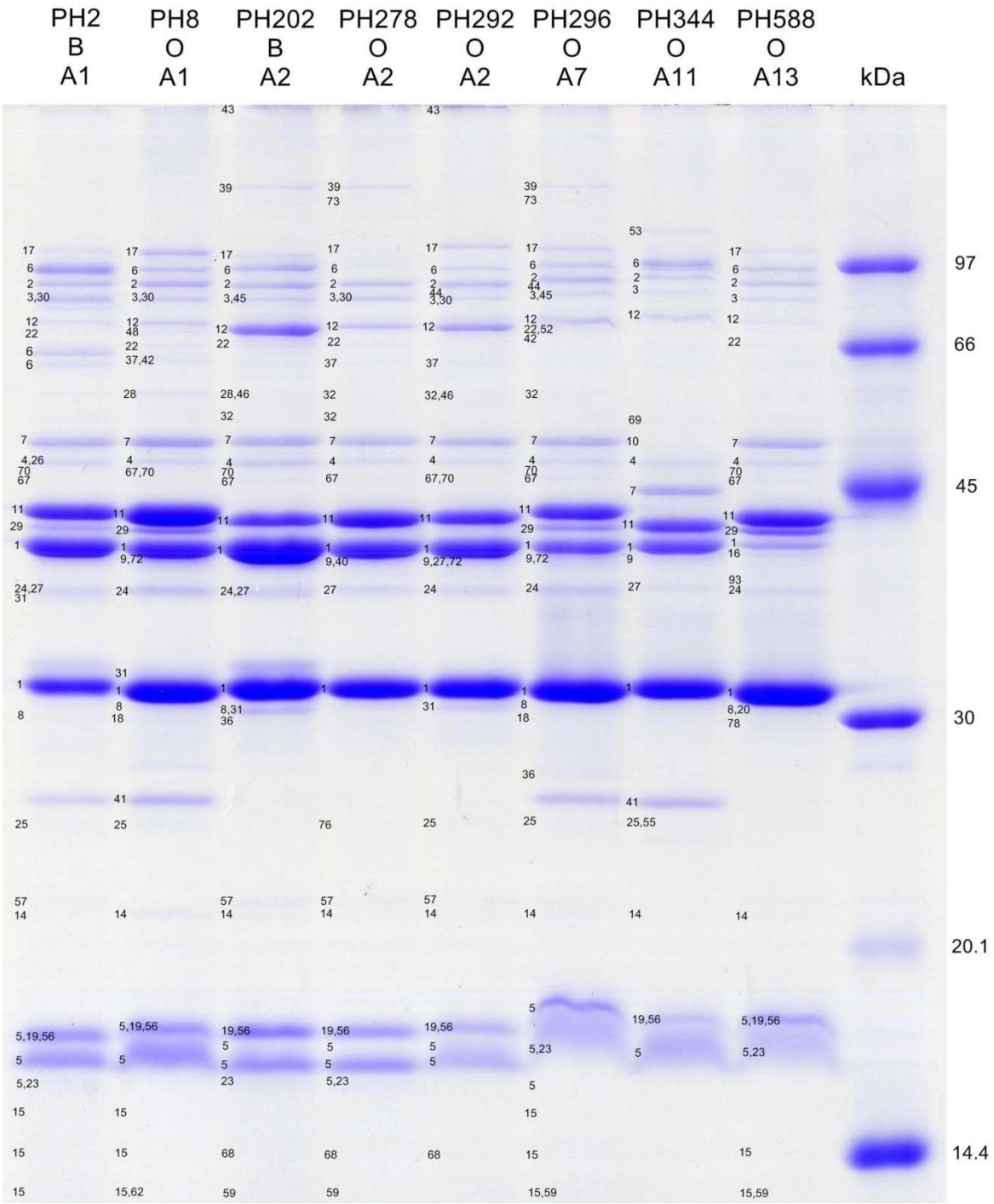


Table 3.5. Proteins identified in the outer membrane fractions of seven representative isolates of *M. haemolytica* and one isolate of *M. glucosida* (PH344) using a combination of gel-based and gel-free proteomic approaches.

No.[a]	Subcellular Localisation[b]	TM[c]	Lipo[c]	Bovine A1	Ovine A2	Bovine A2	Name	Protein identifications in different isolates[d]								
								PH2	PH8	PH202	PH278	PH292	PH296	PH344	PH588	Total
1. Confidently predicted OMPs																
1	OM	+/−	−	MHA_1054	COI_1980	COK_0402	OmpA	+	+	+	+	+	+	+	+	8
2	OM	+	−	MHA_0691	COI_1174	COK_1967	Omp85	+	+	+	+	+	+	+	+	8
3	OM	+	−	MHA_0291	COI_2627	COK_0922	Imp/LptD	+	+	+	+	+	+	+	+	8
4	OM	+	+	MHA_1410	COI_0732	COK_0022	TolC	+	+	+	+	+	+	+	+	8
5	OM	−	+	MHA_0263	COI_2595	COK_1835	Pal	+	+	+	+	+	+	+	+	8
6	OM	+	−	MHA_2492	COI_0850	COK_2411	Ssa	+	+	+	+[1]	+	+	+	+	8
7	OM	+	−	MHA_0639	COI_1221	COK_2014	OmpP1/FadL	+	+	+	+	+	+	+[1]	+	8
8	OM	−	+	MHA_1433	COI_0758	COK_0049	PlpA	+	+	+	+[2]	+[2]	+	+[2]	+	8
9	OM	+	+	MHA_0527	COI_1313	COK_0141	Wza	+[2]	+	+[2]	+	+	+	+	+[2]	8
10	OM	+	−	MHA_0486	COI_0902	COK_2254	Possible OMP	+[2]	+[2]	+[2]	+[2]	+[2]	+[2]	+[1]	+[2]	8
11	OM	+	−	MHA_1793	COI_0051	COK_2166	OmpP2-like	+	+[1]	+[1]	+	+	+	+[1]	+[1]	8
12	OM	+	−	MHA_2109	COI_0085	COK_0218	FrpB	+	+[1]	+	+	+	+	+	+	8
13	OM	−	+	−	COI_1456	COK_1118	*Rickettsia*-like surface antigen	+[2]	+[2]	+[2]	+[2]	+[2]	+[2]	+[2]	+[2]	8

Proteins that were predicted to be putative OMPs by the ten bioinformatic predictors are grey-shaded.
[a]Numbers correspond to the location of the protein on Figure 3.6 if identified by gel-based proteomics.
[b]Confidently predicted subcellular locations; 'OM' = outer membrane; 'P' = periplasm; 'IM' = inner membrane; 'C' = cytoplasm; 'E' = extracellular; 'U' = unknown.
[c]Transmembrane β-barrel and lipoprotein prediction result; '+' = predicted in all genomes; '**−**' **=** not predicted in any genome; '+/−' **=** predicted in one/two genomes (see Supplementary Tables S3.1, S3.2 and S3.3 for specific result).
[d]Two proteomics methods were compared; '+[1]' = proteins identified by gel-based method; '+[2]' = proteins identified by gel-free method; '+' = proteins identified by both methods; '−' = no identification.

Table 3.5. (continued)

No.	Subcellular location	TM	Lipo	Bovine A1	Ovine A2	Bovine A2	Name	Protein identifications in different isolates								
								PH2	PH8	PH202	PH278	PH292	PH296	PH344	PH588	Total
14	OM			MHA_0704	COI_1162	COK_1955	LemA	+[1]	+[1]	+[1]	+	+	+[1]	+[1]	+[1]	8
15	OM	−	+	MHA_0387	COI_1469	COK_1002	Bor/Iss_2	+	+	+[2]	−	+[2]	+	+[2]	+	7
16	OM	−	+	MHA_2152	COI_0845	COK_2238	Lpp38	+[2]	+[2]	+[2]	+[2]	+[2]	+[2]	−	+	7
17	OM	+	−	MHA_0196	COI_2333	COK_1753	TbpA	+	+	+	+[1]	+	+	−	+	7
18	OM	−	+	MHA_2837	COI_1320	COK_1388	VacJ	+[2]	+	−	+[2]	+[2]	+	+[2]	+[2]	7
19	OM	−	−	MHA_2237	COI_2485	COK_1303	OMP18/16	+[1]	+[1]	+[1]	+[1]	+	−	+[1]	+[1]	7
20	OM	−	+	MHA_2158	COI_0840	COK_2233	OmpP4	+[2]	+[2]	+[2]	−	−	+[2]	+[2]	+	6
21	OM	−	+	−	COI_0336	COK_2129	YajG	+[2]	+[2]	+[2]	+[2]	+[2]	+[2]	−	−	6
22	OM	+	−	MHA_0323	COI_0250	COK_0133	YtfM	+[1]	+	+[1]	+	−	+[1]	−	+[1]	6
23	OM	−	+	MHA_0760	COI_1053	COK_0435	Lpp/PCP/SlyB	+[1]	−	+[1]	+[1]	+[2]	+[1]	−	+[1]	6
24	OM	+	−	MHA_0563	COI_2430	COK_0634	Iga1_2	+[1]	+[1]	+[1]	−	+[1]	+[1]	−	+[1]	6
25	OM	−	+	MHA_2282	COI_2279	COK_1699	HlpB	+	+	−	−	+[1]	+	+[1]	−	5
26	OM	−	+	MHA_1514	COI_1139	COK_0733	PlpE	+	+[2]	−	−	+[2]	+[2]	−	+[2]	5
27	OM	−	−	MHA_0718	COI_2021	COK_0360	Possible OMP	+[1]	−	+[1]	+[1]	+[1]	−	+[1]	−	5
28	OM/P/IM			MHA_1007	COI_1369	COK_1206	HbpA	−	+	+	+[2]	−	+[2]	−	+[2]	5
29	OM	+	−	MHA_0735	ctg112	COK_1380	OmpP2	+	+	−	−	−	+	−	+	4
30	OM	+	−	MHA_1532	COI_0667	COK_1504	NanH	+[1]	+	−	+	+[1]	−	−	−	4
31	OM	−	+	MHA_0965	COI_1330	COK_2564/5	fHbp_1	+[1]	+	+[1]	−	+[1]	−	−	−	4
32	OM	+	+	MHA_1804	COI_0885	COK_0647	LppB/NlpD	−	−	+[1]	+[1]	+[1]	+[1]	−	−	4
33	OM	−	+	MHA_0669	COI_1194	COK_1987	RlpB/LptE	+[2]	+[2]	+[2]	−	−	−	−	−	3

Table 3.5. (continued)

No.	Subcellular location	TM	Lipo	Bovine A1	Ovine A2	Bovine A2	Name	Protein identifications in different isolates								
								PH2	PH8	PH202	PH278	PH292	PH296	PH344	PH588	Total
34	OM	−	+/−	MHA_1464	COI_0386	COK_1074	PlpD	+[2]	+[2]	−	−	−	+[2]	−	−	3
35	OM	−	+	MHA_0242	COI_0470	COK_0285	MltC	−	+[2]	−	−	+[2]	−	−	+[2]	3
36	OM	−	+	MHA_1560	COI_0129	COK_2068	ComL	−	+[2]	+[1]	−	−	+	−	−	3
37	OM	+	−	MHA_0867	COI_0226	COK_0335	FhaC	−	+	−	+[1]	+[1]	−	−	−	3
38	OM	−	+	MHA_1435	COI_0760	COK_0051	PlpC	−	−	+[2]	−	−	+[2]	−	+[2]	3
39	OM	+	−	MHA_1965	COI_1820	COK_2480	Iga1_3	−	−	+	+	−	+	−	−	3
40	OM	+	+	MHA_1133	COI_1670	COK_0590	MltA	+[2]	−	−	+	−	−	−	−	2
41	OM	−	−	MHA_2399	COI_1652/3	COK_1583/4	OmpW	−	+	−	−	−	−	+	−	2
42	OM	+	+	MHA_2734	COI_0540	COK_2366/7	LppC	−	+	−	−	−	+[1]	−	−	2
43	OM	+	−	MHA_0302	COI_2651	COK_0898	YadA-like protein	−	−	+	−	+	−	−	−	2
44	OM	+	−	MHA_0860	COI_1565	COK_2304	TonB-dependent receptor	−	−	−	−	+	+	−	−	2
45	OM	+	−	MHA_2261	COI_2258	COK_1624	HmbR1	−	−	+[1]	−	−	+[1]	−	−	2
46	OM	+	−	MHA_1005	COI_1367	COK_1209	HxuB	−	−	+[1]	−	+[1]	−	−	−	2
47	OM			MHA_2702	COI_1630	COK_1108	OapA	−	−	−	+[2]	−	+[2]	−	−	2
48	OM	+/−	+	MHA_0197	COI_2332	COK_1752	TbpB	−	+	−	−	−	−	−	−	1
49	OM	+	−	MHA_0964	COI_1329	COK_2563	Possible OMP	−	+[2]	−	−	−	−	−	−	1
50	OM	−	+	MHA_0386	COI_1470	COK_1001	Bor/Iss_1	−	+[2]	−	−	−	−	−	−	1
51	OM	+	−	ctg59	COI_1368	COK_1207/8	HxuC	−	−	−	−	−	−	−	+[2]	1
52	OM	+	−	MHA_1346	COI_1921	ctg265	TonB-dependent receptor	−	−	−	−	−	+[1]	−	−	1
53	OM	+	−	MHA_2054	COI_1788	COK_2512	Possible OMP	−	−	−	−	−	−	+[1]	−	1

Table 3.5. (continued)

No.	Subcellular location	TM	Lipo	Bovine A1	Ovine A2	Bovine A2	Name	Protein identifications in different isolates								
								PH2	PH8	PH202	PH278	PH292	PH296	PH344	PH588	Total
54	OM			MHA_0470	COI_0918	COK_2270	RlpA	–	–	–	–	–	–	+[2]	–	1
55	OM			–	–	–	CsgG	–	–	–	–	–	–	+[1]	–	1
2. Proteins without confident localisation prediction																
56	U	+	+	MHA_2025	COI_1815	COK_2485	Hypothetical protein	+	+	+	+	+	+[2]	+	+	8
57	U			MHA_2087	COI_1609	COK_1902	Hypothetical protein	+	+[2]	+	+[1]	+[1]	–	–	–	5
58	U			MHA_1782	COI_0313	COK_0888	Hypothetical protein	–	+[2]	+[2]	+[2]	–	+[2]	–	+[2]	5
59	U	–	+	MHA_0761/2	COI_1052	COK_0436	Hypothetical protein	–	–	+[1]	+[1]	–	+[1]	–	+[1]	4
60	U			MHA_2789	COI_2198	COK_1686	PqiB	–	–	+[2]	–	–	+[2]	–	–	2
61	U	–	+	MHA_0804	COI_2411	COK_1455	Patatin	–	–	–	–	–	+[2]	–	–	1
62	U	–	+	MHA_0452	ctg39	ctg44	Hypothetical protein	–	+[1]	–	–	–	–	–	–	1
63	U			MHA_1898	COI_0630	COK_1217	LysM domain protein	–	–	+[2]	–	–	–	–	–	1
64	U			MHA_1828	COI_2056	COK_0536	Hypothetical protein	–	–	–	+[2]	–	–	–	–	1
65	U			–	COI_2639	COK_0910	Hypothetical protein	–	–	–	–	+[2]	–	–	–	1
66	U			MHA_0324	COI_0251	COK_0134	Hypothetical protein	–	–	–	–	–	+[2]	–	–	1
3. Non-outer membrane localised proteins																
67	P	+	–	MHA_0371	COI_1485	COK_0985	AcrA_1	+[1]	+[1]	+	+	+[1]	+	–	+	7
68	IM			MHA_1448	COI_0774	COK_0064	YajC	+[2]	+[2]	+	+	+	+[2]	–	+[2]	7
69	IM			MHA_1228	COI_1149	COK_1941	PntA	–	+[2]	+[2]	+[2]	+[2]	+[2]	+[1]	+[2]	7
70	C			MHA_2176	COI_1521	COK_1096	EF1A	+[1]	+	+[1]	+[2]	+	+	–	+	7
71	C			MHA_1682	COI_0958	COK_1425	RplB	+[2]	–	+[2]	+[2]	–	+[2]	+[2]	+[2]	6

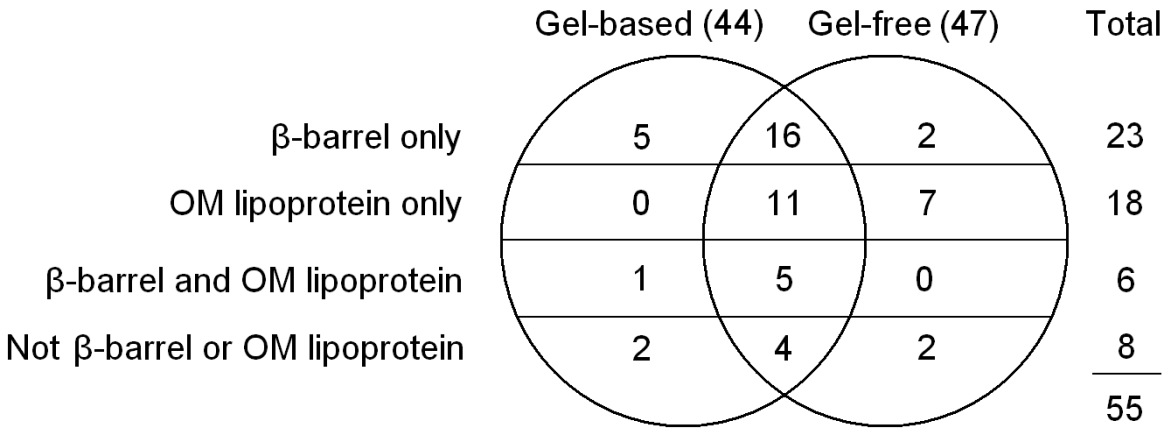
Table 3.5. (continued)

No.	Subcellular location	TM	Lipo	Bovine A1	Ovine A2	Bovine A2	Name	Protein identifications in different isolates								
								PH2	PH8	PH202	PH278	PH292	PH296	PH344	PH588	Total
72	IM	+/−	+	MHA_0528	COI_1312	COK_0142	Wzf	−	+	+[2]	−	+[1]	+	−	−	4
73	IM			MHA_0370	COI_1486	COK_0984	AcrB	−	−	−	+[1]	−	+	+[2]	+[2]	4
74	C			MHA_1040	COI_1994	COK_0388	RplA	−	+[2]	+[2]	−	−	+[2]	−	+[2]	4
75	C			MHA_1552	COI_0122	COK_2061	RplT	−	+[2]	+[2]	−	−	+[2]	+[2]	−	4
76	IM	−	+	MHA_1287	COI_1274	COK_0180	AtpF	−	+[2]	−	+[1]	−	−	−	+[2]	3
77	IM			MHA_1625	COI_0002	COK_0113	LctP	+[2]	+[2]	−	+[2]	−	−	−	−	3
78	C			MHA_0315	COI_0240	COK_0123	RpsB	+[2]	−	−	−	−	−	+[2]	+[1]	3
79	C			MHA_1041	COI_1993	COK_0389	RplK	−	−	−	−	−	+[2]	+[2]	+[2]	3
80	IM			ctg147	COI_1296	COK_0158	GlpT	−	+[2]	−	−	−	−	−	+[2]	2
81	C			MHA_2378	COI_1451	COK_1123	RplE	−	+[2]	−	−	−	−	+[2]	−	2
82	C			MHA_1678	COI_0962	COK_1421	RplP	−	+[2]	−	−	−	−	−	+[2]	2
83	C			MHA_2371	COI_1444	COK_1130	RplO	−	+[2]	−	−	−	−	+[2]	−	2
84	C			MHA_2364	COI_1437	COK_1137	RplQ	−	−	−	−	−	−	+[2]	+[2]	2
85	P	−	+	MHA_0980	COI_1345	COK_2582	YgiW	−	−	−	+[2]	−	−	−	−	1
86	P	−	+	MHA_2224	COI_2497	COK_1291	NikA/DppA/OppA	−	−	−	−	−	+[2]	−	−	1
87	E	+	−	MHA_0254	COI_0481	COK_0274	LktA	−	−	−	−	−	−	+[2]	−	1
88	IM	−	+	−	COI_2478	COK_1310	NhaC	+[2]	−	−	−	−	−	−	−	1
89	IM			MHA_1656	COI_1498	COK_0973	GltP	−	+[2]	−	−	−	−	−	−	1
90	IM			MHA_1795	COI_2038	COK_1899	PtnC	−	+[2]	−	−	−	−	−	−	1
91	IM			MHA_2180	COI_2587	COK_1843	CydA	−	−	−	+[2]	−	−	−	−	1

Table 3.5. (continued)

No.	Subcellular location	TM	Lipo	Bovine A1	Ovine A2	Bovine A2	Name	Protein identifications in different isolates								
								PH2	PH8	PH202	PH278	PH292	PH296	PH344	PH588	Total
92	IM			MHA_1822	COI_1555	COK_1560	TatA	–	–	–	–	–	–	–	+[2]	1
93	IM			MHA_2518	COI_1395	COK_1180	FtsN	–	–	–	–	–	–	–	+[1]	1
94	IM			MHA_1229	COI_1148	COK_1940	PntB	–	+[2]	–	–	–	–	–	–	1
95	C			MHA_0822	COI_0990	COK_0963	RpsA	–	+[2]	–	–	–	–	–	–	1
96	C			MHA_2178	COI_1637	COK_1101	RpsG	–	+[2]	–	–	–	–	–	–	1
97	C			MHA_0991	COI_1354	COK_2591	RpmB	–	+[2]	–	–	–	–	–	–	1
98	C			MHA_1029	COI_2005	COK_0376	RplL	–	–	–	–	–	–	–	+[2]	1
							Total number of proteins identified	42	62	48	44	41	54	35	48	
							Total number of OMPs identified	33	39	34	29	33	37	24	29	

Figure 3.7. Distribution of 55 OMPs identified by proteomic analyses based on gel-based and/or gel-free approaches and bioinformatic prediction of a β-barrel and/or outer membrane (OM) lipoprotein.



The gel-free approach exclusively identified 11 OMPs that were not identified by the gel-based approach (*Rickettsia*-like surface antigen, YajG, RlpB/LptE, PlpD, MltC, PlpC, OapA, possible OMP MHA_0964/COI_1329/COK_2563, Bor/Iss_1, HxuC and RlpA). Of these, two were predicted to be ß-barrel proteins, seven were predicted to be outer membrane lipoproteins and two were not predicted to be either.

The subcellular localisations of eleven identified proteins could not be confidently predicted, and therefore remain unknown (Table 3.2; Supplementary Table S3.4). Four of these proteins (hypothetical protein MHA_2025/COI_1815/ COK_2485, hypothetical protein MHA_0761/COI_1052/COK_0436, patatin, and hypothetical protein MHA_0452) were putative OMPs predicted by the three categories of bioinformatic predictors but could not be confirmed as OMPs or false-positives after additional searches. The remaining seven proteins were not predicted by the bioinformatic approach, and additional searches could not confidently predict their subcellular locations.

Thirty-two identified proteins were confidently predicted to be localised to cellular compartments other than the outer membrane and were therefore classified as false-positives (Table 3.5; Supplementary Table S3.5). Seven of these proteins were putative OMPs predicted by the three categories of bioinformatic predictors, but were confirmed as false positives after additional searches. These included three periplasmic proteins (AcrA_1, YgiW and NikA/DppA/OppA), three inner membrane proteins (Wzf, AtpF and NhaC) and the secreted leukotoxin protein LktA. The remaining twenty-five proteins were not predicted by the bioinformatic approach, but additional searches confirmed their localisation in compartments other than the outer membrane. Thirteen of these were ribosomal proteins located in the cytoplasm (RplB, RplA, RplT, RpsB, RplK, RplE, RplP, RplO, RplQ, RpsA, RpsG, RpmB and RplL). The other twelve were inner membrane proteins (YajC, PntA, AcrB, LctP, GlpT, GltP, PtnC, CydA, TatA, FtsN and PntB) and the cytoplasmic EF1A protein.

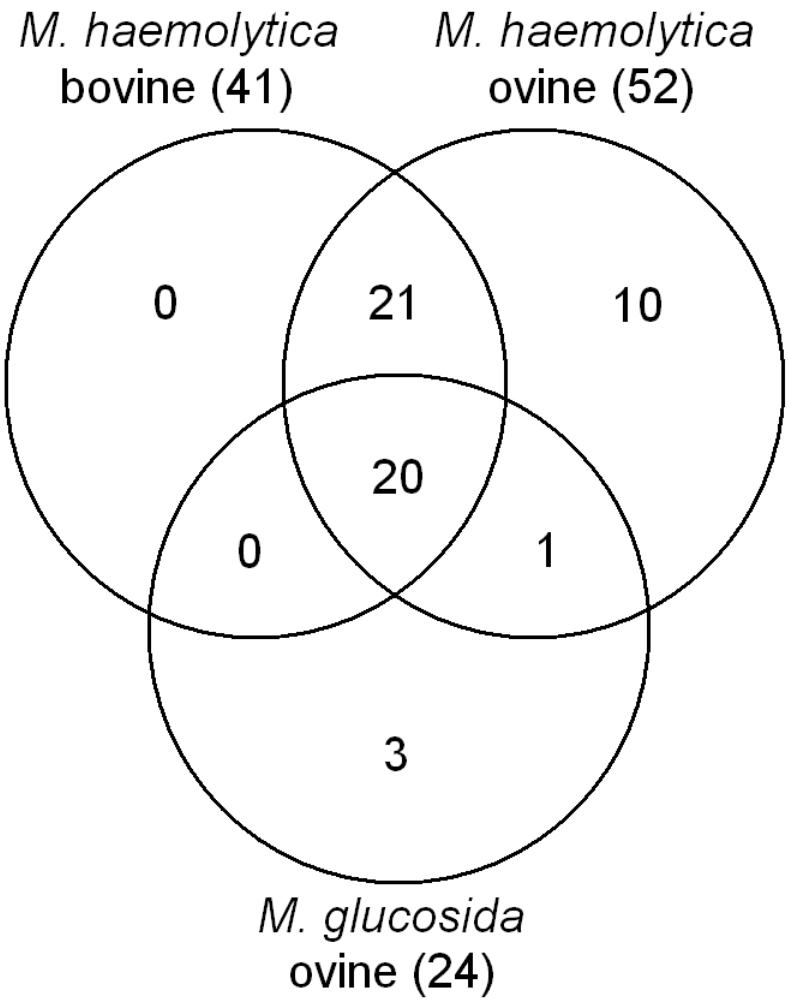
In addition to the identification of OMPs in the outer membrane described above, whole cell envelope fractions of the eight representative isolates were also analysed by the gel free approach to determine whether any OMPs were lost as a result of the inner membrane solubilisation. One hundred and thirty two

unique proteins were identified among whole cell envelope fractions (Supplementary Table 3.6), of which 56 were identified among outer membrane fractions. The remaining 76 proteins were mainly comprised of inner membrane and periplasmic proteins and did not contain any confidently predicted OMPs that were not already identified among outer membrane fractions. This indicates that OMPs are not lost as a result of inner membrane solubilisation.

3.3.5 Comparison of OMPs identified by proteomic approaches that are associated with different host species and phylogenies.

The total number of OMPs identified in each of the seven *M. haemolytica* isolates varied from 29 (isolates PH278 and PH588) to 39 (PH8) (Table 3.5); twenty-four OMPs were identified in the *M. glucosida* isolate (PH344). Forty-one OMPs were identified in both bovine and ovine *M. haemolytica* isolates (Fig. 3.8). Of these, 20 were also identified in the *M. glucosida* isolate. Ten OMPs (MltC, FhaC, LppC, TonB-dependent receptor MHA_0860/COI_1565/COK_2304, OapA, TbpB, possible OMP MHA_0964/COI_1329/COK_2563, Bor/Iss_1, HxuC, TonB-dependent receptor MHA_1346/COI_1921) were identified exclusively in ovine *M. haemolytica* isolates. Three OMPs (possible OMP MHA_2054/COI_1788/ COK_2512, RlpA and CsgG) were identified exclusively in the *M. glucosida* ovine isolate and one OMP (OmpW) was identified exclusively in both an ovine *M. haemolytica* isolate and the *M. glucosida* isolate. No OMPs were identified exclusively in the *M. haemolytica* bovine isolates and there were no proteins identified exclusively in both the bovine *M. haemolytica* isolates and the *M. glucosida* isolate. With the exception of CsgG, which was an *A. pleuropneumoniae* orthologue, all of the OMPs identified in ovine *M. haemolytica* isolates and the *M. glucosida* isolate were present in either the bovine serotype A1 or bovine serotype A2 genomes. Therefore, these proteins may be present in bovine isolates but were not detected by proteomics approach used in this study. Alternatively, they may be expressed under different growth conditions. The putative bovine host-specific protein PulD that was predicted in the bovine serotype A1 genome was not identified in any representative isolates. These findings show that it has not been possible to establish any clear link between any of the identified OMPs and host-specificity.

Figure 3.8. Distribution of 55 OMPs identified by proteomic analyses based on identification in bovine *M. haemolytica* isolates, ovine *M. haemolytica* isolates and/or an *M. glucosida* isolate.



Fourteen OMPs were identified in all eight representative isolates (OmpA, Omp85, Imp/LptD, TolC, Pal, Ssa, OmpP1/FadL, PlpA, Wza, possible OMP MHA_0486/COI_0902/COK_2254, OmpP2-like, FrpB, *Rickettsia*-like surface antigen and LemA), indicating core functions in both *M. haemolytica* and *M. glucosida*. Hypothetical protein MHA_2025/COI_1815/COK_2485 was also identified in all eight isolates but did not have a confidently predicted location. Furthermore, two OMPs were identified in all seven *M. haemolytica* isolates (Lpp38 and TbpA) but not in the *M. glucosida* isolate, indicating core functions in *M. haemolytica* but, not necessarily, in *M. glucosida*.

There appeared to be a clear association between OmpP2 and isolates of serotypes A1, A7 and A13, but not of A2. The OmpP2 porin was identified by both gel-based and gel-free proteomic approaches in the serotype A1 isolates PH2 and PH8, the serotype A7 isolate PH296 and the serotype A13 isolate PH588. However, OmpP2 was not identified in any of the serotype A2 isolates or the *M. glucosida* isolate. In contrast, the OmpP2-like porin was identified in all eight representative isolates and exhibited considerable molecular mass variation.

The four putative trimeric autotransporters, YadA-like, Hsf, Hia and Ahs, were confidently predicted OMPs in all three genomes. However, the Hsf, Hia and Ahs proteins were not detected in any of the isolates. The YadA-like protein was identified in only two serotype A2 isolates, PH202 and PH292, by both gel-based and gel-free proteomic approaches. The protein was identified in the uppermost section of the polyacrylamide gel at the interface between the stacking and resolving gel, indicating that the trimeric ß-barrel domain does not undergo dissociation into its monomeric subunits during boiling in SDS sample buffer and cannot pass into the resolving gel. This is consistent with similar findings for Ahs (Daigneault and Lo, 2009) and other trimeric autotransporters (Sheets *et al.*, 2008).

The three *M. haemolytica* genomes each encoded three confidently predicted OMPs (Iga1_1, Iga1_2 and Iga1_3) that are homologous to the *H. influenzae* and *N. meningitidis* autotransporter Iga1, an IgA protease. A fourth Iga1-like protein, Iga1_4, was present only in the serotype A2 genomes. Iga1_1 and Iga1_4 were not detected in any of the eight representative isolates. Iga1_2 was detected by gel-based methods only in six isolates (PH2, PH8, PH202, PH292, PH296 and

PH588). The expected molecular mass of the Iga1_2 protein after removal of the N-terminal signal sequence is 152.9 kDa (data not shown), but all identified Iga1_2 proteins had an apparent molecular mass of approximately 35 kDa when resolved by 1-D SDS-PAGE. Examination of the mass spectrometry data for Iga1_2 showed better coverage and a greater degree of confidence for identified peptides in the C-terminal ß-barrel domain (data not shown), suggesting that the N-terminal passenger domain might have been proteolytically cleaved. Iga1_3 was identified by both gel-based and gel-free methods in isolates PH202, PH278 and PH296 at an apparent molecular mass of 156 kDa, which is consistent with the expected molecular mass after removal of the N-terminal signal peptide. Iga1_3 of a lower molecular mass was not identified, suggesting that Iga1_3 does not undergo cleavage and remains intact on the cell surface. Similarly, the Ssa protein was identified at 97 kDa in all isolates, but was also identified at 66.4 and 64.0 kDa in isolate PH2. This suggests that Ssa may also undergo a similar cleavage process in this isolate.

Three OMPs (possible OMP MHA_2054/COI_1788/COK_2152, RlpA and CsgG) were identified exclusively in the *M. glucosida* isolate PH344. Rare lipoprotein A (RlpA) was present in the three *M. haemolytica* genomes analysed by bioinformatics. Therefore, it is possible that RlpA is expressed in *M. haemolytica* but was simply not detected by proteomics. CsgG is an outer membrane lipoprotein (Loferer *et al.*, 1997) required for the secretion and stabilisation of two other proteins, CsgA and CsgB (yet to be identified in *M. glucosida*), which form curli amyloid fibres on the extracellular surface. These fibres have been implicated in a number of processes including biofilm formation, attachment and invasion of host cells, interaction with host proteins and activation of the immune system (Barnhart and Chapman, 2006). Given that CsgG was identified by proteomics only in the *M. glucosida* isolate and that it was absent in the three *M. haemolytica* genomes analysed by bioinformatics, it is reasonable to suggest that this protein is expressed solely in *M. glucosida* and not in *M. haemolytica*.

Although 55 OMPs were identified by proteomic approaches, there remained 43 confidently predicted OMPs that were not identified; 15 were predicted to be ß-barrel proteins, 15 were predicted to be outer membrane lipoproteins and 13 were not predicted to be either. Of these 43 unidentified proteins, five are involved in outer membrane biogenesis and integrity (LolB, NlpE, SmpA, WzzB

and MltB), twelve have transport and receptor activities (LamB, ComE, PulD, HxuA, FhuA, FhuE, HmbR2, haemin receptor, haemin-uptake lipoprotein, and three TonB-dependent receptors), five are involved in adherence (Hsf, Hia, Ahs, FhaB_1 and FhaB_2) and seven have enzymatic activity (Iga1_1, Iga1_4, GlpQ, NlpC, NlpD-like_1, NlpD-like_2 and AmiC protein). Fourteen confidently predicted OMPs with other or unknown functions were also not identified (fHbp_2, N1pI, PilF, EcnA, three possible OMPs, Omp26, OapB, PlpB, two autotransporter proteins and two lipoproteins).

3.4 Discussion

A bioinformatic workflow developed for *P. multocida* (E-Komon *et al.*, 2011b) was used to identify outer membrane-localised proteins in the genomes of three *M. haemolytica* isolates (a bovine serotype A1 isolate and bovine and ovine serotype A2 isolates). Using this approach, 93 confidently predicted OMPs were identified across the three genomes, representing 3.3%, 3.4% and 3.6% of the bovine serotype A1, ovine serotype A2 and bovine serotype A2 genomes, respectively. These figures are lower than those obtained for the *P. multocida* avian genome (4.8% of the proteome) (E-Komon *et al.*, 2011b) and that of the closely related *A. pleuropneumoniae* genome (4.3% of the proteome) (Chung *et al.*, 2007); however, the genomes of these organisms contain fewer open reading frames (2015 and 2241, respectively) than any of the three *M. haemolytica* genomes analysed in this study (between 2552 and 2695 open reading frames). Likely functions were assigned to 67 (72%) of the 93 confidently predicted OMPs. Further information is required to characterise the functions of the remaining 26 proteins.

A greater number of confidently predicted OMPs were present exclusively in both the bovine and ovine serotype A2 genomes than between either of these two genomes and the serotype A1 genome. Furthermore, amino acid sequence comparisons demonstrated that OMPs present in the bovine and ovine serotype A2 genomes show a higher degree of identity in relation to each other than to proteins in the bovine serotype A1 genome. The greater similarities between the two serotype A2 outer membrane subproteomes are consistent with a previous study of genetic diversity within the *M. haemolytica* population (Davies *et al.*, 1997) and a previous comparative genomic study (Lawrence *et al.*, 2010b).

These findings provide further evidence to suggest that bovine and ovine serotype A2 isolates of *M. haemolytica* have a common ancestral origin and have recently switched from one host to another. Three OMPs, OmpA, PlpE and Ahs, shared amino acid identity that was significantly greater between the bovine serotype A1 and A2 genomes than between either of these two genomes and the ovine serotype A2 genome. In a previous study, comparative nucleotide sequence analysis of the *ompA* gene revealed hypervariable regions in the distal tips of the surface-exposed loops (Davies and Lee, 2004). These regions vary considerably between bovine and ovine isolates but are highly conserved among isolates from each host species and it was suggested that OmpA is involved in binding to host-specific cell receptor (Davies and Lee, 2004). The exact function of PlpE is currently unknown; however, anti-PlpE antibodies have been demonstrated to promote complement-mediated killing of bovine serotype A1 isolates (Ayalew *et al.*, 2004, Nardini *et al.*, 1998) and the addition of recombinant PlpE to a commercial vaccine also confers enhanced resistance against experimental challenge in cattle compared to the commercial vaccine alone (Confer *et al.*, 2003). Ahs has the predicted structural architecture of a trimeric autotransporters and has been demonstrated to bind collagen *in vitro* (Daigneault and Lo, 2009). A higher percentage similarity in PlpE and Ahs between bovine serotype A1 and bovine serotype A2 genomes, compared to either of these and the ovine serotype A2 genome, suggests that these two proteins may have a role in host adaptation.

Using a combination of MALDI-TOF-TOF and LC-ESI-QqTOF mass spectrometry, a total of 55 OMPs were identified among the eight representative isolates of *M. haemolytica* and *M. glucosida*. Fifty of these proteins were confidently predicted by the bioinformatic prediction approach. The other five identified proteins, LemA, HbpA, OapA, RlpA and CsgG were not predicted by the bioinformatic approach but were indicated to be OMPs after subsequent literature searches. The LemA protein was identified in all eight representative isolates and is a homologue of the LemA protein originally identified in the Gram positive bacterium *Listeria monocytogenes*, a facultative intracellular pathogen (Lenz *et al.*, 1996). The function of this protein is currently unknown, although the N-terminus is thought to be surface-exposed on the outside of the bacterium. The HbpA protein has been identified in association with the outer membrane of

other *Pasteurellaceae* species (Hanson and Hansen, 1991, Hanson *et al.*, 1992, Garrido *et al.*, 2003) and has been implicated in the importation of both haem and glutathione (Vergauwen *et al.*, 2010, Hanson and Hansen, 1991, Hanson *et al.*, 1992). In *H. influenzae*, opacity-associated protein A (OapA) has been demonstrated to be a cell envelope protein that is required for efficient colonisation of the nasopharynx in an infant rat model (Weiser *et al.*, 1995) and contributes to epithelial cell binding (Prasadarao *et al.*, 1999). This protein was identified in only two ovine serotype A2 isolates (PH278 and PH296). In *E.coli*, rare lipoprotein A (RlpA) is thought to link the outer membrane to peptidoglycan during cell division (Arends *et al.*, 2010). The CsgG protein, which was not present in any of the three *M. haemolytica* genomes analysed by bioinformatic analysis, was identified by searching against a genome of the closely related species *A. pleuropneumoniae*. The CsgG protein is involved in the secretion of amyloid fibres and may have a role in biofilm formation (Robinson *et al.*, 2006). Thirty-six OMPs were identified by both gel-based and gel-free methods, whereas eight were identified exclusively by the gel-based method and 11 by the gel-free method. The gel-based method identified a higher proportion of ß-barrel proteins than outer membrane lipoproteins. Conversely, the gel-free method identified a higher proportion of outer membrane lipoproteins than ß-barrel proteins. These findings are consistent with the results of E-Komon *et al.* (E-Komon *et al.*, 2011a) and further reinforce the importance of using complementary approaches to maximise total proteomic coverage of OMPs. Furthermore, no additional OMPs were identified after gel-free analysis of whole cell envelope fractions. This indicated that no OMPs were lost as a result of inner membrane solubilisation and further demonstrated the reliability of Sarkosyl as an appropriate selective solubilisation detergent.

No OMPs were identified exclusively in bovine *M. haemolytica* isolates. This is not surprising given that only one of the 93 confidently predicted OMPs, PulD, was present exclusively in a bovine *M. haemolytica* genome. Fourteen OMPs were identified in the ovine *M. haemolytica* and *M. glucosida* isolates that were not identified in the bovine *M. haemolytica* isolates. Even though these proteins represent potential ovine-specific proteins, all but one (CsgG, an *A. pleuropneumoniae* orthologue detected exclusively in the *M. glucosida* isolate) were present in either the bovine serotype A1 or A2 genomes. Therefore, either

the proteomics approaches failed to identify these proteins or they may be expressed under different growth conditions.

A recent study using immunoproteomic analyses identified 132 immunoreactive proteins in the outer membrane fraction of a serotype A1 *M. haemolytica* isolate from a bovine host (Ayalew *et al.*, 2010). Of these, 35 proteins were identified in the outer membrane fractions of eight representative isolates in the present study (Supplementary Table S3.7). However, according to the bioinformatic analyses performed in this study, 25 of the 35 proteins were OMPs, one was a hypothetical protein of unknown function, and nine were false-positives. The immunoproteomic analyses failed to identify the remaining 30 OMPs identified in the present study or any of the 43 confidently predicted OMPs that were not identified in the present study. However, seven proteins (HbpA, MltC, ComL, OmpW, LppC, TonB-dependent receptor MHA_0860/COI_1565/ COK_2304 and TbpB) were identified that were not identified in the bovine serotype A1 *M. haemolytica* isolate (PH2) used in the present study, although these proteins were identified in one or more of the other representative isolates. The approaches used in the present study and in the study of Ayalew *et al*. (Ayalew *et al.*, 2010) can potentially discriminate between those OMPs which are surface-exposed and immunogenic and those which are not.

Fourteen OMPs (Table 3.5) were identified in all eight representative isolates, suggesting core functions in the biology of *M. haemolytica* and *M. glucosida*. One of these proteins, the Ssa autotransporter protein, is a subtilisin-like serine protease which shares significant identity with similar autotransporters in other species including AasP of *A. pleuropneumoniae* (Ali *et al.*, 2008), NalP of *N. meningitidis* (van Ulsen *et al.*, 2003, Turner *et al.*, 2002) and SphB1 of *B. pertussis* (Coutte *et al.*, 2001), all of which act as surface maturation proteases. This function has not yet been determined for Ssa, or indeed any other *M. haemolytica* OMP, but may be appropriate given that some surface-exposed proteins have not been identified in as many isolates as expected. For example, the transferrin-binding proteins A and B (TbpA and TbpB) are OMPs involved in the uptake of iron from host transferrin. TbpA is an integral TonB-dependent transporter and was identified in all seven representative *M. haemolytica* isolates (but not in *M. glucosida* isolate PH344). However, TbpB is a surface-exposed lipoprotein and was identified in only one isolate. Both OMPs are

encoded in the same operon and are expected to be expressed in similar quantities, but it would appear that TbpB is largely absent from the outer membrane of most isolates. One explanation is that TbpB is lost from the outer membrane during the extraction process. However, the NalP protein of *N. meningitidis* has recently been found to cleave lactoferrin-binding protein B (LbpB), a close homologue of TbpB, from the bacterial cell surface (Roussel-Jazede *et al.*, 2010). Therefore, an alternative explanation for the apparent absence of TbpB is that Ssa is cleaving this protein from the bacterial cell surface, as has been shown for NalP. Interestingly, in isolate PH2, Ssa was identified at 66.4 and 64.0 kDa in addition to its expected molecular mass of 97 kDa. Autocatalytic processing of passenger domains is a characteristic of NalP (van Ulsen *et al.*, 2003) and SphB1 (Coutte *et al.*, 2001), but not of AasP (Ali *et al.*, 2008), therefore it is possible that Ssa in isolate PH2 possesses similar autocatalytic ability. Differences in Ssa amino acid sequences among the representative isolates might explain why this was only observed in one isolate. Furthermore, the *ssa* gene was previously considered to be absent from *M. glucosida* isolates (Gonzalez *et al.*, 1991), yet its expression at the outer membrane has been clearly demonstrated in the present study.

Two other serine protease autotransporters were also identified. These were Iga1_2 (in two bovine and four ovine isolates) and Iga1_3 (in one bovine and two ovine isolates), which are orthologous to Iga1 in the human pathogens *N. meningitidis* and *H. influenzae*. In these pathogens, Iga1 cleaves host mucosal IgA1 antibody, destroying its agglutinating activity and facilitating bacterial colonisation by immunoevasion. While IgA protease activity has not been detected in the supernatants of *M. haemolytica* (Abdullah *et al.*, 1992), IgG-specific protease activity was detected in partially purified culture supernatants (Lee and Shewen, 1996). IgG is also the predominant secretory antibody found in the lower respiratory tract of cattle (Duncan *et al.*, 1972), whereas IgA is the predominant antibody in the upper respiratory tract (Wilkie and Markham, 1981). It is therefore possible that at least one of the Iga1 orthologues identified in *M. haemolytica* actually cleaves IgG instead of IgA. In *N. meningitidis* and *H. influenzae*, the Iga1 protease passenger domain is released from the outer membrane-embedded ß-barrel domain by autoproteolysis. Interestingly, the findings of the present study suggest that proteolysis occurs in Iga1_2, but not in

Iga1_3, even though both contain an active serine protease site. One explanation is that Iga1_3 does not contain a self-recognition sequence for autoproteolytic cleavage and therefore remains as a full length protease anchored to the cell surface by the ß-barrel domain. Alternatively, proteolysis may be mediated by another surface-exposed protein. In *N. meningitidis*, Iga1 can be proteolytically cleaved by NalP (van Ulsen *et al.*, 2003), therefore it is possible that *M. haemolytica* Ssa has a role in the proteolytic cleavage of *M. haemolytica* Iga1-like proteins.

Four trimeric autotransporters were predicted in the bioinformatic analyses (YadA-like, Hsf, Hia and Ahs), all of which have putative roles in adhesion to host molecules. However, only expression of the YadA-like protein was confirmed in one bovine and one ovine serotype A2 isolates (by both gel-based and gel-free approaches). This is the first case of a trimeric autotransporter being detected at the proteomic level in *M. haemolytica*. The Hsf and Hia proteins have been discussed previously in *M. haemolytica* (Gioia *et al.*, 2006), although their exact functions have not yet been characterised. The Ahs protein has previously been reported to be involved in collagen binding (Daigneault and Lo, 2009) and to be transcribed *in vivo* (Lo *et al.*, 2006) in a serotype A1 isolate from a bovine host, although this protein was not identified in any of the representative isolates of the present study.

In the present study, 32 proteins were identified that were predicted to be localised to cellular compartments other than the outer membrane (Table 3.5). The possibility cannot be ruled out that some of these proteins may have secondary functions at the outer membrane, or indeed be true OMPs, even though they were predicted to be located elsewhere. For example, the haem-binding protein HbpA was identified in the outer membrane fractions of five *M. haemolytica* isolates (Table 3.5) but has a C-terminal motif which indicates that it is a lipoprotein anchored to the inner membrane. However, in the closely-related species *H. influenzae*, HbpA has been identified in both inner membrane and outer membrane fractions (Hanson and Hansen, 1991, Hanson *et al.*, 1992), and has been suggested to be involved in the shuttling of material between the two membranes. The identification of HbpA in this study also suggests a likely function at the outer membrane of *M. haemolytica*, hence its inclusion as an OMP. Another protein, Lpp38, was identified in all seven *M. haemolytica* isolates

and has an amino acid sequence that is 55% identical (70% when conservative amino acid substitutions are included) to the periplasmic *E. coli* protein PotD which is involved in polyamine transport. Lpp38 would have been considered to be a periplasmic protein had it not been for experimental evidence that demonstrated partial exposure of Lpp38 on the surface of *M. haemolytica* (Pandher and Murphy, 1996). This illustrates that even though an identified protein is very similar to another protein in a different species it does not necessarily have the same function and/or subcellular location as that protein.

Forty-three of the 93 confidently predicted OMPs across the three genomes were not identified by proteomic analyses. It is possible that some of these proteins are actually present in the outer membrane but only at low levels which are insufficient to permit detection by the methods used in this study. The expression of OMPs also differs when isolates are grown under conditions that mimic the *in vivo* host environment. A previous study examined gene expression changes in a *M. haemolytica* serotype A1 isolate when grown in iron-restricted conditions to mimic the paucity of iron in the host lung (Roehrig *et al.*, 2007). Several genes encoding OMPs were upregulated, including four confidently predicted OMPs that were not identified in the present study: haemin receptor, HmbR2, fHbp-like_2 and possible OMP MHA_2761/COI_0565/COK_2341 (Roehrig *et al.*, 2007). Expression of several *M. haemolytica* OMPs was also affected by the addition of different antibiotics to the growth medium (Nardini *et al.*, 1998). It is therefore important to consider the effect that different growth conditions have on OMP expression when attempting to characterise outer membrane subproteomes.

In summary, a comprehensive bioinformatic workflow was implemented to predict the outer membrane subproteomes of three *M. haemolytica* genomes from isolates with different capsular serotypes and host origins. Subsequently, a total of 55 OMPs were identified from the outer membrane fractions of seven *M. haemolytica* isolates, together with a single *M. glucosida* isolate, which are representative of different phylogenetic lineages (ETs), capsular serotypes and host origins. Many of these OMPs are associated with particular isolates and/or capsular serotypes and have been identified at the protein level for the first time. Despite not being able to establish involvement in host-specific adaptation for the OMPs identified in this study, it will serve as a springboard for further

research into the roles of these diverse proteins in the pathobiology of *M. haemolytica*.

4. COMPARATIVE OUTER MEMBRANE PROTEOMIC ANALYSES OF BOVINE AND OVINE ISOLATES OF *M. HAEMOLYTICA* AND *M. GLUCOSIDA* GROWN UNDER VARIOUS *IN VITRO* CONDITIONS DESIGNED TO MIMIC THE *IN VIVO* HOST ENVIRONMENT

4.1 Introduction

M. haemolytica colonises the nasopharynx and tonsils of healthy cattle and sheep, but under the context of immune suppression (induced by stress or concurrent viral infection) bacteria rapidly increase in number and gain access to the lungs and trachea via aerosolised droplets and cause pneumonia (Gilmour and Gilmour, 1989, Frank, 1989). In most opportunistic pathogens, certain proteins associated with virulence are only expressed during infection in target tissues (Mekalanos, 1992). Therefore, it is likely that *M. haemolytica* only expresses certain virulence-associated proteins when growing in the upper respiratory tract of healthy animals and the lungs of pneumonic hosts, or under conditions which closely mimic this microenvironment.

An essential prerequisite of successful respiratory tract colonisation by *M. haemolytica* is being able to adapt to an environment containing very low levels of free iron. With the exception of some lactobacilli (Weinberg, 1997, Bruyneel *et al.*, 1989, Duhutrel *et al.*, 2010) iron is an essential micronutrient to all bacteria. It is a cofactor in the catalytic core of enzymes involved in a diverse range of cellular processes such as respiration and nucleic acid synthesis and repair. However, iron concentration is tightly regulated in host tissues as it can cause oxidative damage if present in excess amounts. Thus, in host tissues nearly all iron is sequestered by high-affinity iron-binding proteins such as transferrin, lactoferrin, ferritin, haemoglobin and other compounds. To overcome these low iron levels, bacteria which reside on respiratory tract mucosa have evolved a number of iron acquisition systems. These include the secretion of siderophores (small molecules with high affinity for iron) and the expression of outer

membrane receptors specific for iron-containing host proteins (Wandersman and Delepelaire, 2004). In *M. haemolytica*, iron can be obtained from host transferrin via the outer membrane-located transferrin binding proteins A and B (TbpA and TbpB) (Ogunnariwo *et al.*, 1997, Lee and Davies, 2011). Additional OMPs present in the published *M. haemolytica* genomes are also predicted to have roles in iron acquisition, including components of a putative haem-haemopexin acquisition system (Gioia *et al.*, 2006, Lawrence *et al.*, 2010a). Microarray analysis of gene regulation under iron-restricted conditions has been completed in a single bovine serotype A1 *M. haemolytica* isolate which demonstrated the upregulation of transcripts encoding other receptors for the transport of haem, haemoglobin and, possibly, siderophores (Roehrig *et al.*, 2007). However, a comparative proteomic analysis of OMP expression in several *M. haemolytica* isolates under iron-restricted conditions is still lacking.

M. haemolytica is also likely to encounter host serum factors contained in the fibrinous exudate which leaks into the lungs as a result of lung tissue damage caused by virulence factors including leukotoxin (Czuprynski *et al.*, 1991, Yoo *et al.*, 1995b, Stevens and Czuprynski, 1996, Sun *et al.*, 1999) and lipopolysaccharide (Keiss *et al.*, 1964, Li and Clinkenbeard, 1999). These serum factors include complement, antibodies, hormones and other host proteins which may interact with the bacterial cell surface. In other Gram-negative organisms serum components have been demonstrated to interact with and modulate the expression of OMPs (Hellman *et al.*, 2000, Johansson *et al.*, 2003).

It is believed that biofilm formation is a mechanism by which several Gram-negative mucosal pathogens establish long-term colonisation in the host (Luke *et al.*, 2007, Hall-Stoodley *et al.*, 2006, Kaplan *et al.*, 2004, Lam *et al.*, 1980, Greiner *et al.*, 2005, Yi *et al.*, 2004). However, at present there is only limited evidence of biofilm formation in *M. haemolytica* (Olson *et al.*, 2002, Haig, 2011). *M. haemolytica* resides in the respiratory tract via surface-associated growth on host tissues. Previous studies have demonstrated *in vitro* binding of *M. haemolytica* to epithelial cells (Kisiela and Czuprynski, 2009, Clarke and Morton, 2000, Galdiero *et al.*, 2002, Vilela *et al.*, 2004) and implicated several OMPs as host adhesion molecules, including outer membrane protein A (OmpA) (Kisiela and Czuprynski, 2009, Lo and Sorensen, 2007, Davies *et al.*, 2001) and Ahs (Daigneault and Lo, 2009). Some genes that are potentially involved in

adherence and biofilm synthesis have been demonstrated to be upregulated during adhesion to epithelial cells in the closely-related bacteria *A. pleuropneumoniae* (Auger *et al.*, 2009). Surface-associated growth, as opposed to planktonic growth, may result in the differential expression of OMPs involved in biofilm formation and adherence to host tissues.

A comprehensive comparative proteomic analysis of OMPs expressed by several representative *M. haemolytica* isolates and one *M. glucosida* isolate after *in vitro* growth in complex growth medium was completed in the previous chapter. In the present chapter, using a similar comparative proteomic approach, the outer membrane subproteomes of the same representative isolates were characterised after *in vitro* growth under conditions that were designed to mimic the *in vivo* pneumonic lung microenvironment. These conditions included growth in iron-restricted complex growth medium, serum-supplemented tissue culture media and growth on solid-surface agar (in the absence or presence of the dye Congo red). This approach has allowed the identification of several virulence-associated proteins, many of which have not previously been identified in these organisms. Furthermore, an examination of the extracellular subproteome of the representative isolates was undertaken after growth in iron-restricted and iron-replete complex growth media to identify secreted proteins and OMPs fragments that could have been proteolytically cleaved from the bacterial cell surface.

4.2 Materials and methods

4.2.1 Bacterial isolates and growth conditions

The eight bacterial isolates used in this study have been described previously in section 3.2.2. Isolates were stored at -80°C in 50% (v/v) glycerol in brain heart infusion broth (BHIB) and were subcultured on BHI agar (BHIA) containing 5% (v/v) defibrinated sheep blood overnight at 37°C. For growth under iron-restricted conditions, optimum concentrations of the iron chelator 2,2'-dipyridyl were determined (to the nearest 10 μM) for each isolate from growth rate experiments where growth, in the presence of increasing concentrations of 2,2'-dipyridyl, was followed spectrophotometrically at 660 nm. The optimum concentration of 2,2'-dipyridyl for each isolate was deemed to be the maximum

concentration at which a cell density of 0.8 OD_{660nm} was achieved within a 12 h period. For outer membrane preparations after growth in iron-restricted conditions, liquid starter cultures were prepared by inoculating a few colonies into 15 ml volumes of BHIB and incubating overnight at 37°C with shaking at 120 rpm. Eight hundred microlitres of overnight culture were used to inoculate a 2-litre Ehrlenmeyer flask containing 400 ml of BHIB and the optimum final concentration of 2,2'-dipyridyl and incubated at 37°C with shaking at 120 rpm until an OD_{600nm} of 0.8-0.9 was achieved (mid-log phase) or for 18 h (stationary phase). For outer membrane preparations under serum-supplemented conditions, liquid starter cultures were prepared by inoculating a few colonies into 15 ml volumes of BHIB and incubating overnight at 37°C with shaking at 120 rpm. Six hundred microlitres of overnight culture were used to inoculate 1-litre Ehrlenmeyer flasks containing 300 ml of either (a) Dulbecco's Modified Eagle Medium (DMEM) containing 4.5 g glucose/L, 110 mg sodium pyruvate/L and L-glutamine (Sigma), (b) RPMI-1640 containing L-glutamine and $NaHCO_3$ (Sigma) or (c) Medium 199 (M199) containing Earle's salts and L-glutamine (Gibco), (d) 270 ml of the three aforementioned media supplemented with 30 ml of either decomplemented (56 °C for 30 min) foetal calf serum (FCS) (Sigma) or sheep serum (SS) (Sigma), and (e) 300 ml of either complete decomplemented FCS, SS or newborn calf serum (NCS). The flasks were incubated at 37 °C with shaking at 120 rpm until an OD_{600nm} of 0.8-0.9 was achieved. For outer membrane preparations of bacteria after growth on solid surface agar, bacterial suspensions were prepared by resuspending 6-12 colonies in 3 ml of sterile PBS in a bijou. One hundred microlitres of this suspension was spread over each of 24 BHIA plates, or 24 BHIA plates supplemented with Congo red dye at 0.8 g/L ($BHIA_{CR}$) and incubated for 24 h at 37°C. Cells were scraped off with a sterile plastic inoculating loop and resuspended in 50 ml of 20 mM Tris, pH 7.2. For outer membrane preparations after growth in BHIB containing Congo red ($BHIB_{CR}$), liquid starter cultures were prepared by inoculating a few colonies into 15 ml volumes of BHIB and incubating overnight at 37°C with shaking at 120 rpm. Eight hundred microlitres of overnight culture were used to inoculate a 2-litre Ehrlenmeyer flask containing 400 ml of BHIB supplemented with Congo red dye at 0.8 g/L ($BHIB_{CR)}$ and incubated at 37°C with shaking at 120 rpm until an OD_{600nm} of 0.8-0.9 was achieved. For extracellular protein preparations, liquid starter cultures were prepared by inoculating a few colonies into 15 ml volumes

of BHIB and incubating overnight at 37°C with shaking at 120 rpm. Four hundred microlitres of overnight culture were used to inoculate a 1-litre Ehrlenmeyer flask containing 200 ml of BHIB in the absence or presence of the optimum final concentration of 2,2'-dipyridyl and incubated at 37°C with shaking at 120 rpm until stationary phase growth had been achieved (12 h and 18 h for iron-replete and iron-restricted growth, respectively).

4.2.2 Preparation of OMPs.

Outer membrane proteins were prepared by Sarkosyl extraction as previously described in section 2.2.10

4.2.3 Preparation of extracellular proteins

The extracellular proteins were prepared as described previously (Xia *et al.*, 2008, Nandakumar *et al.*, 2006) with some modifications. Cells were removed by centrifugation at 10,000 × *g* for 20 min at 4°C. Forty millilitres of the clear supernatant were collected, passed through a 0.2 µm filter, mixed with an equal volume of ice cold 20% (w/v) TCA in acetone for 3 h at -20°C. The precipitate was collected by centrifugation at 5,500 × *g* for 20 min at 4°C. The precipitated protein pellet was washed twice with ice cold acetone to remove TCA and air-dried. The final pellets were resuspended in approximately 500 µl of 20 mM Tris/HCl (pH 7.2). Fifty microlitre aliquots of these suspensions were transferred to separate tubes and their protein concentrations determined by the modified Lowry procedure. One hundred microlitre aliquots of the extracellular protein suspensions were adjusted to 4 mg/ml with 20 mM Tris/HCl (pH 7.2) and stored at -80°C. Eighty micrograms of each extracellular protein preparation were separated by 1-D SDS-PAGE

4.2.4 Gel-based proteomic analysis of OMPs

Twenty micrograms of each OMP preparation were separated by 1-D SDS-PAGE as previously described in section 3.2.4. Gel pieces were manually excised and digested with trypsin as previously described in 3.2.4. Peptides were analysed by LC-ESI MS/MS as previously described in section 3.2.7 using either the QSTAR XL Hybrid LC/MS/MS system or the Bruker amaZon ETD system (coupled to the Dionex UltiMate 3000 nano LC).

4.2.5 Gel-free proteomic analysis of OMPs

Outer membrane fractions were directly digested with trypsin without prior separation by 1-D SDS-PAGE as previously described in 3.2.5. Peptides were analysed by LC-ESI MS/MS using the QSTAR XL Hybrid LC/MS/MS as previously described in section 3.2.5.

4.2.6 Gel-based analysis of extracellular proteins

Eighty micrograms of each extracellular protein preparation were separated by 1-D SDS-PAGE as previously described in section 3.2.4.

4.2.7 Gel band quantification

Gel images were scanned using the GeneScan (Syngene) gel documentation system. Band quantification was performed using GeneTools (Syngene).

4.3 Results

4.3.1 Identification of *M. haemolytica* and *M. glucosida* OMPs which undergo changes in expression after growth in iron-restricted conditions.

To understand how OMPs are involved in the adaptation of *M. haemolytica* and *M. glucosida* to the low levels of free iron in the host respiratory tract, representative isolates were grown in the presence of 2,2'-dipyridyl, an iron-chelating agent that has been used in several previous studies to create iron-restricted conditions (Paustian *et al.*, 2001, Merrell *et al.*, 2003, McHugh *et al.*, 2003, Klitgaard *et al.*, 2010, Roehrig *et al.*, 2007). In order to obtain sufficient cells for outer membrane extraction, a concentration of 2,2'-dipyridyl was chosen for each representative isolate that gave significant growth-rate reduction (indicating iron-restriction), but which still allowed the growth of relatively high cell numbers. These were determined by following the growth of each representative isolate spectrophotometrically at 660 nm in BHIB in the presence of increasing concentrations of 2,2'-dipyridyl until a concentration had been reached that still allowed mid-log phase growth (OD_{660nm} 0.8-0.9) to be achieved within 12 h. As has been noted previously in *M. haemolytica* (Davies *et al.*, 1992), growth of each representative isolate was inhibited within a very

narrow range of concentrations. Interestingly, the different isolates displayed a broad range of tolerances towards growth in the presence of 2,2'-dipryridyl (Fig. 4.1). The seven *M. haemolytica* isolates grew to mid-log phase within 12 h at 2,2'-dipryridyl concentrations ranging between 120 and 245 μM; the *M. glucosida* isolate grew to mid-log phase within 12 h at a 2,2'-dipryridyl concentration of 310 μM (Table 4.1). OMP profiles of these isolates grown to mid-log phase in iron replete BHIB (Fig. 4.2A) were compared with those of the same isolates grown in presence of the optimum concentrations of 2,2'-dipryridyl (Fig. 4.2B).

In comparison to growth in iron-replete BHIB (Fig. 4.2A), the OMP expression profiles of all isolates were dramatically altered after growth in iron-restricted BHIB (Fig. 4.2B). In particular, upregulation of several high molecular mass proteins was observed. A total of 72 unique proteins were identified among the outer membrane fractions of the representative isolates after growth in iron-restricted BHIB using a combination of gel-based and gel-free approaches (Table 4.2). Fifty-nine of these proteins were previously identified in the previous chapter after growth in iron-replete BHIB, and included 38 OMPs, 5 proteins of unknown location, and 16 proteins predicted to be localised to cellular compartments other than the outer membrane. The other 13 proteins were not previously identified under growth in iron-replete BHIB and are possible virulence-associated proteins. Eight of these proteins were confidently predicted to be OMPs and included a putative haemin receptor, LamB (the substrate-specific maltoporin), FhuA (a putative ferrichrome receptor), autotransporter MHA_0080/COI_1870/ COK_0773 (function unknown), EcnA (possibly involved in programmed cell apoptosis), HmbR2 (a putative haemoglobin receptor), HxuA (a putative haemophore involved in haem-haemopexin uptake) and Iga1_4 (an Iga1-like protease). The subcellular localisations of two identified proteins with unknown functions (hypothetical protein MHA_1307/COI_1292/COK_0162 and lipoprotein MHA_2737/COI_0543/COK_2363) could not be confidently determined. The remaining three identified proteins (PenP, NqrC and RpsP) were predicted to be localised to cellular compartments other than the outer membrane.

The expression of several OMPs was increased in all eight representative isolates after growth in iron-restricted BHIB compared to iron-replete BHIB. There was a 1.1- to 5.1-fold increase in the expression of TbpA (number 17; Fig 4.2B) among

Figure 4.1. **Growth curves of seven *M. haemolytica* isolates and one *M. glucosida* isolate in BHIB in the absence or presence of various concentrations of the iron chelator 2,2'-dipyridyl.**

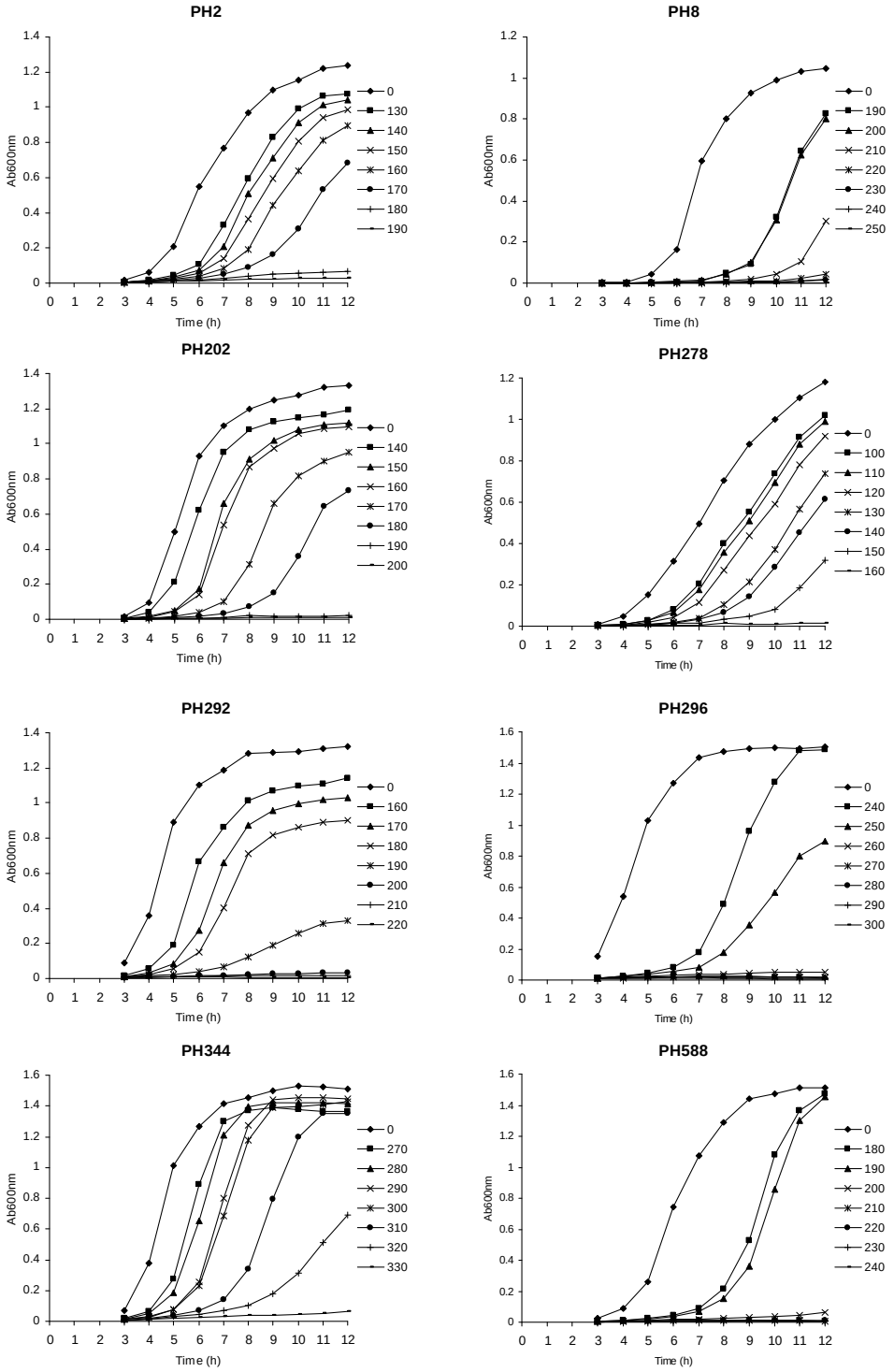


Table 4.1. Properties of seven representative *M. haemolytica* isolates and one *M. glucosida* (PH344) isolate, and the final 2,2'-dipyridyl concentrations used to create iron-restricted growth conditions for each isolate.

Isolate	Animal host	Electro-phoretic type	Capsular serotype	OMP type	Disease status	2,2'-dipryridyl concentration (mM)
M. haemolytica						
PH2	Bovine	1	A1	1.1.1	Pneumonia	160
PH8	Ovine	6	A1	1.2.1	Pneumonia	200
PH202	Bovine	21	A2	2.1.2	Healthy	170
PH278	Ovine	21	A2	2.2.2	Pneumonia	120
PH292	Ovine	22	A2	2.2.1	Pneumonia	175
PH296	Ovine	12	A7	3.1.1	Pneumonia	245
PH588	Ovine	15	A13	3.3.2	Pneumonia	190
M. glucosida						
PH344	Ovine	N/A	A11	3.2.2	Septicaemia	310

Figure 4.2. One-dimensional SDS-PAGE separation of outer membrane fractions from seven *M. haemolytica* isolates and one *M. glucosida* isolate after growth to mid-log phase in (A) iron-replete and (B) iron-restricted BHIB.

Numbered proteins were subsequently identified by proteomic analysis (Table 4.2).

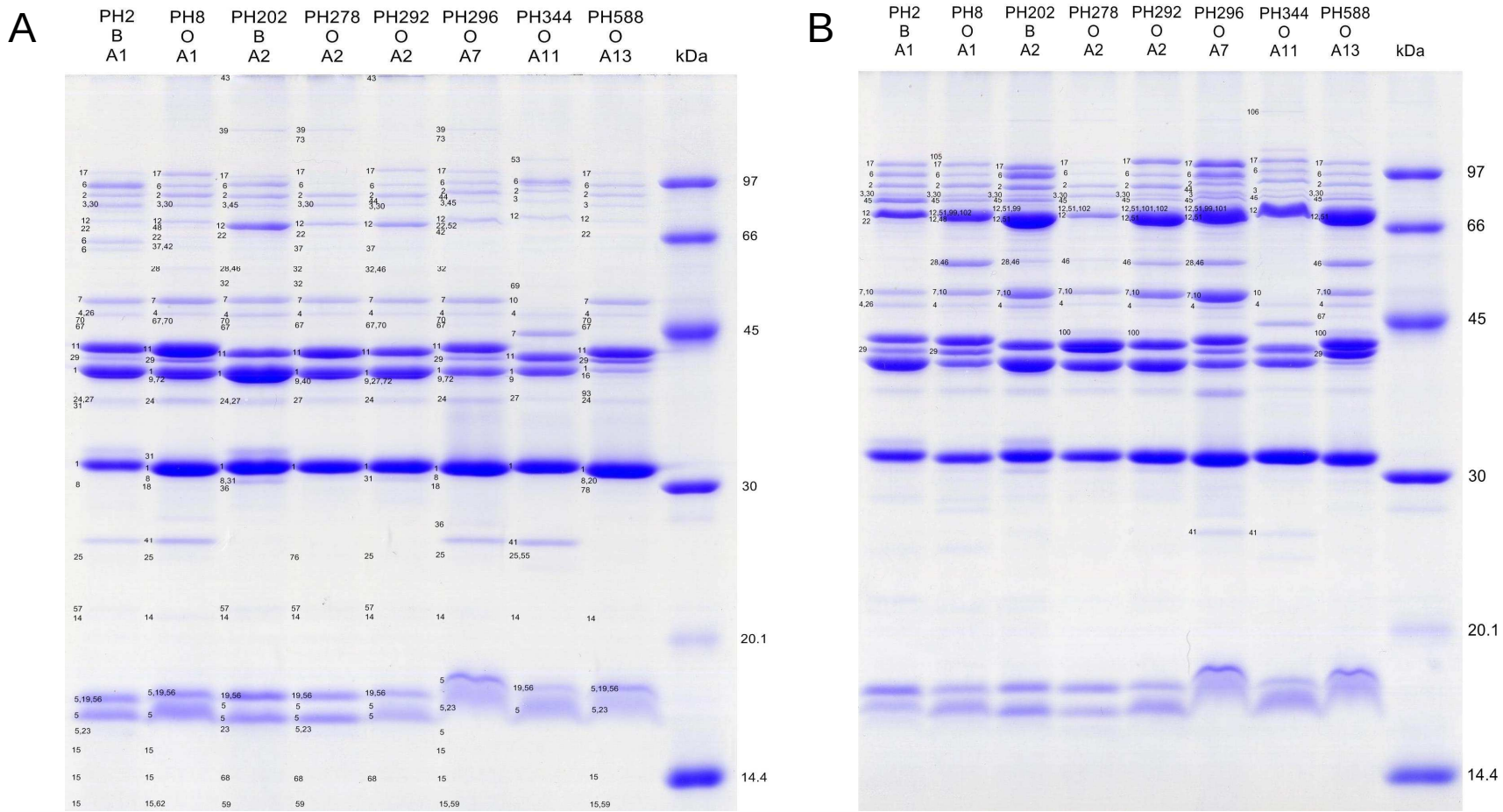


Table 4.2. Proteins identified in the outer membrane fractions of seven representative isolates of *M. haemolytica* and one isolate of *M. glucosida* (PH344) after mid-log phase growth in iron-restricted BHIB using a combination of gel-based and gel-free proteomic approaches.

								Protein identifications[d]							
No.[a]	Sub. Local.[b]	TM[c]	Lipo[c]	Bovine A1	Ovine A2	Bovine A2	Name	PH2	PH8	PH202	PH278	PH292	PH296	PH344	PH588
1. Proteins previously identified in iron-replete BHIB															
1	OM	+/−	−	MHA_1054	COI_1980	COK_0402	OmpA	+[2]	+[2]	+[2]	+[2]	+[2]	+[2]	+[2]	+[2]
2	OM	+	−	MHA_0691	COI_1174	COK_1967	Omp85	+	+	+	+	+	+	+[2]	+
3	OM	+	−	MHA_0291	COI_2627	COK_0922	Imp/LptD	+	+	+	+	+	+	+	+
4	OM	+	+	MHA_1410	COI_0732	COK_0022	TolC	+	+	+	+	+	+	+[1]	+
5	OM	−	+	MHA_0263	COI_2595	COK_1835	Pal	+[2]	+[2]	+[2]	+[2]	+[2]	+[2]	+[2]	+[2]
6	OM	+	−	MHA_2492	COI_0850	COK_2411	Ssa	+	+	+	+[1]	+	+	+	+
7	OM	+	−	MHA_0639	COI_1221	COK_2014	OmpP1/FadL	+	+	+	+	+	+	+[2]	+
8	OM	−	+	MHA_1433	COI_0758	COK_0049	PlpA	+[2]	+[2]	+[2]	+[2]	–	–	+[2]	+[2]
9	OM	+	+	MHA_0527	COI_1313	COK_0141	Wza	+[2]	+[2]	+[2]	+[2]	–	+[2]	–	–
10	OM	+	−	MHA_0486	COI_0902	COK_2254	Possible OMP	+[1]	+[1]	+[1]	+[1]	+[1]	+[1]	+[1]	+[1]
11	OM	+	−	MHA_1793	COI_0051	COK_2166	OmpP2-like	–	–	+[2]	+[2]	+[2]	+[2]	–	+[2]
12	OM	+	−	MHA_2109	COI_0085	COK_0218	FrpB	+	+	+	+	+	+	+	+

Proteins that were bioinformatically predicted to be putative OMPs are grey-shaded.
[a]Numbers correspond to the location of the protein in Figure 4.2B if identified by gel-based proteomics.
[b]Confidently predicted subcellular locations; 'OM' = outer membrane; 'P' = periplasm; 'IM' = inner membrane; 'C' = cytoplasm; 'E' = extracellular; 'U' = unknown
[c]Transmembrane β-barrel and lipoprotein prediction result; '+' = predicted in all genomes; '−' = not predicted in any genome; '+/−' = predicted in one/two genomes (see Supplementary Tables S3.1, S3.2 and S3.3 for specific result).
[d]Two proteomics methods were compared; '+[1]' = proteins identified by gel-based method; '+[2]' = proteins identified by gel-free method; '+' = proteins identified by both methods; '−' = no identification.

Table 4.2. (continued)

No.	Sub. Local.	TM	Lipo	Bovine A1	Ovine A2	Bovine A2	Name	Protein identifications							
								PH2	PH8	PH202	PH278	PH292	PH296	PH344	PH588
13	OM	−	+	−	COI_1456	COK_1118	*Rickettsia*-like antigen	+[2]	+[2]	+[2]	+[2]	+[2]	+[2]	+[2]	+[2]
14	OM			MHA_0704	COI_1162	COK_1955	LemA	−	−	−	−	+[2]	−	−	−
16	OM	−	+	MHA_2152	COI_0845	COK_2238	Lpp38	+[2]	+[2]	+[2]	−	+[2]	+[2]	−	+[2]
17	OM	+	−	MHA_0196	COI_2333	COK_1753	TbpA	+	+	+	+[1]	+	+	+	+
18	OM	−	+	MHA_2837	COI_1320	COK_1388	VacJ	−	−	−	+[2]	+[2]	−	−	−
19	OM	−	−	MHA_2237	COI_2485	COK_1303	OMP18/16	−	−	−	+[2]	−	+[2]	−	−
20	OM	−	+	MHA_2158	COI_0840	COK_2233	OmpP4	+[2]	−	−	−	−	−	+[2]	+[2]
21	OM	−	+	−	COI_0336	COK_2129	YajG	+[2]	+[2]	+[2]	−	+[2]	−	−	+[2]
22	OM	+	−	MHA_0323	COI_0250	COK_0133	YtfM	+[1]	−	−	−	−	−	−	−
25	OM	−	+	MHA_2282	COI_2279	COK_1699	HlpB	+[2]	−	−	−	+[2]	+[2]	−	−
26	OM	−	+	MHA_1514	COI_1139	COK_0733	PlpE	+	+[2]	+[2]	−	+[2]	−	−	+[2]
28	IM/P/OM			MHA_1007	COI_1369	COK_1206	HbpA	−	+	+	+[2]	+[2]	+[1]	−	+[2]
29	OM	+	−	MHA_0735	ctg112	COK_1380	OmpP2	+	+[1]	−	−	−	+[2]	−	+
30	OM	+	−	MHA_1532	COI_0667	COK_1504	NanH	+	+	+[1]	+	+[1]	−	−	+[1]
33	OM	−	+	MHA_0669	COI_1194	COK_1987	RlpB/LptE	+[2]	−	−	+[2]	−	−	−	−
34	OM	−	+/−	MHA_1464	COI_0386	COK_1074	PlpD	+[2]	+[2]	−	−	−	−	−	+[2]
35	OM	−	+	MHA_0242	COI_0470	COK_0285	MltC	−	+[2]	−	−	−	−	−	−
38	OM	−	+	MHA_1435	COI_0760	COK_0051	PlpC	+[2]	+[2]	−	−	−	−	−	−
40	OM	−	+	MHA_1133	COI_1670	COK_0590	MltA	+[2]	+[2]	−	−	−	−	−	−
41	OM	−	−	MHA_2399	COI_1652/3	COK_1583/4	OmpW	−	−	−	−	−	+[1]	+[1]	−

Table 4.2. (continued)

No.	Sub. Local.	TM	Lipo	Bovine A1	Ovine A2	Bovine A2	Name	Protein identifications							
								PH2	PH8	PH202	PH278	PH292	PH296	PH344	PH588
44	OM	−	−	MHA_0860	COI_1565	COK_2304	TBDR	−	−	−	−	−	+[1]	−	−
45	OM	−	−	MHA_2261	COI_2258	COK_1624	HmbR1	+	+	+	+[1]	+	+	+	+
46	OM	+	−	MHA_1005	COI_1367	COK_1209	HxuB	−	+	+	+	+	+	−	+
47	OM			MHA_2702	COI_1630	COK_1108	OapA	−	+[2]	−	−	−	−	−	+[2]
48	OM	+/−	+	MHA_0197	COI_2332	COK_1752	TbpB	+[2]	+	+[2]	−	−	+[2]	+[2]	+[2]
51	OM	+	−	ctg59	COI_1368	COK_1207/8	HxuC	−	+	+	+	+	+	−	+
56	U	+	+	MHA_2025	COI_1815	COK_2485	Hypothetical protein	+[2]	+[2]	+[2]	+[2]	+[2]	+[2]	+[2]	+[2]
57	U			MHA_2087	COI_1609	COK_1902	Hypothetical protein	+[2]	+[2]	−	−	−	−	−	−
58	U			MHA_1782	COI_0313	COK_0888	Hypothetical protein	−	−	−	+[2]	−	−	−	−
59	U	−	+	MHA_0761/2	COI_1052	COK_0436	Hypothetical protein	−	−	−	−	−	−	+[2]	−
63	U			MHA_1898	COI_0630	COK_1217	LysM domain protein	−	+[2]	−	−	−	−	−	−
67	P	+	−	MHA_0371	COI_1485	COK_0985	AcrA_1	+[2]	−	−	−	−	−	−	+
68	IM			MHA_1448	COI_0774	COK_0064	YajC	+[2]	+[2]	+[2]	+[2]	−	+[2]	−	−
69	IM			MHA_1228	COI_1149	COK_1941	PntA	+[2]	+[2]	+[2]	−	−	−	−	−
70	C			MHA_2176	COI_1521	COK_1096	EF1A	+[2]	−	−	−	−	−	−	−
71	C			MHA_1682	COI_0958	COK_1425	RplB	+[2]	+[2]	−	−	−	−	−	+[2]
73	IM			MHA_0370	COI_1486	COK_0984	AcrB	−	−	−	+[2]	−	−	−	−
74	C			MHA_1040	COI_1994	COK_0388	RplA	−	+[2]	−	−	−	−	−	−
75	C			MHA_1552	COI_0122	COK_2061	RplT	+[2]	−	−	−	−	−	−	−
78	C			MHA_0315	COI_0240	COK_0123	RpsB	−	−	−	−	−	−	−	+[2]

Table 4.2. (continued)

No.	Sub. Local.	TM	Lipo	Bovine A1	Ovine A2	Bovine A2	Name	Protein identifications							
								PH2	PH8	PH202	PH278	PH292	PH296	PH344	PH588
80	IM			ctg147	COI_1296	COK_0158	GlpT	+[2]	–	–	–	–	–	–	–
81	C			MHA_2378	COI_1451	COK_1123	RplE	–	+[2]	–	–	–	–	–	–
84	C			MHA_2364	COI_1437	COK_1137	RplQ	–	–	+[2]	–	–	–	–	–
87	E	+	–	MHA_0254	COI_0481	COK_0274	LktA	+[2]	–	–	–	–	–	–	–
89	IM			MHA_1656	COI_1498	COK_0973	GltP	+[2]	+[2]	–	–	–	–	–	+[2]
95	C			MHA_0822	COI_0990	COK_0963	RpsA	+[2]	–	–	–	–	–	–	–
97	C			MHA_0991	COI_1354	COK_2591	RpmB	+[2]	–	–	–	–	–	–	–
2. Proteins identified exclusively in iron-restricted BHIB															
99	OM	+	–	ctg61	COI_2252/3	COK_1629/30	Haemin receptor	–	+[1]	+	–	–	+[1]	+[2]	–
100	OM	+	–	MHA_0232	COI_2297	COK_1718	LamB	–	–	–	+	+	–	–	+[1]
101	OM	+	–	ctg68	COI_1905	COK_2465	FhuA	–	–	–	–	+	+[1]	+[2]	–
102	OM	+	–	MHA_0080	COI_1870	COK_0773	Autotransporter	–	+[1]	–	+[1]	+[1]	–	–	–
103	OM	–	+	–	COI_0131	COK_2070	EcnA	–	–	+[2]	–	–	–	–	–
104	OM	+	–	ctg86	COI_1762/3	COK_2539/40	HmbR2	–	–	–	–	–	–	+[2]	–
105	OM/E	–	+	MHA_1004	COI_1366	COK_1210	HxuA	–	+[1]	–	–	–	–	–	–
106	OM	+	–	–	COI_0585/6	COK_1280/81	Iga1_4	–	–	–	–	–	–	+[1]	–
107	U	–	+	MHA_2737	COI_0543	COK_2363	Lipoprotein	–	+[2]	–	–	–	–	–	–
108	U			MHA_1307	COI_1292	COK_0162	Hypothetical protein	–	+[2]	–	–	–	–	–	–
109	E			–	–	–	PenP	+[2]	–	–	–	–	–	–	–
110	IM			MHA_2767	COI_0571	COK_2335	NqrC	+[2]	–	–	–	–	–	–	–

Table 4.2. (continued)

No.	Sub. Local.	TM	Lipo	Bovine A1	Ovine A2	Bovine A2	Name	Protein identifications							
								PH2	PH8	PH202	PH278	PH292	PH296	PH344	PH588
111	C			MHA_2535	COI_1411	COK_1164	RpsP	–	+[2]	–	–	–	–	–	–
							Total number of proteins identified	42	43	29	28	27	28	22	32
							Total number of OMPs identified	27	31	25	24	26	26	20	27

M. haemolytica isolates and a 23-fold increase in the *M. glucosida* isolate PH344. Expression changes in TbpB could not be quantified due to its close proximity to other proteins on the 1-D SDS PAGE gel; however, it was identified in six isolates after growth in iron-restricted BHIB whereas it was identified in only one isolate after growth in iron-replete BHIB. There was a 4.6 to 14.8-fold increase in the expression of the putative haemoglobin receptor HmbR1 (number 45, Fig. 4.2B) among *M. haemolytica* isolates and an 18.8-fold increase in the *M. glucosida* isolate. Another putative haemoglobin receptor, HmbR2, was also identified by the gel-free approach in the *M. glucosida* isolate PH344 that had not been previously identified after growth in iron-replete BHIB [although transcripts of the gene encoding this protein were previously shown to be upregulated in a bovine serotype A1 *M. haemolytica* isolate under iron-restriction (Roehrig *et al.*, 2007)]. In the previous chapter, the putative siderophore receptor FrpB was identified in a protein band of approximately 69 kDa in all eight representative isolates after growth in iron-replete BHIB (number 12; Fig 4.2A). After growth in iron-restricted BHIB, expression of FrpB (number 12; Fig 4.2B) increased 11.8- and 14.6-fold in isolates PH2 and PH344, respectively. FrpB was also identified in the other six isolates but other proteins were identified alongside it including HxuC, haemin receptor, FhuA and autotransporter MHA_0080/COI_1870/ COK_0773. Despite the presence of these other proteins, FrpB was consistently identified with the high protein scores and emPAI values, indicating that it is a predominantly expressed protein in these bands.

HxuB and HxuC, the outer membrane components of a putative haem-haemopexin uptake system, were identified by both gel-free and gel-based proteomic approaches in all isolates except for the bovine serotype A1 *M. haemolytica* isolate PH2 and the *M. glucosida* isolate PH344. Expression of a band containing HxuB (number 46; Fig. 4.2B) increased 3.5- to 17.4-fold among these isolates. The putative haem/glutathione transporter HbpA (number 28; Fig. 4.2B) was also identified in the same protein band as HxuB in isolates PH8, PH202 and PH296 but at much lower protein scores and emPAI values than HxuB, indicating that HxuB is the predominantly expressed protein in these bands. The expression change in HxuC (number 46; Fig. 4.2B) could not be quantified due to its close proximity to other proteins, including FrpB, on the 1-D SDS-PAGE gel.

HxuA (number 105; Fig. 4.2B) was identified only in isolate PH8 in a very faint protein band.

Expression of a protein band that contained the putative fatty acid transporter FadL (number 7; Fig 4.2B) increased 1.8-, 1.4-, 2.6-, and 3.9-fold in serotype A2 isolates PH202, PH278, PH292 and in serotype A7 isolate PH296, respectively, after mid-log phase growth in iron-restricted BHIB. Possible OMP MHA_0486/COI_0902/COK_2252 (number 10; Fig 4.2B) was also identified in the same protein band as FadL in the seven *M. haemolytica* isolates but at much lower protein scores and emPAI values than FadL, indicating that FadL is the predominantly expressed protein in these bands. Interestingly, after growth to stationary phase in iron-restricted BHIB this protein band was expressed at levels below that observed after mid-log phase growth in iron-replete BHIB in all isolates (Supplementary Fig. S4.1), indicating a more important role for FadL during mid-log phase iron-restricted growth than at stationary phase.

There was a 2.4-, 2.2-, 1.4- and 1.8-fold increase in the expression of the porin OmpP2 (number 29; Fig. 4.2B) in isolates PH2, PH8, PH296 and PH588, respectively. This protein was found to be absent from the outer membranes of serotype A2 isolates PH202, PH278, PH292 and *M. glucosida* isolate PH344 after growth in iron-replete BHIB, and was still absent in the present study after growth in iron-restricted BHIB. This is consistent with the finding that the *ompP2* gene is either absent or truncated in serotype A2 isolates (Lawrence *et al.*, 2010a). Expression of the other major porin OmpP2-like (number 11; Fig. 4.2B) was relatively unchanged in all isolates.

A 157 kDa protein band that was not previously identified under iron-replete conditions in any of the representative isolates was present exclusively in *M. glucosida* isolate PH344 under iron-restricted conditions. This protein was identified as Iga1_4 by gel-based proteomics (number 106; Fig 4.2B), and represents the first Iga1-like protease identified in *M. glucosida*.

4.3.2 Identification of *M. haemolytica* OMPs which undergo changes in expression after growth in serum-supplemented tissue culture media and complete serum.

During an *M. haemolytica* infection, fibrinous exudate leaks into the lungs as a result of vascular and pulmonary damage to host tissue. In order to determine what influence this exudate may have on the expression of OMPs in the pneumonic lung, *M. haemolytica* outer membranes were extracted from four *M. haemolytica* isolates (PH2, PH8, PH202 and PH278) after growth in tissue culture media in the presence or absence of serum (which has a composition similar to that of lung exudate). Fig. 4.3 shows the OMP profiles of the bovine (PH2) and ovine (PH8) serotype A1 isolates (Fig. 4.3A) and the bovine (PH202) and ovine (PH278) serotype A2 isolates (Fig. 4.3B) after growth to mid-log phase in DMEM in the absence or presence of either 10% FCS or SS. Outer membranes were also extracted after growth in M199 and RPMI in the absence or presence of 10% FCS or SS, and also in complete FCS, SS and NCS (Supplementary Figures S4.2 and S4.3). A total of 103 unique proteins were identified among the four isolates under all growth conditions using a combination of gel-based and gel-free proteomic approaches (Table 4.3). Sixty of these proteins were previously identified after growth in iron-replete BHIB, and included 42 OMPs, 3 proteins of unknown location, and 15 proteins predicted to be localised to cellular compartments other than the outer membrane. The remaining 43 proteins that were not previously identified after growth in iron-replete BHIB included five OMPs, seven proteins of unknown location, and 31 proteins predicted to be localised to cellular compartments other than the outer membrane. The five OMPs were the haemin receptor, LamB, Hsf (a putative adhesin/serum-resistance protein), PlpB (function unknown), and possible OMP MHA_0862/COI_1567/COK_2302 (function unknown). Both the haemin receptor and LamB had been previously identified after growth in iron-restricted BHIB. Possible OMP MHA_0862/COI_1567/COK_2302 was identified in isolates PH2, PH8 and PH202 after growth in DMEM alone, and also in isolate PH202 after growth in RPMI alone. PlpB and Hsf were the only OMPs that were identified exclusively after growth in the presence of serum. PlpB was identified in the bovine isolates PH2 and PH202 only after growth in complete FCS.

Figure 4.3. One-dimensional SDS-PAGE separation of outer membrane fractions from (A) bovine (PH2) and ovine (PH8) serotype A1 *M. haemolytica* isolates, and (B) bovine (PH202) and ovine (PH278) serotype A2 *M. haemolytica* isolates after growth in DMEM only, DMEM + 10% FCS and DMEM + 10% SS.

OMP profiles after growth in iron-replete and iron-restricted BHIB are also shown for comparison. Numbered proteins were subsequently identified by proteomic analysis (Table 4.3). Twenty micrograms of protein were loaded into each lane. Protein bands were stained with Coomassie brilliant blue.

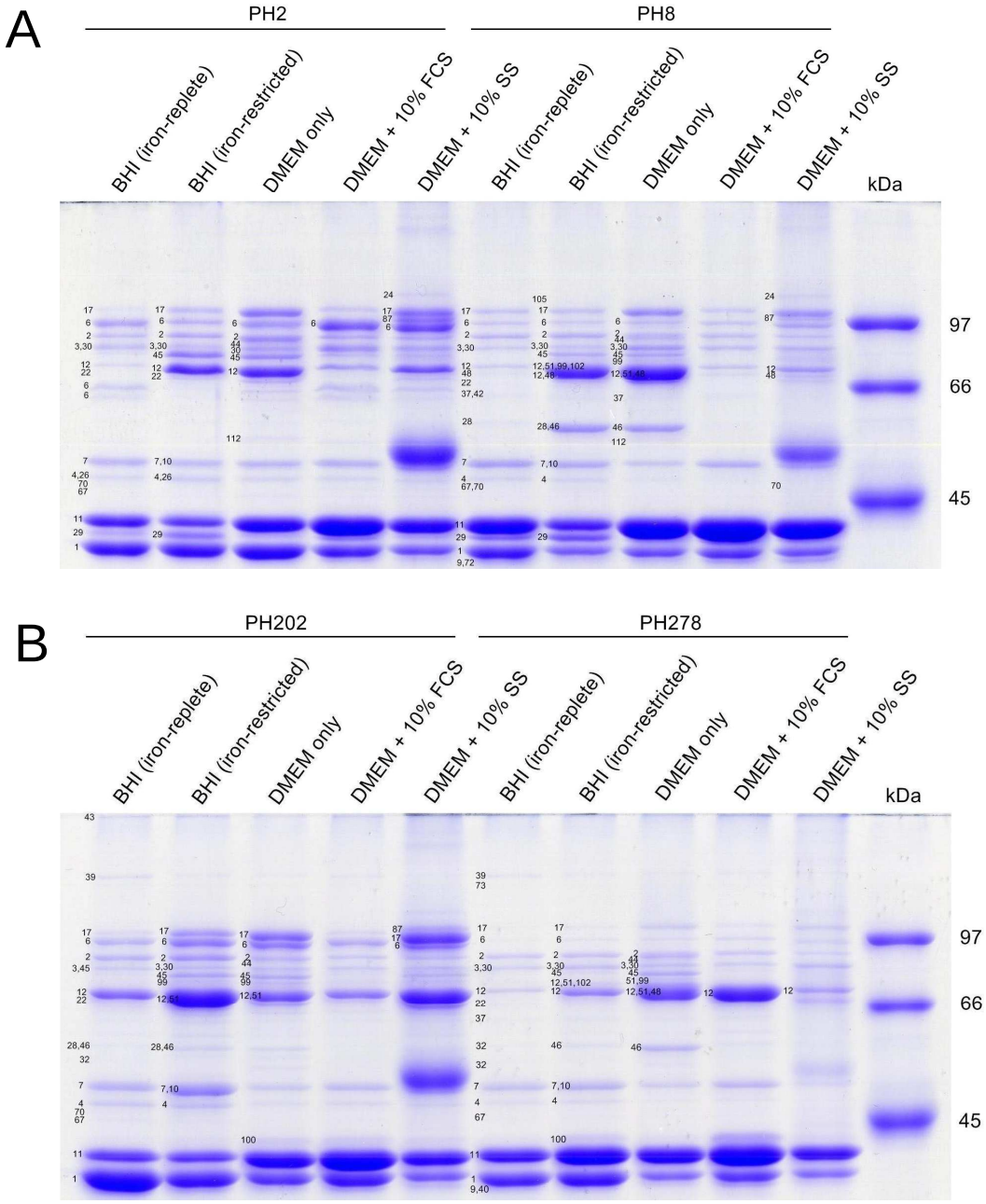


Table 4.3. Proteins identified in the outer membrane fractions of four *M. haemolytica* isolates (PH2, PH8, PH202 and PH278) after growth in DMEM, RPMI and M199 in the absence of presence of 10% FCS or SS, and in total FCS, SS and NCS.

No.[a]	Subcellular Localisation[b]	TM[c]	Lipo[c]	Name	Protein identifications[d]																							
					PH2												PH8											
					DMEM only	DMEM + 10% FCS	DMEM + 10% SS	RPMI only	RPMI + 10% FCS	RPMI + 10% SS	M199 only	M199 + 10% FCS	M199 + 10% SS	FCS only	SS only	NCS only	DMEM only	DMEM + 10% FCS	DMEM + 10% SS	RPMI only	RPMI + 10% FCS	RPMI + 10% SS	M199 only	M199 + 10% FCS	M199 + 10% SS	FCS only	SS only	NCS only
1. Proteins previously identified in iron replete BHIB																												
1	OM	+/−	−	OmpA	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$
2	OM	+	−	Omp85	+	−	$+^2$	$+^2$	−	$+^2$	$+^2$	$+^2$	−	$+^2$	−	$+^2$	+	$+^2$	$+^2$	+	$+^2$	$+^2$	$+^2$	$+^2$	−	$+^2$	−	$+^2$	$+^2$	$+^2$
3	OM	+	−	Imp/LptD	$+^2$	$+^2$	−	−	−	−	−	−	−	$+^2$	−	−	$+^1$	−	−	$+^1$	−	−	−	−	−	$+^2$	−	−	−
4	OM	+	+	TolC	$+^2$	−	−	$+^1$	−	−	−	$+^2$	−	$+^2$	−	−	−	−	−	$+^2$	−	−	−	$+^2$	−	−	−	−	−	−
5	OM	−	+	Pal	$+^2$	−	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$
6	OM	+	−	Ssa	+	+	+	$+^2$	+	+	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	+	$+^2$	$+^2$	−	−	−	−	−	−	−	−	−	−	−
7	OM	+	−	OmpP1/FadL	$+^2$	−	$+^2$	$+^2$	$+^2$	−	$+^2$	$+^2$	−	$+^2$	−	−	−	$+^2$	$+^2$	−	$+^2$	−	$+^2$	$+^2$	−	$+^2$	−	−	−	−
8	OM	−	+	PlpA	−	−	$+^2$	−	−	−	−	−	−	$+^2$	$+^2$	$+^2$	−	$+^2$	$+^2$	−	$+^2$	$+^2$	−	$+^2$	−	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$
9	OM	+	+	Wza	−	−	−	−	−	−	−	−	−	−	−	−	$+^2$	$+^2$	$+^2$	−	$+^2$	−	$+^2$	$+^2$	−	$+^2$	−	−	−	−
10	OM	+	−	Possible OMP	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
11	OM	+	−	OmpP2-like	−	−	−	−	$+^2$	−	$+^2$	$+^2$	−	−	−	−	$+^2$	$+^2$	−	$+^2$	$+^2$	−	$+^2$	$+^2$	−	$+^2$	−	−	−	−
12	OM	+	−	FrpB	+	−	$+^2$	−	$+^2$	$+^2$	−	$+^2$	$+^2$	$+^2$	$+^2$	−	+	−	+	$+^2$	$+^2$	$+^2$	$+^2$	−	−	$+^2$	$+^2$	−	−	−

No.[a]	Subcellular Localisation[b]	TM[c]	Lipo[c]	Name	Protein identifications[d]																							
					PH202												PH278											
					DMEM only	DMEM + 10% FCS	DMEM + 10% SS	RPMI only	RPMI + 10% FCS	RPMI + 10% SS	M199 only	M199 + 10% FCS	M199 + 10% SS	FCS only	SS only	NCS only	DMEM only	DMEM + 10% FCS	DMEM + 10% SS	RPMI only	RPMI + 10% FCS	RPMI + 10% SS	M199 only	M199 + 10% FCS	M199 + 10% SS	FCS only	SS only	NCS only
1. Proteins previously identified in iron replete BHIB																												
1	OM	+/−	−	OmpA	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$
2	OM	+	−	Omp85	+	$+^2$	$+^2$	$+^2$	$+^2$	−	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	+	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	−	$+^2$	−	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$
3	OM	+	−	Imp/LptD	$+^2$	−	−	−	−	−	$+^1$	−	$+^2$	−	$+^2$	−	−	$+^1$	−	−	$+^1$	−	−	$+^2$	−	$+^2$	−	−	$+^1$	$+^2$
4	OM	+	+	TolC	−	$+^2$	−	−	$+^2$	−	−	$+^2$	−	$+^2$	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
5	OM	−	+	Pal	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$
6	OM	+	−	Ssa	−	$+^2$	+	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^1$	$+^2$	−	$+^2$	−	−	−	−	−	−	−	−	−	−	−	−
7	OM	+	−	OmpP1/FadL	−	$+^2$	−	−	$+^2$	−	−	$+^2$	−	$+^2$	−	−	$+^2$	$+^2$	−	−	$+^2$	−	$+^2$	$+^2$	−	$+^2$	−	−	−	−
8	OM	−	+	PlpA	$+^2$	$+^2$	$+^2$	−	$+^2$	−	−	$+^2$	−	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	−	−	$+^2$	−	−	$+^2$	−	$+^2$	$+^1$	−	$+^2$	
9	OM	+	+	Wza	−	−	−	−	$+^2$	−	−	−	−	−	−	−	−	−	−	−	$+^2$	−	−	−	−	$+^2$	−	−	
10	OM	+	−	Possible OMP	−	−	−	−	−	−	−	−	−	$+^2$	−	$+^2$	−	−											
11	OM	+	−	OmpP2-like	−	$+^2$	−	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	−	$+^2$	−	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	−	$+^2$	$+^2$	$+^2$		
12	OM	+	−	FrpB	+	$+^2$	$+^2$	+	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	+	+	+	+	$+^2$	+	$+^2$	$+^2$	$+^2$	$+^2$	$+^1$	$+^2$		

Proteins that were bioinformatically predicted to be putative OMPs are grey-shaded.
[a]Numbers correspond to the location of the protein in Figure 4.3 if identified by gel-based proteomics.
[b]Confidently predicted subcellular locations; 'OM' = outer membrane; 'P' = periplasm; 'IM' = inner membrane; 'C' = cytoplasm; 'E' = extracellular; 'U' = unknown.
[c]Transmembrane β-barrel and lipoprotein prediction result; '+' = predicted in all genomes; '−' = not predicted in any genome; '+/−' = predicted in one/two genomes.
[d]Two proteomics methods were compared; '$+^1$' = proteins identified by gel-based method; '$+^2$' = proteins identified by gel-free method; '+' = proteins identified by both methods; '−' = no identification.

Table 4.3. (continued)

Protein identifications — PH2

No.	Subcellular Localisation	TM	Lipo	Name	DMEM only	DMEM + 10% FCS	DMEM + 10% SS	RPMI only	RPMI + 10% FCS	RPMI + 10% SS	M199 only	M199 + 10% FCS	M199 + 10% SS	FCS only	SS only	NCS only
13	OM	–	+	*Rickettsia*-like	+[2]	–	–	–	–	–	–	–	–	–	–	–
14	OM			LemA	–	–	–	–	–	–	–	–	–	–	–	–
15	OM	–	+	Bor/Iss_2	+[2]	–	–	+[2]	–	–	+[2]	–	–	+[2]	–	+[2]
16	OM	–	+	Lpp38	–	–	–	–	–	–	–	–	–	–	–	–
17	OM	+	–	TbpA	+[2]	+[2]	+	+[2]	+[2]	+[2]	–	+[2]	+[2]	+[2]	+[2]	–
18	OM	–	+	VacJ	–	–	–	–	–	–	–	–	–	–	–	–
19	OM	–	–	OMP18/16	–	–	–	–	–	–	–	–	–	–	–	–
20	OM	–	+	OmpP4	+[2]	–	–	–	–	–	–	–	–	–	–	–
21	OM	–	+	YajG	–	–	–	–	–	–	–	–	–	–	–	–
24	OM	+	–	IgA1_2	–	–	+[1]	–	–	–	–	–	–	–	–	–
25	OM	–	+	HlpB	–	–	–	–	–	–	+[2]	–	–	–	–	–
27	OM	–	–	Possible OMP	–	–	–	–	–	–	–	+[1]	–	–	–	–
28	IM/P/OM			HbpA	–	–	–	–	–	–	–	–	–	+[1]	–	–
29	OM	+	–	OmpP2	–	–	–	+[2]	–	–	–	+[2]	–	+[1]	–	+[2]
30	OM	+	–	NanH	+[1]	–	–	–	–	–	–	+[2]	–	–	–	–
31	OM	–	+	fHbp_1	–	+[2]	–	–	–	–	–	+[1]	–	–	–	–
32	OM	+	+	LppB/NlpD	–	–	–	–	–	–	–	–	–	–	–	–
34	OM	–	+/–	PlpD	–	–	–	–	–	–	–	–	–	–	–	–
36	OM	–	+	ComL	+[2]	–	–	–	–	–	–	–	–	–	–	–
37	OM	+	–	FhaC	–	–	–	–	–	–	–	–	–	–	–	–
38	OM	–	+	PlpC	–	–	–	–	–	–	–	–	–	–	–	–
40	OM	+	+	MltA	–	–	–	–	–	–	–	–	–	–	–	–
41	OM	–	–	OmpW	–	–	–	–	–	–	–	–	–	–	–	–

Protein identifications — PH8

No.	Name	DMEM only	DMEM + 10% FCS	DMEM + 10% SS	RPMI only	RPMI + 10% FCS	RPMI + 10% SS	M199 only	M199 + 10% FCS	M199 + 10% SS	FCS only	SS only	NCS only
13	*Rickettsia*-like	–	–	–	–	–	–	+[2]	+[2]	–	+[2]	–	–
14	LemA	–	–	–	–	–	–	–	+[2]	–	–	–	–
15	Bor/Iss_2	+[2]	+[2]	–	+[2]	+[2]	+[2]	+[2]	+[2]	–	+[2]	–	–
16	Lpp38	–	+[2]	+[2]	–	+[2]	+[2]	+[2]	+[2]	+[2]	+[2]	–	–
17	TbpA	+[2]	+[2]	+[2]	+[2]	+[2]	+	+[2]	–	+	+[2]	+[2]	+[2]
18	VacJ	–	–	–	–	–	–	–	–	–	+[2]	–	–
19	OMP18/16	–	–	–	–	–	–	–	–	–	–	–	–
20	OmpP4	+[2]	+[2]	+[2]	+[2]	+[2]	–	–	–	–	–	–	–
21	YajG	+[2]	+[2]	–	–	+[2]	–	–	+[2]	–	+[2]	–	–
24	IgA1_2	–	–	+[1]	–	–	–	–	–	–	–	–	–
25	HlpB	–	–	–	+[2]	–	–	–	–	–	–	–	–
27	Possible OMP	–	–	–	–	–	–	+[2]	–	–	–	–	–
28	HbpA	–	–	–	–	–	–	–	–	–	+[2]	+[2]	+[2]
29	OmpP2	–	–	–	–	–	–	–	–	–	+[2]	+	+[2]
30	NanH	+	+[2]	+[2]	+[1]	+[2]	+[2]	+[2]	+[2]	–	+[2]	–	–
31	fHbp_1	–	–	–	–	+[2]	–	+[2]	+[2]	–	–	–	–
32	LppB/NlpD	–	–	–	–	–	–	–	–	–	–	–	–
34	PlpD	–	–	–	–	–	–	–	+[2]	–	–	–	–
36	ComL	–	+[2]	–	+[2]	+[2]	+[2]	–	+[2]	–	+[2]	–	+[2]
37	FhaC	+[1]	–	–	–	–	–	–	–	–	–	–	–
38	PlpC	–	–	–	–	–	–	–	–	–	–	–	–
40	MltA	–	–	–	–	–	–	–	+[2]	–	–	–	–
41	OmpW	–	+[2]	–	–	–	–	–	–	–	–	–	–

Protein identifications — PH202

No.	Name	DMEM only	DMEM + 10% FCS	DMEM + 10% SS	RPMI only	RPMI + 10% FCS	RPMI + 10% SS	M199 only	M199 + 10% FCS	M199 + 10% SS	FCS only	SS only	NCS only
13	*Rickettsia*-like	–	–	+[2]	–	–	–	+[2]	–	+[2]	–	–	–
14	LemA	–	–	–	–	–	–	–	–	–	–	–	–
15	Bor/Iss_2	+[2]	+[2]	–	+[2]	+[2]	+[2]	–	+[2]	–	–	–	+[2]
16	Lpp38	–	+[2]	+[2]	–	+[2]	+[2]	–	+[2]	–	–	–	–
17	TbpA	+	+[2]	+	+[2]	+[2]	+	+[2]	+[2]	+	+[2]	+	+
18	VacJ	–	–	–	–	–	–	–	–	–	+[2]	–	–
19	OMP18/16	–	–	–	+[2]	–	–	–	–	–	–	–	–
20	OmpP4	+[2]	+[2]	–	–	+[2]	–	–	–	–	–	–	–
21	YajG	+[2]	+[2]	–	–	+[2]	–	–	+[2]	–	+[2]	–	+[2]
24	IgA1_2	–	–	–	–	–	–	–	–	–	–	–	–
25	HlpB	–	–	–	–	–	–	–	–	–	–	–	–
27	Possible OMP	–	–	–	–	–	–	–	–	–	–	–	–
28	HbpA	–	–	–	–	–	–	–	–	–	+[2]	+[2]	+[2]
29	OmpP2	–	–	–	–	–	–	–	–	–	–	–	–
30	NanH	+[2]	+[2]	–	+[2]	+[2]	+[1]	+[2]	+[2]	+[2]	–	–	–
31	fHbp_1	–	+[2]	–	–	–	–	–	–	–	–	–	–
32	LppB/NlpD	–	–	–	–	–	–	–	–	–	–	–	–
34	PlpD	–	–	–	–	–	–	–	–	–	–	–	–
36	ComL	–	+[2]	–	–	+[2]	–	–	–	+[2]	+[2]	+[2]	–
37	FhaC	–	–	–	–	–	–	–	–	–	–	–	–
38	PlpC	–	–	–	–	–	–	–	–	–	–	–	–
40	MltA	–	–	–	–	–	–	–	+[2]	–	–	–	–
41	OmpW	–	–	–	–	–	–	–	–	–	–	–	–

Protein identifications — PH278

No.	Name	DMEM only	DMEM + 10% FCS	DMEM + 10% SS	RPMI only	RPMI + 10% FCS	RPMI + 10% SS	M199 only	M199 + 10% FCS	M199 + 10% SS	FCS only	SS only	NCS only
13	*Rickettsia*-like	–	–	–	–	–	+[2]	–	–	+[2]	–	–	–
14	LemA	–	–	–	–	–	–	–	+[2]	–	–	–	–
15	Bor/Iss_2	+[2]	+[2]	–	+[2]	–	–	–	+[2]	–	+[2]	–	–
16	Lpp38	+[2]	+[2]	+[2]	–	+[2]	+[1]	+[2]	–	–	+[2]	–	–
17	TbpA	+[2]	–	–	+[2]	–	–	–	+[2]	–	–	–	–
18	VacJ	–	+[2]	–	–	–	–	–	+[2]	–	–	–	–
19	OMP18/16	–	–	–	+[2]	–	–	–	+[2]	–	–	–	–
20	OmpP4	–	+[2]	–	–	–	–	+[2]	–	–	–	–	–
21	YajG	–	+[2]	–	–	+[2]	–	–	+[2]	–	+[2]	–	–
24	IgA1_2	–	–	–	–	–	–	–	–	–	–	–	–
25	HlpB	–	–	–	–	–	–	–	–	–	–	–	–
27	Possible OMP	–	–	–	–	–	–	–	+[2]	–	–	–	–
28	HbpA	+[2]	–	–	–	–	–	–	–	–	+[2]	–	–
29	OmpP2	–	–	–	–	–	–	–	–	–	–	–	–
30	NanH	+[1]	–	+[2]	+	+[1]	+[2]	–	+[2]	–	+[2]	–	–
31	fHbp_1	–	–	–	–	–	–	–	–	–	–	–	–
32	LppB/NlpD	–	–	–	–	+[2]	–	–	–	–	–	–	–
34	PlpD	–	–	–	–	–	–	–	–	–	–	–	–
36	ComL	–	–	+[2]	+[2]	–	–	–	+[2]	–	+	–	–
37	FhaC	–	–	–	–	–	–	–	–	–	–	–	–
38	PlpC	–	–	–	–	–	–	–	–	–	+[1]	–	–
40	MltA	–	+[2]	–	–	+[2]	–	–	+[2]	–	+[2]	–	–
41	OmpW	–	–	–	–	–	–	–	–	–	–	–	–

Table 4.3. (continued)

No.[a]	Subcellular Localisation[b]	TM	Lipo	Name	Protein identifications																													
					PH2												PH8												PH202							
					DMEM only	DMEM + 10% FCS	DMEM + 10% SS	RPMI only	RPMI + 10% FCS	RPMI + 10% SS	M199 only	M199 + 10% FCS	M199 + 10% SS	FCS only	SS only	NCS only	DMEM only	DMEM + 10% FCS	DMEM + 10% SS	RPMI only	RPMI + 10% FCS	RPMI + 10% SS	M199 only	M199 + 10% FCS	M199 + 10% SS	FCS only	SS only	NCS only	DMEM only	DMEM + 10% FCS	DMEM + 10% SS	RPMI only	RPMI + 10% FCS	RPMI + 10% SS	M199 only	M199 + 10% FCS	M199 + 10% SS	FCS only	SS only	NCS only
42	OM	+	+	LppC	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	$+^2$	–	–	–	–	–	$+^1$	–	–	–	–	–	–	–	–
44	OM	+	–	TonB-dep. rece	+	–	–	–	–	–	–	–	–	–	–	–	+	–	–	$+^1$	–	–	–	–	–	–	–	–	+	–	–	$+^2$	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–
45	OM	+	–	HmbR1	+	–	–	–	–	–	–	–	–	–	–	–	+	–	–	+	–	–	–	–	–	–	–	–	+	–	–	$+^2$	$+^2$	–	+	$+^2$	–	–	$+^2$	–	–	+	–	–	+	$+^1$	–	–	–	–	–	–
46	OM	+	–	HxuB	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	$+^2$	–	–	$+^2$	–	–	–	–	–	$+^2$	–	$+^2$	$+^2$	–	$+^2$	–	–	$+^2$	–	$+^2$	–	+	$+^2$	–	$+^2$	+	–	–	–	–	–	$+^2$	–
47	OM			OapA	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	$+^2$	–	$+^2$	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
48	OM	+/–	+	TbpB	$+^2$	$+^2$	$+^2$	–	$+^2$	+	–	$+^2$	–	–	–	–	+	$+^2$	+	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	–	–	–	$+^2$	$+^2$	–	$+^2$	+	–	$+^2$	$+^2$	–	$+^2$	–	$+^2$	–	+	–	–	–	–	–	–	$+^1$	–
51	OM	+	–	HxuC	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	$+^2$	–	$+^2$	$+^2$	–	–	$+^2$	–	–	+	–	–	+	–	–	$+^2$	–	–	–	–	–	+	$+^2$	–	+	$+^2$	–	$+^2$	$+^2$	–	–	–	–
56	U	+	+	Hypothetical pr	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	–	$+^2$	$+^2$	–	$+^2$	–	$+^2$	$+^2$	$+^2$	–	$+^2$	$+^2$	–	$+^2$	$+^2$	–	$+^2$	–	–	$+^2$	$+^2$	–	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	–	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	–	$+^2$	$+^2$	–	$+^2$	–	$+^2$
57	U			Hypothetical pr	$+^2$	–	–	$+^2$	–	–	$+^2$	–	–	$+^2$	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
58	U			Hypothetical pr	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	$+^2$	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
68	IM			YajC	–	–	$+^2$	–	–	$+^2$	$+^2$	–	–	$+^2$	–	–	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	–	–	–	$+^2$	–	$+^2$	–	–	$+^2$	–	–	–	–	–	–	–	–	$+^2$	–	$+^2$	$+^2$	–	$+^2$	$+^2$	–	–	$+^2$	–	–	–	–
69	IM			PntA	$+^2$	–	$+^2$	–	–	–	$+^2$	–	–	$+^2$	–	$+^2$	$+^2$	$+^2$	$+^2$	–	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	$+^2$	–	$+^2$
70	C			EF1A	–	–	–	+	–	–	$+^2$	–	–	–	–	–	–	–	$+^1$	$+^2$	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
71	C			RplB	–	–	–	$+^2$	–	–	$+^2$	–	–	–	–	–	–	–	–	–	–	–	–	$+^2$	–	$+^2$	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
74	C			RplA	–	–	–	$+^2$	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
75	C			RplT	–	–	–	–	–	–	$+^2$	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	$+^2$	–	–	–	–	–	–	–	–	$+^2$	–	$+^2$	–	–	–	–	–	–	–	–	–	–	–	–
78	C			RpsB	–	–	–	$+^2$	–	–	–	$+^2$	–	–	–	$+^2$	–	$+^2$	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	$+^2$	–	–	$+^2$	–	–	–	–	–	–	–	–	$+^2$	–	–	–	–	–	–	–
79	C			RplK	–	–	–	$+^2$	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
81	C			RplE	–	–	–	$+^2$	–	–	$+^2$	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
84	C			RplQ	–	–	–	$+^2$	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
85	P	–	+	YgiW	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
87	E	+	–	LktA	–	–	+	–	–	+	–	–	+	–	+	$+^2$	+	–	+	–	–	+	–	–	+	–	$+^2$	$+^2$	$+^2$	–	+	–	–	$+^2$	–	–	$+^2$	–	+	+	–	–	$+^2$	–	–	$+^2$	–	$+^2$	$+^2$	–	–	$+^2$	$+^2$
89	IM			GltP	–	–	–	–	–	–	–	–	–	$+^2$	$+^2$	$+^2$	–	–	–	–	–	–	–	–	$+^2$	$+^2$	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

Table 4.3. (continued)

No.	Subcellular Localisation	TM	Lipo	Name	Protein identifications																							
					PH2												PH8											
					DMEM only	DMEM + 10% FCS	DMEM + 10% SS	RPMI only	RPMI + 10% FCS	RPMI + 10% SS	M199 only	M199 + 10% FCS	M199 + 10% SS	FCS only	SS only	NCS only	DMEM only	DMEM + 10% FCS	DMEM + 10% SS	RPMI only	RPMI + 10% FCS	RPMI + 10% SS	M199 only	M199 + 10% FCS	M199 + 10% SS	FCS only	SS only	NCS only
94	IM			PntB	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
95	C			RpsA	–	–	–	$+^{2}$	–	–	–	–	–	–	–	–	–	–	–	$+^{2}$	–	–	–	–	–	–	–	–
2. Proteins previously identified in iron-restricted BHIB																												
99	OM	+	–	Haemin recepto	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–
100	OM	+	–	LamB	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
107	U	–	+	Lipoprotein	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	$+^{2}$	–	$+^{2}$	–	–	–
108	U			Hypothetical pr	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
109	E			PenP	–	–	–	–	–	–	–	–	–	$+^{2}$	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
3. Proteins identified exclusively in DMEM, RPMI and M199 in the presence or absence of serum, and in total serum																												
112	OM	–	–	Possible OMP	$+^{1}$	–	–	–	–	–	–	–	–	–	–	–	$+^{1}$	–	–	–	–	–	–	–	–	–	–	–
113	OM	+/–	–	Hsf	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	$+^{2}$	–	–	$+^{2}$	–	–	–	$+^{2}$
114	OM	–	+	PlpB	–	–	–	–	–	–	–	–	–	$+^{2}$	–	–	–	–	–	–	–	–	–	–	–	–	–	–
115	U	–	+	Hypothetical pr	–	–	–	–	–	–	–	$+^{2}$	–	–	–	–	–	–	–	–	–	–	$+^{2}$	–	$+^{2}$	–	–	–
116	U	–	+	Hypothetical pr	$+^{2}$	$+^{2}$	–	–	–	–	–	–	–	–	–	–	$+^{2}$	–	–	$+^{2}$	–	–	–	–	–	–	–	–
117	U	–	+	Hypothetical pr	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
118	U			Hypothetical pr	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
119	U			Hypothetical pr	–	–	–	–	–	–	$+^{2}$	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
120	IM			CstA	–	–	–	–	–	–	–	–	–	–	–	–	–	$+^{2}$	$+^{2}$	–	$+^{2}$	–	–	$+^{2}$	–	$+^{2}$	–	–	–
121	IM			MglC	–	–	–	–	–	$+^{2}$	–	–	–	–	–	–	–	–	$+^{2}$	–	–	$+^{2}$	–	–	–	–	–	–	–
122	IM			WecC	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
123	IM			UraA	–	–	$+^{2}$	–	–	$+^{2}$	–	–	–	–	–	–	–	–	$+^{2}$	–	–	$+^{2}$	–	$+^{2}$	–	–	–	–
124	IM			PTS_EIIB	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	$+^{2}$	–	–	–	–	–
125	IM			NupC	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

No.	Subcellular Localisation	TM	Lipo	Name	Protein identifications																							
					PH202												PH278											
					DMEM only	DMEM + 10% FCS	DMEM + 10% SS	RPMI only	RPMI + 10% FCS	RPMI + 10% SS	M199 only	M199 + 10% FCS	M199 + 10% SS	FCS only	SS only	NCS only	DMEM only	DMEM + 10% FCS	DMEM + 10% SS	RPMI only	RPMI + 10% FCS	RPMI + 10% SS	M199 only	M199 + 10% FCS	M199 + 10% SS	FCS only	SS only	NCS only
94	IM			PntB	–	–	–	–	–	–	–	–	–	–	–	–	–	$+^{2}$	–	–	–	–	–	–	–	–	–	–
95	C			RpsA	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
2. Proteins previously identified in iron-restricted BHIB																												
99	OM	+	–	Haemin recepto	+	–	–	$+^{2}$	–	–	–	–	–	–	–	–	$+^{1}$	–	–	–	–	–	–	–	–	–	–	–
100	OM	+	–	LamB	$+^{1}$	–	–	–	–	–	–	–	–	–	–	–	–	$+^{1}$	–	–	–	–	–	–	–	–	–	–
107	U	–	+	Lipoprotein	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
108	U			Hypothetical pr	–	–	–	–	–	–	$+^{2}$	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
109	E			PenP	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
3. Proteins identified exclusively in DMEM, RPMI and M199 in the presence or absence of serum, and in total serum																												
112	OM	–	–	Possible OMP	$+^{2}$	–	–	$+^{1}$	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
113	OM	+/–	–	Hsf	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
114	OM	–	+	PlpB	–	–	–	–	–	–	–	–	–	$+^{2}$	–	–	–	–	–	–	–	–	–	–	–	–	–	–
115	U	–	+	Hypothetical pr	–	–	–	$+^{2}$	$+^{2}$	–	–	$+^{2}$	–	–	–	–	–	$+^{2}$	–	$+^{2}$	–	–	–	$+^{2}$	–	–	–	–
116	U	–	+	Hypothetical pr	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
117	U	–	+	Hypothetical pr	$+^{2}$	–	–	+	–	–	–	–	–	–	–	–	$+^{2}$	–	–	–	–	–	–	–	–	–	–	–
118	U			Hypothetical pr	–	–	–	$+^{2}$	–	–	–	–	–	–	–	–	$+^{2}$	$+^{2}$	–	$+^{2}$	$+^{2}$	–	–	$+^{2}$	–	–	–	–
119	U			Hypothetical pr	–	–	–	–	–	–	$+^{2}$	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
120	IM			CstA	–	$+^{2}$	–	$+^{2}$	$+^{2}$	–	–	$+^{2}$	–	–	–	–	–	–	–	–	$+^{2}$	–	–	–	–	–	–	–
121	IM			MglC	–	$+^{2}$	$+^{2}$	–	$+^{2}$	$+^{2}$	–	$+^{2}$	$+^{2}$	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
122	IM			WecC	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
123	IM			UraA	–	–	$+^{2}$	–	–	$+^{2}$	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
124	IM			PTS_EIIB	$+^{2}$	–	–	$+^{2}$	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
125	IM			NupC	–	–	–	–	–	–	–	–	–	–	–	–	$+^{2}$	–	–	–	$+^{2}$	–	–	–	–	–	–	–

Table 4.3. (continued)

No.	Subcellular Localisation	TM	Lipo	Name	Protein identifications																							
					PH2												PH8											
					DMEM only	DMEM + 10% FCS	DMEM + 10% SS	RPMI only	RPMI + 10% FCS	RPMI + 10% SS	M199 only	M199 + 10% FCS	M199 + 10% SS	FCS only	SS only	NCS only	DMEM only	DMEM + 10% FCS	DMEM + 10% SS	RPMI only	RPMI + 10% FCS	RPMI + 10% SS	M199 only	M199 + 10% FCS	M199 + 10% SS	FCS only	SS only	NCS only
126	IM			4-aminobutyrat	–	–	–	+[2]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
127	IM			NhaP	–	–	–	–	–	–	–	–	–	–	–	–	+[2]	–	–	–	–	–	–	–	–	–	–	–
128	IM			HflC	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
129	C	–	+	CarB	–	–	–	+[2]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
130	C/IM			Thioredoxin-like	–	–	–	+[2]	–	–	+[2]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
131	C			Pta	–	–	–	+[2]	–	–	+[2]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
132	C			PurL	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	+[2]	–	–	–	–	–	–	–	–	–
133	C			GapA	–	–	–	–	–	–	+[2]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
134	C			RpsC	–	–	–	–	–	–	+[2]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
135	C			AceE	–	–	–	–	–	–	+[2]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
136	C			TktA	–	–	–	–	–	–	+[2]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
137	C			RpsD	–	–	–	+[2]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
138	C			EF2/FusA	–	–	–	+[2]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
139	C			RpoA	–	–	–	+[2]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
140	C			EF1B	–	–	–	+[2]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
141	C			Hsp33	–	–	–	+[2]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
142	C			Electron transp	–	–	–	+[2]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
143	C			LeuB	–	–	–	+[2]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
144	C			AsnA	–	–	–	+[2]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
145	C			FabG	–	–	–	+[2]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
146	C			DnaK	–	–	–	+[2]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
147	C			PurN	–	–	–	+[1]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
148	C			PurA	–	–	–	+[1]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

No.	Subcellular Localisation	TM	Lipo	Name	Protein identifications																							
					PH202												PH278											
					DMEM only	DMEM + 10% FCS	DMEM + 10% SS	RPMI only	RPMI + 10% FCS	RPMI + 10% SS	M199 only	M199 + 10% FCS	M199 + 10% SS	FCS only	SS only	NCS only	DMEM only	DMEM + 10% FCS	DMEM + 10% SS	RPMI only	RPMI + 10% FCS	RPMI + 10% SS	M199 only	M199 + 10% FCS	M199 + 10% SS	FCS only	SS only	NCS only
126	IM			4-aminobutyrat	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
127	IM			NhaP	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
128	IM			HflC	–	–	–	–	–	–	–	–	–	–	–	–	+[2]	–	–	–	–	–	–	–	–	–	–	–
129	C	–	+	CarB	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
130	C/IM			Thioredoxin-like	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
131	C			Pta	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
132	C			PurL	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
133	C			GapA	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
134	C			RpsC	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
135	C			AceE	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
136	C			TktA	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
137	C			RpsD	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
138	C			EF2/FusA	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
139	C			RpoA	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
140	C			EF1B	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
141	C			Hsp33	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
142	C			Electron transp	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
143	C			LeuB	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
144	C			AsnA	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
145	C			FabG	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
146	C			DnaK	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
147	C			PurN	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
148	C			PurA	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

Table 4.3. (continued)

No.	Subcellular Localisation	TM	Lipo	Name	Protein identifications																													
					PH2												PH8												PH202							
					DMEM only	DMEM + 10% FCS	DMEM + 10% SS	RPMI only	RPMI + 10% FCS	RPMI + 10% SS	M199 only	M199 + 10% FCS	M199 + 10% SS	FCS only	SS only	NCS only	DMEM only	DMEM + 10% FCS	DMEM + 10% SS	RPMI only	RPMI + 10% FCS	RPMI + 10% SS	M199 only	M199 + 10% FCS	M199 + 10% SS	FCS only	SS only	NCS only	DMEM only	DMEM + 10% FCS	DMEM + 10% SS	RPMI only	RPMI + 10% FCS	RPMI + 10% SS	M199 only	M199 + 10% FCS	M199 + 10% SS	FCS only	SS only	NCS only
149	C			GlnA	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Total number of proteins identified					22	8	15	37	9	11	23	17	6	20	8	12	26	22	21	25	22	17	19	29	6	30	11	13	25	23	16	25	23	18	15	22	15	22	12	16
Total number of OMPs identified					18	6	10	9	8	7	8	14	5	14	6	7	21	17	14	18	18	12	17	21	4	22	8	10	20	17	12	18	17	13	12	16	11	18	11	11

No.	Subcellular Localisation	TM	Lipo	Name	Protein identifications																													
					PH278																													
					DMEM only	DMEM + 10% FCS	DMEM + 10% SS	RPMI only	RPMI + 10% FCS	RPMI + 10% SS	M199 only	M199 + 10% FCS	M199 + 10% SS	FCS only	SS only	NCS only																			
149	C			GlnA	+[2]	–	–	–	–	–	–	–	–	–	–	–																			
Total number of proteins identified					27	25	11	20	24	10	11	26	6	20	8	9																			
Total number of OMPs identified					19	19	8	15	17	8	8	21	4	18	7	6																			

Hsf was identified only in ovine serotype A1 isolate PH8 after growth in RPMI + 10% FCS, M199 + 10% FCS and complete NCS.

Of the seven identified proteins that were of unknown location, lipoprotein MHA_2737/COI_0543/COK_2363 was the only one to be identified only under growth conditions containing serum. It was identified only in isolate PH8 after growth in M199 containing 10% FCS and also in complete FCS. The remaining six proteins were identified either in tissue culture media or both tissue culture media and serum-supplemented media.

Three of the 31 identified proteins predicted to be localised to cellular compartments other than the outer membrane were identified only under growth conditions containing serum: PenP, MglC and UraA. PenP, a penicillinase, was identified in isolate PH2 after growth in complete FCS; this protein was also identified in the same isolate after growth under iron-restricted conditions. MglC and UraA, inner membrane proteins involved in galactose and uracil transport, respectively, were identified in isolates PH2, PH8 and PH202 only after growth in serum-supplemented conditions.

In the four isolates examined, growth in DMEM resulted in OMP profiles that were similar to those observed after growth in iron-restricted BHIB. This is most likely due to the fact that iron in DMEM is available only in the form of ferric nitrate (Conrad, 2007) and is not a favourable iron source for *M. haemolytica*. In serotype A1 isolate PH8, growth in DMEM supplemented with FCS or SS resulted in an OMP profile which resembled that observed after growth in iron-replete BHIB (Fig 4.2A). This suggests that isolate PH8 is able to acquire iron from serum of both cattle and sheep. A similar observation was made for isolate PH2, although some iron-acquisition proteins including TbpA and FrpB are still expressed at higher levels after growth in the presence of serum from either animal compared to growth in iron-replete BHIB. In the serotype A2 isolates, it appears that there is a correlation between the host of origin and the ability to acquire iron more readily from serum of the same host. In the bovine serotype A2 isolate (PH202), the protein band containing the putative siderophore receptor FrpB (number 12; Fig. 4.3) is expressed at higher levels after growth in the presence of SS than that of FCS, indicating a greater ability to acquire iron from serum of cattle than sheep. Conversely, in the ovine serotype A2 isolate

(PH278), the protein band containing the FrpB protein is expressed at a higher level after growth in the presence of FCS than that of SS, indicating a greater ability to acquire iron from serum of sheep than cattle. This is particularly interesting considering that PH202 and PH278 belong to the same ET and are identical in terms of their housekeeping genes.

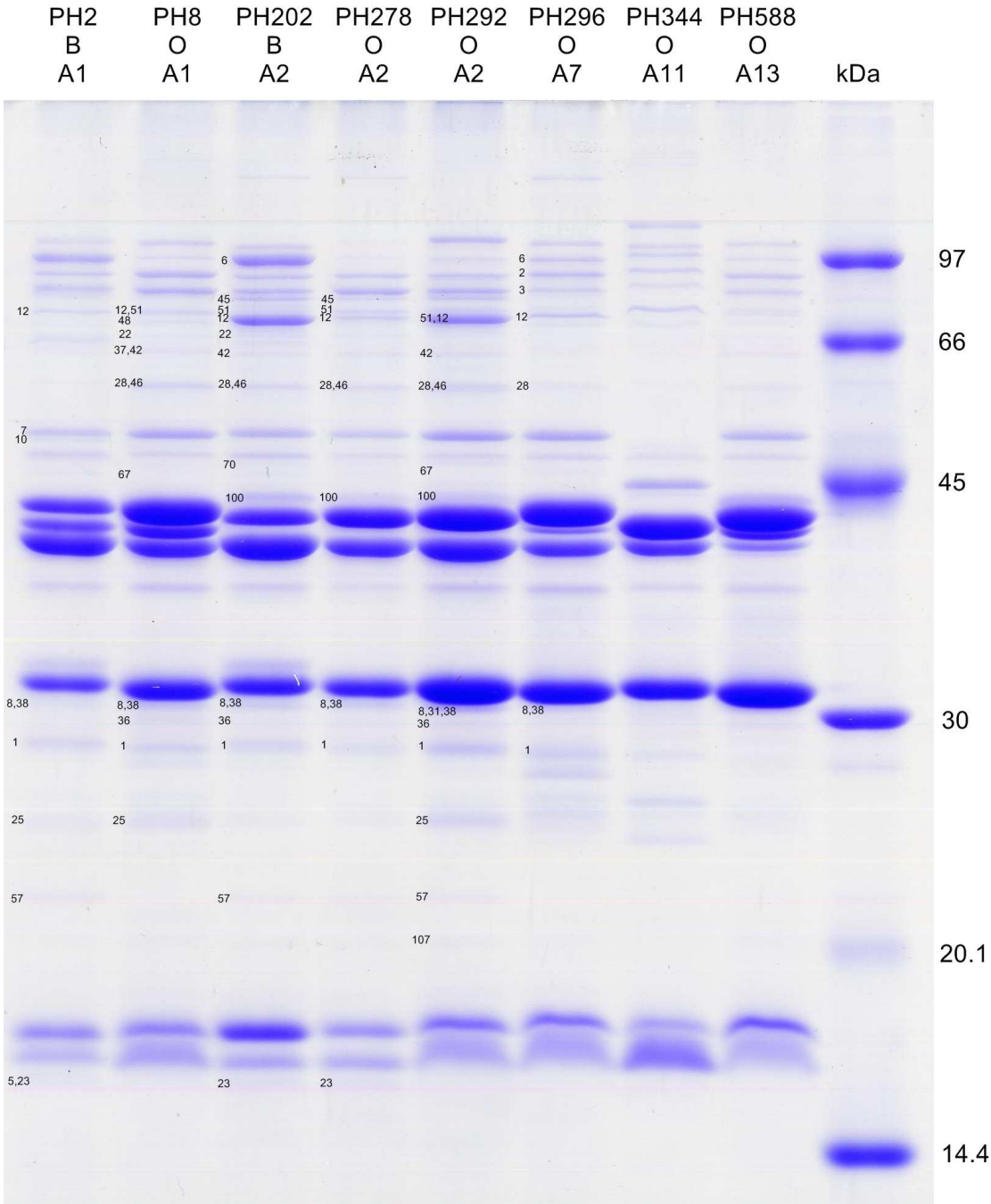
In a previous study, a 98 kDa protein band was identified in the OMP profiles of two *M. haemolytica* isolates after growth in NCS (Davies *et al.*, 1992), although the identity of this protein was not determined. Furthermore, expression of this protein was not repressed after the addition of $FeCl_3$ to the NCS, indicating that its expression was not iron-regulated (Davies *et al.*, 1992). In the present study, this protein was identified as the leukotoxin protein LktA (number 87; Fig. 4.3). It was identified in a 98 kDa protein band in isolates PH2, PH8 and PH202 after growth in tissue culture media containing 10% SS and in complete NCS by gel-based proteomic analysis. It was also identified in isolates PH278 by gel-free proteomic analysis under the same growth conditions.

4.3.3 Identification of *M. haemolytica* and *M. glucosida* OMPs which undergo changes in expression after growth on solid-surface BHIA in the absence or presence of Congo red dye.

In order to identify OMPs that are potentially involved in mediating surface-associated growth and possible biofilm formation, the OMP profiles of the eight representative isolates were examined after 24 h growth on BHIA (Fig. 4.4). The OMP profiles were not too dissimilar to that observed after growth in iron-replete BHIB (Fig. 4.2A). Several iron-acquisition OMPs were identified including HmbR1, HxuB and HxuC, although they were expressed at lower levels than that observed after growth in iron-restricted BHIB. Expression of these OMPs is most likely due to locally available iron being depleted by high cell densities and also the slower diffusion of this nutrient through solid medium compared to liquid medium. Gel-based proteomic analysis revealed no new proteins identified in any of the isolates that had not been identified in iron-replete or iron-restricted BHIB.

Figure 4.4. One-dimensional SDS-PAGE separation of outer membrane fractions from seven *M. haemolytica* isolates and one *M. glucosida* isolate after growth on BHIA for 24 h.

Numbered proteins were subsequently identified by proteomic analysis. Twenty micrograms of protein were loaded into each lane. Protein bands were stained with Coomassie brilliant blue.



Gel-free proteomic analysis resulted in poor coverage of identified proteins and was therefore not included.

The seven *M. haemolytica* isolates and the *M. glucosida* isolate were grown on BHIA supplemented with 0.8 g/L Congo red ($BHIA_{CR}$). Within 24 h, in all isolates, the agar surrounding areas of growth had turned from red to black and there were black dots in the centres of individual colonies (Fig. 4.5). However, after incubation between 48 and 72 h the colonies turned a deep red colour, suggesting that it was only the underlying agar that turned black within 24 h and not the colonies themselves.

To determine any physiological effect that Congo red might be having on OMP expression, outer membrane fractions were extracted after 24 h growth on $BHIA_{CR}$. Outer membrane protein profiles were virtually identical to those observed after growth on BHIB (Fig. 4.4), except for the presence of previously unseen protein bands in the upper molecular mass region of the gel (Fig. 4.6). Two protein bands were present at 131 and 135 kDa in all *M. haemolytica* isolates but not in the *M. glucosida* isolate. Iga1_1 (number 151; Fig. 4.6) and Iga1_2 (number 24; Fig. 4.6) were identified in these bands in one and six isolates, respectively, but given the close proximity of the bands it was not possible to determine which protein was contained in which protein band. Another OMP, the putative filamentous haemagglutinin FhaB_1 (number 150; Fig. 4.6), was identified in a 159 kDa protein band in isolate PH8. Iga1_3 (number 39; Fig. 4.6) was also identified at 146 kDa in isolates PH292 and PH588. This protein had been identified in the previous chapter in isolates PH202, PH278 and PH296 after growth in iron-replete BHIB. The Iga1_1, Iga1_2 and FhaB_1 proteins were also visible when isolates were grown in BHIB supplemented with Congo red dye (Fig. 4.6) indicating that expression of these OMPs is dependent on the presence of Congo red dye and not on solid-surface growth.

Figure 4.5. Colony phenotypes of seven *M. haemolytica* isolates and one *M. glucosida* isolate after growth on $BHIA_{CR}$ for 18, 24, 48 and 72 h.

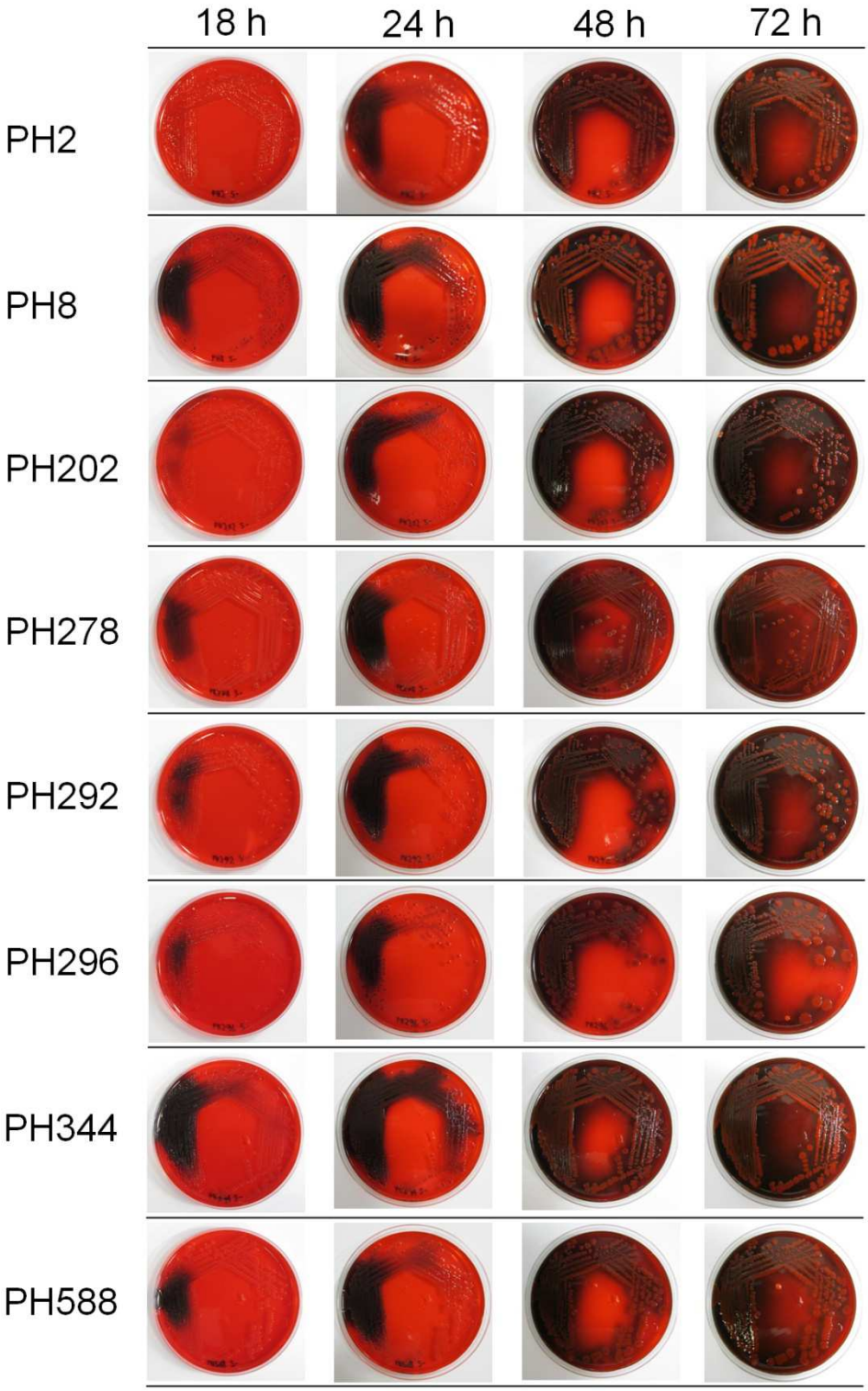
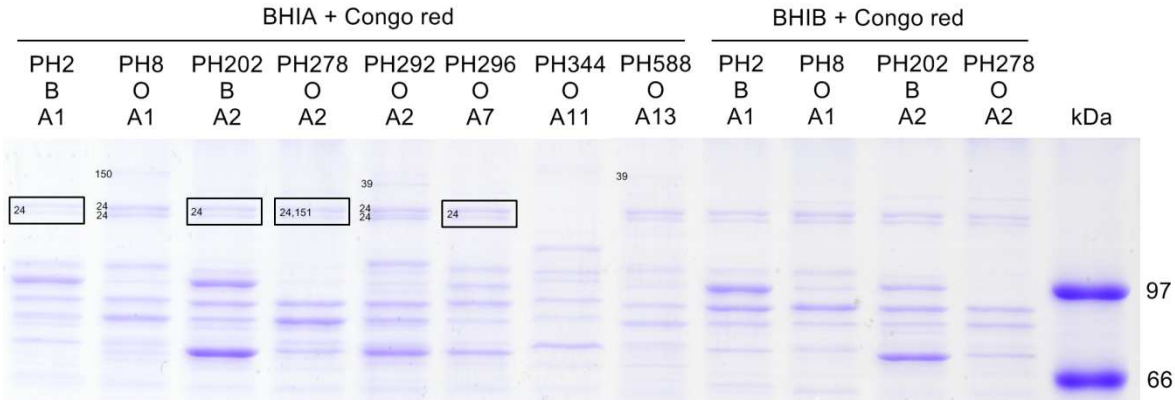


Figure 4.6. One-dimensional SDS-PAGE separation of outer membrane fractions from seven *M. haemolytica* isolates and one *M. glucosida* isolate after growth on $BHIA_{CR}$ for 24 h. The OMP profiles of *M. haemolytica* isolate PH2, PH8, PH202 and PH278 after growth to mid-log phase in $BHIB_{CR}$ are also shown.

Twenty micrograms of protein were loaded into each lane. Protein bands were stained with Coomassie brilliant blue.



4.3.4 Comparison of OMPs identified among different growth conditions.

A total of 55 OMPs were identified in the previous chapter after growth in iron-replete BHIB. In the present study, a further thirteen OMPs were identified using a range of different growth conditions (Fig 4.7; Table 4.4). Six OMPs (FhuA, autotransporter MHA_0080/COI_1870/COK_0773, EcnA, HmbR2, HxuA and Iga1_4) were identified exclusively after growth in iron-restricted BHIB. Possible OMP MHA_0862/COI_1567/COK_2302 was identified exclusively after growth in tissue culture media alone. Two OMPs (Hsf and PlpB), were identified exclusively after growth in media containing serum. The haemin receptor was identified after growth in both iron-restricted BHIB and tissue culture media alone. The maltoporin LamB was identified after growth in iron-restricted BHIB, tissue culture media only and in media containing serum. Iga1_1 and FhaB_1 were identified exclusively after growth in the presence of Congo red dye (not shown in Fig. 4.7).

4.3.5 Extracellular protein expression after growth in iron-replete and iron-restricted BHIB.

The present work has provided evidence to suggest that proteolytic cleavage occurs in the passenger domains of OMPs such as Iga1_2 and Ssa. Furthermore, the finding that HxuA is only present in the outer membrane of one isolate after growth in iron-restricted BHIB suggests that it is a secreted protein. To identify these proteins in the extracellular medium and gain an understanding of the overall extracellular subproteome of *M. haemolytica*, proteins from cell-free culture supernatants were precipitated and analysed by 1-D SDS-PAGE after growth to stationary phase in iron-replete (Fig. 4.8A) and iron-restricted (Fig. 4.8B) BHIB. Supernatants from stationary phase growth were used because only limited protein was obtained from supernatants after growth to mid-log phase (results not shown). This finding is consistent with what has been observed for extracellular proteins of other bacteria in the stationary phase (Voigt *et al.*, 2006, Gohar *et al.*, 2002, Xia *et al.*, 2008, Kim *et al.*, 2005). Similar to OMP expression profiles, the extracellular protein profiles of the representative isolates differ considerably after growth in iron-restricted BHIB compared to iron-replete BHIB. Proteins have not yet been identified by proteomic approaches, but will be in future experiments.

Figure 4.7. Distribution of 66 OMPs identified by proteomic approaches after growth in iron-replete BHIB (grey shaded), iron-restricted BHIB, tissue culture media only and/or media containing serum.

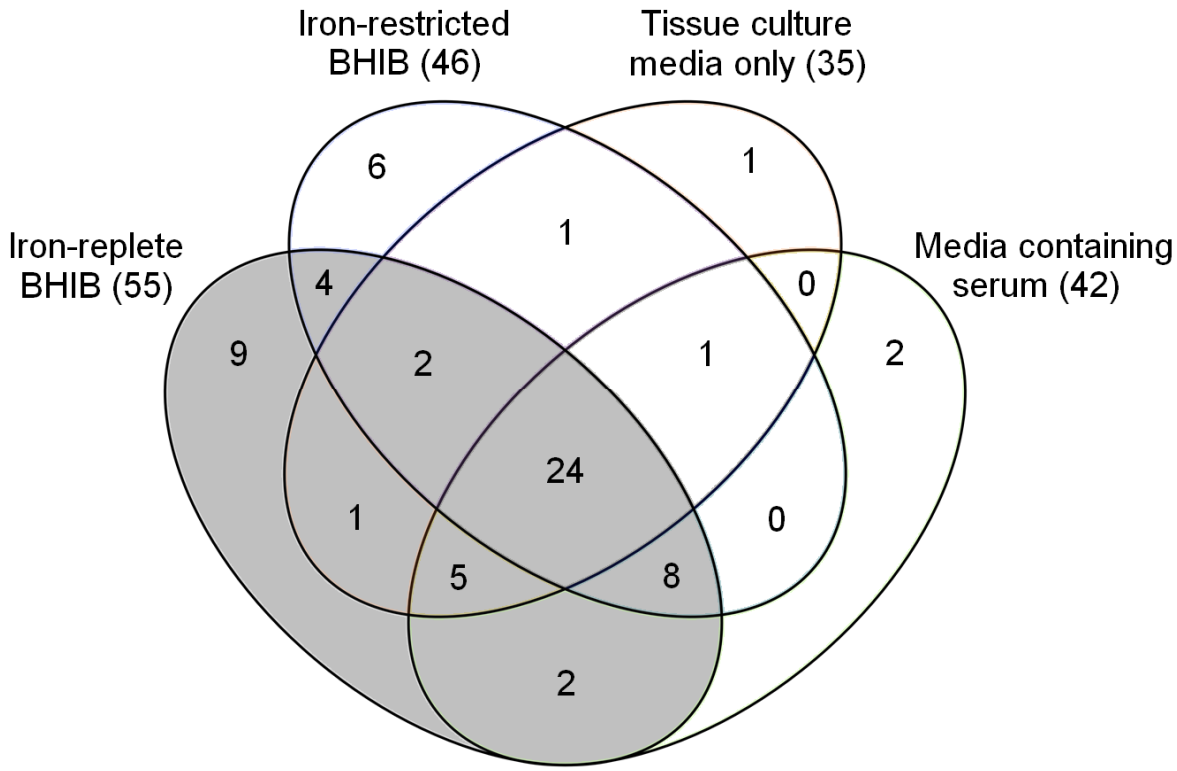


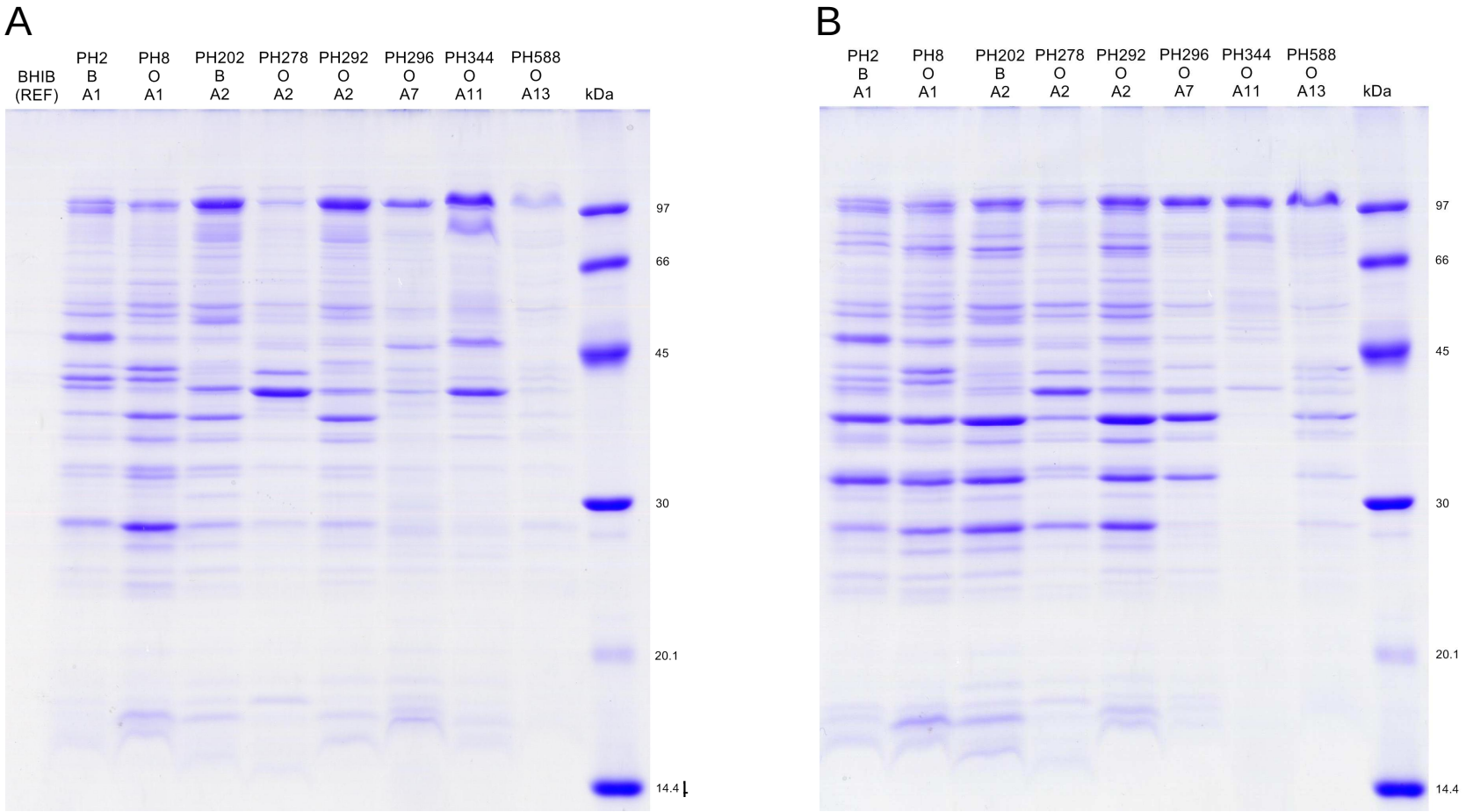
Table 4.4. Names and putative functions of OMPs that were identified under different growth conditions that were not identified after growth in iron-replete BHIB.

No.[a]	Name	Function	Iron-restricted BHIB	Tissue culture media only	Media containing sera	$BHIA_{CR}$
99	Haemin receptor	Haemin receptor	+	+	−	−
100	LamB	Maltoporin transport	+	+	+	−
101	FhuA	Ferric hydroxamate receptor	+	−	−	−
102	Autotransporter	Unknown	+	−	−	−
103	EcnA/B	Programmed cell apoptosis	+	−	−	−
104	HmbR2	Haemoglobin receptor	+	−	−	−
105	HxuA	Haem-haemopexin acquisition	+	−	−	−
106	Iga1_4	Cleavage of host mucosal antibody	+	−	−	−
112	Possible OMP	Unknown	−	+	−	−
113	Hsf	Adherence/serum resistance	−	−	+	−
114	PlpB	Unknown	−	−	+	−
150	FhaB_1	Adherence	−	−	−	+
151	Iga1_1	Cleavage of host mucosal antibody	−	−	−	+

[a]Numbers correspond to the location of the protein in Figures 4.2, 4.3, 4.4 and 4.6 if identified by gel-based proteomics.
'+' = identified in at least one isolate; '−' = not identified in any isolates.

Figure 4.8. One-dimensional SDS-PAGE separation of extracellular proteins from seven *M. haemolytica* isolates and one *M. glucosida* isolate after growth to stationary phase in (A) iron-replete and (B) iron-restricted BHIB.

Eighty micrograms of protein were loaded into each lane. Protein bands were stained with Coomassie brilliant blue.



4.4 Discussion

In the present study, the OMP expression profiles of seven representative *M. haemolytica* isolates and one *M. glucosida* isolate were examined after growth under several *in vitro* conditions designed to mimic the *in vivo* pneumonic lung microenvironment. Subsequently, a combination of proteomic approaches were used to identify new OMPs and determine which OMPs were differentially expressed under these conditions, indicating potential roles in virulence and host-specificity.

Growth in iron-restricted conditions

Firstly, the tolerances of the representative isolates to growth in increasing concentrations of the iron chelator 2,2'-dipyridyl were examined in order to determine the maximum concentration at which each isolate could grow to mid-log phase. Interestingly, isolates PH2 and PH278, which represent groups of pathogenic isolates that cause the majority of disease cases in cattle and sheep, respectively, tolerated the lowest levels of 2,2'-dipyridyl (160 and 120 μM, respectively). One might expect that the most pathogenic isolates would be able to tolerate higher levels of 2,2'-dipyridyl than weakly pathogenic isolates. Also, notably, the weakly pathogenic *M. glucosida* isolate was able to grow at the highest concentration of 2,2'-dipyridyl (310 μM). These findings suggest that the ability to tolerate increasing concentrations of 2,2'-dipyridyl could be inversely correlated with virulence in these species.

Under iron-restricted growth conditions, eight OMPs were identified that were not previously identified under growth in iron-replete BHIB. These were a haemin receptor, LamB, FhuA, autotransporter MHA_0800/COI_1870/COK_0773, EcnA, HmbR2, HxuA and Iga1_4. The haemin receptor and putative haemoglobin receptor HmbR2 have been described previously in a bovine *M. haemolytica* isolate after iron-restricted growth (Roehrig *et al.*, 2007). LamB is a putative maltoporin involved in substrate-specific uptake of maltodextrins. The autotransporter contains a C-terminal translocator domain that is characteristic of other autotransporters but its function is currently unknown. EcnA, shares 45% amino acid identity with the EcnA protein of *E. coli*. In *E. coli*, the *ecnA* and *ecnB* genes comprise the two-gene entericidin locus, whereby EcnB 'the toxin'

and EcnA 'the antitoxin' contribute programmed cell apoptosis (Bishop *et al.*, 1998). During the course of balanced growth, EcnB is constantly neutralised by EcnA; however, metabolic changes brought about by nutrient stress disturbs this balance and EcnB 'poisons' the cell, allowing surviving cells to use the lysis products for nourishment. A counterpart EcnB protein was not predicted in *M. haemolytica* genomes by bioinformatics, therefore it can only be speculated as to whether *M. haemolytica* EcnA functions in a similar manner to *E. coli* EcnA. Iga1_4 was identified exclusively in *M. glucosida* and represents the first Iga1-like protease to be identified in this species under iron-restricted conditions. Members of this protein family were identified in the previous chapter in all *M. haemolytica* isolates after growth in iron-replete BHIB, but not in *M. glucosida*. HxuA and FhuA are described in further detail below in the context of their respective expression systems.

Under iron-restricted growth conditions, protein components of a putative HxuCBA haem-haemopexin utilization system were identified in several *M. haemolytica* isolates. This system was first discovered in *H. influenzae* (Hanson *et al.*, 1992) and is an important virulence determinant in this organism (Morton *et al.*, 2007) and in *H. parasuis* (Melnikow *et al.*, 2005). HxuC is a TonB-dependent receptor protein (Cope *et al.*, 2001, Cope *et al.*, 1995). HxuB and HxuA comprise a two-partner (TPS) system, whereby HxuA (the TpsA protein) is transported across the outer membrane by HxuB (the TpsB protein) (Cope *et al.*, 1995). HxuA binds the haem-haemopexin complex and facilitates haem delivery to the cell surface via HxuC (Cope *et al.*, 1994, Cope *et al.*, 1998). Recent evidence suggests that HxuA might not actually function as a haemophore in *H. influenzae*, but instead releases haem upon interaction with the haem-haemopexin complex (Fournier *et al.*, 2011). Furthermore, the primary activity of HxuA may actually be to sequester haemopexin in its inactive form, thereby decreasing the high-affinity binding of haem by the serum (Fournier *et al.*, 2011). In the present study, HxuB and HxuC were expressed in the outer membranes of all *M. haemolytica* isolates except for the bovine serotype A1 isolate PH2, and the *M. glucosida* isolate PH344, after growth in iron-restricted BHIB. In the previous chapter, HxuB and HxuC were identified after growth in iron-replete BHIB in two (PH202 and PH292) and one (PH588) isolates, respectively, but at much lower expression levels than that observed after

growth in iron-restricted BHIB. HxuA was identified in only one *M. haemolytica* isolate (PH8) under the same conditions, although in a very faint protein band. In *H. influenzae*, HxuA has been identified as both surface-associated (Fournier *et al.*, 2011) and released into the culture medium (Wong *et al.*, 1995). The amino acid sequences of HxuA in the three *M. haemolytica* genomes contain two conserved cysteine residues at their C-terminus that have been demonstrated to be essential for the cell-surface anchoring of TpsA proteins in other microorganisms (Jacob-Dubuisson *et al.*, 2004, Buscher *et al.*, 2006, Fournier *et al.*, 2011). It is therefore surprising that HxuA was absent (with the exception of one isolate) from *M. haemolytica* outer membrane fractions and suggests that HxuA is released from the cell surface. This is the first study to report HxuCBA expression in the outer membrane of *M. haemolytica*. The presence of HxuCBA has not previously been reported in *M. haemolytica* because outer membrane studies have almost exclusively examined the expression of OMPs in bovine serotype A1 isolates (Ayalew *et al.*, 2010, Lo *et al.*, 2006, Roehrig *et al.*, 2007) which, according to the findings presented here, do not express a functional HxuCBA system. Results from the present study, and the failure to detect HxuCBA expression in other bovine serotype A1 isolate studies (Ayalew *et al.*, 2010, Lo *et al.*, 2006, Roehrig *et al.*, 2007), indicate that the HxuCBA system is not expressed in bovine serotype A1 *M. haemolytica* isolates, nor in *M. glucosida* isolates, but is expressed under iron-restricted conditions in all other isolates examined in this study.

Expression of the putative siderophore receptor FrpB was significantly upregulated in two isolates (PH2 and PH344) and was predominantly expressed among other OMPs in an upregulated protein band in the remaining six isolates under iron-restricted growth conditions. Transcription of the *frpB* gene has previously been shown to be upregulated in a bovine serotype A1 *M. haemolytica* isolate (Roehrig *et al.*, 2007) and in the closely related species *A. pleuropneumoniae* (Klitgaard *et al.*, 2010) under iron-restriction conditions. In *A. pleuropneumoniae* FrpB has been demonstrated to be both immunoreactive (Liao *et al.*, 2009) and an essential virulence determinant, as *frpB* deletion mutants are unable to colonise the host or cause clinical disease symptoms upon experimental infection (Buettner *et al.*, 2009). The function of FrpB in *M. haemolytica*, *M. glucosida* and *A. pleuropneumoniae* is currently unknown.

However, in *N. gonorrhoeae* it has been associated with ferric iron uptake from transferrin (Dyer *et al.*, 1988, Beucher and Sparling, 1995) and, more recently, as a receptor for the ferric siderophore enterobactin (Carson *et al.*, 1999). *N. gonorrhoeae* does not synthesise siderophores but can utilise those produced by other bacteria (xenosiderophores), such as *E. coli* aerobactin (West and Sparling, 1987) and enterobactin (Rutz *et al.*, 1991, Carson *et al.*, 1999). *M. haemolytica* and *M. glucosida* do not produce their own siderophores either, suggesting the possibility that these species may utilise xenosiderophores via FrpB in a similar manner to *N. gonorrhoeae* and other Gram-negative bacteria (Thulasiraman *et al.*, 1998). Indeed, some bacterial isolates of the bovine nasopharyngeal flora have been reported to stimulate the growth of *M. haemolytica* and other closely related Gram-negative species, possibly as a result of this mechanism (Corbeil *et al.*, 1985). Another putative siderophore receptor, FhuA, which shares 24% identity with the *A. pleuropneumoniae* FhuA protein, was also identified in three *M. haemolytica* isolates (PH292, PH296 and PH344) under iron-restricted conditions. In *M. haemolytica* serotype A2 genomes, the *fhuA* gene and *fhuCDB* genes that encode attendant proteins are arranged in an operon with the same structure as that in *A. pleuropneumoniae* (Mikael *et al.*, 2002). In *A.* pleuropneumoniae, FhuA is involved in the uptake of the hydroxamate siderophore ferrichrome and, in contrast to FrpB, is not required for virulence (Baltes *et al.*, 2003). Given that the annotations of the FrpB and FhuA proteins of *M. haemolytica* have been allocated solely based on sequence homologies, and the previous finding that *M. haemolytica* is unable to utilise either exogenous enterobactin or ferrichrome under iron-limited conditions (Reissbrodt *et al.*, 1994, Graham and Lo, 2002), it is likely these proteins do not function as enterobactin and ferrichrome receptors, respectively, but are involved in the uptake of other as yet unknown molecules. Indeed, *M. haemolytica* FhuA actually shares greater identity (34%) with the *E. coli* FhuE protein than *A. pleuropneumoniae* FhuA. FhuE is a receptor for ferrioxamine B (Sauer *et al.*, 1990, Hantke, 1983), a siderophore which has been demonstrated to support *M. haemolytica* growth under iron-restricted conditions (Reissbrodt *et al.*, 1994) and may be a more appropriate substrate for *M. haemolytica* FhuA binding.

Expression of the FadL protein increased after growth to mid-log phase under iron-restricted conditions in several isolates; however, expression returned to

below baseline levels after growth to stationary phase under iron-restricted conditions. The function of FadL (also referred to as OmpP1) in *M. haemolytica* is not known. In *E. coli*, FadL is an OMP involved in the uptake of long-chain fatty acids (LCFAs) through the outer membrane (Black *et al.*, 1987). LCFAs and their derivatives are important for a wide variety of cellular processes, including fatty acid and phospholipid synthesis, membrane permeability and enzyme activation (Black and DiRusso, 2003). Upregulation of genes encoding enzymes that metabolise LCFAs have been observed during the early stages of infection with *Salmonella enterica* (Mahan *et al.*, 1995), indicating that transport of LCFAs is also important from a pathophysiological perspective. During bacterial infections, high concentrations of LCFAs (including arachidonic acid) are found in the extracellular inflammatory milieu that are released from host cells. It has been suggested that subsequent uptake of arachidonic acid by FadL might suppress the local inflammatory immune response and hence confer bacteria with an advantage during early colonisation of the host (Black and DiRusso, 2003). If FadL were to have a similar role in *M. haemolytica* then this might explain why expression of FadL is increased during mid-log phase growth (representing early stage infection) in several isolates, but not at stationary phase growth (representing late stage infection). The *M. haemolytica* leukotoxin stimulates the release of arachidonic acid and its derivatives from host neutrophils (Wang *et al.*, 1998, Clinkenbeard *et al.*, 1994). Therefore, an increase in FadL expression may limit inflammatory immune responses and prevent lung tissue damage during early colonisation. The observation that FadL is present in the outer membrane at much lower levels after iron-restricted stationary phase growth compared to iron-restricted mid log phase growth also raises the question of how this OMP and others are removed from the outer membrane when they are no longer required. Several Gram-negative organisms release outer membrane through the production of outer membrane vesicles (OMVs) (Kulp and Kuehn, 2010), and FadL-like proteins have been identified in OMVs released by *N. meningitidis* (Uli *et al.*, 2006, Vaughan *et al.*, 2006, Vipond *et al.*, 2006, Williams *et al.*, 2007), *Pseudomonas aeruginosa* (Bauman and Kuehn, 2006), *Moraxella catarrhalis* (Schaar *et al.*, 2011). OMV release has not yet been demonstrated in *M. haemolytica* but may provide an appropriate mechanism by which FadL and other OMPs are discarded from the outer membrane.

Growth in the presence of serum

Four representative *M. haemolytica* isolates were grown in three different tissue culture media (DMEM, RPMI and M199) in the presence or absence of 10% FCS and 10% SS, and also in complete FCS, SS and NCS. Two OMPs, Hsf (in one ovine isolate) and PlpB (in the two bovine isolates), were identified only after growth in media supplemented with serum. Both of these proteins were not identified after growth in iron-replete and iron-restricted BHIB.

In the human respiratory tract pathogen *H. influenzae*, Hsf is associated with attachment to epithelial cells (Barenkamp and StGeme, 1996) and is considered to be major nonpilus adhesin (StGeme *et al.*, 1996). A gene whose product has high homology to the *H. influenzae* Hsf protein was also found to be upregulated in *A. pleuropneumoniae* after contact with porcine lung epithelial cells (Auger *et al.*, 2009). Hsf also confers serum-resistance on *H. influenzae* isolates by binding the extracellular matrix molecule vitronectin, a regulator of the terminal pathway of complement activation (Hallstrom *et al.*, 2006). Vibronectin-binding proteins have also been identified in other Gram-negative bacteria including DsrA of *Haemophilus ducreyi* (Elkins *et al.*, 2000, Leduc *et al.*, 2009) and Usp of *M. catarrhalis* (Attia *et al.*, 2006). Bacteria are shielded from complement attack when coated with vitronectin and, furthermore, can use the molecule as a bridge to cross-link other bacterial cells and to bind integrin receptors on host cells (Singh *et al.*, 2010). *M. haemolytica* encounters host serum factors, including complement, as a result of lung tissue inflammation caused by the onset of disease (Ackermann and Brogden, 2000). Therefore, the Hsf-like protein identified in the present study under serum-supplemented and complete serum growth conditions may contribute to the protection of *M. haemolytica* in the presence of host serum.

PlpB is encoded in an operon containing the genes for two other similar lipoproteins, PlpA and PlpC (Cooney and Lo, 1993). Each of these proteins are immunogenic and recognised by serum from calves that have been naturally exposed or vaccinated with *M. haemolytica* cells (Dabo *et al.*, 1994). A *M. haemolytica* mutant lacking the *plpABC* operon was also more susceptible to bovine complement-mediated killing (Murphy *et al.*, 1998). Both PlpA and PlpC were identified in the previous chapter in the outer membranes of several *M.*

haemolytica isolates after growth in iron-replete BHIB. It is therefore surprising that PlpB has not identified alongside PlpA and PlpC given that the three proteins are expected to be expressed in equal quantities. The function of PlpB is currently unknown; however, PlpA has recently been demonstrated to be surface-exposed and capable of binding to bovine bronchial epithelial cells *in vitro* (Kisiela and Czuprynski, 2009).

The leukotoxin protein, LktA, was identified in outer membrane fractions of isolates PH2, PH8, PH202 and PH278 when grown in the presence of 10% SS, and in complete SS and NCS. LktA is a member of the repeats-in-toxin (RTX) exoprotein family produced by a variety of Gram-negative bacteria (Linhartova *et al.*, 2010) and is a key virulence factor in the pathogenesis of pneumonic pasteurellosis (Chang *et al.*, 1987, Maheswaran *et al.*, 1993, Petras *et al.*, 1995, Sutherland, 1985, Sutherland and Donachie, 1986). Surface-association of LktA has not previously been demonstrated in *M. haemolytica*, but has been shown in RTX proteins of other Gram-negative bacteria. The adenylate cyclase toxin of *B. pertussis* remains surface-associated following secretion, due to interaction with the filamentous haemagglutinin (Zaretzky *et al.*, 2002). A putative filamentous haemagglutinin (FhaB_1) was identified in the present study in the outer membrane fraction of one isolate (PH8) after growth on $BHIA_{CR}$, but LktA was not concurrently identified. The leukotoxin of the closely-related organism *Actinobacillus actinomycetemcomitans* (LtxA) is both secreted as soluble protein (Kachlany *et al.*, 2000, Kachlany *et al.*, 2002) and retained at the bacteria cell surface within membranous vesicles and by electrostatic association with nucleic acids on the bacterial cell surface (Lally *et al.*, 1991, Berthold *et al.*, 1992, Ohta *et al.*, 1991, Tsai *et al.*, 1984, Kato *et al.*, 2002). Paradoxically, in contrast to the finding in the present study of *M. haemolytica* LktA surface-association in the presence of serum, the LtxA protein of *A. actinomycetemcomitans* is released from the cell surface in the presence of serum (Johansson *et al.*, 2003), although the mechanism of release has not been established. Several possible mechanisms might explain why *M. haemolytica* LktA is surface-associated in the presence of serum. Firstly, serum components which bind to the surface of *M. haemolytica* may also bind LktA and retain it on the surface. Secondly, the presence of serum may somehow disrupt the secretion of LktA into the extracellular medium leading to accumulation of LktA at the cell surface.

Thirdly, the presence of serum may stimulate outer membrane blebbing, whereby vesicles containing LktA are both released from the cell and retained at the cell surface. Furthermore, it is possible that LktA is normally associated with the outer membrane *in vivo* (or under conditions which *in vivo* conditions) but does not associate with the membrane when grown *in vitro* in complex growth media alone.

Growth on solid-surface agar

Surface-associated growth on BHIA resulted in mild expression of OMPs that were previously strongly upregulated under iron-restricted growth conditions (including FrpB, HmbR1, HxuB and HxuC) as has been observed for other Gram-negative bacteria grown on solid surfaces (Wang *et al.*, 2004, Sampathkumar *et al.*, 2006, Pysz *et al.*, 2004). It has previously been suggested that immobilised growth, as opposed to growth in liquid culture, represents more physiologically appropriate baseline growth conditions and that iron-acquisition systems expressed during surface-associated growth may be more appropriately viewed as a repression of these systems during growth in liquid media (Sampathkumar *et al.*, 2006). There were no new OMPs identified in any of the representative isolates after growth on BHIA that had not been identified in iron-replete or iron-restricted BHIB. Furthermore, no OMPs with roles in biofilm formation, such as those encoded in the Tad (tight adherence) locus of *P. multocida* (E-Komon *et al.*, 2011a) and other organisms (Tomich *et al.*, 2007) have been identified in the present study. Therefore, despite limited previous evidence of biofilm formation in *M. haemolytica* (Haig, 2011, Olson *et al.*, 2002), this study has not been able to provide evidence of biofilm-producing ability in the representative isolates examined.

Congo red dye is well known for its ability to bind highly aggregative and insoluble ß-sheet-rich amyloid fibres found in several living organisms (Chapman *et al.*, 2002, Fowler *et al.*, 2007). It is also commonly used to differentiate between slime-producing and non-slime producing strains of Staphylococci, which result in black and pink colonies, respectively, when grown on agar that contains the dye (Freeman *et al.*, 1989, Jain and Agarwal, 2009, Arciola *et al.*, 2002, Arciola *et al.*, 2006). It can also be used to differentiate between virulent and avirulent strains of several Gram-negative bacteria (Deneer and Potter,

1989, Daskaleros and Payne, 1987, Stugard *et al.*, 1989, Ishiguro *et al.*, 1985, Prpic *et al.*, 1983). Notably, in *Shigella flexneri*, the presence of Congo red can stimulate the release of Ipa type III effector proteins (Parsot *et al.*, 1995), regulate membrane protein expression (Sankaran *et al.*, 1989) and is associated with increased infectivity in HeLa cells (Daskaleros and Payne, 1987). Despite these virulence-associated observations made across a broad range of organisms, the mechanism by which this dye achieves these effects is still unclear. A previously unknown effect of Congo red on the expression of high-molecular mass OMPs in *M. haemolytica* was demonstrated in the present study. Two high molecular mass OMPs, Iga1_1 and Iga1_2, were identified in all *M. haemolytica* isolates after growth in the presence of Congo red. Another high molecular mass protein, the filamentous haemagglutinin protein FhaB_1, was also identified in one isolate after growth in the presence of Congo red. These three proteins all contain domains which are expected to extend into the extracellular milieu and undergo subsequent proteolytic cleavage, yet each was identified at their intact molecular mass after growth in the presence of Congo red. Indeed, Iga1_2 was identified in the previous chapter at 37 kDa after growth in iron-replete BHIB in the absence of Congo red, indicating that the extracellular domain of this protein is normally cleaved from the cell surface. This suggests that, rather than regulating the expression of these OMPs at the genetic level, Congo red might be interfering with their post-translational processing at the bacterial cell surface. Consistent with this is the suggestion that the enhanced secretion of *S. flexneri* effector proteins in the presence of Congo red is most likely due to the interference of the dye with the Type III secretion needle tip complex which controls effector protein release (Bahrani *et al.*, 1997). At present, it can only be speculated as to how Congo red interferes with the processing of *M. haemolytica* OMPs. One explanation is that the dye is binding directly to these OMPs, preventing autoproteolysis and allowing them to remain intact at the cell surface. Alternatively, Congo red may bind to and inhibit the function of another as yet undefined surface-associated protein which is involved in the processing of these proteins. In neisserial species, Iga1 proteases are capable of autoproteolysis (Pohlner *et al.*, 1987, Vitovski and Sayers, 2007) and can also be processed by the autotransporter NalP (van Ulsen *et al.*, 2003). Similarly, *B. pertussis* expresses a filamentous haemagglutinin protein which is released from the cell surface by the autotransporter SphB1 (Coutte *et al.*, 2001, Coutte *et al.*,

2003). Both NalP and SphB1 are members of the subtilisin-like protease family (Siezen and Leunissen, 1997) and share significant identity with serotype-specific antigen (Ssa), an autotransporter which has been identified in the outer membrane of all *M. haemolytica* and *M. glucosida* isolates examined to date. It is possible that Ssa could be responsible for releasing the surface-exposed domains of Iga1-like proteases and FhaB_1 from the cell surface. Further experimentation will be required to determine the actual mechanism by which Congo red allows these OMPs to remain surface-associated.

Extracellular protein expression

To complement the information obtained about OMP expression, putative proteolytic cleavage of OMPs and putative secretion of HxuA, an examination of the extracellular subproteomes of the eight representative isolates was performed. Extracellular proteins were precipitated from cell-free culture supernatant after growth to stationary phase in iron-replete and iron-restricted BHIB. The extracellular protein profiles of the eight isolates appear to be as complex as their OMP profiles, and differed considerably after growth in iron-replete and iron-restricted medium. In *E. coli*, a proteomic analysis of the extracellular subproteomes of two isolates demonstrated that periplasmic and OMPs accounted for the majority of released proteins during stationary phase growth (Xia *et al.*, 2008). Furthermore, the expression levels of porins OmpF and OmpC in the outer membrane did not change significantly between different cell growth densities, suggesting that these proteins are continuously secreted into the medium, possibly by OMV blebbing (Xia *et al.*, 2008). Another possibility is that the extracellular proteins were the products of cell lysis, although this was proved not to be the case for *E. coli* (Xia *et al.*, 2008). A proteomic analysis of the *M. haemolytica* extracellular subproteome is still to be completed.

5. FINAL DISCUSSION

The first objective of this study was to examine the surface-exposure of OmpA and to demonstrate whether the OmpA proteins from bovine (OmpA1) and ovine (OmpA2) isolates are antigenically distinct. This was achieved by examining whether antibodies that were raised against recombinant OmpA1 (rOmpA1) and OmpA2 (rOmpA2) proteins could interact with OmpA in a strain-specific manner. Recombinant OmpA1 and rOmpA2 were successfully expressed, purified and used to generate anti-rOmpA1 and anti-rOmpA2 antibodies, respectively. Immunogold-electron microscopy and immunofluorescence techniques clearly demonstrated that OmpA1 and OmpA2 are surface exposed, and are not masked by the polysaccharide capsule, in a selection of *M. haemolytica* isolates of various serotypes and grown under different growth conditions. An examination of the binding specificities of anti-rOmpA antibodies to *M. haemolytica* isolates representing different OmpA subclasses revealed that cross-absorbed anti-rOmpA1 antibodies recognised OmpA1-type proteins but not OmpA2-type proteins; conversely, cross-absorbed anti-rOmpA2 antibodies recognised OmpA2-type proteins but not OmpA1-type proteins. The results of this study have therefore clearly demonstrated that OmpA1 and OmpA2 are surface-exposed and could potentially bind to different receptors in cattle and sheep. This work builds significantly upon what was previously known about *M. haemolytica* OmpA (Davies and Lee, 2004) but also raises some interesting questions to be addressed by future investigations. In particular, how are the loops of OmpA recognised by anti-rOmpA antibodies when the capsule is expected to mask them? This conundrum might be explored in future work by examining the possible roles of phase variable capsule expression and outer membrane vesicle blebbing on the exposure of OMPs to the host environment. Furthermore, the anti-rOmpA antibodies produced in this work could also be used in host epithelial cell binding assays to definitively demonstrate that *M. haemolytica* OmpA1 and OmpA2 are capable of selectively binding to different receptors within cattle and sheep, respectively. Preliminary crystallisation experiments determined a range of conditions under which protein crystals were produced; however, no X-ray diffraction data could be obtained. Further optimisation of these conditions might yield higher quality crystals under which structural data can be obtained.

The second objective of this study was to provide a comparative analysis of the outer membrane subproteomes of several isolates of *M. haemolytica* and *M. glucosida* in order to identify those that are possibly involved in host-specific adaptation and virulence. This objective was achieved in two parts. First, the entire repertoire of OMPs encoded in three *M. haemolytica* genomes was predicted using a simple bioinformatic framework. This yielded a total of 93 confidently predicted OMPs which were assigned to six broad functional categories: outer membrane biogenesis and integrity, transport and receptor, adherence, enzymatic activity, other and unknown. The majority of these proteins (88) were present among all three genomes. There were no proteins present exclusively in the ovine genome, and only one protein (PulD) was exclusively present in a bovine genome (serotype A1). Furthermore, amino acid sequence comparisons were performed to identify OMPs present among bovine and ovine genomes that may be adapted to either cattle or sheep. Only three OMPs, OmpA, PlpE, and Ahs, had greater amino acid sequence identity between bovine genomes than between one of the bovine genomes and the ovine genome. A possible role in host-specific adaptation has already been suggested for OmpA (Davies and Lee, 2004), but has not yet been demonstrated for PlpE or Ahs. Several other OMPs (Table 3.4) also had divergent amino acid sequences among the genomes that were apparently unrelated to host-specificity. The bovine and ovine serotype A2 genomes were more similar both in the number of OMPs present in the two genomes and amino acid conservation of OMPs than between either of these two genomes and the bovine serotype A1 genome. This reflects the common ancestral origin of the bovine and ovine serotype A2 isolates and provides further evidence to support previous work which hypothesised the host-switching of this serotype from cattle to sheep (Davies *et al.*, 1997, Davies *et al.*, 2001, Davies and Lee, 2004, Davies *et al.*, 2002).

Second, complementary proteomic approaches were used to identify and compare the OMPs present in the outer membrane fractions of seven *M. haemolytica* isolates and one *M. glucosida* isolate. Complementary proteomic approaches identified a total of 55 unique OMPs. Fifty of these proteins were confidently predicted by the bioinformatic approach, representing 54% of the confidently predicted outer membrane subproteome. Five identified OMPs (LemA, HbpA, OapA, RlpA and CsgG) were not predicted by the bioinformatic

approach but were considered likely to be OMPs after literature searches on these proteins were carried out. Eleven OMPs (MltC, FhaC, LppC, TonB-dependent receptor MHA_0860/COI_1565/COK_2304, OapA, TbpB, possible OMP MHA_0964/COI_1329/COK_2563, Bor/Iss_1, HxuC, TonB-dependent receptor MHA_1346/COI_1921 and OmpW) were identified among ovine *M. haemolytica* isolates that were not identified among bovine *M. haemolytica* isolates; however, the genes encoding these OMPs were identified in the genomes of bovine isolates, suggesting that they are possibly expressed under different growth conditions. There were no OMPs identified exclusively in bovine *M. haemolytica* isolates. The putative bovine-specific OMP, PulD, was not identified in any of the representative isolates. Despite the lack of evidence for roles in host-specific adaptation of identified OMPs, the results of this objective have produced a comprehensive overview of the outer membrane subproteomes of *M. haemolytica* and *M. glucosida* isolates that can be used to identify individual OMPs to be studied in further detail. Comparative nucleotide sequence analysis of genes encoding selected OMPs, such as PlpE and Ahs, should elucidate further evidence of host-specific adaptation, as has previously been determined for OmpA (Davies and Lee, 2004). Furthermore, the identified Ssa and Iga1_2 autotransporter proteins showed evidence of proteolytic processing, a phenomenon that has not previously been described in *M. haemolytica*. A possible role in proteolytic processing of other OMPs at the bacterial cell surface was hypothesised for Ssa. This was based upon on its similarity to NalP of *N. meningitidis* (Turner *et al.*, 2002, van Ulsen *et al.*, 2003), AasP of *A. pleuropneumoniae* (Ali *et al.*, 2008) and ShpB1 of *B. pertussis* (Coutte *et al.*, 2001), which are involved in proteolytic processing of other OMPs in these species. Studies using *ssa* gene knockout and partial deletion mutants of *M. haemolytica* should further elucidate the exact function of Ssa.

The third and final objective of this study was to characterise the outer membrane subproteomes of the same representative isolates and identify differentially expressed OMPs after *in vitro* growth under conditions that were designed to mimic the *in vivo* host environment. A further 13 confidently predicted OMPs (Table 4.4) were identified under either iron-restricted, serum-supplemented or Congo red-supplemented growth conditions (or a combination of the three), that were not identified under iron-replete growth conditions.

Expression of several OMPs that were previously identified after growth in iron-replete media increased after growth in iron-restricted conditions. One of the most significant findings was that components of an HxuCBA haem-haemopexin acquisition system were upregulated in all representative *M. haemolytica* isolates, except for bovine serotype A1 isolate PH2, after growth under iron-restricted conditions. It is interesting that isolate PH2, a virulent disease isolate, should be lacking this system as it is a known virulence determinant in *H. influenzae* (Morton *et al.*, 2007) and *H. parasuis* (Melnikow *et al.*, 2005). There was also no expression of HxuCBA in the *M. glucosida* isolate after growth under iron-restricted conditions. This study is the first to describe the expression of this system in *M. haemolytica* outer membranes and demonstrates the great advantage of comparative analyses over that of individual isolates. With the exception of studies by this author's research group, the majority of previous *M. haemolytica* outer membrane studies have focused almost exclusively on bovine serotype A1 isolates. This is probably due to the fact that these isolates are responsible for most cases of bovine pneumonic pasteurellosis and are of greater interest to animal health practitioners and vaccine developers. The present study has demonstrated that comparative examination of several isolates, as opposed to individual isolates, provides a broader knowledge of the *M. haemolytica* species as a whole and is more conducive to identifying novel proteins. Further study is warranted for the HxuCBA system, and other putative virulence-associated OMPs which were upregulated after growth in iron-restricted medium including FrpB (a putative siderophore receptor), HmbR1 (a putative haemoglobin receptor) and FadL (a putative LCFA transporter). Biological uptake assays which examine the specificities of different siderophores, haemophores and transferrins might also further elucidate roles in host-specificity for these OMPs.

Association of the leukotoxin with the outer membrane was demonstrated in four *M. haemolytica* isolates (PH2, PH8, PH202 and PH278) grown in the presence of serum. This is a significant finding, as leukotoxin is considered to be the major virulence factor in *M. haemolytica* pathogenesis and has not previously been considered to associate with the outer membrane. The LtxA protein of *A. actinomycetemcomitans* can be retained at the surface of this organism in membrane vesicles and by electrostatic association with the

membrane (Lally *et al.*, 1991, Berthold *et al.*, 1992, Ohta *et al.*, 1991, Tsai *et al.*, 1984, Kato *et al.*, 2002). Interestingly though, in the presence of serum, LtxA is released from the bacterial surface via an unknown mechanism (Johansson *et al.*, 2003). It will be worthwhile to establish if *M. haemolytica* leukotoxin attaches to the outer membrane by a similar mechanism as *A. actinomycetemcomitans* and, alternatively, if normally present in the outer membrane *in vivo*, its mechanism of detachment from the outer membrane *in vitro*.

One of the more serendipitous discoveries of this study was that OMPs with putative cleavable passenger domains appear to be stabilised in the presence of Congo red dye. Two OMPs, Iga1_1 and Fhab_1 were identified on 1-D SDS-PAGE gels at their intact molecular mass after growth in the presence of Congo red. However, these proteins were not identified under identical growth conditions in the absence of the dye. Another protein, Iga1_2, was also identified at its expected intact molecular mass after growth in the presence of Congo red. This protein that had been identified at a much lower-than-expected molecular mass after growth under identical growth conditions in the absence of the dye. These findings suggest that Congo red somehow interrupts extracellular passenger domain processing, allowing the proteins to remain intact at the cell surface. This finding is interesting not only from the point of view of this project, but also from a general microbiological perspective. It is feasible that bacterial growth in the presence of Congo red could be used as a general method of preventing proteolytic changes to OMPs at the cell surface, allowing examination of the outer membrane subproteome prior to these changes occurring. It would be interesting in future investigations to determine the mechanism by which the dye achieves these effects.

An investigation of the extracellular subproteomes of the eight representative isolates under iron-replete and iron-restricted growth conditions was performed. This was prompted by findings that suggested some OMPs, including Iga1_2 and Ssa, undergo proteolytic cleavage of their passenger domains which are released from the bacterial surface. Also, the putative haemophore HxuA was only identified in the outer membrane fraction of one isolate (PH8) after growth under iron-restricted conditions, suggesting that it is secreted. One-dimensional SDS-PAGE separation of extracellular protein fractions demonstrated that the

extracellular subproteome is as complex as the outer membrane subproteome. Due to limitations of resources no MS data were obtained to identify these proteins. Future investigations will use MS to identify these proteins and elucidate whether they are proteolytically-cleaved fragments, secreted proteins, or cell envelope proteins that have blebbed away from the bacterial surface, as has been demonstrated for *E. coli* (Xia *et al.*, 2008).

The work presented in this thesis represents the most comprehensive coverage of *M. haemolytica* and *M. glucosida* outer membrane subproteomes to date. The only other previous study of an *M. haemolytica* outer membrane subproteome identified 25 of the confidently predicted OMPs described in the present work in a single bovine serotype A1 isolate (Ayalew *et al.*, 2010). In this thesis, a total of 68 OMPs were identified among several representative *M. haemolytica* isolates and an *M. glucosida* isolate under a range of different growth conditions. Of these 68 OMPs, 63 were confidently predicted by the bioinformatic approach, representing 67.8% of the confidently outer membrane subproteome. Despite the lack of evidence for roles in host-specificity of the identified OMPs, the work presented in this thesis will serve as a primer for further research into the roles of OMPs in the pathobiology of *M. haemolytica*, and has wide implications for the design of vaccines to treat pneumonic pasteurellosis.

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7. APPENDICES

7.1 Growth media composition

Brain Heart Infusion broth (BHIB)

1 litre	
Brain Heart Infusion, dehydrated (Oxoid)	37.0 g
Distilled water	to 1 litre

Autoclaved at 121°C for 15 min.

Brain Heart Infusion agar (BHIA)

1 litre	
Brain Heart Infusion Agar, dehydrated (Oxoid)	47.2 g
Distilled water	to 1 litre

Autoclaved at 121°C for 15 min.

Luria-Bertani (LB) broth

1 litre	
LB broth powder (Sigma-Aldrich)	20.0 g
Distilled water	to 1 litre

Autoclaved at 121°C for 15 min.

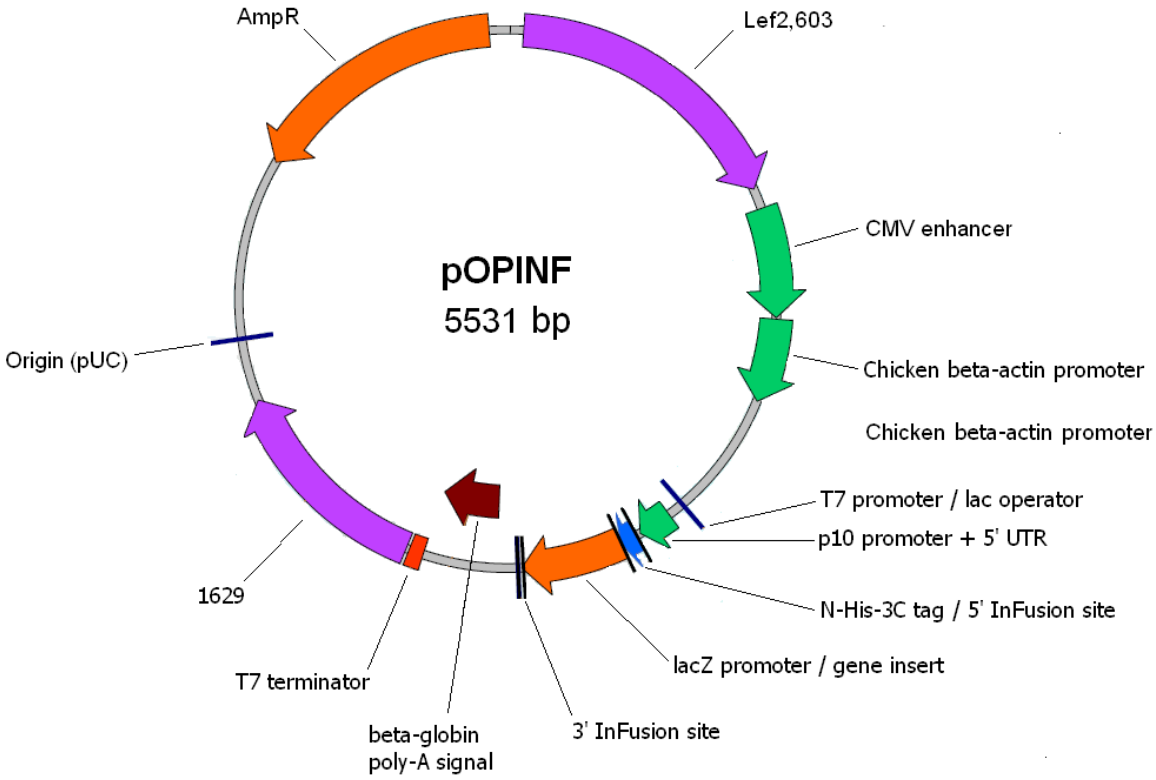
Luria-Bertani (LB) agar

1 litre	
LB agar powder (Sigma-Aldrich)	35.0 g
Distilled water	to 1 litre

Autoclaved at 121°C for 15 min.

7.2 Plasmid DNA

pOPINF (Berrow *et al.*, 2007)



7.3 MemSys screen (Molecular Dimensions, UK)

Tube #	Salt 1	Salt 2	Buffer	pH	Precipitant
1	None	None	0.1 M Na citrate	5.5	2.5 M ammonium sulphate
2	0.1 M sodium chloride	0.1 M lithium sulfate	0.1 M Na citrate	3.5	30% v/v PEG 400
3	0.1 M sodium chloride	0.1 M magnesium chloride	0.1 M Na acetate	4.5	30% v/v PEG 400
4	0.1 M sodium chloride	None	0.1 M Na citrate	5.5	30% v/v PEG 400
5	0.1 M sodium chloride	0.1 M lithium sulfate	0.1 M Na citrate	5.5	30% v/v PEG 400
6	0.1 M sodium chloride	0.1 M magnesium chloride	0.1 M Na citrate	5.5	30% v/v PEG 400
7	None	None	0.1 M MES	6.5	2.5 M ammonium sulphate
8	None	None	0.1 M MES	6.5	30% v/v PEG 400
9	0.1 M sodium chloride	None	0.1 M MES	6.5	30% v/v PEG 400
10	0.1 M sodium chloride	0.1 M lithium sulfate	0.1 M MES	6.5	30% v/v PEG 400
11	0.1 M sodium chloride	0.1 M magnesium chloride	0.1 M MES	6.5	30% v/v PEG 400
12	None	None	0.1 M MOPS	7.0	30% v/v PEG 400
13	None	None	0.1 M Na HEPES	7.5	2.5 M ammonium sulphate
14	0.1 M sodium chloride	None	0.1 M MOPS	7.0	30% v/v PEG 400
15	None	None	0.1 M Na HEPES	7.5	30% v/v PEG 400
16	0.1 M sodium chloride	None	0.1 M Na HEPES	7.5	30% v/v PEG 400
17	0.1 M sodium chloride	0.1 M lithium sulfate	0.1 M Na HEPES	7.5	30% v/v PEG 400
18	0.1 M sodium chloride	0.1 M magnesium chloride	0.1 M Na HEPES	7.5	30% v/v PEG 400
19	None	None	0.1 M Tris	8.5	1.5 M lithium sulphate
20	0.1 M sodium chloride	None	0.1 M Tris	8.5	30% v/v PEG 400
21	0.1 M sodium chloride	0.1 M lithium sulfate	0.1 M Tris	8.5	30% v/v PEG 400
22	0.1 M sodium chloride	0.1 M magnesium chloride	0.1 M Tris	8.5	30% v/v PEG 400
23	0.1 M sodium chloride	0.1 M lithium sulfate	0.1 M CAPSO	9.5	30% v/v PEG 400
24	0.1 M sodium chloride	0.1 M magnesium chloride	0.1 M CAPSO	9.5	30% v/v PEG 400
25	None	None	0.1 M Na citrate	5.5	1.5 M sodium phosphate
26	0.1 M sodium chloride	0.1 M magnesium chloride	0.1 M Na citrate	3.5	12% w/v PEG 4000
27	0.1 M sodium chloride	0.1 M lithium sulfate	0.1 M Na acetate	4.5	12% w/v PEG 4000
28	0.1 M sodium chloride	None	0.1 M Na citrate	5.5	12% w/v PEG 4000
29	0.1 M sodium chloride	0.1 M lithium sulfate	0.1 M Na citrate	5.5	12% w/v PEG 4000
30	0.1 M sodium chloride	0.1 M magnesium chloride	0.1 M Na citrate	5.5	12% w/v PEG 4000
31	None	None	0.1 M MES	6.5	1.5 M sodium phosphate
32	None	None	0.1 M MES	6.5	12% w/v PEG 4000
33	0.1 M sodium chloride	None	0.1 M MES	6.5	12% w/v PEG 4000
34	0.1 M sodium chloride	0.1 M lithium sulfate	0.1 M MES	6.5	12% w/v PEG 4000
35	0.1 M sodium chloride	0.1 M magnesium chloride	0.1 M MES	6.5	12% w/v PEG 4000
36	None	None	0.1 M MOPS	7.0	12% w/v PEG 4000
37	None	None	0.1 M Na HEPES	7.5	1.5 M potassium phosphate
38	0.1 M sodium chloride	None	0.1 M MOPS	7.0	12% w/v PEG 4000
39	None	None	0.1 M Na HEPES	7.5	12% w/v PEG 4000
40	0.1 M sodium chloride	None	0.1 M Na HEPES	7.5	12% w/v PEG 4000
41	0.1 M sodium chloride	0.1 M lithium sulfate	0.1 M Na HEPES	7.5	12% w/v PEG 4000
42	0.1 M sodium chloride	0.1 M magnesium chloride	0.1 M Na HEPES	7.5	12% w/v PEG 4000
43	None	None	0.1 M Tris	8.5	1.5 M potassium phosphate
44	0.1 M sodium chloride	None	0.1 M Tris	8.5	12% w/v PEG 4000
45	0.1 M sodium chloride	0.1 M lithium sulfate	0.1 M Tris	8.5	12% w/v PEG 4000
46	0.1 M sodium chloride	0.1 M magnesium chloride	0.1 M Tris	8.5	12% w/v PEG 4000
47	0.1 M sodium chloride	0.1 M lithium sulfate	0.1 M CAPSO	9.5	12% w/v PEG 4000
48	0.1 M sodium chloride	0.1 M magnesium chloride	0.1 M CAPSO	9.5	12% w/v PEG 4000

Abbreviations:

CAPSO; 3-(Cyclohexylamino)-2-hydroxyl-1-propanesulfonic Acid Sodium Salt, **Na HEPES**; N-(2-hydroxyethyl)-piperazine-N'-2-ethanesulfonic acid sodium salt, **MES**; 2-(N-morpholino)ethanesulfonic acid, **MOPS**; 3-(N-Morpholino)-propanesulfonic acid, **PEG**; Polyethylene glycol, **Tris**; 2-Amino-2-(hydroxymethyl)propane-1,3-diol.

7.4 MemGold screen (Molecular Dimensions, UK)

Tube #	Salt	Buffer	pH	Precipitant
1	None	0.08 M sodium citrate	5.2	2.2 M ammonium sulfate
2	None	0.01 M Tris	8.0	1.2 M tri-sodium citrate
3	None	0.015 M tricine	8.5	24% w/v PEG 4000
4	0.36 M sodium chloride/0.1% w/v sodium azide	0.015 M sodium phosphate	7.0	9.9% w/v PEG 4000
5	0.3 M sodium chloride	0.01 M Tris	8.0	27.5% w/v PEG 4000
6	None	0.225 M MES/bis-tris	6.6	6.6% w/v PEG 6000
7	0.1 M ammonium sulfate	0.1 M HEPES	7.5	12.0% w/v PEG 4000/22% v/v glycerol
8	0.02 M calcium chloride/0.01 M magnesium sulfate/0.02 M sodium chloride	0.02 M MES	6.5	7.7% w/v PEG 1500
9	None	0.05 M HEPES	7.5	2.5 M ammonium sulfate
10	None	0.0665 M HEPES	7.5	1.1 M tri-sodium citrate
11	None	0.15 M potassium phosphate	6.5	3.3 M ammonium sulfate
12	0.1 M magnesium acetate	0.1 M sodium citrate	5.8	14% w/v PEG 5000 MME
13	0.1 M sodium chloride	0.02 M sodium citrate	5.6	11% w/v PEG 3350
14	0.1 M sodium chloride	0.02 M sodium citrate	5.6	5.5% w/v PEG 3350
15	0.05 M calcium chloride/0.05 M barium chloride	0.1 M Tris	8.2	32% v/v PEG 400
16	0.05 M sodium chloride	0.1 M sodium phosphate	6.2	16% w/v PEG 4000
17	0.1 M magnesium chloride	0.03 M Tris-hydrochloride	8.2	19% w/v PEG 4000
18	0.2 M sodium chloride	0.025 M HEPES	7.5	13% w/v PEG 4000
19	None	0.1 M HEPES	7.5	11% w/v PEG 3350
20	0.1 M sodium chloride	0.02 M KMES	6.7	6.6% w/v PEG 4000
21	0.1 M potassium chloride	0.02 M Tris	7.0	20% w/v PEG 4000
22	0.05 M magnesium chloride/0.1% w/v sodium azide	0.1 M sodium cacodylate	6.7	6.6% w/v PEG 3350
23	0.2 M potassium chloride	0.1 M sodium citrate	5.5	37% v/v pentaerythritol propoxylate (5/4 PO/OH)
24	None	0.1 M Tris	8.0	5.5% w/v PEG 4000
25	0.1 M sodium chloride	0.02 M Tris	7.0	7.7% w/v PEG 4000
26	0.1 M magnesium chloride	0.1 M Tris	7.5	22% v/v PEG 400
27	0.04 M sodium chloride	0.04 M Tris	8.0	27% v/v PEG 350 MME
28	0.05 M sodium chloride/0.02 M magnesium chloride	0.1 M sodium citrate	6.0	22% v/v PEG 400
29	None	0.1 M sodium acetate	5.5	8.8% w/v PEG 2000 MME
30	None	0.4 M ammonium acetate	8.0	13% w/v PEG 2000 MME
31	None	0.02 M bis Tris	7.0	15% w/v PEG 2000
32	0.1 M sodium chloride/0.1 M magnesium chloride	0.02 M Tris	7.5	11% w/v PEG 1500
33	0.1 M sodium chloride/0.1 M magnesium chloride	0.1 M HEPES	8.0	11% w/v PEG 1500
34	0.2 M sodium acetate/0.2 M potassium chloride	0.1 M HEPES	7.0	22% w/v PEG 3000
35	0.02 M nickel sulfate	0.01 M HEPES	7.0	33% v/v Jeffamine-M600
36	0.15 M sodium chloride	0.1 M Tris	8.0	13% w/v PEG 6000
37	0.2 M calcium chloride	0.1 M HEPES	7.5	53% v/v PEG 400
38	0.05 M magnesium acetate	0.05 M sodium acetate	5.0	28% v/v PEG 400
39	None	0.05 M HEPES	7.5	22% v/v PEG 4000
40	0.2 M calcium chloride	0.1 M Tris hydrochloride	8.0	44% v/v PEG 400
41	0.05 M magnesium acetate	0.05 M sodium acetate	5.4	24% v/v PEG 400
42	0.2 M calcium chloride	0.1 M MES	6.5	26% v/v PEG 350 MME
43	0.1 M potassium chloride	0.1 M Tris	8.5	39% v/v PEG 400
44	0.05 M magnesium chloride	0.1 M glycine	9.0	22% v/v PEG 400
45	0.1 M ammonium sulfate	0.1 M glycine	3.8	28% w/v tri-ethylene glycol
46	0.15 M sodium formate	0.1 M HEPES	7.2	18% w/v PEG 3350
47	None	0.2 M sodium acetate	6.8	8.8% w/v PEG 6000
48	0.2 M potassium chloride	0.1 M MES	6.5	18% w/v PEG 6000
49	0.22 M sodium citrate	0.1 M Tris	8.0	35% v/v PEG 400
50	None	0.1 M sodium acetate	4.5	17% v/v PEG 400

51	None	0.02 M Tris	8.5	1.0 M lithium sulfate/1.8% w/v PEG 8000
52	None	0.02 M Tris	7.5	22% v/v PEG 550 MME
53	0.05 M sodium chloride	0.02 M glycine	10.0	33% w/v PEG 1000
54	0.2 M magnesium chloride	0.1 M Tris	8.5	25% w/v PEG 4000
55	0.2 M magnesium chloride	0.1 M sodium cacodylate	6.5	31% w/v PEG 2000
56	None	0.64 M sodium acetate	4.6	18% w/v PEG 3350
57	0.1 M sodium chloride/0.1 M cadmium chloride	0.1 M Tris hydrochloride	8.0	33% v/v PEG 400
58	None	0.1 M Bicine	8.9	31% w/v PEG 2000
59	0.05 M sodium sulfate/0.05 M lithium sulfate	0.05 M Tris	8.5	35% v/v PEG 400
60	0.1 M sodium chloride	0.05 M glycine	9.5	33% v/v PEG 300
61	0.3 M magnesium nitrate	0.1 M Tris	8.0	23% w/v PEG 2000
62	0.12 M lithium sulfate	0.02 M Tris/0.1 M sodium citrate	7.5/5.0	20% v/v PEG 300
63	0.1 M sodium chloride	0.12 M Tris	9.4	20% v/v PEG 400
64	0.2 M sodium chloride	0.1 M HEPES	7.0	22% v/v PEG 550 MME
65	0.1 M sodium chloride/0.325 M sodium acetate	0.1 M Tris	8.0	21% v/v PEG 400
66	0.02 M sodium citrate	0.08 M sodium phosphate	6.2	18% w/v PEG 2000
67	0.02 M potassium nitrate	0.03 M potassium citrate	6.5	7.7% w/v PEG 4000
68	0.1 M sodium chloride/0.005 M magnesium chloride	0.1 M Tris	8.5	30% w/v PEG 2000 MME
69	0.2 M calcium chloride	0.1 M HEPES	7.0	33% v/v PEG 400
70	0.1 M calcium chloride	0.1 M Tris	6.5	13% w/v PEG 2000 MME
71	0.2 M ammonium sulfate/0.02 M sodium chloride	0.02 M sodium acetate	4.0	33% v/v PEG 200
72	0.07 M sodium chloride	0.05 M sodium citrate	4.5	22% v/v PEG 400
73	0.2 M ammonium sulfate	0.1 M sodium acetate	4.6	28% v/v PEG 550 MME
74	None	0.05 M glycine	9.0	55% v/v PEG 400
75	0.1 M magnesium chloride/0.1 M sodium chloride	0.1 M Tris	8.5	33% v/v PEG 400
76	0.1 M lithium sulfate/0.05 M disodium hydrogen phosphate	0.05 M citric acid	None	19% w/v PEG 1000
77	0.2 M magnesium chloride/0.1 M potassium chloride	0.025 M sodium citrate	4.0	33% v/v PEG 400
78	0.05 M zinc acetate	0.05 M MES	6.1	11% w/v PEG 8000
79	0.3 M magnesium nitrate	0.1 M Tris	8.0	22% w/v PEG 8000
80	0.1 M sodium chloride/4% v/v ethylene glycol	0.1 M MES	6.5	33% v/v PEG 400
81	0.05 M sodium chloride	0.1 M sodium citrate	5.5	26% v/v PEG 400
82	0.1 M lithium sulfate	0.1 M glycine	9.3	30% v/v PEG 400
83	0.15 M potassium citrate/0.05 M lithium citrate	0.1 M sodium phosphate	–	22% w/v PEG 6000
84	0.001 M zinc sulfate	0.05 HEPES	7.8	28% v/v PEG 600
85	0.1 M sodium chloride	0.1 M sodium phosphate	7.0	33% v/v PEG 300
86	0.1 M sodium chloride	0.05 M Bicine	9.0	33% v/v PEG 300
87	0.05 M zinc acetate/6% v/v ethylene glycol	0.1 M sodium cacodylate	6.0	6.6% w/v PEG 8000
88	0.2 M lithium sulfate	0.1 M sodium citrate	3.5	28% v/v PEG 400
89	0.1 M sodium chloride	0.1 M Tris	7.5	11% w/v PEG 4000
90	0.05 M lithium sulfate	0.1 M tricine	7.4	7% w/v PEG 3000
91	0.2 M calcium chloride	0.1 M MES	6.5	33% v/v PEG 400
92	1 M sodium chloride	0.1 M sodium citrate	6.0	28% w/v PEG 4000
93	None	0.1 M HEPES	7.5	11% w/v PEG 4000
94	0.002 M zinc sulfate	0.08 M HEPES	7.0	25% v/v Jeffamine ED2001
95	0.001 M cadmium chloride/0.03 M magnesium chloride	0.1 M MES	6.5	30% v/v PEG 400
96	None	0.1 M bis-tris-propane	7.0	3.0 M sodium chloride

Abbreviations:

ADA; N-(2-Acetamido)iminodiacetic Acid, **Bicine**; N,N-Bis(2-hydroxyethyl)glycine, **CHES**; 2-(N-Cyclohexylamino)ethane sulfonic Acid, **HEPES**; N-(2-hydroxyethyl)-piperazine-N'-2-ethanesulfonic acid, **KMES**; 2-(N-morpholino)ethanesulfonic acid potassium salt, **MES**; 2-(N-morpholino)ethanesulfonic acid, **MME**; Monomethylether, **PEG**; Polyethylene glycol, **Tricine**; N-[Tris(hydroxymethyl)methyl]glycine, **Tris**; 2-Amino-2-(hydroxymethyl)propane-1,3-diol,**Tris HCl**; 2-Amino-2-(hydroxymethyl)propane-1,3-diol, hydrochloride]

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Outer Membrane Protein A of Bovine and Ovine Isolates of *Mannheimia haemolytica* Is Surface Exposed and Contains Host Species-Specific Epitopes[▽]

Jonathan D. A. Hounsome,[1] Susan Baillie,[1] Mojtaba Noofeli,[1] Alan Riboldi-Tunnicliffe,[2] Richard J. S. Burchmore,[1] Neil W. Isaacs,[2] and Robert L. Davies[1]*

Institute of Infection, Immunity and Inflammation, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, United Kingdom,[1] and School of Chemistry, College of Science and Engineering, University of Glasgow, Glasgow, United Kingdom[2]

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***Mannheimia haemolytica* is the etiological agent of pneumonic pasteurellosis of cattle and sheep; two different OmpA subclasses, OmpA1 and OmpA2, are associated with bovine and ovine isolates, respectively. These proteins differ at the distal ends of four external loops, are involved in adherence, and are likely to play important roles in host adaptation. *M. haemolytica* is surrounded by a polysaccharide capsule, and the degree of OmpA surface exposure is unknown. To investigate surface exposure and immune specificity of OmpA among bovine and ovine *M. haemolytica* isolates, recombinant proteins representing the transmembrane domain of OmpA from a bovine serotype A1 isolate (rOmpA1) and an ovine serotype A2 isolate (rOmpA2) were overexpressed, purified, and used to generate anti-rOmpA1 and anti-rOmpA2 antibodies, respectively. Immunogold electron microscopy and immunofluorescence techniques demonstrated that OmpA1 and OmpA2 are surface exposed, and are not masked by the polysaccharide capsule, in a selection of *M. haemolytica* isolates of various serotypes and grown under different growth conditions. To explore epitope specificity, anti-rOmpA1 and anti-rOmpA2 antibodies were cross-absorbed with the heterologous isolate to remove cross-reacting antibodies. These cross-absorbed antibodies were highly specific and recognized only the OmpA protein of the homologous isolate in Western blot assays. A wider examination of the binding specificities of these antibodies for *M. haemolytica* isolates representing different OmpA subclasses revealed that cross-absorbed anti-rOmpA1 antibodies recognized OmpA1-type proteins but not OmpA2-type proteins; conversely, cross-absorbed anti-rOmpA2 antibodies recognized OmpA2-type proteins but not OmpA1-type proteins. Our results demonstrate that OmpA1 and OmpA2 are surface exposed and could potentially bind to different receptors in cattle and sheep.**

The Gram-negative bacterium *Mannheimia haemolytica* is a commensal of cattle, sheep, and other ruminants but also causes bovine and ovine pneumonic pasteurellosis; these infections are responsible for considerable economic losses to the livestock industries (33, 35). Twelve different capsular serotypes of *M. haemolytica* have been identified to date, but A1 and A2 are the most prevalent (37), and strains of these serotypes are responsible for the majority of pneumonia cases worldwide in cattle and sheep, respectively. *M. haemolytica* consists of genetically distinct subpopulations that are differentially adapted to, and elicit disease in, either cattle or sheep (20, 21). The molecular basis of host adaptation and host specificity in *M. haemolytica* is not understood, but it is likely that outer membrane proteins (OMPs) play important roles in these processes. The publication of the genome sequence of a bovine serotype A1 *M. haemolytica* isolate (36) and, more recently, of the genome sequences of bovine and ovine serotype A2 isolates (45) have revealed the presence of genes that encode various OMPs. Many of these proteins serve as adhesins that are involved in host receptor-specific binding (19) or as iron transport proteins (69).

There is growing evidence to suggest that the OmpA protein of *M. haemolytica* functions as an adhesin (41, 48). OmpA is a highly conserved, integral, outer membrane protein of Gram-negative bacteria that has been implicated in a diverse range of functions in different species (reviewed in reference 72). It comprises an N-terminal transmembrane β-barrel domain embedded in the outer membrane and a C-terminal globular domain which extends into the periplasm to interact with the underlying peptidoglycan (28). The N-terminal domain consists of eight membrane-traversing antiparallel β-sheets and four relatively long, mobile, hydrophilic external loops (62). In previous studies, molecular mass heterogeneity of OmpA was observed among bovine and ovine *M. haemolytica* isolates that correlated with the host of origin (21). Subsequently, comparative nucleotide sequence analysis of the *ompA* gene from 31 *M. haemolytica* isolates revealed the presence of hypervariable domains within the four surface-exposed loops (22). The amino acid sequences of these domains are very different in bovine and ovine isolates but are highly conserved among isolates recovered from the same host species (22). The *ompA* gene can be categorized into four distinct allelic classes, I to IV. The class I (*ompA1*) alleles are associated almost exclu-

* Corresponding author. Mailing address: Institute of Infection, Immunity and Inflammation, College of Medical, Veterinary and Life Sciences, Sir Graeme Davies Building, 120 University Place, Glasgow G12 8TA, United Kingdom. Phone: 44 141 330 6685. Fax: ●●●●. E-mail: robert.davies@glasgow.ac.uk.

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TABLE 1. Properties of bovine and ovine *M. haemolytica* isolates

Isolate	ET[a]	Serotype	Host species	Clinical status	Site of origin	*ompA* allele	Immunogold labeling		Immunofluorescence	
							Anti-rOmpA1	Anti-rOmpA2	Anti-rOmpA1	Anti-rOmpA2
PH2	1	A1	Bovine	Pneumonia	Lung	*ompA1.1*	+	−	+	−
PH30	1	A1	Bovine	Healthy	Nasopharynx	*ompA1.1*	+	−	+	+/−
PH376	1	A6	Bovine	Pneumonia	Lung	*ompA1.1*	+	−	+	−
PH540	2	A1	Bovine		Nasopharynx	*ompA1.2*	+	−	+	−
PH202	21	A2	Bovine	Healthy	Nasopharynx	*ompA1.3*	+	−	+	+/−
PH470	21	A2	Bovine	Pneumonia	Lung	*ompA1.3*	+	−	+	−
PH494	16	A2	Ovine	Pneumonia	Lung	*ompA1.4*	+	−	+	−
PH550	17	A2	Bovine	Healthy	Nasopharynx	*ompA1.5*	+	−	+	+/−
PH8	6	A1	Ovine	Pneumonia	Lung	*ompA2.1*	−	+	+/−	+
PH284	8	A6	Ovine	Pneumonia	Lung	*ompA2.1*	−	+	+/−	+
PH66	10	A14	Ovine		Lung	*ompA2.1*	−	+	+/−	+
PH56	5	A8	Ovine	Pneumonia	Lung	*ompA2.2*	−	+	+/−	+
PH278	21	A2	Ovine	Pneumonia	Lung	*ompA2.3*	−	+	−	+
PH292	22	A2	Ovine	Pneumonia	Lung	*ompA2.3*	−	+	−	+
PH196	18	A2	Bovine	Healthy	Nasopharynx	*ompA3.1*	−	+/−	−	+/−
PH296	12	A7	Ovine	Pneumonia	Lung	*ompA4.1*	−	+/−	−	+/−
PH484	14	A7	Ovine	Pneumonia	Lung	*ompA4.1*	−	+/−	−	+/−
PH588	15	A13	Ovine	Pneumonia	Lung	*ompA4.2*	−	+/−	−	+/−

[a] ET, electrophoretic type (16).

sively with bovine *M. haemolytica* isolates, whereas the class II to IV (*ompA2* to *ompA4*) alleles occur only in ovine *M. haemolytica* isolates (22). Significantly, the *ompA1*- and *ompA2*-type alleles are associated with the major pathogenic lineages of bovine (electrophoretic type [ET] 1) and ovine (ETs 21 and 22) isolates, respectively (16). Based on the distribution of the OmpA1 and OmpA2 proteins among bovine and ovine isolates, respectively, and the localization of the amino acid variation to the tips of the four loops, it was hypothesized that OmpA acts as a ligand, plays an important role in adherence, and is involved in host specificity (22). Subsequently, it was demonstrated that OmpA is involved in the binding of serotype A1 *M. haemolytica* to bovine bronchial epithelial cells (41) and that fibronectin is a potential host receptor molecule in cattle (48).

The cell envelope of *M. haemolytica* is surrounded by a layer of capsular polysaccharide (CPS) (1, 47) which has been implicated in a number of functions, including the adherence of the bacterium to alveolar surfaces (10, 79), inhibition of complement-mediated serum killing (11), and inhibition of the phagocytic and bactericidal activities of neutrophils (17, 77). Visibly thicker capsules have been observed in *M. haemolytica* during *in vitro* early-log-phase growth than during stationary-phase growth in both capsular serotype A1 (16) and A2 (73) isolates. Crucially, polysaccharide capsules have been shown to inhibit outer membrane adhesin function in a range of capsular types in different bacterial species (32, 70, 71, 76). Indeed, an acapsular serotype A1 *M. haemolytica* mutant was shown to have greater fibronectin-binding activity than that of the capsular parental strain, suggesting a shielding role of the capsule. In other species, CPS may be downregulated upon contact with host cells (2, 15, 26) or as a consequence of phase-variable expression (4, 29, 43), thus allowing transient exposure of outer membrane adhesins. The shielding of OMPs, including OmpA, by CPS is likely to have important implications for the function of surface proteins but has yet to be investigated in *M. haemolytica*.

The objectives of this study were 2-fold. First, we wished to determine if the *M. haemolytcia* OmpA protein is surface exposed or whether it is masked by the polysaccharide capsule under various growth conditions. Second, we wanted to demonstrate whether the bovine OmpA1 and ovine OmpA2 proteins are antigenically distinct, i.e., whether antibodies raised against each of these proteins interact in a strain-specific manner. This would provide clues as to whether these proteins are likely to interact with host cell receptors in a host-specific manner. To achieve these aims, recombinant OmpA1 (rOmpA1) and OmpA2 (rOmpA2) proteins were overexpressed, purified, and used to generate anti-OmpA1 and anti-OmpA2 antibodies, respectively. These antibodies were used to explore OmpA surface exposure and epitope specificity using electron microscopy and immunofluorescence techniques.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *M. haemolytica* isolates used in this study are shown in Table 1. *Escherichia coli* strains DH5α and Rosetta 2(DE3)pLysS were obtained from Invitrogen and Novagen, respectively. The *M. haemolytica* isolates were stored at −80°C in 50% (vol/vol) glycerol in brain heart infusion (BHI) broth and were subcultured on BHI agar containing 5% (vol/vol) defibrinated sheep's blood (blood agar) overnight at 37°C. Liquid starter cultures were prepared by inoculating a few colonies into 10-ml volumes of BHI broth and incubating them overnight at 37°C with shaking at 120 rpm. *E. coli* strain DH5α was grown in Luria-Bertani (LB) broth containing 54 μg/ml carbenicillin. *E. coli* Rosetta 2(DE3)pLysS was grown in LB broth containing 54 μg/ml carbenicillin and 34 μg/ml chloramphenicol. Both *E. coli* strains were grown at 37°C with shaking at 120 rpm.

Capsule staining. The polysaccharide capsule of *M. haemolytica* was demonstrated using the Maneval method (16).

Preparation of chromosomal DNAs. Bacterial cells from 1 ml of overnight culture were harvested by centrifugation for 1 min at 13,000 × *g* and washed once with 1 ml of sterile distilled H_2O (dH_2O). DNAs were prepared using an InstaGene Matrix kit (Bio-Rad) according to the manufacturer's instructions and were stored at −20°C.

PCR amplification and plasmid construction. The *M. haemolytica ompA* gene fragments corresponding to the transmembrane domain of OmpA1 (amino acid positions 19 to 217) and OmpA2 (amino acid positions 19 to 211) were amplified by PCR from total genomic DNA from *M. haemolytica* isolates PH2 and PH278, respectively, using the following forward and reverse primers: 5′-AAGTTCTG

TTTCAGGGCCCGCAAGCTAACACTTTCTACGCAGG-3′ and 5′-ATGGT CTAGAAAGCTTTAACCTTGACCGAAACGGTATG-3′. PCR products containing the *ompA1* or *ompA2* gene fragments were amplified from chromosomal DNA in 50-μl reaction mixtures using Platinum Pfx DNA polymerase (Invitrogen) with 2 mM $MgSO_4$ and 50 pmol of each forward and reverse primer. PCRs were carried out in a GeneAmp PCR System 9700 (Applied Biosystems) thermal cycler using the following amplification parameters: initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 15 s, annealing at 65°C for 30 s, and extension at 68°C for 1 min 30 s. The production of PCR amplicons of the expected size was confirmed by electrophoresis in a 1% (wt/vol) agarose gel and visualization with SybrSafe (Invitrogen). The PCR amplicons were purified with a QIAquick PCR purification kit (Qiagen) and eluted in 30 μl of sterile distilled H_2O. Yields were assessed by agarose gel electrophoresis, and the DNA was stored at −20°C. The PCR products were cloned into separate pOPINF vectors (Oxford Protein Production Facility) (6) according to the manufacturer's instructions. The resulting plasmids, designated *ompA1*/His-pOPINF and *ompA2*/His-pOPINF, were verified by DNA sequencing. Five microliters of *ompA1*/His-pOPINF or *ompA2*/His-pOPINF was added to a tube containing 50 μl *E. coli* DH5α cells (as supplied by the manufacturer) and incubated on ice for 30 min. The mixtures were heated in a water bath at 42°C for 30 s and returned to ice for 2 min. Four hundred fifty microliters of GS96 broth (QbioGene) was added to each tube, and the contents were mixed thoroughly and incubated at 37°C for 1 h. Transformant colonies were selected after plating onto LB agar containing 54 μg/ml carbenicillin and incubation overnight at 37°C. An individual colony was used to inoculate 50 ml of LB containing 54 μg/ml carbenicillin and grown overnight at 37°C with shaking at 170 rpm. Five milliliters of the overnight culture was centrifuged at 5,000 × *g* for 15 min at 4°C to pellet the cells. Plasmids were recovered using a QIAprep Spin Miniprep kit (Qiagen) according to the manufacturer's instructions and eluted in 50 μl of sterile distilled H_2O. Plasmid size was confirmed by electrophoresis on a 1% (wt/vol) agarose gel and visualization with SybrSafe (Invitrogen). The DNA was stored at −20°C.

Expression of rOmpA. Two microliters of *ompA1*/His-pOPINF or *ompA2*/His-pOPINF plasmids recovered from *E. coli* DH5α transformant cultures was added to a tube containing 100 μl *E. coli* Rosetta 2(DE3)pLysS cells (Novagen) as supplied by the manufacturer and incubated on ice for 30 min. The bacterial cells were heated in a water bath at 42°C for 30 s and returned to ice for 2 min. Four hundred fifty microliters of GS96 broth was added to each tube, and the contents were mixed thoroughly and incubated at 37°C for 1 h. Transformant colonies were selected after plating onto LB agar containing 54 μg/ml carbenicillin and 34 μg/ml chloramphenicol and incubation overnight at 37°C. An individual colony was used to inoculate 15 ml LB and grown overnight at 37°C with shaking at 120 rpm. One hundred fifty microliters of overnight culture was inoculated into 30 ml LB broth containing 54 μg/ml carbenicillin and 34 μg/ml chloramphenicol and incubated at 37°C with shaking at 170 rpm until an optical density at 600 nm (OD_{600}) of 0.7 to 1.0 was achieved. Thirty microliters of 1 M isopropyl-β-D-thiogalactopyranoside (IPTG) was added, and the culture was incubated at 25°C with shaking at 170 rpm for a further 18 h. One and one-half milliliters of overnight culture was centrifuged for 1 min at 13,000 × *g*, and the pellet was resuspended in 100 μl of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. The sample was heated at 100°C for 5 min and analyzed by SDS-PAGE to check for the presence of recombinant protein. A second colony from the *E. coli* Rosetta 2(DE3)pLysS transformant plate was used to inoculate 15 ml LB broth and grown overnight at 37°C with shaking at 120 rpm. Two milliliters of overnight culture was used to inoculate each of eight 2-liter Erlenmeyer flasks containing 1 liter of LB broth supplemented with 54 μg/ml carbenicillin and 34 μg/ml chloramphenicol and incubated at 37°C with shaking at 170 rpm until an OD_{600} of 0.7 to 1.0 was achieved. One milliliter of 1 M IPTG was added, and the culture was incubated at 25°C with shaking at 170 rpm for a further 18 h.

Purification of recombinant OmpA proteins (rOmpA1 and rOmpA2). The protein purification protocol of Zhu et al. (82) was followed, with modifications. Bacterial cells from each 1 liter of culture were harvested by centrifugation at 10,000 × *g* for 20 min at 4°C, resuspended in 25 ml of buffer A (25 mM Tris, 200 mM NaCl [pH 8.0]), and stored at −20°C. The cells from 2 liters of culture medium were disrupted by sonication (six cycles of 30 s on and 30 s off), and the lysates were centrifuged at 4,700 × *g* for 30 min at 4°C to pellet the inclusion bodies. These were resuspended in 1% Triton X-100 in buffer A and centrifuged at 4,700 × *g* for 30 min at 4°C. The inclusion bodies were solubilized overnight at 4°C in 50 ml of 6 M urea. The solubilized protein was added dropwise to 500 ml of 50 mM HEPES, 300 mM NaCl, 3% *N,N*-dimethyldodecylamine-*N*-oxide (LDAO), 5 mM dithiothreitol (DTT) (pH 8.0) at 4°C; stirred slowly at 4°C for 72 h; and dialyzed against buffer B (50 mM Tris, 300 mM NaCl, 0.1% LDAO [pH 7.6]). The protein solution was loaded onto a 5-ml HiTrap (GE Healthcare) column equilibrated with buffer B. Bound recombinant protein was eluted from the column using an imidazole gradient (0 to 300 mM imidazole in buffer B) and collected in 5-ml fractions. Protein concentrations were quantified using a Perkin-Elmer Lambda 40 UV/VIS spectrophotometer. Ten milligrams of eluted protein was loaded onto a Superdex G-200 gel filtration column (Pharmacia Biotech) equilibrated with buffer B and collected in 5-ml fractions after running at 0.25 ml/min overnight. Concentrations of rOmpA in the collected fractions were determined by the modified Lowry procedure (52).

Circular dichroism spectroscopy. Circular dichroism spectroscopy (40) was performed to examine the secondary structure of refolded PH2 rOmpA1 and PH278 rOmpA2. Mean residue ellipticity was measured between 190 and 240 nm on a Jasco J-810 spectropolarimeter (Jasco, United Kingdom). Percentage estimates of secondary structure content (α-helix, β-strands, turn, unordered) were calculated using the modified CONTINLL algorithm (66, 75) provided by the online server DICHROWEB (49, 80).

Anti-OmpA antibody preparation. Purified rOmpA1 and rOmpA2 were sent to Eurogentec (Belgium) and used to raise antibodies in rabbits using their 87-day classic polyclonal antibody protocol. This protocol involves the injection of 0.2 mg of protein into each of two rabbits at days 0, 14, 28, and 56. Serum bleeds were obtained before the first injection (preimmune) and 38, 66, and 87 (final bleed) days after the initial injection.

Serum cross-absorption. Two hundred fifty milliliters of overnight cultures of PH2 and PH278 was centrifuged at 10,000 × *g* for 20 min at 4°C, and the cells were washed three times in 50 ml phosphate-buffered saline (PBS). The bacterial cells were resuspended in 5 ml PBS, and 1 ml was aliquoted into each of five microcentrifuge tubes. Each microcentrifuge tube was centrifuged (13,000 × *g*, 3 min) to pellet the cells, and the supernatants were removed. Cells from one tube were resuspended in 1 ml of heterologous final bleed serum and incubated at 37°C for 30 min on an orbital shaker. The cells were pelleted by centrifugation, and the supernatant was used to resuspend the cells in a second microcentrifuge tube. These were incubated at 37°C for 30 min on an orbital shaker. This process was repeated for the remaining three tubes. The cross-absorbed sera were diluted 1:5 with distilled water (dH_2O), filter sterilized, and stored at −80°C.

Isolation of OMPs. The OMPs of *M. haemolytica* isolates PH2 and PH278 were prepared as previously described (24).

SDS-PAGE. OMPs were separated by SDS-PAGE in a 12% polyacrylamide gel as previously described (24).

Western blotting. Briefly, Western blotting was performed essentially as described by Davies et al. (23). SDS-PAGE gels were equilibrated in transfer buffer (25 mM Tris, 192 mM glycine [pH 8.3]) for 30 min, and the proteins were transferred to nitrocellulose membranes overnight at 30 V. The nitrocellulose membranes were washed twice in TBS (20 mM Tris, 50 mM NaCl [pH 7.5]) for 5 min, blocked with 3% gelatin in TBS for 1 h, and washed twice in TTBS (0.05% Tween 20 in TBS) for 5 min each. The membranes were incubated with final bleed anti-rOmpA antibody diluted 1:100 in antibody buffer (1% gelatin in TTBS) for 1 h at room temperature and washed twice in TTBS for 5 min each. This was followed by incubation with secondary horseradish peroxidase-conjugated anti-rabbit antibody diluted 1:1,000 in antibody buffer for 1 h at room temperature, two 5-min washes in TTBS, and one 5-min wash in TBS. The membranes were developed in a substrate solution containing 0.05% (wt/vol) 4-chloro-naphthol (dissolved in 20 ml of ice-cold methanol) and 0.05% (vol/vol) hydrogen peroxide in 100 ml of TBS. Development was stopped by washing the membranes in distilled H_2O.

Immunogold electron microscopy. Bacterial cells from 25-ml volumes of 6- or 8-h BHI broth cultures, or 6-h BHI broth cultures containing 100 μM 2,2-dipyridyl, were harvested by centrifugation at 3,500 × *g* for 5 min and washed once with PBS. Alternatively, overnight cultures of bacteria were scraped off blood agar plates and washed once in PBS. The washed cells were resuspended in PBS to an OD_{600} of 0.4 equivalent to 1.0×10^8 CFU/ml. One milliliter of this suspension was centrifuged at 13,000 × *g* for 1 min, and the pellet was resuspended in 1 ml 4% paraformaldehyde in PBS (pH 7.2) for 30 min at room temperature. The cells were washed twice in 50 mM glycine in PBS and resuspended in 0.5 ml of 0.2% bovine serum albumin (BSA) in PBS for 30 min. The cells were centrifuged and resuspended in 100 μl primary anti-rOmpA antibody (1:10 dilution) in 0.2% BSA in PBS, incubated for 1 h, washed three times in 0.2% BSA in PBS, and finally resuspended in 200 μl of 0.2% BSA in PBS. Twenty microliters of this suspension was dropped onto a freshly prepared poly-L-lysine-coated Formvar carbon-coated nickel grid and allowed to stand for 15 min, and the excess fluid was removed. The grids were floated facedown on 50 μl of secondary goat anti-rabbit IgG antibody conjugated to 10-nm gold particles (1:20 dilution) in 0.2% BSA in PBS and incubated for 1 h at room temperature. The grids were washed once in 0.2% BSA in PBS, three times in PBS (1 min each), and once in 1% glutaraldehyde in PBS (pH 7.4) for 5 min. The grids were

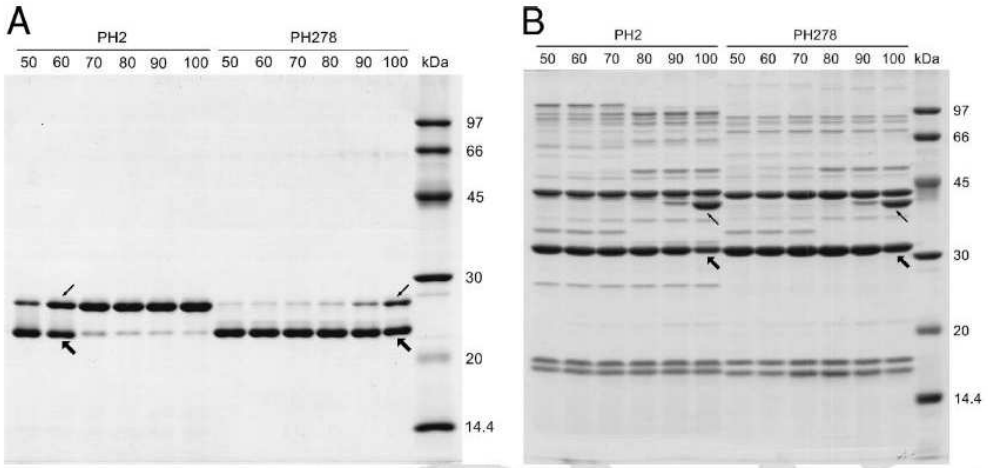


FIG. 1. Heat modifiability of recombinant and full-length OmpA proteins of *M. haemolytica* isolates PH2 and PH278. Panels A and B represent purified recombinant proteins rOmpA1 (PH2) and rOmpA2 (PH278) and Sarkosyl-extracted OMPs of bovine isolate PH2 (OmpA1) and ovine isolate PH278 (OmpA2), respectively. Samples were heated for 5 min at 50, 60, 70, 80, 90, and 100°C prior to separation by SDS-PAGE. Unmodified low-molecular-mass and heat-modified high-molecular-mass forms of rOmpA (A) and full-length OmpA (B) of isolates PH2 and PH278 are indicated by thick and thin arrows, respectively. rOmpA1 (PH2) and rOmpA2 (PH278) underwent heat modification at 70°C and 100°C, respectively, whereas full-length OmpA of PH2 and PH278 underwent heat modification at 100°C.

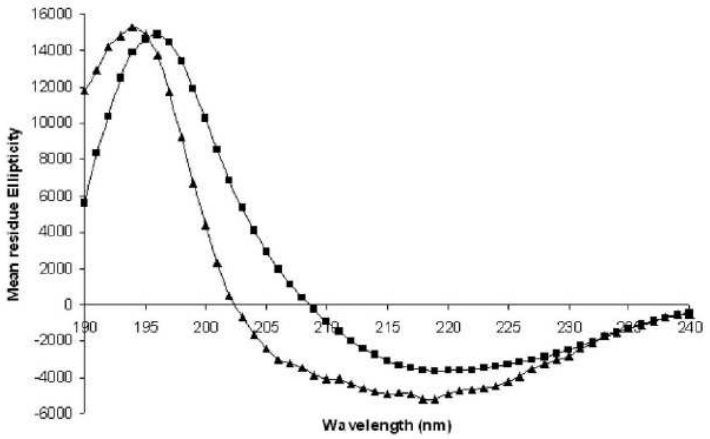
finally washed three times with filtered deionized water (1 min each) and allowed to air dry, and the bacteria were visualized using a Zeiss 912 AB energy-filtering transmission electron microscope operating under standard conditions at 80 kV.

Immunofluorescence staining. Bacteria were grown overnight on blood agar, resuspended in 5 ml PBS, and adjusted to an OD_{600} of 0.5. The bacterial cells were mixed 1:1 with 8% paraformaldehyde in PBS (2×) for 10 min. Five microliters of bacterial suspension was added to each well of a multiwell microscope slide (Hendley-Essex, United Kingdom), allowed to air dry, and incubated with 20 μl of primary anti-rOmpA antibody (1:100 dilution) in 0.1% BSA in PBS for 60 min at room temperature. The slides were washed three times in 0.1% BSA in PBS and incubated with 20 μl of Alexa Fluor 488 (Invitrogen) goat anti-rabbit IgG antibody (15 μg/ml in 0.1% BSA in PBS) for 30 min. The slides were washed three times in 0.1% BSA in PBS and allowed to air dry. One or two drops of fluorescence mounting medium (Dako, Sweden) were added to each slide, covered with a coverslip, and sealed with clear nail varnish. Bacteria were visualized with a Zeiss Axioskop fluorescence microscope.

RESULTS

Recombinant OmpA proteins are comprised predominantly of β-sheets and exhibit heat modifiability after heat treatment in the presence of SDS. Heat modifiability is a characteristic property of the OmpA family of proteins (5, 58, 68). The native form of the complete OmpA protein unfolds in the presence of SDS only after heat treatment at 100°C, which exposes additional SDS-binding sites (30, 59). When analyzed by SDS-PAGE, OmpA migrates through the gel according to its structural compactness, with the native protein migrating faster than the fully denatured polypeptide (30, 42). Recombinant OmpA proteins from the *M. haemolytica* bovine isolate PH2 (rOmpA1) and ovine isolate PH278 (rOmpA2) were successfully expressed, purified, and refolded after cloning the *ompA* gene fragments encoding the OmpA transmembrane domains into an *E. coli* protein expression strain. Mass spectrometric analysis of gel-extracted protein bands confirmed that native and heat-modified proteins (described below) represented *M. haemolytica* OmpA (results not shown). The rOmpA1 and rOmpA2 proteins were heated at a range of temperatures between 50 and 100°C for 5 min prior to SDS-PAGE, and both proteins exhibited heat modifiability when analyzed by SDS-PAGE, although this occurred at different temperatures (Fig. 1A). The native and heat-modified proteins migrated at approximately 24 and 22.5 kDa, respectively; however, PH2 rOmpA1 unfolded at 70°C whereas PH278 rOmpA2 unfolded at 100°C (Fig. 1A, arrows). In contrast, the native and heat-modified forms of the complete OmpA protein recovered from the outer membrane of *M. haemolytica* isolates PH2 and PH278 migrated at approximately 32 and 38 kDa, respectively, on SDS-polyacrylamide gels (Fig. 1B). In this case, the full-length OmpA protein of both isolates underwent heat modification at 100°C (Fig. 1B, arrows). The finding that rOmpA1 unfolds more readily at a lower temperature than does rOmpA2 indicates a less stable structure for rOmpA1. Circular dichroism spectroscopy analysis predicted that the secondary structures of both recombinant proteins comprised predominantly β-sheets and turns, indicating that they have folded into a β-barrel conformation similar to that of native *M. haemolytica* OmpA (Fig. 2). However, PH2 rOmpA1 has a lower β-sheet composition than does PH278 rOmpA2 (33.6% and 40.6%, respectively), which is consistent with the heat modifiability results indicating a less stable structure for PH2 rOmpA1.

Anti-rOmpA1 and anti-rOmpA2 antibodies exhibit immune specificity for the homologous proteins. The binding specificities of the anti-rOmpA1 and anti-rOmpA2 antibodies to Sarkosyl-extracted OMPs and rOmpA from the bovine isolate PH2 and the ovine isolate PH278 were assessed by Western blotting (Fig. 3). Unabsorbed anti-rOmpA1 antibodies bound to the full-length OmpA and rOmpA proteins of both PH2 (Fig. 3A, panel ii) and PH278 (Fig. 3B, panel ii). Similarly, unabsorbed anti-rOmpA2 antibodies bound to the full-length



Protein	α-helix	β-strand	Turn	Unordered	Total
rOmpA1	0.109	0.336	0.228	0.327	1
rOmpA2	0.08	0.406	0.213	0.301	1

FIG. 2. Circular dichroism spectroscopy analysis of purified rOmpA proteins. Mean residue ellipticity was measured between 190 and 240 nm for PH2 rOmpA1 (▲) and PH278 rOmpA2 (■). Percentage estimates of secondary structure content (α-helix, β-strands, turn, unordered) for each recombinant protein were calculated using the modified CONTINLL algorithm (66, 75) provided by the online server DICHROWEB (49, 80).

OmpA and rOmpA proteins of both PH2 (Fig. 3A, panel iii) and PH278 (Fig. 3B, panel iii). Significantly, no other *M. haemolytica* proteins were recognized by either antibody in the complete Sarkosyl-extracted outer membrane protein samples. After cross-absorption with the heterologous isolate PH278, anti-rOmpA1 antibodies bound to PH2 OmpA and rOmpA1 (Fig. 3A, panel iv) but not PH278 OmpA and rOmpA2 (Fig. 3B, panel iv). Similarly, anti-rOmpA2 antibodies cross-absorbed with the heterologous isolate PH2 bound to PH278 OmpA and rOmpA2 (Fig. 3B, panel v) but not PH2 OmpA and rOmpA1 (Fig. 3A, panel v). However, the intensity of staining with the cross-absorbed antisera was noticeably weaker than that for the unabsorbed antisera. These results indicate that the cross-absorbed anti-rOmpA1 and -rOmpA2 antibodies bind specifically to OmpA1 and OmpA2, respectively.

***M. haemolytica* OmpA is surface exposed and recognized by anti-rOmpA antibodies *in vitro* in a strain-specific manner.** The ability of anti-rOmpA antibodies to recognize OmpA on the surface of *M. haemolytica* was assessed by immunogold labeling (Fig. 4) and immunofluorescent staining (Fig. 5). Immunogold labeling demonstrated that the OmpA proteins of *M. haemolytica* isolates PH2 and PH278 are both recognized by unabsorbed anti-rOmpA1 (Fig. 4A and B, panel i) and anti-rOmpA2 (Fig. 4A and B, panel ii) antibodies. However, cross-absorbed anti-rOmpA1 antibodies recognized OmpA of isolate PH2 (Fig. 4A, panel iii) but not of isolate PH278 (Fig. 4B, panel iii); conversely, cross-absorbed anti-rOmpA2 antibodies recognized OmpA of isolate PH278 (Fig. 4B, panel iv) but not of isolate PH2 (Fig. 4A, panel iv). Antibody binding was evenly distributed over the surface of the bacterial cells and was not concentrated in any particular area. In addition, the density of labeling of isolate PH278 by the cross-absorbed anti-rOmpA2 antibody (Fig. 4B, panel iv) was greater than that of PH2 by the cross-absorbed anti-rOmpA1 antibody (Fig. 4A, panel iii), suggesting greater exposure of the OmpA2 protein in isolate PH278. Immunofluorescent staining confirmed that cross-absorbed anti-rOmpA1 antibodies recognized OmpA in isolate PH2 (Fig. 5A, panel i) but not in isolate PH278 (Fig. 5B, panel i) and that cross-absorbed anti-rOmpA2 antibodies recognized OmpA in isolate PH278 (Fig. 5B, panel ii) but not in isolate PH2 (Fig. 5A, panel ii).

OmpA is surface exposed at different stages of the growth cycle and after growth in different media. Isolates PH2 and PH278 were grown for 6 h (logarithmic phase) and 18 h (stationary phase) in BHI broth, and cells were incubated with cross-absorbed anti-rOmpA1 or anti-rOmpA2 antibodies. No differences were observed in the degree of antibody binding between bacteria grown for 6 h and those grown for 18 h (results not shown). These results demonstrated that there was no appreciable difference in OmpA surface exposure between logarithmic- and stationary-phase cells. Isolates PH2 and PH278 were also grown for 18 h on blood agar or in iron-restricted (100 μM dipyridyl) BHI broth, and cells were incubated with cross-absorbed anti-rOmpA1 or anti-rOmpA2 antibodies. Again, no differences were observed in antibody binding between bacteria grown in iron-restricted BHI broth or on blood agar plates and bacteria grown in BHI broth (results not shown). We also examined the capsulation of bacteria grown under these various conditions by Maneval staining (results not shown); there were no major differences between isolates grown under different conditions with the exception that 18-h stationary-phase cultures contained cells that were less consistent in overall size and shape. Taken together, our results demonstrated that OmpA is not masked by the polysaccharide capsule and that the degree of surface exposure

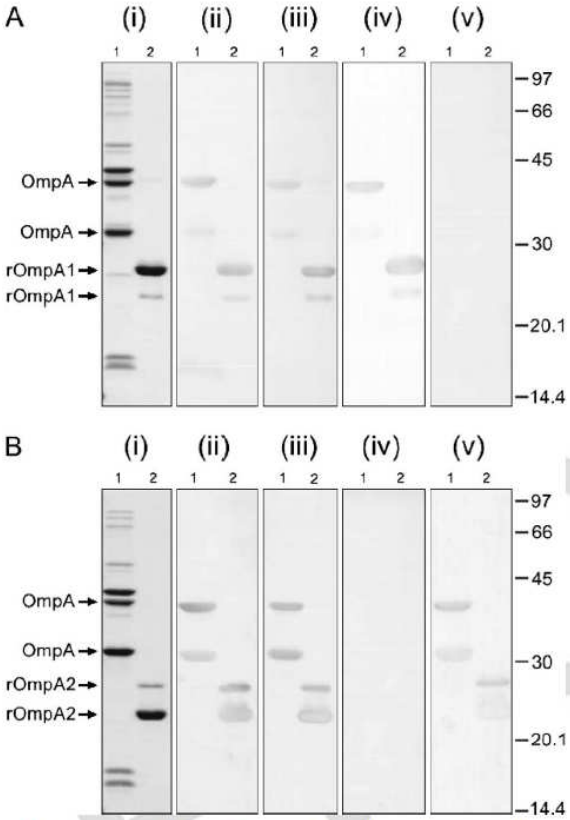


FIG. 3. Western blot analysis of anti-rOmpA antibody binding to full-length OmpA and rOmpA of *M. haemolytica* isolates PH2 and PH278. Panels A and B represent protein samples from bovine isolate PH2 and ovine isolate PH278, respectively. Lanes 1 and 2 represent Sarkosyl-extracted OMPs and purified rOmpA, respectively. The protein samples were separated by SDS-PAGE and either stained with Coomassie blue (i) or transferred to nitrocellulose (ii to v). The blotted proteins were probed with anti-rOmpA1 antibodies (ii), anti-rOmpA2 antibodies (iii), anti-rOmpA1 antibodies after cross-absorption with isolate PH278 (iv), and anti-rOmpA2 antibodies after cross-absorption with isolate PH2 (v). This was followed by incubation with secondary goat horseradish peroxidase-conjugated anti-rabbit antibody. Molecular mass markers (in kDa) are shown on the right.

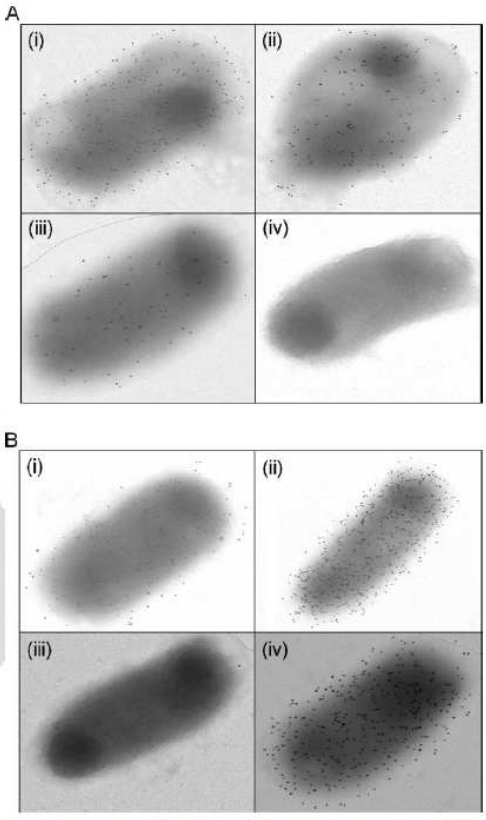


FIG. 4. Immunogold labeling of the OmpA protein of *M. haemolytica* isolates PH2 and PH278 using anti-rOmpA antibodies. Panels A and B represent bovine isolate PH2 and ovine isolate PH278, respectively. Isolates were incubated with anti-rOmpA1 antibodies (i), anti-rOmpA2 antibodies (ii), anti-rOmpA1 antibodies after cross-absorption with isolate PH278 (iii), and anti-rOmpA2 antibodies after cross-absorption with isolate PH2 (iv). This was followed by incubation with secondary goat anti-rabbit IgG antibody conjugated with 10-nm gold particles. Isolates were visualized by electron microscopy. Magnification, ×10,000.

remains relatively unchanged when bacteria are grown under different growth conditions. For subsequent experiments, bacterial cells were grown in BHI broth for 6 h.

Binding specificities of cross-absorbed anti-rOmpA1 and anti-rOmpA2 antibodies to *M. haemolytica* isolates representing different OmpA subclasses. Eleven distinct *ompA* alleles have previously been identified among 31 *M. haemolytica* isolates; these alleles were assigned to four subclasses, *ompA1* to *ompA4* (22). Individual alleles within each subclass were designated *ompA1.1*, *ompA1.2*, etc. The binding specificities of cross-absorbed anti-rOmpA1 and anti-rOmpA2 antibodies to 18 *M. haemolytica* isolates representing all 11 *ompA* alleles were determined by immunogold labeling and immunofluorescent staining (Table 1). Five *ompA1*-type alleles, representing the class I lineage, have been identified in *M. haemolytica*, and these are associated almost exclusively with bovine isolates (22). The single exception, ovine isolate PH494, possesses a bovine-like LktA2-type leukotoxin (25) in addition to a bovine-like OmpA1-type protein and most likely represents a strain of bovine origin that has recently transferred to sheep. Anti-rOmpA1 antibodies were raised against bovine isolate PH2 OmpA, which possesses the *ompA1.1* allele. At the amino acid level, OmpA1.1 is identical to OmpA1.2 and OmpA1.3; OmpA1.4 differs from OmpA1.1 at two amino acid positions in hypervariable region 1 (HV1) and OmpA1.5 differs from OmpA1.1 in having four amino acid deletions in HV1. Cross-absorbed anti-rOmpA1 antibodies bound to all eight OmpA1-type *M. haemolytica* isolates when examined by both immunogold labeling and immunofluorescent staining (Table 1). Cross-absorbed anti-rOmpA2 antibodies showed a negative binding response to all OmpA1-type isolates when examined by immunogold labeling, although weak fluorescence was de-

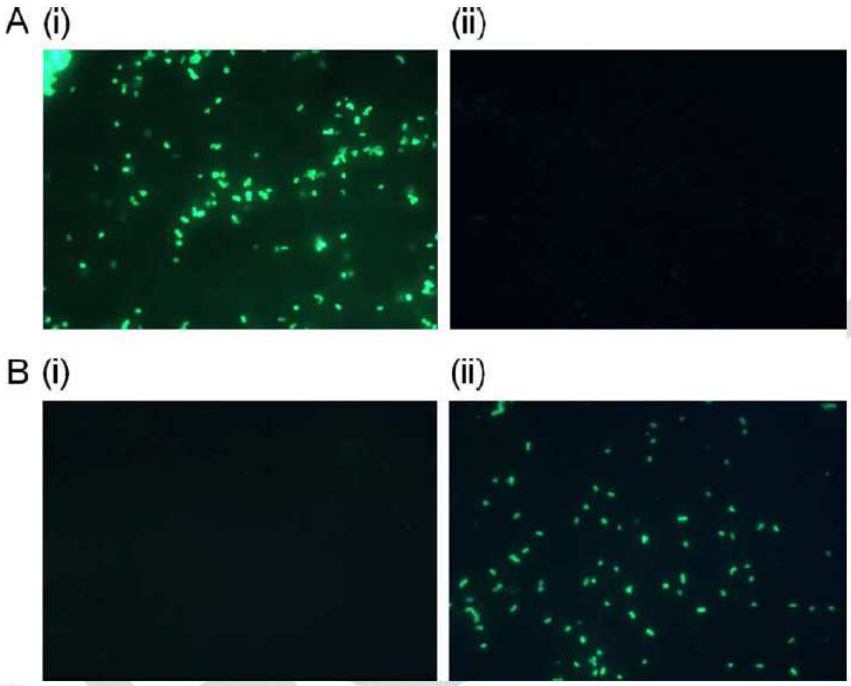


FIG. 5. Immunofluorescent staining of *M. haemolytica* isolates PH2 and PH278 using cross-absorbed anti-rOmpA1 and anti-rOmpA2 antibodies. Panels A and B represent bovine isolate PH2 and ovine isolate PH278, respectively. Isolates were incubated with anti-rOmpA1 antibodies after cross-absorption with isolate PH278 (i) or anti-rOmpA2 antibodies after cross-absorption with isolate PH2 (ii). These were then incubated with Alexa Fluor 488 (Invitrogen) goat anti-rabbit IgG antibody. Bound antibodies were visualized by immunofluorescence microscopy.

tected in three isolates when examined by immunofluorescent staining (Table 1).

Three *ompA2*-type alleles, representing the class II lineage, have been identified in *M. haemolytica* and these are associated exclusively with ovine isolates (22). Anti-rOmpA2 antibodies were raised against ovine isolate PH278 OmpA, which possesses the *ompA2.3* allele. OmpA2.1 and OmpA2.2 are identical and differ from OmpA2.3 at one amino acid position in HV1 and one in HV3. Cross-absorbed anti-rOmpA2 antibodies showed positive binding to all six OmpA2-type *M. haemolytica* isolates when examined by both immunogold labeling and immunofluorescent staining (Table 1). There was a negative binding response to all OmpA2-type isolates with cross-absorbed anti-rOmpA1 antibodies when examined by immunogold labeling, although weak fluorescence was detected in four isolates by immunofluorescent staining (Table 1).

The *ompA3.1* allele, also belonging to the class II lineage, is associated with the bovine isolate PH196. The OmpA3.1 protein was recognized by cross-absorbed anti-rOmpA2 (weakly) but not by anti-rOmpA1 antibodies when examined by immunogold labeling and immunofluorescent staining. Two *ompA4*-type alleles, representing the class III lineage, have been identified in *M. haemolytica*, and these are associated with ovine serotype A7 and A13 isolates. The OmpA4-type proteins (OmpA4.1 and OmpA4.2) were recognized by cross-absorbed anti-rOmpA2 (weakly) but not by anti-rOmpA1 antibodies when examined by immunogold labeling and immunofluorescent staining.

DISCUSSION

In the present study, we have clearly demonstrated that the OmpA protein of *M. haemolytica* is surface exposed and able to bind anti-rOmpA antibodies *in vitro*. This was initially demonstrated in the bovine and ovine isolates PH2 and PH278 (Fig. 4 and 5) but was subsequently confirmed in a wide range of *M. haemolytica* isolates (Table 1). The degree of surface exposure of OmpA in PH2 and PH278 was not affected by the stage of growth (i.e., logarithmic versus stationary phase) or the growth medium (i.e., blood agar and iron-replete or iron-restricted broth). Since a well-developed capsule was observed by Maneval staining in these isolates under different growth conditions, our findings strongly suggest that the capsule does not mask the OmpA protein since antibody binding seemed unaffected. However, in a previous study the OmpA protein of an acapsular mutant was shown to have higher fibronectin-binding activity than that of the capsular parental strain (48), suggesting that the capsule may partially mask the protein.

The loops of the *M. haemolytica* OmpA protein range from 25 to 31 amino acids in length (22) and are estimated to extend less than 10 nm from the bacterial surface (unpublished data). However, the capsule of the parental strain of the acapsular

mutant described above has been observed by electron microscopy to extend almost 200 nm from the bacterial surface (54). It is therefore puzzling as to how the loops of OmpA are recognized by antibodies and, presumably, by host cell molecules through a capsule of this thickness. The *M. haemolytica* capsule is structurally fragile, and it is possible that *in vitro* manipulation of bacterial cells causes sloughing of the capsule in some areas which exposes the tips of the loops and allows antibody binding. However, uniform immunogold labeling clearly demonstrated that OmpA is evenly distributed over the bacterial cell surface. The loops of the OmpA protein of *M. haemolytica* are longer than those of the OmpA protein of *E. coli* (62) but not as long as the loops of other OMPs such as neisserial opacity (Opa) proteins, which can extend to over 50 amino acids in length (27). There are a number of reasons why OMPs might evolve loops of different lengths. The neisserial Opa proteins are involved in binding to host receptors, and longer loops could be seen as advantageous for increasing the distance range to which they can bind host molecules. It is also reasonable to hypothesize that OMPs involved in host molecule binding might evolve loops which are long enough to traverse the capsule. However, this is contradicted by findings that long loops are still present in unencapsulated species such as *Neisseria gonorrhoeae* and *Haemophilus influenzae*. Longer loops have also been correlated with virulence in neisserial species, where commensal organisms have shorter hypervariable domains (and thus shorter loops) than pathogenic species (51). There is evidence to suggest that in a number of bacterial species the capsule can be downregulated upon contact with host cells (2, 15, 26) and as a consequence of phase-variable expression (4, 29, 43), thus allowing transient exposure of OMP adhesins. Also, blebbing of outer membrane vesicles has been observed in several Gram-negative bacteria (reviewed in reference 44), allowing OMPs to traverse the capsule and reach the extracellular environment. These phenomena have not been demonstrated in *M. haemolytica*, although they would provide appropriate mechanisms for the transient exposure of OmpA and other adhesin molecules for host molecule binding.

A wider examination of the binding specificities of the cross-absorbed antibodies to *M. haemolytica* isolates representing different *ompA* subclasses demonstrated that anti-rOmpA1 antibodies recognize OmpA1-type (class I) proteins present in bovine isolates but not OmpA2-type (class II) proteins that occur in ovine isolates. Conversely, cross-absorbed anti-rOmpA2 antibodies recognize OmpA2- but not OmpA1-type proteins. The ability of cross-absorbed anti-rOmpA antibodies to discriminate between OmpA proteins of the OmpA1 and OmpA2 subclasses is due to the greater degree of amino acid sequence variation (within the hypervariable domains) between each of the two subclasses than of the variation within each subclass (22). There are only two variable amino acid sites within the OmpA1-type proteins; similarly, there are only two variable amino acid sites within the OmpA2-type proteins. However, there are 18 variable amino acid sites within the transmembrane domains of OmpA1 and OmpA2, all of which occur within the hypervariable regions (i.e., HV1 to HV4) located at the distal ends of the four external loops. The greater degree of variation between the two OmpA subclasses, compared to that within each subclass, is sufficient to allow the cross-absorbed antibodies to discriminate between the bovine OmpA1- and ovine OmpA2-type subclasses. Overall, these results clearly demonstrate that the OmpA1 and OmpA2 proteins are sufficiently different in structure and epitope specificity that they are potentially capable of recognizing host receptor molecules of differing specificities in cattle and sheep.

Previous studies have demonstrated that OmpA functions as a ligand, is involved in binding to specific host cell receptor molecules, and plays an important role in adherence and colonization in a number of Gram-negative bacteria (8, 18, 38, 55, 64, 65, 67, 74). In particular, it has been shown that the OmpA protein of bovine serotype A1 isolates of *M. haemolytica* binds to fibronectin (48) and to bovine bronchial epithelial cells (41). However, a potential role of OmpA as an adhesin has yet to be investigated in ovine isolates of *M. haemolytica*. We have previously shown that the OmpA1 and OmpA2 proteins are associated exclusively with bovine and ovine isolates, respectively, which led to the hypothesis that they are involved in binding to host-specific receptors in the upper respiratory tracts of these animals (22). This, in turn, might partially account for the different host specificities of strains carrying the OmpA1 and OmpA2 protein types. Importantly, we have now extended the previous study (22) by demonstrating that the binding specificities of the OmpA1 and OmpA2 proteins are very different from each other but are conserved within bovine and ovine *M. haemolytica* isolates representing different capsular serotypes and evolutionary lineages; there is no association between allele/protein type and clinical status (Table 1). From our findings, it is reasonable to conclude that OmpA1 and OmpA2 are capable of binding to different receptors within cattle and sheep, respectively. Although bovine fibronectin has been demonstrated to be a potential receptor for the OmpA protein of a bovine serotype A1 strain (48), it is interesting that the OmpA (P5) protein of the related species *H. influenzae* targets a different molecule, namely, the carcinoembryonic antigen (CEA) family of cell adhesion molecules. Thus, it is reasonable to speculate that different molecular variants or regions of fibronectin, or even different molecules, are targeted in cattle and sheep. The production of antibodies against OmpA1 and OmpA2, and the demonstration that they are highly strain and protein specific, will allow further studies to be performed designed to investigate the role of OmpA in the adherence and colonization of *M. haemolytica* in cattle and sheep.

Commercial vaccines have been produced and are important for the prevention of respiratory disease in both cattle and sheep (9, 39). Several studies have demonstrated the importance of *M. haemolytica* OMPs as surface antigens and their potential as vaccine components (3, 14, 56, 57, 61). Antibodies against the immunogenic and surface-exposed lipoprotein PlpE contribute toward host defense (60), and the addition of recombinant PlpE to commercial vaccines significantly enhances protection against experimental challenge (12, 13). The incorporation of iron-regulated proteins into a vaccine was shown to enhance protection against experimental pasteurellosis in lambs (34). An edible vaccine that expresses a fragment of the outer membrane antigen GS60 has also been developed (46). Immune sera from cattle vaccinated with live or killed *M. haemolytica* cells (50), and immune sera from naturally infected cattle (81), contain antibodies that recognize OmpA. In addition, in the closely related species *Pasteurella multocida*,

OmpA is the major protein that cattle produce antibodies to after experimental challenge (63). Clearly, OmpA is an important immunogen that should be considered for inclusion in *M. haemolytica* subunit vaccines. However, a critical property of any vaccine candidate is a high degree of amino acid conservation throughout bacterial populations. Hypervariability of surface-exposed loop regions is a common occurrence within OMPs of many bacterial species and poses a serious problem for vaccine design (7, 31, 53, 78). We previously demonstrated that four distinct OmpA classes occur within bovine and ovine *M. haemolytica* strains; OmpA1 and OmpA2 are associated with those strains that are responsible for the majority of disease in cattle and sheep, respectively, and have very different hypervariable domains within their surface-exposed loops (22). In the present study, we have now shown that antibodies specific for the surface-exposed loops of OmpA1 will not recognize the same regions of OmpA2 and *vice versa*. Therefore, an animal exposed to OmpA1 will generate anti-OmpA antibodies that are unlikely to confer protection against *M. haemolytica* isolates possessing OmpA2 and *vice versa* (although some cross-reacting antibodies may be present). These findings clearly have important implications with regard to vaccine development and, in particular, to the inclusion of OmpA in subunit vaccines that are designed to provide universal protection against heterologous *M. haemolytica* isolates. Essentially, an OmpA-based *M. haemolytica* subunit vaccine would need to include OmpA from more than one class to provide universal protection against heterologous strains.

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