Molecular characterisation of lipoprotein processing

in *Streptococcus uberis*

Thesis submitted for the degree of

Doctor of Philosophy

at the University of Leicester

by

Emma Louise Denham BSc (Leicester)

Department of Infection, Immunity and Inflammation

University of Leicester

April 2007

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Abstract

Molecular characterisation of lipoprotein processing in

Streptococcus uberis

Emma Louise Denham

Streptococcus uberis is a common cause of bovine mastitis and the lipoprotein MtuA has been shown to be essential for growth in milk and virulence. The enzymes responsible for processing lipoproteins in other Gram positive bacteria are lipoprotein diacylglyceryl transferase (Lgt) which acts to anchor lipoproteins to the membrane and lipoprotein signal peptidase (Lsp) which cleaves the signal peptide. *S. uberis* mutants containing lesions in *lgt* and *lsp* uncovered several novel phenotypes. A number of additional proteins were shown to be present in extracellular fractions prepared from *lgt*$^-$ and *lgt*$^-$*/lsp*$^-$ mutants when compared to the equivalent fraction prepared from wild type bacteria. Atypical processing of MtuA and other lipoproteins within the signal peptide was shown to occur, indicating the presence of an activity capable of shaving such proteins from the membrane in the absence of Lgt and Lsp activity. MtuA was shown to be released into the extracellular space and detection by Western blot, the size closely resembled that of wild type protein in both the *lgt*$^-$ and *lgt*$^-$*/lsp*$^-$ mutants. A metallopeptidase that can be inhibited by phosphoramidon and metal ion chelating agents may be responsible for the activity that results in these proteins being alternatively processed.

MtuA in the *lsp*$^-$ mutant had a molecular weight that corresponded to full length MtuA and remained localised in the membrane as seen in the wild type. During late log phase a second form of MtuA with a lower molecular weight was detected. The study of a mutant containing insertions in both *lsp* and the gene encoding the *S. uberis* homologue to the *Enterococcus faecalis* Enhanced expression of pheromone (*eep*) suggested that this metallopeptidase was also able to cleave the signal peptide of MtuA.

Statement of Originality

The accompanying thesis submitted for the degree of PhD entitled Molecular characterisation of lipoprotein processing in *Streptococcus uberis* is based on work conducted by the author Emma Louise Denham during the period October 2003 – March 2007.

All the work recorded in this thesis is original unless otherwise acknowledged in the text or by references.

None of the work has been submitted for another degree in this or any other university.

E Denham
E Denham

03-04-2008

Emma Louise Denham Date

Acknowledgements

I would like to thank the Institute for Animal Health for allowing me the opportunity to carry out my PhD and the BBSRC for providing me with funding.

I am grateful to my supervisors James Leigh and Philip Ward for providing support and guidance throughout the length of my studies. I am indebted to Andrew Gill for rescuing me in my hour of need and for all his help throughout the project, it has been much appreciated. I would also like to thank Tracey Coffey for her help and advice. I would like to thank the members of the two laboratories that I have been a member. In particular from the Streptococcus research group Terry Field for collecting milk and for answering all my questions and to Andrew Green for his help in the lab and for reading drafts of this thesis. In the TSE research group I would like to thank Steven Banner for help with everything protein, Lawrence Hunt for carrying out the N-terminal sequencing and for all his help with ShvU (I am sure that he is glad that I have now shoved off!), James Graham for keeping me company in the office and Louise Kirby for female company and useful discussions. I would like to thank the members of media services past and present and wash up for providing the litres of media and clean glass wear that have been required throughout this project.

I would like to thank my many friends; both those made before and during my time in Compton. I would especially like to thank the boys and girls hostels - I will miss Sunday night dinners! I would especially like to express my gratitude to Hannah, Kirsty, Caroline and Maddi for always being there for me. Special thanks must go to Tjarda for providing me with much needed respite and relaxation from the lab wandering over the Berkshire downs.

I would also like to show my gratitude to members of my family; Granddad and Doffy for always being interested in what I am doing. I am extremely thankful for the love and support that my parents have provided me, without whom I would have not got this far and to whom this thesis is dedicated.

Table of Contents

Table of Figures

Table of Tables

List of Abbreviations

Ω	Ohm
°C	Degrees Celsius
µF	Micro-farad
µg	Microgram
µl	Microlitre
µM	Micromolar
A	Alanine
ABC	ATP binding cassette
AEBSF	4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride
BCA	Bicinchoninic acid
BLAST	Basic Local Alignment Search Tool
bp	Base pair
C	Cysteine
Ca	Calcium
c.f.u.	Colony forming unit
Co	Cobalt
CO_2	Carbon dioxide
Cu	Copper
D	Aspartic acid
DABCYL	4-(dimethylaminoazo)benzene-4-carboxylic acid
DIG	Dioxygenin

DMSO	Dimethylsulphoxide
DNA	Deoxyribose nucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide triphosphate
E	Glutamic acid
EDANS	5-[(2-aminoethyl)amino]naphthalene-1-sulfonic acid
EDTA	Ethylene diamine tetra acetic acid
Eep	Enhanced expression of pheromone
EGTA	ethylene glycol tetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ExPASy	Expert protein analysis system
F	Phenylalanine
Fe	Iron
FPLC	Fast protein liquid chromatography
FRET	Fluorescence resonance energy transfer
g	Grams
G	Glycine
h	Hour
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulphuric acid
HAP	Hyaluronate associated protein
HCl	Hydrochloric acid
H	Histidine

HPLC	High performance liquid chromatography
HRP	Horse radish peroxidase
I	Isoleucine
IEF	Isoelectric focussing
IPG	Immobilised pH gradient
IS*S1*	Bacterial insertion sequence *S1*
K	Lysine
Kb	Kilobase
kDa	Kilo dalton
KeV	Kiloelectron Volt
l	Litre
L	Leucine
Lgt	Lipoprotein diacylglyceryl transferase
Lsp	Lipoprotein signal peptidase
M	Methionine
M	Molar
mbar	Millibar
MEROPS	The peptidase database (http://merops.sanger.ac.uk/)
Mg	Magnesium
min	Minute
ml	Millilitre
mM	Millimolar
Mn	Manganese

MS/MS	Tandem mass spectroscopy
MtuA	Manganese transporter uberis A
MtuC	Manganese transporter uberis C
MtuR	Manganese transporter uberis Regulator
m/z	Mass to charge ratio
NBT/BCIP	Nitroblue tetrazolium chloride/5-Bromo-4-chloro-3-indolyl phosphate, toluidine salt
N	Asparagine
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NCBI	National Centre for Biotechnology Information
ng	Nanogram
Ni	Nickel
nm	Nanometre
nM	Nanomolar
O.D.	Optical density
ORF	Open reading frame
P	Proline
PAGE	Polyacrylamide gel electrophoresis
PauA	Plasminogen activator uberis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pI	Isoelectric point

PLGS	Protein lynx global server
PMSF	Phenylmethylsulphonyl fluoride
PVDF	Polyvinylidene fluoride
Q	Glutamine
Q-tof	Quadrapole time of flight
R	Arginine
RIP	Regulated intramembrane proteolysis
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
RT PCR	Reverse transcription PCR
S	Serine
SAE	Sheep esculin agar
SDS	Sodium dodecyl sulphate
ShvU	Shavase Uberis
SO_4	Sulphate
Spy	*Streptococcus pyogenes* protein
SSC	Stock (20X): 3.0 Sodium chloride, 0.3 M Sodium citrate
T	Threonine
TAE	Tris acetate buffer
TCA	Trichloracaetic acid
TE buffer	Tris-EDTA buffer
THB	Todd Hewitt Broth

TMHMM	Transmembrane helix Markov model
TMB	3,3',5,5'-tetramethylbenzidine
U	Unit
UPLC	Ultra performance liquid chromatography
V	Valine
V	Volts
W	Tryptophan
WT	Wild type
v/v	Volume per volume
w/v	Weight per volume
Y	Tyrosine
Zn	Zinc

Chapter 1

Introduction

1.1 Streptococci

Disease caused by Streptococci was first described by Hippocrates in the 5th century BC when he described a Scarlet fever epidemic, however it was in 1884 when Rosenbach officially described the genus Streptococcus (Rosenbach, 1884). To date there are approximately 113 different species of Streptococcus (as listed by the German Collection of Microorganisms and Cell Cultures) and these are found in a wide range of plant, animal and human hosts. Streptococci have been classified to the order Lactobacillales, the class Bacilli and the phylum Firmicutes.

Streptococci are Gram positive, non-motile, non-sporeforming bacteria that occur in chains or pairs of cells. Individual cells are round-to-ovoid cocci in shape and are between 0.6-1.0 μm in diameter. The name Streptococcus originates from the Greek Streptos meaning easily bent or twisted like a chain, this name was given due to the mechanism by which the cells divide; division occurs in one plane and therefore the bacteria occur in pairs or in chains of varying lengths.

Clinically Streptococci are distinguished by their haemolytic properties and are subdivided into three different groups. Alpha haemolytic Streptococci reduce the iron in haemoglobin and leave a green colour when cultured on blood agar and include *S. pneumoniae* and the Viridans group. Beta haemolytic Streptococci cause complete rupture of red blood cells leaving a distinct clear area around the bacterial colonies on blood agar plates and include *S.*

agalactiae and *S. pyogenes*. The final group is gamma haemolytic and these bacteria do not cause haemolysis. In the 1930's the work of Lancefield subdivided the family of the beta haemolytic Streptococci into serogroups A-S according to the carbohydrate composition of the bacterial antigen (Lancefield, 1933).

Bacteria belonging to the genus Streptococcus. of bacteria are considered a major worldwide problem. Infections caused by Streptococci may cause localised or systemic disease with a wide range of severities. *S. pneumoniae* is a major human pathogen effecting young children, the elderly and the immunocompromised, this pathogen causes pneumonia, meningitis and sepsis (Mitchell, 2006). *S. agalactiae* is not only able to cause mastitis in dairy cattle (Keefe, 1997), but also neonatal sepsis that develops as a result of pre-existing maternal colonisation of the female urogenital tract. *S. agalactiae* causes pneumonia, meningitis and sepsis (Schuchat, 2001). *S. pyogenes* (GAS) is able to cause a number of different diseases including pharyngitis, scarlet fever, impetigo, toxic shock syndrome and necrotizing faciitis in addition to the autoimmune sequelae rheumatic fever and rheumatic heart disease following GAS infection (Carapetis *et al.*, 2005). *S. suis* is able to cause meningitis, septicaemia, arthritis and sudden death in pigs and occasionally humans as a result of close contact with infected swine (Huang *et al.*, 2005). *S. equi* is the cause of strangles, an upper respiratory tract infection, complications of which lead to up to 10% mortality in horses. *S. uberis* is a common cause of bovine mastitis. Few vaccine options are available for these organisms and antibiotics are most frequently the chosen choice of treatment (Leigh, 1999).

1.1.1 *Streptococcus uberis*

In the late 19th century Streptococcus was shown to cause mastitis in dairy cattle and in 1932 Diernhofer described the species *Streptococcus uberis* (Diernhofer, 1932). *S. uberis* was shown to be divided into two discrete genotypes; types I and II, the latter was later called *S. parauberis* and rarely causes mastitis (Williams and Collins, 1990). *S. uberis* was shown to have similarities to pyogenic Streptococci (Slot, 1958) and was grouped with parapyogenic strains by Bridge and Sneath (Bridge and Sneath, 1983). Subsequently, molecular methods confirmed *S. uberis* belonged to the pyogenic group within the genus Streptococcus that includes *S. pyogenes*, *S. dysgalactiae* and *S. agalactiae* (Bentley *et al.*, 1991).

Mastitis is an inflammatory disease of the bovine udder and in the United Kingdom as much as 33% of all cases are due to infection with *S. uberis* (Hillerton *et al.*, 1993), although a recent survey of 97 dairy farms in the United Kingdom suggested that 23.5% of clinical cases of mastitis were caused by *S. uberis* (Bradley *et al.*, 2007). Mastitis is the most costly disease that affects dairy cattle; infections cause loss in revenue due to a reduction in milk production, treating affected animals and through the loss of animals that are repeatedly infected and are culled. The annual loss caused by this disease in the United Kingdom is approximately £170 million (Kossaibati and Esslemont, 1997) and in the United States between $1.5 and $2 billion (Wells *et al.*, 1998). There are two routes that mastitis-causing bacteria can be transmitted; those that show a contagious route of transmission such as *Staphylococcus aureus* and *S. agalactiae* and those that cause infection from an environmental reservoir, which is the route used by *Escherichia coli* and *S. uberis*. Efforts to control and prevent infection have been implemented successfully for the contagious route of transmission using the five point control

programme (Bramley and Dodd, 1984), which brought about improved milking practices, post milking teat disinfection and routine intramammary antibiotics after each lactation period. However, these practices have had very little effect on bacteria that cause infection through the environmental route of transmission. No vaccine is currently commercially available to help in the control of infection by this microorganism.

1.1.1.1 Epidemiology

Bovine mastitis caused by *S. uberis* is prevalent in all geographical regions of the world (Hogan and Smith, 1997). However, there are likely to be major differences in infection incidence between different regions and also between herds. As previously mentioned intramammary infection caused by *S. uberis* has not been reduced by the implementation of the five point control programme for the management of bovine mastitis (Bramley and Dodd, 1984), due to the environmental nature of this pathogen. The bacteria has been isolated from many locations around the cow, including tonsils, genital tract, rumen, rectum, and coat (Leigh, 1999). It can also be isolated in high numbers from bedding used by cattle (Bramley, 1982).

Cattle that are kept at pasture are generally considered to be at a lower risk of infection by *S. uberis* than housed cattle (Faull *et al.*, 1983). This is because bedding materials act as a reservoir for the transmission of this organism. It has been shown that *S. uberis* can be isolated from the teat apex immediately before milking despite the application of a disinfectant teat-dip after the previous milking (NIRD, 1970). This contamination is thought to come from the material that the dairy cattle lie on. The literature is inconclusive as to which bedding

material is most beneficial for cows to be housed on. Zdanowicz *et al.*, 2004 (Zdanowicz *et al.*, 2004) reported that sand bedding after 6 days was more contaminated with *Streptococcus* spp. than saw dust bedding. However, Janzen *et al.*, 1982 (Janzen *et al.*, 1982), reported that bedding made of organic materials was likely to have a higher level of bacterial contamination compared to inorganic bedding materials such as sand. The levels of bacteria increase in these materials with the increase in level of organic contamination (Zdanowicz *et al.*, 2004).

Epidemiological investigation of *S. uberis* strains has been carried out using different typing schemes (Coffey *et al.*, 2006; Douglas *et al.*, 2000; Gilbert *et al.*, 2006; Wieliczko *et al.*, 2002), the most recent of these being Multilocus Sequence Typing (MLST) (Coffey *et al.*, 2006). MLST allows the investigation of populations and population dynamics on a global scale as data is accessed through the internet. This typing scheme is based on the DNA sequence of seven different housekeeping genes, the evolution of which were thought likely to be constrained due to encoding gene-products being essential for cellular function (Maiden *et al.*, 1998). The 160 isolates of *S. uberis* that had been collected from six different farms in the United Kingdom split into 57 different sequence types, which showed that the population was heterogeneous. However, it was also shown that the majority of isolates were closely related and that these grouped together into a single clonal complex which was designated ST-5 (Coffey *et al.*, 2006). A comparison of populations from the United Kingdom and New Zealand allowed determination of distinct populations. ST-5 was shown to be present in New Zealand, but the clonal complex ST-143 was shown to be dominant in this location. ST-86 was a second clonal complex identified in New Zealand. The *S. uberis* isolates from the United Kingdom were all isolated from milk samples, where as those from New Zealand were

isolated from milk, areas of the cow and the environment. All three major clonal complexes (ST-5, ST-143 and ST-86) are all capable of establishing intramammary infection as well as surviving in the environment and colonizing other bovine sites (Pullinger *et al.*, 2006). It is of interest to note that the carriage of the *hasA* locus which encodes for capsule formation and has been shown to be associated with disease-causing isolates, correlates with clonal complex and isolation from clinical cases of mastitis (Coffey *et al.*, 2006; Pullinger *et al.*, 2006).

1.1.1.2 Pathogenesis

The bovine mammary gland varies in its susceptibility to infection depending on the phase of lactation. Colonisation of the lactating bovine mammary gland is thought to occur due to a combination of bacterial growth and a failure to remove the bacterium by milking. When a dairy cow is dried-off (withdrawn from regular milking), susceptibility to infection is comparatively low (McDonald and Anderson, 1981), but the susceptibility increases throughout the course of the dry period (Cousins *et al.*, 1980). For the colonisation of the bovine mammary gland to occur during lactation or the dry period the bacteria must gain access to the gland and then multiply at a rate sufficient to prevent its elimination during milking or adhere to host tissues. *S. uberis* was found predominantly in the luminal areas of the secretory alveoli and ductular tissues of experimentally infected animals (Thomas *et al.*, 1994), indicating that much of the bacterial growth occurs in residual and newly formed milk. Bacterial numbers can reach up to 10^9 c.f.u./ml of milk, however typically bacterial numbers of 10^6 to 10^8 c.f.u./ml are more typically seen in clinical disease.

S. uberis is able to bind to cultured bovine mammary epithelial cells and may be internalized (Almeida and Oliver, 2006; Matthews *et al.*, 1994). The bacteria is preferentially taken up into the host cell by exploiting the caveolae dependent pathway (Almeida and Oliver, 2006). The bacterium is able to do this by inducing host cell cytoskeletal rearrangement and expressing its own proteins. Use of protein kinase inhibitors has led to the speculation that *S. uberis* is able to exploit host cell protein kinases to induce its own uptake signal (Almeida *et al.*, 2000). The bacterium becomes enclosed in vesicles once it has entered the cell, however after 24 hour incubation in the eukaryotic cell *S. uberis* may be found visible in the host cell cytoplasm (Tamilselvam *et al.*, 2006). The bacteria are able to remain within the cell for extended periods of time without inducing host cellular damage (Almeida *et al.*, 2000; Almeida and Oliver, 2001).

1.1.1.3 Virulence factors

S. uberis infection causes an inflammatory response which leads to an influx of neutrophils, macrophages and lymphocytes into the infected gland (Leigh, 1999). An important aspect of bacterial pathogenesis is the acquisition of nutrients; this is likely to be executed through proteins expressed on the cell surface. A 30 kDa secreted plasminogen activator (PauA) is able to activate bovine and ovine plasminogen to plasmin, a potent serine protease (Leigh, 1994). PauA mediates the acquisition of plasmin at the bacterial surface following growth in media containing plasminogen. PauB is a second plasminogen activator of *S. uberis*, the strain that this gene was isolated from does not produce PauA, which is expressed by the majority of strains. PauB is able to activate a broad spectrum of plasminogen including bovine, ovine and human. The protein sequence for PauB contains a candidate signal peptide sequence and

cleavage site (Ward and Leigh, 2002). The presence of these proteins in the bacterium has been hypothesized to facilitate the growth and survival of these auxotrophic bacteria in milk due to the release of peptides and amino acids from host proteins (Leigh, 1999). A recent study using a PauA mutant that failed to activate bovine plasminogen was able to grow normally in milk and within the mammary gland of a dairy cow similar to the wild type, disproving the above hypothesis (Ward *et al.*, 2003).

MtuA is an extracytoplasmic protein of S. uberis and a putative lipoprotein receptor antigen (LraI) that is responsible for in the acquisition of manganese during growth in milk. MtuA is also essential for infection of the lactating bovine mammary gland (Smith *et al.*, 2003). Localization studies have shown that MtuA is located within the cell membrane and not exposed on the bacterial surface, therefore this protein is not a good vaccine target (Jones *et al.*, 2004). A further two putative lipoproteins; OppA1 and OppA2 have been predicted to be oligopeptide binding proteins (Taylor *et al.*, 2003). OppA1 shown to be essential for efficient growth of *S. uberis* in milk, as a bacteria carrying an insertion in the *oppA1* gene had a lower rate of growth than the wildtype in this medium. The expression of OppA2 was shown to be upregulated during growth in milk.

Obtaining a competitive advantage over other bacteria is a method that many bacteria have developed to help in colonisation of a new niche. *S. uberis* has been shown to produce a novel cyclic bacteriocin Uberolysin, a proteinacious antibiotic (Wirawan *et al.*, 2007). Uberolysin is encoded by *ublA* which is part of a 6 open reading frame operon. Within this operon are genes involved in the regulation of expression, circularisation of the bacteriocin, an accessory factor

for transport and a protein that transports the bacteriocin. Uberolysin is thought to have a bactericidal mechanism of action and is particularly effective against *Lactococcus lactis,* decreasing the viability of the population by 6-logs after only 10 minutes exposure. The bacteriocin is able to inhibit an extensive spectrum of Gram positive bacteria including most Streptococci, Listeria, Enterococci and Staphylococcal species.

Some strains of *S. uberis* have been shown to be able to resist phagocytosis, hence these are more capable of establishing infections in the lactating bovine mammary gland (Leigh *et al.*, 1990). The loss of the hyaluronic acid capsule does not prevent phagocytosis from occurring (Leigh *et al.*, 1990). Ward et al., 2001 investigated the role of capsule in virulence and showed that acapsular mutants were less resistant to phagocytosis by bovine neutrophils *in vitro* compared with the wild type. This suggests that the capsule may have a role in virulence of *S. uberis* (Ward *et al.*, 2001). However, a *hasA* mutant that had a acapsular phenotype was found to be as virulent as the wild type strain that the mutant was derived from, suggesting that capsule played none or a limited role in the virulence of *S. uberis* (Field *et al.*, 2003).

Hyaluronidase is a 54 kDa protein that has been shown to be exported into the extracellular space (Lammler, 1991). The role of this protein has been suggested in facilitating bacterial dissemination into the mucosal barrier and other tissues leading to a spread of the *S. uberis* infection. Hyaluronic acid capsule and hyaluronidase are able to influence proliferation of mammary epithelial cells *in vitro*. Whether this proliferation is active *in vivo* and the effect of it is unknown (Segura and Gottschalk, 2004).

Uberis factor (Skalka *et al.*, 1980) is a protein that is similar to the CAMP-factor described for group B streptococci that is able to lyse erythrocytes (Christie *et al.*, 1944). This is achieved by the interaction of this protein with the *S. aureus* beta-toxin. The protein is 28 kDa and is highly homologous to the protein expressed by group B streptococci (Jiang *et al.*, 1996). The protein has been shown to have lethal effects when administered parenterally to rabbits and mice. CAMP factor may be a significant factor in the pathogenesis of this bacterium, but this role has not been established and it has been suggested that the gene encoding this protein may be restricted to a small number of isolates (Leigh, 1999).

Streptococcus uberis adhesion molecule (SUAM) was identified by its affinity for bovine lactoferrin. Antibodies and a 17 amino acid long N-terminal peptide sequence of SUAM were both able to inhibit adherence to and internalization of *S. uberis* into bovine mammary epithelial cells (Almeida *et al.*, 2006). These data suggest that SUAM may be a useful candidate in vaccine development.

The virulence factors so far identified in *S. uberis* are all extracytoplasmic proteins, the mechanisms of their translocation to the surface and or external environment of the cell is very poorly understood. It may be possible by identifying the specific cellular apparatus involved in the movement of proteins to better understand the disease-causing nature of these and other related bacteria.

1.1.1.4 Vaccines and Treatment

1.1.1.4.1 Killed bacterial vaccines

Repeated immunisation of dairy cattle with killed whole cell *S. uberis* is able to reduce the numbers of bacteria present in the milk following experimental homologous challenge (Finch *et al.*, 1994). However, this form of immunisation did not prevent the inflammatory response of the mammary gland following bacterial challenge.

1.1.1.4.2 Live bacterial vaccines

Immunisation with live *S. uberis* by the sub-cutaneous route along with administration of a soluble preparation of bacteria cell wall antigens by the intramammary route has been shown to confer some protection against experimental challenge (Hill *et al.*, 1994). Non-immunised animals shed high numbers of bacteria (10^6 to 10^7 c.f.u./ml of milk) and all challenged quarters developed clinical mastitis by 5 days post challenge. In contrast, animals which had received the vaccine shed considerably fewer bacteria ($<10^2$ c.f.u./ml of milk). By day 15 post challenge only 25% of challenged quarters exhibited clinical signs. However, this regime was not as effective against heterologous strains of *S. uberis*.

1.1.1.4.3 Sub-unit vaccines

Traditionally, bacterial proteins directly involved in colonisation and pathogenesis have been targeted for their use in subunit vaccines. PauA is currently the only component that has been tested as a candidate for a sub-unit vaccine antigen. Bacterial culture supernatants where the presence of PauA was confirmed were immunised sub-cutaneously. This mixture of proteins from a single strain were found to confer some protection from clinical disease following

challenge with a different strain (Finch *et al.*, 1997; Leigh *et al.*, 1999). The bacterial recovery from milk of immunised animals was typically 3-logs lower than that from non-immunised animals following experimental challenge. Protection correlated with the production of neutralising activity towards PauA since immunisation with a similar preparation from which the plasminogen activator had been removed, showed no protective effect (Leigh *et al.*, 1999).

By restricting bacterial colonisation it has been hypothesised that there will be a reduction in the inflammatory stimulus, a reduction in the influx of neutrophils, maintenance of milk quality and a reduction in the local tissue damage associated with the inflammatory reaction. Therefore, vaccines aimed at reducing intramammary colonisation would appear an attractive option for the control of *S. uberis* caused mastitis which has not been controlled by the implementation of the five point control plan.

1.2 Protein export in bacteria

Identification of the proteins that may be potential vaccine targets can be identified by studying the process of protein translocation within the bacteria. A common theme that occurs in nature is the movement of proteins translated in the cytosol, through the membrane to the cell wall, where they may be attached or secreted into the extracellular space. The process of protein translocation can be separated into five distinct parts; a) export, b) retention, c) cleavage, d) linkage and e) cell wall or membrane incorporation (Tjalsma *et al.*, 2004). The last two parts are only applicable to those proteins that are retained on the cell surface. For a protein to be translocated in general it must have a signal peptide that is recognised by the

cellular sorting and translocation machinery, this will enable the protein to pass the hydrophobic physical barrier of the membrane (Tjalsma *et al.*, 2000a). Distinctions can be made between the different types of proteins that are translocated depending on their signal peptide sequence. A signal peptide can be defined as a short stretch of amino acids that directs delivery of the protein to the correct subcellular compartment and is subsequently removed by a signal peptidase (Antelmann *et al.*, 2001). The translocated proteins that are retained on the cytoplasmic membrane or the cell wall will contain signals for their attachment. The proteins involved in processing of secreted proteins are shown in Table 1.

Protein	Function
SecA	Translocation motor
SecY	Translocation channel
SecE	Translocation channel
SecG	Translocation channel
SecB	Chaperone (restricted to Gram negative bacteria)
CsaA	Chaperone like activities and affinity for SecA and preproteins
Lgt	Anchoring lipoproteins to cell membrane
Lsp	Specific signal peptidase for lipoproteins
Signal peptidase type I	Removal of signal peptides from secreted proteins
Signal peptide peptidase	Breaks down signal peptide
PrsA	Correct folding of proteins
Signal Recognition Particle (SRP)	Secretion dedicated chaperone
FtsY	Bacterial signal-recognition particle receptor

Table 1 Components of bacterial secretion machinery

1.2.1 Signal peptide sequences of secreted proteins

The signal peptide sequence of a protein has several functions, it enables the protein to be recognised by the secretory machinery, it is a topological determinant and the presence of the signal peptide can inhibit the folding of nascent chains which can avoid the activation of potentially harmful enzymes until they are correctly localised (Driessen *et al.*, 1998). The signal peptide is likely to be found at the amino terminus of the protein and is composed of three distinct domains (Tjalsma *et al.*, 2000a). The amino terminal N-domain contains at least one arginine or lysine residue and overall is positively charged. This domain has been predicted to interact with the translocation machinery and also the negatively charged lipid bilayer of the membrane during translocation (von Heijne and Manoil, 1990). The H-domain is so named due to the stretch of hydrophobic residues that assume an α-helical structure within the membrane (Tjalsma *et al.*, 2000a). Amino acids such as glycine and proline often appear in the middle of the hydrophobic core. It has been proposed that these cause a hairpin like structure to form within the peptide chain and allow the complete signal peptide to become inserted within the membrane. A further hypothesis has been made suggesting that the helix-breaking residues within the H-domain facilitate cleavage with a specific signal peptidase (Dalbey *et al.*, 1997). The final part of the signal peptide is the C-domain, which contains the signal peptide cleavage site. Once cleaved, the mature part of the protein can adopt its native structure. The signal peptide is degraded by a signal peptide peptidase, this enzyme has been described in *Bacillus subtilis* (Bolhuis *et al.*, 1999) and has been shown to be required for efficient translocation and processing of secreted proteins. However, no homologous protein has yet to be described in other Gram positive bacteria. The average length of a signal peptide in Gram positive bacteria is 32 amino acids (Nielsen *et al.*, 1997).

However, there is very little conservation of sequence between Gram positive species. Gram positive signal peptides are significantly longer and have a much longer hydrophobic region compared to those from Gram negative species. Small differences between individual signal peptides can cause cleavage by different signal peptidases, export via different pathways and transport to different cellular destinations.

1.2.2 The Sec protein-translocation pathway

One of the main features of the translocation apparatus that operates in a bacterial cell is the Sec machinery (Figure 1). The Sec pathway recognises exported proteins at the membrane and catalyzes there export out of the cytosol, before they acquire their final structures. This process is achieved without compromising the permeability barrier of the membrane. The Sec apparatus is composed of several proteins and is a dynamic membrane embedded nanomachine (Papanikou *et al.*, 2007). SecA is an ATPase that drives protein movement into and across the membrane. This protein is able to interact with chaperones dedicated to protein export, in *E. coli* this is named SecB, however in *B. subtilis* and many other Gram positive bacteria there is no SecB homologue (Tjalsma *et al.*, 2000a). SecA contains a SecB docking site that is conserved in the protein found in *B. subtilis*. A candidate for a SecB analogue in *B. subtilis* is the CsaA protein; this protein was identified through expression of a *B. subtilis* chromosomal gene library in an *E. coli* SecA temperature sensitive mutant. This protein was found able to reverse the growth defects of this mutant and it was able to relieve the detrimental accumulation of precursors of exported proteins (Muller *et al.*, 1992). CsaA has chaperone like properties and an affinity for SecA and preproteins (Tjalsma *et al.*, 2000a).

CsaA and SecB share functional similarities, but have no sequence homology (Muller *et al.*, 1992; Muller *et al.*, 2000).

The SecB interaction with nascent polypeptide chains has been shown to occur independently of the presence of a signal peptide sequence (Randall *et al.*, 1990). The principles governing substrate recognition for SecB were determined using a cellulose-bound peptide scan. Interaction of SecB with the peptides was inhibited by the presence of acidic residues; SecB is an acidic protein. The presence of a motif containing approximately 9 residues enriched in basic and aromatic amino acids has high affinity to the SecB peptide-binding site (Knoblauch *et al.*, 1999). Thus, SecB was unable to distinguish between secretory and non-secretory proteins and therefore discrimination between these two types of protein must be made at another point during translocation of the protein. Substrate similarities between CsaA of *B. subtilis* and SecB were shown using the secreted protein YvaY. The protein was broken down into a peptide library and analyzed for CsaA binding. CsaA was shown to have affinity for multiple peptides and the majority of these had a net positive charge (Linde *et al.*, 2003). The presence of acidic-residues did not abolish binding of CsaA, unlike SecB (Knoblauch *et al.*, 1999). CsaA has a greater affinity to peptides that are in the unfolded state than the folded state. The signal peptide region of YvaY does not contain the preferred binding site of CsaA, suggesting that there may be other chaperones responsible for chaperoning proteins to the translocation machinery (Linde *et al.*, 2003).

A second chaperone protein that has been associated with secretion is the signal recognition particle (SRP). This comprises of a 4.5S RNA species and the protein Ffh (Economou, 2002).

SRP operates through recognition of the signal sequence of the pre-protein being translocated. This chaperone shows greater specificity in the proteins that it targets to the cell membrane. The SRP binds to the nascent chains that are emerging from the ribosome; this causes the ribosome to stop translation of the polypeptide. The SRP targets the complex to the FtsY protein to form a novel assembly at the membrane where it was able to dock and interact with the Sec translocon (Angelini *et al.*, 2005). FtsY is partially located in the cytosol and partially in the membrane (Luirink and Sinning, 2004). Within the SRP and FtsY are two GTPases that control the various steps of the targeting reaction. There is a reciprocal activation of the other GTPase activity upon formation of the SRP-FtsY complex. Therefore there is no need for GTPase activating proteins as is the case in most other known GTPase proteins. The complexities of the interaction between SRP and FtsY are discussed in detail in Shan et al., 2004 (Shan *et al.*, 2004). Once the interaction at the membrane has taken place the SRP is released and the ribosome continues translation of the polypeptide (Tjalsma *et al.*, 2000a).

Formation of the two different complexes at the membrane engages the translocase machinery in a specific manner (Economou, 2002). The three proteins SecY, SecE and SecG form a hetero-trimeric protein channel complex within the cytoplasmic membrane enabling polypeptides to be transported (Mori and Ito, 2001). These proteins span the membrane ten, three and two times respectively. Medium and high resolution structures of SecYEG have been resolved and these have allowed several more details of the translocation process to be described in an excellent review of the translocation process (Papanikou *et al.*, 2007). SecYEG has been shown to have a closed state, where a plug that is present on the SecY protein blocks the channel (Saparov *et al.*, 2007). SecE and SecG bind to SecY peripherally

and SecE acts as a clamp, keeping the complex together. Several of the residues that are present within the helix and the plug appear to be responsible for the specificity of the proteins that are being translocated (Saparov *et al.*, 2007). It has been proposed that signal peptides of the preproteins bind to a hydrophobic patch of amino acids that are between the transmembrane 2 and 7, when this occurs the opening of the channel is triggered. The pore that is formed by SecYEG must dilate from its inactive state in order to allow proteins across the membrane. The size of the channel is approximately 5-8 Å, which is too small to accommodate a preprotein chain that has a minimal width of approximately 12 Å (Tani *et al.*, 1990). Evidence is available for this dilation has been provided by the presence of several tilted helices on the SecY protein, the topological inversion of SecG, specific binding of lipid molecules, the partial detachment of SecE and by SecA which induces ATP driven conformational changes (Nishiyama *et al.*, 1996).

Several different propositions have been put forward as to the number of SecYEG molecules that are necessary for protein translocation (Papanikou *et al.*, 2007). However, it has been shown that a single SecYEG trimer can form an export pore. SecYEG complexes have also been seen in dimeric or higher order assemblies (Mori *et al.*, 2003). These larger extended forms of the preprotein might facilitate the docking of other translocase subunits and the ribosome or other roles that remain poorly understood (Papanikou *et al.*, 2007).

SecA has high affinity for the SecYEG complex and this protein complex supports the translocation of the preproteins across the membrane (van Wely *et al.*, 2001). Each SecA molecule contains a central core, the DEAD box is the helicase motor and these contain a nucleotide binding domain and an intramolecular regulator of ATPase 2. The central core is

lined with these helicase motifs that have been suggested to link ATP binding and hydrolysis to translocation. SecY is potentially linked to SecA through interaction of residues that are present on TM4. The C domain of the SecA is also likely to play a large role in either binding directly to the SecYEG complex or by allosteric modulation of the DEAD motor. The ATPase activity of SecA becomes activated when contact has been made with SecB interacting with a preprotein that has a functional and recognisable signal peptide region. The SecA subunit must be in contact with lipid and in the presence of SecYEG for translocation to occur (Triplett *et al.*, 2001). It is likely that SecA must also be in contact with part of the mature protein for translocation to proceed (Triplett *et al.*, 2001). SecD and SecF are required for efficient protein export, but are not essential for cell viability (Tjalsma *et al.*, 2000a).

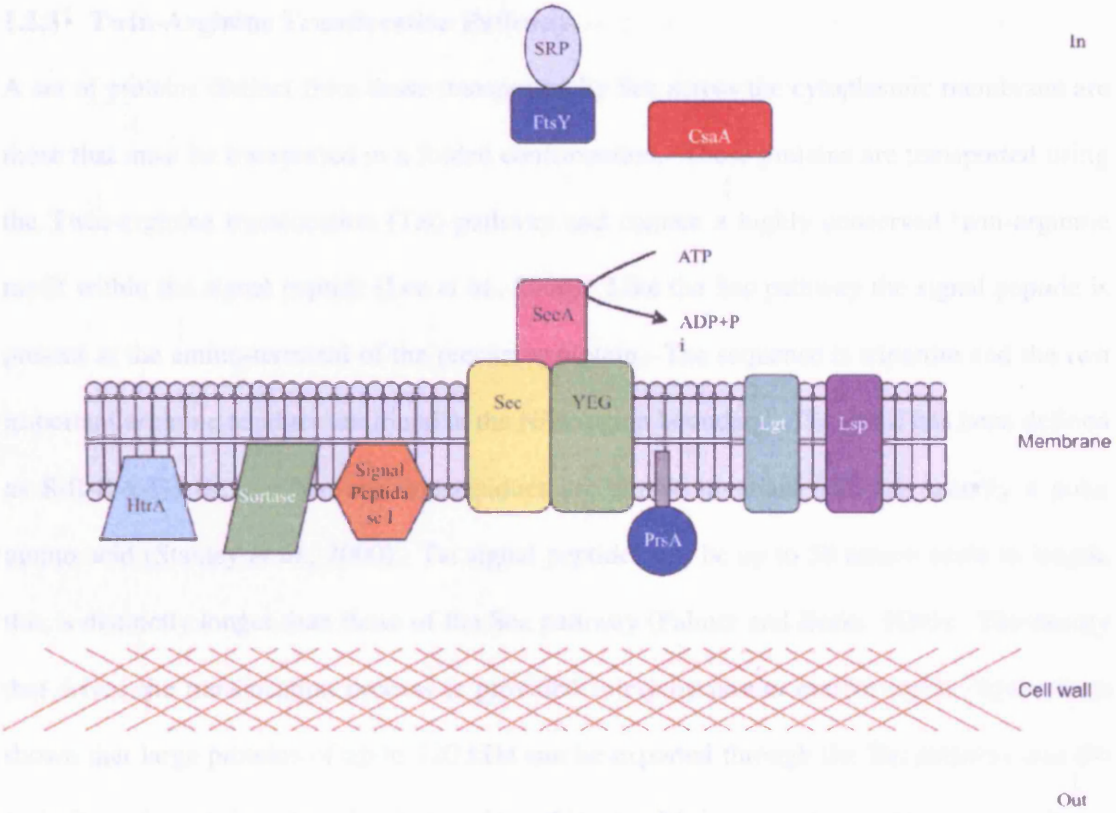


Figure 1 The main components of the Sec-dependent protein secretion machinery in Streptococcus spp.

Adapted from (Tjalsma *et al.*, 2000a).

1.2.3 Twin-Arginine Translocation Pathway

A set of proteins distinct from those transported by Sec across the cytoplasmic membrane are those that must be transported in a folded conformation. These proteins are transported using the Twin-arginine translocation (Tat) pathway and contain a highly conserved twin-arginine motif within the signal peptide (Lee *et al.*, 2006). Like the Sec pathway the signal peptide is present at the amino-terminal of the precursor protein. The sequence is tripartite and the two important arginine residues are found at the N/H region boundary. The motif has been defined as S-R-R-x-F-L-K, the two arginine residues are almost invariant and x is usually a polar amino acid (Stanley *et al.*, 2000). Tat signal peptides can be up to 58 amino acids in length, this is distinctly longer than those of the Sec pathway (Palmer and Berks, 2003). The energy that drives the translocation process is provided solely by proton motive force. It has been shown that large proteins of up to 120 kDa can be exported through the Tat pathway and the majority of proteins translocated using this mechanism contain cofactors such as hydrogenases, formate dehydrogenases, nitrate reductases, trimethylamine N-oxide reductase and dimethyl sulfoxide reductases (Palmer *et al.*, 2005; Rose *et al.*, 2002; Stanley *et al.*, 2001). The degree to which organisms use the Tat apparatus to export proteins is varied. Some bacteria have very low numbers of substrates, where as others such as *Streptomyces coelicolor* is predicted to have 129, although only a small number of these have been confirmed (Li *et al.*, 2005).

Within the *E. coli* genome two genetic loci have been identified that encode components of the Tat machinery. A single operon contains *tatABCD* and *tatE* is located at a distinct position on the chromosome. All transcription units are expressed constitutively, indicating that the

translocation pathway is likely to be required under all growth conditions (Jack *et al.*, 2001). TatA, TatB and TatE are sequence-related proteins and each has been predicted to contain a membrane spanning α-helix at the amino terminus which are all followed immediately by an amphipathic helix located on the cytoplasmic side of the membrane and a carboxyl-terminal region of variable length (Palmer and Berks, 2003). TatA was shown to be a fully integral membrane protein that was accessible to protease digestion on the cytoplasmic side of the membrane, but not on the periplasmic side (Palmer and Berks, 2003; Porcelli *et al.*, 2002). TatC is a highly hydrophobic protein that has been predicted to contain six transmembrane helices with both the amino and carboxyl termini located at the cytoplasmic face of the membrane. TatC shows the most sequence conservation of all the components of the Tat translocation machinery (Bogsch *et al.*, 1998; Buchanan *et al.*, 2002). Site-directed mutagenesis experiments have revealed that some of the residues within the predicted cytoplasmic domain are essential for the operation of the Tat pathway (Buchanan *et al.*, 2002).

Until recently it was thought that the majority of integral membrane proteins were assembled via the Sec pathway, however it became apparent that the Tat pathway was involved in the post-translational biogenesis of certain integral membrane proteins (Hatzixanthis *et al.*, 2003). These included HybO, HybaA, HybA, FdnH and FdoH which are all members of larger multisubunit Ni/Fe hydrogenase or formate dehydrogenase respiratory complexes. Tat signal peptides are not universally recognized by different Tat translocases. The Tat-dependent precursor proteins have evolved to undergo export optimally via their own translocation apparatus. This is the opposite to the Sec-dependent translocation mechanism, where proteins from different organisms can be translocated (Blaudeck *et al.*, 2001).

Several different complexes of the Tat machinery have been purified. TatB and TatC have been purified in a complex of 350 – 600 kDa with a 1:1 stoichiometry (Bolhuis *et al.*, 2001; de Leeuw *et al.*, 2002; Oates *et al.*, 2003). It has been shown that the Tat complex must be located in the membrane for translocation of the substrates to take place. The complex will still form in the absence of the membrane anchoring region of TatB (Mangels *et al.*, 2005). However, the TatC component of the complex is unstable in the absence of TatB (Sargent *et al.*, 1999). A second complex has been purified composed of TatA and TatB that is approximately 600 kDa (Bolhuis *et al.*, 2000; de Leeuw *et al.*, 2002), the two proteins can be immunoprecipitated together (Sargent *et al.*, 2001). TatA has been purified in a pure complex of sizes ranging from 100-600 kDa (De Leeuw *et al.*, 2001; de Leeuw *et al.*, 2002; Oates *et al.*, 2005; Porcelli *et al.*, 2002). TatA has been shown to be in large molar excess to the other components of the translocation machinery (Jack *et al.*, 2001; Sargent *et al.*, 2001). The Tat system is likely to be modular and highly dynamic, each of the complexes that has been described is likely to exist in nature in a resting state and come together to form a gated aqueous membrane channel across the cytoplasmic membrane (Alami *et al.*, 2003; Mori and Cline, 2002). Multiple copies of the protein must be present within the channel due to the large substrates that it accommodates (Lee *et al.*, 2006).

A Tat substrate is translocated across the membrane in several steps. The first step is the binding of the TatBC complex to the substrate in an energy independent step (Cline and Mori, 2001; de Leeuw *et al.*, 2002). Site specific photoaffinity crosslinking experiments strongly suggest that TatC binds the consensus motif of the Tat signal peptide and therefore TatC is the

primary site of signal peptide recognition (Alami *et al.*, 2003). Once this has occurred energy is required to bind the complex to TatA. The complex of TatABC and the TAT substrate remain associated until the substrate molecule has been transported across the membrane. The signal peptide is removed once the protein has reached the periplasm and it has been shown that this even occurs late in the transport cycle (Mori and Cline, 2002).

In a genomic survey of the prokaryotic utilization of the twin-arginine translocation pathway, 84 genome-sequenced organisms were analyzed using TATFIND 1.2, an algorithm for predicting substrates of the Tat translocation apparatus (Rose *et al.*, 2002). Two *S. pneumoniae* and two *S. pyogenes* strains were incorporated in the search. Both the *S. pyogenes* strains and the R6 *S. pneumoniae* strain each contain one putative Tat translocated protein, but the *S. pneumoniae* strain TIGR4 did not appear to contain any Tat translocated proteins. None of the four strains appeared to encode the Tat translocation machinery (Dilks *et al.*, 2003). In *B. subtilis* secretion via Tat has been shown to only have a selective contribution to the total protein secretion of the organism. 14 of the 69 proteins with the predicted RR/KR-signal peptide motif were shown detectable in the growth medium. Only PhoD was secreted in a strictly Tat-dependent manner. Thirteen proteins of the RR/KR category were able to be secreted by strains containing mutations in several of the genes encoding the Tat machinery and secretion of three of these proteins was shown dependent on SecA for their secretion (Jongbloed *et al.*, 2002). TatABC appear to be absent from the genome of *S. uberis* as determined by BLAST searches using the amino acid sequences of the proteins from *E. coli* and *B. subtilis*. The genome also does not appear to encode for any

proteins containing the twin arginine motif as determined using the TATFIND server (Rose *et al.*, 2002).

1.2.4 Cellular location of secretion

Microscopy has allowed the location of secretion sites to be determined. These have been shown to vary between different bacterial species. The cellular location of the secretion machinery in *S. pyogenes* has potentially been determined (Rosch and Caparon, 2004), although there is literature contradicting the findings of Caparon and Rosch (Rosch and Caparon, 2004) by Carlsson et al., 2006 (Carlsson *et al.*, 2006). Caparon and Rosch determined the location of the site of secretion by analysing the cysteine protease, SpeB. Cells grown to late exponential phase were prepared for immunogold electron microscopy after they had been reacted with SpeB-specific polyclonal antiserum. A single intense focus of gold particles was seen located adjacent to the membrane suggesting that protein secretion occurs in a single microdomain in the cellular membrane. HtrA, a heat shock induced serine protease localised in a similar pattern (Rosch and Caparon, 2005), as did PhoZ, obtained from *Enterococcus faecalis*. These data indicated this phenomenon is not restricted to SpeB. The SecA subunit was also shown to be located exclusively within this site of secretion which was given the term ExPortal (Rosch and Caparon, 2004). Caparon and Rosch in both papers describing the ExPortal report that protein secretion machinery in *S. pyogenes* does not localize at the poles of the bacteria, but that a mechanism restricts their positioning on the cell (Rosch and Caparon, 2004; Rosch and Caparon, 2005). This possibly involves other factors involved in the processing of a fully functional secreted protein. How the secreted proteins circumnavigate around the cell after secretion is unknown. As previously mentioned evidence

contradicting the localisation of SecA to the ExPortal has been published by Carlsson et al., 2006 (Carlsson *et al.*, 2006). SecA was detected using immunogold electron microscopy employing anti-SecA raised against the *B. subtilis* protein which has been shown to detect SecA from *S. pyogenes*. SecA was found throughout the periphery of *S. pyogenes* and also in the cytoplasm. There was no evidence suggesting that the protein was located at a single site on the cell. It was suggested that the Sec machinery may be circumferentially distributed in *S. pyogenes* (Carlsson *et al.*, 2006), as has been described in *B. subtilis* (Campo *et al.*, 2004).

The correct localisation of the components to the site of the ExPortal has been shown to be essential for function of at least some proteins that are processed through it. HtrA is normally found anchored to the site of the ExPortal. The coding sequence of this protein was modified and instead of it becoming anchored to the membrane it was released into the extracellular space. In the presence of the modified form of HtrA it was found that the maturation of SpeB did not occur, indicating the essentiality of the correct localisation of HtrA to the ExPortal for function (Rosch and Caparon, 2005).

In contrast to the ExPortal model proposed for *S. pyogenes* (Rosch and Caparon, 2004; Rosch and Caparon, 2005), *B. subtilis* has been shown to contain multiple sites dedicated to Sec mediated protein export (Campo *et al.*, 2004). GFP fusions were used to show that the major components of the Sec machinery SecA and SecY localise on average to 3-10 sites per cell. These sites are near or in the cytoplasmic membrane. Detection of pre-AmyQ, a substrate of the Sec machinery, results in a similar pattern of localisation. The localisation pattern suggests that proteins responsible for translocation of secreted proteins in *B. subtilis* are

located in spiral like patterns along the cell. Results published for *E. coli* show a similar localisation pattern (Shiomi *et al.*, 2006). A complete overview of subcellular location of secretome components in Gram positive bacteria is described in Buist et al., 2006 (Buist *et al.*, 2006).

1.2.5 Retention and Cleavage

Once a protein has passed through the Sec complex, it is on the extracytoplasmic side of the membrane. From here the protein can be released in to the extracellular space, in which case the signal peptide is removed or the protein may be anchored to the membrane or cell wall depending on the motifs that are present within the protein.

1.2.5.1 Lipoproteins

Primary amino acid sequence can be used to distinguish between types of signal peptides. Lipoproteins become anchored to the cell membrane due to the presence of a well conserved four amino-acid motif which has been named a lipobox in the C-region (von Heijne, 1989). The lipobox motif contains an invariable cysteine residue that is lipid modified by the enzyme lipoprotein diacylglyceryl transferase (Lgt) (Sankaran and Wu, 1994) prior to cleavage of the signal peptide on the amino-terminal side of the cysteine by lipoprotein signal peptidase (Lsp) (Tokunaga *et al.*, 1985). The modification of the cysteine residue permits membrane retention and ensures that the protein is correctly anchored.

Lgt and Lsp are integral membrane proteins that are likely to be located within the sites described for protein secretion. Lsp is a novel aspartic peptidase (Dev and Ray, 1984; Tjalsma

et al., 1999b). Alignment of Lsp amino acid sequences from various Gram positive bacteria identified the presence of 15 conserved amino acid residues (Pragai *et al.*, 1997). Mutagenesis of Asp 102 and Asp 129 identified these two residues as being essential for the activity of the *B. subtilis* Lsp enzyme (Tjalsma *et al.*, 1999b).

In Gram positive bacteria, lipoproteins play vital roles in protein secretion; the uptake of nutrients, sporulation and germination, conferring resistance to certain antibiotics and targeting of the bacteria to different substrates such as host tissues (Sutcliffe and Russell, 1995). Several Gram positive bacterial lipoproteins have been shown to be involved in pathogenesis including *L. monocytogenes*, *S. pneumoniae* and *S. equi* (Berry and Paton, 1996; Hamilton *et al.*, 2006; Reglier-Poupet *et al.*, 2003b). The *S. uberis* protein MtuA is a predicted lipoprotein and carries a signal peptide sequence with a lipobox motif and this protein is also essential for virulence (Smith *et al.*, 2003).

Several studies of Lgt and Lsp in Gram positive bacteria have revealed that these proteins, or proteins processed through them play different roles and may affect infection scenarios in different ways. Studies using *lgt* mutants have shown very different results depending on the bacterial species studied. A study of an *lgt* knockout mutant in *S. pneumoniae* showed that this enzyme was not essential to this bacterium for cell growth, but was essential for viability during infection (Petit *et al.*, 2001). The maturase protein of *S. equi* was found to be required for causing disease in a pony challenge, but the *lgt* mutant was able to cause disease (Hamilton *et al.*, 2006). This suggests that for *S. equi* the proteins processed through Lgt are essential for allowing the establishment of infection compared to Lgt itself. This result would also suggest

that the correct processing of the maturase lipoprotein is not essential for the activity of this protein and the role that it plays in the infection model. An *lgt* mutant of *S. aureus* was shown to be attenuated in its growth abilities and in its activation of the immune system (Stoll *et al.*, 2005). An *B. subtilis lgt* mutant abolishes the modification of lipoproteins, but remains fully viable (Antelmann *et al.*, 2001; Leskela *et al.*, 1999). Interestingly, in a proteomic investigation of *B. subtilis* lipoproteins were found in the growth medium of the wild type bacteria. These proteins were shown to have been processed on the C-terminal side of the cysteine residue found within the lipobox motif(Antelmann *et al.*, 2001). In the *B. subtilis lgt* mutant other lipoproteins were also identified in the growth medium, which is not unexpected considering that the mechanism to anchor lipoproteins to the membrane has been removed.

Lsp mutants from various species have also revealed some interesting phenotypes. An *L. monocytogenes lsp* knockout mutant showed a reduced ability to escape the phagosome of macrophages (Reglier-Poupet *et al.*, 2003a). Whereas in *S. suis* an *lsp* mutant was shown to be as virulent as the wild type in an infection model (De Greeff *et al.*, 2003). However, when Lsp was studied in *M. tuberculosis* the *lsp* mutant was shown to be attenuated *in vivo* (Sander *et al.*, 2004). Lsp in *B. subtilis* has been shown to be required for growth at higher temperatures, but is not essential for cell viability (Tjalsma *et al.*, 1999a). Interestingly in this mutant the lipoprotein PrsA was not only visualised in the pre-form, but also in the processed form, suggesting that another enzyme is able to cleave the signal peptide from lipoproteins. A processed from of the *L. monocytogenes* lipoprotein LpeA was also seen in the *lsp* mutant of this species (Reglier-Poupet *et al.*, 2003a).

Processing of bacterial lipoproteins has been shown to be dependent upon both the signal peptide features that direct the proteins export and the lipobox motif. The lipobox sequence is typically seen as Leucine-3-Alanine/Serine-2-Glycine/Alanine-1-Cysteine+1. Analysis of Gram negative and Gram positive lipoprotein signal peptides reveal that these sequences are typically shorter than those of secretory signal peptides. This reflects the finding that the C-regions of these proteins are shorter and typically contain apolar amino acids. A consensus pattern has been described for proven lipoproteins of Gram positive bacteria, G+LPP. G+LPP is a computer assisted sequence analysis method and is highly discriminatory against sequences that have been shown to be false positives (Sutcliffe and Harrington, 2002).

1.2.5.2 Cleavage of the signal peptide sequence by the Signal Peptidases of the Type I class

The second type of signal peptidase that has been discovered within Gram positive bacterium is the Type I signal peptidase that recognises the signal peptides of all secreted proteins except lipoproteins. The type I signal peptidase contains an amino-terminal transmembrane anchor and a carboxyl-terminal catalytic domain. The signal peptide is cleaved by the signal peptidase when the C-domain of the secreted protein emerges on the extracytoplasmic side of the membrane (Tjalsma *et al.*, 2000a). Gram positive bacteria are likely to contain multiple type I signal peptidases, for example *B. subtilis* has five chromosomal and two plasmid encoded signal peptidases (Paetzel *et al.*, 2000). It is recognized that the different type I signal peptidases of *B. subtilis* have different substrate specificities. However, the molecular basis of these differences with respect to the composition of the signal peptide C-domain and the structure of the active site of the signal peptidase is currently unknown (Tjalsma *et al.*, 2000a).

In bacteria that contain a single signal peptidase type I, it has been shown that this is an essential enzyme to cell viability (Inada *et al.*, 1989). Conditional lethal temperature sensitive mutants were created in *E. coli* within the gene encoding the signal peptidase to facilitate the study of the structure-function relationship of the peptidase (Inada *et al.*, 1989).

The cleavage site for signal peptidase I has been termed Ala-X-Ala, this cleavage site is usually made up of small aliphatic residues that are often alanines, but other amino acids also appear in this site. The catalytic mechanism employed by type I signal peptidases is unconventional in the class of serine peptidases. These proteins instead of using the classical serine-histidine-aspartic acid triad for catalysis, use strictly conserved serine, lysine and aspartic acid residues and these have been shown to be essential for enzymatic activity (Tjalsma *et al.*, 2000b; Tschantz *et al.*, 1993; van Dijl *et al.*, 1995). Type I signal peptidases belong to a novel group of serine peptidases. A complete overview of type I signal peptidases is Van Roosmalen *et al.*, 2004 (van Roosmalen *et al.*, 2004)

1.2.5.3 Cell wall associated proteins

1.2.5.3.1 Sortase-catalyzed anchoring of surface proteins to the cell wall

Sortase is an enzyme that covalently attaches specific proteins at their carboxyl-terminal to the cell wall. The enzyme also cleaves a carboxyl-terminal cell wall sorting signal at a sequence that is generally made up of the amino acids L-P-X-T-G (where X can be any amino-acid). This cell wall sorting signal also requires a carboxyl-terminal hydrophobic domain; this is a putative membrane segment and a positively charged tail. Sortase is encoded by the *srtA* gene. However, in several bacterial species other sortases have been found (for example *S.*

suis; *srtB*, *srtC*, *srtD* and *srtE* (Osaki *et al.*, 2002)). The protein products of these genes are membrane-bound transpeptidases (Mazmanian *et al.*, 1999), which cleave the peptide bond between the threonine and glycine residues within the motif (Navarre and Schneewind, 1999; Ton-That *et al.*, 1999). The carboxyl group of the threonine residue is concurrently covalently linked to the cell wall by a branched anchor peptide. Approximately 30 carboxyl-terminal residues are cleaved and the rest of the protein remains exposed to the extracellular space.

Proteins that are attached to the cell wall by sortase are likely to contain an amino-terminal signal peptide that will direct the protein to the Sec-dependent secretion pathway (Navarre and Schneewind, 1999). Truncation of the cell wall sorting signal results in secretion of the mutant protein into the extracellular medium, whereas deletion of the LPXTG motif prevents cleavage and cell wall anchoring; the mutant polypeptides fractionate with the cell wall and the cytoplasmic membrane compartments (Mazmanian *et al.*, 2001).

Protein-A of *S. aureus* is proteolytically cleaved by sortase between the glycine and threonine residues in the classical sortase motif. This is initially tethered as a thioester-linked intermediate to the active site sulfhydryl residue of the sortase enzyme (Ton-That *et al.*, 1999). The intermediate is resolved by nucleophilic attack by the amino group of the pentaglycine cross-bridges within the peptidoglycan. This tethers the carboxyl-terminal end of Protein-A to the bacterial cell wall peptidoglycan (Perry *et al.*, 2002). Wild-type peptidoglycan which contains the NH_2-Gly_5 is the preferred substrate for the sorting reaction, however sortase is able to recognize some, but not all cross bridges as substrates for the sorting reaction (Mazmanian *et al.*, 2001). However, by altering the cross-bridges by mutagenesis of the genes

that encode the formation of crossbridges (*fem* operon), it has been shown that some altered cell wall cross-bridges can be linked to the carboxyl-terminal end of surface proteins (Ton-That *et al.*, 1998). There was a reduction in the anchoring to the cell wall of proteins with the LPXTG motif in the presence of a *femB* mutation which produces NH_2-Gly_3, but no NH_2-Gly_5 and also in the presence of *femA* and *femX* mutations which synthesise NH_2-Gly_1 or NH_2Gly_1/NH_{2L}-Lys crossbridges respectively. This result suggested that NH_2-Gly_5 is the preferred substrate of the sorting reaction. However, surface protein was found to be linked to the surface of proteins carrying mutations within the cell wall encoding genes (Mazmanian *et al.*, 2001). In *S. aureus* the precursor has been shown to be part of Lipid II (Ruzin *et al.*, 2002), however for *L. monocytogenes* further detailed experiments have shown that the protein is actually attached to meso-diaminopimelic acid which is part of Lipid II (Dhar *et al.*, 2000).

Sortase contains a single cysteine residue that has been shown to be essential for cleavage of the LPXTG motif *in vitro* (Ton-That *et al.*, 1999). A form of sortase carrying an amino acid substitution of this cysteine residue to alanine was unable to complement a sortase mutant (Ton-That *et al.*, 2002). A second essential amino acid was identified as histidine 120, if this amino acid was substituted the activity of the enzyme is abrogated (Ton-That *et al.*, 2002). This histidine residue was thought to hold the cysteine residue in its active state through a thiolate imidazolium pair (Ton-That *et al.*, 2002). However, using an irreversible inhibitor the Pk_as were predicted for the cysteine and histidine residues of approximately 9.4 and 7.0 respectively. These figures are inconsistent with the existence of a thiolate imidazolium pair and this has suggested a mechanism of general base catalysis during transpeptidation (Connolly *et al.*, 2003). The cysteine and histidine are conserved across species and suggest a

universal mechanism, although there must be adaptations across species to allow the attachment of sortase substrates to the different cell wall cross bridges (Dilks *et al.*, 2003; Ton-That *et al.*, 1999). Arginine 197 has also been shown to be essential for activity; this amino acid is located in close proximity to the cysteine residue and the scissile T-G peptide bond of the LPXTG motif has been shown to be positioned in the active site between these two residues (Marraffini *et al.*, 2004; Zong *et al.*, 2004).

Microbial genomes can be screened for the presence of motifs that identify them as being part of a group or class of proteins (Boekhorst *et al.*, 2005; Janulczyk and Rasmussen, 2001). The sensitivity of an LPXTGX pattern search for sortase cleaved proteins in Gram positive bacteria was improved by designing a tripartite pattern (Janulczyk and Rasmussen, 2001). This was designed by accumulating data from the carboxyl-terminal end sorting signals of 65 cell wall proteins. The three parts were designated the sortase target region, a membrane spanning region and a charged tail region. This pattern was shown to be 98% sensitive compared to the 80% sensitivity that was shown when the LPXTGX motif alone was used to search for sortase cleaved proteins. The three parts of the sequence are the sortase target region, a membrane-spanning region and a charged tail region. By using a pattern search a list of atypical sequences can be compiled that may lead to a more detailed understanding of the sortase mechanism of action (Janulczyk and Rasmussen, 2001). A second search using LPXTG HMM which when used in combination with the hframe algorithm over 99% of the substrates, which have been detected by a series of other methods were predicted (Boekhorst *et al.*, 2005). A different search pattern was used to characterize novel LPXTG-containing proteins of *S. aureus*. Twenty-one genes were identified *in silico* from six *S. aureus* genomes;

ten genes had not yet been described. Two of the proteins SasF and SasD contain an LPXAG motif and were shown to undergo the normal sorting reaction (Roche *et al.*, 2003). This provides evidence that under some circumstances the sortase enzyme allows for leniency in the motif or the presence of multiple sortase enzymes that recognize slight differences within the motifs.

Multiple sortases have been shown to be present in many of the genomes that have been sequenced so far. All *S. aureus* strains that have been examined to date contain two sortase genes, *srtA* and *srtB*. SrtA is responsible for anchoring regular LPXTG-motif containing proteins to the cell wall, for example Surface Protein A (Mazmanian *et al.*, 2001), whereas SrtB has been shown to be necessary for anchoring IsdC which contains an NPQTN motif. The gene is part of a locus that is iron regulated called iron-responsive determinants (isd). The locus contains a ferrichrome transporter and surface proteins containing NPQTN and LPXTG motifs. Upstream of the transcriptional unit is a Fur box, which allows for transcription to be terminated when the bacteria is in the presence of high extracellular iron concentrations. Two LPXTG containing proteins are adjacent to this operon; these two proteins are regulated by Fur and are transcribed from independent promoters. *isdC* is the first gene in the operon and the protein which it encodes contains the SrtB sorting signals. SrtB has been shown to be required for *S. aureus* persistence during host infection (Mazmanian *et al.*, 2002). The genome of *S. suis* strain NCTC 10234 contains five *srtA* homologues (Osaki *et al.*, 2002). This is more than has been described for other Streptococci, for example four in *S. pneumoniae* and one in *S. mutans*. Across the sortase family there is a low level of homology (Pallen *et al.*, 2001). This could be explained due to the different substrates that sortases

process and also due to the diversity of cross bridge structures that differ between species due to the variation of compounds that make up the cell wall.

In *S. pyogenes* an unusual enzyme has been identified and purified that is able to cleave the LPXTG anchor motif (Lee *et al.*, 2002). It is very different from the sortase enzyme of *S. aureus* due to its glycosylation and the presence of unusual amino acids. The enzyme has been given the term LPXTGase (Lee *et al.*, 2002). The enzyme is glycosylated and the carbohydrate moiety appears to be essential for the enzymes activity. The backbone of the enzyme is very hydrophobic and it has been hypothesized that this part is embedded in the cell membrane. The LPXTG motif is present within the peptide KRPLPSTGETANPFY; Lee et al., 2002 (Lee *et al.*, 2002) used this peptide to show that the LPXTGase cleaves this sequence twice, after the serine and glutamic acid residues. A small molecule is produced by *S. pyogenes* that is able to inhibit the activity of the LPXTGase; it is likely to have an important regulatory function within the cell. *S. pyogenes* grown in the presence of the inhibitor fails to display M protein and fibronectin binding protein on the cell surface. Similar enzymes and inhibitors have been hypothesized to appear in other Gram positive proteomes (Lee *et al.*, 2002).

Sortase has been shown to be important for the virulence of some pathogenic bacteria; this is likely due to the importance of the proteins carrying LPXTG motifs and they must be correctly processed for their activity. An *S. aureus srtA* mutant in a mouse infection model was found to be reduced in its virulence properties compared to the wild type (Weiss *et al.*, 2004). Interestingly the *srtB* mutant of *S. aureus* is able to cause infection in a renal abscess model to

the same extent as the wild type, but is attenuated in its ability to persist in the infection (Mazmanian *et al.*, 2002). The *S. pneumoniae srtA* mutant is the only *srtA* mutant to date that is not effected in an animal infection model, however this mutant was shown to release cell wall proteins and it was also reduced in its ability to adhere to human pharyngeal cells *in vitro* (Kharat and Tomasz, 2003). There is currently much interest in sortase as a drug target to allow the control of Gram positive infections.

Within staphylococcal lipases the motif YSIRK-G/S was first observed and is situated in the signal peptide region of the preprotein (Rosenstein and Gotz, 2000). The same motif has also been identified in proteins of other Streptococci, including *S. pneumoniae* (Bae and Schneewind, 2003). However, the motif is absent in Listeriae and Bacilli genus (Bae and Schneewind, 2003). The motif was found within signal peptides of proteins bearing C-terminal cell wall sorting signals with the LPXTG motif. The YSIRK-G/S motif has been shown to play a role in the efficiency of the secretion of Protein A of *S. aureus*, but is not absolutely essential for precursor protein translocation. The motif is dispensable for the cell wall anchoring of surface proteins (Bae and Schneewind, 2003). It is feasible that proteins containing the YSIRK-G/S motif represent a subset of secretion substrates that require dedicated factors for efficient translocation.

1.2.5.3.2 Choline binding proteins

Terminal choline residues of the teichoic/lipoteichoic acids present on the surface of Gram positive bacteria bind a group of proteins that contain two to ten copies of an approximately 20 amino acid repeat found in the carboxyl-terminal region of the protein. This repeat region is

usually found connected to the main part of the protein via a proline-rich, flexible linker. The rest of the protein is made up of an amino-terminal active domain and a short tail on the carboxyl terminal. Binding of these proteins is through a non-covalent bond, which can be removed. Choline binding proteins have been found in *S. pneumoniae* and include Pneumococcal Surface Protein A (PspA), the bacterial peptidoglycan hydrolases; LytA, LytB and LytC and also CbpA a protein adhesin responsible for binding to human cell glyconjugates (Rosenow *et al.*, 1997). This method of displaying proteins on the cell surface is essential for many features of bacterial virulence (Gosink *et al.*, 2000). The amino-terminal region of the protein PspA is made up of an α-helical coiled coil structure to which the host is able to raise antibodies. The use of this protein as a vaccine against pneumococcal infections is currently under consideration (Jedrzejas *et al.*, 2001).

LytA is the major pneumococcal autolysin and responsible for the release of toxic substances into the extracellular environment. The carboxyl-terminal of the enzyme is constructed of six repeated units of 20 or 21 residues; this can be isolated from the protein and has been shown to specifically bind to the choline residues present in the pneumococcal envelope. Serial deletions of the repeat units have been made and biochemical characterization of the truncated proteins revealed that LytA must contain at least four of the units to efficiently recognize the choline residues of the cell wall (Garcia *et al.*, 1994).

Choline binding proteins were identified from the genome of *S. pneumoniae* using a search with the 180 amino acid choline-binding domain from CbpA (Gosink *et al.*, 2000). The six previously known proteins were identified along with seven other open reading frames

containing sequences with homology to the choline-binding domain. None of these sequences, like most other choline-binding proteins, contain a recognizable secretion signal sequence. Consequently, the mechanism of secretion of this type of protein is uncertain (Gosink *et al.*, 2000). Mutations in the newly discovered choline-binding proteins of *S. pneumoniae* have shown that these proteins play roles in adhesion and colonization of the nasopharynx. The six new proteins discovered have unique amino-terminal domains that indicate varied functions (Gosink *et al.*, 2000).

Choline binding proteins have a large effect on the chemical nature of the subcapsular compartment of the pneumococcal cell and enhance the interaction with eukaryotic cells. This is due to the hydrophobic nature of an unencapsulated cell that does not display choline-binding proteins on their cell surface. A cell that lacks the choline-binding proteins is negatively charged; the net positive charge of the choline-binding proteins may have the effect of stabilizing the negatively charged polysaccharide polymers. PspA is known to have an overall positive charge on the surface exposed region and this may allow its functional domain to appear above the capsular surface (Swiatlo *et al.*, 2002). These results show how important it is for a cell to correctly process the proteins that it requires for interacting with host cells.

1.2.5.3.3 Cell wall binding of Type 2

The cell wall binding domain of the type 2 are found in multiple copies, approximately 100. They are present in the *B. subtilis* autolysin CwlB (Ishikawa *et al.*, 1998a; Ishikawa *et al.*, 1998b) and the *Clostridium difficile* Cwp66 (Waligora *et al.*, 2001). The domain is responsible for binding the protein to the cell wall, but the components that are responsible for

this binding have yet to be identified. This type of protein has only been shown to be present in the Bacillacae and Clostridiacae species (Desvaux *et al.*, 2006).

1.2.5.3.4 Lysin motif domain

The Lysin motif domain (LysM) binds directly to the peptidoglycan and is 40 residues in length (Steen *et al.*, 2003). They are present as multiple tandem copies of 1-6 and are generally located in the N-terminal or C-terminal regions of the protein. Over 1500 proteins have been found that contain LysM domains, including both eukaryotic and prokaryotic cells. The enzyme activities of these proteins include cell wall degradation, peptidases, chitinases, esterases, reductases and nucleotidases. These proteins can also act as antigens or binding proteins to albumin, elastin or immunoglobulin (Desvaux *et al.*, 2006).

1.2.5.3.5 GW modules

The *L. monocytogenes* protein InlB contains a GW domain that is approximately 80 amino acids long and contains the dipeptide Gly-Trp (Braun *et al.*, 1997). The motif is found in multiple copies, three GW modules are found in the C-terminal region of InlB. However, there are 8 GW modules in the Ami protein which has an amidase activity. The higher the number of GW modules present within the protein the stronger the attachment to the bacterial cell wall (Desvaux *et al.*, 2006).

1.2.6 Extracytoplasmic Folding Factors

After a protein has been translocated and the signal peptide removed, it must be folded into its native conformation. Many folding factors have been identified including chaperones,

peptidyl prolyl cis/trans isomerases and thiol-disulphide oxidoreductases. The major folding factor in *B. subtilis* is the PrsA protein; this belongs to the parvulin family of PPIase's (Rahfeld *et al.*, 1994). This protein is a lipoprotein anchored to the cytoplasmic membrane. PrsA and its homologues, (PmpA *L. lactis* (Drouault *et al.*, 2002)) catalyse the cis/trans isomerisation of the peptidyl-proline bonds in oligopeptides and proteins. Over production of these proteins significantly increases the amounts of correctly folded proteins. In bacteria where PrsA has been mutagenised, extensive degradation of protein was seen (Wahlstrom *et al.*, 2003).

1.3 Exported proteins and in particular lipoproteins are required in colonisation and virulence

Many exported proteins have been found to be required or are essential for colonisation and virulence of Gram positive pathogens as can be seen from the above description of the mechanisms that these microorganisms employ to ensure the correct localisation of these proteins. Lipoproteins in particular have raised interest, as they have been implicated not only in being essential to causing infection, but are also responsible for raising an immune response to the infecting pathogen (Brown *et al.*, 2001; Jomaa *et al.*, 2005; Jomaa *et al.*, 2006; Salim *et al.*, 2005).

Many lipoproteins belong to operons involved in the acquisition of essential nutrients. Iron uptake systems are virulence factors in many bacterial pathogens, making them attractive vaccine targets. Several iron uptake systems have been characterised in Streptococcal species. HtsA is a cell surface exposed lipoprotein and a member of an operon involved in heme

acquisition in *S. pyogenes* with homologues in *S. equi* (Lei *et al.*, 2003; Nygaard *et al.*, 2006). *S. pyogenes* has a second operon involved in the uptake of iron; FtsABCD involved in the uptake of iron Fe^{3+} (Hanks *et al.*, 2005). The sirABC operon of *S. aureus* is involved in the import of siderophores. Two loci in *S. pneumoniae* have been identified as being responsible for uptake of iron, PiaA and PiuA, both have been shown to be involved in the virulence of this pathogen (Brown *et al.*, 2001). Active or passive immunisation with recombinant PiaA and PiuA protected mice against invasive *S. pneumoniae* disease due to increased levels of opsonophagocytosis (Jomaa *et al.*, 2005; Jomaa *et al.*, 2006).

Other nutrient or metal transport systems that include lipoproteins have been studied in several Gram positive pathogens. PsaA of *S. pneumoniae* is the lipoprotein of a manganese transportation system and essential for the virulence of *S. pneumoniae* in mice (Berry and Paton, 1996). Homologues of this protein have been studied in *S. pyogenes* (MtsA) (Janulczyk *et al.*, 2003), *S. uberis* (MtuA) (Smith *et al.*, 2003) and *S. gordonii* (Sca) (Jakubovics *et al.*, 2002). This protein has been shown to be able to illicit an immune response, but is not surface expressed and has therefore been disregarded as a vaccine candidate in *S. pneumoniae* (Briles *et al.*, 2000; Johnston *et al.*, 2004). MtuA has also been shown not to be expressed on the cell surface of *S. uberis* (Jones *et al.*, 2004). A mutant in the manganese transporter, MntA of *Bacillus anthracis* is severely attenuated in virulence and this mutant is being investigated as an attenuated vaccine (Gat *et al.*, 2005). Ami AliA/AliB is an oligopeptide permease ATP binding cassette of *S. pneumoniae* that has been shown to be involved in nutrient uptake and is required for the successful colonisation of the nasopharynx. However, in animals where these mutants persisted, survival times, bacterial loads and

inflammatory cytokine production levels were similar to those of animals infected with the wild type bacteria (Kerr *et al.*, 2004).

Correct folding of proteins is essential for their function. Streptococcal Lipoprotein Rotamase A (SlrA) of *S. pneumoniae* is a functional peptidyl prolyl isomerase involved in pneumococcal colonisation (Hermans *et al.*, 2006). A rapid elimination of *slrA* mutants from the upper airways of a mouse infection model was seen (Hermans *et al.*, 2006). The numbers of bacteria seen in the lungs, blood and spleen were comparable to the wild type suggesting that this protein is not involved in invasive pneumococcal disease. The *S. equi* putative maturase PrtM which is homologous to the *S. pneumoniae* Putative Proteinase Maturation protein A (PpmA), a potential vaccine target (Overweg *et al.*, 2000), is unable to cause disease in a pony challenge model, but an *lgt* mutant is virulent suggesting that this protein is essential for disease, but its correct processing by the lipoprotein processing pathway is not essential for its function (Hamilton *et al.*, 2006). DsbB a lipoprotein of *S. aureus* has been shown to be responsible for the formation of disulphide bonds, however no phenotype for this mutant *in vivo* or *in vitro* has yet been identified (Dumoulin *et al.*, 2005).

Lipoproteins have been identified as antigenic determinants using *in vivo* induced antigen technology (IVIAT). The IVIAT technique creates a genomic expression library of the pathogen of interest. Convalescent serum is then adsorbed against *in vitro* grown organisms; the genomic expression library is then probed against this adsorbed serum. Reactive clones are identified, the DNA insert sequenced and the gene product of this insert identified. The *in vivo* or *in vitro* expression of the reactive genes is measured and the potential role for these

within virulence and pathogenesis can be evaluated (Rollins *et al.*, 2005). In *S. pyogenes*, the lipoprotein AtmB was identified and has been shown to be involved in virulence (Salim *et al.*, 2005). AtmB has been implicated to have a role in evasion or defence against the immune system and is expressed early in the infection process (Salim *et al.*, 2005).

Colonisation of new surfaces is beneficial for bacterial survival. Many bacteria form biofilms and mutational analysis of the *adcCBA* operon of *S. gordonii* leads to a reduced ability to form biofilms and the competence of these mutants was also shown to be affected (Mitrakul *et al.*, 2005).

Several other lipoproteins currently being investigated as potential vaccine targets include VapA of *Rhodococcus equi*; immunisation of pregnant mares resulted in passive antibody mediated protection of foals (Cauchard *et al.*, 2004). The genome of *S. pyogenes* was analysed for potential lipoproteins, 16 of these were investigated as vaccine antigens and of these, 5 had significant inhibitory activity compared to the serum of non-vaccinated mice (Lei *et al.*, 2004). These five proteins included the lipoprotein component of a ferrichrome uptake ABC transporter, protease maturation protein PrtM, a thioredoxin homologue that is probably involved in the repair of oxidative damage to proteins, the lipoprotein component of an ABC transporter predicted to be involved in the uptake of amino acids and a member of phosphate transport system.

Immune responses that are raised against pathogenic organisms occur due to recognition of bacterial components. Pathogen associated molecule patterns (PAMP's) bind to dedicated

Toll like receptors (TLR's) or Nod proteins, leading to specific signalling events of the host's response to the infecting bacteria. TLR2 knockout mice are hugely susceptible to intravenous infection by *S. aureus* (Takeuchi *et al.*, 1999). Bacterial lipoproteins can act as PAMPS (Bubeck Wardenburg *et al.*, 2006) and a mutant in *lgt,* the enzyme essential for anchoring these proteins to the cell membrane is able to escape recognition by the immune system. Mice infected with this mutant displayed disseminated abscess formation with increased lethality compared to the wild type during the infection process (Bubeck Wardenburg *et al.*, 2006). This is due to the lipoproteins being essential in initiating and sustaining an immune response to the infecting *S. aureus*. The NFκB response is not induced in the *lgt* mutant; because bacterial lipoproteins function through TLR2 in an NFκB dependent manner to induce the production of inflammatory cytokines essential for clearing the infection. Fewer inflammatory cytokines were induced when mice were infected with an *lgt* mutant or a crude lysate prepared from the mutant (Stoll *et al.*, 2005). An *lsp* mutant in this study did not develop acute lethal disease and was attenuated compared to the wild type (Bubeck Wardenburg *et al.*, 2006). This mutant had a severe defect in its ability to multiply in liver tissue. Suggestions as to why the *lsp* mutant in this study is attenuated in virulence were not offered.

The above description of lipoproteins clearly shows that they have a fundamental role in the pathogenesis of disease and are worth investigating as vaccine or drug targets. Many lipoproteins appear to be involved in colonisation of a new host niche; therefore vaccination with these proteins that are expressed early in the infection process by these pathogens are likely to prevent an infection in taking hold of the host. However, identification of proteins for new vaccination strategies is challenging. Approximately 2% of bacterial genomes are

made up of potential lipoproteins (Antelmann *et al.*, 2001; Sutcliffe and Harrington, 2002), the majority of which are yet to be studied. In *S. uberis* the predicted lipoprotein manganese transporter MtuA has been previously studied and is essential for virulence in the dairy cow challenge model (Smith *et al.*, 2003). As a predicted lipoprotein, it is expected that MtuA will be processed through Lgt and Lsp. It is possible that by studying the enzymes responsible for processing lipoproteins, one could identify further lipoproteins required for the pathogenesis of *S. uberis*. Growing mutants in bovine skimmed milk provides a guide as to whether a particular mutant may be impaired in a challenge model. Anti-MtuA sera are available and would provide a useful tool with which to investigate the phenotypes of lipoprotein processing mutants and the location of lipoproteins processed through this pathway.

1.4 Aims and Objectives

The aim of this project was to investigate and thus further understand the processing of lipoproteins in the economically important pathogen, *Streptococcus uberis*.

1. Identify potential lipoproteins from the genome of *S. uberis* strain 0140J through the use of bioinformatics.
2. Characterise *lgt* and *lsp* mutants with respect to processing of lipoproteins processed using MtuA as an example lipoprotein.
3. Identify other proteins that may be involved in the processing of lipoproteins.

Chapter 2

Materials and Methods

2.1 Bacteria growth conditions

S. uberis was routinely grown on Todd Hewitt Agar plates and in Todd Hewitt broth (Oxoid, Basingstoke, United Kingdom) at 37°C as standing cultures. Uncured pGh9$^+$::IS*S1* mutants were grown in the same media with the addition of 1 μg/ml erythromycin.

2.2 Bacterial Strains

Table 2 describes the bacterial strains used in this study.

Strain	Characteristics	Reference
S. uberis 0140J	Isolated from a clinical case of bovine mastitis, UK. Genome sequenced strain, Sanger Centre, UK.	ATCC reference number BAA854
S. uberis IS*S1*::*lgt*$_1$	IS*S1* element 416 bp from start codon	This study
S. uberis IS*S1*::*lgt*$_2$	IS*S1* element 729 bp from start codon	This study
S. uberis IS*S1*::*lgt*$_3$	IS*S1* element 13 bp outside *lgt* coding sequence	This study
S. uberis lsp::IS*S1*	IS*S1* element 132 bp from start codon	This study
S. uberis lgt/lsp::IS*S1*	IS*S1* elements 546 bp and 132 bp from start codon of *lgt* and *lsp* respectively	This study
S. uberis eep::IS*S1*	IS*S1* element 34 bp from start codon	This study
S. uberis lsp/eep::IS*S1*	IS*S1* element 132 bp and 362 bp from start codon of *lsp* and *eep* respectively	This study
S. uberis 0140J/pGh9$^+$::IS*S1*		(Ward *et al.*, 2001)

Table 2 Bacterial strains used in this study

2.3 Oligonucleotide primers used in this study

Primer	Sequence 5' – 3'	Use for Primer
P82 Tm 55.0°C	CCAACAGCGACAATAATCACATC	IS*S1*, antisense 5' region for flanking sequences
P247 54.0°C	GCTCTTCGGATTTTCGGTATC	IS*S1*, sense, 697bp from 5' end 112bp from 3'
P348 Tm 53.2°C	AGGACGAAATGTTTCAGTTG	*lgt* from 5' end
P349 Tm 55.3°C	AGATAACCAGTGCCACAAAG	*lgt* from 3' end
P350 Tm 54.7°C	TCAATCGTCTTAATCATGTAGC	Alternative *lsp* from 5' end
P351 Tm 55.3°C	TGGTATTTCTTGAGCTAGGC	Alternative *lsp* from 3' end
P432 Tm 54.0°C	GCTCTTCGGATTTTCGGTATC	*lsp* from 5' end
P571 Tm 55.2°C	AATATCTTCAGCTTCATAATCC	*lsp* from 3' end
P358 Tm 57.0°C	CATTTTCCACGAATAGAAGGACTGTC	IS*S1*, antisense, 298bp from 5' end
IS*S1*-F Tm 54.0°C	TGTGATTATTGTCGCTGTTGG	IS*S1* sense probe for Southern blotting
IS*S1*-R Tm 54°C	GATACCGAAAATCCGAAGAGC	IS*S1* antisense probe for Southern blotting
eep Fwd Tm 50°C	TCAGTTTGGAGACTTAGTGG	*eep* from 5' end
eep Rev Tm 46°C	TTTCATTTATCTGTATTTCTCC	*eep* from 3' end
pauA Fwd Tm 55°C	ATAAGGGAGGATCCATGAAAAAATGG	*S. uberis pauA* 5' end
pauA Rev Tm 55°C	CTCCAATGGATCCTATGACTG	*S. uberis pauA* 3' end

Table 3 Oligonucleotide primers used in this study

2.4 *S. uberis* mutagenesis techniques

2.4.1 Isolation of mutants from an *S. uberis* strain 0140J pGh9$^+$::IS*S1* mutant bank

PCR amplification was carried out on DNA from pools of 96 mutants representing a single microtitre tray from an *S. uberis* strain 0140J pGh9$^+$::IS*S1* mutant bank, comprised of 8800

individual clones (Taylor *et al.*, 2003). A screen for IS*S1* insertions at a specific locus was carried out by PCR using IS*S1* and gene specific primers (Table 3) as first described by Taylor et al., 2003 (Taylor *et al.*, 2003). Amplification was carried out using 2 U Taq Polymerase (New England Biolabs). Each pooled DNA sample was screened twice using the gene specific primer with either P247 or P358, to enable identification of either orientation of IS*S1* insertions. Following identification of a pool containing an appropriate candidate mutant, a location (well coordinate) was identified by the same PCR using smaller overlapping pools of mutant clones comprised from the appropriate tray's rows and columns. Once an individual well had been identified, the clone from that well was cultured to obtain single colonies the presence of the insertion checked by the same PCR procedure described above.

Excision of the pGh9 vector was promoted by growth at the permissive temperature (28°C) without antibiotic selection. Loss of the vector was assessed by repetition plating at 37°C in the presence and absence of erythromycin. Loss of the vector and retention of a single copy of IS*S1* were confirmed by Southern blotting with a probe complementary to IS*S1* and generated with primers IS*S1*-F and IS*S1*-R. The exact location of IS*S1* was determined by sequencing PCR products that spanned the insertion with the IS*S1* specific primer (P247 or P358). Sequence analysis was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, United Kingdom), according to the manufacturer's instructions. Sequence determination was carried out by L. Richardson (University of Oxford, UK).

2.4.2 Preparation of Chromosomal DNA from *S. uberis*

Chromosomal DNA from 3 ml of culture was prepared by using a variation of the method of Hill and Leigh (Hill and Leigh, 1989). Bacteria were harvested by centrifugation (10,000 x g, 5 min), and the cell pellet washed with 500 µl of 10 mM Tris-Cl, 5 mM EDTA (pH 7.8). Bacterial cell walls were disrupted by resuspending in 375 µl of 10 mM Tris, Cl-5 mM EDTA (pH 7.8) containing 30 U/ml mutanolysin (Sigma) and 10 mg/ml lysozyme (Sigma). The bacterial suspension was incubated at 37°C for 30 min. Total cell lysis was achieved by addition of 20 µl of a sodium dodecyl sulphate (SDS) solution (20% [wt/vol] in 50 mM Tris-Cl, 20 mM EDTA pH 7.8) and Proteinase K (Sigma) at a final concentration of 150 µg/ml and incubated at 37°C for 1 h. Cell wall material was precipitated by the addition of 200 µl of saturated NaCl solution and subsequent centrifugation (13,000 x g, 15 min, 4°C). The cleared supernatant was transferred to a clean tube and contaminating proteins were removed by the addition and subsequent removal of phenol-chloroform. The DNA was precipitated by addition of 2 volumes of absolute ethanol. DNA pellets were washed with 70% aqueous ethanol and air dried prior to resuspension in Tris-EDTA buffer containing 20 µg/ml of RNase A (Sigma).

2.4.3 Southern Blotting to determine location and random distribution of pGh9::IS*S1* insertion

Chromosomal DNA was prepared from mutants as previously described in 2.4.2. The quantity of DNA was quantified spectrophotometrically and typically 10 µg of DNA was digested using either the restriction enzyme *Hind*III or *Eco*RI overnight at 37°C. Half of the digested DNA (5 µg) was visualized on 0.8% (w/v) agarose in TAE. The agarose gel was immersed in

denaturing solution (1.5M NaCl, 0.5N NaOH) for 30 minutes. The digested DNA was transferred overnight on to Hybond N+ nitrocellulose membrane (GE Healthcare, Life Sciences), prewetted with 3X SSC (20 X SSC: 3M NaCl, 0.3 M sodium citrate (citric acid); 1 litre H_2O). This was overlaid with 3 sheets of 3MM prewetted with 3X SSC; wicking towels and a light weight placed on top of the blot. The membrane was washed with 3X SSC. Using 1ml pre-hybridisation solution per 20 cm^2 (6X SSC, 5X Denhardt's solution (Sambrook et al., 1989), 0.5% SDS and 50 µg/ml final concentration denatured salmon sperm DNA (Sigma)) the membrane was pre-hybridised at 65°C with rotation in a hybridisation oven for 2 hours. The pre-hybridisation solution was replaced with 1-1.5X volume of fresh pre-hybridisation solution pre-warmed to 65°C containing denatured DIG-labelled IS*51* probe at approximately 10 ng/ml and left overnight in a hybridisation oven with rotation at 65°C. The membrane was washed as follows; 2X SSC, 0.1% SDS 250 ml 2 x 5 minutes at room temperature and 0.2X SSC, 0.1% SDS 50 ml 2 x 10 minutes at 68°C. The membrane was probed as follows; Malate buffer (100 mM Maleic acid (11.61 g/l), 150 mM NaCl (8.77 g/l), pH adjusted to 7.5 with NaOH) (1 minute, room temperature), Blocking solution (Roche Diagnostics Ltd) (Diluted stock solution 1/10 with Malate buffer. Stock blocking solution 10% w/v Boehringer Blocking Reagent (Roche) in Malate buffer) (30 minutes, RT), Anti-DIG-AP conjugate (Roche) 1/5000 dilution in Blocking solution (30 minutes, room temperature), Malate buffer (2 x 15 minutes, room temperature). The blot was equilibrated in developing solution (0.1 M Tris-HCl, 0.1 M NaCl pH 9.5, autoclaved and filter sterile $MgCl_2$ added to 50mM final concentration) (2 minutes, room temperature). The blot was developed using NBT/BCIP Stock Solution 200 µl/10 ml (Roche Diagnostics Ltd) developing solution without shaking and in subdued light until bands were at appropriate intensity. The development of the blot was

terminated using Stop solution (50 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0) for 30 minutes prior to air drying.

2.4.4 Construction of pGh9$^+$::IS*S1* mutant bank in mutant background

2.4.4.1 Purification of pGh9$^+$::IS*S1* plasmid from *S. uberis* carrying the plasmid extrachromosomally

Bacteria from a 100 ml overnight culture (grown at 28°C with antibiotic selection) of *S. uberis* carrying the pGh9$^+$::IS*S1* plasmid extrachromosomally were harvested by centrifugation (10,000 g, 10 minutes). The pellet was washed in 10 ml 10 mM Tris-Cl-5 mM EDTA (pH 7.8). Bacterial cell walls were disrupted by resuspension in 5 ml of 10 mM Tris-Cl-5 mM EDTA (pH 7.8) containing mutanolysin (Sigma) 30 U/ml and lysozyme (Sigma) 10 mg/ml and incubated (37°C, 30 minutes). The plasmid was extracted from the suspension using the Miniprep kit (Qiagen) procedure as described by the manual. Briefly, 1 ml P1 buffer containing RNase A was mixed thoroughly with the bacterial suspension. Cell lysis was achieved by adding 1 ml P2 buffer and inverting the tube 4-6 times. To precipitate cell debris 1.5 ml N3 buffer was added and the tube was inverted 4-6 times. The cell debris was pelleted by centrifugation (13,000 x g, 10 minutes, 4°C). The resulting supernatant was applied to 6 QIAprep Spin Column and centrifuged (13,000 x g, 1 minute), the collection tube was emptied and the column washed with 0.5 ml PB buffer and centrifuged (13,000 x g, 1 minute). The column was washed further with 750 µl PE buffer, centrifuged and the flow through discarded. The column was centrifuged again before being placed in a 1.5 ml Eppendorf tube. The plasmid DNA was eluted by adding 30 µl of elution buffer (EB buffer, Qiagen) to the

centre of the column. The column was allowed to stand for 1 minute before being centrifuged (13,000 x g, 1 minute) and stored at -20°C.

2.4.4.2 Transformation of pGh9^{+}::IS*S1* into *S. uberis* cured mutants

2.4.4.2.1 Production of Electrocompetent *S. uberis*

S. uberis to be electroporated were grown in THB broth to mid-exponential phase, $O.D._{550nm}$ of 0.5. Cells were harvested by centrifugation (4000 x g, 15 minutes, 4°C). The cell pellet was resuspended in an equal volume of chilled sterile 10% glycerol on ice. The centrifugation and resuspension was repeated in successively smaller volumes of 10% glycerol until desired concentration of cells was achieved. Aliquots of cells were flash-frozen on dry ice/ethanol and stored at -70°C.

2.4.4.2.2 Electroporation of *S. uberis*

Electrocompetent bacterial cells were defrosted on ice before being transformed with 1-10 ng plasmid DNA in a volume of 2-10 µl of EB buffer. The DNA was mixed with the electrocompetent bacteria on ice and transferred into a chilled 0.1 cm electroporation cuvette (Biorad). The following electroporation conditions were used in the Gene Pulser (Biorad) 25 µF, 10 Ω, 2.4 KeV which gave time constants of approximately 2.1 ms. The electroporated cells were resuspended in 0.5 ml warmed THB and transferred to a sterile tube. The bacteria were put at 28°C to recover for 2 hours without antibiotic. A range of dilutions were plated on THA in the presence and absence of erythromycin and grown at 28°C to determine the transformation efficiencies.

2.4.4.2.3 Transposition of pGh9$^+$::IS*S1* into target strain

A single colony of *S. uberis* carrying pGh9$^+$::IS*S1* was inoculated into 10 ml THB containing 1 µg/ml erythromycin and grown overnight at 28°C. The bacteria were diluted 1/100 in THB without antibiotic and grown at 28°C until an O.D.$_{550nm}$ of 0.2 was reached. The culture was shifted to 37.5°C in a water bath and grown for a further 2.5 hours, before being stored at -70°C with the addition of 15% final volume glycerol. To check for transposition efficiency bacteria were diluted in THB and plated on THA plus and minus erythromycin and grown overnight at 37°C. The random nature of insertion was determined by Southern blotting as previously described.

2.4.4.3 Gridding of Random mutant bank

A randomly mutagenised population of bacteria were diluted in THB to 10^{-4}/ml and 800 µl spread on to pre-warmed THA plates in 245 x 245 x 25 mm bio-assay dishes (Nunc). Plates were incubated at 37°C for 24 h. Colonies were transferred into individual wells of 96-well microtitre trays (Nunc) containing 150 µl THB with 1 µg erythromycin and 10% glycerol by using a Flexys robotic workstation (Genomic Solutions, Ann Arbor, Mich.). Cultures were incubated at 37°C for 24 h before being stored at -70°C.

2.5 Growth Curves

2.5.1 Growth curves in Todd Hewitt Broth

Overnight cultures of *S. uberis* were transferred as 1:100 dilutions to fresh THB. The culture was incubated at 37°C without shaking. The absorbance of the culture was measured at O.D.$_{550nm}$ on the Pharmacia Ultrospec III or at O.D.$_{540nm}$ on the Bioscreen C Reader (Growth

Curves Ltd, Finland). Growth curves were determined by time course measurements from 0 to 24 h.

2.5.2 Growth Curve in Milk

An overnight culture of *S. uberis* was transferred to skimmed milk at a starting concentration of 10^3 to 10^4 c.f.u./ml. The culture was incubated at 37°C without shaking. Bacteria were serially diluted and plated on to SAE. Growth curves were determined by time course measurements of viable counts from 0 to 24 h.

2.6 Preparation of proteins from *S. uberis*

2.6.1 Preparation of Whole Cell Lysates

Bacteria were harvested from cultures (2 ml – 100 ml) at required time point by centrifugation (12,000 x g, 5 min), and the pellets washed three times in an equal volume of PBS. Washed cells were suspended in 100 - 500 µl of PBS and mixed with 170- to 180-µm-diameter glass beads (0.5 g; Braun Biotech International). Bacteria were disrupted by rapid agitation (40 s x 2) by using a Cell Homogenizer-MSK (Braun Biotech International). During disruption, samples were cooled with liquid CO_2. Following disruption, samples were kept on ice for 1 h in the presence of 1% (vol/vol) Triton X-100 (BDH). Cell debris and unbroken cells were removed by centrifugation (12,000 x g, 5 min), and the supernatant was stored at -20°C.

2.6.2 Methods for Preparation of culture supernatants of *S. uberis*

2.6.2.1 Preparation of proteins from bacterial growth media by TCA precipitation

Bacteria were grown to an appropriate density. Protease inhibitor was added to a 1X concentration from a 25X stock of Complete EDTA free protease inhibitor (Roche). Bacteria were harvested by centrifugation (10,000 x g, 10 minutes). The culture supernatants were transferred to a fresh centrifuge vessel, before being centrifuged as previously described to remove remaining bacteria. The culture supernatants were filtered through a 0.22 μm filter into a fresh centrifuge vessel and placed on ice. Trichloracaetic acid was added (10% v/v from a 100% w/v stock solution) and mixed thoroughly. Proteins were precipitated on ice for 30 minutes before being harvested by centrifugation (13,000 x g, 30 minutes, 4°C). The supernatant was discarded and the precipitate washed with 5 ml cold acetone, the tube was vortexed well. The precipitate was pelleted by centrifugation as previously described, before being washed and repelleted again to remove traces of TCA. The remaining precipitate was air dried thoroughly before being resuspended in either SDS-PAGE loading buffer pH 8.0 or Tris-HCl pH 8.0 and frozen at –20°C.

2.6.2.2 Preparation of proteins from bacterial growth media by methanol chloroform precipitation

Culture supernatants were cleared as previously described in 2.6.2.1. The culture supernatant was concentrated to 50 times the original volume in a 10 kDa molecular weight exclusion Centricon centrifugal filter device (Millipore Corporation) (4,000 x g, room temperature). The concentrated supernatant was transferred to a 50 ml falcon tube before the addition of 3 times the concentrated volume of methanol and 0.75 times the concentrated volume of chloroform.

The mixture was vortexed before the addition of 2.25 times the starting volume of water. The mixture was separated in to discrete phases by centrifugation (4000 x g, 15 minutes). The upper layer was discarded and 2.25 times starting volume of methanol added, the solution was vortexed and the precipitated protein pelleted by centrifugation (4000 x g, 30 minutes). The supernatant was discarded and the pellet air dried before being resuspended in sodium phosphate buffer 20 mM, pH 7.8 and frozen at -20°C.

2.6.3 Subcellular fractionation of *S. uberis*

The following fractionation procedures were used sequentially on 100 ml cultures. All buffers contained 1x Complete EDTA free protease inhibitors (Roche Diagnostics Ltd, UK).

2.6.3.1 Preparation of Capsule Fraction

Bacteria were harvested by centrifugation (11,500 x g, 10 minutes). The supernatant was discarded and the pelleted bacteria were washed three times in 10ml ice cold phosphate buffered saline (PBS, 0.01 M phosphate buffer, 0.0027 M potassium chloride, and 0.137 M sodium chloride, pH 7.4 at 25°C) and resuspended in 250 µl PBS containing hyaluronidase (100 U ml^{-1}; Sigma). Bacteria were incubated for 2 hours, 37°C and cells collected by centrifugation (9500 x g, 5 minutes). The supernatant fraction (capsular extract) was removed and stored at -20°C.

2.6.3.2 Preparation of Cell Wall Fraction

Cells harvested from the capsule preparation procedure (above), were washed three times in 1ml ice cold PBS before being resuspended in 250 µl PBS containing 40% (w/v) sucrose and

170 U mutanolysin (Sigma). Bacteria were incubated for 2 hours, 37°C and cells harvested by centrifugation (400 x g 15 minutes, 4°C) and the supernatant fraction (cell wall extract) removed and stored at –20°C.

2.6.3.3 Preparation of Cell Contents Fraction

The pellet from the cell wall preparation (above) was washed three times in 1ml ice cold PBS containing 40% (w/v) sucrose and resuspended in 250 µl membrane buffer (100mM Tris-HCl pH 7.5, 10 mM MgCl2, 100 mM NaCl). The cells were freeze-thawed (-80°C to +37°C) three times to promote cell lysis. Intact cells and debris were removed by centrifugation (9,500 x g, 5 min, 4°C) and the supernatant fraction (cell contents) removed and stored at –20°C.

2.6.3.4 Preparation of Membrane Fraction

The pellet remaining from the cell contents procedure was washed three times in 1ml cold PBS, resuspended in 250 µl membrane buffer containing Triton-X 100 (1% v/v) and incubated at room temperature for 1 hour to solubilise the membrane components. Insoluble debris was removed by centrifugation (9,500 x g, 5 min, 4°C) and the supernatant fraction (membrane extract) was removed and stored at -20°C.

2.6.4 Protein Assay to determine levels of protein within cell fractions

Levels of protein within the cell fractions were determined using the BCA Protein assay kit (Pierce). $O.D_{550\text{-}620nm}$, values for individual samples were measured (Anthos 2001 plate reader, Anthos Labtec Instruments GmbH, Salzburg, Austria) and concentrations calculated from mean values of triplicate readings for each sample using a standard curve.

2.7 Determination of proteins present within individual fractions

2.7.1 Visualisation of proteins on SDS PAGE

Proteins (10 μg) were prepared and separated by SDS PAGE on 12 % gels (Laemmli, 1970) or 10 % Bis tris gels (Invitrogen) and stained with Coomassie Blue or PlusOne Silver Stain kit method as detailed in Table 4. Reagents were obtained from Sigma Aldrich and were all of the highest available quality.

Step	Components	Quantity	Duration
Fixing	Ethanol	120 ml	2-24 h
	Glacial Acetic Acid	40 ml	
	ddH_2O	240 ml	
	Total volume	400 ml	
Sensitizing	Ethanol	120 ml	1-24 h
	25% Gluteraldehyde	2 ml	
	Sodium thiosulphate	0.8 g	
	Sodium acetate	27.2 g	
	ddH_2O	278 ml	
	Total volume	400 ml	
Washing	ddH_2O	400 ml	4 x 15 m
Silver	Silver nitrate	1 g	1 h
	Formaldehyde (37%)	160 µl	
	ddH_2O	400 ml	
	Total volume	400.16 ml	
Washing	ddH_2O	400 ml	2 x 1 m
Developing	Sodium carbonate	10 g	Time to be
	Formaldehyde (37%)	80 µl	determined
	ddH_2O	400 ml	empirically
	Total volume	400.08 ml	
Stop	EDTA	5.84 g	1 h – indefinitely
	ddH_2O	400 ml	
	Total volume	400 ml	

Table 4 Silver staining methodology

2.7.2 Detection of MtuA within cell fractions prepared from *S. uberis*

The proteins present in cell fractions (1 μg) were separated by SDS PAGE on 12.5 % gels or 10 % Bis tris gels and detected by Western blotting using MtuA antiserum (Jones *et al.*, 2004) at a concentration of 1:2500 and a secondary goat anti-rabbit-horseradish peroxidase conjugate (Sigma) at a concentration 1:2500.

2.7.3 ELISA to detect levels of MtuA within different cell fractions

ELISA was performed as previously described (Jones *et al.*, 2004), briefly a MaxiSorp plate (Corning Incorporated) containing triplicate protein samples was prepared. Samples were diluted to 1 μg/ml in PBS and 20 μl (20 ng protein) and were diluted to 100 μl with coating buffer (0.05 M carbonate buffer pH 9.6). No antigen control wells were filled with coating buffer only. Plates were incubated overnight in a humid environment at 4°C to promote binding. Wells were washed three times with ELISA wash buffer [0.05% (v/v) Tween 20 in PBS]. Non-specific binding capacity was blocked by adding blocking solution [1% (w/v) Marvel 0.1% (v/v) Tween 20 in PBS] at a volume of 100 μl per well and incubated for 1 hour at room temperature. The well contents were removed and the wells were washed three times as previously described and then incubated for 1 hour at room temperature with MtuA antisera (1 in 1000, in blocking solution, 100 μl per well). Wells were washed as previously described to remove unbound antibody and then incubated for 1 hour with anti-rabbit HRP conjugate antibody (1 in 1000 in blocking solution; Sigma). Wells were again washed prior to the addition of TMB reagent [0.1 ml TMB dissolved in dimethyl sulfoxide and 0.03% (v/v) H_2O_2 in 10 ml TMB substrate buffer (0.1 M sodium acetate, pH 6.0), 100 μl per well]. After approximately 3 min, 1 M H_2SO_4 was added (100 μl per well) to stop further colour

generation. Absorbance readings were taken at an $O.D._{450nm}$ (Anthos 2001 plate reader, Anthos Labtec Instruments GmbH, Salzburg, Austria).

2.7.4 Detection of MtuA within whole cell ELISA

Bacteria were grown to mid–exponential phase ($O.D._{550nm}$) before the suspensions were adjusted to an $O.D._{550nm}$ of 0.3 with growth media (THB). Suspensions were mixed with equal volumes of coating buffer (carbonate buffer 0.05 M, pH 9.6) and applied in triplicate to the wells of a MaxiSorp plate. No antigen controls contained only coating buffer in the wells. The plate was incubated overnight in a humid environment at 4°C to promote binding. Wells were washed three times with ELISA wash buffer and blocking solution added (200 µl per well), the plate was incubated for 90 minutes at RT. Wells were washed again prior to addition of MtuA antisera (1 in 1000 diluted in blocking solution, 100 µl per well) and incubated for 90 minutes at RT. Wells were washed as previously described to remove unbound antibody and then incubated for 1 hour with anti-rabbit HRP conjugate antibody (Sigma) (1 in 1000 dilution in blocking solution). Antibodies were detected using TMB reagent, as previously described in 2.7.3.

2.8 Growth of *S. uberis* in the presence of globomycin

A stock of globomycin (kindly supplied by Shunichi Miyakoshi, Sankyo Company Ltd, Japan) was prepared at a concentration of 10 mg/ml in ethanol and stored at -20°C. Bacteria were grown overnight in THB, before being diluted into fresh THB to an $O.D._{550\ nm}$ of 0.01. Bacteria were grown to the start of exponential phase ($O.D._{550nm}$ 0.1) before the addition of 100 µg/ml globomycin. Bacteria were harvested at appropriate time points by centrifugation

(12,000 x g, 5 minutes, 4°C) before whole cell lysates and TCA or methanol chloroform supernatant precipitates were prepared as previously described in 2.6.1 and 2.6.2.

2.9 Preparation of samples for N-terminal Sequencing

Samples were prepared for N-terminal sequencing according to the method of Coligan et al., (Coligan, 2001). Briefly, samples were prepared for SDS-PAGE in the concentration range of 100 to 200 pmol. Polyacrylamide gels (4% stacking; 10% resolving) were prerun with 2 mM mercaptoacetic acid present in the upper buffer reservoir to scavenge amino-terminal-blocking free radicals. Reduced and denatured samples were resolved at 15 V/cm and then transblotted to Immobilon-PSQ (polyvinylidene fluoride) transfer membrane (Millipore) with 10 mM cyclohexylamino propane sulphonic acid (pH 11) 1 mM dithiothreitol 10% (vol/vol) methanol as transfer buffer at 100 V for 1 h. Transferred protein was visualized on the membrane by rinsing with distilled water followed by immersion in 0.1% (wt/vol) amido black for 5 seconds and extensive destaining in distilled water. Heavily stained bands at the appropriate molecular weights were excised and prepared for amino acid sequence analysis by Edman degradation, performed by Mr L. Hunt (IAH, Compton, UK).

2.10 Identification of Lipoprotein sequences by pattern searching

Lipoproteins from the genome of *S. uberis* strain 0140J were found using a slight modification of the G+Lpp published by Sutcliffe and Harrington (Sutcliffe and Harrington, 2002). The pattern [MV]-X(0,13)-[RK]-{DERK}(6,20)-[LIVMFESTAG]-[LVIAMF]-[IVMSTAFGC]-[AG]-C (personal communication, I. C. Sutcliffe) was entered in the pattern search option of the PEDANT website (http://kulan.gsf.de/cgi-

bin/wwwfly.pl?Set=Streptococcus_uberis&Page=index) (Frishman *et al.*, 2001; Frishman *et al.*, 2003). Proteins were checked to determine that the sequence was located at the N-terminus of the protein and that a signal peptide sequence could be predicted using SignalP (Bendtsen *et al.*, 2004). Putative identification of function for each of the predicted lipoproteins was carried out using BLAST searches (Altschul *et al.*, 1990) using the National Centre for Biotechnology Information BLASTP server (http://www.ncbi.nlm.nih.gov/BLAST/).

2.11 Assay to determine Shaving Factor Uberis (ShvU)

2.11.1 Synthesis of the Fluorogenic Peptide Substrate

The Fluorescence Resonance Energy Transfer FRET peptide EDANS-LGACSVGN-DABSYL was synthesized based upon the lipobox motif and the 4 amino acids downstream of the invariable cysteine residue of MtuA. Two variations on this peptide were also produced EDANS-LGAKSVGN-DABSYL which would allow cleavage by trypsin and EDANS-LGASSVGN-DABSYL where the invariable cysteine residue of the lipobox is modified to a serine residue to allow preliminary determination of the specificities of the enzyme.

2.11.2 Fluorometric assay to determine cleavage of fluorogenic substrate by putative Shavase enzyme

Standard assays were carried out in solid black microtitre plates (Nunc, Thermo Fisher Scientific) in 150 µl reaction volumes containing 20 mM Phosphate buffer pH 6.25 and 6 µM peptide substrate. Stock solutions of peptide substrate were prepared in DMSO. The increase in fluorescence upon hydrolysis of the substrate was monitored with a fluorescent microplate

reader Wallac 1420 Victor^2 (Perkin Elmer) with excitation at 355 nm and emission at 460 nm. Reactions were incubated overnight at 37°C and were carried out in triplicate.

2.11.3 Inhibition assay of proteolytic activity

The inhibition assays were performed using a protease inhibitors set (Sigma, UK). Whole cell lysates (10 μg/ml) were mixed with the appropriate amount of protease inhibitor and incubated for 30 minutes before the addition of the fluorogenic peptide substrate. The final concentration of inhibitors was set according to the manufacturers guidelines and is shown in Table 5.

Protease Inhibitor	Final Concentration
AEBSF	1 mM
Antipain Hydrochloride	75 μM
Bestatin Hydrochloride	115 μM
E64	20 μM
EDTA	1 mM
EGTA	1 mM
Pepstatin	10 μM
Phosphoramidon	570 μM
1.10 Phenanthroline	2 mM

Table 5 Concentration of Protease Inhibitors

2.11.4 Addition of ions to ShvU

Whole cell lysate (10 μg/ml) was incubated with 1 mM final concentration ion (stock solutions of 100 mM $CaCl_2$, $FeCl_3$, $FeSO_4$, $MnCl_2$, $MgCl_2$, $ZnCl_2$, $NiCl_2$ and $CoCl_2$) for 30 minutes before the addition of the fluorogenic peptide substrate.

2.11.5 Addition of ions to ShvU treated extensively with EDTA

Whole cell lysate was dialyzed (12 kDa exclusion) against 10 mM EDTA dissolved in HPLC grade H_2O overnight. The EDTA was removed by extensive dialysis of proteins in HPLC grade H_2O overnight, the H_2O was changed three times.

2.11.6 Purification of ShvU from *S. uberis*

The methods used to purify ShvU from *S. uberis* are described in Appendix 3 of this thesis.

2.11.7 Preparation of proteins for Mass Spectrometry to determine identity of ShvU

Samples to be analysed by mass spectrometry from the purified fractions were treated with 1 mM DL-Dithiothreitol (Sigma) (4 hours, 37°C) followed by 4 mM iodoacetamide (Sigma) (1.5 hours, room temperature, in the dark). The proteins were dialyzed in membrane with a molecular weight cut off of 12 kDa (2 M urea (Sigma), 100 mM Ammonium Bicarbonate (Sigma) 2 hours, room temperature). The proteins were digested with 100 ng high grade trypsin (Sigma) (37°C, overnight). Digested peptides were stored at -20°C and were analysed by Mass Spectrometry by Dr A. C. Gill, IAH Compton. HPLC-MS and HPLC-MS/MS were performed by use of a Q-ToF Premier (Waters Plc, Altrincham, UK) mass spectrometer equipped with a nanoAcquity UPLC system, interfaced to the mass spectrometer by way of a continuous flow nanospray source. Samples (10 µl) were pre-concentrated on a peptide trap (Symmetry C18, 5 µm particle size, 180 µm id x 20 mm length, Waters Plc) and were separated by use of a capillary UPLC column (BEH130 C18, 1.7 µm particle size, 100 µm id x 100 mm length, Waters Plc). Eluted peptides were passed to the mass spectrometer which was operated in data-dependant acquisition mode. Full scan MS spectra (m/z 300-2000) were acquired with 1 sec scan time and components were selected for MS/MS based on charge (2+, 3+ and 4+ only) and intensity. MS/MS spectra were acquired for a maximum of 20 seconds, with a collision cell pressure of approximately 1.4×10^{-4} mbar and a variable collision energy that depended on mass and charge.

MS/MS spectra were processed by background subtracting, smoothing and deisotoping using generic algorithms (Waters Plc). The resulting processed spectra were searched against a *S. uberis* protein database by use of ProteinLynx Global Server (PLGS, Waters Plc). The database was constructed by use of amino acid sequences from the predicted ORF's of the genome of *S. uberis* strain 0140J which was obtained with permission from the Sanger Centre. Peptide sequence matches were filtered by PLGS generic quality filters and all results had at least 2 matching peptides associated with them.

Chapter 3

Characterisation of the lipoprotein signal peptidase (Lsp) of *Streptococcus uberis*

3.1 Introduction

Many secreted proteins are produced with a signal peptide at the amino terminus which is then removed by a signal peptidase during the secretion process (Tjalsma *et al.*, 2000a; van Roosmalen *et al.*, 2004). Lipoproteins typically contain a lipoprotein signal peptide consisting of a lipobox motif, present in the carboxyl region of the signal peptide (Sutcliffe and Harrington, 2002; Tjalsma *et al.*, 2000a), that targets the protein to the correct post translational processing pathway (von Heijne, 1989). The consensus motif of the lipobox is LxxC. The cysteine residue is the invariable target of the enzyme lipoprotein diacylglyceryl transferase (Lgt), this lipidates the protein which serves to anchor the lipoprotein to the membrane (Sutcliffe and Harrington, 2002; Tokunaga *et al.*, 1982). The lipidation of the lipoprotein to the membrane is thought to be a prerequisite activity for the action of lipoprotein signal peptidase (Lsp), a specific signal peptidase for lipoproteins which removes the signal peptide and leaves the cysteine of the lipobox as the new amino terminal residue of the mature lipoprotein (Tokunaga *et al.*, 1982).

Lsp was first identified in *E. coli*, (Innis *et al.*, 1984; Regue *et al.*, 1984; Tokunaga *et al.*, 1985; Yamagata, 1983) and subsequently in Gram positive bacteria, where it was first identified in *S. aureus* (Zhao and Wu, 1992). Since then, Lsp has been found to be a ubiquitous enzyme as the gene for which is present in the genomes of all sequenced bacteria.

The enzyme has been specifically studied in several species including *Mycobacterium tuberculosis* (Sander *et al.*, 2004), *Listeria monocytogenes* (Reglier-Poupet *et al.*, 2003a), *Enterobacter aerogenes* (Isaki *et al.*, 1990), and *Streptococcus suis* (De Greeff *et al.*, 2003).

The role of Lsp in *B. subtilis* has been studied in much detail and shown that, while not essential for cell viability, it was required for growth at higher temperatures (Tjalsma *et al.*, 1999a). The lipoprotein PrsA, which functions to fold proteins was not processed to its mature form in an *lsp* mutant, as was seen in the wild type, during observation over a short time-course (15 minutes). However, only 50% of the PrsA was visualized in the pre-form, the remaining 50% was shown to have been processed, but had a slightly lower mobility, and therefore a higher molecular weight, than the mature form when visualised on Western blots. It was suggested therefore that an alternative pathway existed for processing lipoproteins from *B. subtilis*. Both forms of the PrsA protein present in the *lsp* mutant of *B. subtilis* were correctly localised to the membrane and it was concluded by Tjalsma et al., 1999 (Tjalsma *et al.*, 1999a) that it was possible that both forms of the PrsA could retain their biological function . Interestingly, it was also shown that the non-lipoprotein of *B. amyloliquefaciens* α-amylase (AmyQ) was also affected by Lsp (Tjalsma *et al.*, 1999a). A fivefold reduction in the amount of AmyQ was seen in the background of an *lsp* mutation. This was paralleled by the level of accumulation of pre-PrsA and mature-like forms of PrsA. A reduced level of secretion of AmyQ is indicative of reduced levels of PrsA activity, PrsA is required to fold the protein into a protease resistant conformation (Kontinen and Sarvas, 1993). However, in cells lacking PrsA, pre-AmyQ does not accumulate, suggesting that any accumulation was due to loss of functional Lsp, rather than reduced amounts of PrsA. Whether this result is due to the

accumulation of lipoprotein precursors, the action of Lsp directly on AmyQ, or the malfunction of an, as yet unidentified, lipoprotein is unknown.

Lsp enzymes are predicted to have four transmembrane spanning regions (Munoa *et al.*, 1991) and there are five regions of conserved sequence which were identified by comparison of 18 amino acid sequences of Lsp (Tjalsma *et al.*, 1999b). Point mutagenesis was used to determine that six individual residues within the Lsp amino acid sequence are important for the functional activity of this enzyme in *B. subtilis*. The residues shown to be important are all predicted to be localized close to the external surface of the cytoplasmic membrane. The Asp-14 was shown to be important for the structural stability of the enzyme while the other five residues were directly involved in the catalysis of the signal peptide removal.

Lsp lack conserved serine residues, as seen in type I signal peptidases that process other secreted proteins (Paetzel *et al.*, 1998). There is also only one conserved lysine residue (as shown by the alignments of the Lsp amino acid sequences (Tjalsma *et al.*, 1999b)), which is not required for activity, so this has ruled out the possibility that the two classes of signal peptidases make use of similar catalytic mechanisms. Lsp enzymes do not utilize thiol or metallopeptidase mechanisms of activity, instead two strictly conserved aspartic acid residues are essential for the activity of this enzyme. This suggests that Lsp belongs to a group of enzymes known as aspartic peptidases. This data is reinforced by the observation that Lsp was inhibited by pepstatin, a known inhibitor of aspartic peptidases (Dev and Ray, 1984). Observations by Tjalsma *et al.* in 1999 (Tjalsma *et al.*, 1999b) suggested that Lsp enzymes belonged to a novel class of aspartic peptidases that evolved exclusively in eubacteria. The

Lsp of *B. subtilis* is thought to employ Asp-102 and Asp-129 as a catalytic dyad. The other highly conserved residues could be involved in stabilising the active site or recognising the diacyl-glyceryl-modified cysteine residue in the lipobox of preproteins (Tjalsma *et al.*, 1999b).

The antibiotic globomycin has been invaluable in studying the activity and function of Lsp enzymes. This antibiotic is a potent, reversible and non-competitive inhibitor of Lsp (Inukai *et al.*, 1978). It functions by binding to the enzyme and in doing so prevents the cleavage of signal peptides from target lipoproteins (Dev *et al.*, 1985).

In this chapter the properties of Lsp in *S. uberis* are described through the use of comparative analysis of the wild type bacteria with an isogenic mutant.

3.2 Results

3.2.1 Bioinformatic analysis of the complete genome of *S. uberis* strain 0140J for genes encoding putative lipoproteins.

The search for lipoproteins within genomes has been carried out for several bacteria (Baumgartner *et al.*, 2007; Stoll *et al.*, 2005; Sutcliffe and Harrington, 2002; Sutcliffe and Harrington, 2004a; Tjalsma *et al.*, 2000a). The prosite pattern PS00013; {DERK(6)-[LIVMFWSTAG](2)-[LIVMFYSTAGCQ]-[AGS]-C, predicts lipoproteins. This pattern avoids acidic amino acids in the sequence preceding the cysteine residue of the lipobox motif, but it does not limit the sequence prediction to the amino terminus of the protein where the signal peptide is located. The G+LPP pattern (below) is more specific to Gram positive bacterial lipoproteins (Sutcliffe and Harrington, 2002) as it has been shown that Gram positive lipoproteins have a slightly different consensus sequence compared to Gram negative organisms. In the G+LPP pattern, the amino acid restrictions at -2 and -3 are more constrained than the prosite pattern. The G+LPP pattern was produced on a list of experimentally verified Gram positive lipoproteins, an approach that was aimed to prevent the prediction of false positives while being more likely to select for true lipoproteins. Previously, the lipoproteins of *S. pyogenes* and *S. agalactiae* were predicted using G+LPP (Sutcliffe and Harrington, 2002, , 2004b). The complete genomic sequence of *S. uberis* strain 0140J (http://www.sanger.ac.uk/Projects/S_*uberis*/) was screened for genes encoding putative lipoproteins by using a modified version of the G+LPP pattern [MV]-X(0,13)-[RK]-{DERK}(6,20)-[LIVMFESTAG]-[LVIAMF]-[IVMSTAFGC]-[AG]-C (personal communication, I. C. Sutcliffe). Thirty individual open reading frames were predicted using the G+LPP pattern. Each contained this motif at the amino terminus of the protein and

contained a signal peptide sequence that was predicted by SignalP (Bendtsen *et al.*, 2004) (Table 6). A pattern search was carried out using the Prosite pattern PS00013 for bacterial lipoproteins which identified a further 55 proteins, however all but one of these sequences were considered to be false positives due to the positioning of the lipobox motif or the absence of a signal peptide sequence predicted by SignalP. SUB0586 remained from the search using the PS00013 pattern which encodes OppA2, an oligopeptide transport protein (Taylor *et al.*, 2003). However, when compared to the G+LPP sequence, it was shown to fit with this pattern. It remains unclear as to why this lipoprotein was not selected using the primary search. Where possible, a potential function was ascribed to the open reading frame identified by using blast-p (Altschul *et al.*, 1990); these are shown (Table 6) along with the source and accession number to which the alignment was made. Many of the proteins identified from *S. uberis* displayed homology to known lipoproteins such as ABC transporters and the folding protein PrsA.

Locus name[a]	Signal Peptide	Predicted SignalP*	Putative function/homology (Source, accession number or reference)
SUB0098	MKKTFSTLVLLSALMLTACS	28-29	Hypothetical protein
SUB0137	MKKGMRISLILLALMLLTACR	33-34	ABC transporter permease protein (*S. suis*, YP_001200911)
SUB0138	MKLKTYLLLGLTSLLLVGCR	22-23	Hypothetical Protein
SUB0248	MMSKIVKKIFFLTAFLIMFFLSACA	25-26	Putative Lipoprotein (*S. equi*, AAN18300)
SUB0306	MKKRWIASSVIVLASTIVLGACG	30-31	Putative ABC transporter periplasmic component (*S. pyogenes*, YP_001200911)
SUB0365	MMTLKKNLGILSLTLGTLAILAACG	25-26	ABC transporter substrate-binding protein (*S. pyogenes*, YP_597876)
SUB0366	MSYKKILGLIGLTLVSSVLVACG	23-24	Putative ABC transporter, substrate binding protein (*S. pyogenes*, YP_601760)
SUB0393	MKIKLNRILFSGLALSILITLTGCV	31-32	Putative lipoprotein OxaA like protein (*S. pyogenes*, YP_279755)
SUB0423	MKKLLVTLVLIFSTLSLIACS	28-29	Putative ferrichrome binding protein (*S. pyogenes*, YP_059667)
SUB0432	MKKKLLVSTIACLSLLSLAACD	32-33	Putative ABC transporter, branched amino acids (*S. mutans*, NP_722002)
SUB0473	MKKKLSLAIMAFLGLLMLGACS	29-30	MtuA (*S. uberis*, (Jones *et al.*, 2004; Jones, 2006; Smith *et al.*, 2003))
SUB0477	MKKIISFALLTLSLFSLSACE	19-20	Peptidyl prolyl cis trans isomerase (*S. pyogenes*, NP_664125)
SUB0569	MKMNKMLTLAVLTLSSFGLAACG	29-30	Putative ABC transporter, substrate binding protein (*S. pneumoniae*, NP_346504)
SUB0586	MKNLKKYSSLGLLVISATTLVACG	30-31	OppA2 (*S. uberis*, (Taylor *et al.*, 2003))
SUB0600	MGNYFKSLCLLLFSFLLVACS	25-26	Hypothetical protein
SUB0638	MKTKKILKAAIGLMTLVSMTACS	24-25	Putative ABC transporter Amino Acids (*S. agalactiae*, NP_688248)
SUB0688	MKKFKLIFTALSLSSLMLLTACS	28-29	ABC transporter amino acid binding protein (*S. agalactiae*, YP_688428)
SUB0842	MTFEKKHVFYLILLFVACL	23-24	Hypothetical membrane spanning protein (*S. pyogenes*, NP_608045)
SUB0884	MKRKFLSFILVLTFFLPFLVGLSACQ	24-25	Putative laminin binding protein
SUB0885	MKHKKLIGTGTVLALLFSACG	27-28	Similar to Streptococcal histidine triad family (*S. pyogenes*, YP_001129196)
SUB0950	MNKKFIGLGLASVAILSLAACG	40-41	Putative lipoprotein (*S. pyogenes*, NP_802330)
SUB0965	MNMKKFFLVGMLTLSMLTLTACS	29-30	Putative ABC transporter phosphate (*S. pyogenes*, NP_664685)
SUB0990	MNLKKILLTTLALASTLFLVACG	27-28	Putative amino acid binding protein (*S. uberis*, ACC35851, (Jiang *et al.*, 1998))
SUB1007	MKTQKSITLLLLSVLCFSLGACS	29-30	Hypothetical protein

SUB1019	MKKLFIYLSLAFSLLVLGACS	22-23	Putative amino acid transporter amino acids (S. mutans, NP_721596)
SUB1143	MKSWQKIIVSGASLTLASTLLVGCA	32-33	Putative ABC transporter maltose/maltodextrin (*S. pyogenes*, YP_280507)
SUB1231	MNTSKKIVTGFVTLASVLTLAACS	22-23	PrsA homologue (B. subtilis, P24327)
SUB1307	MKCIKKLGFLALFLSMLLLLGACG	24-25	Putative ABC transporter Ribose sugar (S. agalactiae, NP_00785901)
SUB1625	MTVAQKSTFKRFGLGAVTLASAALLMACG	34-35	Hyaluronate Associated Protein (*S. equi*, AAD17886) or OppA1 (Taylor *et al.*, 2003)
SUB1655	MSKKILKLATLAILPFVGLTACS	39-30	Putative extracellular solute binding protein (*S. pyogenes*, NP_268607)
SUB1666	MKKNIKIAALLPMLTLLAACS	27-28	60 kDa inner membrane protein YidC (*S. pyogenes*, YP_601697)

Table 6 Putative lipoproteins of *S. uberis* strain 0140J with putative functions/homologies

The lipobox motif is shown in bold within the signal peptides. The SUB numbers cross reference to the completed genome of *S. uberis* 0140J. Potential functions have been ascribed by Blast homology searches. The bacteria and the ascension number to which the protein was ascribed are also shown. * SignalP prediction, amino acids in the signal peptide sequence where cleavage by a signal peptidase is predicted to take place.

3.2.2 The processing of the essential lipoprotein MtuA is affected by the presence of the antibiotic globomycin

The presence of Lsp activity in wild type *S. uberis* was indicated by growing the bacteria in THB medium in the presence of globomycin. No difference in $O.D._{550nm}$ at any point in the growth curve could be seen (data not shown). MtuA is a putative lipoprotein of *S. uberis* (Table 6) that has been studied previously (Jones *et al.*, 2004; Smith *et al.*, 2003) and in this analysis was used as a candidate lipoprotein to follow the processing of such proteins. Whole cell lysates were prepared from cultures grown to either the start of stationary phase or for 24 hours, and analysed by Western blotting with anti-MtuA serum (Figure 2). In the absence of globomycin a single band of 37 kDa was seen. However, in the presence of globomycin, samples taken at the start of stationary phase yielded a single band that migrated more slowly than that detected in the absence of the antibiotic. The size difference was approximately 2 kDa, and corresponded to the size of the signal peptide sequence of MtuA. Two bands were detected in the sample prepared from bacteria grown for 24 hours, the band of a smaller molecular weight appeared to be of a similar size to that detected in the absence of globomycin while the other, larger protein, was approximately 2 kDa greater than the mature form of MtuA. MtuA was not detectable in the supernatant of *S. uberis* wild type bacteria in the presence or absence of globomycin (data not shown).

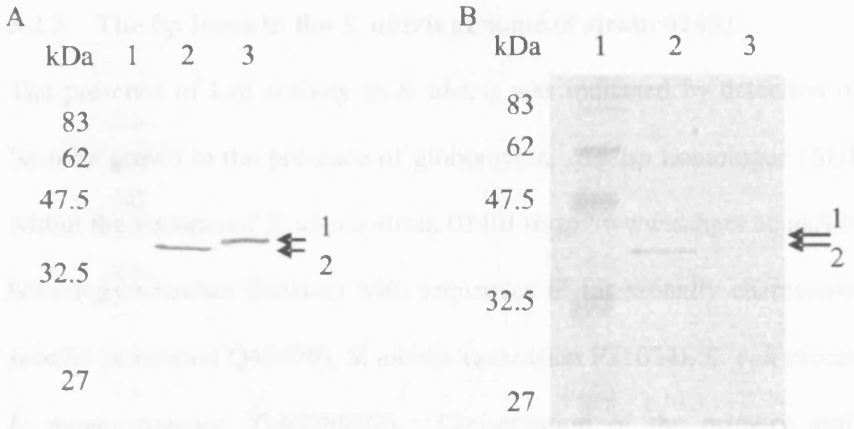


Figure 2 Western blot analysis of wild type *S. uberis* grown in the presence and absence of the Lsp inhibitor globomycin

Globomycin (100 µg/ml) was added, where appropriate to bacterial cultures at the start of exponential phase. Whole cell lysates were prepared from bacteria cultured in the presence and absence of globomycin grown to the start of stationary phase (A) or for 24 hours (B). Lane 1, marker shown with molecular weights; Lane 2, wild type without globomycin; Lane 3, wild type with globomycin.

3.2.3 The *lsp* locus in the *S. uberis* genome of strain 0140J

The presence of Lsp activity in *S. uberis* was indicated by detection of full length MtuA in bacteria grown in the presence of globomycin. An *lsp* homologue (SUB0729) was identified within the genome of *S. uberis* strain 0140J (http://www.sanger.ac.uk/Projects/S_*uberis*) using homology searches (blast-n) with sequences of functionally characterised *lsp* genes from *B. subtilis* (accession Q45479), *S. aureus* (accession P31024), *E. coli* (accession AAA24092) and *L. monocytogenes* (CAC99922). Conservation of the primary amino acid sequence to functionally described Lsp encoded by other bacterial species was determined (Table 7). Lsp of *S. uberis* was found to be 148 amino acids in length and the gene was flanked upstream by a transcriptional regulator, *lysR* (SUB0728), and downstream by the ribosomal large subunit, pseudouridine synthase, *rluD* (SUB0730) (Figure 3).

Analysis of the amino acid sequence of *S. uberis* Lsp suggested the presence of the 4 trans-membrane domains present in other Lsp proteins. The Lsp enzyme of *B. subtilis* was predicted to contain five highly conserved domains, and six residues were shown to be functionally important for the activity of Lsp (Tjalsma *et al.*, 1999b) (these residues and domains are shown in (Figure 4). The Asn, Asp and Ala residues in domains III and V, which are critical for the activity of Lsp in *B. subtilis,* are conserved in *S. uberis* Lsp (Figure 4).

Species	Amino acids	% identity	% conserved
B. subtilis	154	40	61
L. monocytogenes	164	42	64
S. aureus	154	36	61
S. suis	202	51	69
M. tuberculosis	154	29	49
E. coli	163	33	53

Table 7 Homologies of the predicted Lsp of *S. uberis* to characterised Lsp proteins

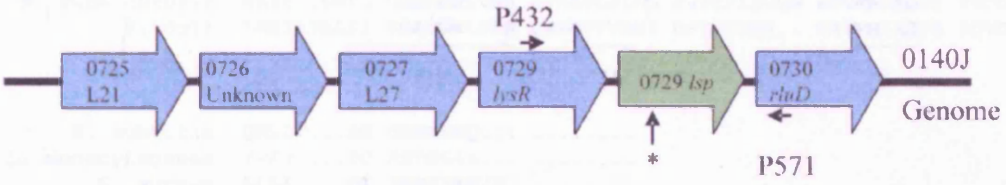


Figure 3 Context of the *lsp* locus of *S. uberis* strain 0140J

The flanking regions of *lsp* are shown, upstream are four genes; SUB0725 encodes the 50s ribosomal protein L21; SUB0726 is of unknown function; SUB0727 encodes the 50s ribosomal protein L27; and SUB0728 encodes the transcriptional regulator LysR. Downstream of the gene encoding Lsp (0729) is SUB0730 which encodes the ribosomal large subunit, pseudouridine synthase. A single mutant was identified in the *lsp* locus (*), through PCR screening of a pGh9^{+}::IS*S1* mutant bank; *S. uberis lsp*::IS*S1* has an IS*S1* element 132 bp from the start codon (3.2.4).

Figure 4 Conserved domains of lipoprotein signal peptidase across species

The amino acid sequences of *B. subtilis*, *L. monocytogenes*, *S. aureus*, *S. suis*, *S. uberis*, *M. tuberculosis* and *E. coli* lipoprotein signal peptidases were compared. Five conserved domains are shown in blue (Venema *et al.*, 2003), and residues in bold are conserved across the majority of Lsp enzymes identified. Residues that have been described to be important for activity (●) and stability (⬇) of *B. subtilis* Lsp (Tjalsma *et al.*, 1999b) are indicated.

3.2.4 Genotypic detection of *lsp* mutants within the *S. uberis* strain 0140J pGh9$^+$::IS*S1* mutant bank

The *lsp* gene of *S. uberis* is 447 bp in length. A mutant carrying an insertion in *lsp* was detected in a bank of approximately 8,800 random insertion mutants (Ward *et al.*, 2001) by PCR screening (Figure 5). One mutant designated *S. uberis lsp*::IS*S1* was identified with an insertion in the *lsp* gene locus. DNA sequence analysis was used to define the IS*S1* insertion to 132 bp from the start codon of the *lsp* coding sequence (Figure 3). Excision of the pGh9$^+$ vector from *S. uberis lsp* mutant and subsequent PCR amplification across the *lsp* locus was performed. An 800 bp band shift was evident corresponding to the presence of the residual IS*S1* element within the *lsp* gene (Figure 6). Southern blotting was used to verify that the mutant carried only a single IS*S1* insertion (Figure 7). The mutant from this point forward will be termed *lsp*$^-$.

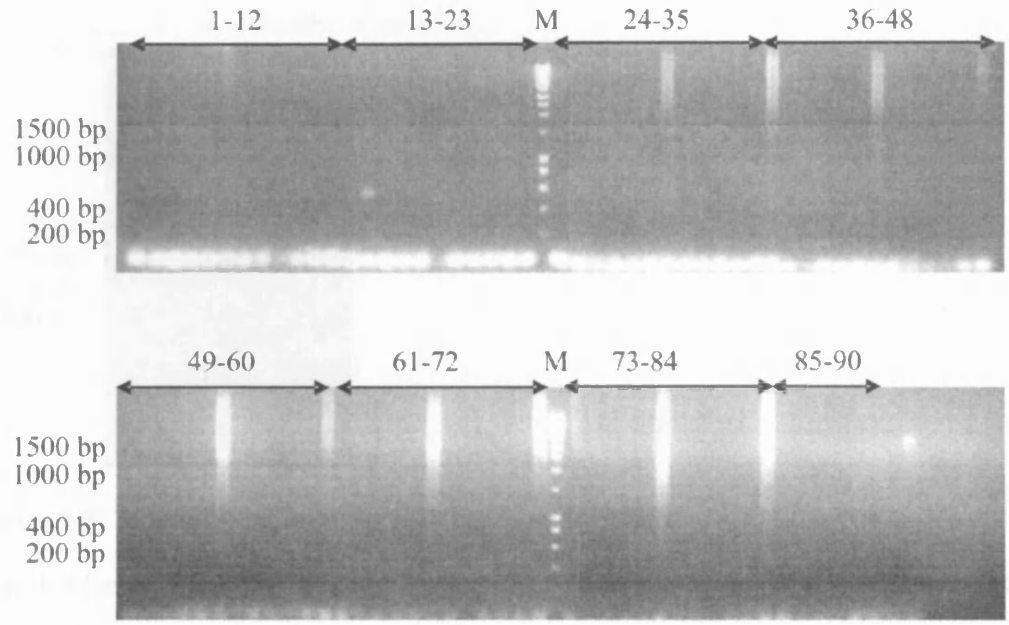


Figure 5 Identification of *lsp* mutants within pGh9$^+$::IS*S1* mutant bank

PCR was carried out using P432 and P370 on pools of DNA prepared from pGh9$^+$::IS*S1* mutant bank. Plate 14 was identified as containing an *lsp* insertion mutant. DNA preparations from rows and columns of plate 14 identified well 7H as the location of the *lsp* mutant (data not shown). Size of molecular weight marker is shown in base pairs.

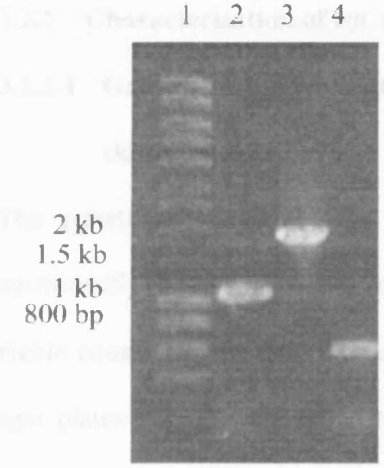


Figure 6 PCR amplification from wild type and *lsp*::IS*51*

Lane 1, Marker; Lane 2, PCR amplification of *lsp* from wild type; Lane 3, PCR amplification of *S. uberis lsp*::IS*51* (an 800 bp size difference was seen compared to the wild type); Lane 4, PCR amplification of *S. uberis lsp*::IS*51* using *lsp* and IS*51* specific primers.

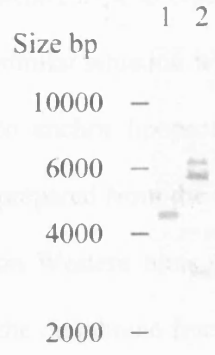


Figure 7 Southern blot probing for IS*51* in *lsp*⁻ mutant

Chromosomal DNA was prepared from the cured (Lane 1) and uncured (Lane 2) *lsp*⁻ mutant, and digested with the restriction endonuclease *Hind*III. *Hind*III cuts the pGh9+::IS*51* vector once, splitting the two IS*51* elements into two discreet DNA fragments allowing the visualisation of either two or three bands.

3.2.5 Characterisation of *lsp*⁻ mutant

3.2.5.1 Growth of *lsp*⁻ mutant compared to wild type in nutritionally rich media and skimmed milk

The growth of the wild type bacteria and the *lsp*⁻ mutant were compared in THB, a nutritionally rich media measured by $O.D._{540nm}$ (Figure 8) and also in skimmed bovine milk by viable counts (Figure 9). Bacterial colonies from the 24 hour time point were picked from agar plates and all were found to correspond genetically to the *lsp*⁻ mutant. The *lsp*⁻ mutant grew as well as the wild type in both the rate of growth and the yield of bacteria in both growth medium.

3.2.5.2 The lipoprotein MtuA is localised in the membrane of the *lsp* mutant

MtuA in wild type *S. uberis* is seen predominantly in the membrane (Jones *et al.*, 2004). A similar situation was predicted for the *lsp*⁻ mutant, as the action of Lgt would still be expected to anchor lipoproteins to the cell membrane. MtuA was visualised within cell fractions prepared from the wild type and *lsp*⁻ mutant grown to mid-exponential phase using anti-MtuA on Western blots (Figure 10). A single band of approximately 37 kDa was detected only in the membrane fraction of the wild type (as shown previously (Jones, 2006)). However, in the *lsp*⁻ mutant, grown to mid-exponential phase, a single band was seen approximately 2 kDa larger than that observed in the wild type. One explanation for this apparent size difference was the possible retention of the MtuA signal peptide on the protein. Intriguingly, in a sample produced from bacteria grown to 24 hours an additional immunoreactive band was detected in the *lsp*⁻ mutant preparation. The additional protein was slightly larger than the mature form

seen in the wild type, but smaller than that detected in the *lsp*⁻ mutant, postulated to be full length MtuA, at the earlier time point (Figure 10).

3.2.5.3 ELISA using MtuA-anti serum to show location of MtuA in *lsp*⁻ mutant

MtuA was detected by ELISA within the wild type membrane fraction and also at slightly lower levels within the cell contents (Figure 11). In the *lsp*⁻ mutant MtuA was also detected primarily within the membrane fraction, confirming the anchoring of this putative lipoprotein to the membrane.

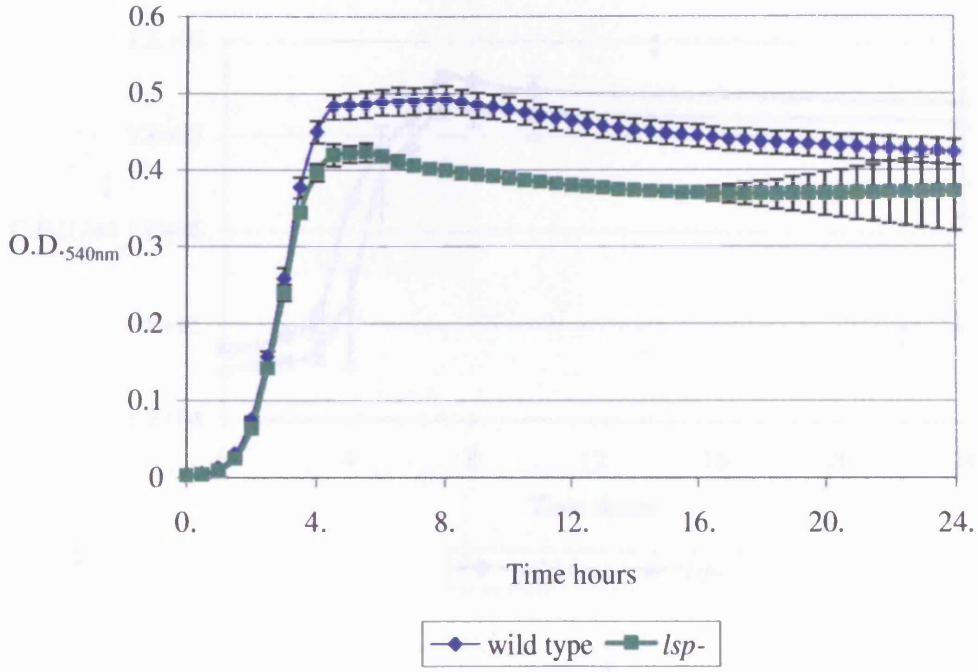
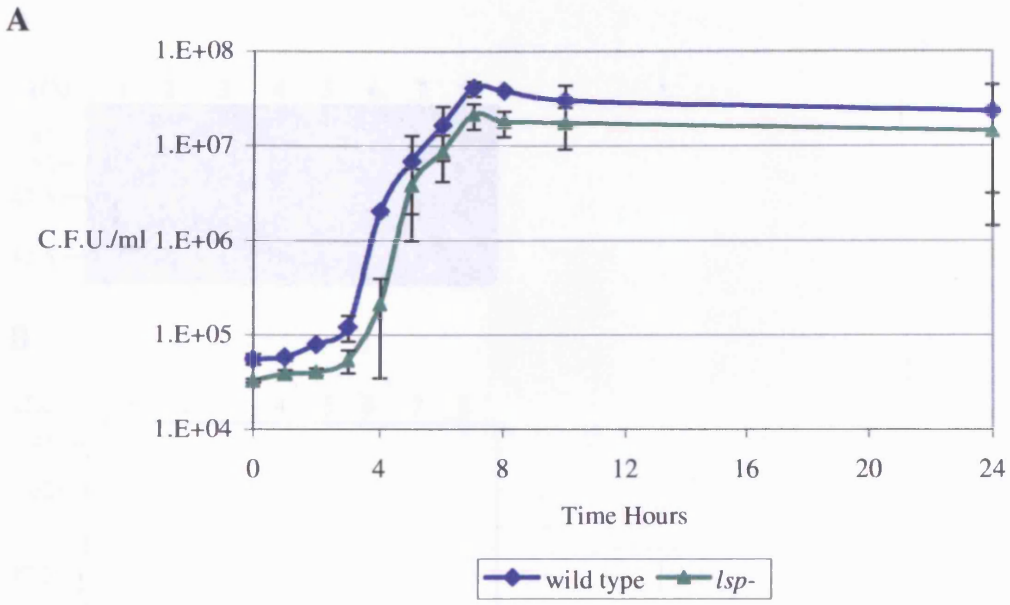


Figure 8 Growth of wild type *S. uberis* strain 0140J was compared with growth of the *lsp*⁻ mutant over 24 hours in Todd Hewitt Broth.

Error bars represent a standard deviation between results performed in triplicate (P at .



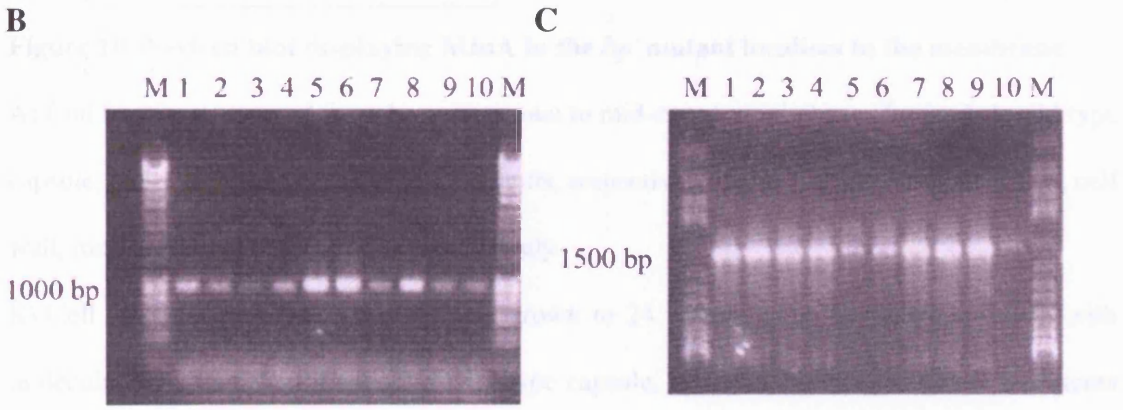


Figure 9 Growth of *S. uberis* wild type compared to *lsp*⁻ mutant in milk over 24 hours

The growth of the *lsp*⁻ mutant was compared to the wild type in milk (A). Error bars represent a standard deviation between results performed in triplicate. PCR was carried out on 9 colonies and genomic DNA from 24 hour plates of the milk grown cultures. Wild type using primers pauA Fwd and PauA Rev (B) and the *lsp*⁻, primers P432 and P571 (C) confirmed that the bacteria were free from contamination.

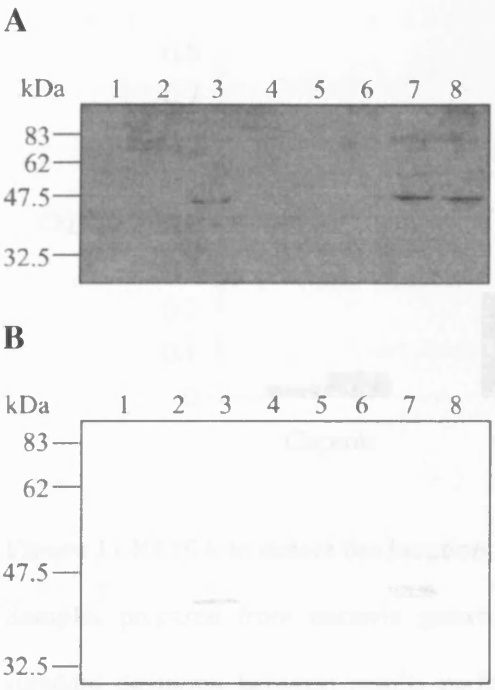


Figure 10 Western blot displaying MtuA in the *lsp*⁻ mutant localises to the membrane

A) Cell fractions prepared from bacteria grown to mid-exponential phase. Lanes 1-4 wild type capsule, cell wall, membrane and cell contents, respectively; lanes 5-8 *lsp*⁻ mutant capsule, cell wall, membrane and cell contents, respectively.

B) Cell fractions prepared from bacteria grown to 24 hours. Lane 1 – Marker shown with molecular weights (kDa). Lanes 2-5 wild type capsule, cell wall, membrane and cell contents respectively. Lanes 6-9 *lsp*⁻ mutant capsule, cell wall, membrane and cell contents respectively.

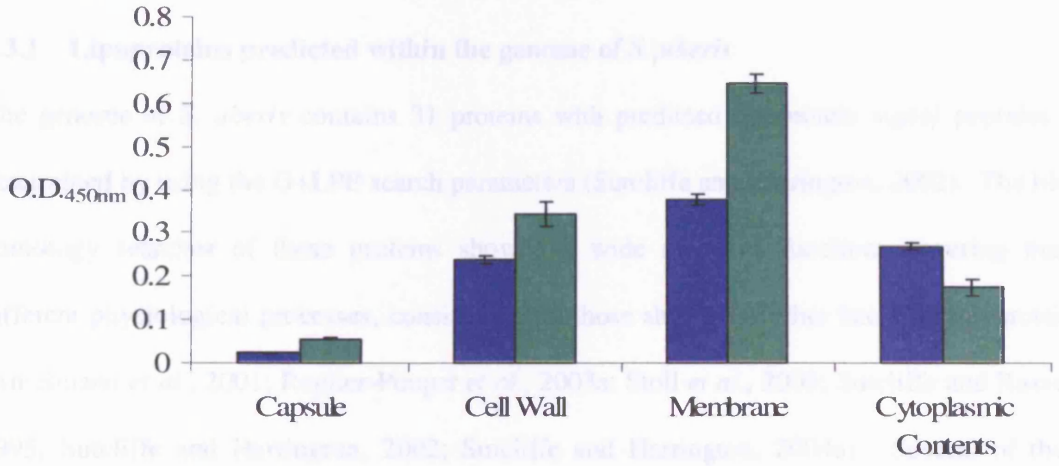


Figure 11 ELISA to detect the location of MtuA in the *lsp*⁻ mutant.

Samples prepared from bacteria grown to mid-exponential phase. Error bars represent a standard deviation between results performed in triplicate. Wild type (blue bars) and *lsp*⁻ mutant (green bars).

3.3 Discussion

3.3.1 Lipoproteins predicted within the genome of *S. uberis*

The genome of *S. uberis* contains 31 proteins with predicted lipoprotein signal peptides as determined by using the G+LPP search parameters (Sutcliffe and Harrington, 2002). The blast homology searches of these proteins showed a wide range of functions covering many different physiological processes, consistent with those shown for other bacterial lipoproteins (Antelmann *et al.*, 2001; Reglier-Poupet *et al.*, 2003a; Stoll *et al.*, 2005; Sutcliffe and Russell, 1995; Sutcliffe and Harrington, 2002; Sutcliffe and Harrington, 2004a). Several of these lipoproteins have been implicated as being essential for virulence including MtuA, which plays a role during intramammary infection by *S. uberis* (Smith *et al.*, 2003). This makes not only the enzymes involved in processing lipoproteins, but the lipoproteins themselves attractive for further study as potential targets for therapeutic agents.

Processing of lipoproteins in Gram positive bacteria requires the activity of two enzymes; lipoprotein diacylglyceryl transferase (Lgt) and lipoprotein signal peptidase (Lsp). Modification of lipoproteins by Lgt is thought to be a prerequisite for the activity of Lsp (Tokunaga *et al.*, 1982). In the presence of a mutation inactivating *lsp*, Lgt would be predicted to remain functional (and therefore still anchor the lipoprotein to the membrane) but the signal peptide of these proteins would remain intact. As in other Gram positive species of bacteria, an *lnt* homologue can not be found within the genome. *lnt* encodes apolipoprotein N-acyltransferase in Gram negative bacteria which has been shown to be essential for the correct localisation of lipoproteins to the outer membrane (Fukuda *et al.*, 2002). As Gram positive

bacteria only have a single membrane it is not surprising that theses species are lacking in this enzyme.

3.3.2 Lsp (SUB0729) of *S. uberis* is responsible for cleavage of signal peptides from the putative lipoprotein MtuA

Detection of MtuA in wild type *S. uberis* by Western blotting of whole cell lysates displayed two different sized bands depending upon the presence or absence of globomycin in the growth medium. This antibiotic has an inhibitory activity on Lsp (Dev *et al.*, 1985), therefore suggesting that the larger of the two bands was likely to be unprocessed MtuA containing an intact signal peptide, while the smaller band is of an equivalent size to processed MtuA (lacking signal peptide). This suggested that *S. uberis* contained an Lsp enzyme whose activity could be inhibited by globomycin (similar to other, previously characterised members of the Lsp enzyme group (Dev *et al.*, 1985)), and it is this Lsp enzyme that is responsible for the cleavage of lipoproteins. SUB0729 was identified from the genome of strain 0140J by BLAST alignment as being homologous to proteins previously characterised as Lsp enzymes. An *lsp*$^-$ mutant was selected from the *S. uberis* strain 0140J random pGh9$^+$::IS*S1* mutant bank using PCR. Whole cell lysates from this mutant were analysed using Western blot to detect MtuA to determine the processing of the signal peptide. A noticeable size difference in molecular weight of the protein was seen between the wild type and the *lsp*$^-$ mutant, suggesting that the signal peptide remained attached to MtuA in the presence of the insertion mutation.

3.3.3 Lsp is not essential in allowing *S. uberis* to grow in skimmed milk

The role of Lsp in determining growth rates and virulence is varied in the bacteria where this protein has been studied. The *S. uberis lsp*⁻ mutant was able to grow in skimmed milk to levels that were comparable to the wild type. The growth of the mutant was not prevented in the low manganese environment of milk, in which the *mtuA* mutant was unable to grow (Smith *et al.*, 2003). Suggesting that MutA was able to function correctly in the absence of the correct processing of the signal peptide by Lsp. The literature for a *L. lactis lsp* mutant describes similar results to the experiments described above; the mutant was viable in skimmed milk (Venema *et al.*, 2003). An *S. suis lsp* mutant shows unaffected growth rates in nutrient rich media and retained its virulence in pigs (De Greeff *et al.*, 2003). In contrast, an *lsp* mutant of *L. monocytogenes* not only failed to correctly process some lipoproteins, but also exhibited reduced efficiency for phagosomal escape during infection of eukaryotic cells, leading to an attenuation in virulence (Reglier-Poupet *et al.*, 2003a). It was suggested that the lipoproteins in *L. monocytogenes* may interact with components of the phagosomal membrane to facilitate its disruption. In *M. tuberculosis* growth of an *lsp* mutant was not affected *in vitro*, but virulence was attenuated in a mouse model of infection (Sander *et al.*, 2004). A mutation within the *lsp* gene of *S. uberis* did not effect the growth of this bacterium in nutrient rich media or in skimmed milk. This suggests that the signal peptide of MtuA does not effect the correct folding of this protein or the activity, as functional MtuA is required for growth in milk (Smith *et al.*, 2003). It could be hypothesised that the *lsp*⁻ mutant would be as equally virulent as the wild type in a dairy cow challenge due to the mutant being able to grow well in skimmed milk. However, this will depend on the roles that lipoproteins play within the infection of the bovine mammary gland. It may be that some lipoproteins play a role in

attachment to host tissues or other essential roles in nutrient uptake that have yet to be identified. It is unknown as to whether all the lipoproteins are functional in the presence of an intact signal peptide sequence.

3.3.4 An *S. uberis* insertion mutant of *lsp*⁻ revealed alternative processing of the signal peptide of MtuA

Western blotting and ELISA of cell fractions prepared from the *lsp*⁻ mutant with anti-MtuA revealed that the protein, comparable to the wild type strain, remained localised to the membrane. This indicated that the absence of Lsp does not alter anchoring, suggesting that Lgt was functioning correctly, and also that the diglyceride anchor on the lipobox cysteine residue to the membrane was still present. This means that the main mechanism of anchoring lipoprotein to the membrane is via the signal peptide and the lipid group as has been described for other bacteria (Hamilton *et al.*, 2006; Leskela *et al.*, 1999; Petit *et al.*, 2001; Sankaran and Wu, 1994; Stoll *et al.*, 2005). Using Western blot no MtuA protein is detected within the cell contents fraction, however in the ELISA data a small amount of protein is detectable within the this fraction. This discrepancy between the two data sets can be explained by the use of two different preparations to carry out the two techniques to analyse the location of MtuA. The ELISA data was carried out on samples that had been prepared before the complete optimisation of the cell fractionation procedure. This procedure is described by Jones *et al.*, 2004 (Jones *et al.*, 2004) and involved slightly different methods to separate the membrane and cell contents. It was found that the methods described in this thesis resulted in better separation of the two fractions.

It was interesting to see that two different forms of MtuA were detected by Western blotting of fractionated samples from the *lsp*⁻ mutant. The upper band corresponded to that of the full length, unprocessed MtuA but the smaller protein appeared to migrate slightly less than in comparable wild type preparations, suggesting that MtuA was cleaved at an alternative position. An equivalent result has been observed in several other bacterial species. In *B. subtilis* alternative processing of the lipoprotein PrsA was seen in a *lsp* mutant (Tjalsma *et al.*, 1999a) and in *L. monocytogenes* Western blot detected a processed form of the LpeA lipoprotein in the *lsp* mutant as well as a band of unprocessed size (Reglier-Poupet *et al.*, 2003a). The mature like form has also been described in *S. suis* (De Greeff *et al.*, 2003) and in *M. tuberculosis* (Sander *et al.*, 2004)The identity of the activities responsible for the alternate processing of lipoproteins in *B. subtilis* and *L. monocytogenes* has not been described.

In light of the discovery of the ExPortal, a single site for protein secretion on the bacterial cell (Rosch and Caparon, 2004; Rosch and Caparon, 2005) it could be imagined that proteins are produced, targeted and exported through the Sec machinery and end up on the extracytoplasmic side of the membrane. Individual proteins could then be targeted to their individual pathways to either anchor and/or remove the signal peptide sequences e.g. Sortase substrates, signal peptidase I substrates, lipoprotein processing substrates. If the active components of these pathways are absent then it could be hypothesised that the ExPortal becomes blocked with proteins that are not being correctly processed. Such a scenario may prevent the processing of other proteins and therefore a "rescue mechanism" may have evolved to clear the ExPortal of aberrantly processed secreted proteins. In the case of the *lsp*⁻ mutation, the cysteine residue from the lipoprotein's lipobox is likely to be modified by Lgt

and data presented here shows the signal peptides remain attached. The hydrophobic nature of the signal peptides may result in the retention of the protein on this location, causing a blockage of the ExPortal and reducing secretion from the cell. Removal of the signal peptide from the protein may assist in clearance of any blockage thus restoring secretion. It is interesting to note that the alternative processing, in *S. uberis* was only detected during stationary phase when the bacteria would not be proliferating rapidly, if at all.

An enzyme that has been implicated in cleavage of the signal peptides of lipoproteins is Enhanced Expression of Pheromone (Eep). Eep has been characterised in *Enterococcus faecalis* (An *et al.*, 1999) and shown to cleave the signal peptides of lipoproteins to yield octapeptides that are subsequently involved in inducing conjugation between different strains of *E. faecalis.*

3.3.5 The Lsp sequence of *S. uberis*

In *S. uberis* the ORF homologous with other known members of the Lsp family was SUB0729. The amino acid sequence of SUB0729 appeared to contain the usual four membrane-spanning domains and the conserved residues predicted for stability and activity. During the BLAST homology searches that were carried out to identify an ORF responsible for the Lsp activity, a second ORF was identified in the genome that had homology to protein sequences, labelled Signal peptidase type II, which is an alternative name for Lsp in the NCBI protein database (Appendix 1) (Figure 56). This protein was identified using the Lsp amino acid sequence of *L. monocytogenes* (accession number ZP_01942712) where the E-number was 1.2e-45. This protein appeared to be conserved across many bacterial species including

Streptococci, Staphylococci, Enterococci, Bacilli and Listeriae but, importantly, the amino acid sequence has none of the conserved functional domains associated with proteins known to possess lipoprotein signal peptidase activity. Searches using PFAM (Finn *et al.*, 2006) propose that the alternative SPase II protein contains a PSP1 C-terminal conserved region, an Nrap domain and an uncharacterised bacterial protein domain, which are not associated with sequences of functional Lsp enzymes. Analysis of the amino acid sequence with those of characterised Lsp sequences such as *B. subtilis* and *E. coli*, show there to be very little homology between these sequences. Furthermore, a search of the MEROPS peptidase database (Rawlings *et al.*, 2006) finds no likeness to signal peptidases, or peptidases in general. There are no reports within the literature ascribing functions to such proteins. An attempt to find a mutant within the gene encoding for this protein within the *S. uberis* pGh9^{+}::IS*S1* mutant bank was unsuccessful, implying that it might encode an essential function within the cell. It is also possible that the annotation of this protein within the databases is incorrect, but only a functional study would confirm this.

MtuA is an essential lipoprotein of *S. uberis* and is required for growth in milk and *in vivo*. This data suggests that correct processing of MtuA by Lsp is not essential for the function of this protein.

Chapter 4

Characterisation of the lipoprotein diacylglyceryl transferase (Lgt) Enzyme

4.1 Introduction

Lipoproteins are a major class of membrane bound proteins, that were first identified in *Escherichia coli*, but have been shown to be present within many bacterial pathogens including *Staphylococcus aureus*, *Mycobacterium tuberculosis* and Streptococcal species (Hamilton *et al.*, 2006; Leskela *et al.*, 1999; Petit *et al.*, 2001; Stoll *et al.*, 2005; Sutcliffe and Russell, 1995; Sutcliffe and Harrington, 2004a). Lipoproteins perform critical roles within bacteria; these include facilitating nutrient uptake (Janulczyk *et al.*, 2003), mediating antibiotic resistance (Sutcliffe and Russell, 1995), protein folding and the processing of extracytoplasmic proteins emerging from the cell (Kontinen *et al.*, 1991; Wahlstrom *et al.*, 2003). Lipoproteins are synthesized with an N-terminal signal sequence that directs the protein into the lipoprotein processing pathway. Within this signal sequence a specific motif, the Lipobox (von Heijne, 1989) (Sutcliffe and Harrington, 2002), is present which acts as a target for the lipoprotein diacylglyceryl transferase enzyme (Lgt), permitting lipid modification and the consequent anchoring of the protein to the membrane (Hantke and Braun, 1973; Leskela *et al.*, 1999). In a number of bacteria it has been demonstrated that lipid modification is a pre-requisite for cleavage of the signal peptide by the lipoprotein signal peptidase (Lsp) (Hussain *et al.*, 1982; Tjalsma *et al.*, 1999a; Tokunaga *et al.*, 1982).

Lgt is ubiquitous across bacterial species. The understanding of the biosynthetic anchoring pathway of lipoproteins in Gram negative bacteria has been elucidated in considerable detail

(Braun and Hantke, 1975; Chattopadhyay and Wu, 1977; Dev and Ray, 1984; Hantke and Braun, 1973; Hussain *et al.*, 1982; Innis *et al.*, 1984; Inukai *et al.*, 1984; Lee *et al.*, 1983; Tokunaga and Wu, 1984; Tokunaga *et al.*, 1982; Tokunaga *et al.*, 1985; Yamagata *et al.*, 1982; Yamagata, 1983; Yamaguchi *et al.*, 1988). In Gram positive species of bacteria Lgt was first reported in *S. aureus*, where the gene encoding Lgt was identified by complementation of a temperature sensitive defect in an *E. coli lgt* mutant (Qi *et al.*, 1995). A high level of homology was found between the sequences of Lgt from *E. coli*, *S. typhimurium*, *H. influenzae* and *S. aureus* at the protein level (Qi *et al.*, 1995).

The lipobox motif is present in the C terminal portion of the signal peptide sequence. The consensus sequence of the lipobox is LxxC, where the cysteine residue is the invariable target used by Lgt to lipid-anchor lipoproteins to the membrane. In a study using the signal peptide region of Braun's lipoprotein from *E. coli*, it was revealed that Lgt used diacylglycerol derived from phosphatidylglycerol to anchor the protein to the membrane while other, similar, molecules such as phosphatidylethanolamine or cardiolipin were not suitable substrates for this enzyme (Sankaran and Wu, 1994). Lgt transfers the diacylglycerol moiety from phosphatidylglycerol to the sulfhydryl group of the cysteine residue with the formation of N-acyl-S-diacylglyceryl cysteine. Once lipidation has occurred Lsp recognizes the modified cysteine residue and cleaves the signal peptide sequence leaving the lipid modified cysteine residue as the new amino terminus of the protein (Hussain *et al.*, 1982).

The functional role of Lgt has been studied in *Bacillus subtilis* (Antelmann *et al.*, 2001; Leskela *et al.*, 1999). A mutant was identified that abolished the modification of lipoproteins

however, unlike in Gram negative species, bacteria carrying the *lgt* mutation remained fully viable (Gan *et al.*, 1993; Leskela *et al.*, 1999; Yamagata *et al.*, 1982). By use of tritiated palmitic acid and via SDS PAGE analysis, multiple labelled lipoprotein bands were visualised from the *B. subtilis* wild type but no labelled lipoproteins were evident in the *lgt* mutant. Western blotting of the major lipoprotein, PrsA, displayed a larger protein in the *lgt* mutant compared to the form seen in the wild type, suggesting that its signal peptide sequence remained uncleaved. These findings provide evidence suggesting that the pathway for lipoprotein processing in Gram positive species of bacteria was similar to that detected in Gram negative bacteria.

An *lgt* deletion mutant has been partially characterized in *S. aureus* where the mutation caused altered anchoring of at least 20 membrane associated proteins (Stoll *et al.*, 2005). Several lipoproteins were released into the culture supernatant (SitC, PrsA, and OppA) along with non-lipoproteins, for example AltE, a major autolysin, and N-acetylglucosaminidase. The altered localisation of these non-lipoproteins is not fully understood (Stoll *et al.*, 2005). A similar result was found in an *lgt* deletion mutant in *Listeria monocytogenes*, where putative lipoproteins were found in the supernatant. These proteins were not apparent in the supernatant when the mutant was grown in the presence of globomycin, an antibiotic that inhibits Lsp activity (Baumgartner *et al.*, 2007). This suggests that the lack of anchoring and release of the proteins was dependent not only on the absence of Lgt, but also the presence of Lsp.

Lgt homologues are present in all streptococcal species sequenced to date and lipoproteins represent an abundant group of streptococcal proteins with a wide range of functions (Sutcliffe and Russell, 1995). An *S. pneumoniae lgt* mutant was shown to be viable, but exhibited reduced virulence (Petit *et al.*, 2001). The pathogenicity of an *lgt* mutant in *S. equi* was reduced but not ablated indicating that it is not an absolute requirement for virulence in a challenge model in the horse (Hamilton *et al.*, 2006). In *Streptococcus uberis*, a solute receptor involved in manganese transport (MtuA) showed high homology to other lipoprotein receptor antigens (LraI), most notably PsaA of *S. pneumoniae* and MtsA of *S. pyogenes*. A mutant lacking MtuA was identified from a random mutant bank due to its inability to grow in milk and functional MtuA was essential for infection of the bovine mammary gland (Smith *et al.*, 2003). MtuA is part of a 4 gene operon; MtuR, A, B and C. MtuB encodes an ATP-binding protein, MtuC encodes a hydrophobic membrane protein and MtuR regulates the expression of the operon (Smith *et al.*, 2003). MtuA has been localised to the cell membrane using ELISA and Western blotting techniques (Jones *et al.*, 2004). The amino acid sequence of MtuA contains a typical lipoprotein sequence and, within the signal peptide sequence there is a lipobox motif. Its localisation to the membrane and the observation that it is cleaved by Lsp (Jones, 2006) (Chapter 3) suggests that MtuA is likely to be a typical lipoprotein modified by Lgt. As such, MtuA represents a useful candidate with which to study the function of Lgt in *S. uberis*.

This chapter reports the properties of Lgt in *S. uberis* through the use of a comparative analysis of the wild type bacteria with an isogenic mutant.

4.2 Results

4.2.1 The *lgt* locus in the *S. uberis* strain 0140J genome

The *lgt* gene (SUB0578) of *S. uberis* strain 0140J was identified using homology searches (blast-n (Altschul *et al.*, 1990)) with the sequences of functionally characterised *lgt* genes of *B. subtilis* (accession AAC67287) and *E. coli* (accession NP417305). Homology of the primary amino acid sequence to functionally described Lgt encoded by other bacterial species was determined (Table 8). The amino acid sequences of characterised Lgt proteins were aligned (Figure 13) and the residues shown to be critical in *E. coli* analysed (Qi *et al.*, 1995; Sankaran *et al.*, 1997). His-103 and Tyr-235 have been shown to be essential for the activity of Lgt (Sankaran *et al.*, 1997). His-103 is not conserved, but Tyr-235 is conserved in *S. uberis*. Two of four of the other residues present in *E. coli* shown to be important for the structure function of Lgt are conserved in *S. uberis*. Lgt of *S. uberis* is 259 amino acids in length and, as in *S. aureus* (Stoll *et al.*, 2005), is flanked upstream by an ORF (SUB0577) with homology to *hprK* (an ATP dependent, metabolite-activated protein kinase/phosphorylase (Reizer *et al.*, 1998)) and downstream by an ORF (SUB0579) encoding an unknown hypothetical protein (Figure 12).

4.2.2 Genotypic detection of *lgt* mutants within the *S. uberis* strain 0140J pGh9^{+}::IS*S1* mutant bank

The *lgt* gene of *S. uberis* is 777 bp in length and, to isolate a mutant carrying an insertion in *lgt*, a bank of approximately 8,800 random insertion mutants (Ward *et al.*, 2001) was screened by PCR (Figure 14). Correct mutants were identified by PCR carried using the reverse primer for *lgt* (P349) or by DNA sequencing. Many of the positive bands that can be seen in figure

13 were shown to be false positive. Three mutants were identified with insertions in the region of the *lgt* locus. Sequence analysis revealed that two mutations mapped within the open reading frame (*S. uberis lgt*::IS*S1*$_1$ had an IS*S1* insertion 416 bp from start codon, and *S. uberis lgt*::IS*S1*$_2$ had an IS*S1* insertion 729 bp from start codon). A further mutant carried an insertion that mapped outside the open reading frame (*S. uberis lgt*::IS*S1*$_3$ has an IS*S1* insertion 13 bp after the stop codon) (Figure 12). The insertion in *S. uberis lgt*::IS*S1*$_1$ disrupts the Lgt signature sequence (PS01311) as described by Prosite (Hulo *et al.*, 2006). The amino acids in this motif are highly conserved between species and are in a central part of the protein.

Southern blotting was used to verify that each mutant carried a single insertion (Figure 16). Excision of the pGh9$^+$ vector from *S. uberis lgt*::IS*S1*$_1$ (cured mutant) allowed PCR amplification across the *lgt* locus of this mutant to be performed (primers P348 and P349). An 800 bp band shift was evident reflecting the presence of the residual IS*S1* element within the *lgt* gene (Figure 15). The excision of pGh9$^+$::IS*S1* from the *lgt*::IS*S1*$_1$ mutant was confirmed by Southern blot using a probe complementary to IS*S1* (Figure 16).

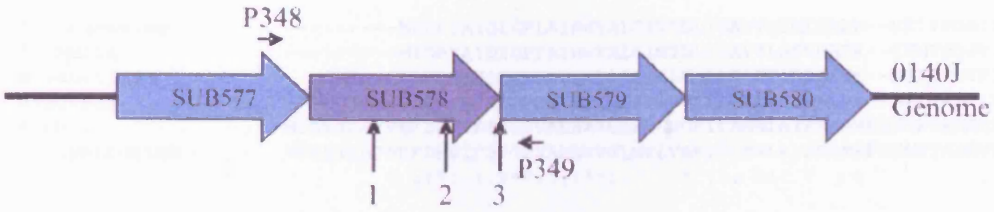


Figure 12 Context of *lgt* in the genome of *S. uberis* strain 0140J

The flanking region of *lgt* are shown, upstream is *hprK* (HPr kinase/phosphatase) and downstream are two genes with unknown function. Three mutants were identified in the *lgt* locus, *S. uberis lgt*::IS*S1*$_1$ is 416 bp from the start codon, *S. uberis lgt*::IS*S1*$_2$ is 729 bp from start codon and *S. uberis lgt*::IS*S1*$_3$ is 13 bp after the stop codon.

Bacteria	**Protein length (amino acids)**	**% identity with *S. uberis* Lgt**	**% conserved with *S. uberis* Lgt**
E. coli	291	30	52
S. typhimurium	291	31	51
B. subtilis	269	46	68
S. aureus	279	45	68
S. pneumoniae	262	65	82

Table 8 Homologies of the predicted Lgt of *S. uberis* to characterised Lgt proteins.

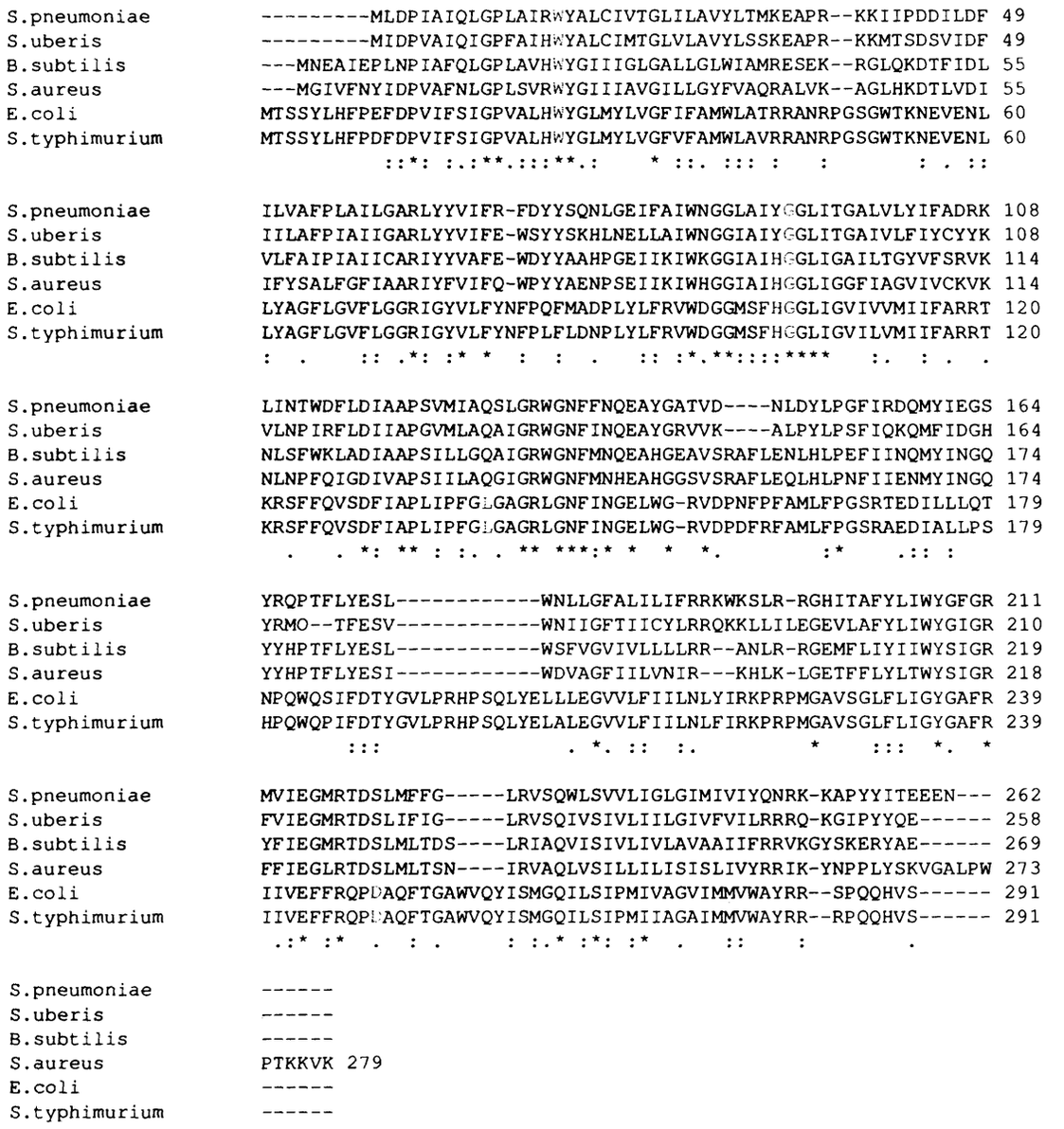


Figure 13 Conservation of critical residues of lipoprotein diacylglyceryl transferase

The amino acid sequences of *S. pneumoniae*, *S. uberis*, *B. subtilis*, *S. aureus*, *E. coli* and *S. typhimurium* were compared. The residues shown to be essential in *E. coli* are marked in blue (Sankaran *et al.*, 1997) and those residues shown to be important for the structure and function of the enzyme are shown in green (Qi *et al.*, 1995).

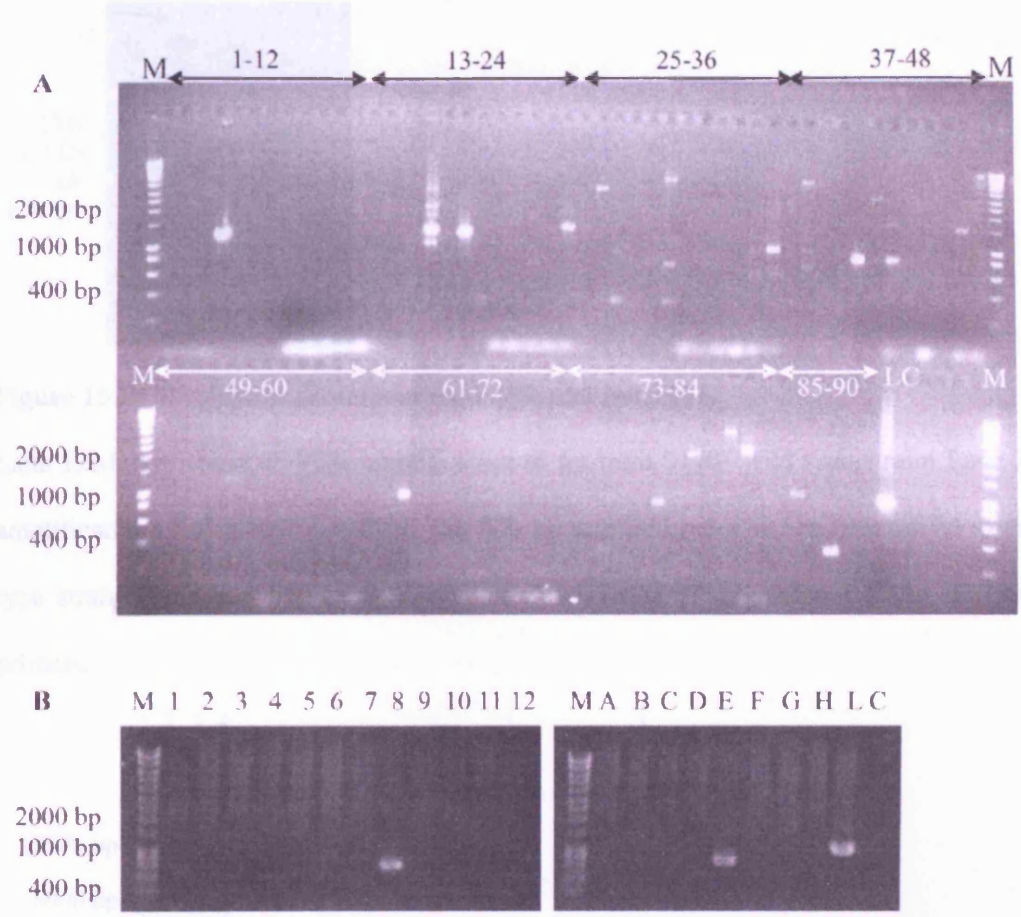


Figure 14 Identification of *lgt* mutants within pGh9$^+$::IS*S1* mutant bank.

PCR was carried out using P349 and P370 on pools of DNA prepared from pGh9$^+$::IS*S1* mutant bank. Plate 45 was identified as containing an *lgt* insertion mutant (A) and DNA preparations from rows and columns of plate 45 identified the mutant in well 8E (B). Size of molecular weight marker is shown in base pairs, *lgt* wild type gene (L), control, no template DNA (C).

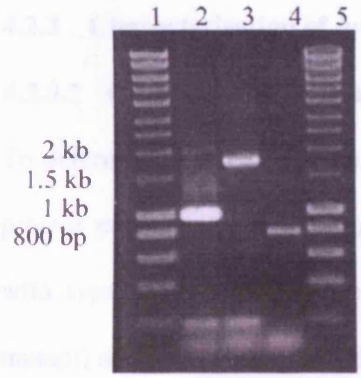


Figure 15 PCR amplification from wild type and *lgt*::IS*S1*.

Lane 1, Marker; Lane 2, PCR amplification of *lgt* from 0140J wild type strain; Lane 3, PCR amplification of *S. uberis lgt*::IS*S1*$_1$ (an 800 bp size difference is seen compared to the wild type strain); lane 4, PCR amplification of *S. uberis lgt*::IS*S1*$_1$ using *lgt* and IS*S1* specific primers.

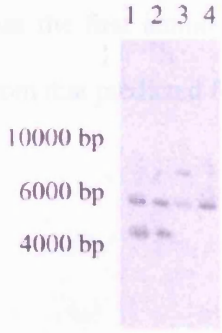


Figure 16 Southern blot probing for IS*S1* in *lgt* insertion mutants.

Chromosomal DNA was prepared from each of the three uncured *lgt* mutants (Lane 1, *lgt*::IS*S1*$_1$; Lane 2, *lgt*::IS*S1*$_2$; Lane 3, *lgt*::IS*S1*$_3$) and from the cured *lgt*::IS*S1*$_1$ mutant (Lane 4), digested with the restriction endonuclease *Hind*III (which cuts the pGh9$^+$::IS*S1* vector once), splitting the two IS*S1* elements into two discreet DNA fragments, allowing the visualisation of two or three bands when the DNA is subjected to alkali Southern blotting using a probe complementary to the IS*S1* element of the pGh9$^+$::IS*S1* construct.

4.2.3 Characterisation of *lgt* mutants

4.2.3.1 Characterisation of the extracellular profile of the *lgt* mutant

To determine whether a mutation in the *lgt* gene of *S. uberis* had any effect on the secreted protein profile, capsule fractions were prepared from mid-exponential phase cultures of the wild type strain and the three *lgt* mutants (*lgt*::IS*S1*$_1$ (cured mutant), *lgt*::IS*S1*$_2$ (uncured mutant) and *lgt*::IS*S1*$_3$ (uncured mutant)). Several proteins that were seen in the protein profile for *lgt*::IS*S1*$_1$ were not evident in the wild type strain, *lgt*::IS*S1*$_2$ or *lgt*::IS*S1*$_3$ when analysed on SDS PAGE (Figure 17). These proteins were immobilised on PVDF membrane and subjected to N-terminal sequencing by Edman degradation. Three proteins were identified, including MtuA and corresponded to those predicted to be lipoproteins in the bioinformatic analysis (Chapter 3, Table 6), (Table 9). The N-terminal residue of each protein sequenced was the first amino acid following the cysteine residue of the lipobox motif, a site distinct from that predicted for the cleavage of lipoproteins by Lsp.

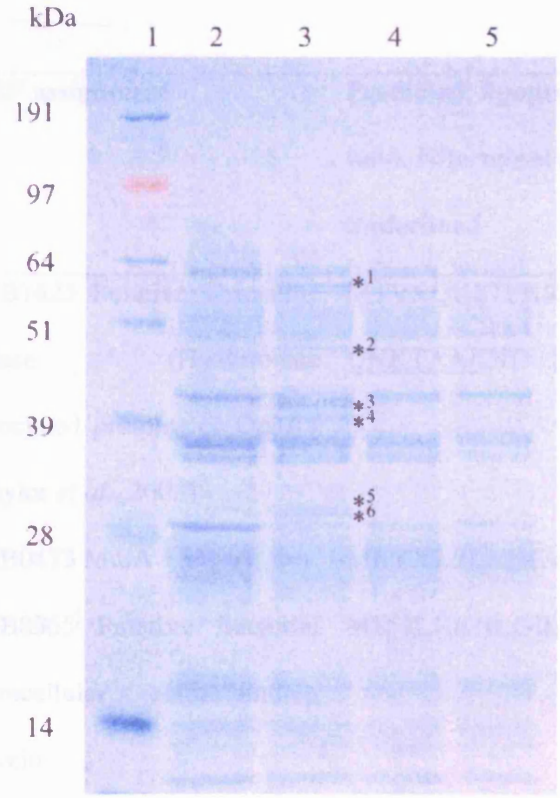


Figure 17 Protein profiles of capsule fractions.

Lane 1, molecular weight marker. Proteins from capsule fraction prepared from mid-exponential phase *S. uberis* cultures of wild type (lane 2) and three *lgt*⁻ mutants (IS*S1*::lgt_1 cured and IS*S1*::$lgt_{2\text{-}3}$ uncured) (lanes 3-5). Proteins were separated by SDS-PAGE and stained with Simply blue safe stain reagent (Invitrogen). At least 6 additional protein bands were detected in *lgt*::IS$S1_1$ (marked with asterisks 1-6). Three of these proteins were identified by N-terminal sequencing; 2 - Hyaluronate associated protein SUB1625, 4 - Manganese transporter *uberis* (MtuA) SUB0473, and 6 - an extracellular solute binding protein SUB0365.

ORF assignment	**Predicted lipoprotein leader sequence, lipobox in bold. N-terminal sequence derived from *lgt*::IS*S1*$_1$ is underlined**
SUB1625 Putative Threonine kinase (Hyaluronate associated protein) or OppA1 (Taylor *et al.*, 2003)	MTVAQKSTFKRFGLGAVTLASAALL**MAC** GNKTAAKND
SUB0473 MtuA	MKKKLSLAIMAFLGLLM**LGAC** SVGNGRKAT
SUB0365 Putative Bacterial extracellular solute-binding protein	MMTLKKNLGILSLTLGTLAI**LAAC** GNKASNNSG

Table 9 Sequence of proteins identified by N-terminal sequencing.

4.2.3.2 Differential localisation of MtuA in wild type and *lgt* mutants

MtuA was used as an example lipoprotein and studied to determine the effect of the *lgt* mutations. Anti-MtuA serum (Smith *et al.*, 2003) was used in an ELISA to detect any exposed MtuA protein on the surface of whole wild type and *lgt* mutant derivative cells (uncured mutants) (Figure 18). MtuA was found in very low levels on the surface of the wild type strain, *lgt*::IS*S1*$_2$ and *lgt*::IS*S1*$_3$, but was clearly detectable on the surface of *lgt*::IS*S1*$_1$. This observation coupled with the different extra-cytoplasmic protein profile shown for *lgt*::IS*S1*$_1$ suggested that this mutant, unlike the wild type, was unable to conventionally anchor lipoproteins to the membrane. Furthermore the findings for *lgt*::IS*S1*$_2$ and *lgt*::IS*S1*$_3$ suggested that the phenotype observed for *lgt*::IS*S1*$_1$ was not due to polar or downstream effects. Further analysis focused upon the *S. uberis lgt*::IS*S1*$_1$ mutant, from this point referred to as *lgt*$^-$ mutant.

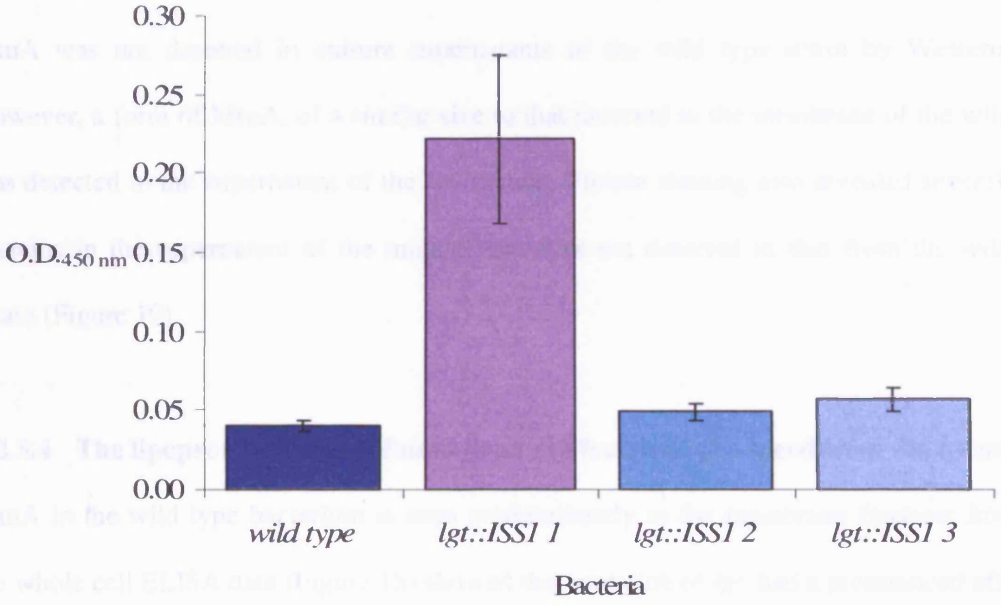


Figure 18 Detection of MtuA by whole cell ELISA on wild type and *lgt*⁻ mutants.

Bacteria were grown to mid-exponential phase and the suspensions adjusted to an $O.D._{550nm}$ of 0.3 with THB. MtuA is only found on the surface of *lgt*::IS*S1*$_1$ cells. Wild type blue bar, *lgt*::IS*S1*$_1$ (uncured) purple bar, *lgt*::IS*S1*$_2$ (uncured) aqua bar and *lgt*::IS*S1*$_3$ (uncured) light blue bar. Error bars represent a standard deviation between results performed in triplicate.

4.2.3.3 Additional proteins are present in the supernatant of the *lgt*⁻ mutant including MtuA

MtuA was not detected in culture supernatants of the wild type strain by Western blot. However, a form of MtuA, of a similar size to that detected in the membrane of the wild type was detected in the supernatant of the *lgt*⁻ mutant. Protein staining also revealed several other proteins in the supernatant of the mutant that were not detected in that from the wild type strain (Figure 19).

4.2.3.4 The lipoprotein MtuA is found in all cell fractions produced from the *lgt*⁻ mutant

MtuA in the wild type bacterium is seen predominantly in the membrane fraction; however, the whole cell ELISA data (Figure 18) showed that mutation of *lgt*⁻ had a pronounced effect on the localisation of MtuA. Anti-MtuA serum was used to detect MtuA by Western blotting of cell fractions prepared from the wild strain and *lgt*⁻ mutant (Figure 20). A single band of approximately 37 kDa was seen only in the membrane fraction of the wild type strain as shown previously (Jones *et al.*, 2004). In the *lgt*⁻ mutant a similar sized protein band was detected in the capsule, cell wall, membrane and cytoplasmic contents. The apparent size of the band seen in the wild type strain and the *lgt*⁻ mutant is indistinguishable, which corresponds with the N-terminal sequencing data of lipoproteins released by the *lgt*⁻ mutant, whereby the signal peptide was removed from the mature protein only one residue further into the sequence than would be predicted for conventional lipoprotein processing (i.e. after the cysteine of the predicted lipobox). Therefore, it was surmised that there was only one amino acid difference between the predicted mature MtuA anchored in the wild type and that released by the *lgt*⁻ mutant.

MtuA was detected by ELISA within the membrane fraction and at a slightly reduced level within the cytoplasmic fraction of the wild type strain (Figure 21). In the *lgt*⁻ mutant a higher level of MtuA was detected in cell contents, membrane, cell wall and capsule. The ELISA detected a MtuA within the cell contents of the wild type strain, where as in the Western blot, MtuA was only detected within the membrane of the wild type and not the cell contents. It should be noted that these two data sets were carried out on samples prepared at different times and, therefore the fractions may have been treated slightly differently. These fractions are enrichments of the portion of the cell that they represent. This is likely to account for the slight discrepancy between the two data sets.

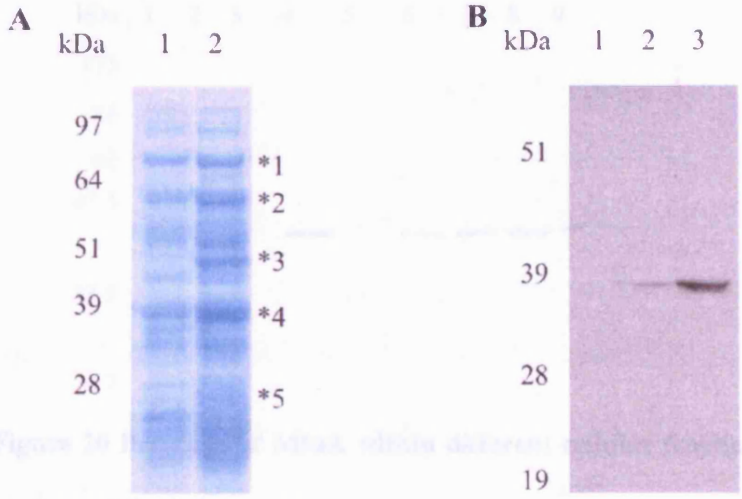


Figure 19 MtuA and several other proteins were detected in the *lgt*⁻ mutant supernatant

Proteins from the supernatant prepared from mid-exponential phase grown *S. uberis* wild type strain (Lane 1) and *lgt*⁻ mutant (Lane 2) were separated by SDS-PAGE and stained with Simply blue safe stain reagent (Invitrogen) (A). At least 5 additional protein bands were visually detected in *lgt*⁻ (marked with numbered asterisks). A second identical gel was transferred to blotting membrane before being incubated with anti-MtuA. MtuA can be found in the supernatant of the *lgt*⁻ mutant, but not in the wild type strain, lane 3 is wild type strain membrane (B). Asterisk 4 on the stained gel is MtuA. Molecular weights of markers are shown in kDa.

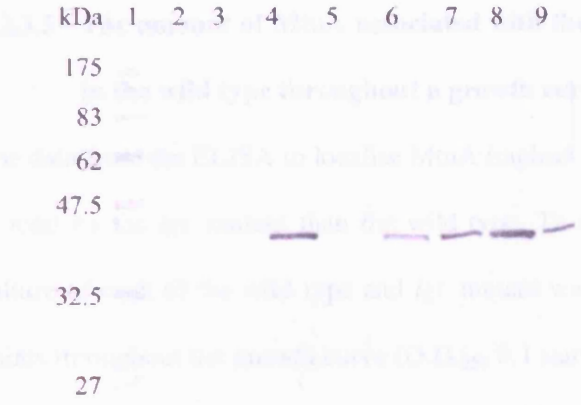


Figure 20 Presence of MtuA within different cellular fractions prepared from wild type strain and the *lgt*⁻ mutant.

Lane 1 – Marker shown with molecular weights (kDa). Lanes 2-5, wild type capsule, cell wall, membrane and cell contents, respectively. Lanes 6-9 *lgt*⁻ mutant capsule, cell wall, membrane and cell contents, respectively.

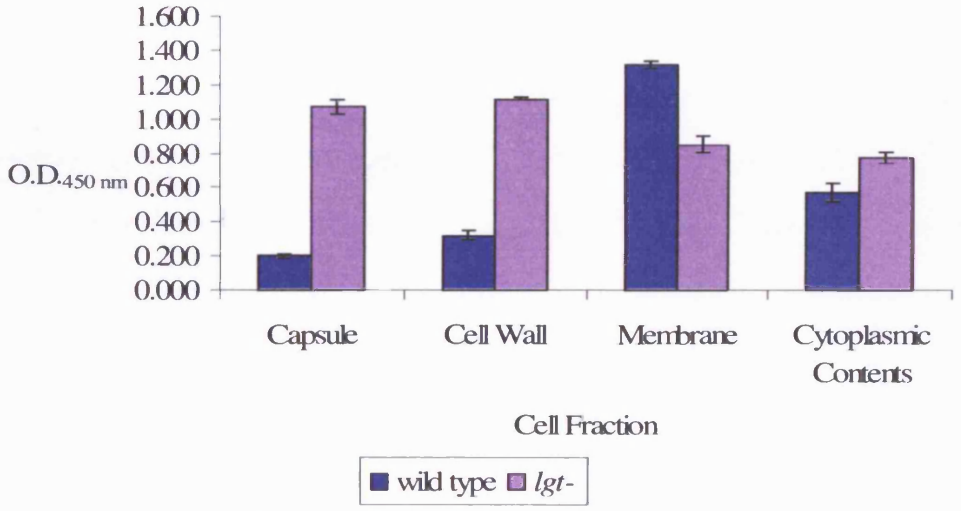


Figure 21 Detection of MtuA within cellular fractions by ELISA.

ELISA performed on samples prepared from mid-exponentially grown *S. uberis*. Error bars represent a standard deviation between results performed in triplicate for wild type (blue bars) and *lgt*⁻ mutant (purple bars).

4.2.3.5 The amount of MtuA associated with the *lgt*⁻ mutant appeared 50% higher than in the wild type throughout a growth curve

The data from the ELISA to localise MtuA implied that there was more MtuA being produced in total by the *lgt*⁻ mutant than the wild type. To determine if this was the case, a 2.5 litre culture of each of the wild type and *lgt*⁻ mutant was grown and bacteria harvested at various points throughout the growth curve ($O.D._{550}$ 0.1 start of exponential, 0.5 middle of exponential phase, 0.8 start of stationary phase and also at 24 hours). Whole cell lysates were prepared and the protein concentrations determined. ELISA carried out on the whole cell lysates containing equivalent amounts of total protein showed that the amount of MtuA detected within each lysate at each point in the growth curve from the *lgt*⁻ mutant was approximately 50% higher compared with the equivalent lysate from the wild type (Figure 22).

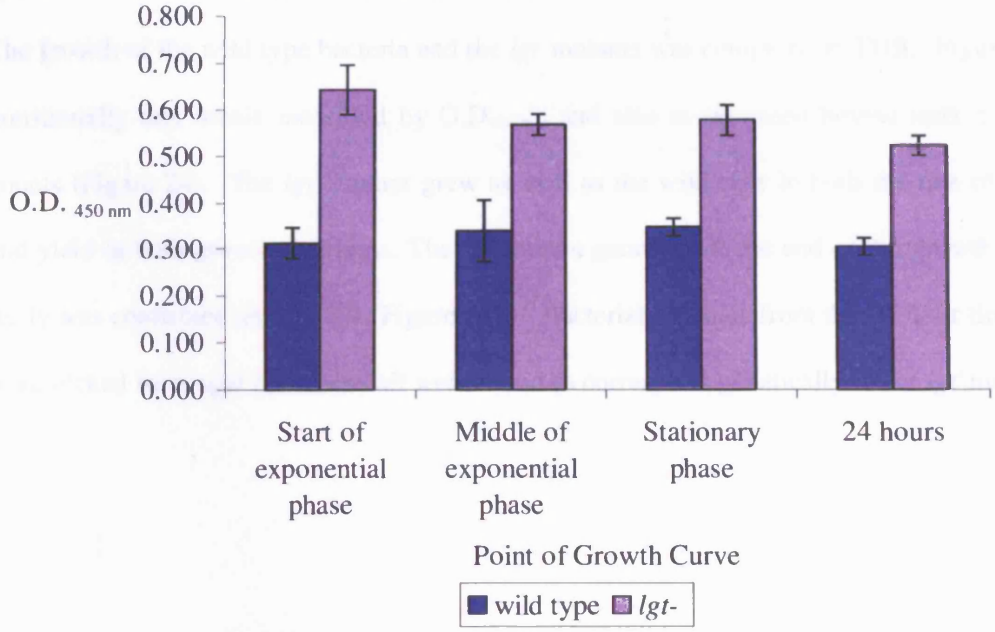


Figure 22 ELISA to determine the relative levels of MtuA through a growth curve in the wild type strain and *lgt*⁻ mutant.

ELISA performed on whole cell lysates prepared from the wild type strain (blue) and *lgt*⁻ mutant (purple) grown in THB to an O.D. $_{550\ nm}$ of 0.1 (start of exponential phase), 0.5 (middle of exponential phase), 0.8 (stationary phase) and at 24 hours. Equivalent amounts of total protein (20 ng/well) were used for each data point. Error bars represent a standard deviation between results performed in triplicate.

4.2.3.6 Growth of the *lgt*⁻ mutants in Todd Hewitt Broth and Skimmed Milk

The growth of the wild type bacteria and the *lgt*⁻ mutants was compared in THB, (Figure 23), a nutritionally rich media measured by $O.D._{540nm}$ and also in skimmed bovine milk by viable counts (Figure 24). The *lgt*⁻ mutant grew as well as the wild type in both the rate of growth and yield in both growth mediums. The *lgt*⁻ mutant genotype at the end of the growth in milk study was confirmed using PCR (Figure 24b). Bacterial colonies from the 24 hour time point were picked from agar plates and all were found to correspond genetically to the *lgt*⁻ mutant.

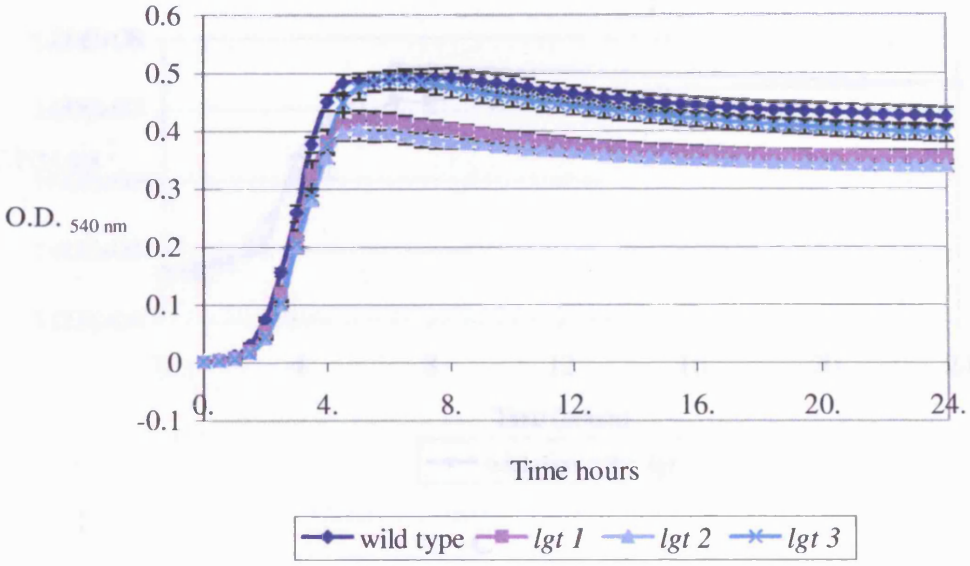


Figure 23 Growth of *S. uberis* wild type was compared with that of the *lgt*⁻ mutants over 24 hours in THB

Error bars represent a standard deviation between results performed in triplicate.

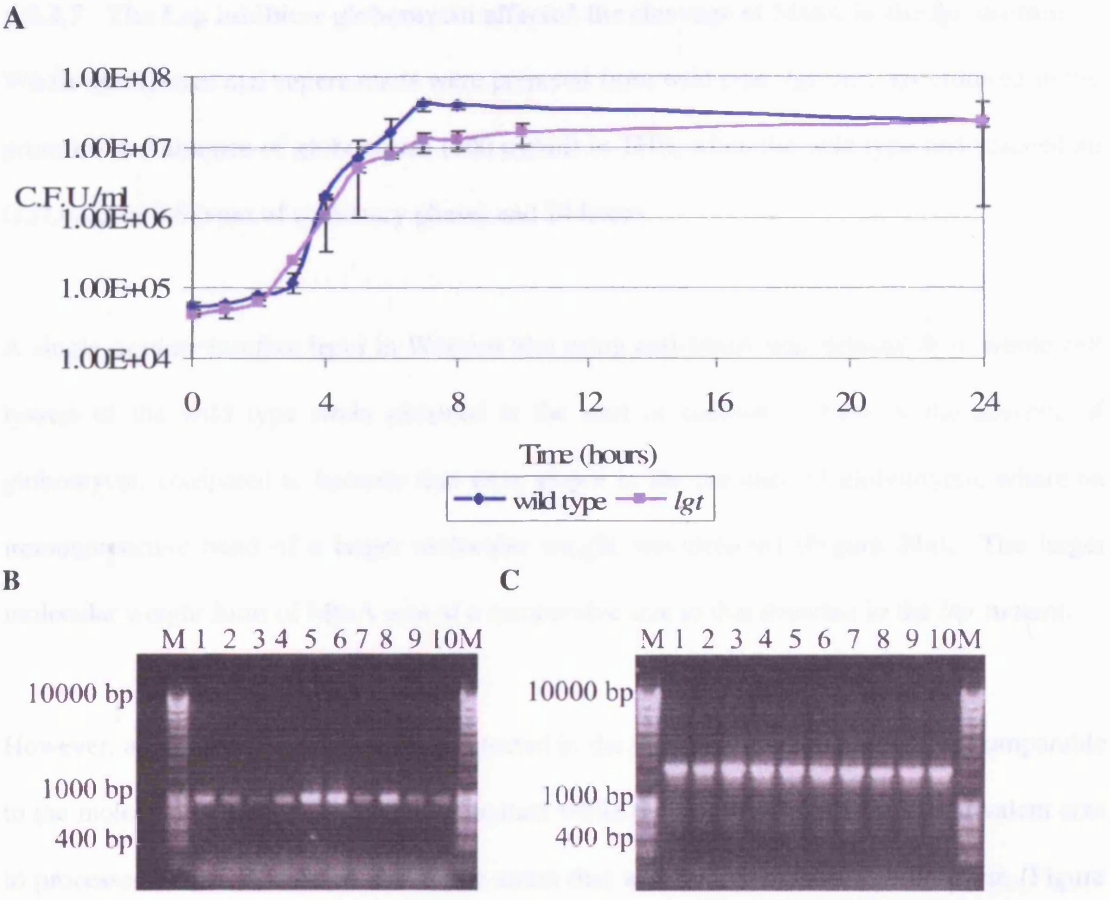


Figure 24 Growth of *S. uberis* wild type compared to *lgt*⁻ mutant in milk over 24 hours.

The growth of *lgt*⁻ mutant was compared to the wild type in milk (A). Error bars represent a standard deviation between results performed in triplicate. PCR was carried out on 9 colonies from milk grown cultures of wild type and *lgt*⁻ to check that the bacterial cultures were what free of contamination. (B) Wild type using PauA Fwd and Rev primers, (C) *lgt*⁻ mutant using P348 and P349. Lane 10 PCR was carried out on genomic DNA from the wild type or the *lgt*⁻ mutant.

4.2.3.7 The Lsp inhibitor globomycin affected the cleavage of MtuA in the *lgt*⁻ mutant

Whole cell lysates and supernatants were prepared from wild type, *lgt*⁻ and *lsp*⁻ cultured in the presence and absence of globomycin (100 μg/ml) in THB, when the wild type had reached an $O.D._{550nm}$ of 0.8 (start of stationary phase) and 24 hours.

A single immunoreactive band in Western blot using anti-MtuA was detectable in whole cell lysates of the wild type strain obtained at the start of stationary phase in the absence of globomycin, compared to bacteria that were grown in the presence of globomycin, where an immunoreactive band of a larger molecular weight was detected (Figure 24a). The larger molecular weight form of MtuA was of a comparable size to that detected in the *lsp*⁻ mutant.

However, at 24 hours two bands were detected in the wild type; the larger band is comparable to the molecular weight seen in the *lsp*⁻ mutant while the smaller band is of an equivalent size to processed MtuA seen in the wild type strain that was not treated with globomycin (Figure 24b).

No immunoreactive band in the whole cell lysate was detected at the start of stationary phase when the *lgt*⁻ mutant was treated with globomycin (Figure 24a). However, at the same time point, MtuA was detectable in the supernatant, and at a similar size in both the *lgt*⁻ mutant with and without globomycin Figure 24c). The amount of MtuA detected in the *lgt*⁻ mutant that had been grown in the presence of globomycin was reduced compared to the mutant that had not been treated. It is worthwhile to note that the *lgt*⁻ and *lsp*⁻ mutants grown in the presence of globomycin were slowed in their growth compared to the wild type (Figure 26). The *lgt*

and *lsp* mutants that were not treated with globomycin grew slower on this occasion than the wild type, which is different to the data that is presented in figure 8 (chapter 3) and figure 22 (chapter 4). The difference is likely to be due to the wild type strain having grown particularly rapidly on this occasion. MtuA is detectable in the *lsp*⁻ mutant at the start of stationary phase compared to the *lgt*⁻ mutant where it remains undetectable even when additional protein (the whole sample, approximately 25-50 μg) was separated by SDS PAGE and blotted to membrane.

At 24 hours two immunoreactive bands to anti-MtuA were detected in the *lgt*⁻ mutant that had been grown in the presence of globomycin (Figure 24b). The band of a larger molecular weight was of an equivalent size to MtuA that was detected at the start of stationary phase in the *lsp*⁻ mutant, and the smaller band was of a comparable size to that of MtuA from the wild type. A band of an analogous size to that seen in the *lgt*⁻ mutant cell lysate not treated with globomycin, was seen in the supernatant of the *lgt*⁻ mutant grown in the presence of globomycin (Figure 24d). However, as seen in the sample prepared from the mutant at stationary phase there was a reduced amount of MtuA detected.

The protein profiles in the supernatant of the wild type bacterium treated with globomycin at both time points were similar to the profiles of the supernatant prepared from wild type bacteria not treated with globomycin (Figure 24e and f). The level of extracytoplasmic proteins detected in the supernatant of the *lgt*⁻ mutant treated with globomycin was much reduced compared to the *lgt*⁻ mutant not treated with globomycin. The level of proteins present in the supernatant prepared from the *lsp*⁻ mutant was not reduced, and therefore it can

be hypothesised that this reduction in extracytoplasmic protein profile is not growth phase related as both were at similar O.D. when the samples were taken. However, by 24 hours the protein profile of the *lgt*⁻ mutant treated with globomycin appeared to be comparable to the mutant not treated with globomycin.

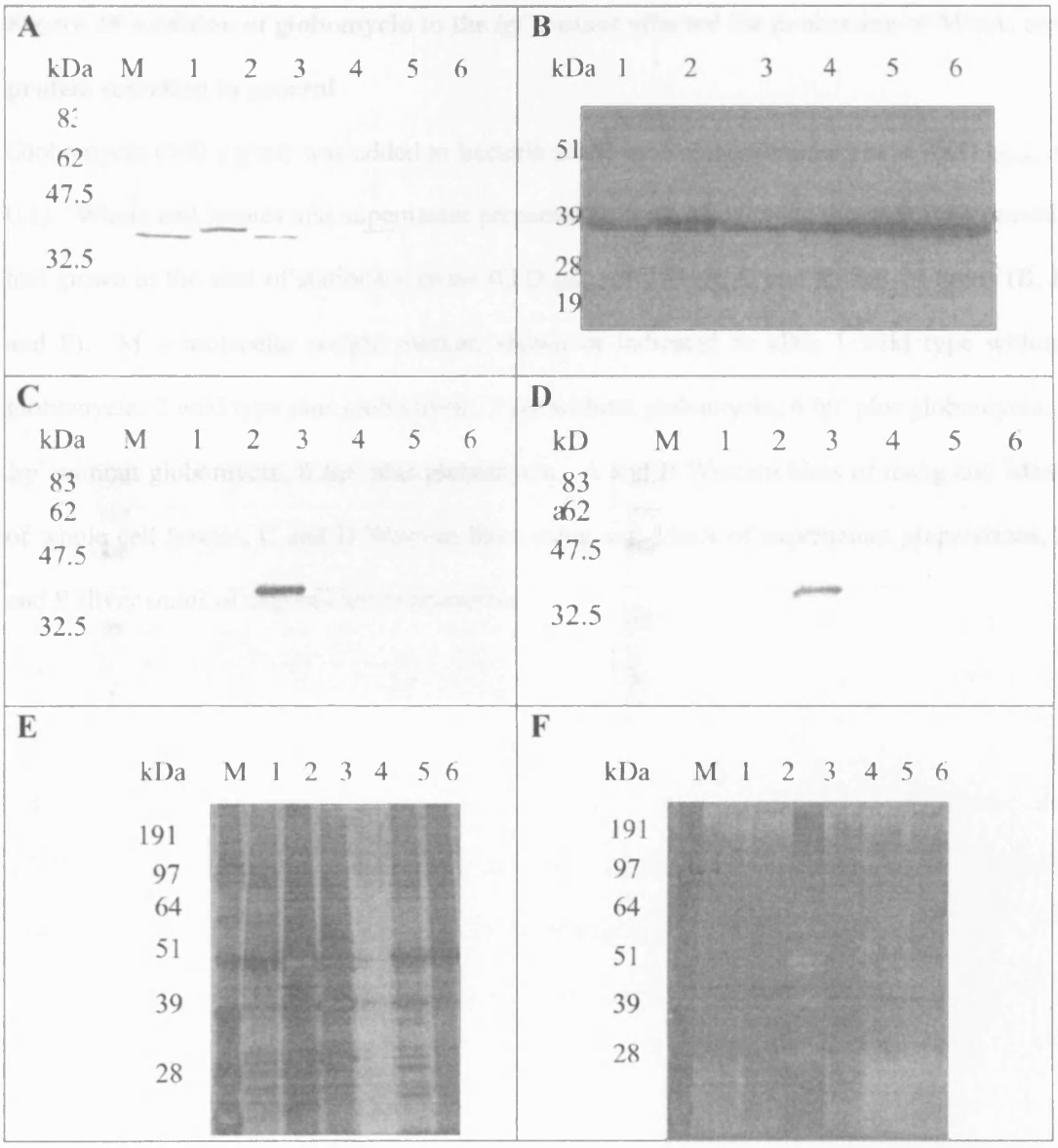

A
kDa M 1 2 3 4 5 6
62
47.5
32.5
B
kDa 1 2 3 4 5 6
51
39
28
19
C
kDa M 1 2 3 4 5 6
83
62
47.5
32.5
D
kD M 1 2 3 4 5 6
83
62
47.5
32.5
E
kDa M 1 2 3 4 5 6
191
97
64
51
39
28
F
kDa M 1 2 3 4 5 6
191
97
64
51
39
28

Figure 25 Addition of globomycin to the *lgt*⁻ mutant affected the processing of MtuA, and protein secretion in general

Globomycin (100 μg/ml) was added to bacteria at the start of exponential phase ($O.D._{550nm}$ of 0.1). Whole cell lysates and supernatant preparations were made when the wild type bacteria had grown to the start of stationary phase ($O.D._{550nm}$ of 0.8) (A, C and E) and 24 hours (B, D and F). M – molecular weight marker, shown or indicated in kDa, 1 wild type without globomycin, 2 wild type plus globomycin, 3 *lgt*⁻ without globomycin, 4 *lgt*⁻ plus globomycin, 5 *lsp*⁻ without globomycin, 6 *lsp*⁻ plus globomycin. A and B Western blots of using anti MtuA of whole cell lysates, C and D Western blots using anti-MtuA of supernatant preparations, E and F silver stains of supernatant preparations.

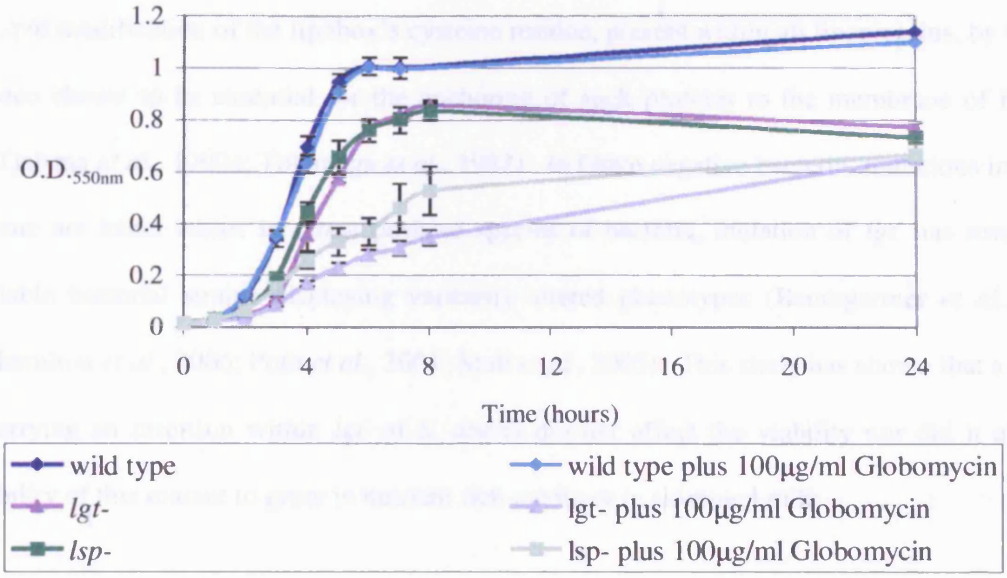


Figure 26 Growth of the *S. uberis* wild type and lipoprotein processing mutants in the presence and absence of the Lsp inhibitor globomycin.

Bacteria were grown to the start of exponential phase ($O.D._{550nm}$ of 0.1) in THB before the addition of globomycin 100 µg/ml. Growth was measured at an $O.D._{550nm}$ over 24 hours. Error bars represent a standard deviation between results performed in triplicate.

4.3 Discussion

Lipid modification of the lipobox's cysteine residue, present within all lipoproteins, by Lgt has been shown to be essential for the anchoring of such proteins to the membrane of bacteria (Tjalsma *et al.*, 1999a; Tokunaga *et al.*, 1982). In Gram negative bacteria, mutations in the *lgt* gene are lethal while, in Gram positive species of bacteria, mutation of *lgt*⁻ has resulted in viable bacterial strains displaying variously altered phenotypes (Baumgartner *et al.*, 2007; Hamilton *et al.*, 2006; Petit *et al.*, 2001; Stoll *et al.*, 2005). This study has shown that a mutant carrying an insertion within *lgt*⁻ of *S. uberis* did not affect the viability nor did it alter the ability of this mutant to grow in nutrient rich media or in skimmed milk.

4.3.1 An *lgt*⁻ insertion mutant of *S. uberis* affects the extracellular profile and some, if not all, of the extra proteins have been aberrantly processed

Three mutants were found in the area surrounding the *lgt* locus in the pGh9^{+}::IS*S1* mutant bank of *S. uberis* strain 0140J. The location of the insertion in the *lgt*::IS*S1*$_1$ mutant mapped to 416 bp from the start codon which is in the middle of the ORF and likely to completely disrupt the function of the protein. Lgt is predicted to have seven transmembrane domains (as predicted by HMMTOP (Tusnady and Simon, 2001), TMHMM misses the 5th helix that has been previously predicted in *B. subtilis* and *E. coli* (http://www.cbs.dtu.dk/services/TMHMM/). The insertion in *lgt*::IS*S1*$_1$ maps immediately after the fourth transmembrane domain and in the middle of the prosite PS01311 motif that was selected on the basis of being highly conserved amino acid sequence within the protein. The conserved amino acids within this motif are likely to play a role in either the catalysis of the diglyceride bond or in the stability of the enzyme. Therefore *lgt*::IS*S1*$_1$ would not be

predicted to be functional. The two other mutants *lgt*::IS*S1*$_2$ and *lgt*::IS*S1*$_3$ mapped 729 bp from the start codon and 13 bp after the stop codon, respectively. The amino acid sequence of Lgt predicts that the last transmembrane domain ends at 741 bp from the start codon; this would suggest that the insertion for *lgt*::IS*S1*$_2$ is within the last transmembrane domain. No difference to the wild type could be seen when the extracellular protein profiles of the mutant and the wild type were compared, this would suggest that disruption of the ORF at this point has no major effect on the activity of Lgt. An *lgt* mutant of *B. subtilis* was selected from an transposon mutant library from a screen to detect mutants that were defective in the sporulation pathway (Dartois *et al.*, 1997). This mutation prevented the correct localisation of the lipoprotein KapB, which is required for KinB signal transduction and activation of the phosphorelay to sporulation. The mutation is 87 bp upstream of the insertion for *lgt*::IS*S1*$_2$. Suggesting that there is a divide somewhere between the locations of these two points where the essential region ends and the non-essential region starts. The third mutant, *lgt*::IS*S1*$_3$ was mapped to outside of the ORF encoding Lgt, comparison of the extracellular profile of the mutant with the wild type found no difference suggesting that the phenotypes seen in *lgt*::IS*S1*$_1$ were due to the insertion of IS*S1* in the gene and not due to downstream effects of the mutation.

The two downstream mutants (*lgt*::IS*S1*$_2$ and *lgt*::IS*S1*$_3$) within the *lgt* locus proved highly useful, as complementation of the *lgt*::IS*S1*$_1$ was not feasible, nor was the construction of inframe deletion mutants. No effect upon lipoprotein processing was detected within these two mutants, which suggested that there was no involvement of the genes immediately downstream of these mutations that influenced lipoprotein processing, and that the mutation in

lgt::IS*S1*$_1$ had inactivated the only gene responsible for modifying lipoproteins to enable anchoring to the membrane.

Analysis of the proteins found within the capsule fractions from the three *S. uberis lgt*::IS*S1* mutants revealed at least 6 additional proteins in *lgt*::IS*S1*$_1$ that were present neither in the wild type nor in the other *lgt* mutants. Three of these were confirmed as putative lipoproteins following protein identification via Edman degradation N-terminal sequencing data. Interestingly each of the three lipoprotein sequences was the amino acid immediately following the cysteine residue of the lipobox sequence motif rather than the predicted N-terminus of the full length protein. This is surprising as modification of the cysteine within the lipobox motif by Lgt has been shown to be a prerequisite for the cleavage of the signal peptide from lipoproteins (Tokunaga *et al.*, 1982) and consequently in the absence of Lgt cleavage of the signal peptide would not be predicted. This finding was supported by Western blotting data with anti-MtuA, where a band of the same size as the wild type was detected in the supernatant, capsule, cell wall, membrane and cell contents of the *lgt* mutant. Interestingly, in the extracellular proteome of *B. subtilis* wild type (Antelmann *et al.*, 2001) 7 lipoproteins were shed into the extracellular medium and 6 of these were processed in an alternate manner similar to that of the *S. uberis lgt*$^-$ mutant. The *B. subtilis lgt* mutant released 11 other lipoproteins into the growth media, although it was not shown how these proteins were processed (Antelmann *et al.*, 2001). This data suggests the presence of an enzyme "Shavase" capable of removing lipoproteins from the surface of the bacteria.

Rosch and Caparon (Rosch and Caparon, 2005) reported the presence of a single site for the export of secreted proteins in *S. pyogenes*; the ExPortal. It is hypothesised that it is this point on the cell from which all proteins are secreted and where Lgt and Lsp are most likely to be located. In the *lgt*⁻ mutant, lipoproteins could be secreted through the ExPortal, but conceivably get stuck due to the absence of a system to anchor them into the membrane and subsequently distribute around the whole cell. The activity of Lsp requires this anchoring modification. The action of a hypothesised "Shavase" enzyme could cleave the protein away from the cell, thus unblocking the secretion site. It would be of interest to not only determine which protein encodes the activity of the Shavase, but to also determine its function in the wild type.

A search of the *S. uberis* strain 0140J genome for this hypothetical Shavase, using the MEROPS peptidase database (Rawlings *et al.*, 2006), suggested the presence of approximately 90 different peptidase enzymes (Appendix 2). Twenty-seven of these have potential membrane spanning domains, which would be expected if this enzyme has similarity to the other proteins predicted to be present in the ExPortal (Lgt having 7 predicted transmembrane domains, Lsp having 4 predicted transmembrane domains, SecY which interacts with SecA (Mori and Ito, 2006) is predicted to have 10 transmembrane domains as predicted by TMHMM (Finn *et al.*, 2006)).

4.3.2 The activity of MtuA, an essential lipoprotein of *S. uberis*, is not influenced by aberrant processing due to an insertion mutation in *lgt*

This study has shown that the *lgt* mutant of *S. uberis* was still viable, growing in skimmed milk to levels comparable to that of the wild type strain. This finding was unexpected since *S. uberis* lacking MtuA was unable to grow in milk (Smith *et al.*, 2003) and in the absence of Lgt, the protein is somewhat mislocalised and it could be envisaged unlikely to be in the correct location in the membrane. Mutation of the *S. pneumoniae lgt* gene produced bacteria that were 5-fold reduced in their ability to cause infection (Petit *et al.*, 2001), and in an *S. aureus lgt* mutant, both the whole bacteria and a crude lysate induced fewer inflammatory cytokines. This suggested that lipoproteins themselves, or proteins processed by lipoproteins, may be having an effect on the immune response (Stoll *et al.*, 2005). An *lgt* mutant and a maturase lipoprotein mutant have both been studied in *S. equi* where it was found that the maturase mutant was less virulent than both the wild type and *lgt* mutant (Hamilton *et al.*, 2006). As a whole, this data indicates that some Gram positive pathogens are constrained in their pathogenic abilities when the Lgt function is absent, but that in some cases (such as in *S. equi*) mutation of essential lipoproteins has a much greater effect on the virulence of the pathogen. The experimental evidence presented above for the *S. uberis lgt* mutant indicates that infection of the lactating mammary gland would not be predicted to be compromised due to the ability of the mutant to grow in milk. It is likely that the situation described for *S. equi* (Hamilton *et al.*, 2006) where disruption of individual lipoproteins has a greater effect of on the virulence of the bacteria will be comparable for *S. uberis*, as has been seen in the use of an *mtuA* mutant in an infection model of dairy cattle (Smith *et al.*, 2003). However it is far from clear what effect the absence of Lgt may have on other virulence related lipoproteins. The

hypothesis that this mutant would be as virulent as the wild type would reinforce the data suggesting an *lgt*⁻ mutation has no constraint on the bacterium's virulence and that any or all virulence related lipoproteins can function adequately in the absence of a membrane anchor.

Analysis of the Western blotting and ELISA data detecting MtuA in *lgt*⁻ cell fractions showed that MtuA was present in the membrane and cytoplasmic contents, as in the wild type, but was also present in the supernatant, capsule and cell wall fractions. The level of MtuA protein appeared to be increased compared to the level in the wild type. This data implies that MtuA could retain some functionality as it was still likely to be transiently associated with the membrane, possibly by virtue of hydrophobic amino acids in the H-region of the signal peptide. This transient anchoring could allow sufficient manganese to enter the bacteria to meet requirements for growth. However, full length MtuA protein is not seen via Western blot analysis, which suggests that full length MtuA is either present in very small amounts within the cell and is not detectable, or it is processed as soon as it is made.

MtuA may have other protein-protein interactions with other members of the Mtu operon. MtuC is a hydrophobic membrane-protein and earlier studies have revealed that a mutation within the gene encoding this protein resulted in an altered location of MtuA; MtuA was detected in the cell wall and capsule as well as the membrane and cell contents of *mtuC* mutants (Jones, 2006). The apparent size of MtuA detected in the *mtuC* mutant was comparable to that seen in the wild type strain (Jones, 2006) and also to that in the *lgt*⁻ mutant in the present study, suggesting that MtuA had undergone some form of alternative processing to release it from the membrane. It was hypothesised that an *lgt*⁻/*mtuC*⁻ double mutant may

behave differently in its localisation of MtuA compared to the *lgt*⁻ mutant in that more of the MtuA was expected to be less associated with the membrane. Unfortunately, an *mtuC* mutant could not be identified in an *lgt*⁻::IS*S1* double mutant bank of approximately 6000 mutants (data not shown). Assuming an even distribution of insertion events throughout the genome and no lethality the IS*S1* would be anticipated to insert every 300 base pairs, within a bank of 6000 mutants. Therefore, with an MtuC coding sequence of 840 bp, screening 6000 clones would be anticipated to be sufficient to identify the desired mutant on two occasions. The inability to find an *lgt*⁻/*mtuC*⁻ double mutant could be explained by two possibilities; firstly, that insertion was not totally random and consequently an insufficient number of mutants were screened; or that the double mutant is not viable. The last scenario was deemed unlikely due to the previous isolation of individual *lgt*⁻ (this study) and *mtuC* insertion mutants (Jones, 2006). Lack of viability may result from the absence of MtuC not in the membrane thus preventing interaction with MtuA and other members of the Mtu operon, resulting in insufficient uptake of manganese to support growth. However, as individual mutants in any of the genes in the Mtu operon are viable in THB, it would appear unlikely that lack of viability could account for the apparent absence of a double mutant within the second mutant bank.

A marked increase of the MtuA protein was detectable in the *lgt*⁻ mutant at all points in the growth curve. A quantitative method for determining the levels of mRNA that encode MtuA would provide evidence as to whether the regulation of this increase was at the transcription or translational level. It could be hypothesised that there is a regulatory system in the wild type bacteria that prevents MtuA being produced to the levels seen in the *lgt*⁻ mutant, due to sufficient levels of manganese accumulation. This would suggest that the regulation of the

levels of MtuA protein in the *lgt*⁻ mutant is likely to be carried out at a translational level. A preliminary investigation revealed no gross difference in mRNA from *mtuA* from the wild type and mutant strains. MntA of *B. subtilis* is found in the supernatant of the *lgt* mutant and was shown to be in an increased amount compared to the wild type strain (Antelmann *et al.*, 2001). Analysis of mRNA levels of *mntA* in the *lgt* mutant and the wild type strain showed no difference in levels of transcript of this gene. This suggested that there is another mechanism in that is able to alter the level of protein at the level of translation rather than at the level of transcription. It could be hypothesised that this mechanism is also in process in the *lgt* mutant of *S. uberis*.

The addition of globomycin to the *lgt*⁻ mutant revealed several unexpected phenotypes; MtuA was not detectable at the first time point (when wild type bacteria had reached the start of stationary phase); full length as well as processed MtuA was detectable at 24 hours in the whole cell lysate of the mutant; the growth rate of the *lgt*⁻ and *lsp*⁻ mutants treated with globomycin was initially reduced (although both mutants reached optical densities comparable to those detected in the absence of globomycin); and that the secretion profile of the *lgt*⁻ mutant was reduced at the start of stationary phase. Analysis by Western blot showed that, at the first time point, a small amount of MtuA was detectable in the supernatant of bacteria grown in the presence of globomycin, but that there was no MtuA detectable in the whole cell lysate in the *lgt*⁻ mutant. This suggested that MtuA was being produced but was also being degraded. It could be predicted that the antibiotic is placing the bacteria under an unidentified stress that would result in this phenotype.

The secretion profile of bacteria at the start of stationary phase is dramatically reduced, both in lipoproteins and in proteins normally present in the wild type strain. It could be hypothesised that the globomycin is preventing normal function of the bacteria due to some kind of blocking effect at the site of secretion. Such a dramatically altered phenotype could be explained if *S. uberis* only has a single site for all secretion, such as the ExPortal (Rosch and Caparon, 2005). It is unlikely to be due to an inability to traffic the protein to Lsp as an *S. uberis lsp*⁻ mutant is fully viable (Chapter 3).

At 24 hours two forms of MtuA were detectable in whole cell lysates prepared from the *lgt*⁻ mutant treated with globomycin. The smaller form is likely to be alternatively processed MtuA as it runs at a size that cannot be discriminated between that of processed MtuA in the wild type, and that of the *lgt*⁻ mutant without globomycin treatment. The form of MtuA with a larger molecular weight appears to be the full length form, which leads to the hypothesis that unlipidated lipoproteins are able to be processed by Lsp or that a hypothetical Shavase enzyme requires the presence of Lsp for activity in *S. uberis*. In *L. monocytogenes* a study suggested that Lsp was able to cleave unlipidated lipoproteins and that these appeared in the supernatant of an *lgt* deletion mutant (Baumgartner *et al.*, 2007). Addition of globomycin to this mutant affected the release of these proteins present in the supernatant fraction to appear similar to that of the wild type strain. To determine whether Lsp is able to cleave unlipidated lipoproteins, a double mutant in *lgt* and *lsp* needs to be studied as globomycin may effect other unknown cellular components.

It is intriguing that when globomycin is added to the *lsp*⁻ mutant the ability of this mutant is drastically reduced in its ability to grow. This is unexpected due to the fact that the target of globomycin is not present within this mutant. It would be interesting to determine if there was a secondary protein/s that this antibiotic is able to bind to that reduce the ability of this mutant to grow. It would also be interesting to determine if the reduction in the growth rate of the *lgt*⁻ mutant grown in the presence of globomycin is also due to the binding of the globomycin to Lsp or to this secondary target. The literature does not allude to any other target of globomycin.

In conclusion the *lgt*⁻ mutant of *S. uberis* has several novel and interesting phenotypes, most notably the presence of a so called shaving activity on the unmodified lipoproteins. It will be of interest to identify the other, additional, proteins present in the extracellular space in the *lgt*⁻ mutant and also to determine if any lipoproteins are released into the extracellular space in the wild type strain.

Chapter 5

Characterisation of an *lgt*⁻/*lsp*⁻ mutant in *S. uberis*

5.1 Introduction

5.1.1 Aberrant processing of lipoproteins in the presence of an *lgt*⁻ mutation

The lipoprotein processing pathway has been analysed in several bacterial species. Consistently, lipoprotein diacylglyceryl transferase (Lgt) has been observed to add lipid at the invariable cysteine residue of the lipobox, thus anchoring lipoproteins to the membrane (Leskela *et al.*, 1999; Petit *et al.*, 2001; Sankaran and Wu, 1994; Stoll *et al.*, 2005). Once the protein has been lipidated, lipoprotein signal peptidase (Lsp) was shown to cleave the signal peptide from the lipoprotein leaving the cysteine residue as the amino terminus of the mature protein. It has been reported that signal peptidase enzymes are not able to cleave the signal peptide from such proteins unless lipidation has first taken place (Tokunaga *et al.*, 1982).

Alternative processing around the lipobox sequence has been reported in *B. subtilis* (Antelmann *et al.*, 2001). In the wild type, several lipoproteins appear extracellularly and the amino terminus of these proteins is the amino acid immediately after the cysteine residue of the lipobox. A similar observation was made for *S. uberis* lacking functional Lgt (Chapter 4), where additional proteins corresponding to similarly cleaved lipoproteins, appeared in the culture supernatant. Several hypotheses were put forward to explain the phenomenon in *B. subtilis*. These included proteolytic shaving of lipoproteins to release lipoproteins from the membrane, or to release the lipoprotein from the cells after emission of the protein from the membrane (Antelmann *et al.*, 2001). A lipoprotein release determinant has been proposed for

B. subtilis, G-[NSTF]-x-[SGN]-x-[SGKAE]-x-x-[SGA]. This pattern was present in 6 of the 7 lipoproteins that have been shown to be shaved from wild type *B. subtilis*. This pattern was also present in 4 of the proteins that are found in the extracellular space of the *lgt* mutant and is completely absent from any of the lipoproteins that do not appear in the extracellular space. This motif was shown not to be conserved in the sequences of the three lipoproteins shown to be alternatively processed within the *lgt*⁻ mutant of *S. uberis* (chapter 4). Suggesting that this motif is different for different species of bacteria or there are other residues important for the activity of the putative Shavase enzyme.

Analysis of the extracellular proteins prepared from supernatants of wild type *L. monocytogenes*, an *lgt* mutant and the *lgt* mutant grown in the presence of globomycin (an antibiotic that inhibits the activity of Lsp), were compared by 2D PAGE (Baumgartner *et al.*, 2007). Additional lipoproteins were identified in the *lgt* mutant and the amino terminus of these proteins was determined to be the cysteine residue present in the lipobox but, in the presence of globomycin, these additional proteins were not evident. This suggested that Lsp was able to cleave the unlipidated lipoproteins and that this event was responsible for the release of lipoproteins into the supernatant (Baumgartner *et al.*, 2007). An *lgt*⁻/*lsp*⁻ double mutant of *L. monocytogenes* was not studied to confirm that Lsp was responsible for cleaving the signal peptides from the lipoproteins in the *lgt* mutant. An alternative proteolytic processing procedure for the lipoprotein SitC of *S. aureus* has been indicated, as in the absence of Lgt this protein appeared extracellularly and at a size consistent with such cleavage in close proximity to the lipobox (Stoll *et al.*, 2005). Lipoproteins of *M. tuberculosis* have been shown to be surface associated, which is unexpected given the nature of the diglyceride

bond to the cysteine that keeps the protein localised to the membrane. This observation may reflect that shaving may also take place in *M. tuberculosis* (Sutcliffe and Harrington, 2004a).

The data presented for *B. subtilis*, *L. monocytogenes* and *S. aureus* suggest that alternative mechanisms for processing lipoproteins occur in the absence of an active Lgt (and also in the presence of Lgt as described for *B. subtilis* (Antelmann *et al.*, 2001)). If Lsp is indeed able to cleave unlipidated lipoproteins, as suggested in *L. monocytogenes* (Baumgartner *et al.*, 2007), it would be interesting to determine which amino acids of the Lsp sequence allow this to occur as the detail of this process are, to date, unknown.

5.1.2 Alternative processing of Lipoproteins occurs in the presence of an *lgt*⁻ mutation in *S. uberis*

Three of the additional proteins (including MtuA) that were found to be present in an extracellular fraction of *S. uberis* lacking Lgt have sequences consistent with that of putative lipoproteins. These were atypically processed with respect to Lsp, as cleavage of the signal peptide was shown to have taken place on the carboxyl side of the cysteine residue of the lipobox motif (Chapter 4). This suggested the presence of activity other than Lsp that is able to cleave the signal peptides from lipoproteins. However, Western blotting of whole cell lysates showed that when the *lgt*⁻ mutant was grown in the presence of globomycin (a reversible inhibitor of Lsp), MtuA was detected at a size comparable to that seen in the *lsp*⁻ mutant and the wild type cultured in the presence of globomycin (Chapter 4). This suggested that Lsp might be able to cleave the signal peptide from unlipidated lipoproteins, or that a protein that is processed through Lsp is involved in the alternative processing phenomenon in

S. uberis. It is possible that there are two enzymes that are able to cleave signal peptides from lipoproteins; Lsp and an as yet unidentified factor. In order to clarify this matter, this chapter describes the construction of an *lgt*$^{-}$/*lsp*$^{-}$ double mutant and its characterisation with regard to lipoprotein processing.

5.2 Results

5.2.1 Production of *lsp*⁻::pGh9⁺::IS*S1* mutant bank

Targeted mutagenesis in *S. uberis* using a variety of vectors and cloning approaches has been attempted without success (data not shown). Therefore, a strategy to produce a random mutant bank in the *lsp*⁻ mutant background was undertaken. The cured, erythromycin sensitive *lsp*⁻ mutant was randomly mutagenised by pGh9⁺::IS*S1* (Maguin *et al.*, 1996) and clones picked into individual wells of 72 96-well microtitre plates. Pools of DNA were prepared from individual plates to allow genotypic screening (Taylor *et al.*, 2003).

5.2.2 Southern blot to determine the random nature of insertions

DNA was prepared from individual double *lsp*⁻::pGh9⁺::IS*S1* mutants, digested with the restriction endonuclease *Hind*III, and analysed by Southern blot using a probe complementary to IS*S1* (Figure 27). A common band from the *lsp*⁻ mutant was seen to migrate at 2.8 kb. The profiles of the mutants appeared different. Similar profiles have been seen within the parent ISS1 mutant bank and these were found to give acceptable genome-wide coverage on sequencing. Mutants 20 and 21 have very similar profiles, but it is not possible to say that they are identical as a Southern blot is very unlikely to resolve differences of less than 50 bp. Sibling mutants have been found within primary mutant bank at a frequency substantially lower than 1 in 100, so it is possible that this is what these two profiles are. Within lanes 3 and 22, the insertion for *lsp* appears to be missing, this may be due to the new insertion of the pGh9⁺::IS*S1* plasmid having inserted between the IS*S1* element of the disrupted *lsp*⁻ and the *Hind*III restriction enzyme site used to detect the band at 2.8 kb. This will make the band for the insertion within the *lsp* gene to appear slightly larger or smaller depending on the

orientation of the plasmid insertion. Within mutants 3 and 22, 3 and 4 bands are detectable, which is what is expected for a single or double insertion of the pGh9^{+}::IS*51* plasmid in the presence of an already established IS*51* insertion.

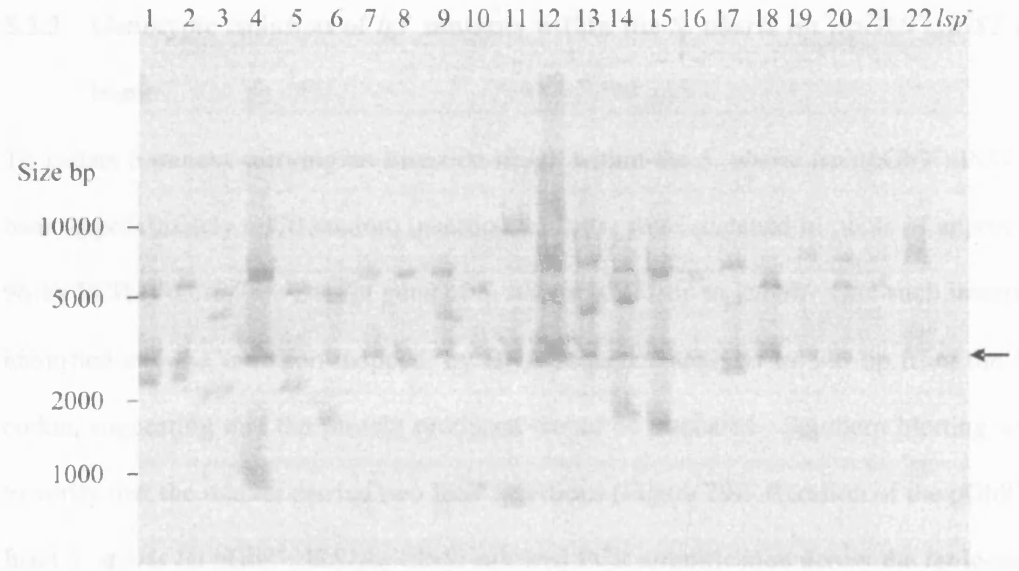


Figure 27 Southern blot of *lsp*⁻::pGh9⁺::IS*S1* double mutant bank

Chromosomal DNA was prepared from 22 *lsp*⁻pGh9⁺::IS*S1* double mutants and the cured *lsp*⁻ mutant, digested with the restriction endonuclease *Hind*III and subjected to alkali Southern blotting using a probe complimentary to the IS*S1* element of the pGh9⁺::IS*S1* vector. The IS*S1* element from the *lsp*⁻ mutant migrated at a size of approximately 2.8 kb and this was common to all double mutants, indicated by arrow.

5.2.3 Genotypic isolation of *lgt*⁻ mutants within the *S. uberis lsp*⁻::pGh9⁺::IS*S1* mutant bank

To isolate a mutant carrying an insertion in *lgt*⁻ within the *S. uberis lsp*⁻::pGh9⁺::IS*S1* mutant bank approximately 6000 random insertion mutants were screened in pools of approximately 96 by PCR (Figure 28). The *lgt* gene of *S. uberis* is 777 bp in length. One such insertion was identified and the insertion mapped, by DNA sequence analysis to 546 bp from the *lgt* start codon, suggesting that the protein produced would be truncated. Southern blotting was used to verify that the mutant carried two IS*S1* insertions (Figure 29). Excision of the pGh9⁺ vector from *S. uberis lgt*⁻pGh9⁺::IS*S1lsp*⁻::IS*S1* allowed PCR amplification across the *lgt* locus of this mutant to be performed using *S. uberis* specific primers. An 800 bp band shift was evident reflecting the presence of the residual IS*S1* element within the *lgt* gene (Figure 30). The location of the primers for each of the mutants is shown in Figure 31. The excision of pGh9⁺ from the *lgt*⁻::IS*S1lsp*⁻::IS*S1* (*lgt*⁻*/lsp*⁻)was also confirmed using Southern blot (Figure 29). The original IS*S1* element inserted at the *lsp* locus was confirmed by DNA sequence analysis and was found to be as in the single *lsp*⁻ insertion mutant, 132 bp from the start codon.

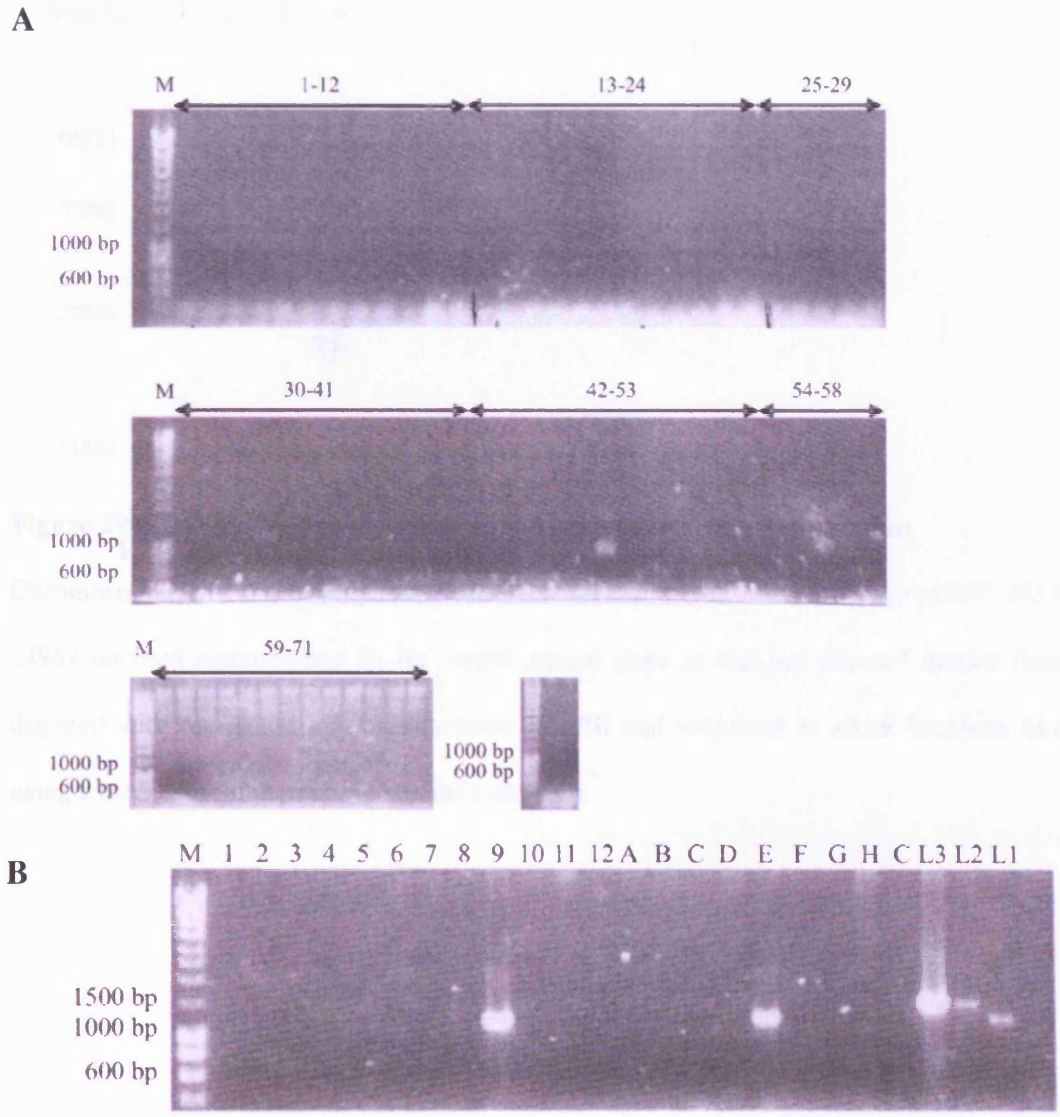


Figure 28 PCR screen for *lgt* mutant in *lsp*⁻ background mutant bank

PCR was carried out using primers P349 and P370 on pools of DNA prepared from the *lsp*⁻ ::pGh9⁺::IS*1* mutant bank. Plate 47 was identified as containing an *lgt* insertion mutant (A). DNA preparations were made from rows and columns of plate 47 and the mutant's location was identified as well 9E (B). Size of molecular weight marker is shown in base pairs. L1, L2 and L3 correspond to lgt_{1-3} respectively which are described in chapter 4, C – control PCR.

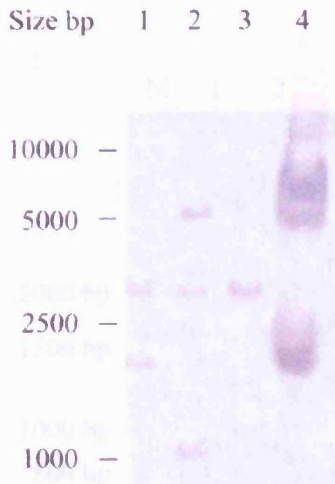


Figure 29 Southern blot probed with IS*S1* in the *lgt*⁻/*lsp*⁻ insertion mutant

Chromosomal DNA was prepared from each *lgt*⁻/*lsp*⁻ cured mutant 1), *lgt*⁻pGh9⁺::IS*S1*/*lsp*⁻ ::IS*S1* uncured mutant (lane 2), *lsp*⁻ cured mutant (lane 3) and *lsp*⁻ uncured mutant (lane 4), digested with the restriction endonuclease *Hind*III and subjected to alkali Southern blotting using a probe complimentary to the IS*S1* element.

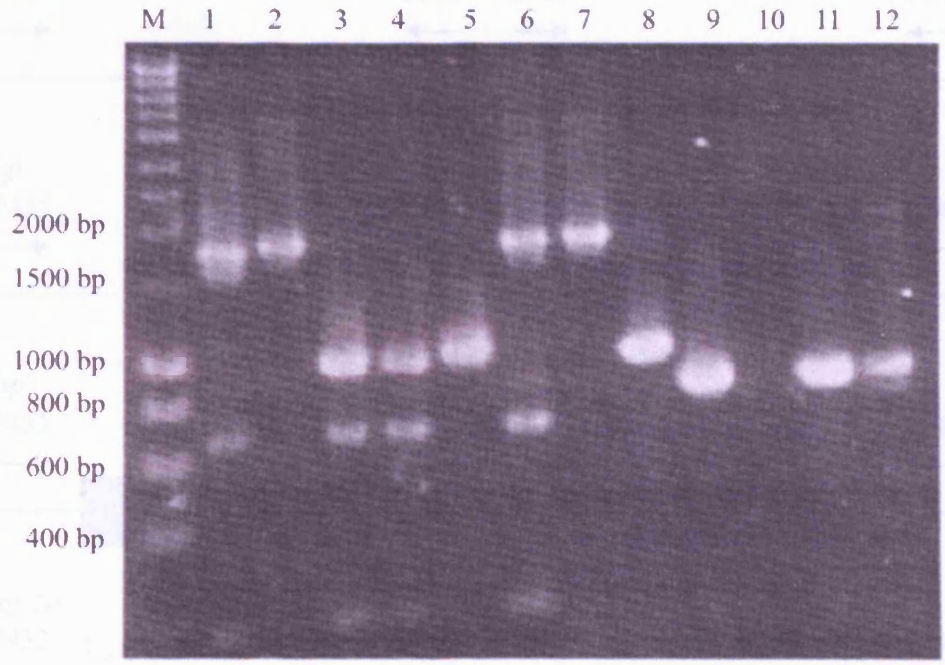


Figure 30 Confirmatory PCR on *lgt*⁻/*lsp*⁻ mutant

PCR carried out on DNA prepared from 0140J, *lsp*⁻, *lgt*⁻ and *lgt*⁻/*lsp*⁻ mutants. M – Molecular marker (bp), 1, *lgt*⁻ cured mutant DNA P348 and P349; 2, *lsp*⁻ cured mutant DNA P432 and P571; 3, 0140J DNA P348 and P349; 4, *lsp*⁻ cured mutant P348 and P349; 5, 0140J DNA primers P432 and P571; 6, *lgt*⁻ *lsp*⁻ cured mutant DNA P348 and P349; 7, *lgt*⁻/*lsp*⁻ cured mutant DNA P432 and P571; 8, *lgt*⁻/*lsp*⁻ cured mutant DNA P348 – 370; 9, *lgt*⁻/*lsp*⁻ cured mutant DNA P432 – 370; 10, Control (no template); 11, *lgt*⁻ cured mutant DNA P348 – 370; 12, *lsp*⁻ cured mutant P432-370

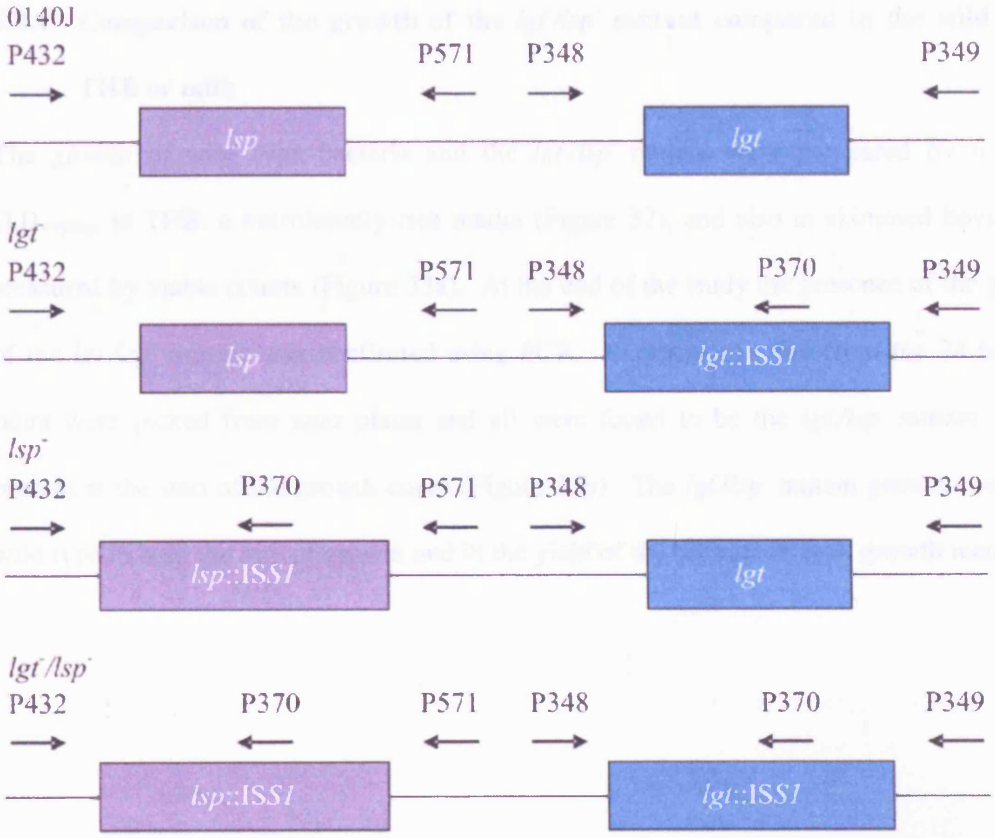


Figure 31 Location of primers within lipoprotein processing mutants

5.2.4 Comparison of the growth of the *lgt⁻/lsp⁻* mutant compared to the wild type in THB or milk

The growth of wild type bacteria and the *lgt⁻/lsp⁻* mutant were compared by measuring $O.D._{540nm}$ in THB, a nutritionally rich media (Figure 32), and also in skimmed bovine milk, measured by viable counts (Figure 33a). At the end of the study the presence of the genotype of the *lgt⁻/lsp⁻* mutant was confirmed using PCR. Bacterial colonies from the 24 hour time point were picked from agar plates and all were found to be the *lgt⁻/lsp⁻* mutant that was present at the start of the growth curve (Figure 33b). The *lgt⁻/lsp⁻* mutant grew as well as the wild type in both the rate of growth and in the yield of the bacteria in both growth medium.

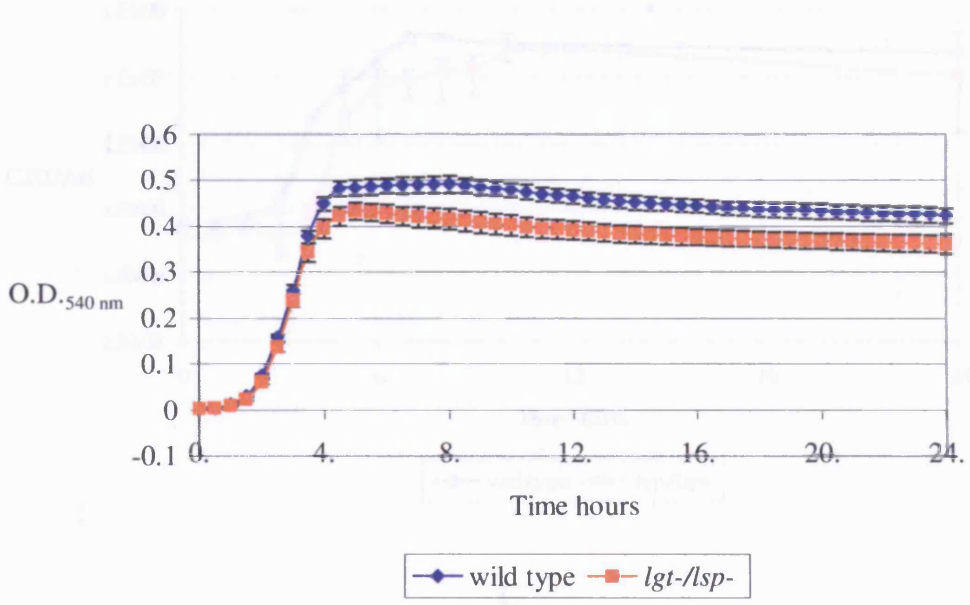
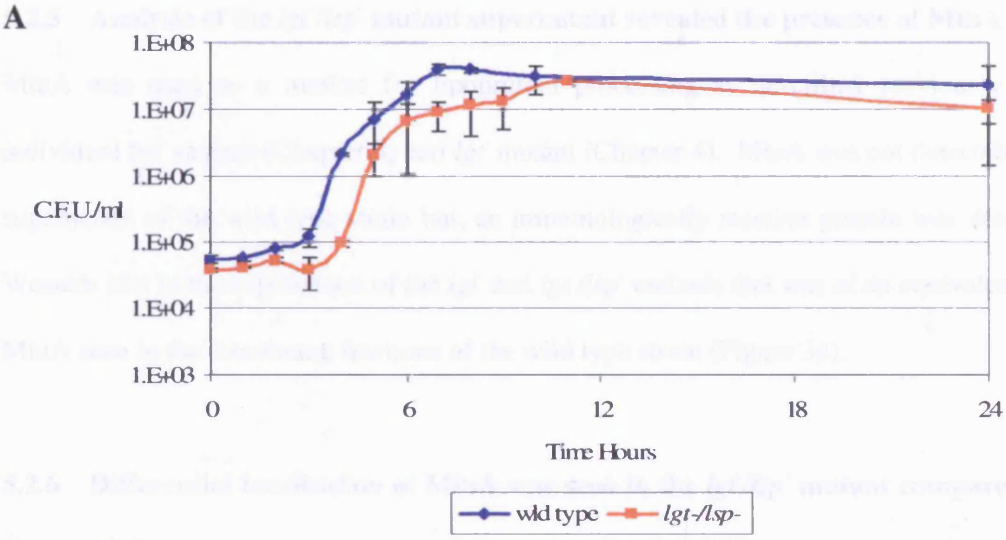


Figure 32 Growth of *S. uberis* wild type was compared with the *lgt*⁻/*lsp*⁻ over 24 hours in THB.

Error bars represent a standard deviation between results performed in triplicate.



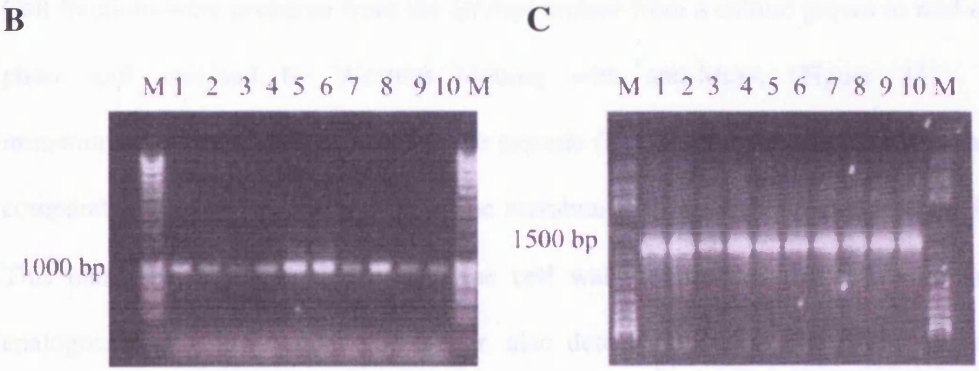


Figure 33 Growth of wild type compared to *lgt*⁻/*lsp*⁻ in milk over 24 hours

The growth of the *lgt*⁻/*lsp*⁻ was compared to wild type in milk (A). Error bars represent a standard deviation between results performed in triplicate. PCR was carried out on 9 colonies from the milk grown cultures of wild type and *lgt*⁻/*lsp*⁻ to check the bacterial culture's purity and composition. Primers PauA fwd and rev were used for the wild type (B), while *lgt*⁻/*lsp*⁻ used P348 and P349 (C). Lane 10 was carried out on genomic DNA from the wild type or *lgt*⁻/*lsp*⁻ mutant. Molecular marker shown in bp.

5.2.5 Analysis of the *lgt⁻/lsp⁻* mutant supernatant revealed the presence of MtuA

MtuA was used as a marker for lipoprotein processing as described previously for the individual *lsp⁻* mutant (Chapter 3) and *lgt⁻* mutant (Chapter 4). MtuA was not detectable in the supernatant of the wild type strain but, an immunologically reactive protein was detected by Western blot in the supernatant of the *lgt⁻* and *lgt⁻/lsp⁻* mutants that was of an equivalent size to MtuA seen in the membrane fractions of the wild type strain (Figure 34).

5.2.6 Differential localisation of MtuA was seen in the *lgt⁻/lsp⁻* mutant compared to the wild type

Cell fractions were prepared from the *lgt⁻/lsp⁻* mutant from a culture grown to mid-exponential phase and analysed by Western blotting with anti-MtuA (Figure 35). A single immunoreactive band was detected in the capsule fraction of the *lgt⁻/lsp⁻* mutant, this was of a comparable size to MtuA detected in the membrane of the wild type and in the *lgt⁻* mutant. This band was also detected within the cell wall, membrane and cell contents which is analogous to the *lgt⁻* mutant. However, also detected in these three fractions is a second immunoreactive band of a larger molecular weight that is of a comparable size to that of the unprocessed MtuA detected in the *lsp⁻* mutant.

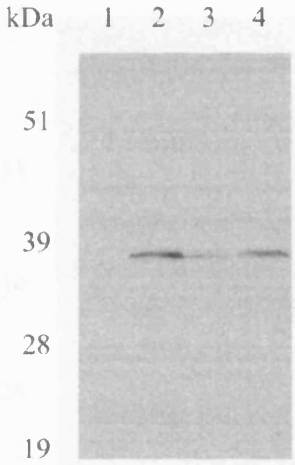


Figure 34 MtuA was detectable in the supernatant from the *lgt*⁻/*lsp*⁻ mutant

Proteins from the supernatant prepared from mid exponentially grown *S. uberis* wild type (lane 1), *lgt*⁻ mutant (lane 2), *lgt*⁻/*lsp*⁻ mutant (lane 3). Membrane from the wild type strain was included as a control (lane 4). MtuA was found in the supernatant of the two lipoprotein processing mutants.

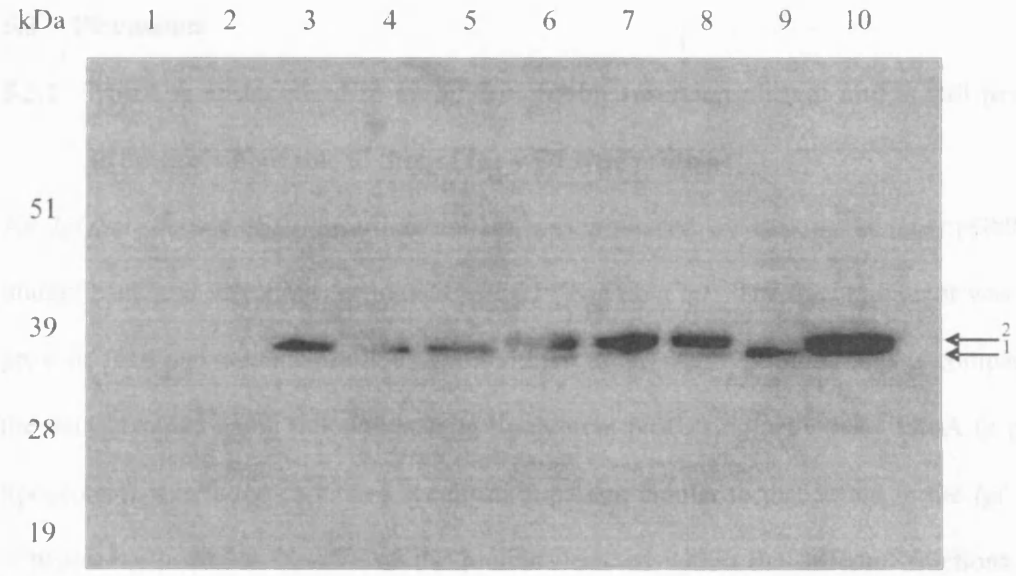


Figure 35 MtuA is mislocalised in the *lgt*⁻/*lsp*⁻ mutant

Lanes 1-4, wild type capsule, cell wall, membrane and cell contents, respectively; lanes 5-8 *lgt*⁻/*lsp*⁻ mutant capsule, cell wall, membrane and cell contents, respectively; lane 9, *lgt*⁻ capsule; lane 10, *lsp*⁻ mutant membrane prepared from 24 hour culture. Arrow 1 indicates the size of MtuA processed in the wild type, *lgt*⁻ mutant and *lgt*⁻/*lsp*⁻ mutants and arrow 2 indicates unprocessed MtuA detected in the *lsp*⁻ and *lgt*⁻/*lsp*⁻ mutants. Molecular weights shown in kDa.

5.3 Discussion

5.3.1 MtuA is mislocalised in an *lgt*⁻/*lsp*⁻ double insertion mutant and is still processed to an equivalent size to that of the wild type protein

An *lgt*⁻/*lsp*⁻ double IS*S1* insertion mutant was produced by making an *lsp*⁻::pGh9⁺::IS*S1* mutant bank and screening for mutations by PCR within *lgt*. The double mutant was able to grow in THB and skimmed milk to levels similar to the wild type strain; this is comparable to the data obtained using mutants lacking lipoprotein processing activities. MtuA (a putative lipoprotein) was found to have a localisation pattern similar to that found in the *lgt*⁻ mutant (Chapter 4); however the size of the protein detected within the different fractions varied. Within the cell wall, membrane, and cell contents a band was present that migrated to a size equivalent to MtuA detected within the *lsp*⁻ mutant (full length lipidated protein). This result is different to the *lgt*⁻ mutant where protein is seen of an equivalent size to processed MtuA in all four fractions (Figure 20). The lgt mutant treated with globomycin (Figure 25) also shows the same banding pattern as described here for the *lgt*⁻/*lsp*⁻ mutant. This data together suggests that Lsp has either a direct or indirect role in processing MtuA in the *lgt*⁻ mutant.

A second form of MtuA was present within the capsule, cell wall, membrane and cell contents that was hypothesized to be protein processed on the carboxyl side of the cysteine residue of the lipobox, as is seen in the individual *lgt*⁻ mutant. The size of the band detected is comparable to the size of the band detected in the wild type and in the *lgt*⁻ mutant. In the absence of Lsp, which processes lipoproteins on the amino terminal side of the cysteine residue, processing by this enzyme or activity cannot take place; therefore it is likely that the processing of MtuA occurs on the carboxyl terminal side of the cysteine residue as described

in Chapter 4 for the individual *lgt*⁻ mutant. The presence of this second band indicates strongly the presence of an enzyme that is not Lsp that is able to process MtuA. Lipoproteins are unlikely to be anchored in the presence of an *lgt*⁻ mutant as MtuA has been found in an extracellular location. The signal peptide of lipoprotein that is not anchored or associated with the membrane or cell wall is processed. A proportion of the lipoprotein is cleaved in the presence of the two lipoprotein processing mutations, however some remains uncleaved and this lipoprotein has been shown to be cell associated. It is possible that the hydrophobic nature of the signal peptide prevents the release of the full length lipoprotein. The literature does not suggest what is likely to be responsible for this phenomenon, but it is most likely to be one of the 90 peptidases predicted in the MEROPS peptidase database (Rawlings *et al.*, 2006) from the *S. uberis* strain 0140J genome (appendix 2).

As described above there appear to be a number of different mature like forms of the MtuA lipoprotein that appear within the different cell fractions. Within the *lgt*⁻ mutant a form of the protein that is not lipidated and not cleaved at the signal peptide would be expected to build up within the cell, however mature like form 1 is detected, this form is processed after the cysteine residue of the lipobox as shown by N-terminal sequencing. This form is seen in the supernatant, capsule, cell wall membrane and cell contents. This form of the protein can be generated from full length and mature lipoprotein. Mature like form 2 is seen in the *lsp*⁻ mutant as a doublet of lipoprotein that is seen at 24 hours. This form of lipoprotein remains membrane anchored due to the activity of Lgt within the cell. This form of the protein suggests intra signal peptide cleavage. The third mature like form suggested due to the presence of a new band appearing in the *lgt*⁻/*lsp*⁻ double mutant. Mature like 1 and mature like

3 are likely to only differ by one amino acid and therefore it is unlikely that these two forms of the protein can be resolved by SDS PAGE. The lgt mutant treated with globomycin shows a doublet that matched the profile in the *lgt⁻/lsp⁻* mutant. This suggests that Lsp is also able to process unlipidated lipoprotein. It is of interest to note that mature like form 3 was not detected when the N-terminal sequence analysis was carried out on the *lgt-* mutant. It could be possible that the cysteine at the N-terminus of the protein in mature like form 3 keeps the protein located in the membrane. Mature like form 1 may be in excess to the mature like form 3, thus preventing the detection of this form during the N-terminal sequencing.

The Lsp enzyme of *L. monocytogenes* was reported to be able to process unlipidated lipoproteins (Baumgartner *et al.*, 2007). This was discovered through the use of the reversible inhibitor of Lsp, globomycin. The extra proteins that appeared in the supernatant of the *lgt* mutant, did not appear when the mutant was grown in the presence of this inhibitor. It was suggested that the extra proteins, identified as lipoproteins, had been processed to leave the cysteine residue as the amino terminus but, due to the inhibitor, had became stuck within the membrane when their hydrophobic signal peptide sequence was not removed. An *lgt/lsp* double mutant was not studied by Baumgartner *et al.*, 2007 (Baumgartner *et al.*, 2007), therefore it could only be hypothesised that Lsp was responsible for processing the unlipidated lipoproteins in the *lgt* mutant. A comparable result was found for the *S. uberis lgt⁻* mutant that was grown in the presence of globomycin; in Western blot using anti-MtuA, two immunoreactive bands were detected. The band with the smaller molecular weight was of a comparable size to that found in the membrane of the wild type and in the *lgt⁻* mutant that had not been grown in the presence of globomycin, and the second band was of a larger molecular

weight and comparable in size to that detected in the membrane of the *lsp*⁻ mutant, suggesting that either Lsp is able to process unlipidated lipoprotein or that the cellular component that alternatively processes MtuA requires Lsp for its activity. However, the data presented in this chapter clearly shows that an enzyme that is not Lsp is able to process lipoproteins as shown using MtuA as a marker for lipoprotein processing. It is unclear at present as to whether Lsp is able to process unlipidated lipoprotein as well as the unidentified factor that processes lipoprotein in the *lgt*⁻ and *lgt*⁻*/lsp*⁻ mutants.

Not only is the cleavage site for unlipidated lipoproteins in *S. uberis* different to the normal processing site of lipoproteins, but there is an enzyme present within the proteome of this bacterium that is responsible for the alternatively processing of some, if not all lipoproteins. It would be of interest to determine if any of the lipoproteins present within the *lgt*⁻ mutant are of the form where the cysteine of the lipobox is the amino-terminus of the protein. This would give further indication as to whether Lsp is able to cleave unlipidated lipoprotein, or if a protein processed through Lsp is involved in this alternative processing of lipoproteins. If Lsp is responsible for processing lipoproteins in the absence of lipidation in the *lgt* mutant of *L. monocytogenes*, it would appear that different bacteria have evolved different mechanisms to overcome the effects of the incorrect processing of these proteins. Further investigation as to whether this is true is required.

In conclusion, the *lgt*⁻*/lsp*⁻ mutant of *S. uberis* shows alternative processing of MtuA that is not due to Lsp. MtuA is found within the supernatant of this double lipoprotein processing mutant

and it would be of interest to identify if there are other, additional proteins present in the extracellular space and whether they are comparable to those of the *lgt*⁻ mutant alone.

Chapter 6

Characterisation of the *Streptococcus uberis* homologue of the *Enterococcus faecalis* protein Enhanced Expression of Pheromone (Eep)

6.1 Introduction

6.1.1 Alternative Lipoprotein processing in the *lsp* mutant

An *lsp*$^-$ mutant of *S. uberis* displayed an alternatively cleaved form of MtuA during stationary phase of growth (Chapter 3). It is not known where in the sequence of MtuA that this alternative processing occurred. As MtuA was not detected in fractions other than the membrane and the cell contents, it was hypothesised that the amino terminus of the protein was located in the region preceding the cysteine residue of the lipobox, as this would be required to permit anchoring of the protein to the membrane through the formation of a protein-lipid diglyceride bond formed by Lgt (Tokunaga *et al.*, 1982). A small size difference between the form of MtuA present in Western blot of extracts prepared from the wild type strain and that observed in the *lsp*$^-$ mutant. This suggests the alternatively cleaved form of MtuA from the *lsp*$^-$ mutant was several amino acids longer than that from the wild type strain.

6.1.2 Processing of lipoprotein signal peptides in *Enterococcus faecalis* by the metallopeptidase Eep (Enhanced Expression of Pheromone)

The metallopeptidase Eep from *E. faecalis* has been shown to cleave the signal peptides from certain lipoproteins with the consequent release of short peptides that can act as aggregation pheromones (Chandler and Dunny, 2004; Flannagan and Clewell, 2002) (An *et al.*, 1999). This includes the peptide cAD1 (An and Clewell, 2002), the sequence of which appears to

consist of the eight amino-terminal amino acids preceding the cysteine residue of the lipobox, the predicted cleavage point for Lsp (Clewell *et al.*, 2000).

Eep has been characterised as a Regulated Intramembrane Proteolysis (RIP) metallopeptidase (Kanehara and Akiyama, 2003) with at least three predicted transmembrane spanning regions, suggesting that the protein is located within the membrane. This is a common feature with other members of the lipoprotein processing pathway. Both Lgt and Lsp have predicted transmembrane domains (Tjalsma *et al.*, 1999b). Eep belongs to the M50A family of metallopeptidases, homologues of which have been observed in all genomes that have been sequenced so far except fungi. The wide ranging nature of such proteins suggests they may have an essential cellular house-keeping function (Barrett *et al.*, 2004).

A similar activity to Eep has been described in *S. aureus*, where cAM373 is the pheromone derived from the signal peptide of the lipoprotein CamA (Flannagan and Clewell, 2002). It is interesting to note that *E. faecalis* can sense and respond to this pheromone, leading to the aggregation of bacteria required as an initial step in the processes leading to the efficient transfer of plasmid DNA between bacteria of different species.

The *traH* gene is encoded by a transfer-associated region of the staphylococcal conjugative plasmid pSK41 and encodes a lipoprotein. Seven of the eight carboxyl terminal amino acids of the TraH signal peptide are identical to the enterococcal pheromone cAD1. It has been shown that cells responsive to cAD1 signalling can also respond to *S. aureus* (and *E. coli*) cells encoding the TraH signal peptide (Firth *et al.*, 1994). The maturation of the *traH* derived

peptide pheromone of *S. aureus* was examined in *E. coli* using a strain lacking Lsp. TraH was expressed from a plasmid within the mutant *E. coli lsp* background but this strain failed to express the *traH*-encoded pheromone-like peptide, suggesting a role for Lsp in its production (Berg *et al.*, 1997). The data provided from the study of Eep in *E. faecalis* and that from the *E. coli lsp* mutant together suggest that these two peptidases are responsible for correctly processing the signal peptides of certain lipoproteins to produce bacterial peptide pheromones.

This chapter describes the characterisation of an Eep homologue in *S. uberis* and its role in the alternative processing of the lipoprotein signal peptide of MtuA.

6.2 Results

6.2.1 A homologue of the gene encoding the *E. faecalis* protein Eep (Enhanced expression of pheromone) is present within the genome of *S. uberis* strain 0140J

Eep of *E. faecalis* has been implicated in the cleavage of signal peptides from certain lipoproteins (An *et al.*, 1999). A homologue of *eep* was found within the genome of *S. uberis* strain 0140J (ORF SUB0254) using BLAST homology searches (Altschul *et al.*, 1990) of the *E. faecalis* strain V538 Eep protein sequence against the genome sequence of *S. uberis* (Figure 36). The percentage identity shared between the predicted amino acid sequence of *S. uberis* SUB0254 and *E. faecalis* Eep protein is 73%.

The Eep protein belongs to the M50A group of peptidases and the conserved HEXXH zinc binding signature motif (Jongeneel *et al.*, 1989) is present within both sequences (shown in blue in Figure 36). Eep of *E. faecalis* is a predicted membrane protein with at least three regions expected to span the membrane. Five membrane spanning domains for the *S. uberis* equivalent Eep could be predicted using TMHMM (Kall *et al.*, 2004). The genes in proximity to SUB0254 share some similarities with those close to *eep* (ORF2380) in *E. faecalis* however there is a difference in the order of genes (Paulsen *et al.*, 2003) (Figure 37).

6.2.2 Identification of *eep* mutant clones within the *S. uberis* 0140J and *S. uberis* 0140J *lsp*⁻ mutant banks

PCR screening of the 0140J pGh9$^+$::IS*S1* and *lsp*⁻ pGh9$^+$::IS*S1* mutant bank (Chapter 5) was carried out using an *eep* gene specific primer and an IS*S1* specific primer (Figure 38). Plate 45 was shown to contain an *eep*::IS*S1* mutant by carrying out PCR using the reverse eep

primer and P247. The other positive plates from the primary screen where shown to be false positives using the same PCR. An *eep*::IS*S1* mutant (*eep*$^-$) was identified with an insertion of the pGh9$^+$::IS*S1* plasmid 362 bp from the start codon, and a double *lsp*$^-$/*eep*$^-$::IS*S1* (*lsp*$^-$/*eep*$^-$) mutant was identified carrying an insertion of the pGh9$^+$::IS*S1* plasmid 34 bp from the start codon, suggesting that the gene would have been disrupted in both these insertion mutants (Figure 37). In the *lsp*$^-$/*eep*$^-$ double mutant the ion binding motif (starting at amino acid 18) should not be translated due to the insertion site corresponding to amino acid 12. A complete absence of peptidase activity would be predicted from such a mutant. The translation of Eep in the *eep*$^-$ mutant would be interrupted at amino acid 120 due to the insertion of the pGh9$^+$::IS*S1* plasmid at the corresponding point in the gene. This is in the middle of the predicted intracellular region between the first and second transmembrane domain. Even though the ion binding motif of the Eep protein might be translated, the resultant protein is not likely to be functional due to the remaining 4 transmembrane domains remaining untranslated. These domains are considered likely to regions essential regions for activity. A Southern blot of chromosomal DNA prepared from the uncured *lsp*$^-$/*eep*$^-$ and *eep*$^-$ mutants was carried out (Figure 39); the *eep*$^-$ mutant carries a single insertion of the pGh9$^+$::IS*S1* vector and the *lsp*$^-$/*eep*$^-$ mutant carries the expected two insertions; one at the *lsp* locus and a single insertion of the pGh9$^+$::IS*S1* vector in the *eep* homologue locus.

```
            1                                                   50
E. faecalis MKTIITFIIV FGILVLVHEF GHFYFAKRAG ILVREFAIGM GPKIFAHRGK
S. uberis   MLGLITFIIV FGILVIVHEF GHFYFAKKSG ILVREFAIGM GPKLYSHVDK
Consensus   M   ITFIIV FGILV VHEF GHFYFAK  G ILVREFAIGM GPK   H  K
            51                                                  100
E. faecalis DGTTYTIRLL PIGGYVRMAG MGEDMTEITP GMPLSVELNA VGNVVKINTS
S. uberis   EGTLYTIRSL PLGGYVRMAG WGDDSTEIKT GTPASLSLDA SGKVTRINLS
Consensus    GT  TIR L PLGGYVRMAG  G  DTEI   G P S    A  G V   N S
            101                                                 150
E. faecalis KKVQLPHSIP MEVVDFDLEK ELFIKGYVNG NEEEETVYKV DHDATIIESD
S. uberis   QNRLDPNSLP MHVTAYDLED KLTITGLVL. ..EETKEFDV AHDATIVEED
Consensus        P S P M     DLE                 EE     V  HDATI EED
            151                                                 200
E. faecalis GTEVRIAPLD VQFQSAKLSQ RILTNFAGPM NNFILGFILF TLAVFLQGGV
S. uberis   GTEVRIAPLD VQYQNASIWG RLITNFAGPM NNFILGLLVF IFLVFLQGGA
Consensus   GTEVRIAPLD VQ Q A     R  TNFAGPM NNFILG   F    VFLQGG
            201                                                 250
E. faecalis TDLNTNQIGQ VIPNGPAAEA GLKENDKVLS INNQKIKKYE DFTTIVQKNP
S. uberis   LDTNSNHI.K VVDNGAAAKA GIKSNDQILQ IENIPVSNWQ ELTGAVASST
Consensus      N       V  NG AA A G K ND  L  I N            V
            251                                                 300
E. faecalis EKP.....LT FVVERNGKEE QLTVTPEKQK VEKQTIGKVG VYPYMKTDLP
S. uberis   KDLKEGQSLT VKVKSQGKVK ELSLKP..QK ..VGGKFAIG VQCRLKTGFK
Consensus           LT   V   GK    L   P  QK          G V    KT
            301                                                 350
E. faecalis SKLMGGIQDT LNSTTQIFKA LGSLFTGFSL NKLGGPVMMF KLSEEASNAG
S. uberis   DKLLGGFEMA INGALLIITA LKNLMTGFSL DKLGGPVAMY QMSSQAAASG
Consensus    KL GG              A L  L TGFSL  KLGGPV M       A   G
            351                                                 400
E. faecalis VSTVVFLMAM LSMNLGIINL LPIPALDGGK IVLNIIEGVR GKPISPEKEG
S. uberis   IETVLSMMAM LSINLGIFNL IPIPALDGGK ILMNLIEAIR RKPLKRETET
Consensus      V   MAM LS NLGI NL  PIPALDGGK I    IE  R  KP   E E
            401                 427
E. faecalis IITLIGFGFV MVLMVLVTWN DIQRFFF
S. uberis   YITFVGVVIM LVLMVAVTWN DIMRAFF
Consensus    IT  G      VLMV VTWN DI R FF
```

Figure 36 Alignment of the amino acid sequence of *E. faecalis* Eep (Enhanced expression of pheromone), strain V538, and *S. uberis* SUB0254, strain 0140J

The *S. uberis* homologue has 73 % identity with the amino acid sequence of the *E. faecalis* protein. The HEXXH motif of both species is shown in blue.

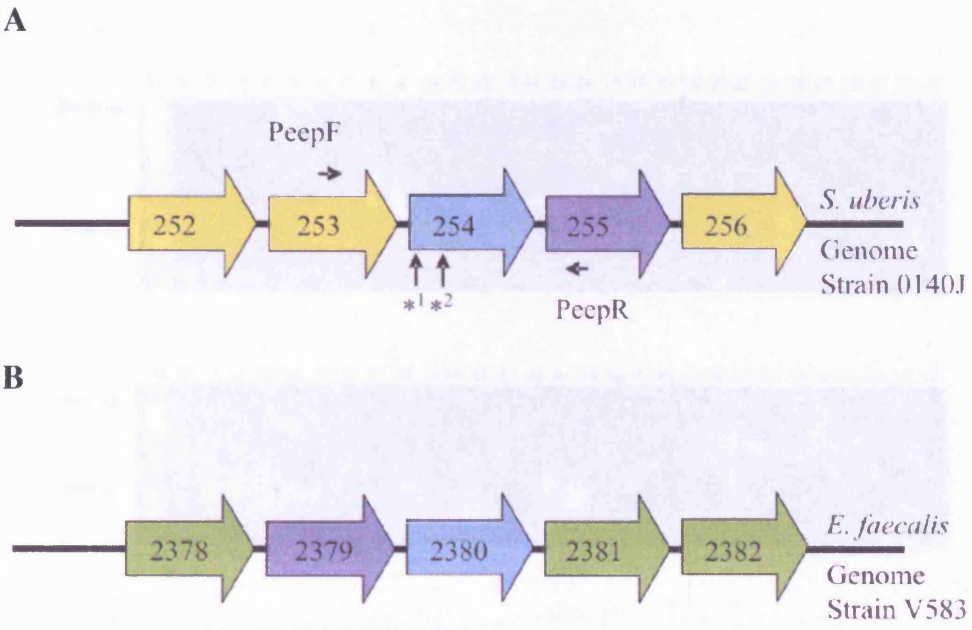
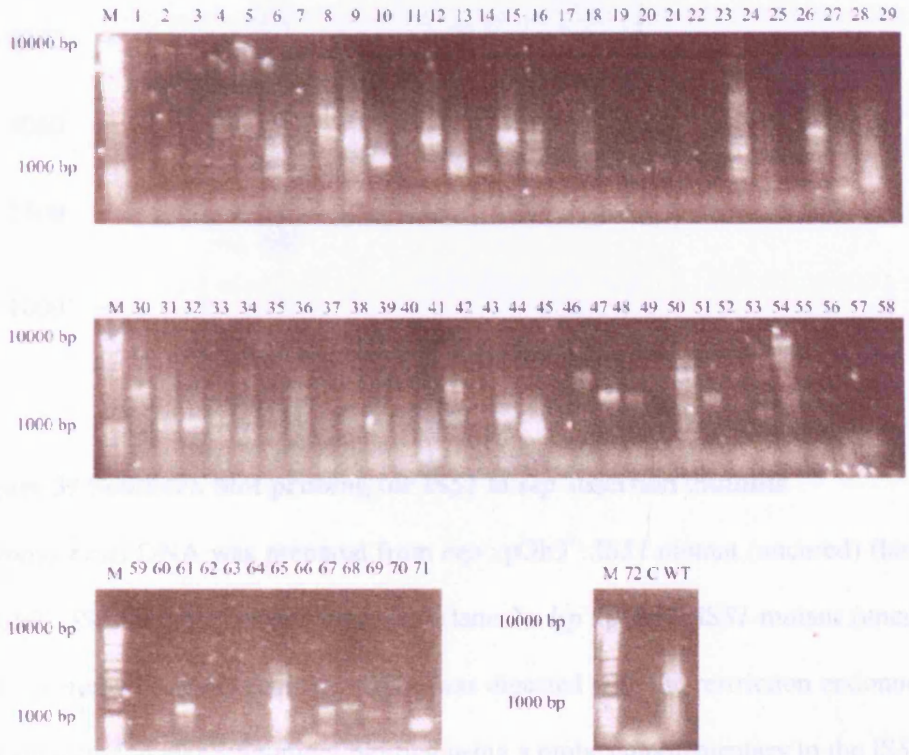


Figure 37 Context of the *S. uberis* homologue *E. faecalis eep*

The flanking regions of *eep* are shown; upstream SUB252 - undecaprenyl pyrophosphate synthetase, SUB253 - cytidyl transferase, 254 - *eep*, downstream SUB255 - prolyl tRNA synthetase, SUB256 Unknown product (A). The locus in the genome of *E. faecalis* strain V583 is different to that in *S. uberis* strain 0140J; 2378 – DNA polymerase III α subunit G+ type, 2379 - Prolyl tRNA synthetase, 2380 - *eep*, 2381 – Hypothetical protein, 2382 – Glucose 1 dehydrogenase (B). Homologues between the two species are shown in the same colour. The *lsp/eep*$^-$ ($*^1$) and *eep*$^-$ ($*^2$) insertion mutants were identified through PCR screening. The location of the *eep*$^-$ mutant in the *S. uberis* pGh9$^+$::IS*S1* mutant bank is 362 bp from the start codon, and in the *lsp*$^-$::pGh9$^+$::IS*S1* mutant is 34 bp from the start codon.

A



B

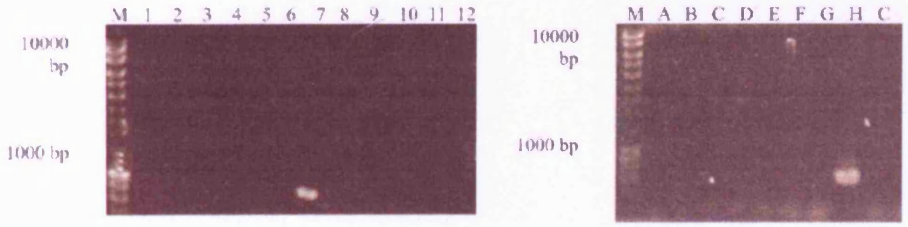


Figure 38 Identification of *lsp*⁻/*eep*⁻ mutant within *lsp*⁻::pGh9::IS*S1* mutant bank.

PCR was carried out using P*eep*Fwd and P370 on pools of DNA prepared from *lsp*⁻ ::pGh9⁺::IS*S1* mutant bank, plate 45 was identified as containing an *lsp*⁻/*eep*⁻ mutant candidate (A). DNA preparations were made from rows and columns of plate 45 and the well location for the mutant was identified as 7H (B).

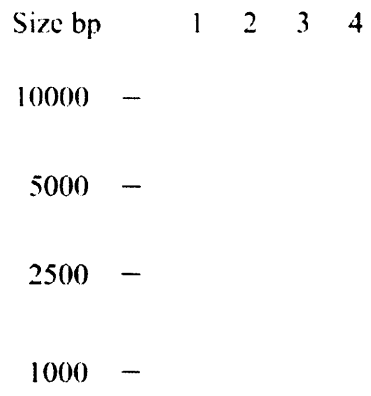


Figure 39 Southern blot probing for IS*S1* in *eep*⁻ insertion mutants

Chromosomal DNA was prepared from *eep*⁻::pGh9⁺::IS*S1* mutant (uncured) (lane 1), *lsp*⁻/*eep*⁻ ::pGh9⁺::IS*S1* double mutant (uncured) (lane 2), *lsp*⁻::pGh9⁺::IS*S1* mutant (uncured) (lane 3), and *lsp*⁻ mutant (cured) (lane 4). DNA was digested with the restriction endonuclease *Hind*III and subjected to alkali Southern blotting using a probe complementary to the IS*S1* element.

6.2.3 Characterisation of *eep*⁻ and *lsp*⁻/*eep*⁻ mutants

6.2.3.1 The *lsp*⁻/*eep*⁻ mutant is able to grow in THB and milk to levels comparable with the wild type

The growth of the wild type bacteria and the *lsp*⁻/*eep*⁻ mutant were compared in THB, a nutritionally rich media measured by $O.D._{540nm}$ (Figure 40) and also in skimmed bovine milk, measured by viable counts. The presence of the wild type and *lsp*⁻/*eep*⁻ mutant bacteria at the end of the 24 hour period after growth in milk was confirmed using colony PCR (Figure 41b). The *lsp*⁻/*eep*⁻ mutant grew as well as the wild type in both the rate of growth and in the yield of the bacteria in both growth medium. The growth of the single *eep*⁻ mutant was not analysed.

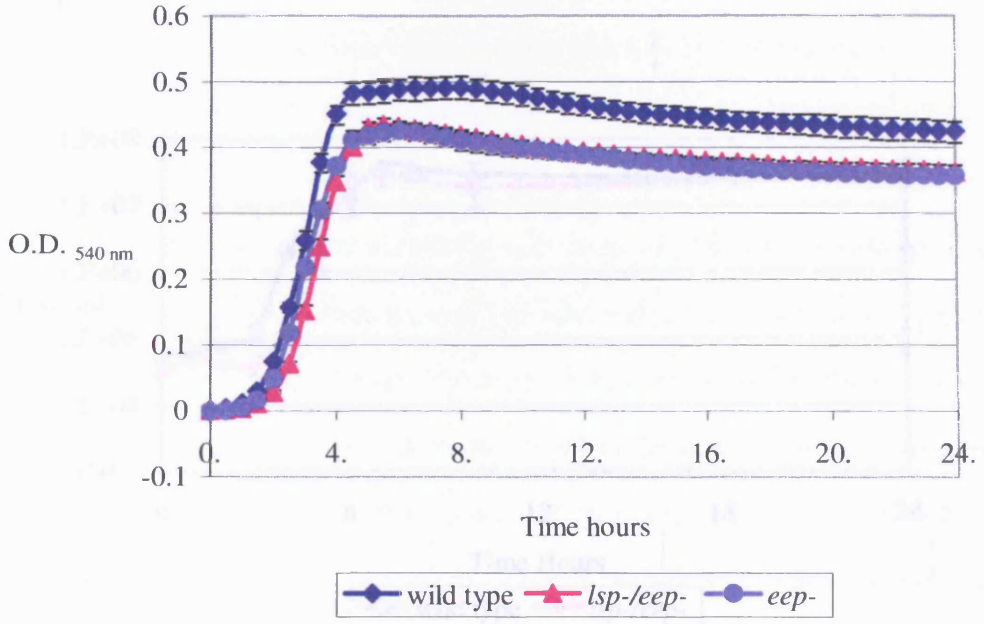
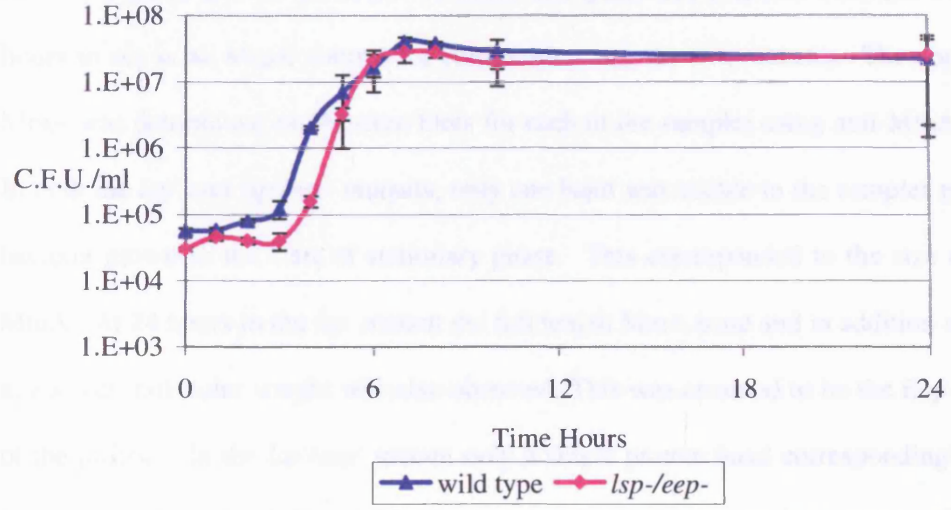


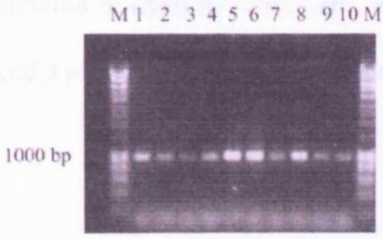
Figure 40 Growth of wild type was compared to *lsp*⁻/*eep*⁻ and *eep*⁻ mutants over 24 hours in THB

Error bars represent a standard deviation between results performed in triplicate.

A



B



C

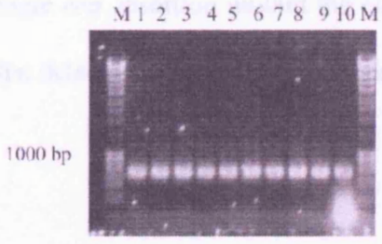


Figure 41 Growth of wild type compared to *lsp⁻/eep⁻* mutant in milk

The growth of *lsp⁻/eep⁻* mutant was compared to wild type in milk and no appreciable difference was observed (A). Error bars represent a standard deviation between results performed in triplicate. PCR was carried out on 9 colonies picked randomly from milk grown cultures of wild type and *lsp⁻/eep⁻* to verify the genotypes present; wild type using PauA Fwd and Rev primers (B), *lsp⁻/eep⁻* using P*eep* Fwd and P370 (C). Lane 10 PCR was carried out on wild type or *lsp⁻/eep⁻* stock genomic DNA.

6.2.3.2 MtuA is not processed in the *lsp⁻/eep⁻* mutant over a 24 hour period

Whole cell lysates were prepared from the *lsp⁻* and *lsp/eep⁻* mutants grown to the start of stationary phase and for 24 hours. A whole cell lysate was prepared from the wild type at 24 hours to act as an MtuA control for comparison with the two mutants. The apparent size of MtuA was determined on Western blots for each of the samples using anti-MtuA (Figure 42). In both the *lsp⁻* and *lsp⁻/eep⁻* mutants, only one band was visible in the samples prepared from bacteria grown to the start of stationary phase. This corresponded to the size of full length MtuA. At 24 hours in the *lsp⁻* mutant the full length MtuA band and in addition a second band at a lower molecular weight was also observed. This was assumed to be the Eep cleaved form of the protein. In the *lsp⁻/eep⁻* mutant only a single protein band corresponding to the higher molecular weight band detected in the Lsp mutant was found to be present. This suggested that Eep was responsible for cleavage of MtuA, but this activity could be detected only against a background of dysfunctional Lsp. In the single *eep⁻* insertion mutant the cleavage of MtuA produced a product of a size consistent with that detected in the wild type strain (Figure 43).

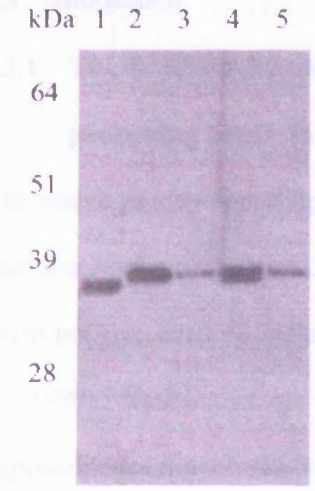


Figure 42 Presence of the different forms of MtuA in the *lsp*⁻ mutant and the *lsp*⁻/*eep*⁻ mutant

Lane 1, wild type whole cell lysate 24 hours; lane 2, *lsp*⁻ start of stationary phase; lane 3 *lsp*⁻ /*eep*⁻ start of stationary phase; lane 4, *lsp*⁻ 24 hours; and lane 5, *lsp*⁻/*eep*⁻ 24 hours. Molecular weights indicated in kDa.

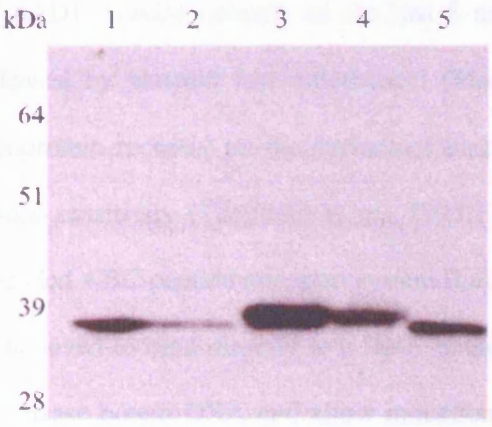


Figure 43 MtuA is correctly processed in an individual *eep*⁻ insertion mutant

Lane 1 wild type 24 hour whole cell lysate; lane 2 *eep*⁻ 24 hour whole cell lysate; lane 3 *lsp*⁻ 24 hours; lane 4 *lsp*⁻/*eep*⁻ 24 hours; and lane 5 wild type 24 hour whole cell lysates. Molecular weights indicated in kDa.

6.3 Discussion

6.3.1 The *S. uberis* homologue of Eep from *E. faecalis* is responsible for alternatively processing MtuA in the absence of an active Lsp

Alternative processing of lipoproteins in *S. uberis* has been shown in both an *lgt*⁻ and an *lsp*⁻ insertion mutant (Chapters 3-5). Such phenomena have also been reported in several other Gram positive bacteria including *B. subtilis* and *L. lactis* (Antelmann *et al.*, 2001; Tjalsma *et al.*, 1999a; Venema *et al.*, 2003), however no explanation has been offered as to what is responsible for the alternative processing of these proteins. A protein, Enhanced expression of Pheromone (Eep), characterised in *E. faecalis* where it was reported as being able to cleave the signal peptide of lipoproteins (An *et al.*, 1999), and appears to be highly conserved in Streptococci and Lactococci, as determined by BLAST alignment (Altschul *et al.*, 1990). This metallopeptidase was shown to be able to increase production of the competence peptide cAD1 when expressed from the enterococcal plasmid pAM2011E; a mini plasmid derivative of pAD1. cAD1 consists of the last 8 amino acids of a lipoprotein signal peptide and is released by plasmid free enterococci (Mori *et al.*, 1984). This peptide binds to the TraC lipoprotein receptor on the surface of bacteria carrying the pAD1 plasmid (which enhances donor sensitivity (Tanimoto *et al.*, 1993)) and allows the peptide to be taken up via a host encoded ABC peptide transport system (Leonard *et al.*, 1996). Once inside the cell the peptide is believed to bind directly to a DNA binding, negative regulator protein, TraA, permitting it to release bound DNA and allow induction of the mating response and transfer of the donor plasmid (Fujimoto and Clewell, 1998).

It is hypothesised that Eep is responsible for cleaving the lipoprotein's signal peptide between the eighth and ninth amino acids preceding the cysteine residue of the lipobox, after it has been cleaved by Lsp. Lsp has been previously associated with the production of the TraH derived pheromone from *S. aureus* which has high homology to cAD1. An *E. coli lsp* mutant was unable to correctly process this peptide (Berg *et al.*, 1997); suggesting that both Eep and Lsp are required for the correct processing of the TraA derived pheromone.

The processing of MtuA in an individual *eep*⁻ insertion mutant of *S. uberis* appeared unaffected, presumably due to the removal of the signal peptide by Lsp following lipidation of MtuA by Lgt. It is possible that Eep functions to process lipoprotein signal peptide in the wild type strain, but this was not resolved in this investigation. It could be hypothesised from the data presented that the order of events that occurs to process the signal peptides of lipoproteins is as follows, first Lgt acts to add lipid to the cysteine residue, this is followed by cleavage of the signal peptide by Lsp and finally the signal peptide that has been removed from the protein is processed by Eep. The order is likely to be as described due to the form of MtuA that has been cleaved in the lsp mutant only occurs in stationary phase, where as forms that have been processed by Lsp appear at all time points in a growth curve as seen in chapter 3 and 5.

Following the cultivation of the *lsp*⁻ mutant of *S. uberis* for 24 hours a form of the protein of a lower molecular weight than the full length (unprocessed) MtuA was detected. In corresponding cultures of the mutant carrying lesions in both *lsp* and *eep* this alternatively processed form of MtuA was not detected suggesting that Eep was responsible for the alternative processing of the protein at this time point. Eep was not required for growth in

milk, which was comparable to other proteins studied in the lipoprotein processing pathway of *S. uberis*, providing further evidence that MtuA functions correctly despite the presence of an intact signal peptide and in the absence of lipidation as discussed in Chapters 3-5 of this thesis.

Two interesting questions have arisen in the study of Eep in *S. uberis*; firstly *S. uberis* is not naturally competent, is not known to take up plasmids through the system described for *E. faecalis*, and there are no reports in the literature of a plasmid DNA transfer system in Streptococcus species comparable to that found in *E. faecalis* therefore why are parts of this conjugation induction process present. It could be hypothesised that Eep is an evolutionary remnant that has remained in the genome of *S. uberis* from a time when it possibly was able to take up plasmid DNA. A further point of interest regarding the uptake of foreign DNA into *S. uberis* was raised by the multi locus sequence typing project that provides evidence that *S. uberis* is evolving by recombination, suggesting that it must be able to take up foreign DNA for this process to occur (Coffey *et al.*, 2006). Secondly this alternatively processed form of the MtuA only appears after 24 hours of growth. At this point in the growth very little if any cell division will be occurring. It may be interesting to determine the effect on processing of MtuA of the *lsp*$^-$ and *lsp/eep*$^-$ mutations when the mutants have been grown in skimmed milk rather than in nutrient rich media. It would also be interesting to determine what the normal role of Eep is in *S. uberis*, as it may well be that the normal role is in alternative processing of the signal peptide of MtuA and possibly other lipoproteins. The role of these peptides if they exist in *S. uberis* is undetermined. It could be hypothesised that Eep is the missing Signal peptide peptidase that as yet has remained undescribed in Streptococci species. SppA of B. subtilis has been shown to be at least partially responsible for the degredation of signal

peptides and some of these degredation products have been shown to have signalling properties (Bolhuis *et al.*, 1999). This shows some similarities with the system that has been described for Eep in E. faecalis (An *et al.*, 1999; An and Clewell, 2002). However, this protein has only ever been shown to be active on lipoproteins. Therefore, if it was to be the so far elusive signal peptide peptidase, it is likely not to be the only signal peptide peptidase encoded by these bacteria. It is likely that there will be another that is dedicated to signal peptides cleaved by the signal peptidase of the type I class.

Alternative processing of lipoproteins in the presence of an *lsp* mutation has been described in *B. subtilis*, *L. monocytogenes* and *L. lactis* (Reglier-Poupet *et al.*, 2003a; Tjalsma *et al.*, 1999a; Venema *et al.*, 2003) but the mechanism by which these lipoproteins are alternatively processed was not identified in any of the studies. *L. monocytogenes* strain EGDe contains a protein that is a member of the M50 metallopeptidases (lmo1318) and shares 49% and 41% amino acid identity with Eep of *E. faecalis* and *S. uberis*, respectively. The metal ion binding site is conserved. YluC of *B. subtilis* has been characterised as a RIP peptidase and degrades the anti-sigma factor RsiW through intramembrane proteolysis (Schobel *et al.*, 2004). This protein has the highest homology in the *B. subtilis* genome to Eep; amino acid identity of 44% and 39% for *E. faecalis* and *S. uberis* Eep, respectively. The protein with the highest homology to Eep in *L. lactis* is L181494 with homologies of 54% and 52% with *E. faecalis* and *S. uberis* respectively. These levels of homology to Eep suggest that the proteins (YluC, L181494 and lmo1318) may constitute a conserved activity for alternatively processing lipoproteins.

In conclusion, this chapter describes for the first time a bacterium carrying *lsp⁻/eep⁻* mutations that appears not to show alternative processing of lipoproteins. This suggests that Eep and Lsp in conjunction are responsible for the production of peptides derived from lipoproteins. Eep is the factor responsible for alternatively processing this protein in the absence of Lsp. The activity observed is currently restricted to MtuA which has been used as a candidate for lipoprotein processing in *S. uberis*. It would be of interest to determine the role of the Eep protein in the wild type strain, whether it is active on other lipoprotein substrates, and why the alternatively processed form of MtuA only appears during stationary phase of growth.

Chapter 7

Characterisation of the Shavase *uberis* - ShvU

7.1 Introduction

7.1.1 Determination of the enzyme responsible for cleaving the lipoprotein signal peptide in the presence of an *lgt*⁻ and *lgt*⁻/*lsp*⁻ mutation in *S. uberis*

There are reports in the literature discussing the phenomenon of alternative processing of lipoprotein signal peptides between the cysteine residue and C-terminal portion of the remaining protein in wild type bacteria and in mutants (Antelmann *et al.*, 2001; Sibbald *et al.*, 2006). I have shown this phenomenon in *S. uberis lgt*⁻ and *lgt*⁻/*lsp*⁻ mutants and this work is described in Chapters 4 and 5 of this thesis, respectively. However, the factor that is responsible for this activity has yet to be identified.

7.1.2 Types of peptidase

Peptidase enzymes are responsible for the cleavage of peptide bonds and therefore a member of this category of proteins is likely to be responsible for the alternative processing of lipoproteins. Peptidases fall into six different mechanistic classes depending on their active site; Cysteine, Serine, Threonine, Aspartic and Metallopeptidases and those of unknown mechanism. The means by which the cysteine, serine and threonine peptidases cleave peptide bonds is very different compared to that used by the aspartic and metallopeptidases. In the first group of peptidases an amino acid is used as the nucleophile to allow the cleavage of the peptide bond, whereas in the second group of peptidases an activated water molecule acts as the nucleophile.

7.1.2.1 Cysteine peptidases

A wide range of cysteine peptidases exist in nature and many of these have evolved through different routes. These peptidases exist in 9 clans and within these there are 60 different families. The majority of studies on the mechanism of action used by this class of peptidase have been carried out on the enzyme Papain. Papain forms an acyl-enzyme intermediate in the form of a thiolester. The active site residues involved in the cleavage of the peptide bond are a pair of cysteine and histidine residues. The histidine becomes mono protonated between the pH values of 4 and 8.5. Nucleophilic attack on the peptide bond to be broken by the sulphur atom of the cysteine residue is aided by the formation of this thiolate-imidazolium pair, where the proton is already on the imidazolium when the thiolate ion attacks the substrate. This differs to the mechanism of serine peptidases that proceed by general base and, consequently, general acid catalysis (Barrett *et al.*, 2004).

7.1.2.2 Serine peptidases

Serine peptidases are the most thoroughly studied class and their mechanism of cleavage relies upon the hydroxyl group of the active serine acting as the nucleophile. There are approximately 50 different families of serine peptidases belonging to 16 different clans that can be distinguished based on their different amino acid sequences. The proteolytic cleavage in this group of enzymes involves the formation of a covalent intermediate between the enzyme and the point of cleavage. The reaction proceeds due to the active site serine, histidine and aspartic acid residues forming a catalytic triad. The acyl intermediate is formed between the substrate and the serine residue. The formation of this intermediate proceeds through a negatively charged tetrahedral transition state and leads to the cleavage of the

peptide bond by the enzyme. The removal of the enzyme from the intermediate form requires a deacylation step, where the intermediate is hydrolyzed by a water molecule to release the peptide and to restore the serine-hydroxyl of the enzyme. The histidine residue provides a general base and accepts the OH group of the reactive serine residue (Barrett *et al.*, 2004).

7.1.2.3 Threonine peptidases

Threonine peptidases have a similar catalytic mechanism to serine peptidases, but a threonine residue replaces the serine residue of the active site. Mutation of the threonine residue to alanine inactivates the enzyme, whereas the enzyme remains active when this residue is mutated to serine and it becomes more sensitive to serine protease inhibitors such as 3,4-dichloroisocoumarin (Seemuller *et al.*, 1995). Threonine peptidases have been reported in both eukaryotes and prokaryotes. In eukaryotes these peptidases make up subunits of proteasomes, which are catalytic, ATP dependent complexes responsible for the breakdown of ubiquitin-conjugated proteins. The biological activity of prokaryotic proteasomes is poorly understood (Barrett *et al.*, 2004).

7.1.2.4 Aspartic peptidases

Aspartic peptidases do not use nucleophilic attack to cleave the peptide bond. In this class of peptidase, two aspartic acid residues are involved in catalysing the peptide bond and these residues have optimal activity at low pH. Members of this class of enzyme belong to seven different clans and these are divided into 19 families. All aspartic peptidase enzymes that have been described so far are endopeptidases and they include the lipoprotein signal peptidase, which is a member of the AC clan and A8 family (Barrett *et al.*, 2004). During the

cleavage event the pair of aspartic acid residues act together to bind and activate the catalytic water molecule that acts as a ligand. The second aspartic acid residue in some aspartic peptidases is replaced by a different amino acid. The proteolytic cleavage event occurs through the transfer of two protons. The first proton is transferred from the catalytic water molecule to the diad of the two carboxyl groups and the second is transferred from the diad to the carbonyl oxygen of the substrate with the simultaneous cleavage of the peptide bond (Barrett *et al.*, 2004).

7.1.2.5 Metallopeptidases

Metallopeptidases use a metal ion, usually a zinc ion at the active site, creating a strong electrophilic pull to assist in attack by a water molecule. Metallopeptidases can be broadly subdivided into two groups and subdivided further into 16 different clans which are subdivided into 53 different families. The first group of metallopeptidases have a single metal ion at the active site and the second have two ions that act co-catalytically in order to break the peptide bond. In peptidases where two ions are required either cobalt or manganese is likely to be present, however there are families where there are two zinc ions that act co-catalytically. Metallopeptidases that contain two ions have only been found to be exopeptidase, where as those that contain single ions have been found to be endo and exopeptidases. The active sites of metallopeptidases that hold a single ion are made up of three amino acids. In proteins that contain two ions, five amino acids are responsible for holding the ions in place and one amino acid of these five acts to bring the two ions together. Amino acids involved in holding metal ions in place are histidine, glutamic acid, aspartic acid and lysine residues. Additionally to the metal ions one other amino acid is required for the

catalysis to proceed; glutamic acid has been implied in many metallopeptidases, (however for the activity of leucylaminopeptidase lysine and arginine residues have been inferred to be required for activity). The glutamic acid residue is thought to transfer a proton to the leaving nitrogen atom during the cleavage event. The catalytic mechanism leads to the formation of a non-covalent tetrahedral intermediate being formed as a result of the attack of the ion bound water molecule on the carbonyl group of the scissile bond. This intermediate is further decomposed by the transfer of the glutamic acid proton to the leaving group (Figure 44) (Barrett *et al.*, 2004).

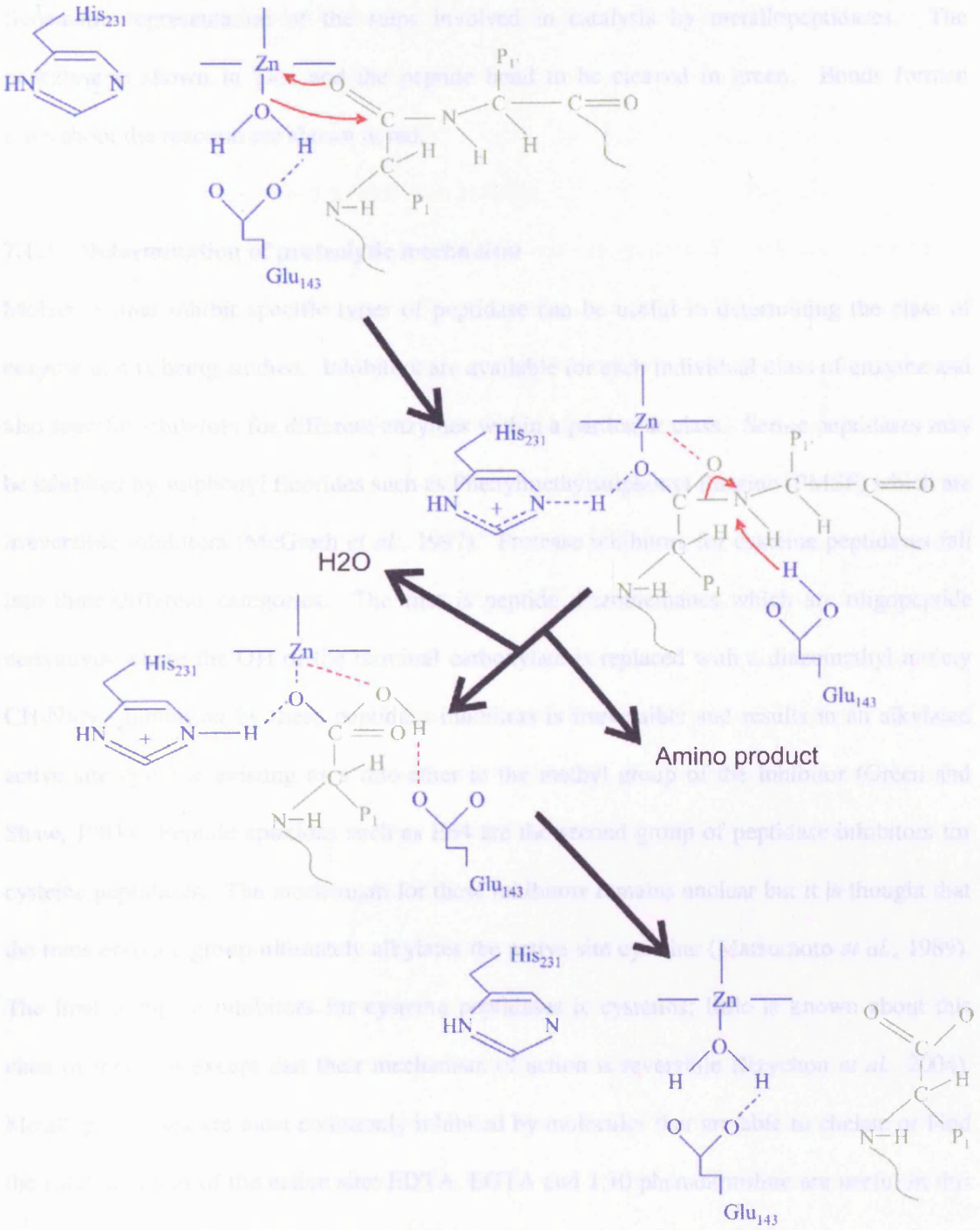


Figure 44 Mechanism of metallopeptidase

Schematic representation of the steps involved in catalysis by metallopeptidases. The peptidase is shown in blue and the peptide bond to be cleaved in green. Bonds formed throughout the reaction are shown in red.

7.1.3 Determination of proteolytic mechanism

Molecules that inhibit specific types of peptidase can be useful in determining the class of enzyme that is being studied. Inhibitors are available for each individual class of enzyme and also specific inhibitors for different enzymes within a particular class. Serine peptidases may be inhibited by sulphonyl fluorides such as Phenylmethylsulphonyl fluoride (PMSF) which are irreversible inhibitors (McGrath *et al.*, 1997). Protease inhibitors for cysteine peptidases fall into three different categories. The first is peptide diazomethanes which are oligopeptide derivatives where the OH of the terminal carboxylate is replaced with a diazomethyl moiety CH-N=N. Inhibition by these peptidase inhibitors is irreversible and results in an alkylated active site cysteine existing as a thio-ether to the methyl group of the inhibitor (Green and Shaw, 1981). Peptide epoxides such as E64 are the second group of peptidase inhibitors for cysteine peptidases. The mechanism for these inhibitors remains unclear but it is thought that the trans-epoxide group ultimately alkylates the active site cysteine (Matsumoto *et al.*, 1989). The final group of inhibitors for cysteine peptidases is cystatins; little is known about this class of inhibitor except that their mechanism of action is reversible (Rzychon *et al.*, 2004). Metallopeptidases are most commonly inhibited by molecules that are able to chelate or bind the catalytic atom of the active site; EDTA, EGTA and 1,10 phenanthroline are useful in this respect. Phosphoramidon is a reversible inhibitor and is able to inhibit the activity of

thermolysin, neprilysin and endothelin converting enzyme-1 (Balwierczak *et al.*, 1995; Oefner *et al.*, 2000).

7.1.4 Methods for measuring peptidase activity

Peptidases are a complex group of enzymes and each is specific for cleavage of particular peptide bonds. Peptide assays have been developed using both natural and synthetic substrates. The choice of substrate depends on the activity of the unknown peptidase, whether it is involved in the cleavage of a specific peptide bond or more general proteolysis. A natural substrate is useful for detection of general proteolysis of endopeptidases without regard for the specificity of its action (Beynon and Bond, 1989). Detection of proteolysis of natural substrates depends on an ability to detect the products of the proteolysis e.g. using a U.V. spectrophotometer to measure changes in absorbance or separation of the products by SDS-PAGE (Beynon and Bond, 1989). Synthetic substrates have been useful in the study of peptidases that recognise specific amino acid sequences. Synthetic substrates are made by adding compounds to the amino and/or carboxyl termini. The addition of this group may add a chromogenic nature to the substrate which allows for quantitation of the proteolysis (Dunn *et al.*, 1994). One of the most common chromogenic groups conjugated to peptides is 4-nitroanaline. This substrate, when conjugated to the peptide, has a low absorbance at wavelengths around 400 nm. Free amine is highly absorbent at this wavelength. A second type of peptide useful in peptidase assays are those with fluorophores conjugated to the peptide. Fluorescence resonance energy transfer (FRET) involves the conjugation of two fluorophores to the peptide, one to the amino terminus and the second to the carboxyl terminus. The fluorophores added to peptides for FRET must be carefully paired together to

allow the detection of fluorescence once the peptide has been cleaved (Grahn *et al.*, 1998). A common pair of fluorophores includes EDANS and DABCYL. The mechanism by which FRET operates is by having one fluorophore that becomes excited at a specific excitation wavelength and by a long range dipole-dipole coupling mechanism this excited state is transferred to the second fluorophore which acts as an acceptor. When the two fluorophores are in close proximity to each other, as they would be in a peptide, the fluorescence of the donor is quenched by the acceptor molecule and no fluorescence can be measured on a fluorimeter. When the peptide is cut by a peptidase, the two fluorophores are no longer in close proximity to one another and the fluorescence that is emitted from the donor fluorophore when it is excited at the correct wavelength can be measured by the fluorescence reader.

7.1.5 Aims and Objective

The aim of the investigation described in this chapter was to identify a protein responsible of the Shavase activity that has been described in chapters 4 and 5 of this thesis.

1) Produce an assay suitable for screening for the Shavase activity
2) Characterise the Shavase activity with regards to location and protease inhibitor profile.
3) Purify the enzyme from *S. uberis* and determine its identity.

7.2 Results

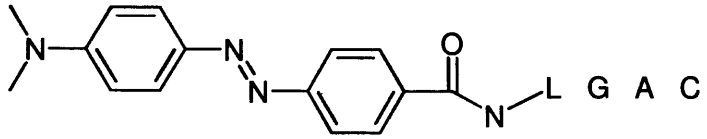
Alternative processing of lipoproteins has been shown to occur in the presence of *lgt*⁻ and *lgt*⁻ */lsp*⁻ mutations in *S. uberis* (Chapters 4 and 5). The enzyme that is responsible for this activity is likely to be a peptidase and this may be partially characterised through the use of peptidase assays.

7.2.1 Design of fluorogenic peptide and mechanism for fluorogenic peptidase assays

A peptide was designed based on the lipobox motif within the signal peptide of MtuA and the proceeding four amino acids. The alternative cleavage site for the hypothetical shaving enzyme of *S. uberis*, ShvU is present in the middle of the peptide. At the amino terminus of the peptide the fluorogenic marker DABCYL was added and at the carboxyl terminus EDANS was linked (Figure 45a). When these two fluorogenic markers are in close proximity to each other no fluorescence is detectable due to the quenching ability of the DABCYL, but when the peptide is proteolytically cleaved the fluorescence of EDANS is no longer quenched and may be measured using a fluorimeter (Figure 45b and c).

A

B



C

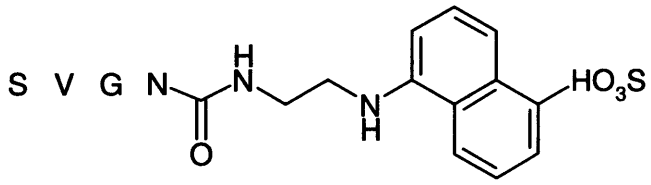


Figure 45 Fluorogenic peptide for the identification of ShvU from *S. uberis*

DABCYL was conjugated to the amino terminus of the peptide and EDANS added to the carboxyl terminus (A). When the peptidase ShvU is mixed with the peptide, it is cut into two smaller peptides (B and C) allowing fluorescence to be measured from the EDANS portion (C).

7.2.2 Determination of the presence of an enzyme able to cleave the fluorogenic peptide

Whole cell lysates were prepared from *S. uberis* strain 0140J, *lgt*⁻ and *lsp*⁻ mutants, 10 μg of protein was added to a peptidase assay in Hepes buffer pH 8.0. A second peptide was included where the cysteine was replaced with a lysine to enable cleavage by the enzyme trypsin. The control peptide was included in the experiment to act as a comparison control to ensure that the cleaved lipoprotein peptide was being detected. The peptide in the presence of buffer and the absence of whole cell lysate was included as a negative control. This also acted as a background fluorescence control. The reaction was monitored by use of a fluorimeter (Wallac 1420 VICTOR^2) after overnight incubation at 37°C (Figure 46). An increase in fluorescence was detectable for the wild type and the two mutants and this increase was comparable to that shown by the cleavage of trypsin of the peptide containing the lysine residue instead of the cysteine residue.

To determine that the peptide had been processed in the correct place, cleaved and uncleaved peptides were separated by reverse phase HPLC (Figure 47). Differences can be seen in the HPLC traces of the cleaved and uncleaved peptides, suggesting that the peptide that had been incubated in the presence of *S. uberis* whole cell lysate had been cleaved. The amino terminus of the EDANS portion of the cleavage product was SVGN showing that the peptide had been cleaved on the carboxyl side of the cysteine residue of the lipobox as is described in the *lgt*⁻ mutant (Chapter 4). The increase in absorbance at five minutes is likely to be an artefact from the injection of the sample on to the reverse HPLC column. It is likely to have caused a pressure change that has led to a change of the refractive index with in the flow cell. This

fluctuation registers as a signal on the trace. The identity of the small shoulder in trace B could not be identified. The carboxyl portion was subjected to Edman degradation amino-terminal protein sequencing.

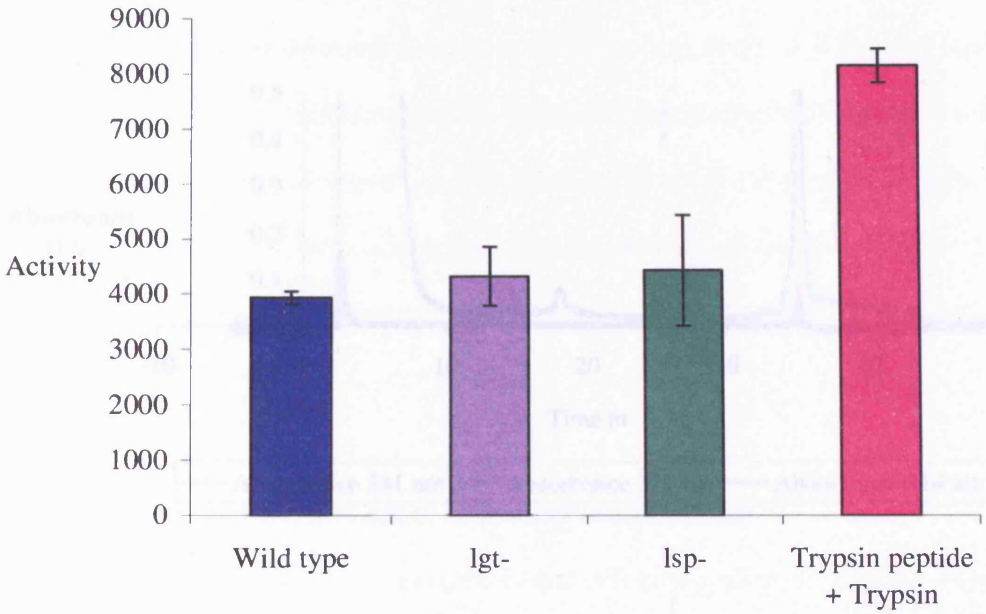
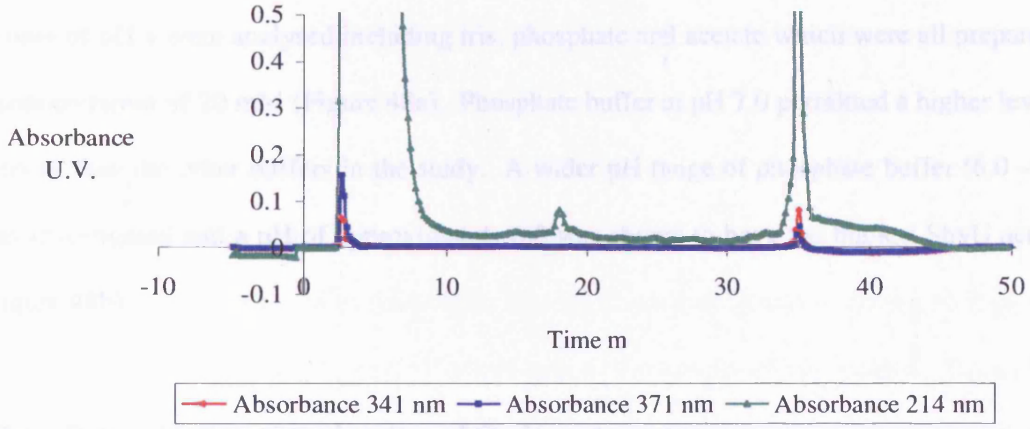


Figure 46 Determination of the presence of a peptidase in the *S. uberis* proteome that is able to cut the FRET peptide

Whole cell lysate (10 µg) prepared from wild type, *lgt*⁻ and *lsp*⁻ mutants was added to peptide and incubated overnight at 37°C before the fluorescence was measured. The trypsin peptide contains a lysine residue instead of the cysteine residue and was incorporated as control to determine that cleavage of the peptides had occurred. Background fluorescence was removed (peptide in the presence of buffer) and error bars represent a standard deviation between results performed in triplicate.

A



B

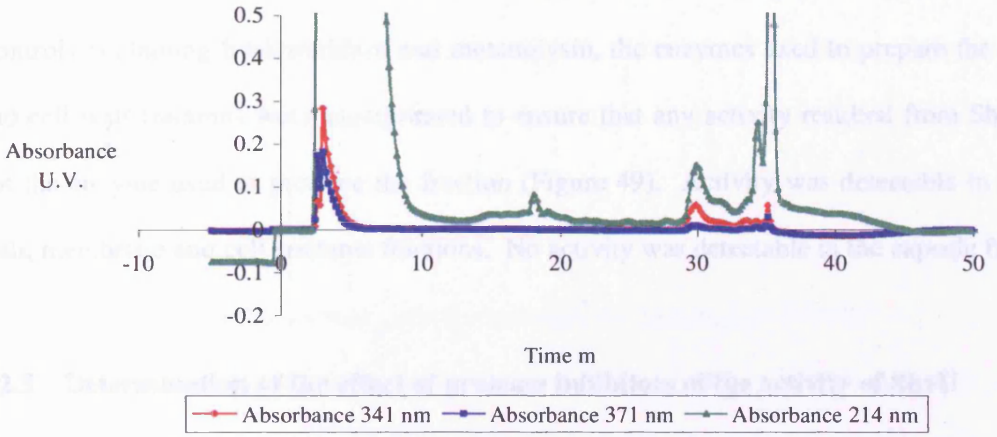


Figure 47 Determination of the cleavage point of fluorescence peptide

The uncleaved peptide (A) and cleaved peptide (B) were separated by reverse phase HPLC. An increase in U.V. absorbance at approximately 30 minutes can be seen in the cut traces which correspond to the two portions of the peptide. Absorbance 341 nm measures the absorbance of EDANS, absorbance 371 nm measures the absorbance of DABCYL and absorbance 214 nm measures the absorbance of the peptide bond.

7.2.3 Determination of the optimum buffer conditions for ShvU activity

In order to determine the optimum conditions for the activity of ShvU, a range of buffers at a variety of pH's were analysed including tris, phosphate and acetate which were all prepared at a concentration of 20 mM (Figure 48a). Phosphate buffer at pH 7.0 permitted a higher level of activity than the other buffers in the study. A wider pH range of phosphate buffer (6.0 – 8.0) was investigated and a pH of approximately 6.0 was shown to have the highest ShvU activity (Figure 48b).

7.2.4 Determination of the location of ShvU protease enzyme

Cell fractions (capsule, cell wall, membrane, and cell contents) were prepared from the *lgt*⁻ mutant and 10 μg of protein from each fraction added to individual peptide assays in triplicate. Controls containing hyaluronidase and mutanolysin, the enzymes used to prepare the capsule and cell wall fractions were incorporated to ensure that any activity resulted from ShvU and not the enzyme used to produce the fraction (Figure 49). Activity was detectable in the cell wall, membrane and cell contents fractions. No activity was detectable in the capsule fraction.

7.2.5 Determination of the effect of protease inhibitors of the activity of ShvU

A range of protease inhibitors with inhibitory activities for the main range of peptidases (cysteine, serine, metallo and aspartic) were added to the extracts (10 μg/ml protein) 30 minutes before addition to the peptide assay. Protease inhibitors with specificity for metallopeptidases (EDTA, EGTA, 1,10 phenanthroline and phosphoramidon) reduced the proteolytic activity of ShvU, suggesting that ShvU is a metallopeptidase (Figure 50). To confirm that the same peptidase was active in all fractions the protease inhibition profile was

determined for each active cell fraction. Each fraction produced a similar inhibition profile as previously described for the whole cell lysates (Figure 51). This result suggested a single peptidase is responsible for cleaving the fluorogenic peptide and that this is most likely a metallopeptidase.

7.2.6 Determination of the metal ion specificity of ShvU

The effect of the addition of various metal ions to whole cell lysates is shown in Figure 52. Addition of Zn^{2+}, Mg^{2+}, Mn^{2+} and Co^{2+} all stimulated the activity of the enzyme. The addition of Cu^{2+}, Fe^{3+} and Fe^{2+} all inhibited the activity of the enzyme. To determine which ion was at the active site of ShvU whole cell lysate was dialysed in the presence of EDTA as described in Chapter 2. The protein was incubated with the ions for 30 minutes before being added to the peptide (Figure 53). The addition of Fe^{2+}, Ni^{2+}, Fe^{3+}, and Cu^{2+} all inhibited the activity of ShvU, where as Zn^{2+}, Ca^{2+}, Mg^{2+}, Mn^{2+} and Co^{2+} all had little or no effect on the activity of ShvU.

7.2.7 Alteration of the cleavage site to establish the specificity of ShvU

The ShvU fluorogenic peptide as previously described is based on the lipobox motif of MtuA and the four amino acids immediately after. The cysteine residue of the lipobox has been shown to be the invariable target of lipoprotein diacylglyceryl transferase (Hantke and Braun, 1973). It could be hypothesised that this cysteine residue is one of the determinants that allows ShvU to recognise its substrate. By changing this for an alternative amino acid, such as serine, it may be able to start preliminary determination of the specificity of this peptidase for its substrate. The original peptide and the peptide containing the serine residue instead of the

cysteine were made up to the same concentration and doubling dilutions made. Whole cell lysate (10 μg) was added to the peptide and the reaction was incubated overnight and the resulting fluorescence was measured on the fluorescence reader (Figure 54). The peptide containing the cysteine residue at the active site of peptide was cleaved, but the peptide containing the modification to the serine residue remained uncleaved at all dilutions.

7.2.8 Purification of ShvU from cell fractions of *S. uberis*

The purification strategies that have been used to purify ShvU from cell fractions are shown in appendix 3. Purification of ShvU was achieved, but not to a sufficient level to obtain an identification.

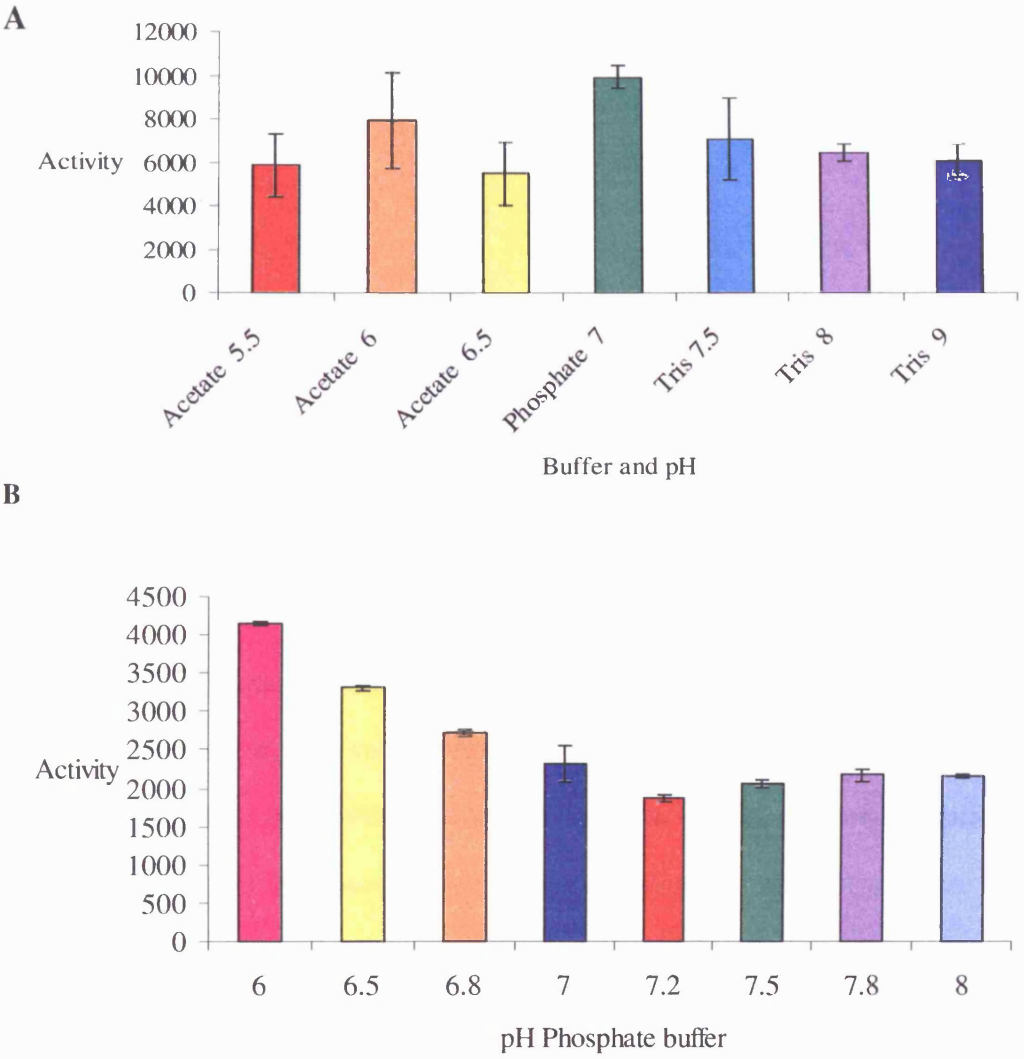


Figure 48 Determination of the correct buffer and pH conditions for ShvU

Whole cell lysate (10 µg) was added to peptide in each of the acetate, phosphate and tris buffers which were each prepared at a concentration of 20 mM (A). Phosphate was determined to have the greatest activity. Phosphate buffer (20 mM) was prepared at a range of pH and included in peptidase reactions. The fluorescence after overnight incubation was measured (B). Background fluorescence was removed and error bars represent a standard deviation between results performed in triplicate.

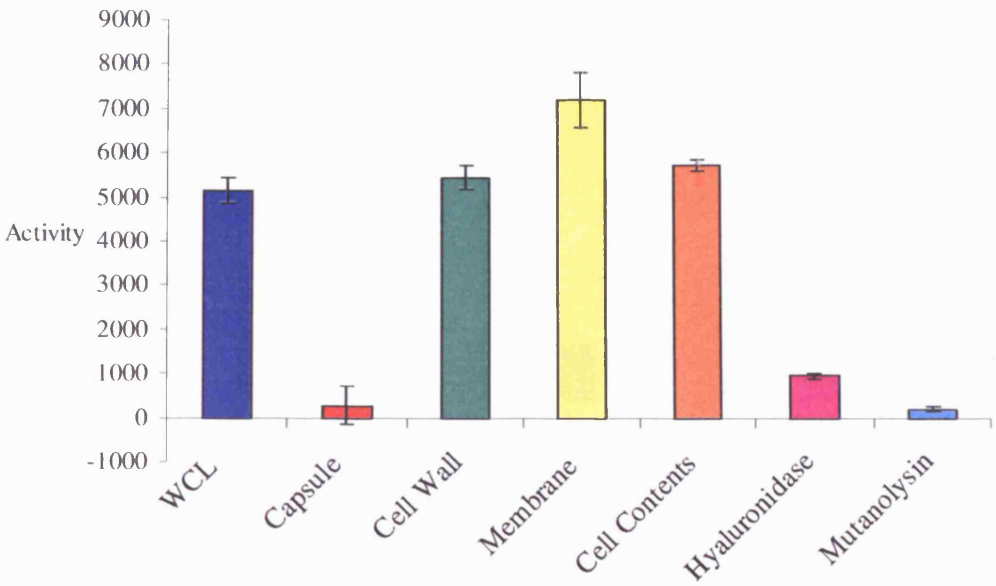


Figure 49 Identification of the location of ShvU in *S. uberis*

Cell fractions (capsule, cell wall, membrane and cell contents) were prepared from wild type and 10 µg of each fraction added to the FRET peptide. Reactions were incubated overnight and measured on the fluorescence reader. Hyaluronidase and mutanolysin were included as controls to demonstrate that activity in the capsule and cell wall respectively was from proteins within the cell fraction and not due to the activity of the enzyme used to prepare the fraction. Background fluorescence was removed and error bars represent a standard deviation between results performed in triplicate.

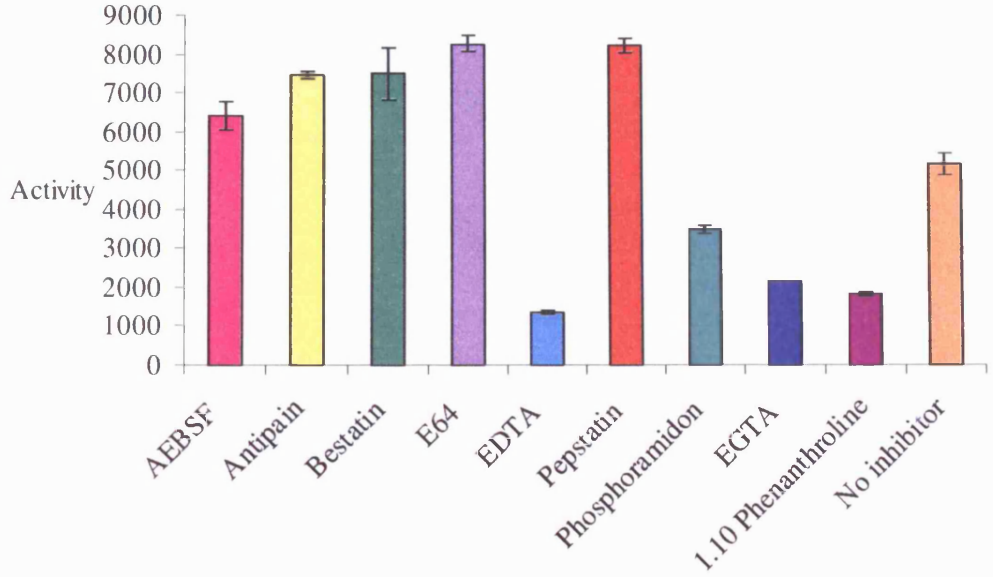


Figure 50 Determination of the protease inhibitor profile of ShvU

Whole cell lysate (10 µg) was incubated with each of the protease inhibitors for 30 minutes before being added to the FRET peptide, the reaction was incubated overnight and the fluorescence measured. Background fluorescence was removed and error bars represent a standard deviation between results performed in triplicate.

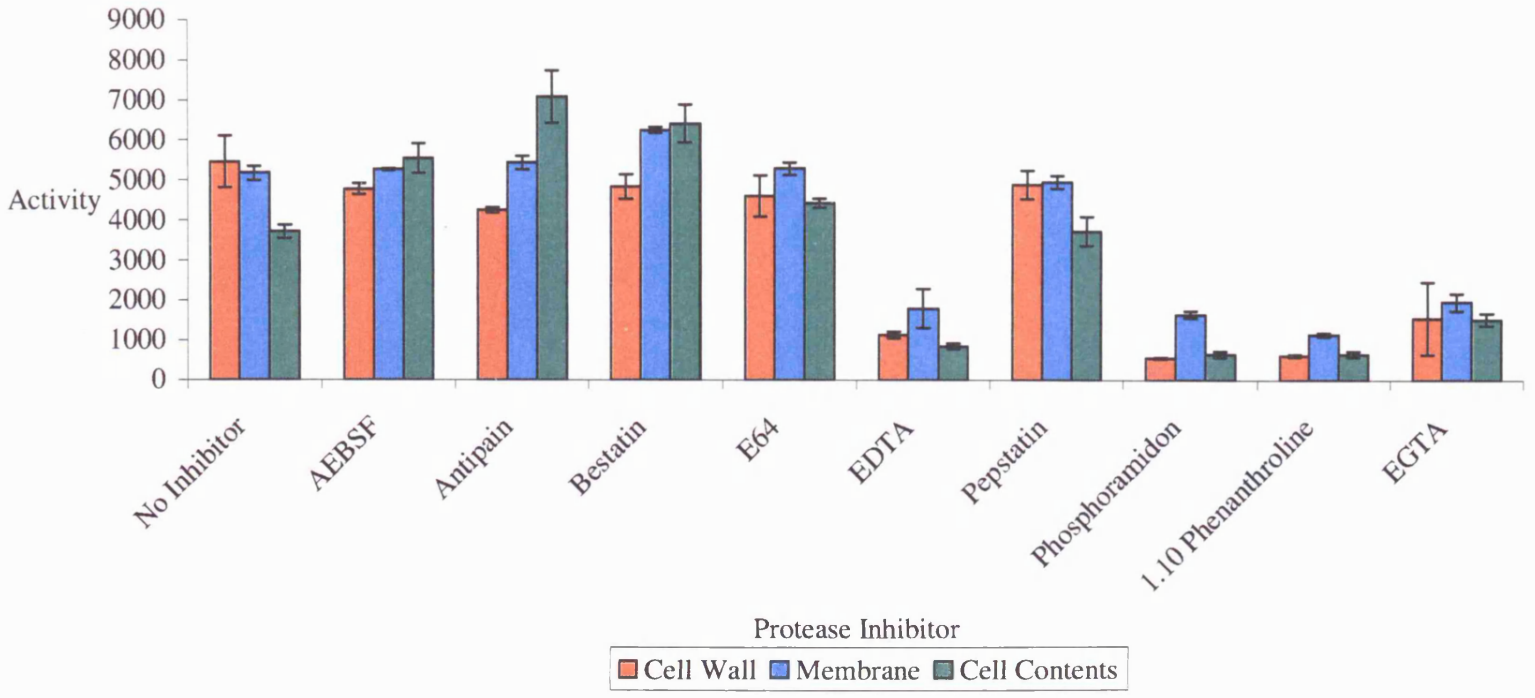


Figure 51 Determination of the protease inhibitor profile of the different ShvU active cell fractions

The cell fractions that have ShvU activity were prepared from the *lgt* mutant (cell wall, membrane and cell contents). 10 µg of each cell fraction was incubated with each protease inhibitor for 30 minutes before being added to the peptide, incubated overnight at 37°C and the fluorescence measured. Background fluorescence was removed and error bars represent results performed in triplicate.

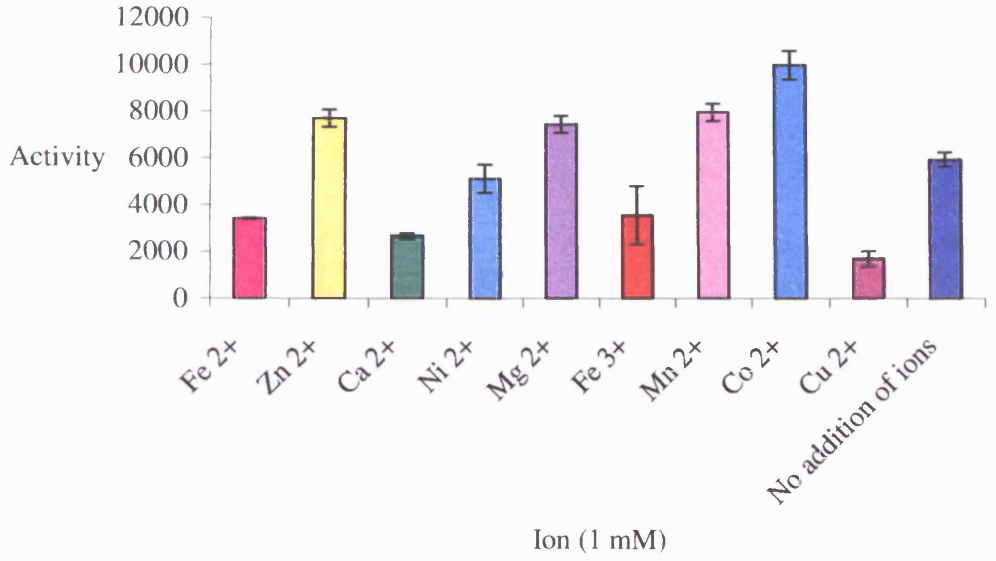


Figure 52 Addition of ions to ShvU

Whole cell lysate was incubated with each ion (1mM) for 30 minutes before being added to the peptide. The reaction was incubated overnight at 37°C before the fluorescence was measured. The background fluorescence was deleted and error bars represent results performed in triplicate.

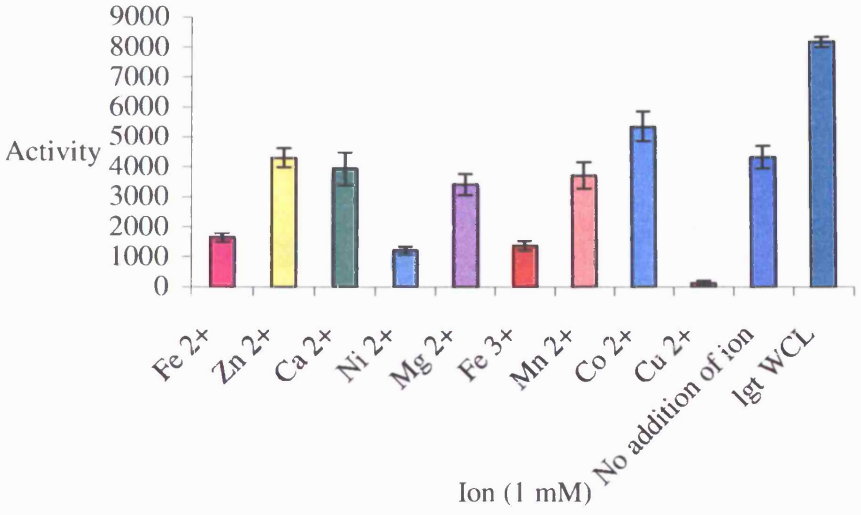


Figure 53 Determination of the effect of metal ions on ShvU treated with EDTA

Whole cell lysate was dialyzed for 24 hours in HPLC grade H_2O containing 10 mM EDTA, before being dialysed in to HPLC grade H_2O, which was changed three times over a period of 24 hours. The EDTA treated whole cell lysate was incubated with each ion (1mM) for 30 minutes before being added to the peptide, incubated overnight at 37°C and the fluorescence was measured. Controls included EDTA treated protein with no ions added and the *lgt* mutant whole cell lysate before it was treated with EDTA. Background fluorescence was deleted and error bars represent results performed in triplicate.

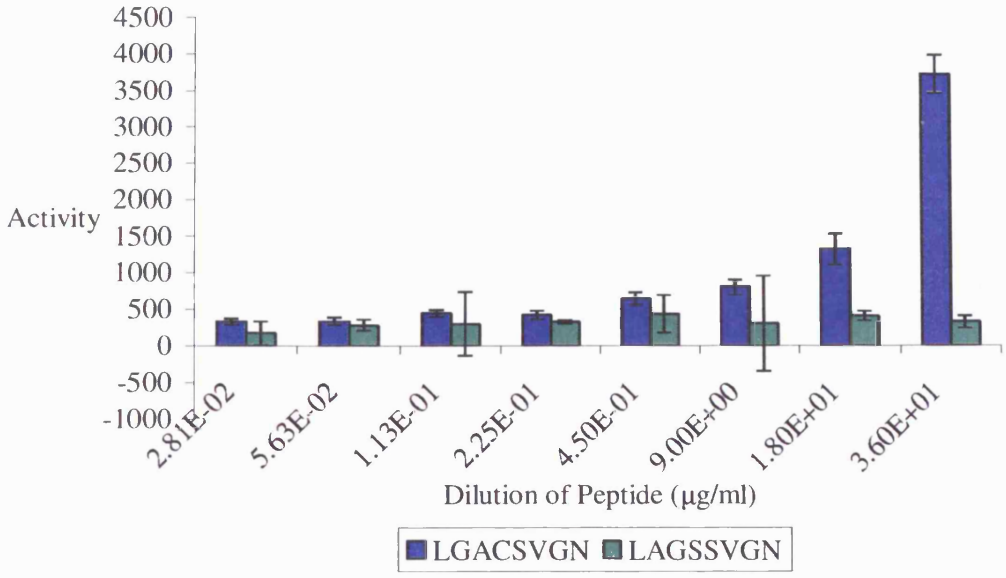


Figure 54 Modification of the cleavage site of lipoprotein peptide from cysteine to serine

Background fluorescence was deleted and error bars represent results performed in triplicate.

7.3 Discussion

7.3.1 ShvU activity in *S. uberis*

The alternative processing of lipoproteins is evident in the *S. uberis lgt*⁻ and *lgt*⁻*/lsp*⁻ insertion mutants. This study describes an investigation that attempted to characterise and determine the enzyme responsible for this phenotype that has been provisionally termed Shavase Uberis (ShvU). The investigation was based on a FRET peptide assay as a marker for the activity of this enzyme. The activity of an enzyme shown to be able to process this peptide immediately after the cysteine residue of the lipobox motif, as in the alternative processing phenotype shown for three lipoproteins in Chapter 4, was found to be present in a whole cell lysate of wild type, *lgt*⁻ and *lsp*⁻ insertion mutants. The *lsp*⁻ mutant was able to process the peptide; this provides further evidence that an enzyme that is not Lsp is present in *S. uberis* that is able to process the non-lipidated signal peptides of potential lipoproteins. The activity of the enzyme able to process the peptide was shown to be located in the cell wall, membrane and cell contents. The highest activity for the enzyme was shown to be in phosphate buffer at a pH of 6.0. There is likely to be an acidic pH at the cell wall membrane interface (Calamita *et al.*, 2001), where ShvU is likely to be active, as the highest activity of the protein was seen in the membrane. Therefore, the low pH optimum of this enzyme is not unexpected. The active site residues for Lgt and Lsp, the two characterised enzymes in the lipoprotein processing pathway (Sankaran *et al.*, 1997; Tjalsma *et al.*, 1999b) are also predicted to be present at this interface. The substrate for ShvU is also likely to be present at this cellular location. It could be hypothesised that the protein responsible for this activity may have a signal peptide as activity is found located in the cell wall. However, alternative methods for locating this enzyme in the cell wall cannot be excluded, since several proteomic studies have identified proteins without

signal peptide sequences to be found on the cell surface (Severin *et al.*, 2007). It is likely that this protein will have a motif that anchors it to either the cell wall or to the membrane, consequently keeping the protein out of the capsule and extracellular space. Of the metallopeptidases that were predicted by searching the open reading frames of *S. uberis* strain 0140J in the MEROPS database, one metallopeptidase has a signal peptide sequence, three have signal peptide sequences and transmembrane helices and four have just transmembrane helices (appendix 2).

7.3.2 The M13 class of metallopeptidases

Characterisation of the behaviour of ShvU in the presence of protease inhibitors allowed classification of the enzyme able to cleave the FRET peptide as a metallopeptidase. EDTA, EGTA and 1, 10 phenanthroline are all metal ion chelating agents and these compounds all strongly inhibited the activity of ShvU. Phosphoramidon was a second type of protease inhibitor that was able to inhibit the activity of ShvU. This inhibitor has been shown to inhibit members of the M4 and M13 families of metallopeptidases. Genome analysis of *S. uberis* strain 0140J with the MEROPS database determined that there are no known homologues of M4 metallopeptidases present within this strain, but the ORF SUB1756 shows homology with members of the M13 family. Several members of the M13 family of metallopeptidases have been characterised in Gram positive bacteria including oligoendopeptidaseO (PepO). This protein has high homology to mammalian metalloendopeptidases including neutral endopeptidase (NEP) and endothelin converting enzyme-1 (ECE-1), enzymes responsible for modulating peptide receptor signalling in eukaryotic cells (Barrett *et al.*, 2004; Turner *et al.*, 2001). In the oral cavity bacterium, *Streptococcus parasanguinis,* PepO was shown to be

localised to the cytosol and protein extracts from this bacteria were shown to be able to cleave metenkephalin, a pentapeptide present in the brain and a natural substrate of NEP (Oetjen *et al.*, 2001). This enzyme was also shown to be inhibited by phosphoramidon, EDTA and 1, 10 phenanthroline. The actual function of this protein has not been described. Mutants of *pepO* of *L. lactis* and *Lactobacillus helveticus* have been investigated to determine if they have a role in the development of cheese flavour (Chen and Steele, 1998; Mierau *et al.*, 1993). They showed reduced levels of endopeptidase activity compared to the wild type strains, but were able to grow to similar levels as the wild type in milk and were not reduced in their level of acidification. It has been suggested that PepO is not involved in hydrolysis of milk proteins and as this protein does not carry a signal sequence is likely to be located in the cytosol. The PepO protein of *Lactobacillus rhamnosus* showed unique cleavage specificity for α_{S1} casein fragment 1-23 and it was suggested that if this protein is released by autolysis, it may act in controlling the bitterness of cheese during ripening (Christensson *et al.*, 2002). PepO2 is a second protein expressed by *L. helveticus* and shows specificity for post proline bonds and distinguishes it from the other PepO homologues that have been characterised to date (Chen *et al.*, 2003). There has been no suggestion in the literature that proteins homologous to SUB1756 are involved in processing of secreted proteins. As SUB1756 is the only gene with homology to M13 peptidases, this protein may be worth investigating to determine if it is responsible for the alternative processing of lipoproteins. It should be noted that this protein does not contain a signal peptide sequence and no transmembrane helices can be predicted using TMHMM (Kall *et al.*, 2004), which may suggest there is another protein within the genome of S. uberis that is a member of the M4 or M13 classes of peptidase. However, TepA of *B. subtilis* is a peptidase that has been shown to be necessary to allow protein secretion and

is likely to be involved in the degredation of signal peptidases. This protein is likely to have a cytoplasmic location (Bolhuis *et al.*, 1999).

In conclusion a peptidase activity has been confirmed within *S. uberis* that is able to process the peptide based on the lipobox motif of MtuA between the cysteine and serine residue as was shown in the *lgt* mutant (Chapter 4). The peptidase that is able to process this peptide is likely to be a metallopeptidase as it can be inhibited by a range of metal ion chelating agents and also by phosphoramidon. Partial purification of the protein responsible for this activity has been successful, but further purification needs to be carried out to identify the protein that is actually ShvU and is able to alternatively process lipoproteins.

Chapter 8

General Discussion

The translocation of proteins from the cytosol where they are made across the cell membrane to the cell surface is essential for bacteria to grow and cause disease in host tissues. These proteins allow attachment to cells and the uptake of nutrients. At the cell surface they may become anchored to either the cell wall or the membrane, or they may pass out into the extracellular space.

Lipoproteins are a major group of membrane anchored proteins and have been identified in many bacteria to have essential functions, including the manganese transporter, MtuA of *S. uberis* (Smith *et al.*, 2003). In this thesis the lipoprotein diacylglyceryl transferase (Lgt) and lipoprotein signal peptidase (Lsp) of the pathogen *S. uberis* have been described through the study of bacteria carrying insertion mutations within the genes encoding these two enzymes. The correct processing of lipoproteins is dependent on the activity of these two enzymes.

8.1 Characterisation of an *lgt*⁻ mutant of *S. uberis*

Lgt was shown to be responsible for the anchoring of proteins to the membrane. It can be predicted from this observation that this was due to the removal of the bacteriums ability to modify the invariable cysteine residue of the lipobox motif which lipoproteins carry within their signal peptide sequence. This modification allows the protein to be anchored to the membrane by the formation of a diacyl-glyceryl bond between the sulfhydral group of the cysteine residue and the membrane (Hussain *et al.*, 1982). This has been described in a

number of other bacteria (Baumgartner *et al.*, 2007; Petit *et al.*, 2001; Stoll *et al.*, 2005). The presence of additional proteins in the supernatant and capsule fractions was seen in the *lgt*⁻ mutant, these proteins were not detectable in the equivalent fractions prepared from the wild type. It was hypothesised that these proteins were likely to be lipoproteins. Analysis of three of the proteins by N-terminal sequence determination confirmed their identity as typical lipoproteins. Mass spectrometry analysis of the supernatant and capsule fractions identified several other lipoproteins that were not present within the equivalent wild type fraction (data not shown). These two independent methods for identification of proteins show that the *lgt*⁻ mutant is not able to anchor lipoproteins correctly. This is comparable to *lgt* mutants that have been studied in *B. subtilis*, *L. monocytogenes*, *S. equi* and *S. aureus* (Antelmann *et al.*, 2001; Baumgartner *et al.*, 2007; Hamilton *et al.*, 2006; Stoll *et al.*, 2005).

Alternative processing of lipoproteins was shown to be evident at the amino terminal amino acid of the three identified lipoproteins. It was determined that the proteins had been processed on the carboxyl side of the cysteine residue and this included the essential lipoprotein MtuA. The MtuA that was detected in the *lgt*⁻ mutant was of a size that could not be discriminated between that detected in the membrane of the wild type and that presumed to be correctly processed lipoprotein. This suggested that there is an activity within the *lgt*⁻ mutant whereby the signal peptides of non-lipidated lipoproteins are removed. This phenomenon has been shown to occur in *B. subtilis* wild type and *lgt* mutant and *S. aureus* wild type and has been described as proteolytic shaving (Antelmann *et al.*, 2001; Sibbald *et al.*, 2006). It is unknown as to whether proteolytic shaving occurs in wild type *S. uberis*. Lipoproteins in the presence of an *lgt*⁻ mutation may get stuck at the site of secretion, possibly

by virtue of their hydrophobic signal peptide sequence. The presence of an enzyme (termed "Shavase") may unblock the site of secretion and allow other proteins to be transported correctly. It is unknown as to why proteolytic shaving occurs in wild type bacteria (Antelmann *et al.*, 2001).

The sequences of the three lipoproteins shown to be alternatively processed in the presence of the mutation within *lgt*⁻ are all slightly different in amino acid sequence at the cleavage site. It would be of interest to determine the specificity of the putative Shavase enzyme that has been hypothesised to be responsible for this activity. The identity of the Shavase enzyme was studied using a FRET peptide assay based on the lipobox of MtuA and the four amino acids immediately following. A peptidase activity was identified where the peptide was processed on the carboxyl side of the cysteine residue as shown in the *lgt*⁻ mutant. Alteration of the cysteine residue within the FRET peptide to a serine residue has determined that this enzyme will not cleave the peptide, showing that the invariable cysteine residue of the lipobox is a major factor in the specificity of this peptidase. A lipoprotein release determinant sequence has been described for *B. subtilis* (Tjalsma and van Dijl, 2005). The consensus sequence for the lipoprotein release determinant is G-[NSTF]-x-[SGN]-x-[SGKAE]-x-x-[SGA], this sequence was shown to be present within 6 of the 7 lipoproteins found in the supernatant of the wild type bacteria and also in 4 of the 9 lipoproteins released into the supernatant of the *lgt* mutant. The pattern is absent from all lipoproteins that remain membrane anchored. The three lipoproteins that were released by *S. uberis* do not fit with this motif. If the metallopeptidase activity identified in this project is the putative Shavase and is responsible for the proteolytic shaving of lipoproteins from the surface of cells, the lipobox motif and the

four amino acids following this would appear sufficient for these proteins to act as a substrate thus permitting the metallopeptidase activity to remove non-lipidated lipoproteins from the surface of the cell.

Two different sized immunoreactive bands of MtuA were detected in whole cell lysates prepared from the wild type cultured in the presence of the Lsp inhibitor globomycin. The protein with the higher molecular weight was surmised to be unprocessed MtuA with the signal peptide remaining attached and the smaller form is the mature lipoprotein. A comparable result was seen in the equivalent preparation from the *lgt*⁻ mutant; where by a form of MtuA was detected that appeared to be full length protein. This suggested that either Lsp was able to process unlipidated lipoprotein or that the enzyme that does alternatively processes some if not all lipoproteins was affected by the presence of inactivated Lsp.

An enzyme that was not Lsp was, for the first time shown to be able to process the signal peptides of unlipidated lipoprotein. MtuA of a size comparable to that seen in the wild type and *lgt*⁻ mutant was shown to be present in an *lgt*⁻/*lsp*⁻ double mutant. As in the single *lgt*⁻ mutant, MtuA was detected in all cell fractions, showing that the anchoring mechanism of lipoproteins was not functioning. In the capsule fraction a single band was detected of a size comparable to that seen in the wild type, however in the cell wall, membrane and cell contents two bands were exposed. The band of a smaller molecular weight was the same size as in the capsule fraction, but the larger band appears to be equivalent to unprocessed MtuA. As previously mentioned this result confirmed the presence of an enzyme that was able to process lipoproteins that was not Lsp, but poses the question as to whether Lsp is able to process

unlipidated lipoproteins. The amino terminus of lipoproteins in a *L. monocytogenes lgt* mutant was shown to be the cysteine residue of the lipobox (Baumgartner *et al.*, 2007). The addition of globomycin to the *L. monocytogenes* mutant, removed the presence of the extra proteins that had been identified in the supernatant of the *lgt*⁻ mutant. This was possibly due to Lsp having been inactivated by the antibiotic and removal of the signal peptide from the proteins was therefore prevented, allowing the proteins to become stuck in the membrane. However, a double mutant was not studied to confirm this result. These observations suggest that the specificity of Lsp in Gram positive bacteria may be different to that in Gram negative bacteria where this enzyme is not able to process unlipidated lipoproteins (Tokunaga *et al.*, 1982).

8.2 Identification of the peptidase responsible for the alternative processing of lipoproteins in the presence of *lgt*⁻ and *lgt*⁻/*lsp*⁻ mutants

A peptidase activity was identified within *S. uberis* cell wall, membrane and cell contents fractions, which was able to process a FRET peptide made up of the lipobox of MtuA and the four proceeding amino acids. The processing was shown to occur between the cysteine residue of the lipobox and the amino acid immediately after as shown in the *lgt*⁻ mutant. The peptidase activity was shown to occur in fractions prepared from the *lsp*⁻ mutant, which provides further evidence that Lsp and another enzyme are able to process the signal peptides of lipoproteins. This peptidase activity was inhibited by metal ion chelators and phosphoramidon, which are metallopeptidase inhibitors. Phosphoramidon has specificity for two classes of metallopeptidases; M4 and M13. The genome of *S. uberis* does not encode any homologues to known members of the M4 class of metallopeptidase, but SUB1756 is homologous to members of the M13 class, so this may be a potential candidate for Shavase

Uberis (ShvU). However, a signal sequence and membrane spanning domains cannot be predicted for this protein. This suggests that another protein within the genome of *S. uberis* is likely to be responsible for the activity of ShvU that fits with the M4 or M13 families of metallopeptidase.

8.3 Characterisation of *lsp*⁻ and *lsp*⁻/*eep*⁻ mutants of *S. uberis*

Two forms of membrane anchored MtuA can be detected in the *lsp*⁻ mutant of *S. uberis*. The form of the protein with a larger molecular weight was present in all growth phases and appears to be MtuA containing the signal peptide sequence; therefore confirming that Lsp is responsible for processing the signal peptide of lipoproteins as has been described in other bacteria (De Greeff *et al.*, 2003; Pragai *et al.*, 1997; Reglier-Poupet *et al.*, 2003a; Sander *et al.*, 2004; Tjalsma *et al.*, 1999b). The form of MtuA with the smaller molecular weight was detectable by Western blot at 24 hours and appears to be slightly larger in molecular weight than the form seen in the wild type. This suggests that MtuA was able to be processed in a third position (the first being between the third and fourth amino acids of the lipobox and the second on the carboxyl side of the cysteine residue of the lipobox). The literature reports that competence peptides of *E. faecalis* are the last eight carboxyl amino acids of the signal peptide of some lipoproteins (An and Clewell, 2002) and that their production depends on the peptidase Enhanced expression of pheromone (Eep). This is a predicted metallopeptidase located in the membrane (An *et al.*, 1999). *S. uberis* contains a homologue to Eep (SUB254). The smaller form of MtuA seen in the *lsp*⁻ mutant was no longer detectable in a double *lsp*⁻ */eep*⁻ insertion mutant, suggesting that the peptidase is responsible for processing the signal peptide of lipoproteins at this third position.

8.4 Overall Conclusion

In conclusion the research presented in this thesis has shown that lipoproteins in the pathogen *S. uberis* are processed by the two enzymes; Lgt, that acts to anchor the protein to the membrane, and Lsp which removes the signal peptide, as has been described for other bacteria. This work has described two novel phenotypes; alternative processing of lipoproteins on the carboxyl side of the cysteine residue that may be being carried out by a metallopeptidase and also alternative processing of the signal peptide carried out by the metallopeptidase Eep. It will be of interest to determine the function of these two enzymes and their roles in the wild type *S. uberis*.

8.5 Future work and investigation

8.5.1 Determination of the alternative lipoprotein processing occurring in *S. uberis* by using a Two Dimensional Polyacrylamide Gel electrophoresis

Alternative processing in the three lipoprotein processing mutants is evident (Chapters 3-5). MtuA in the Lsp mutant remains in the membrane, which is very complex in its nature, approximately 600 predicted proteins in the *S. uberis* genome are thought to contain a membrane anchor of some sort. For this reason it would be impossible to blot a 1-dimensional gel to PVDF and cut out a single band, contamination would be a large issue and the protein would not be able to be sequenced. Immunoprecipitation using anti-MtuA to pull out the different forms of MtuA was also unsuccessful. The isoelectric points for the different forms of MtuA are shown in Figure 55. As can be seen the predicted isoelectric points of the different forms of MtuA vary by a whole pI unit. This is a useful feature that would allow separation of the membrane proteome using two dimensional gel electrophoresis followed by Western blotting to determine the location of MtuA. Once the location of MtuA has been identified a separate gel could then be blotted to PVDF, stained with amido black and the individual spots cut out before subjecting them to N-terminal sequencing to determine were in the signal peptide sequence these proteins are processed.

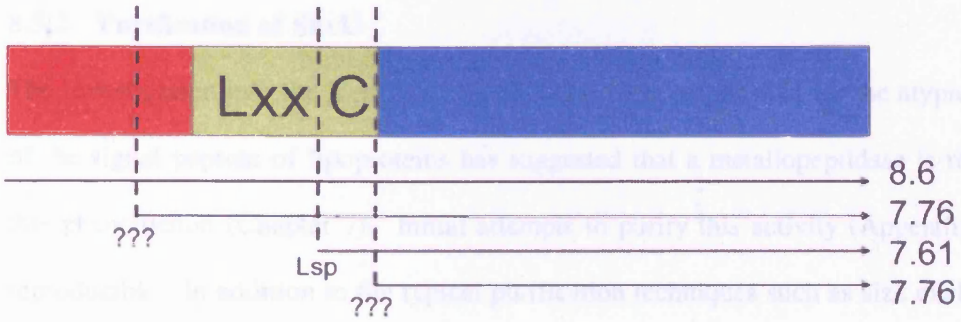


Figure 55 The alternative forms of MtuA have different isoelectric points

The lipoprotein MtuA in the wild type bacteria is predicted to be processed by Lsp at the point shown leaving the cysteine residue as the amino terminus of the protein, the pI of this protein is predicted to be 7.61. Full length MtuA as is seen in the *lsp*$^-$ and *lgt*$^-$/*lsp*$^-$ mutants has a predicted pI of 8.6. Cleavage point (a) is 8 amino acids from the Lsp cleavage point and is predicted to be the alternatively processed form seen in the *lsp*::IS*S1* mutant and has a pI of 7.76 (Chapter 6). Cleavage point (b) is the form that has been N-terminally sequenced in the *lgt*$^-$ mutant (Chapter 4) and this has a predicted pI of 7.76.

8.5.2 Purification of ShvU

The investigation into the identity of ShvU, the protein responsible for the atypical processing of the signal peptide of lipoproteins has suggested that a metallopeptidase is responsible for this phenomenon (Chapter 7). Initial attempts to purify this activity (Appendix 3) were not reproducible. In addition to the typical purification techniques such as size exclusion and ion exchange, the use of purification techniques that exploit the demonstrated properties of the ShvU activity, for example using a metal ion affinity column may prove effective. This technique would require investigation as to which metal ion would be most suitable, however from the metal ion investigation Co^{2+}, Mg^{2+}, Mn^{2+} or Zn^{2+} would be a suitable starting point. Chromatofocusing separates proteins at their isoelectric points. It is a high resolving technique and concentrates the sample. It is possible that such a technique may also be useful in the purification of ShvU which has been problematic thus far. A further that should be taken into consideration is the location of ShvU. The protein had the most activity in the membrane fraction and therefore techniques for the selection and enrichment of membrane proteins should be investigated.

Appendix 1

```
         1                                                          60
SPase_IIa  .......... ........MR LMKLCLGSLV LIALDQLSKL WIVTHIGLGQ VKSFLPG...
SPase_IIb  MTKTIGLHFP EFNAIKYVKT DKDFPLGTWL LVKDAKGSRL AQVKQGMKQL QQAQLPGDMP

          61                                                         120
SPase_IIa .......... ....LVSLTY LQNRGAAFSI LQNQ.RWFFT IITCIVVSAA IIYYIKRAPM
SPase_IIb YVIGKASDMD MKAYRANLAY AEASKADVKA LIKQNQLSMK VIDIIFPLDR SYVLITFSAE

         121                                                         180
SPase_IIa  SKLKEWALIL IISGAIGNFI DRMRLSYVVD MIHLDFMNFA IFNVADSYLS IGVVLLMIIL
SPase_IIb  ERVDFRQLLK DLAALFKTRI ELRQLNNREE AKVYGGIGPC GRPICCSTFL GEFPAVSIKM

         181                                                         240
SPase_IIa  WKEE...... .......... .......... .......... .......... ..........
SPase_IIb  LKNQDLSLNS GKSLGYCGRF LCCLQYEDAF YSDRKKRFPD YGTLVETKEG KGRVVSVNIF

         241                        266
SPase_IIa  .......... .......... ......
SPase_IIb  EETVKVAFED KQSCMTYILE EIKIGQ
```

Figure 56 Alignment of Lsp of *S. uberis* to a possible alternative Signal peptidase II of *S. uberis*

The lipoprotein signal peptidase of *S. uberis* (SPase II a) aligned to a possible alternative Signal peptidase II (SPase II b). The blue residues are the five conserved domains of Lsp as previously identified (Tjalsma *et al.*, 1999b).

Appendix 2

SUB	MEROPS Family/Sub family	Catalytic Residues	Metal ion binding residues	E-Value	Features
SUB0012	S11	S231, K234, S299		5.00E-06	
SUB0015	M41	K48E	I47H, K51H, A117D	1.00E-152	FtsH, 2 transmembrane helices
SUB0027	C44	C23		1.00E-101	
SUB0129	U48			2.00E-15	
SUB0131	M20A	D68, >E	H66, I77D, >E, >D/E, >H	6.00E-05	
SUB0131	M42	D68, E213	H66, D181, E214, D236, H318	1.00E-129	
SUB0175	S66	S109, E249, H315		6.00E-23	
SUB0178	U48			1.00E-09	
SUB0239	M42	D75, E225	H73, D191, E226, D246, H326	5.00E-19	
SUB0239	M42	D75, E225	H73, D191, E226, D246, H326	5.00E-19	
SUB0254	M50B	<E	<H, <H, D369	2.00E-34	Eep, 5 transmembrane helices

SUB0254	M50B	E19	H18, H22, >D	4.00E-16	
SUB0302	S33	S96, D188, H215		2.00E-33	
SUB0302	S33	S96, D188, H215		2.00E-33	
SUB0310	C26	C115, H210		3.00E-56	
SUB0310	S49	<S		1.00E-07	
SUB0326	C26	C381, H508		4.00E-06	
SUB0329	S09X	S166, D255, H284		3.00E-70	
SUB0349	S11	S75, K78, S139		3.00E-33	
SUB0350	S11	S59, K62, S122		8.00E-56	
SUB0351	S11	S60, K63, N123S		1.00E-52	
SUB0367	M20A	D92, E157	H90, D123, E158, Q185D/E, >H	9.00E-35	
SUB0376	M48B	E144	H143, H147, E225	6.00E-48	Signal peptide, 4 transmembrane helices
SUB0429	S14	S96, H121, D172		5.00E-75	
SUB0437	M50B	<E	<H, <H, <D	9.00E-07	
SUB0501	C39	Q11, C17, H96, S116D		1.00E-41	
SUB0535	M24B	H201, H289, H300	D218, D229, H293, E322, E336	5.00E-63	
SUB0582	U32			3.00E-22	
SUB0583	U32			0.00E+00	

SUB0601	M03B	E386	H385, H389, E413	8.00E-29	
SUB0633	C69	C3		1.00E-123	
SUB0706	M20B	D84, E177	H82, D143, E178, D200, H382	1.00E-105	
SUB0729	A08	D105, D130		4.00E-28	Lipoprotein signal peptidase
SUB0734	C26	E45C, V129H		1.00E-122	
SUB0734	S49	I43S		3.00E-76	
SUB0826	S08A	D113, H166, N261, S508		9.00E-29	
SUB0827	M50X	G295E	G294H, L298H, L371D	1.00E-15	10 transmembrane helices
SUB0827	M50X	G89E	S88H, H92, S160D	1.00E-05	
SUB0844	C26	C116, H213		2.00E-96	
SUB0881	C60A	H144, C210		1.00E-14	Sortase
SUB0891	C26	C89, H179		1.00E-67	
SUB0891	S49	<S		9.00E-10	
SUB0899	S24	S150, K186		2.00E-24	
SUB0909	M20D	D78, E145	D76, D113, E146, H375	3.00E-82	
SUB0959	M01	E293, Y379	H292, H296, E315	4.00E-52	
SUB0998	C44	C2		2.00E-52	
SUB0999	S26A	A37S, S73K		1.00E-16	

SUB1016	M38	T303D	H60, H62, A145K, N191H, H228	2.00E-68	
SUB1052	U48			1.00E-07	
SUB1052	U48			1.00E-07	
SUB1054	M20A	D88, E151	H86, D117, E152, D177, >H	7.00E-17	
SUB1070	S09X	S230, D329, H361		4.00E-29	
SUB1070	S09X	S230, D329, H361		4.00E-29	
SUB1105	M10X	E201	H200, H204, H210	4.00E-26	1 transmembrane helix
SUB1106	S09X	S123, D205, H234		1.00E-12	
SUB1106	S33	S123, >D, >H		4.00E-05	
SUB1154	S08A	D112, H176, N278, S496		8.00E-30	
SUB1159	M29	Y355	E253, E319, H348, H381, D383	9.00E-95	
SUB1172	C39	Q14, C20, H103, D119		5.00E-16	
SUB1194	M24A	H81	D98, D111, H199, E232, E264	5.00E-47	
SUB1229	M50X	G282E	N281H, G285H, I357D	1.00E-13	Signal peptide, 10 transmembrane helices
SUB1235	M03B	E391	H390, H394, E418	1.00E-115	

SUB1241	S11	S134, K137, S196		4.00E-09	
SUB1300	A24A	D110, D159		7.00E-36	
SUB1313	S16	S235, K280		6.00E-17	
SUB1325	M20A	D85, E150	H83, D116, E151, D174, >H	2.00E-16	
SUB1370	M14X	R410E	<H, <E, H449	2.00E-07	Signal peptide
SUB1408	C01B	Q62, C68, H362, N384		7.00E-96	
SUB1443	M38	S311D	H66, H68, K172, H201, H222	1.00E-60	
SUB1508	M15B	E215	H164, D171, H218	2.00E-24	Signal peptide, 1 transmembrane helix
SUB1550	M24B	H227, H315, H326	D244, D255, H319, E347, E361	5.00E-60	
SUB1564	S26A	S35, K75		4.00E-25	Signal peptidase type I
SUB1570	S12	S55, K58, Y137		3.00E-30	
SUB1574	S15	S349, D469, H499		0.00E+00	
SUB1616	M22		H114, H118	1.00E-101	
SUB1618	M22		S95H, L99H	2.00E-45	
SUB1651	S09C	S180, D270, H299		5.00E-07	
SUB1682	S16	A365S, R408		2.00E-65	
SUB1693	S54	N76, S124, H175		8.00E-49	

SUB1698	M20D	D71, E127	D69, D97, E128, H348	4.00E-64	
SUB1738	C69	C26		4.00E-66	
SUB1756	M13	E480, D543	H479, H483, E539	2.00E-64	
SUB1857	M16B	E71, E137	H68, H72, E144	1.00E-22	
SUB1858	M16B	E131	Y138E	2.00E-19	
SUB1864	S54	L63N, G109S, D167H		1.00E-05	
SUB1868	S01C	H127, D157, S239		4.00E-49	

Table 10 Predicted peptidases of *S. uberis* strain 0140J

Appendix 3

Purification	Step	Result - active fractions
Purification 1 1.5 litre media *lgt* mutant was grown to the start of stationary phase (OD $_{550nm}$ 0.8). The capsule was removed using hyaluronidase and a cell wall preparation was produced using mutanolysin, the proteins were dialyzed into 20 mM Phosphate pH 6.25 MW cut off 12 kDa	Size Exclusion Superdex 200 column Buffer pH 6.25 20 mM phosphate, 150 mM NaCl 5 ml fractions	Active fractions 12-15. Active fractions pooled and dialyzed into pH 6.25 20 mM Phosphate buffer overnight, molecular weight cut off 12 kDa, pH then increased to 7.5.
	Cation Exchange SP sepharose Buffer A pH 7.5 20 mM Phosphate, buffer B pH 7.5 20 mM Phosphate, 1M NaCl Proteins eluted over 0-50% gradient B 2 ml fractions	Activity in flow through
	Anion Exchange Q Sepharose Buffer A pH 7.5 20 mM Phosphate, buffer B pH 7.5 20 mM Phosphate, 1M NaCl Proteins eluted over 0-50% gradient B 2 ml fractions	Active fractions 11-15
	MiniQ Anion Exchange Buffer A pH 7.5 20 mM Phosphate, buffer B pH 7.5 20 mM Phosphate, 1M NaCl Proteins diluted 1:3 before being applied to	Active fractions 43-44 approximately 10 bands on Silver Stained SDS gel.

	column and eluted over 0-50% gradient B 1 ml fractions	
Purification 2 – As purification 1, except 6 litres media	Size Exclusion Superdex 200 pH 6.25 20 mM phosphate, 150 mM NaCl 5 ml fractions	Preparation split into 3 and each portion run through size exclusion column Active fractions 8-16
	Anion Exchange Fraction 9 Vydac Column Buffer A pH 7.5 20 mM Phosphate Buffer B pH 7.5 20 mM Phosphate, 1M NaCl 0-50% gradient B 1 ml fractions	Active fractions 23-26
	Anion Exchange Fraction 10 Vydac Column Buffer A pH 7.5 20 mM Phosphate Buffer B pH 7.5 20 mM Phosphate, 1M NaCl 0-50% gradient B 1 ml fractions	Active fractions 24-26 and FT
	Anion Exchange Fraction 11 Vydac Column Buffer A pH 7.5 20 mM Phosphate Buffer B pH 7.5 20 mM Phosphate, 1M	Active fractions 24-26 and FT

	NaCl 0-50% gradient B 1 ml fractions	
	Anion Exchange Fraction 12 Vydac Column Buffer A pH 7.5 20 mM Phosphate Buffer B pH 7.5 20 mM Phosphate, 1M NaCl 0-50% gradient B 1 ml fractions	Active fractions 24-26 and FT
	Anion Exchange Fraction 13 MiniQ column Buffer A pH 7.5 20 mM Phosphate Buffer B pH 7.5 20 mM Phosphate, 1M NaCl 0-50% gradient B 1 ml fractions	Activity in all fractions, very little activity in the FT
	Anion Exchange Fraction 14 MiniQ column Buffer A pH 7.5 20 mM Phosphate Buffer B pH 7.5 20 mM Phosphate, 1M NaCl 0-50% gradient B 1 ml fractions	FT

	Anion Exchange Fraction 15 MiniQ column FT Buffer A pH 7.5 20 mM Phosphate Buffer B pH 7.5 20 mM Phosphate, 1M NaCl 0-50% gradient B 1 ml fractions
	Anion Exchange Fraction 16 MiniQ column FT Buffer A pH 7.5 20 mM Phosphate Buffer B pH 7.5 20 mM Phosphate, 1M NaCl 0-50% gradient B 1 ml fractions

Purification 3 – 2.4 litres media *lgt* grown to the start of stationary phase (O.D. $_{550\ nm}$ 0.8) - the capsule was removed using hyaluronidase and the cells pelleted, before removing the cell wall using mutanolysin and freeze thawing the protoplasts three times, the protein was harvested by pelleting the cell debris. The proteins were dialyzed in to pH 6.25, 20 mM phosphate buffer with a molecular weight cut off 12 kDa	Size exclusion Hiload™ 26/60 Superdex 200 Prep grade pH 6.25 20 mM phosphate, 150 mM NaCl 5 ml fractions	Active fractions 21-26, 27-35 and 40-50
	Anion exchange MonoQ purification a fractions 21-26 dialyzed in to buffer A Buffer A pH 7.5 20 mM Phosphate, Buffer B pH 7.5 20 mM Phosphate, 1M NaCl 0-50% gradient B 2 ml fractions	Active fractions 20-23
	Anion exchange MonoQ b Fractions 27-35, dialyzed in to buffer A Buffer A pH 7.5 20 mM Phosphate, Buffer B pH 7.5 20 mM Phosphate, 1M NaCl 0-50% gradient B 2 ml fractions	Active fraction 21
	Anion exchange MonoQ c Fractions 40-50 dialyzed in to buffer A Buffer A pH 7.5 20 mM Phosphate, Buffer	Active fraction 21 Proteins had to be applied to anion exchange column twice as didn't stick

	B pH 7.5 20 mM Phosphate, 1M NaCl 0-50% gradient B 2 ml fractions	first time
	Anion Exchange MiniQ 1 active fractions 20-23 from MonoQ purification a Buffer A pH 7.5 20 mM Phosphate Buffer B pH 7.5 20 mM Phosphate, 1M NaCl 0-50% gradient B 1ml fractions	Active fractions 1-6
	Anion Exchange MiniQ 2 active fraction 21 from MonoQ purification b Buffer A pH 7.5 20 mM Phosphate Buffer B pH 7.5 20 mM Phosphate, 1M NaCl 0-50% gradient B 1ml fractions	Active fraction 11-16
Purification 4 – 6 litres media *lgt* culture grown to start of stationary phase (O.D. $_{550nm}$ 0.8) - capsule removed using hyaluronidase	Ammonium Sulphate Precipitation Salt cut at 40% for 2 hours, 4°C, precipitate pelleted by centrifugation 13,000 x g, 10 minutes, 4°C and then ammonium sulphate	

and discarded. The cell wall was removed using mutanolysin and the resulting proteins dialyzed in to pH 7.5 20 mM Phosphate 12kDa cut off	concentration adjusted to 70%, proteins precipitated as for 40% and then resuspended in pH 7.5, 20 mM Phosphate buffer.	
	Size exclusion Hiload™ 26/60 Superdex 200 Prep grade pH 7.5, 20 mM Phosphate, 150 mM NaCl 5 ml fractions	Active fractions 28-34
Purification 5 – Protoplasts from Purification 4 freeze thawed three times before harvesting all the protein by pelleting cell debris and dialysis in to pH 7.5 20 mM Phosphate 12 kDa cut off	Ammonium Sulphate Precipitation Salt cut at 40% and then at 70% as described for purification 4	Activity in precipitate at 70%
	Size exclusion Hiload™ 26/60 Superdex 200 Prep grade pH 7.5, 20 mM Phosphate, 150 mM NaCl 5 ml fractions	Active fractions 22-32
	Anion Exchange MonoQ fractions 23-25 from size exclusion Buffer A pH 7.5 20 mM Phosphate Buffer B pH 7.5 20 mM Phosphate, 1M	Active fractions 37-40

	NaCl Proteins dialyzed into buffer A 12 kDa cut off 0-50% gradient B 2 ml fractions	
	Anion Exchange MiniQ fractions 37-39 from MonoQ anion exchange Buffer A pH 7.5 20 mM Phosphate Buffer B pH 7.5 20 mM Phosphate, 1M NaCl Dialyzed into buffer A 12 kDa cut off 0-50% gradient B 1 ml fractions	Active fractions 33-35

Purification 6 – 3.6 litres culture Cell Wall preparation of *lgt* mutant grown to the start of stationary phase pH 7.5 20 mM phosphate buffer dialyzed 12 kDa cut off	Ammonium Sulphate Cut 40% then 70% cut as described for purification 4.	
	Size exclusion Hiload™ 26/60 Superdex 200 Prep grade pH 7.5, 20 mM Phosphate, 150 mM NaCl 5 ml fractions	Active fractions 22-34
	Anion Exchange MonoQ fractions 22-34 from size exclusion Buffer A pH 7.5 20 mM Phosphate Buffer B pH 7.5 20 mM Phosphate, 1M NaCl Proteins dialyzed into buffer A 12 kDa cut off 0-50% gradient B 2 ml fractions	Activity in flow through
	Anion Exchange Mono Q flow through as described for fractions 22-34	Activity in flow through

Purification 6 – 3.6 litres culture Cell Wall preparation of *lgt* mutant grown to the start of stationary phase pH 7.5 20 mM phosphate buffer dialyzed 12 kDa cut off	Ammonium Sulphate Cut 40% then 70% cut as described for purification 4.	
	Size exclusion Hiload™ 26/60 Superdex 200 Prep grade pH 7.5, 20 mM Phosphate, 150 mM NaCl 5 ml fractions	Active fractions 22-34
	Anion Exchange MonoQ fractions 22-34 from size exclusion Buffer A pH 7.5 20 mM Phosphate Buffer B pH 7.5 20 mM Phosphate, 1M NaCl Proteins dialyzed into buffer A 12 kDa cut off 0-50% gradient B 2 ml fractions	Activity in flow through
	Anion Exchange Mono Q flow through as described for fractions 22-34	Activity in flow through

Purification 7 – Whole Cell Lysate from prep 5 dialyzed in to pH 7.5 20 mM phosphate buffer 12 kDa cut off	Ammonium Sulphate Cut 40% then 70% as described in purification 4	
	Size exclusion Hiload™ 26/60 Superdex 200 Prep grade pH 7.5, 20 mM Phosphate, 150 mM NaCl 5 ml fractions	Active fractions 22-32
	Anion Exchange MonoQ fractions 22-32 from size exclusion Buffer A pH 7.5 20 mM Phosphate Buffer B pH 7.5 20 mM Phosphate, 1M NaCl Proteins dialyzed into buffer A 12 kDa cut off 0-50% gradient B 2 ml fractions	Activity in flow through
	Mono Q anion Exchange of flow through as described above for fractions 22-23	Activity in flow through

Purification 8 Overnight *lgt* mutant culture 2 litres THB media, cell wall protein prepared as previously described and dialyzed in to pH 7.5 phosphate cut off 12 kDa	Size Exclusion Superdex 200 pH 7.5, 20 mM Phosphate, 150 mM NaCl 5 ml fractions	Active fractions 13-19 dialyzed into pH 7.5
	Anion Exchange Q sepharose fractions 13-19 from size exclusion Buffer A pH 7.5 20 mM Phosphate Buffer B pH 7.5 20 mM Phosphate, 1M NaCl Proteins dialyzed into buffer A 12 kDa cut off 0-50% gradient B 2 ml fractions	Activity in flow through and Wash
Purification 9 – Overnight *lgt* mutant culture 2 litres THB media, cell wall prepared as previously described and dialyzed into pH 6.25 20 mM phosphate buffer, 350 mM NaCl	Size Exclusion Superdex 200 pH 7.5, 20 mM Phosphate, 350 mM NaCl 5 ml fractions	Active fractions 11-21, pooled and split into to 2, one half dialyzed into pH 8.5 20 mM Phosphate and the other dialyzed into pH 6.5 20 mM Phosphate
	Anion exchange Q sepharose 1 pH 8.5 Buffer A pH 7.5 20 mM Phosphate Buffer B pH 7.5 20 mM Phosphate, 1M NaCl	Active fractions 9-12 plus flow through and wash contained very low levels of activity

	Proteins dialyzed into buffer A 12 kDa cut off 0-50% gradient B 2 ml fractions	
	Anion exchange Q Sepharose 2 dialyzed into 6.5 and adjusted to 8.5 as Anion exchange 1 appeared to work (Buffer A 0 M NaCl, Buffer B 1 M NaCl), run over 50 % gradient)	Activity in flow through and wash
	Cation exchange all active fractions pooled, adjusted to pH 6 and applied to SP sepharose (Buffer A 0 M NaCl, Buffer B 1 M NaCl), run over 50 % gradient)	Activity in flow through and wash
	Mini Q Anion Exchange Dialyzed into pH 7 20 mM Phosphate (Buffer A 0 M NaCl, Buffer B 1 M NaCl), run over 50 % gradient)	Activity in flow through and wash
	Mini Q Anion Exchange adjusted to pH 7.5 with NaOH (Buffer A 0 M NaCl, Buffer B 1 M NaCl), run over 50 % gradient)	Activity in flow through and wash, sample concentrated and analyzed by SDS PAGE, no proteins detectable

Purification 10 Cell Wall Prep 2 litres of overnight culture lgt:ISS1, dialyzed into pH 7.0 20 mM Phosphate buffer 12 kDa Cut off	Mini Q Anion exchange pH 7.0 20 mM Phosphate ((Buffer A 0 M NaCl, Buffer B 1 M NaCl), run over 50 % gradient over 60 ml)	Active fractions 15-23, Flow through and Wash (Proteins identified by Mass Spectrometry after reduction of disulphide bonds, alkylation and digestion with trypsin.
	Size exclusion superdex 200 Flow Through and Wash pH 7.0 20 mM Phosphate, 150 mM NaCl	Active fractions 13-16 Fractions slightly cleaned up, fraction 14 concentrated for Mass spectrometry analysis.
	Size exclusion superdex 200 Fractions 15-23 pH 7 20 mM Phosphate, 150 mM NaCl	Active fractions 13-15 Activity very weak nothing detectable of SDS PAGE
Purification 11 Cell Wall Prep 2 litres of overnight culture lgt:ISS1, dialyzed into pH 7.0 20 mM Phosphate buffer 12 kDa Cut off	Size exclusion superdex 200 pH 7.0 20 mM Phosphate, 150 mM NaCl	Active fractions 11-24

Purification 12 Cell Wall Preparation 2.4 litres of overnight 0140J culture, dialyzed into pH 7.0 20 mM Phosphate buffer 12 kDa Cut off	Mini Q Anion exchange pH 7.0 20 mM Phosphate Buffer A 0 M NaCl, Buffer B 1 M NaCl, Run over 50 % gradient over 60 ml	Active fractions 7-17, flow through and wash
	Size exclusion superdex 200 Flow Through pH 7.0 20 mM Phosphate, 150 mM NaCl	Flow through separated in size exclusion on 4 separate runs. Active fractions 14-18, Each individually concentrated using Ultracel membrane 10 KMWCO.
	Size exclusion superdex 200 Fraction 11 pH 7.0 20 mM Phosphate, 150 mM NaCl	Active fractions 13-18 active
	Size exclusion superdex 200 Wash pH 7.0 20 mM Phosphate, 150 mM NaCl	
	Size exclusion superdex 200 Fraction 9 and 10 pH 7.0 20 mM Phosphate, 150 mM NaCl	

Table 11 Purification of ShvU

References

Alami, M., Luke, I., Deitermann, S., Eisner, G., Koch, H.G., Brunner, J., and Muller, M. (2003) Differential interactions between a twin-arginine signal peptide and its translocase in Escherichia coli. *Mol Cell* **12**: 937-946.

Almeida, R.A., Calvinho, L.F., and Oliver, S.P. (2000) Influence of protein kinase inhibitors on Streptococcus uberis internalization into bovine mammary epithelial cells. *Microb Pathog* **28**: 9-16.

Almeida, R.A., and Oliver, S.P. (2001) Role of collagen in adherence of Streptococcus uberis to bovine mammary epithelial cells. *J Vet Med B Infect Dis Vet Public Health* **48**: 759-763.

Almeida, R.A., Luther, D.A., Park, H.M., and Oliver, S.P. (2006) Identification, isolation, and partial characterization of a novel Streptococcus uberis adhesion molecule (SUAM). *Vet Microbiol* **115**: 183-191.

Almeida, R.A., and Oliver, S.P. (2006) Trafficking of Streptococcus uberis in bovine mammary epithelial cells. *Microb Pathog* **41**: 80-89.

Altschul, S.F., Gish, W., Miller, W., Meyers, E.W., and Lipman, D.J. (1990) Basic Local Alignment Search Tool. *Journal of Molecular Biology* **215**: 403-410.

An, F.Y., Sulavik, M.C., and Clewell, D.B. (1999) Identification and characterization of a determinant (eep) on the Enterococcus faecalis chromosome that is involved in production of the peptide sex pheromone cAD1. *J Bacteriol* **181**: 5915-5921.

An, F.Y., and Clewell, D.B. (2002) Identification of the cAD1 sex pheromone precursor in Enterococcus faecalis. *J Bacteriol* **184**: 1880-1887.

Angelini, S., Deitermann, S., and Koch, H.G. (2005) FtsY, the bacterial signal-recognition particle receptor, interacts functionally and physically with the SecYEG translocon. *EMBO Rep* **6**: 476-481.

Antelmann, H., Tjalsma, H., Voigt, B., Ohlmeier, S., Bron, S., van Dijl, J.M., and Hecker, M. (2001) A proteomic view on genome-based signal peptide predictions. *Genome Research* **11**: 1484-1502.

Bae, T., and Schneewind, O. (2003) The YSIRK-G/S motif of staphylococcal protein A and its role in efficiency of signal peptide processing. *Journal of Bacteriology* **185**: 2910-2919.

Balwierczak, J.L., Kukkola, P.J., Savage, P., and Jeng, A.Y. (1995) Effects of metalloprotease inhibitors on smooth muscle endothelin-converting enzyme activity. *Biochem Pharmacol* **49**: 291-296.

Barrett, A.J., Rawlings, N.D., and Woessner, J.F. (2004) Handbook of Proteolytic Enzymes Second edition **1**.

Baumgartner, M., Karst, U., Gerstel, B., Loessner, M., Wehland, J., and Jansch, L. (2007) Inactivation of Lgt allows systematic characterization of lipoproteins from Listeria monocytogenes. *J Bacteriol* **189**: 313-324.

Bendtsen, J.D., Nielsen, H., von Heijne, G., and Brunak, S. (2004) Improved prediction of signal peptides: SignalP 3.0. *J Mol Biol* **340**: 783-795.

Bentley, R.W., Leigh, J.A., and Collins, M.D. (1991) Intrageneric structure of Streptococcus based on comparative analysis of small-subunit rRNA sequences. *Int J Syst Bacteriol* **41**: 487-494.

Berg, T., Firth, N., and Skurray, R.A. (1997) Enterococcal pheromone-like activity derived from a lipoprotein signal peptide encoded by a Staphylococcus aureus plasmid. *Adv Exp Med Biol* **418**: 1041-1044.

Berry, A.M., and Paton, J.C. (1996) Sequence heterogeneity of PsaA, a 37-kilodalton putative adhesin essential for virulence of Streptococcus pneumoniae. *Infect Immun* **64**: 5255-5262.

Beynon, R., and Bond, J. (1989) *Proteolytic Enzymes - A pratical approach*: IRL Press at Oxford University Press, Oxford.

Blaudeck, N., Sprenger, G.A., Freudl, R., and Wiegert, T. (2001) Specificity of signal peptide recognition in tat-dependent bacterial protein translocation. *Journal of Bacteriology* **183**: 604-610.

Boekhorst, J., de Been, M.W.H.J., Kleerebezem, M., and Siezen, R.J. (2005) Genome-Wide Detection and Analysis of Cell Wall-Bound Proteins with LPxTG-Like Sorting Motifs. *J. Bacteriol.* **187**: 4928-4934.

Bogsch, E.G., Sargent, F., Stanley, N.R., Berks, B.C., Robinson, C., and Palmer, T. (1998) An essential component of a novel bacterial protein export system with homologues in plastids and mitochondria. *J Biol Chem* **273**: 18003-18006.

Bolhuis, A., Matzen, A., Hyyrylainen, H.L., Kontinen, V.P., Meima, R., Chapuis, J., Venema, G., Bron, S., Freudl, R., and van Dijl, J.M. (1999) Signal peptide peptidase- and ClpP-like proteins of Bacillus subtilis required for efficient translocation and processing of secretory proteins. *J Biol Chem* **274**: 24585-24592.

Bolhuis, A., Bogsch, E.G., and Robinson, C. (2000) Subunit interactions in the twin-arginine translocase complex of Escherichia coli. *FEBS Lett* **472**: 88-92.

Bolhuis, A., Mathers, J.E., Thomas, J.D., Barrett, C.M., and Robinson, C. (2001) TatB and TatC form a functional and structural unit of the twin-arginine translocase from Escherichia coli. *J Biol Chem* **276**: 20213-20219.

Bradley, A.J., Leach, K.A., Breen, J.E., Green, L.E., and Green, M.J. (2007) Survey of the incidence and aetiology of mastitis on dairy farms in England and Wales. *Vet Rec* **160**: 253-257.

Bramley, A.J. (1982) Sources of Streptococcus uberis in the dairy herd. I. Isolation from bovine faeces and from straw bedding of cattle. *J Dairy Res* **49**: 369-373.

Bramley, A.J., and Dodd, F.H. (1984) Reviews of the progress of dairy science: mastitis control--progress and prospects. *J Dairy Res* **51**: 481-512.

Braun, L., Dramsi, S., Dehoux, P., Bierne, H., Lindahl, G., and Cossart, P. (1997) InlB: an invasion protein of Listeria monocytogenes with a novel type of surface association. *Mol Microbiol* **25**: 285-294.

Braun, V., and Hantke, K. (1975) Characterization of the free form of murein-lipoprotein from the outer membrane of Escherichia coli B/r. *FEBS Lett* **60**: 26-28.

Bridge, P.D., and Sneath, P.H. (1983) Numerical taxonomy of Streptococcus. *J Gen Microbiol* **129**: 565-597.

Briles, D.E., Ades, E., Paton, J.C., Sampson, J.S., Carlone, G.M., Huebner, R.C., Virolainen, A., Swiatlo, E., and Hollingshead, S.K. (2000) Intranasal immunization of mice with a mixture of the pneumococcal proteins PsaA and PspA is highly protective against nasopharyngeal carriage of Streptococcus pneumoniae. *Infect Immun* **68**: 796-800.

Brown, J.S., Gilliland, S.M., and Holden, D.W. (2001) A Streptococcus pneumoniae pathogenicity island encoding an ABC transporter involved in iron uptake and virulence. *Mol Microbiol* **40**: 572-585.

Bubeck Wardenburg, J., Williams, W.A., and Missiakas, D. (2006) Host defenses against Staphylococcus aureus infection require recognition of bacterial lipoproteins. *Proc Natl Acad Sci U S A* **103**: 13831-13836.

Buchanan, G., Leeuw, E., Stanley, N.R., Wexler, M., Berks, B.C., Sargent, F., and Palmer, T. (2002) Functional complexity of the twin-arginine translocase TatC component revealed by site-directed mutagenesis. *Molecular Microbiology* **43**: 1457-1470.

Buist, G., Ridder, A.N., Kok, J., and Kuipers, O.P. (2006) Different subcellular locations of secretome components of Gram-positive bacteria. *Microbiology* **152**: 2867-2874.

Calamita, H.G., Ehringer, W.D., Koch, A.L., and Doyle, R.J. (2001) Evidence that the cell wall of Bacillus subtilis is protonated during respiration. *Proceedings of the National Academy of Sciences* **98**: 15260-15263.

Campo, N., Tjalsma, H., Buist, G., Stepniak, D., Meijer, M., Veenhuis, M., Westermann, M., Muller, J.P., Bron, S., Kok, J., Kuipers, O.P., and Jongbloed, J.D. (2004) Subcellular sites for bacterial protein export. *Mol Microbiol* **53**: 1583-1599.

Carapetis, J.R., Steer, A.C., Mulholland, E.K., and Weber, M. (2005) The global burden of group A streptococcal diseases. *Lancet Infect Dis* **5**: 685-694.

Carlsson, F., Stalhammar-Carlemalm, M., Flardh, K., Sandin, C., Carlemalm, E., and Lindahl, G. (2006) Signal sequence directs localized secretion of bacterial surface proteins. *Nature* **442**: 943-946.

Cauchard, J., Sevin, C., Ballet, J.J., and Taouji, S. (2004) Foal IgG and opsonizing anti-Rhodococcus equi antibodies after immunization of pregnant mares with a protective VapA candidate vaccine. *Vet Microbiol* **104**: 73-81.

Chandler, J.R., and Dunny, G.M. (2004) Enterococcal peptide sex pheromones: synthesis and control of biological activity. *Peptides* **25**: 1377-1388.

Chattopadhyay, P.K., and Wu, H.C. (1977) Biosynthesis of the covalently linked diglyceride in murein lipoprotein of Escherichia coli. *Proc Natl Acad Sci U S A* **74**: 5318-5322.

Chen, Y.-S., and Steele, J.L. (1998) Genetic Characterization and Physiological Role of Endopeptidase O from Lactobacillus helveticus CNRZ32. *Appl. Environ. Microbiol.* **64**: 3411-3415.

Chen, Y.-S., Christensen, J.E., Broadbent, J.R., and Steele, J.L. (2003) Identification and Characterization of Lactobacillus helveticus PepO2, an Endopeptidase with Post-Proline Specificity. *Appl. Environ. Microbiol.* **69**: 1276-1282.

Christensson, C., Bratt, H., Collins, L.J., Coolbear, T., Holland, R., Lubbers, M.W., O'Toole, P.W., and Reid, J.R. (2002) Cloning and Expression of an Oligopeptidase, PepO, with Novel Specificity from Lactobacillus rhamnosus HN001 (DR20). *Appl. Environ. Microbiol.* **68**: 254-262.

Christie, R., Atkins, N., and Munch-Petersoen, E. (1944) A Note on a lytic phenomenon shown by group B streptococci. *Australian Journal of Experimental Biology and Medical Science* **22**: 197-200.

Clewell, D.B., An, F.Y., Flannagan, S.E., Antiporta, M., and Dunny, G.M. (2000) Enterococcal sex pheromone precursors are part of signal sequences for surface lipoproteins. *Mol Microbiol* **35**: 246-247.

Cline, K., and Mori, H. (2001) Thylakoid DeltapH-dependent precursor proteins bind to a cpTatC-Hcf106 complex before Tha4-dependent transport. *J Cell Biol* **154**: 719-729.

Coffey, T.J., Pullinger, G.D., Urwin, R., Jolley, K.A., Wilson, S.M., Maiden, M.C., and Leigh, J.A. (2006) First insights into the evolution of Streptococcus uberis: a multilocus sequence typing scheme that enables investigation of its population biology. *Appl Environ Microbiol* **72**: 1420-1428.

Coligan, J.E., B. M. Dunn, H. L. Ploegh, D. W. Speicher, (2001) *Current protocols in protein science*: John Wiley & Sons, Inc., New York, N.Y.

Connolly, K.M., Smith, B.T., Pilpa, R., Ilangovan, U., Jung, M.E., and Clubb, R.T. (2003) Sortase from Staphylococcus aureus Does Not Contain a Thiolate-Imidazolium Ion Pair in Its Active Site. *J. Biol. Chem.* **278**: 34061-34065.

Cousins, C.L., Higgs, T.M., Jackson, E.R., Neave, F.K., and Dodd, F.H. (1980) Susceptibility of the bovine udder to bacterial infection in the dry period. *J Dairy Res* **47**: 11-18.

Dalbey, R.E., Lively, M.O., Bron, S., and van Dijl, J.M. (1997) The chemistry and enzymology of the type I signal peptidases. *Protein Sci* **6**: 1129-1138.

Dartois, V., Djavakhishvili, T., and Hoch, J.A. (1997) KapB is a lipoprotein required for KinB signal transduction and activation of the phosphorelay to sporulation in Bacillus subtilis. *Mol Microbiol* **26**: 1097-1108.

De Greeff, A., Hamilton, A., Sutcliffe, I.C., Buys, H., Van Alphen, L., and Smith, H.E. (2003) Lipoprotein signal peptidase of Streptococcus suis serotype 2. *Microbiology* **149**: 1399-1407.

De Leeuw, E., Porcelli, I., Sargent, F., Palmer, T., and Berks, B.C. (2001) Membrane interactions and self-association of the TatA and TatB components of the twin-arginine translocation pathway. *FEBS Lett* **506**: 143-148.

de Leeuw, E., Granjon, T., Porcelli, I., Alami, M., Carr, S.B., Muller, M., Sargent, F., Palmer, T., and Berks, B.C. (2002) Oligomeric properties and signal peptide binding by Escherichia coli Tat protein transport complexes. *J Mol Biol* **322**: 1135-1146.

Desvaux, M., Dumas, E., Chafsey, I., and Hebraud, M. (2006) Protein cell surface display in Gram-positive bacteria: from single protein to macromolecular protein structure. *FEMS Microbiol Lett* **256**: 1-15.

Dev, I.K., and Ray, P.H. (1984) Rapid assay and purification of a unique signal peptidase that processes the prolipoprotein from Escherichia coli B. *J Biol Chem* **259**: 11114-11120.

Dev, I.K., Harvey, R.J., and Ray, P.H. (1985) Inhibition of prolipoprotein signal peptidase by globomycin. *J Biol Chem* **260**: 5891-5894.

Dhar, G., Faull, K.F., and Schneewind, O. (2000) Anchor structure of cell wall surface proteins in Listeria monocytogenes. *Biochemistry* **39**: 3725-3733.

Diernhofer, K. (1932) Asculinbullon als Hilfsmittel fur die Differenzierung von Euter und Milchstreptokokken bei Massenunterschungen. *Milchw. Forsch* **13**: 368-374.

Dilks, K., Rose, R.W., Hartmann, E., and Pohlschroder, M. (2003) Prokaryotic utilization of the twin-arginine translocation pathway: a genomic survey. *Journal of Bacteriology* **185**: 1478-1483.

Douglas, V.L., Fenwick, S.G., Pfeiffer, D.U., Williamson, N.B., and Holmes, C.W. (2000) Genomic typing of Streptococcus uberis isolates from cases of mastitis, in New Zealand dairy cows, using pulsed-field gel electrophoresis. *Vet Microbiol* **75**: 27-41.

Driessen, A.J., Fekkes, P., and van der Wolk, J.P. (1998) The Sec system. *Current Opinon in Microbiology* **1**: 216-222.

Drouault, S., Anba, J., Bonneau, S., Bolotin, A., Ehrlich, S.D., and Renault, P. (2002) The peptidyl-prolyl isomerase motif is lacking in PmpA, the PrsA-like protein involved in the secretion machinery of Lactococcus lactis. *Applied Environmental Microbiology* **68**: 3932-3942.

Dumoulin, A., Grauschopf, U., Bischoff, M., Thony-Meyer, L., and Berger-Bachi, B. (2005) Staphylococcus aureus DsbA is a membrane-bound lipoprotein with thiol-disulfide oxidoreductase activity. *Arch Microbiol* **184**: 117-128.

Dunn, B.M., Scarborough, P.E., Davenport, R., and Swietnicki, W. (1994) Analysis of proteinase specificity by studies of peptide substrates. The use of UV and fluorescence spectroscopy to quantitate rates of enzymatic cleavage. *Methods Mol Biol* **36**: 225-243.

Economou, A. (2002) Bacterial secretome: the assembly manual and operating instructions (Review). *Molecular Membrane Biology* **19**: 159-169.

Faull, W.B., Walton, J.R., Bramley, A.J., and Hughes, J.W. (1983) Mastitis in a large, zero-grazed dairy herd. *Vet Rec* **113**: 415-420.

Field, T.R., Ward, P.N., Pedersen, L.H., and Leigh, J.A. (2003) The hyaluronic acid capsule of Streptococcus uberis is not required for the development of infection and clinical mastitis. *Infection and Immunity* **71**: 132-139.

Finch, J.M., Hill, A.W., Field, T.R., and Leigh, J.A. (1994) Local vaccination with killed Streptococcus uberis protects the bovine mammary gland against experimental intramammary challenge with the homologous strain. *Infect Immun* **62**: 3599-3603.

Finch, J.M., Winter, A., Walton, A.W., and Leigh, J.A. (1997) Further studies on the efficacy of a live vaccine against mastitis caused by Streptococcus uberis. *Vaccine* **15**: 1138-1143.

Finn, R.D., Mistry, J., Schuster-Bockler, B., Griffiths-Jones, S., Hollich, V., Lassmann, T., Moxon, S., Marshall, M., Khanna, A., Durbin, R., Eddy, S.R., Sonnhammer, E.L., and Bateman, A. (2006) Pfam: clans, web tools and services. *Nucleic Acids Res* **34**: D247-251.

Firth, N., Fink, P.D., Johnson, L., and Skurray, R.A. (1994) A lipoprotein signal peptide encoded by the staphylococcal conjugative plasmid pSK41 exhibits an activity resembling that of Enterococcus faecalis pheromone cAD1. *J Bacteriol* **176**: 5871-5873.

Flannagan, S.E., and Clewell, D.B. (2002) Identification and characterization of genes encoding sex pheromone cAM373 activity in Enterococcus faecalis and Staphylococcus aureus. *Mol Microbiol* **44**: 803-817.

Frishman, D., Albermann, K., Hani, J., Heumann, K., Metanomski, A., Zollner, A., and Mewes, H.W. (2001) Functional and structural genomics using PEDANT. *Bioinformatics* **17**: 44-57.

Frishman, D., Mokrejs, M., Kosykh, D., Kastenmuller, G., Kolesov, G., Zubrzycki, I., Gruber, C., Geier, B., Kaps, A., Albermann, K., Volz, A., Wagner, C., Fellenberg, M., Heumann, K., and Mewes, H.W. (2003) The PEDANT genome database. *Nucleic Acids Res* **31**: 207-211.

Fujimoto, S., and Clewell, D.B. (1998) Regulation of the pAD1 sex pheromone response of Enterococcus faecalis by direct interaction between the cAD1 peptide mating signal and the negatively regulating, DNA-binding TraA protein. *Proc Natl Acad Sci U S A* **95**: 6430-6435.

Fukuda, A., Matsuyama, S., Hara, T., Nakayama, J., Nagasawa, H., and Tokuda, H. (2002) Aminoacylation of the N-terminal cysteine is essential for Lol-dependent release of lipoproteins from membranes but does not depend on lipoprotein sorting signals. *J Biol Chem* **277**: 43512-43518.

Gan, K., Gupta, S.D., Sankaran, K., Schmid, M.B., and Wu, H.C. (1993) Isolation and characterization of a temperature-sensitive mutant of Salmonella typhimurium defective in prolipoprotein modification. *Journal of Biological Chemistry* **268**: 16544-16550.

Garcia, J.L., Diaz, E., Romero, A., and Garcia, P. (1994) Carboxy-terminal deletion analysis of the major pneumococcal autolysin. *Journal of Bacteriology* **176**: 4066-4072.

Gat, O., Mendelson, I., Chitlaru, T., Ariel, N., Altboum, Z., Levy, H., Weiss, S., Grosfeld, H., Cohen, S., and Shafferman, A. (2005) The solute-binding component of a putative Mn(II)

ABC transporter (MntA) is a novel Bacillus anthracis virulence determinant. *Mol Microbiol* **58**: 533-551.

Gilbert, F.B., Fromageau, A., Lamoureux, J., and Poutrel, B. (2006) Evaluation of tandem repeats for MLVA typing of Streptococcus uberis isolated from bovine mastitis. *BMC Vet Res* **2**: 33.

Gosink, K.K., Mann, E.R., Guglielmo, C., Tuomanen, E.I., and Masure, H.R. (2000) Role of novel choline binding proteins in virulence of Streptococcus pneumoniae. *Infection and Immunity* **68**: 5690-5695.

Grahn, S., Ullmann, D., and Jakubke, H. (1998) Design and synthesis of fluorogenic trypsin peptide substrates based on resonance energy transfer. *Anal Biochem* **265**: 225-231.

Green, G.D., and Shaw, E. (1981) Peptidyl diazomethyl ketones are specific inactivators of thiol proteinases. *J Biol Chem* **256**: 1923-1928.

Hamilton, A., Robinson, C., Sutcliffe, I.C., Slater, J., Maskell, D.J., Davis-Poynter, N., Smith, K., Waller, A., and Harrington, D.J. (2006) Mutation of the maturase lipoprotein attenuates the virulence of Streptococcus equi to a greater extent than does loss of general lipoprotein lipidation. *Infect Immun* **74**: 6907-6919.

Hanks, T.S., Liu, M., McClure, M.J., and Lei, B. (2005) ABC transporter FtsABCD of Streptococcus pyogenes mediates uptake of ferric ferrichrome. *BMC Microbiol* **5**: 62.

Hantke, K., and Braun, V. (1973) Covalent binding of lipid to protein. Diglyceride and amide-linked fatty acid at the N-terminal end of the murein-lipoprotein of the Escherichia coli outer membrane. *Eur J Biochem* **34**: 284-296.

Hatzixanthis, K., Palmer, T., and Sargent, F. (2003) A subset of bacterial inner membrane proteins integrated by the twin-arginine translocase. *Mol Microbiol* **49**: 1377-1390.

Hermans, P.W., Adrian, P.V., Albert, C., Estevao, S., Hoogenboezem, T., Luijendijk, I.H., Kamphausen, T., and Hammerschmidt, S. (2006) The streptococcal lipoprotein rotamase A

(SlrA) is a functional peptidyl-prolyl isomerase involved in pneumococcal colonization. *J Biol Chem* **281**: 968-976.

Hill, A.W., and Leigh, J.A. (1989) DNA fingerprinting of Streptococcus uberis: a useful tool for epidemiology of bovine mastitis. *Epidemiol Infect* **103**: 165-171.

Hill, A.W., Finch, J.M., Field, T.R., and Leigh, J.A. (1994) Immune modification of the pathogenesis of Streptococcus uberis mastitis in the dairy cow. *FEMS Immunol Med Microbiol* **8**: 109-117.

Hillerton, J.E., Shearn, M.F., Teverson, R.M., Langridge, S., and Booth, J.M. (1993) Effect of pre-milking teat dipping on clinical mastitis on dairy farms in England. *J Dairy Res* **60**: 31-41.

Hogan, J.S., and Smith, K.L. (1997) Occurrence of clinical and subclinical environmental streptococcal mastitis. In *Proceedings of the Symposium on Udder Health Management for Environmental Streptococci*, pp. 59-75.

Huang, Y.T., Teng, L.J., Ho, S.W., and Hsueh, P.R. (2005) Streptococcus suis infection. *J Microbiol Immunol Infect* **38**: 306-313.

Hulo, N., Bairoch, A., Bulliard, V., Cerutti, L., De Castro, E., Langendijk-Genevaux, P.S., Pagni, M., and Sigrist, C.J. (2006) The PROSITE database. *Nucleic Acids Res* **34**: D227-230.

Hussain, M., Ichihara, S., and Mizushima, S. (1982) Mechanism of signal peptide cleavage in the biosynthesis of the major lipoprotein of the Escherichia coli outer membrane. *J Biol Chem* **257**: 5177-5182.

Inada, T., Court, D.L., Ito, K., and Nakamura, Y. (1989) Conditionally lethal amber mutations in the leader peptidase gene of Escherichia coli. *J. Bacteriol.* **171**: 585-587.

Innis, M.A., Tokunaga, M., Williams, M.E., Loranger, J.M., Chang, S.Y., Chang, S., and Wu, H.C. (1984) Nucleotide sequence of the Escherichia coli prolipoprotein signal peptidase (lsp) gene. *Proc Natl Acad Sci U S A* **81**: 3708-3712.

Inukai, M., Takeuchi, M., Shimizu, K., and Arai, M. (1978) Mechanism of action of globomycin. *J Antibiot (Tokyo)* **31**: 1203-1205.

Inukai, M., Ghrayeb, J., Nakamura, K., and Inouye, M. (1984) Apolipoprotein, an intermediate in the processing of the major lipoprotein of the Escherichia coli outer membrane. *J Biol Chem* **259**: 757-760.

Isaki, L., Kawakami, M., Beers, R., Hom, R., and Wu, H.C. (1990) Cloning and nucleotide sequence of the Enterobacter aerogenes signal peptidase II (lsp) gene. *J Bacteriol* **172**: 469-472.

Ishikawa, S., Hara, Y., Ohnishi, R., and Sekiguchi, J. (1998a) Regulation of a new cell wall hydrolase gene, cwlF, which affects cell separation in Bacillus subtilis. *J Bacteriol* **180**: 2549-2555.

Ishikawa, S., Yamane, K., and Sekiguchi, J. (1998b) Regulation and characterization of a newly deduced cell wall hydrolase gene (cwlJ) which affects germination of Bacillus subtilis spores. *J Bacteriol* **180**: 1375-1380.

Jack, R.L., Sargent, F., Berks, B.C., Sawers, G., and Palmer, T. (2001) Constitutive expression of Escherichia coli tat genes indicates an important role for the twin-arginine translocase during aerobic and anaerobic growth. *J Bacteriol* **183**: 1801-1804.

Jakubovics, N.S., Smith, A.W., and Jenkinson, H.F. (2002) Oxidative stress tolerance is manganese (Mn(2+)) regulated in Streptococcus gordonii. *Microbiology* **148**: 3255-3263.

Janulczyk, R., and Rasmussen, M. (2001) Improved pattern for genome-based screening identifies novel cell wall-attached proteins in gram-positive bacteria. *Infection and Immunity* **69**: 4019-4026.

Janulczyk, R., Ricci, S., and Bjorck, L. (2003) MtsABC is important for manganese and iron transport, oxidative stress resistance, and virulence of Streptococcus pyogenes. *Infect Immun* **71**: 2656-2664.

Janzen, J.J., J. R. Bishop, A. B. Bodine, C. A. Caldwell, and Johnson, D.W. (1982) Composted dairy waste solids and crushed limestone as a bedding in freestalls. *Journal of Dairy Science* **65**: 1025-1028.

Jedrzejas, M.J., Lamani, E., and Becker, R.S. (2001) Characterization of selected strains of pneumococcal surface protein A. *J Biol Chem* **276**: 33121-33128.

Jiang, M., Babiuk, L.A., and Potter, A.A. (1996) Cloning, sequencing and expression of the CAMP factor gene of Streptococcus uberis. *Microb Pathog* **20**: 297-307.

Jiang, M., MacLachlan, P.R., Babiuk, L.A., Bolton, A.J., and Potter, A.A. (1998) The abp locus of Streptococcus uberis encodes a protein homologous to polar amino acid and opine binding proteins of gram-negative bacteria. *Can J Microbiol* **44**: 784-788.

Johnston, J.W., Myers, L.E., Ochs, M.M., Benjamin, W.H., Jr., Briles, D.E., and Hollingshead, S.K. (2004) Lipoprotein PsaA in virulence of Streptococcus pneumoniae: surface accessibility and role in protection from superoxide. *Infect Immun* **72**: 5858-5867.

Jomaa, M., Yuste, J., Paton, J.C., Jones, C., Dougan, G., and Brown, J.S. (2005) Antibodies to the iron uptake ABC transporter lipoproteins PiaA and PiuA promote opsonophagocytosis of Streptococcus pneumoniae. *Infect Immun* **73**: 6852-6859.

Jomaa, M., Terry, S., Hale, C., Jones, C., Dougan, G., and Brown, J. (2006) Immunization with the iron uptake ABC transporter proteins PiaA and PiuA prevents respiratory infection with Streptococcus pneumoniae. *Vaccine* **24**: 5133-5139.

Jones, C.L., Monaghan, P., Field, T.R., Smith, A.J., Ward, P.N., and Leigh, J.A. (2004) Localization of MtuA, an LraI homologue in Streptococcus uberis. *J Appl Microbiol* **97**: 149-157.

Jones, C.L. (2006) Ph.D. Thesis. Vol. PhD: University of Sussex, UK.

Jongbloed, J.D., Antelmann, H., Hecker, M., Nijland, R., Bron, S., Airaksinen, U., Pries, F., Quax, W.J., van Dijl, J.M., and Braun, P.G. (2002) Selective contribution of the twin-arginine translocation pathway to protein secretion in Bacillus subtilis. *J Biol Chem* **277**: 44068-44078.

Jongeneel, C.V., Bouvier, J., and Bairoch, A. (1989) A unique signature identifies a family of zinc-dependent metallopeptidases. *FEBS Lett* **242**: 211-214.

Kall, L., Krogh, A., and Sonnhammer, E.L. (2004) A combined transmembrane topology and signal peptide prediction method. *J Mol Biol* **338**: 1027-1036.

Kanehara, K., and Akiyama, Y. (2003) [RIP(regulated intramembrane proteolysis): from bacteria to higher organism]. *Tanpakushitsu Kakusan Koso* **48**: 836-841.

Keefe, G.P. (1997) Streptococcus agalactiae mastitis: a review. *Can Vet J* **38**: 429-437.

Kerr, A.R., Adrian, P.V., Estevao, S., de Groot, R., Alloing, G., Claverys, J.P., Mitchell, T.J., and Hermans, P.W. (2004) The Ami-AliA/AliB permease of Streptococcus pneumoniae is involved in nasopharyngeal colonization but not in invasive disease. *Infect Immun* **72**: 3902-3906.

Kharat, A.S., and Tomasz, A. (2003) Inactivation of the srtA gene affects localization of surface proteins and decreases adhesion of Streptococcus pneumoniae to human pharyngeal cells in vitro. *Infect Immun* **71**: 2758-2765.

Knoblauch, N.T., Rudiger, S., Schonfeld, H.J., Driessen, A.J., Schneider-Mergener, J., and Bukau, B. (1999) Substrate specificity of the SecB chaperone. *J Biol Chem* **274**: 34219-34225.

Kontinen, V.P., Saris, P., and Sarvas, M. (1991) A gene (prsA) of Bacillus subtilis involved in a novel, late stage of protein export. *Mol Microbiol* **5**: 1273-1283.

Kontinen, V.P., and Sarvas, M. (1993) The PrsA lipoprotein is essential for protein secretion in Bacillus subtilis and sets a limit for high-level secretion. *Mol Microbiol* **8**: 727-737.

Kossaibati, M.A., and Esslemont, R.J. (1997) The costs of production diseases in dairy herds in England. *Vet J* **154**: 41-51.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680-685.

Lammler, C. (1991) Biochemical and serological properties of Streptococcus uberis. *Zentralbl Veterinarmed B* **38**: 737-742.

Lancefield, R.C. (1933) A Seriological differentiation of human and other groups of hemolytic Streptococci *J. Exp. Med.* **57**: 571-595.

Lee, N., Yamagata, H., and Inouye, M. (1983) Inhibition of secretion of a mutant lipoprotein across the cytoplasmic membrane by the wild-type lipoprotein of the Escherichia coli outer membrane. *J Bacteriol* **155**: 407-411.

Lee, P.A., Tullman-Ercek, D., and Georgiou, G. (2006) The bacterial twin-arginine translocation pathway. *Annu Rev Microbiol* **60**: 373-395.

Lee, S.G., Pancholi, V., and Fischetti, V.A. (2002) Characterization of a unique glycosylated anchor endopeptidase that cleaves the LPXTG sequence motif of cell surface proteins of Gram-positive bacteria. *J Biol Chem* **277**: 46912-46922.

Lei, B., Liu, M., Voyich, J.M., Prater, C.I., Kala, S.V., DeLeo, F.R., and Musser, J.M. (2003) Identification and characterization of HtsA, a second heme-binding protein made by Streptococcus pyogenes. *Infect Immun* **71**: 5962-5969.

Lei, B., Liu, M., Chesney, G.L., and Musser, J.M. (2004) Identification of new candidate vaccine antigens made by Streptococcus pyogenes: purification and characterization of 16 putative extracellular lipoproteins. *J Infect Dis* **189**: 79-89.

Leigh, J.A., Field, T.R., and Williams, M.R. (1990) Two strains of Streptococcus uberis, of differing ability to cause clinical mastitis, differ in their ability to resist some host defence factors. *Res Vet Sci* **49**: 85-87.

Leigh, J.A. (1994) Purification of a plasminogen activator from Streptococcus uberis. *FEMS Microbiol Lett* **118**: 153-158.

Leigh, J.A. (1999) Streptococcus uberis: a permanent barrier to the control of bovine mastitis? *Vet J* **157**: 225-238.

Leigh, J.A., Finch, J.M., Field, T.R., Real, N.C., Winter, A., Walton, A.W., and Hodgkinson, S.M. (1999) Vaccination with the plasminogen activator from Streptococcus uberis induces an inhibitory response and protects against experimental infection in the dairy cow. *Vaccine* **17**: 851-857.

Leonard, B.A., Podbielski, A., Hedberg, P.J., and Dunny, G.M. (1996) Enterococcus faecalis pheromone binding protein, PrgZ, recruits a chromosomal oligopeptide permease system to import sex pheromone cCF10 for induction of conjugation. *Proc Natl Acad Sci U S A* **93**: 260-264.

Leskela, S., Wahlstrom, E., Kontinen, V.P., and Sarvas, M. (1999) Lipid modification of prelipoproteins is dispensable for growth but essential for efficient protein secretion in Bacillus subtilis: characterization of the Lgt gene. *Mol Microbiol* **31**: 1075-1085.

Li, H., Jacques, P.E., Ghinet, M.G., Brzezinski, R., and Morosoli, R. (2005) Determining the functionality of putative Tat-dependent signal peptides in Streptomyces coelicolor A3(2) by using two different reporter proteins. *Microbiology* **151**: 2189-2198.

Linde, D., Volkmer-Engert, R., Schreiber, S., and Muller, J.P. (2003) Interaction of the Bacillus subtilis chaperone CsaA with the secretory protein YvaY. *FEMS Microbiol Lett* **226**: 93-100.

Luirink, J., and Sinning, I. (2004) SRP-mediated protein targeting: structure and function revisited. *Biochim Biophys Acta* **1694**: 17-35.

Maguin, E., Prevost, H., Ehrlich, S.D., and Gruss, A. (1996) Efficient insertional mutagenesis in lactococci and other gram-positive bacteria. *J Bacteriol* **178**: 931-935.

Maiden, M.C., Bygraves, J.A., Feil, E., Morelli, G., Russell, J.E., Urwin, R., Zhang, Q., Zhou, J., Zurth, K., Caugant, D.A., Feavers, I.M., Achtman, M., and Spratt, B.G. (1998) Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. *Proc Natl Acad Sci U S A* **95**: 3140-3145.

Mangels, D., Mathers, J., Bolhuis, A., and Robinson, C. (2005) The core TatABC complex of the twin-arginine translocase in Escherichia coli: TatC drives assembly whereas TatA is essential for stability. *J Mol Biol* **345**: 415-423.

Marraffini, L.A., Ton-That, H., Zong, Y., Narayana, S.V., and Schneewind, O. (2004) Anchoring of surface proteins to the cell wall of Staphylococcus aureus. A conserved arginine residue is required for efficient catalysis of sortase A. *J Biol Chem* **279**: 37763-37770.

Matsumoto, K., Yamamoto, D., Ohishi, H., Tomoo, K., Ishida, T., Inoue, M., Sadatome, T., Kitamura, K., and Mizuno, H. (1989) Mode of binding of E-64-c, a potent thiol protease inhibitor, to papain as determined by X-ray crystal analysis of the complex. *FEBS Letters* **245**: 177-180.

Matthews, K.R., Almeida, R.A., and Oliver, S.P. (1994) Bovine mammary epithelial cell invasion by Streptococcus uberis. *Infect Immun* **62**: 5641-5646.

Mazmanian, S.K., Liu, G., Ton-That, H., and Schneewind, O. (1999) Staphylococcus aureus sortase, an enzyme that anchors surface proteins to the cell wall. *Science* **285**: 760-763.

Mazmanian, S.K., Ton-That, H., and Schneewind, O. (2001) Sortase-catalysed anchoring of surface proteins to the cell wall of Staphylococcus aureus. *Mol Microbiol* **40**: 1049-1057.

Mazmanian, S.K., Ton-That, H., Su, K., and Schneewind, O. (2002) An iron-regulated sortase anchors a class of surface protein during Staphylococcus aureus pathogenesis. *Proc Natl Acad Sci U S A* **99**: 2293-2298.

McDonald, J.S., and Anderson, A.J. (1981) Experimental infection of bovine mammary glands with Streptococcus uberis during the nonlactating period. *Am J Vet Res* **42**: 465-467.

McGrath, M.E., Mirzadegan, T., and Schmidt, B.F. (1997) Crystal Structure of Phenylmethanesulfonyl Fluoride-Treated Human Chymase at 1.9 &#197. *Biochemistry* **36**: 14318-14324.

Mierau, I., Tan, P.S., Haandrikman, A.J., Mayo, B., Kok, J., Leenhouts, K.J., Konings, W.N., and Venema, G. (1993) Cloning and sequencing of the gene for a lactococcal endopeptidase,

an enzyme with sequence similarity to mammalian enkephalinase. *J Bacteriol* **175**: 2087-2096.

Mitchell, T.J. (2006) Streptococcus pneumoniae: infection, inflammation and disease. *Adv Exp Med Biol* **582**: 111-124.

Mitrakul, K., Loo, C.Y., Gyurko, C., Hughes, C.V., and Ganeshkumar, N. (2005) Mutational analysis of the adcCBA genes in Streptococcus gordonii biofilm formation. *Oral Microbiol Immunol* **20**: 122-127.

Mori, H., and Ito, K. (2001) The Sec protein-translocation pathway. *Trends Microbiol* **9**: 494-500.

Mori, H., and Cline, K. (2002) A twin arginine signal peptide and the pH gradient trigger reversible assembly of the thylakoid [Delta]pH/Tat translocase. *J Cell Biol* **157**: 205-210.

Mori, H., Tsukazaki, T., Masui, R., Kuramitsu, S., Yokoyama, S., Johnson, A.E., Kimura, Y., Akiyama, Y., and Ito, K. (2003) Fluorescence resonance energy transfer analysis of protein translocase. SecYE from Thermus thermophilus HB8 forms a constitutive oligomer in membranes. *J Biol Chem* **278**: 14257-14264.

Mori, H., and Ito, K. (2006) Different modes of SecY-SecA interactions revealed by site-directed in vivo photo-cross-linking. *Proc Natl Acad Sci U S A* **103**: 16159-16164.

Mori, M., Sakagami, Y., Narita, M., Isogai, A., Fujino, M., Kitada, C., Craig, R.A., Clewell, D.B., and Suzuki, A. (1984) Isolation and structure of the bacterial sex pheromone, cAD1, that induces plasmid transfer in Streptococcus faecalis. *FEBS Lett* **178**: 97-100.

Muller, J., Walter, F., van Dijl, J.M., and Behnke, D. (1992) Suppression of the growth and export defects of an Escherichia coli secA(Ts) mutant by a gene cloned from Bacillus subtilis. *Mol Gen Genet* **235**: 89-96.

Muller, J.P., Ozegowski, J., Vettermann, S., Swaving, J., Van Wely, K.H., and Driessen, A.J. (2000) Interaction of Bacillus subtilis CsaA with SecA and precursor proteins. *Biochem J* **348 Pt 2**: 367-373.

Munoa, F.J., Miller, K.W., Beers, R., Graham, M., and Wu, H.C. (1991) Membrane topology of Escherichia coli prolipoprotein signal peptidase (signal peptidase II). *J Biol Chem* **266**: 17667-17672.

Navarre, W.W., and Schneewind, O. (1999) Surface proteins of gram-positive bacteria and mechanisms of their targeting to the cell wall envelope. *Microbiol Mol Biol Rev* **63**: 174-229.

Nielsen, H., Engelbrecht, J., Brunak, S., and von Heijne, G. (1997) Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Eng* **10**: 1-6.

NIRD (1970) National Institute for Research in Dairying Annual Report: 65.

Nishiyama, K., Suzuki, T., and Tokuda, H. (1996) Inversion of the membrane topology of SecG coupled with SecA-dependent preprotein translocation. *Cell* **85**: 71-81.

Nygaard, T.K., Liu, M., McClure, M.J., and Lei, B. (2006) Identification and characterization of the heme-binding proteins SeShp and SeHtsA of Streptococcus equi subspecies equi. *BMC Microbiol* **6**: 82.

Oates, J., Mathers, J., Mangels, D., Kuhlbrandt, W., Robinson, C., and Model, K. (2003) Consensus structural features of purified bacterial TatABC complexes. *J Mol Biol* **330**: 277-286.

Oates, J., Barrett, C.M., Barnett, J.P., Byrne, K.G., Bolhuis, A., and Robinson, C. (2005) The Escherichia coli twin-arginine translocation apparatus incorporates a distinct form of TatABC complex, spectrum of modular TatA complexes and minor TatAB complex. *J Mol Biol* **346**: 295-305.

Oefner, C., D'Arcy, A., Hennig, M., Winkler, F.K., and Dale, G.E. (2000) Structure of human neutral endopeptidase (Neprilysin) complexed with phosphoramidon. *J Mol Biol* **296**: 341-349.

Oetjen, J., Fives-Taylor, P., and Froeliger, E. (2001) Characterization of a streptococcal endopeptidase with homology to human endothelin-converting enzyme. *Infect Immun* **69**: 58-64.

Osaki, M., Takamatsu, D., Shimoji, Y., and Sekizaki, T. (2002) Characterization of Streptococcus suis genes encoding proteins homologous to sortase of gram-positive bacteria. *J Bacteriol* **184**: 971-982.

Overweg, K., Kerr, A., Sluijter, M., Jackson, M.H., Mitchell, T.J., de Jong, A.P., de Groot, R., and Hermans, P.W. (2000) The putative proteinase maturation protein A of Streptococcus pneumoniae is a conserved surface protein with potential to elicit protective immune responses. *Infect Immun* **68**: 4180-4188.

Paetzel, M., Dalbey, R.E., and Strynadka, N.C. (1998) Crystal structure of a bacterial signal peptidase in complex with a beta-lactam inhibitor. *Nature* **396**: 186-190.

Paetzel, M., Dalbey, R.E., and Strynadka, N.C. (2000) The structure and mechanism of bacterial type I signal peptidases. A novel antibiotic target. *Pharmacol Ther* **87**: 27-49.

Pallen, M.J., Lam, A.C., Antonio, M., and Dunbar, K. (2001) An embarrassment of sortases - a richness of substrates? *Trends Microbiol* **9**: 97-102.

Palmer, T., and Berks, B.C. (2003) Moving folded proteins across the bacterial cell membrane. *Microbiology* **149**: 547-556.

Palmer, T., Sargent, F., and Berks, B.C. (2005) Export of complex cofactor-containing proteins by the bacterial Tat pathway. *Trends Microbiol* **13**: 175-180.

Papanikou, E., Karamanou, S., and Economou, A. (2007) Bacterial protein secretion through the translocase nanomachine. *Nat Rev Microbiol* **5**: 839-851.

Paulsen, I.T., Banerjei, L., Myers, G.S., Nelson, K.E., Seshadri, R., Read, T.D., Fouts, D.E., Eisen, J.A., Gill, S.R., Heidelberg, J.F., Tettelin, H., Dodson, R.J., Umayam, L., Brinkac, L., Beanan, M., Daugherty, S., DeBoy, R.T., Durkin, S., Kolonay, J., Madupu, R., Nelson, W., Vamathevan, J., Tran, B., Upton, J., Hansen, T., Shetty, J., Khouri, H., Utterback, T., Radune,

D., Ketchum, K.A., Dougherty, B.A., and Fraser, C.M. (2003) Role of mobile DNA in the evolution of vancomycin-resistant Enterococcus faecalis. *Science* **299**: 2071-2074.

Perry, A.M., Ton-That, H., Mazmanian, S.K., and Schneewind, O. (2002) Anchoring of surface proteins to the cell wall of Staphylococcus aureus. III. Lipid II is an in vivo peptidoglycan substrate for sortase-catalyzed surface protein anchoring. *J Biol Chem* **277**: 16241-16248.

Petit, C.M., Brown, J.R., Ingraham, K., Bryant, A.P., and Holmes, D.J. (2001) Lipid modification of prelipoproteins is dispensable for growth in vitro but essential for virulence in Streptococcus pneumoniae. *FEMS Microbiol Lett* **200**: 229-233.

Porcelli, I., de Leeuw, E., Wallis, R., van den Brink-van der Laan, E., de Kruijff, B., Wallace, B.A., Palmer, T., and Berks, B.C. (2002) Characterization and membrane assembly of the TatA component of the Escherichia coli twin-arginine protein transport system. *Biochemistry* **41**: 13690-13697.

Pragai, Z., Tjalsma, H., Bolhuis, A., van Dijl, J.M., Venema, G., and Bron, S. (1997) The signal peptidase II (Isp) gene of Bacillus subtilis. *Microbiology* **143 (Pt 4)**: 1327-1333.

Pullinger, G.D., Lopez-Benavides, M., Coffey, T.J., Williamson, J.H., Cursons, R.T., Summers, E., Lacy-Hulbert, J., Maiden, M.C., and Leigh, J.A. (2006) Application of Streptococcus uberis multilocus sequence typing: analysis of the population structure detected among environmental and bovine isolates from New Zealand and the United Kingdom. *Appl Environ Microbiol* **72**: 1429-1436.

Qi, H.Y., Sankaran, K., Gan, K., and Wu, H.C. (1995) Structure-function relationship of bacterial prolipoprotein diacylglyceryl transferase: functionally significant conserved regions. *J Bacteriol* **177**: 6820-6824.

Rahfeld, J.U., Rucknagel, K.P., Schelbert, B., Ludwig, B., Hacker, J., Mann, K., and Fischer, G. (1994) Confirmation of the existence of a third family among peptidyl-prolyl cis/trans isomerases. Amino acid sequence and recombinant production of parvulin. *FEBS Lett* **352**: 180-184.

Randall, L.L., Topping, T.B., and Hardy, S.J. (1990) No specific recognition of leader peptide by SecB, a chaperone involved in protein export. *Science* **248**: 860-863.

Rawlings, N.D., Morton, F.R., and Barrett, A.J. (2006) MEROPS: the peptidase database. *Nucleic Acids Res* **34**: D270-272.

Reglier-Poupet, H., Frehel, C., Dubail, I., Beretti, J.L., Berche, P., Charbit, A., and Raynaud, C. (2003a) Maturation of lipoproteins by type II signal peptidase is required for phagosomal escape of Listeria monocytogenes. *J Biol Chem* **278**: 49469-49477.

Reglier-Poupet, H., Pellegrini, E., Charbit, A., and Berche, P. (2003b) Identification of LpeA, a PsaA-like membrane protein that promotes cell entry by Listeria monocytogenes. *Infect Immun* **71**: 474-482.

Regue, M., Remenick, J., Tokunaga, M., Mackie, G.A., and Wu, H.C. (1984) Mapping of the lipoprotein signal peptidase gene (lsp). *J Bacteriol* **158**: 632-635.

Reizer, J., Hoischen, C., Titgemeyer, F., Rivolta, C., Rabus, R., Stulke, J., Karamata, D., Saier Jr, M.H., and Hillen, W. (1998) A novel protein kinase that controls carbon catabolite repression in bacteria. *Molecular Microbiology* **27**: 1157-1169.

Roche, F.M., Massey, R., Peacock, S.J., Day, N.P., Visai, L., Speziale, P., Lam, A., Pallen, M., and Foster, T.J. (2003) Characterization of novel LPXTG-containing proteins of Staphylococcus aureus identified from genome sequences. *Microbiology* **149**: 643-654.

Rollins, S.M., Peppercorn, A., Hang, L., Hillman, J.D., Calderwood, S.B., Handfield, M., and Ryan, E.T. (2005) In vivo induced antigen technology (IVIAT). *Cell Microbiol* **7**: 1-9.

Rosch, J., and Caparon, M. (2004) A microdomain for protein secretion in Gram-positive bacteria. *Science* **304**: 1513-1515.

Rosch, J.W., and Caparon, M.G. (2005) The ExPortal: an organelle dedicated to the biogenesis of secreted proteins in Streptococcus pyogenes. *Mol Microbiol* **58**: 959-968.

Rose, R.W., Bruser, T., Kissinger, J.C., and Pohlschroder, M. (2002) Adaptation of protein secretion to extremely high-salt conditions by extensive use of the twin-arginine translocation pathway. *Mol Microbiol* **45**: 943-950.

Rosenbach, F.J. (1884) Mikroorganismen bei Wundinfektionskrankheiten des Menschen.

Rosenow, C., Ryan, P., Weiser, J.N., Johnson, S., Fontan, P., Ortqvist, A., and Masure, H.R. (1997) Contribution of novel choline-binding proteins to adherence, colonization and immunogenicity of Streptococcus pneumoniae. *Mol Microbiol* **25**: 819-829.

Rosenstein, R., and Gotz, F. (2000) Staphylococcal lipases: biochemical and molecular characterization. *Biochimie* **82**: 1005-1014.

Ruzin, A., Severin, A., Ritacco, F., Tabei, K., Singh, G., Bradford, P.A., Siegel, M.M., Projan, S.J., and Shlaes, D.M. (2002) Further evidence that a cell wall precursor [C(55)-MurNAc-(peptide)-GlcNAc] serves as an acceptor in a sorting reaction. *J Bacteriol* **184**: 2141-2147.

Rzychon, M., Chmiel, D., and Stec-Niemczyk, J. (2004) Modes of inhibition of cysteine proteases. *Acta Biochim Pol* **51**: 861-873.

Salim, K.Y., Cvitkovitch, D.G., Chang, P., Bast, D.J., Handfield, M., Hillman, J.D., and de Azavedo, J.C. (2005) Identification of group A Streptococcus antigenic determinants upregulated in vivo. *Infect Immun* **73**: 6026-6038.

Sander, P., Rezwan, M., Walker, B., Rampini, S.K., Kroppenstedt, R.M., Ehlers, S., Keller, C., Keeble, J.R., Hagemeier, M., Colston, M.J., Springer, B., and Bottger, E.C. (2004) Lipoprotein processing is required for virulence of Mycobacterium tuberculosis. *Mol Microbiol* **52**: 1543-1552.

Sankaran, K., and Wu, H.C. (1994) Lipid modification of bacterial prolipoprotein. Transfer of diacylglyceryl moiety from phosphatidylglycerol. *Journal of Biological Chemistry* **269**: 19701-19706.

Sankaran, K., Gan, K., Rash, B., Qi, H.Y., Wu, H.C., and Rick, P.D. (1997) Roles of histidine-103 and tyrosine-235 in the function of the prolipoprotein diacylglyceryl transferase of Escherichia coli. *J Bacteriol* **179**: 2944-2948.

Saparov, S.M., Erlandson, K., Cannon, K., Schaletzky, J., Schulman, S., Rapoport, T.A., and Pohl, P. (2007) Determining the conductance of the SecY protein translocation channel for small molecules. *Mol Cell* **26**: 501-509.

Sargent, F., Stanley, N.R., Berks, B.C., and Palmer, T. (1999) Sec-independent protein translocation in Escherichia coli. A distinct and pivotal role for the TatB protein. *J Biol Chem* **274**: 36073-36082.

Sargent, F., Gohlke, U., De Leeuw, E., Stanley, N.R., Palmer, T., Saibil, H.R., and Berks, B.C. (2001) Purified components of the Escherichia coli Tat protein transport system form a double-layered ring structure. *Eur J Biochem* **268**: 3361-3367.

Schobel, S., Zellmeier, S., Schumann, W., and Wiegert, T. (2004) The Bacillus subtilisσW anti-sigma factor RsiW is degraded by intramembrane proteolysis through YluC. *Molecular Microbiology* **52**: 1091-1105.

Schuchat, A. (2001) Group B streptococcal disease: from trials and tribulations to triumph and trepidation. *Clin Infect Dis* **33**: 751-756.

Seemuller, E., Lupas, A., Stock, D., Lowe, J., Huber, R., and Baumeister, W. (1995) Proteasome from Thermoplasma acidophilum: a threonine protease. *Science* **268**: 579-582.

Segura, M., and Gottschalk, M. (2004) Extracellular virulence factors of streptococci associated with animal diseases. *Front Biosci* **9**: 1157-1188.

Severin, A., Nickbarg, E., Wooters, J., Quazi, S.A., Matsuka, Y.V., Murphy, E., Moutsatsos, I.K., Zagursky, R.J., and Olmsted, S.B. (2007) Proteomic analysis and identification of Streptococcus pyogenes surface-associated proteins. *J Bacteriol* **189**: 1514-1522.

Shan, S.O., Stroud, R.M., and Walter, P. (2004) Mechanism of association and reciprocal activation of two GTPases. *PLoS Biol* **2**: e320.

Shiomi, D., Yoshimoto, M., Homma, M., and Kawagishi, I. (2006) Helical distribution of the bacterial chemoreceptor via colocalization with the Sec protein translocation machinery. *Mol Microbiol* **60**: 894-906.

Sibbald, M.J., Ziebandt, A.K., Engelmann, S., Hecker, M., de Jong, A., Harmsen, H.J., Raangs, G.C., Stokroos, I., Arends, J.P., Dubois, J.Y., and van Dijl, J.M. (2006) Mapping the pathways to staphylococcal pathogenesis by comparative secretomics. *Microbiol Mol Biol Rev* **70**: 755-788.

Skalka, B., Smola, J., and Pillich, J. (1980) Comparison of some properties of the CAMP-factor from Streptococcus agalactiae with the haemolytically latent active exosubstance from Streptococcus uberis. *Zentralbl Veterinarmed B* **27**: 559-566.

Slot, P.A. (1958) Classification systematics and occurence. *Nordisk Veterinaermedicin* **10**: 143-152.

Smith, A.J., Ward, P.N., Field, T.R., Jones, C.L., Lincoln, R.A., and Leigh, J.A. (2003) MtuA, a lipoprotein receptor antigen from Streptococcus uberis, is responsible for acquisition of manganese during growth in milk and is essential for infection of the lactating bovine mammary gland. *Infection and Immunity* **71**: 4842-4849.

Stanley, N.R., Palmer, T., and Berks, B.C. (2000) The twin arginine consensus motif of Tat signal peptides is involved in Sec-independent protein targeting in Escherichia coli. *J Biol Chem* **275**: 11591-11596.

Stanley, N.R., Findlay, K., Berks, B.C., and Palmer, T. (2001) Escherichia coli strains blocked in Tat-dependent protein export exhibit pleiotropic defects in the cell envelope. *J Bacteriol* **183**: 139-144.

Steen, A., Buist, G., Leenhouts, K.J., El Khattabi, M., Grijpstra, F., Zomer, A.L., Venema, G., Kuipers, O.P., and Kok, J. (2003) Cell wall attachment of a widely distributed peptidoglycan binding domain is hindered by cell wall constituents. *J Biol Chem* **278**: 23874-23881.

Stoll, H., Dengjel, J., Nerz, C., and Gotz, F. (2005) Staphylococcus aureus deficient in lipidation of prelipoproteins is attenuated in growth and immune activation. *Infect Immun* **73**: 2411-2423.

Sutcliffe, I.C., and Russell, R.R. (1995) Lipoproteins of gram-positive bacteria. *J Bacteriol* **177**: 1123-1128.

Sutcliffe, I.C., and Harrington, D.J. (2002) Pattern searches for the identification of putative lipoprotein genes in Gram-positive bacterial genomes. *Microbiology* **148**: 2065-2077.

Sutcliffe, I.C., and Harrington, D.J. (2004a) Lipoproteins of Mycobacterium tuberculosis: an abundant and functionally diverse class of cell envelope components. *FEMS Microbiology Reviews* **28**: 645-659.

Sutcliffe, I.C., and Harrington, D.J. (2004b) Putative lipoproteins of Streptococcus agalactiae identified by bioinformatic genome analysis. *Antonie Van Leeuwenhoek* **85**: 305-315.

Swiatlo, E., Champlin, F.R., Holman, S.C., Wilson, W.W., and Watt, J.M. (2002) Contribution of choline-binding proteins to cell surface properties of Streptococcus pneumoniae. *Infection and Immunity* **70**: 412-415.

Takeuchi, O., Hoshino, K., Kawai, T., Sanjo, H., Takada, H., Ogawa, T., Takeda, K., and Akira, S. (1999) Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity* **11**: 443-451.

Tamilselvam, B., Almeida, R.A., Dunlap, J.R., and Oliver, S.P. (2006) Streptococcus uberis internalizes and persists in bovine mammary epithelial cells. *Microb Pathog* **40**: 279-285.

Tani, K., Tokuda, H., and Mizushima, S. (1990) Translocation of ProOmpA possessing an intramolecular disulfide bridge into membrane vesicles of Escherichia coli. Effect of membrane energization. *J Biol Chem* **265**: 17341-17347.

Tanimoto, K., An, F.Y., and Clewell, D.B. (1993) Characterization of the traC determinant of the Enterococcus faecalis hemolysin-bacteriocin plasmid pAD1: binding of sex pheromone. *J Bacteriol* **175**: 5260-5264.

Taylor, D.L., Ward, P.N., Rapier, C.D., Leigh, J.A., and Bowler, L.D. (2003) Identification of a differentially expressed oligopeptide binding protein (OppA2) in Streptococcus uberis by representational difference analysis of cDNA. *J Bacteriol* **185**: 5210-5219.

Thomas, L.H., Haider, W., Hill, A.W., and Cook, R.S. (1994) Pathologic findings of experimentally induced Streptococcus uberis infection in the mammary gland of cows. *Am J Vet Res* **55**: 1723-1728.

Tjalsma, H., Kontinen, V.P., Pragai, Z., Wu, H., Meima, R., Venema, G., Bron, S., Sarvas, M., and van Dijl, J.M. (1999a) The role of lipoprotein processing by signal peptidase II in the Gram-positive eubacterium bacillus subtilis. Signal peptidase II is required for the efficient secretion of alpha-amylase, a non-lipoprotein. *J Biol Chem* **274**: 1698-1707.

Tjalsma, H., Zanen, G., Venema, G., Bron, S., and van Dijl, J.M. (1999b) The potential active site of the lipoprotein-specific (type II) signal peptidase of Bacillus subtilis. *J Biol Chem* **274**: 28191-28197.

Tjalsma, H., Bolhuis, A., Jongbloed, J.D., Bron, S., and van Dijl, J.M. (2000a) Signal peptide-dependent protein transport in Bacillus subtilis: a genome-based survey of the secretome. *Microbiol Mol Biol Rev* **64**: 515-547.

Tjalsma, H., Stover, A.G., Driks, A., Venema, G., Bron, S., and van Dijl, J.M. (2000b) Conserved serine and histidine residues are critical for activity of the ER-type signal peptidase SipW of Bacillus subtilis. *J Biol Chem* **275**: 25102-25108.

Tjalsma, H., Antelmann, H., Jongbloed, J.D., Braun, P.G., Darmon, E., Dorenbos, R., Dubois, J.Y., Westers, H., Zanen, G., Quax, W.J., Kuipers, O.P., Bron, S., Hecker, M., and van Dijl, J.M. (2004) Proteomics of protein secretion by Bacillus subtilis: separating the "secrets" of the secretome. *Microbiol Mol Biol Rev* **68**: 207-233.

Tjalsma, H., and van Dijl, J.M. (2005) Proteomics-based consensus prediction of protein retention in a bacterial membrane. *Proteomics* **5**: 4472-4482.

Tokunaga, H., and Wu, H.C. (1984) Studies on the modification and processing of prolipoprotein in Escherichia coli. Effects of structural alterations in prolipoprotein on its maturation in wild type and lpp mutants. *J Biol Chem* **259**: 6098-6104.

Tokunaga, M., Tokunaga, H., and Wu, H.C. (1982) Post-translational modification and processing of Escherichia coli prolipoprotein in vitro. *Proc Natl Acad Sci U S A* **79**: 2255-2259.

Tokunaga, M., Loranger, J.M., Chang, S.Y., Regue, M., Chang, S., and Wu, H.C. (1985) Identification of prolipoprotein signal peptidase and genomic organization of the lsp gene in Escherichia coli. *J Biol Chem* **260**: 5610-5615.

Ton-That, H., Labischinski, H., Berger-Bachi, B., and Schneewind, O. (1998) Anchor structure of staphylococcal surface proteins. III. Role of the FemA, FemB, and FemX factors in anchoring surface proteins to the bacterial cell wall. *J Biol Chem* **273**: 29143-29149.

Ton-That, H., Liu, G., Mazmanian, S.K., Faull, K.F., and Schneewind, O. (1999) Purification and characterization of sortase, the transpeptidase that cleaves surface proteins of Staphylococcus aureus at the LPXTG motif. *Proc Natl Acad Sci U S A* **96**: 12424-12429.

Ton-That, H., Mazmanian, S.K., Alksne, L., and Schneewind, O. (2002) Anchoring of surface proteins to the cell wall of Staphylococcus aureus. Cysteine 184 and histidine 120 of sortase form a thiolate-imidazolium ion pair for catalysis. *J Biol Chem* **277**: 7447-7452.

Triplett, T.L., Sgrignoli, A.R., Gao, F.B., Yang, Y.B., Tai, P.C., and Gierasch, L.M. (2001) Functional signal peptides bind a soluble N-terminal fragment of SecA and inhibit its ATPase activity. *J Biol Chem* **276**: 19648-19655.

Tschantz, W.R., Sung, M., Delgado-Partin, V.M., and Dalbey, R.E. (1993) A serine and a lysine residue implicated in the catalytic mechanism of the Escherichia coli leader peptidase. *J Biol Chem* **268**: 27349-27354.

Turner, A.J., Isaac, R.E., and Coates, D. (2001) The neprilysin (NEP) family of zinc metalloendopeptidases: genomics and function. *Bioessays* **23**: 261-269.

Tusnady, G.E., and Simon, I. (2001) The HMMTOP transmembrane topology prediction server. *Bioinformatics* **17**: 849-850.

van Dijl, J.M., de Jong, A., Venema, G., and Bron, S. (1995) Identification of the Potential Active Site of the Signal Peptidase SipS of Bacillus subtilis. *J. Biol. Chem.* **270**: 3611-3618.

van Roosmalen, M.L., Geukens, N., Jongbloed, J.D., Tjalsma, H., Dubois, J.Y., Bron, S., van Dijl, J.M., and Anne, J. (2004) Type I signal peptidases of Gram-positive bacteria. *Biochim Biophys Acta* **1694**: 279-297.

van Wely, K.H., Swaving, J., Freudl, R., and Driessen, A.J. (2001) Translocation of proteins across the cell envelope of Gram-positive bacteria. *FEMS Microbiol Rev* **25**: 437-454.

Venema, R., Tjalsma, H., van Dijl, J.M., de Jong, A., Leenhouts, K., Buist, G., and Venema, G. (2003) Active lipoprotein precursors in the Gram-positive eubacterium Lactococcus lactis. *J Biol Chem* **278**: 14739-14746.

von Heijne, G. (1989) The structure of signal peptides from bacterial lipoproteins. *Protein Eng* **2**: 531-534.

von Heijne, G., and Manoil, C. (1990) Membrane proteins: from sequence to structure. *Protein Eng* **4**: 109-112.

Wahlstrom, E., Vitikainen, M., Kontinen, V.P., and Sarvas, M. (2003) The extracytoplasmic folding factor PrsA is required for protein secretion only in the presence of the cell wall in Bacillus subtilis. *Microbiology* **149**: 569-577.

Waligora, A.J., Hennequin, C., Mullany, P., Bourlioux, P., Collignon, A., and Karjalainen, T. (2001) Characterization of a cell surface protein of Clostridium difficile with adhesive properties. *Infect Immun* **69**: 2144-2153.

Ward, P.N., Field, T.R., Ditcham, W.G., Maguin, E., and Leigh, J.A. (2001) Identification and disruption of two discrete loci encoding hyaluronic acid capsule biosynthesis genes hasA, hasB, and hasC in Streptococcus uberis. *Infection and Immunity* **69**: 392-399.

Ward, P.N., and Leigh, J.A. (2002) Characterization of PauB, a novel broad-spectrum plasminogen activator from Streptococcus uberis. *J Bacteriol* **184**: 119-125.

Ward, P.N., Field, T.R., Rapier, C.D., and Leigh, J.A. (2003) The activation of bovine plasminogen by PauA is not required for virulence of Streptococcus uberis. *Infection and Immunity* **71**: 7193-7196.

Weiss, W.J., Lenoy, E., Murphy, T., Tardio, L., Burgio, P., Projan, S.J., Schneewind, O., and Alksne, L. (2004) Effect of srtA and srtB gene expression on the virulence of Staphylococcus aureus in animal models of infection. *J Antimicrob Chemother* **53**: 480-486.

Wells, S.J., Ott, S.L., and Seitzinger, A.H. (1998) Key health issues for dairy cattle--new and old. *J Dairy Sci* **81**: 3029-3035.

Wieliczko, R.J., Williamson, J.H., Cursons, R.T., Lacy-Hulbert, S.J., and Woolford, M.W. (2002) Molecular typing of Streptococcus uberis strains isolated from cases of bovine mastitis. *J Dairy Sci* **85**: 2149-2154.

Williams, A.M., and Collins, M.D. (1990) Molecular taxonomic studies on Streptococcus uberis types I and II. Description of Streptococcus parauberis sp. nov. *J Appl Bacteriol* **68**: 485-490.

Wirawan, R.E., Swanson, K.M., Kleffmann, T., Jack, R.W., and Tagg, J.R. (2007) Uberolysin: a novel cyclic bacteriocin produced by Streptococcus uberis. *Microbiology* **153**: 1619-1630.

Yamagata, H., Ippolito, C., Inukai, M., and Inouye, M. (1982) Temperature-sensitive processing of outer membrane lipoprotein in an Escherichia coli mutant. *J Bacteriol* **152**: 1163-1168.

Yamagata, H. (1983) Temperature-sensitive prolipoprotein signal peptidase in an Escherichia coli mutant: use of the mutant for an efficient and convenient assay system. *J Biochem (Tokyo)* **93**: 1509-1515.

Yamaguchi, K., Yu, F., and Inouye, M. (1988) A single amino acid determinant of the membrane localization of lipoproteins in E. coli. *Cell* **53**: 423-432.

Zdanowicz, M., Shelford, J.A., Tucker, C.B., Weary, D.M., and von Keyserlingk, M.A.G. (2004) Bacterial Populations on Teat Ends of Dairy Cows Housed in Free Stalls and Bedded with Either Sand or Sawdust. *J. Dairy Sci.* **87**: 1694-1701.

Zhao, X.J., and Wu, H.C. (1992) Nucleotide sequence of the Staphylococcus aureus signal peptidase II (lsp) gene. *FEBS Lett* **299**: 80-84.

Zong, Y., Bice, T.W., Ton-That, H., Schneewind, O., and Narayana, S.V.L. (2004) Crystal Structures of Staphylococcus aureus Sortase A and Its Substrate Complex. *J. Biol. Chem.* **279**: 31383-31389.