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Functional Characterisation of
*Staphylococcus pseudintermedius*
Cell Wall-Associated Proteins

Amy C. Richards

Doctor of Philosophy
The University of Edinburgh
2015
Declaration

This is to certify that the work contained within this thesis has been composed by me and is entirely my own work. No part of this thesis has been submitted for any other degree or professional qualification.

Amy Richards  August 2015
Acknowledgements

I would like to thank my supervisors Professor Ross Fitzgerald and Dr Andreas Lengeling for their guidance and support. Particularly I would like to thank Ross for his critical analysis of my work throughout my PhD. The murine infection experiments would not have been possible without Dr Marie O’Shea’s excellent technical assistance and Dr Pip Beard’s pathology expertise. I would also like to thank Dr Andy Gill and Dr Jo Stevens for providing me with temporary lab space.

I am very grateful to Professor Magnus Höök for the invitation to develop my proteomic techniques at Texas A&M University. Special thanks to Dr Brandon Garcia who provided fantastic technical support during my visit and taught me how to collect and analyse surface plasmon resonance data. I would like to thank the Microbiology Society for funding this research visit. I am also very grateful to Dr Ian Monk, University of Melbourne, who provided me with the protocols and strains required to genetically manipulate S. pseudintermedius. I would also like to thank Professor Pietro Speziale and Dr Giampiero Pietrocola, University of Pavia, for providing me with recombinant expression constructs and antibodies aiding my investigation of the interaction of SpsL with fibrinogen.

I would like to thank the current and past members of LBEP who have all helped me in various ways over the years including Stephen Nutbeam-Tuffs, Chriselle Mendonca, Mariya Goncheva, Marta Rodriguez, Gordon Gong, Emily Richardson, Rodrigo Bacigalupe, Emma Raftis, Laura Spoor, Paul McAdam, Melissa Ward, and Cheryl Gibbons. I would particularly like to thank Mariya Goncheva for her help in the murine infection experiments. I would also like to thank non-LBEP coffee-time buddies for their friendship including members of ZAP, CMG, MMBP, Louise Nicol, Hazel Wilkie, Preyna Vohra, Laura Vezza, and Iain MacArthur. Thanks for reminding me to socialise every now and then!

I would not have completed the PhD process without my brilliant partner Siôn Pickering who has been my rock and my cheerleader. Thank you to Mum, Dad, Kylie, and Mark for all your love and care.
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<tr>
<td>aa</td>
<td>amino acids</td>
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<td>AD</td>
<td>atopic dermatitis</td>
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<td>AFM</td>
<td>atomic force microscopy</td>
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<td>Amp</td>
<td>ampicillin</td>
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<td>anhydrotetracycline</td>
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<td>bone sialoprotein-binding protein</td>
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<td>dock, lock, and latch</td>
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<td>dNTP</td>
<td>deoxyribonucleotide triphosphate</td>
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<td>ECM</td>
<td>extracellular matrix</td>
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<td>Fg</td>
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<td>K_D</td>
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<td>M</td>
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<td>MDR</td>
<td>multidrug resistant</td>
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<td>mg</td>
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<td>MGE</td>
<td>mobile genetic element</td>
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<td>min</td>
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<tr>
<td>MRSA</td>
<td>methicillin resistant <em>S. aureus</em></td>
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<tr>
<td>MRSP</td>
<td>methicillin resistant <em>S. pseudintermedius</em></td>
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<tr>
<td>MSCRAMM</td>
<td>microbial surface components recognising adhesive matrix molecules</td>
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<td>OD&lt;sub&gt;600&lt;/sub&gt;</td>
<td>optical density at 600 nm</td>
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<td>OLR</td>
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Abstract

*Staphylococcus pseudintermedius* is the major cause of the common canine skin disease, pyoderma, and is a zoonotic pathogen of humans. Multidrug resistant strains of *S. pseudintermedius* have emerged and are spreading globally leading to decreased therapeutic success. The development of novel therapeutics is hindered by the lack of understanding of critical host-pathogen interactions mediating *S. pseudintermedius* colonization and pathogenesis. For the major human pathogen *Staphylococcus aureus*, interactions with host fibrinogen play a fundamental role in pathogenesis. The aim of the current study was to genetically and functionally characterise 2 cell wall-associated proteins of *S. pseudintermedius*, SpsD and SpsL, which mediate binding to multiple host extracellular matrix proteins including fibrinogen and fibronectin. DNA sequencing of the A- (ligand binding) domains of *spsD* and *spsL* genes for 37 phylogenetically diverse isolates revealed a highly conserved sequence for SpsL (97.1% derived amino acid identity), in contrast to more extensive variation for SpsD (76.7% derived amino acid identity). Further, recombination events were identified to have contributed to the evolution of *spsD* diversity. Functional analysis with gene deletion mutants of *S. pseudintermedius* strain ED99, constructed in the current study, demonstrated that SpsL is a major cell wall-associated fibrinogen-binding protein with enhanced affinity for canine fibrinogen. Using recombinant chains of fibrinogen it was determined that SpsL binds to the α-chain of fibrinogen similar to clumping factor B (ClfB) of *S. aureus*. However, ELISA and surface plasmon resonance analyses of recombinant truncated derivatives of SpsL indicated that the predicted ligand-binding N2N3 subdomains of the A-domain of SpsL are not sufficient for high-affinity fibrinogen-binding suggesting that either additional domains or post-translational modifications are required for fibrinogen-binding. Furthermore, development of a murine skin infection model allowed an investigation of the contribution of SpsD and SpsL to pathogenesis revealing a role for SpsL in focal abscess pathology. Overall these studies have provided new insights into the diversity, function and therapeutic potential of *S. pseudintermedius* fibrinogen-binding proteins.
Lay Summary

The bacterium *Staphylococcus pseudintermedius* is the major cause of skin infections in dogs and can cause human disease usually via dog bites. Treating these infections is becoming increasingly difficult due to increased drug resistance and new treatment options are required. Before new therapies can be developed more research is needed to allow increased understanding of how the infection starts, develops, and resolves. The aim of this study is to increase the understanding of how this bacteria binds to canine fibrinogen, a protein that forms part of the skin barrier as well as being involved in normal host defence during a bacterial infection. In order to do this, bacterial proteins that bind to canine fibrinogen were removed from the surface of the bacteria by gene deletion allowing the role of these proteins to be analysed. This identified that one protein, called SpsL, is particularly important for fibrinogen-binding and that this protein can only bind to fibrinogen from particular hosts – it shows a host specific binding interaction. The region of SpsL that binds to fibrinogen could not be fully determined suggesting that SpsL uses a novel binding mechanism to adhere to fibrinogen not yet identified in other bacteria. The role of SpsL during skin infection was also investigated by injecting the bacteria under the surface of mouse skin. This skin infection model identified that SpsL is key to the development of murine skin abscesses suggesting that SpsL could be involved in the development of skin abscesses in dogs. The ability of SpsL to be used as a novel therapeutic was also investigated by identifying how many infection-causing bacterial strains of *S. pseudintermedius* express SpsL and how the gene sequence of this protein alters between infection-causing strains. This identified that SpsL is at least 97.1 % similar in the 37 strains tested and is expressed in most strains analysed. This high gene similarity and protein expression suggests that SpsL could be a good therapeutic target but more research is needed to investigate this. Overall this study has increased our understanding of how *S. pseudintermedius* binds to fibrinogen and the potential role that fibrinogen-binding bacterial proteins play in the development of skin infections.
Chapter 1 Introduction
1.1 Introduction to *Staphylococcus pseudintermedius*

1.1.1 The *Staphylococcus intermedius* Group: Population Genetics

Traditionally, *Staphylococcus intermedius* is a coagulase-positive bacterium first isolated in 1976 from the anterior nares of healthy pigeons, dogs, minks, and horses, with cell wall structure and phenotypic characteristics distinct from *Staphylococcus aureus* and *Staphylococcus epidermidis* (Hajek, 1976). This Staphylococcal species demonstrated diverse characteristics suggesting that the species may be composed of closely related subspecies with canine and human isolates appearing most closely related (Aarestrup, 2001; Bes et al., 2002). In 2005, a novel species with high similarity to *S. intermedius*, named *Staphylococcus pseudintermedius* was identified (Devriese et al., 2005). The clinical importance of this novel species in canine pyoderma was demonstrated in 2 independent phylogenetic studies into the population structure of *S. intermedius* isolates (Bannoehr et al., 2007; Sasaki et al., 2007b). Both of these studies revealed that large numbers of clinical isolates had been misidentified as *S. intermedius* and were in fact *S. pseudintermedius*. This provided strong support for a *S. intermedius* group (SIG) made up of 3 coagulase-positive staphylococcal species including *S. intermedius*, *S. pseudintermedius*, and another closely-related species *Staphylococcus delphini* (Bannoehr et al., 2007; Sasaki et al., 2007b). These new taxonomic classifications clearly aligned to host origins with *S. intermedius* being isolated from wild pigeons, *S. pseudintermedius* being primarily isolated from dogs but also from cats and humans, and *S. delphini* being more diversely isolated from domestic pigeons, horses, minks, badgers, ferrets, camels and dolphins (Bannoehr et al., 2007; Gary et al., 2014; Guardabassi et al., 2012; Sasaki et al., 2007b). The high correlation between *S. pseudintermedius* and dogs meant that all canine isolates of *S. intermedius* have now been reclassified as *S. pseudintermedius* (Bannoehr et al., 2007; Sasaki et al., 2007a).

To allow rapid and accurate differentiation of each species within the SIG non-phenotypic techniques have now been established. These techniques include polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), multiplex PCR, matrix-assisted laser desorption/ionisation-time of flight mass spectrometry, and loop-mediated isothermal amplification of *spsL* (Bannoehr et al.,...
2009; Blaiotta et al., 2010; Decristophoris et al., 2011; Diribe et al., 2014; Murugaiyan et al., 2014; Sasaki et al., 2010; Silva et al., 2015). Until these techniques become common-place in medical and veterinary clinics there is a risk of misidentification of coagulase-positive staphylococci, with *S. delphini* particularly hard to differentiate. Typing methods have also been established for *S. pseudintermedius* including staphylococcal cassette chromosome mec (SCCmec) typing, *spa* typing and multi-locus sequence typing (MLST) that uses combinations of alleles of 7 housekeeping genes to characterise a particular sequence type (ST) (Kondo et al., 2007; Moodley et al., 2009; Solyman et al., 2013). These standardised techniques, alongside whole genome sequencing, allow the diversity of *S. pseudintermedius* to be investigated.

### 1.1.2 *S. pseudintermedius* Carriage

*S. pseudintermedius* is a natural commensal of dogs that colonises primarily the skin and mucosal membranes such as the nose, mouth and anus (Allaker et al., 1992; Devriese and De Pelsmaecker, 1987; Iverson et al., 2015). Reports of carriage rates in healthy dogs range from 46% to 92%, much higher than the rate of carriage of *S. aureus* in healthy humans, which is roughly 20% (Bannoehr and Guardabassi, 2012; Kluytmans et al., 1997). Strain diversity is present within multiple colonisation sites of the same dog with longitudinal studies demonstrating that colonisation of healthy dogs can be either intermittent or persistent with some dogs identified as non-carriers (Fazakerley et al., 2010; Gómez-Sanz et al., 2013c; Hartmann et al., 2005; Paul et al., 2012; Pinchbeck et al., 2006; Sasaki et al., 2005). Strain diversity within dogs suffering from atopic dermatitis (AD) is lower than in healthy dogs with generally the same strain cultured from infection and carriage sites (Fazakerley et al., 2010; Pinchbeck et al., 2006; Sasaki et al., 2005). Investigation of the skin microbiome in both healthy and allergic dogs confirmed large bacterial diversity between multiple skin sites that was reduced in dogs with allergic skin diseases (Rodrigues Hoffmann et al., 2014).
1.1.3 Clinical Features of Canine Pyoderma

Healthy mammalian skin consists of multiple layers including the outermost epidermis, the dermis, and the panniculus (subcutaneous fat). The epidermis is constantly renewed due to the differentiation of layered keratinocytes from the dermis and desquamation (the detaching of cells from the epidermis) (Le Lamer et al., 2015; Watt and Fujiwara, 2011). As keratinocytes mature they produce large quantities of intracellular keratin filaments as well as the extracellular matrix (ECM) proteins involucrin and loricrin. The very outermost layer of the epidermis consists of dead keratinocytes called corneocytes that form a cornified envelope and contain large quantities of involucrin (Simon and Green, 1985). Linking the epidermis and the dermis is the basement membrane, a specialised ECM structure containing distinct networks of collagen and laminin (Yurchenco et al., 1992). The dermis also contains collagen fibres and provides the strength and elasticity of the skin as well as containing sweat glands and hair roots (Watt and Fujiwara, 2011). Traditionally it was assumed that the dermis was free from commensal bacteria, with the cornified envelope creating a barrier against bacterial entry. However, microbiome analysis has now identified bacteria present throughout the skin layers including the dermis (Nakatsuji et al., 2013).

*S. pseudintermedius* has been implicated in a range of opportunistic infections including urinary tract, ear, and wound infections but its most clinically significant role is in canine pyoderma (van Duijkeren et al., 2011). This chronic inflammatory disease is more common in canines than in any other mammal (Bloom, 2014). Superficial bacterial folliculitis (SBF) and AD are the most common forms of canine pyoderma presented and are primarily caused by *S. pseudintermedius* (Hillier et al., 2014). However, the role of *Staphylococcus schleiferi* subsp *schleiferi* in canine pyoderma is increasingly being documented (Gold et al., 2013). Classical features of SBF and AD include pustules, scaling, erythematous papules, erythema, epidermal collarettes, alopecia, hypo- or hyper-pigmentation, crusting, and pruritus (Hillier et al., 2014). Some clinical symptoms are more common in particular canine breeds with the most common breeds affected by canine pyoderma including Labradors, terriers,
German shepherds, cocker spaniels, and golden retrievers (Horvath and Neuber, 2007; Summers et al., 2014).

Canine pyoderma is an umbrella term referring to infections associated with multiple underlying conditions such as hypersensitivity reactions, hypothyroidism, endocrinopathy, and the presence of ectoparasites (Bloom, 2014; Gold et al., 2013; Ihrke, 2005). These conditions alter the skin environment predisposing to opportunistic bacterial infection (Bloom, 2014). In the case of AD (Figure 1.1), high levels of allergen specific IgE induce skin inflammation leading to the degranulation of mast cells that release histamine and cause intense itching (pruritus) (Abramo et al., 2014; Bexley et al., 2013). AD is dominated by an acute T helper 2 (Th2) immune response that is induced by interleukin-17A (IL-17A) expression by dermal γδ T cells (Nakajima et al., 2014). Mutations in both the innate and adaptive immune responses are genetically linked to AD (Narbutt et al., 2014; Sun et al., 2011).

In both human and canine AD, defects in the filaggrin gene (FLG) are strongly associated with but not essential for disease (Barnes, 2010; Kanda et al., 2013; Seguchi et al., 1996). Filaggrin is instrumental to keratinocyte development with hereditary mutations leading to defects in the skin barrier (Agrawal and Woodfolk, 2014; Inman et al., 2001; Mu et al., 2014; Samuelov et al., 2013; Santoro et al., 2015). A defective skin barrier is thought to be central to the development of AD (Figure 1.1) with patients characteristically having decreased skin thickness, increased pH, decreased lipid levels and increased transepidermal water loss as well as decreased levels of the ECM proteins loricrin and involucrin (Abramo et al., 2014; Baumer et al., 2011; Inman et al., 2001; Ishikawa et al., 2010; Kim et al., 2008; Reiter et al., 2009). These classic clinical features can be replicated in canine models of AD with defects in the skin barrier correlating with reduced lipid barrier ceramides and decreased expression of tight-junction proteins allowing increased sensitisation to allergens (Olivry and Dunston, 2014; Olivry et al., 2011). The importance of both filaggrin and allergens during AD is highlighted in filaggrin-deficient mice that display decreased thickness of the skin barrier and altered moisture levels (Pendaries et al., 2014). Filaggrin-deficient mice do not develop AD spontaneously but the presence of allergens, to which they have increased sensitisation, leads to AD (Kawasaki et al., 2012).
Figure 1.1. The Multiple Immune Mechanisms Implicated in Atopic Dermatitis.

Stage I, mutations in filaggrin affect the skin barrier allowing allergens, such as *S. aureus* superantigens (SAg), to penetrate through the skin. Dendritic cells (DC) present the allergens to both T cells and B cells inducing IgE and IL-4 production. IL-4 promotes a Th2 dominant acute inflammatory response. Stage II, IgE bound mast cells and basophils (Bas) degranulate in the presence of allergen releasing more IL-4 and histamine, producing pruritus. *S. aureus* δ-toxin also mediates mast cell degranulation. Stage III, itching, IL-4-mediated inflammation, and α-toxin-mediated keratinocyte cytotoxicity allow bacteria to penetrate the skin. Simultaneous activation of TLR2 and IL-4R suppresses IL-10 and promotes a chronic Th1 inflammatory response.
1.1.4 The Role of Staphylococci in Skin Infections

As *S. pseudintermedius* is the predominant pathogen causing canine pyoderma, *S. aureus* is the predominant organism associated with human pyoderma. Until recently it was assumed that the alterations in the skin barrier described above are key to the development of AD with the bacterial infection being secondary to these defects. Recent research using a disintegrin and metalloproteinase 17- (ADAM17) deficient model of disease demonstrated that the natural microbiota are required for the initiation of AD (Kobayashi et al., 2015b). These ADAM-17 deficient mice mimic the clinical features of AD including decreased microbiota diversity compared to wild type mice (Kobayashi et al., 2015b). Analysis of disease initiation demonstrated that microbial imbalance, including increased levels of *S. aureus* and *Corynebacterium bovis*, superseded the development of eczematous inflammation and intense pruritus (Kobayashi et al., 2015b). Antibiotic treatment prevented development of skin inflammation suggesting that microbial dysbiosis drives the development of disease symptoms (Kobayashi et al., 2015b). The presence of *S. aureus* alone was sufficient to induce AD symptoms but only when pre-existing skin barrier defects were also present (Kobayashi et al., 2015b). The mechanism responsible for microbial dysbiosis could not be determined but the conclusions point to a prominent role of *S. aureus* colonisation in the initiation of AD in this murine model (Kobayashi et al., 2015b).

Mice deficient in toll-like receptor 2 (TLR2), an innate immune receptor capable of recognising *S. aureus* antigens such as lipoteichoic acid, have also demonstrated the role of *S. aureus* in the progression of chronic dermatitis (Kaesler et al., 2014; Travers et al., 2010). *S. aureus* activation of TLR2 converts an acute self-limiting inflammation to a chronic persistent inflammation that could be suppressed by neutralising IL-4 (Figure 1.1) (Kaesler et al., 2014). In line with this Dupilumab (monoclonal antibody to IL-4 and IL-13) is likely to become the first systemic treatment available for human AD after passing both phase I and II clinical trials (Cully, 2014). This highlights the importance of *S. aureus*-induced IL-4 in the development of chronic dermatitis.

*S. aureus* toxins also influence the progression of AD with α-toxin mediated keratinocyte cytotoxicity shown to be increased in filaggrin-deficient murine skin and in skin with reduced ceramide levels biopsied from AD patients (Figure 1.1)
(Brauweiler et al., 2013; Brauweiler et al., 2014). *S. aureus* δ-toxin is a potent inducer of mast cell degranulation leading to increased IgE production and therefore worsening of skin lesions in a murine epicutaneous skin infection model (Figure 1.1) (Nakamura et al., 2013). This function of δ-toxin can occur in the absence of IgE-specific antigen demonstrating that the expression of this toxin is sufficient to initiate AD-related inflammation (Nakamura et al., 2013). Superantigens (SAg) of *S. aureus* may act as allergens in AD patients, as staphylococcal exotoxin A (SEA), SEB, and toxic shock syndrome toxin-1 (TSST-1) can react with patient IgE and activate basophils to promote skin inflammation (Figure 1.1) (Leung et al., 1993). SEB can also activate eosinophils producing inflammation during AD (Minai-Fleminger et al., 2014). Staphylococcal Protein A (SpA) is also a potent inducer of inflammation and direct interactions with tumour necrosis factor receptor 1 (TNFR1) are sufficient to induce a potent inflammatory response (Giai et al., 2013).

In contrast, our understanding of the role of *S. pseudintermedius* in the development of AD is very limited. Research into the pathogenic potential of *S. pseudintermedius* has failed to identify markers differentiating disease-causing and non-disease-causing isolates with common toxins and virulence factors identified in both highlighting the opportunistic nature of this pathogen (Allaker et al., 1993; Allaker et al., 1991; Sasaki et al., 2005; Yoon et al., 2010; Youn et al., 2011). The whole genome sequence of clinical isolate ED99 identified multiple virulence factors encoded by *S. pseudintermedius* (Ben Zakour et al., 2011). These encoded virulence factors include putative lipases, proteases, toxins, a coagulase, a thermonuclease, iron-transport system proteins, adhesive proteins including those involved in biofilm formation and the global regulators *agr, sae, sar,* and *rot* (Ben Zakour et al., 2012). Most of these putative virulence factors have not been investigated in detail but *S. pseudintermedius* exfoliative toxins have been implicated in the development of pyoderma with subcutaneous or intradermal injection of 3 exfoliative toxins SIET, ExpA, or ExpB into dogs resulting in clinical signs of AD such as crusting, erythema, or epidermal splitting (Futagawa-Saito et al., 2009; Iyori et al., 2010; Terauchi et al., 2003). Both ExpA and ExpB function by cleaving desmoglein 1 in canine skin to produce cell splitting, strongly implicating both proteins in the pathogenesis of canine impetigo (Futagawa-Saito et al., 2009; Iyori et al., 2011). A novel exfoliative toxin, SPETA,
was identified from the genome sequence of ED99 but the function of this putative toxin remains uncharacterised (Ben Zakour et al., 2012). Only two SAggs have been identified in S. pseudintermedius, SEC canine and SE-INT, with SAg activity and emesis reported for only SEC canine (Edwards et al., 1997; Futagawa-Saito et al., 2004b; Garbacz et al., 2013). These S. pseudintermedius SAg may act as antigens during AD with increased IgE responses against Staphylococcal antigens identified in AD patients compared to non-AD cases of canine pyoderma (Bexley et al., 2013). The β-toxin of S. pseudintermedius is known to cause the haemolysis of rabbit erythrocytes while the leukotoxins LukS-I and LuF-I are highly cytotoxic to rabbit leukocytes (Dziewanowska et al., 1996; Futagawa-Saito et al., 2004a; Prevost et al., 1995). However, the role of these toxins in the context of canine pyoderma has not been investigated. Little is also known about the function of biofilm formation or the development of small colony variants during canine pyoderma but these are documented features of methicillin-resistant S. pseudintermedius (MRSP) (Savini et al., 2014; Singh et al., 2013).

1.1.5 Treatment of Canine Pyoderma

Canine pyoderma is divided into 3 clinical categories including surface, superficial, and deep. Current treatments of surface and superficial canine pyoderma include the use of topical creams and shampoos containing antimicrobial agents, with shampoos containing chlorhexidine exhibiting enhanced efficacy (Young et al., 2012). These treatments are generally successful by reducing skin pH to prevent bacterial growth, but the high level of owner compliance required can lead to treatment failure and accordingly shampoos are often prescribed as adjunct therapy alongside antibiotics (Horvath and Neuber, 2007). A UK-based study identified that 40% of dogs treated for a superficial pyoderma were not prescribed an adequate course of antibiotics, potentially increasing the likelihood of MRSP development (Summers et al., 2014). Antibiotic prescriptions of 4 to 6 weeks are required for the resolution of deep or recurrent pyoderma with amoxicillin-clavulanic acid, cephalexin, clindamycin, or potentiated sulphonamides most commonly prescribed (Beco et al., 2013; Gold et al., 2013). Relapse of infection often occurs upon removal of antibiotic treatment as the underlying conditions of canine pyoderma are not resolved. This has promoted
research into the use of immunomodulators (drugs that suppress the innate immune response) to treat the underlying cause of pyoderma. Combinations of antibiotics and immunomodulators or sole treatment with injections of bacterin significantly reduce the incidence of relapse (Borku et al., 2007; Curtis et al., 2006; Špruček et al., 2007). Ideally a therapeutic option would be available that eliminates the risk of bacterial infection allowing the underlying causes of pyoderma to be effectively managed.

Treatment is also complicated by the high prevalence of resistant *S. pseudintermedius* with MRSP infections requiring aminoglycosides, tetracyclines, rifampicin, or chloramphenicol with vancomycin and linezolid reserved for human treatment (Gold et al., 2013; Weese, 2008). MRSP isolates have been identified that are resistant to all commonly used antimicrobials in veterinary practice (Detwiler et al., 2013; Papich, 2013). Correct dosing can be difficult as highlighted by the observation that heterogeneous expression of *mecA* can be present in a single MRSP strain (Savini et al., 2013b). The development of novel therapeutics to treat *S. pseudintermedius* canine pyoderma is therefore warranted. With this in mind, 6 short synthetic antimicrobial peptides (AMPs) have been identified that demonstrate rapid bactericidal activity against *S. pseudintermedius* *in vitro* producing pore formation and membrane disruption (Mohamed et al., 2014). Such peptides could be used as a topical treatment but this has not been tested (Mohamed et al., 2014). DispersinB, a protease of the bacterial surface component poly-N-acetylglucosamine (PNAG), demonstrates the ability to decrease MRSP biofilm formation in both static and microfluidic systems *in vitro* (Terry and Neethirajan, 2014; Turk et al., 2013). The development of DispersinB within a topical gel provided increased antimicrobial efficacy in combination with a broad-spectrum cationic antimicrobial decapeptide KSL-W suggesting that this could be a valuable therapeutic agent in the future (Gawande et al., 2014). Research into novel therapeutics is warranted but the development of a vaccine to prevent *S. pseudintermedius* related pyoderma could be the ideal approach for controlling infection.

### 1.1.6 The Rapid Emergence of Methicillin Resistant *S. pseudintermedius*

The widespread use of antibiotics has selected for antibiotic resistant strains of bacteria leading to reduced therapeutic options. Multi-drug resistance is a common feature of
S. pseudintermedius strains and the incidence of MRSP has rapidly increased in recent years (Beever et al., 2015; Chrobak et al., 2011; De Lucia et al., 2011; Gomez-Sanz et al., 2011; Moodley et al., 2014; Onuma et al., 2012; Penna et al., 2009; Rantala et al., 2004). MRSP is now reported as being the most common methicillin-resistant coagulase-positive staphylococci isolated from dogs with up to 25% of healthy dogs carrying MRSP (Fulham et al., 2011; Wang et al., 2012). Risk factor analysis studies have demonstrated that previous antimicrobial use and the presence of chronic skin disease increase the incidence of MRSP infections, with isolates from a recurrent infection demonstrating higher resistance levels than isolates from a first-time infection (Eckholm et al., 2013; Holm et al., 2002; Larsen et al., 2015; Lehner et al., 2014; Rota et al., 2013; Weese et al., 2012). Treatment of MRSP pyoderma has also been documented to increase the likelihood of MRSP being present at carriage sites (Beck et al., 2012). MLST analysis has revealed a diverse population structure of S. pseudintermedius with large numbers of STs being identified (Bannoehr et al., 2007; Black et al., 2009; McCarthy et al., 2015; Solyman et al., 2013). In contrast, analysis of MRSP isolates identified 2 lineages, ST71 predominating in Europe and ST68 predominating in North America, undergoing clonal expansion and geographic dissemination suggesting multiple acquisitions of meca through horizontal gene transfer (HGT) (Bannoehr et al., 2007; McCarthy et al., 2015; Perreten et al., 2010; Ruscher et al., 2010). Genetic diversity has been identified in the ST68 lineage but ST71 remains highly clonal (McCarthy et al., 2015; Ruscher et al., 2010; Solyman et al., 2013).

The defining characteristic of MRSP strains is the presence of the meca gene within the SCCmec mobile genetic element (MGE) (Loeffler et al., 2007). This gene encodes a truncated penicillin binding protein 2a of 78 kDa that confers resistance to all β-lactam antibiotics. Various SCCmec elements have been characterised in S. pseudintermedius with ST71 characterised by the SCCmecII-III element and ST68 characterised by the SCCmecVT (Black et al., 2009; Descloux et al., 2008). SCCmecVT was first reported in a community associated methicillin resistant S. aureus (CA-MRSA) strain suggesting that HGT of resistance cassettes can occur between human and canine pathogens (Black et al., 2009).
Investigation of the distribution of drug resistance genes among 15 published genome sequences of *S. pseudintermedius* has identified a Tn5405-like element present in all MDR strains encoding up to 5 antimicrobial resistance genes \( \text{aphA3-sat-aadE-dfrG-erm(B)} \) (providing aminoglycosides, streptothricin, trimethoprim, and erythromycin resistance) (McCarthy et al., 2015). HGT of plasmids encoding antimicrobial resistance in *S. pseudintermedius* is rare with additional resistance genes present on transposons or insertion sequence elements (McCarthy et al., 2015). Only 3 genomic events have led to the antimicrobial resistance profiles of both MRSP ST68 and ST71 with the acquisitions of an SCCmec element, a Tn5405-like element, and single nucleotide polymorphisms within \( \text{gyrA/grlA} \) (providing fluoroquinolone resistance) (McCarthy et al., 2015).

1.1.7 Restriction Modification Systems of *S. pseudintermedius*

The lack of plasmid-mediated antimicrobial resistance and the identification of restriction-modification (RM) systems in all of the available genome sequences of *S. pseudintermedius* suggest poor natural transformation of this bacterium (McCarthy et al., 2015). Restriction modification systems limit HGT both within a bacterial species and between species by encoding endonucleases (Monk and Foster, 2012). There are 3 major classes of RM systems which have been identified to date among Staphylococci. Type I RM systems encode 3 host specificity of DNA (hsd) genes including \( \text{hsdS} \) (specificity), \( \text{hsdR} \) (restriction), and \( \text{hsdM} \) (methylase) (Murray, 2000). The HsdS-HsdM complex recognises specific sequences of DNA and initiates full methylation, preventing DNA cleavage by the HsdS-HsdM-HsdR complex (Murray, 2000). DNA cleavage by Type I RM does not occur at the site of DNA recognition (Murray, 2000). Type II RM systems encode a methyltransferase as well as a classical restriction endonuclease such as \( \text{Sau3AI} \) that cleaves DNA at a sequence specific recognition site (Murray, 2000). Type IV RM systems encode \( \text{SauSI} \), a type IV endonuclease, which recognises methylated cytosine in the motif \( C/G^{\text{m}}CNGC/G \) (Xu et al., 2011).

Type I RM systems are encoded in all examined *S. pseudintermedius* genomes analysed but the presence of incomplete systems, which do not contain a full complement of \( \text{hsdR}, \text{hsdM}, \) and \( \text{hsdS} \) genes, were identified in ED99, HKU10-03,
GL118B and 4639949 (McCarthy et al., 2015). The presence of 2 hsdR variants suggest that HGT among the S. pseudintermedius population may be restricted into 2 lineages (McCarthy et al., 2015). The presence of a Type II RM system was also identified in 6 of the genomes with the genes encoded at 4 different loci depending on the S. pseudintermedius strain (McCarthy et al., 2015). Type IV RM SauSI homologues have also been identified within S. pseudintermedius ED99 (Monk and Foster, 2012). All of these RM systems could restrict gene flow and therefore genetic manipulation of S. pseudintermedius.

1.1.8 Zoonotic Transmission of S. pseudintermedius

S. pseudintermedius is adapted to the family Canidae composed of dogs, wolves, and foxes and clinically has low zoonotic potential (Bannoehr et al., 2009; Guardabassi et al., 2012). Isolation of S. pseudintermedius from cases of feline pyoderma, as well as from horses and humans are presumed to be due to direct contact with dogs (Gómez-Sanz et al., 2013a; Kadlec et al., 2010; Loeffler et al., 2007; Nienhoff et al., 2011; Ruscher et al., 2010; Stull et al., 2014). Longitudinal studies demonstrate transient colonisation of humans with S. pseudintermedius with persistent human colonisation rarely documented (Gómez-Sanz et al., 2013b; Guardabassi et al., 2004; Laarhoven et al., 2011; Windahl et al., 2012). Veterinary staff are more frequently colonised with MRSP than methicillin sensitive S. pseudintermedius (MSSP) suggesting that the main zoonotic concern for human health is through the transmission of antimicrobial resistance elements to human-related pathogens such as S. aureus (Paul et al., 2011). However, these concerns may be unfounded with few antimicrobial resistance elements shared between S. aureus and S. pseudintermedius (Damborg et al., 2015; Guardabassi et al., 2004).

Despite of this low zoonotic potential, increasingly S. pseudintermedius mediated human infections do not have obvious canine links (Börjesson et al., 2015; Lee et al., 2015; Savini et al., 2013a). In particular, ST71 MRSP has been demonstrated to have increased virulence with high levels of human and canine corneocyte binding in comparison to other MRSP and MSSP strains and biofilm formation (Latronico et al., 2014; Osland et al., 2012). Of note, the ST71 clone was responsible for a 14-month nosocomial outbreak in a veterinary teaching hospital characterised by patient-to-
patient transmission that was difficult to control (Grönthal et al., 2014). This is particularly concerning as *S. pseudintermedius* is an opportunistic pathogen that is not thought to be associated commonly with contagious infections (Simou et al., 2005). Evidence that ST71 MRSP has evolved for human-to-human spread has been demonstrated by a hospital outbreak in which 4 elderly patients were MRSP positive with 3 of the patients presenting with MRSP culture positive skin infections (Starlander et al., 2014). No canine link was determined in this outbreak with one patient remaining persistently colonised and presenting with recurrent skin infections (Starlander et al., 2014). Taken together, these data suggest an increased zoonotic potential of ST71 MRSP.

1.1.9 Host Specificity of *Staphylococcus aureus*

*S. aureus* has co-evolved with the human host and causes a range of opportunistic human infections including skin infections, toxic shock, and pneumonia (Tenover et al., 2006). *S. aureus* is also a veterinary pathogen capable of mediating disease in multiple hosts including mastitis in ruminants, skin abscesses in chickens, and subcutaneous abscesses in rabbits (Bergonier et al., 2003; Hermans et al., 2003). Genomic analysis of *S. aureus* populations have identified specific clones associated with particular hosts. For instance, clonal complex 133 (CC133) strains are ruminant-adapted while CC385 strains are avian-adapted (Ben Zakour et al., 2008; Lowder et al., 2009). Other clonal complexes are associated with multiple hosts including CC5, which is a major clade of both human and poultry strains, and CC97, which contains both human and bovine strains (Lowder et al., 2009; Spoor et al., 2013). The ability of *S. aureus* to adapt to multiple hosts is mostly dependent on the acquisition of host specific genes encoded by MGEs (Ben Zakour et al., 2008). Both poultry- and bovine-adapted strains contain unique *S. aureus* pathogenicity islands (SaPIs) and prophages that encode genes proposed to mediate host adaptation (Guinane et al., 2010; Lowder et al., 2009). One example is SaPI-encoded von Willebrand factor-binding protein (vWbp) that is required to coagulate ruminant and equine plasma, a fundamental pathogenic characteristic of *S. aureus* infection (Guinane et al., 2010; Viana et al., 2010). Host adaptation can also occur due to mutations in the core genome with a single mutation in the dltB gene sufficient to provide host adaptation of *S. aureus* to
rabbits (Viana et al., 2015). The presence of pseudogenes is often linked to host adaptation with genes specific for human infection no longer required (Guinane et al., 2010; Herron-Olson et al., 2007; Lowder et al., 2009). These pseudogenes are often virulence factors central to S. aureus pathogenesis in humans such as toxins and cell surface proteins as well as metabolic and regulatory genes (Guinane et al., 2010; Herron-Olson et al., 2007; Lowder et al., 2009). Cell surface proteins involved in host adhesion, such as clumping factor A (ClfA), have been identified as pseudogenes in bovine and ovine mastitis isolates suggesting that adhesins are less important during mastitis infections (Guinane et al., 2010; Herron-Olson et al., 2007). Variation in cell surface proteins is common among S. aureus isolates with particular lineages exhibiting a unique repertoire of surface proteins (Lindsay et al., 2006).

1.2 Extracellular Matrix Binding of S. pseudintermedius

1.2.1 Interaction of S. pseudintermedius to Extracellular Matrix Proteins

The ability of S. pseudintermedius to adhere to and colonise canine skin may be important in the development of canine pyoderma. S. pseudintermedius isolates bind more strongly to skin from atopic patients compared to healthy controls with adherence to canine corneocytes being greater than that of S. aureus (Forsythe et al., 2002; McEwan et al., 2006; Simou et al., 2005). Four genetically diverse strains also demonstrated increased binding to corneocytes isolated from persistent carriers compared to non-carriers (Paul et al., 2013). These observations suggest that S. pseudintermedius strains are capable of interacting with extracellular matrix (ECM) proteins that may be differentially expressed in atopic versus healthy and carrier versus non-carrier hosts. This is supported by the ability of strains isolated from canine pyoderma to bind to the ECM proteins fibronectin (Fn), vitronectin, and laminin with increased affinity, in comparison to strains isolated from healthy carriers (Cree and Noble, 1995). In support of this study, the variable ability of S. pseudintermedius strains to adhere to human fibrinogen (Fg), Fn, and cytokeratin-10 (CK-10) was observed in 2 other studies (Geoghegan et al., 2009; Schmidt et al., 2009). Schmidt et al (2009) found that poor adherence to one ECM protein could predict poor adherence to all tested ECM proteins, supporting the earlier research, except that the binding did
not correlate with isolation from healthy or pyodermic skin (Schmidt et al., 2009). Contradictory to this, Geoghegan et al (2009) found that poor binding to one ECM protein could not predict universally poor binding. Further, the investigation found that strain 326 adhered to the Fg α-chain and the N-terminal of Fn, suggesting the presence of proteins in *S. pseudintermedius* that resemble ECM-binding proteins of *S. aureus* (Geoghegan et al., 2009). Importantly, it is known that *S. aureus* ECM-binding proteins are centrally involved in *S. aureus* pathogenesis (Foster et al., 2014).

1.2.2 Identification of Cell Wall Associated Proteins encoded by *S. pseudintermedius* ED99

Cell wall associated (CWA) proteins encode an N-terminal secretory signal sequence that targets the protein for secretion by the Sec apparatus. Many signal sequences contain a conserved YSIRK-G/S motif that promotes secretion at the cell division site, rather than the cell pole, allowing distribution of the protein over the whole cell surface (DeDent et al., 2008). After membrane translocation, the signal sequence is cleaved by the signal peptidase I and the protein becomes anchored to the cell wall due to the C-terminal sorting signal composed of an LPXTG motif, a hydrophobic domain and a positively charged tail (Cregg et al., 1996; Schneewind et al., 1992). The hydrophobic domain remains cell membrane-associated where the LPXTG motif is recognised by the membrane-bound sortase A enzyme and undergoes cleavage between the threonine and glycine residues (Mazmanian et al., 1999; Schneewind et al., 1995). This LPXTG motif cleavage transfers the protein to a glycine residue in a pentaglycine cross-link of peptidoglycan covalently attaching it to the cell wall (Schneewind et al., 1995). CWA proteins can be released from the peptidoglycan by the endopeptidase LytM (Ramadurai and Jayaswal, 1997). Variations in either the signal peptide, for instance the SraP contains a long signal peptide, or the sorting signal, for instance IsdC contains an NPQTN motif, can require secretion by the accessory Sec system or processing by sortase B respectively (Marraffini and Schneewind, 2005; Siboo et al., 2008).

These common secretion and sorting characteristics of CWA proteins allow their coding sequences to be identified from whole genome sequences (Roche et al., 2003a). This bioinformatic approach was used to identify predicted CWA proteins of *S. pseudintermedius*, using the whole genome sequence of ED99 (Ben Zakour et al.,
Eighteen genes encoding CWA proteins were identified by the presence of an N-terminal signal peptide, a C-terminal LPXTG motif, as well as repeat regions typical of *S. aureus* CWA proteins (Bannoehr et al., 2011). The family of proteins were named *S. pseudintermedius* surface (Sps) proteins A-R (Figure 1.2) (Bannoehr et al., 2011). SpsP and SpsQ contained protein domains characteristic of the CWA SpA of *S. aureus* (Bannoehr et al., 2011). SpA is a key virulence factor of *S. aureus* exhibiting numerous roles in immune evasion and prevents an effective host immune response being developed during *S. aureus* infection (Kim et al., 2015b).

### 1.2.3 Identification of Extracellular Matrix Binding Proteins of *S. pseudintermedius* ED99

One family of CWA proteins present in all Gram positive bacteria are the Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMMs) (Patti et al., 1994a). This family of proteins interact with ECM proteins such as Fg, Fn, and collagen and are encoded by all Staphylococcal species with the 9 MSCRAMMs encoded by *S. aureus* being extensively studied (Foster et al., 2014). To identify potential MSCRAMMs in *S. pseudintermedius*, each of the Sps proteins identified in ED99 were analysed for the archetypal MSCRAMM domains including an N-terminal signal sequence, a non-repeated A-domain with 2 IgG-like folds, C-terminal tandem repeat regions, and a LPXTG anchor (Bannoehr et al., 2011). Three of the 18 Sps proteins contain all these features, SpsD, SpsL (Figure 1.3), and SpsO, with SpsI lacking a signal peptide (Bannoehr et al., 2011). Southern blot analysis and PCR amplification demonstrated that the *spsD* gene is present in all *S. pseudintermedius* strains as well as strains of *S. intermedius* and *S. delphini*, the *spsL* gene is specific to all *S. pseudintermedius* strains, and the *spsO* gene is only present in certain *S. pseudintermedius* strains (Bannoehr et al., 2011). The C-terminal repeat region of SpsL shares weak sequence homology to the Fn binding proteins (FnBPs) of *S. aureus* while the N-terminal repeat region of SpsD contains a novel connecting (C) region and B_{SDR} repeat domains (Figure 1.3) (Bannoehr et al., 2011).
Figure 1.2. *S. pseudintermedius* Surface Proteins Identified from the Whole Genome Sequence of ED99. Schematic representation of the *S. pseudintermedius* ED99 genome (~2.6 Mb) highlighting the location of 18 genes encoding CWA proteins SpsA-SpsR. Genes encoding all the characteristic features of MSCRAMMs are highlighted in red. The origin of replication (~250 kb) is also labelled. Adapted from (Bannoehr et al., 2011).
**Figure 1.3. Schematic Representation of the Domain Structure of Typical S. aureus MSCRAMMs and S. pseudintermedius SpsD and SpsL proteins.** All MSCRAMMs encode an N-terminal signal peptide (SP) (red) and C-terminal cell wall spanning fragment (W), LPXTG anchor, and membrane spanning fragment (M). The proteins contain an A-domain with N1, N2, and N3 subdomains of roughly 500 residues (yellow) and a C-terminal repeat region. All Sdr proteins contain serine-aspartic acid repeats with some Sdr proteins, and SpsD, containing B_{SDR} repeats, of unknown function (green). FnBPs and SpsL contain Fn-binding repeats (FnBRs) with FnBPA containing 11 repeats and FnBPB containing 10 repeats (blue). Cna contains B_{CNA} repeats (green) of unknown function with varying numbers between strains. SpSD contains a novel region C (purple) and Pro rich repeats (light blue).
To determine if SpsD and SpsL are capable of binding to the ECM proteins Fg, Fn, and CK-10, the surrogate host *Lactococcus lactis* subsp. *cremoris*, which does not express native proteins capable of mediating adherence to ECM proteins, was employed (Que et al., 2000). Expression of full-length SpsD and SpsL using this system identified that SpsD and SpsL are both sufficient to mediate adherence to Fg and Fn and SpsD also provides adherence to CK-10 (Bannoehr et al., 2011). Expression of SpsD on the surface of *L. lactis* was sufficient to promote adherence to canine corneocytes whereas expression of SpsL was not (Bannoehr et al., 2012). Antibodies present within convalescent sera of canine pyoderma patients reacted with recombinant proteins of both SpsD and SpsL A-domains (Bannoehr et al., 2011). This indicates that these proteins are expressed during infection and induce a humoral immune response.

1.3 *Staphylococcus aureus* MSCRAMMs

As mentioned, MSCRAMMs are characterised by the presence of 2 IgG-like folds in the N-terminal A-domain that promote ligand-binding via a conserved binding mechanism (Foster et al., 2014). Even though all MSCRAMMs contain these structurally similar folds, the A-domain of different proteins contain limited sequence homology. For example, ClfA and ClfB share only 26% amino acid sequence identity within the A-domain (Ní Eidhin et al., 1998). Most MSCRAMMs are capable of binding to multiple ligands using these conserved structures with high levels of ligand-binding redundancy in the MSCRAMM family. For example, 5 of the 9 MSCRAMMs encoded by *S. aureus* exhibit Fg-binding activity (Clarke et al., 2009; Ganesh et al., 2011; Keane et al., 2007a).

1.3.1 The SD Repeat Family

The serine-aspartic acid (SD) repeat (Sdr) family of *S. aureus* is made up of ClfA, ClfB, SdrC, SdrD, SdrE, and bone sialoprotein-binding protein (Bbp). This family of proteins are differentiated from other MSCRAMMs in that they contain a C-terminal stretch of SD dipeptide repeats called the R region (McDevitt et al., 1994). This R region varies in length between bacterial isolates and acts as a ‘stalk’ to project the functional N-terminal A-domain away from the cell membrane (Hartford et al., 1997;
McDevitt and Foster, 1995). The presence of capsular polysaccharides can prevent ClfA ligand-interactions with longer R regions capable of overcoming this masking effect (Risley et al., 2007). SdrC, SdrD, SdrE, and Bbp also contain additional repeat domains called B_{SDR} domains that separate the SD repeats and the N-terminal A-domain (Josefsson et al., 1998a). SdrC contains 2, SdrD contains 5, and both SdrE and Bbp contain 3 B_{SDR} domains that show high sequence similarity (Josefsson et al., 1998a; Tung et al., 2000). High affinity calcium binding via EF-hand motifs provides stable secondary structures to these B_{SDR} domains creating a barrel of 3 β-strands (Josefsson et al., 1998b; Wang et al., 2013). The function of these B_{SDR} domains is unknown.

1.3.1.1 Ligand-Binding Mechanism

Of the SD family of proteins in *S. aureus* ClfA and ClfB are the most extensively characterised and are regarded as archetypal MSCRAMMs (McDevitt et al., 1992; Ní Eidhin et al., 1998). Both of these proteins interact with Fg via the N-terminal A-domain with the recombinant N2N3 subdomains sufficient for ligand-binding (McDevitt et al., 1995; Perkins et al., 2001). Residues 211 to 220 of the N1 subdomain of MSCRAMMs are not directly involved in ligand-binding but display chaperone-like activity essential for export and expression of all MSCRAMMs on the surface of *S. aureus* (McCormack et al., 2014). The N2N3 subdomains of ClfA adhere to the C-terminal Fg γ-chain at the same site as the α_{IIb}β_{3} platelet receptor with the N2N3 subdomains of ClfB adhering specifically to tandem repeat 5 of the Fg α-chain (McDevitt et al., 1997; Walsh et al., 2008). The N2N3 subdomains of ClfB also exhibit adherence to the C-terminal glycine-serine-rich Ω loops of CK-10 and multiple sites within loricrin (Mulcahy et al., 2012; O'Brien et al., 2002; Walsh et al., 2004).

Crystal structures of ClfA A-domain confirmed the presence of 3 independent subdomains N1, N2, and N3 (Deivanayagam et al., 2002; Perkins et al., 2001). The N1 subdomain is highly hydrophilic and disordered whereas the N2 and N3 subdomains contain novel DEv-IgG folds composed of anti-parallel β-sheets (Deivanayagam et al., 2002; Perkins et al., 2001). Connecting the 2 subdomains is a hydrophobic trench that directly interacts with the Fg γ-chain in the case of ClfA or the Fg α-chain and CK-10 in the case of ClfB (Ganesh et al., 2011; Ganesh et al., 2008; Xiang et al., 2012). The
binding interactions of both ClfA and ClfB mimicked the binding mechanism previously determined for SdrG of *Staphylococcus epidermidis* that binds to the Fg β-chain thrombin cleavage site (Davis et al., 2001; Ponnuraj et al., 2003). ClfA, ClfB, and SdrG interact with their ligands using a “dock, lock, and latch” (DLL) binding mechanism (Figure 1.4) (Ponnuraj et al., 2003). In SdrG this binding mechanism requires an open conformation with ligand binding occurring via β-strand complementation and conformational changes in the C-terminal of the N3 subdomain that lock the ligand in place (Figure 1.4) (Ponnuraj et al., 2003). Latching of the ligand then occurs with C-terminal disordered residues of the N3 subdomain creating an additional β-strand with the N2 subdomain to stabilise the ligand-bound structure (Figure 1.4) (Ponnuraj et al., 2003). This structural model identified a linker sequence (Ile581 to Ser586) that makes up the binding trench as well as a latch sequence (Ser586 to Gly592) (Ponnuraj et al., 2003). The TYTFTDYVD sequence conserved in MSCRAMMs is demonstrated, within the ligand-bound complex, to make up the back of the latching cleft suggesting that this conserved motif may be required for the structural preservation of MSCRAMM A-domains (Josefsson et al., 1998a; Ponnuraj et al., 2003).

A variation of this binding mechanism is utilised by ClfA and ClfB with the orientation of the bound ligand reversed with β-strand complementation occurring in a parallel rather than anti-parallel orientation (Ganesh et al., 2011; Ganesh et al., 2008; Xiang et al., 2012). ClfA is able to bind to the Fg γ-chain in both an open or closed state but for ClfB an open state is required for ligand-binding with both Fg and CK-10 competing for the same binding trench (Ganesh et al., 2011; Ganesh et al., 2008; Xiang et al., 2012). Site-directed mutagenesis confirmed the requirement of specific residues in the DLL binding mechanism in both ClfA and ClfB (Ganesh et al., 2011; Ganesh et al., 2008; Xiang et al., 2012). The presence of Ca²⁺ ions reduce the ligand-binding of both ClfA and ClfB (Ní Eidhin et al., 1998; O’Connell et al., 1998). In the case of ClfB, ligand binding is also effected by the cleavage of the N1 subdomain by the zinc metalloprotease aureolysin (McAleese et al., 2001; Perkins et al., 2001). The level of cleaved protein increases with bacterial growth explaining the loss of functional ClfB in late exponential and stationary phases (McAleese et al., 2001).
Figure 1.4. Dock, Lock, and Latch Binding Mechanism of SdrG. Schematic of the DLL binding mechanism utilised by SdrG of *S. epidermidis* to interact with Fg. The open form allows ligand binding into the trench between the N2 and N3 subdomains. Upon ligand binding the C-terminal of the N3 subdomain locks the ligand in place with the latch creating β-strand complementation with the N2 subdomain. Image taken from (Foster et al., 2014).
The crystal structure of the N2N3 subdomains of SdrD demonstrate the same DEv-IgG folds suggesting ligand binding via DLL (Wang et al., 2013). A ligand for SdrD has not yet been identified but high structural similarity and conservation of binding site residues to ClfB suggest an interaction with the Fg α-chain (Wang et al., 2013). Structural studies have not been performed for SdrC, SdrE, or Bbp but it is likely that they also contain globular subdomains.

1.3.1.2 Role in Pathogenesis and Immune Evasion

Fg is a 340 kDa glycoprotein that is predominantly found in the blood and is involved in clot formation (Mosesson, 2005). It is a dimer of trimers composed of 2 α-, 2 β-, and 2 γ-chains that can be differentiated into outer D- and central E-domains (Figure 1.5) (Mosesson, 2005). During the coagulation cascade, thrombin cleavage of Fg releases fibrinopeptide A and B (FpA and FpB) allowing cross-linking of Fg and fibrin formation (Figure 1.5) (Mosesson, 2005). Direct interactions of the C-terminal Fg γ-chain with the integrin αIIbβ3 platelet receptor allow effective clot formation and wound healing (Mosesson, 2005). The significance of the interaction of ClfA to Fg has been directly analysed in murine models using strains that express Fg-binding deficient ClfA (Flick et al., 2013; Josefsson et al., 2008). Mutations in P336 and Y338 ablate Fg-binding of ClfA and S. aureus strains containing these mutations exhibit decreased virulence in both a sepsis and septic arthritis murine model of infection (Josefsson et al., 2008; Loughman et al., 2005). Murine infection models in mice lacking the Fg γ-chain binding site of ClfA demonstrate that bacterial Fg-binding in the acute phase of sepsis is detrimental to the host whereas bacterial Fg-binding in an intra-peritoneal model is detrimental to the bacteria (Flick et al., 2013). Both of these studies highlight the significance of ClfA Fg-binding in the development of S. aureus infections in vivo.
Figure 1.5. Schematic Representation of Fibrinogen and the Formation of Fibrin.  
(A) Fg is a dimer of trimers made up of 2 α-, β-, and γ-chains. The central region of Fg is termed the E-domain and is flanked by 2 symmetrical D-domains. (B) Fibrin formation occurs when fibrinopeptides A and B (FpA and FpB) are cleaved from the N-terminal α- and β-chains by thrombin. Fibrin formation then occurs through cross-linking of the C-terminal γ-chain with the N-terminal of the α-chain and the C-terminal β-chain with the N-terminal of the β-chain (Everse et al., 1998). Adapted from (Chernysh et al., 2012).
Infective endocarditis is defined by the development of platelet-bacteria thrombi on heart valves due to bacterial-mediated platelet aggregation. ClfA, ClfB, and SdrE are all sufficient to induce platelet activation and aggregation in vitro with ClfA shown to be essential for platelet aggregation under high shear conditions (Kerrigan et al., 2008; McDevitt et al., 1997; Miajlovic et al., 2007; O’Brien et al., 2002). L. lactis expressing ClfA induces rapid platelet aggregation through 2 indirect platelet interactions binding to the αIIbβ3 platelet receptor via Fg, and binding to the FcγRIIa platelet receptor via antibodies directed against ClfA A-domain (Loughman et al., 2005). Both of these pathways were required for rapid platelet aggregation of L. lactis expressing ClfA with the expression of Fg-binding deficient ClfA displaying reduced platelet aggregation (Loughman et al., 2005). ClfA expressed on the surface of L. lactis is also sufficient to promote fibrin-platelet clot binding and infective endocarditis in a murine model and adherence to ventricular assist devices coated in Fg (Arrecubieta et al., 2006; Que et al., 2001).

The importance of ClfB mediated Fg-binding to S. aureus pathogenesis cannot be investigated using murine models due to the lack of murine Fg-binding but the relevance of CK-10 and loricrin interactions can be investigated (Walsh et al., 2008). CK-10 and loricrin are both components of the skin with CK-10 abundant in epidermal cells and loricrin an important component of the cornified cell envelope (Mulcahy et al., 2012). ClfB mediates adherence to keratinocytes and nasal epithelial cells with strains lacking the clfB gene exhibiting reduced murine and human colonisation (O’Brien et al., 2002; Schaffer et al., 2006; Wertheim et al., 2008). Human colonisation decreased from a median of 7 d in the wild type to 3 d in the clfB isogenic mutant (Wertheim et al., 2008). Loricrin is the key ligand of ClfB mediating nasal colonisation with loricrin-deficient mice displaying low levels of S. aureus colonisation (Mulcahy et al., 2012). Further analysis demonstrated that ClfB is required for sustained colonisation of murine nares with wall teichoic acid required for the initial adherence to nasal epithelial cells (Baur et al., 2014; Mulcahy et al., 2012). L. lactis expressing SdrC and SdrD also exhibit binding to squamous cells suggesting a role in nasal colonisation in an as yet undefined manner (Corrigan et al., 2009).
Definitive roles in the pathogenesis of *S. aureus* have not been defined for the other Sdr proteins with the ligand for SdrE not yet reported. SdrC adheres to the N-terminal of β-neurexin but the significance of this interaction during *S. aureus* infection is unknown (Barbu et al., 2010). As the name suggests, Bbp was originally identified as a virulence factor in orthopaedic infections mediating binding to bone sialoprotein (Tung et al., 2000). Bbp can also interfere with thrombin-induced Fg coagulation by direct binding of residues 561 to 575 of the human Fg α-chain (Vazquez et al., 2011).

The Sdr family of *S. aureus* MSCRAMMs also has immune evasion characteristics. The serine of SD repeats of all Sdr proteins are glycosylated by the glycosyltransferases SdgA and SdgB that are encoded adjacent to the *sdrCDE* locus (Hazzenbos et al., 2013). SdgB provides N-acetylglucosamine glycosylation with SdgA producing disaccharide decoration of each serine (Hazzenbos et al., 2013). This glycosylation inhibits cleavage of ClfA by the neutrophil protease cathepsin G that could otherwise interfere with the adhesive potential of *S. aureus* during infection (Hazzenbos et al., 2013). SD glycosylation also effects *S. aureus* mediated agglutination via an unknown mechanism (Thomer et al., 2014). ClfA, Bbp, and SdrE also evade the innate immune system by directly binding to complement factor I for ClfA and factor H and C4BP for both Bbp and SdrE (Hair et al., 2013; Hair et al., 2008; Sharp et al., 2012). These interactions with complement components decrease complement-mediated phagocytosis of *S. aureus* (Hair et al., 2010; Hair et al., 2013; Higgins et al., 2006; Sharp et al., 2012). In the case of ClfA, Fg-binding is required to activate factor I to allow immune evasion but ClfA is able to interact with factor I in the absence of Fg (Hair et al., 2010).

1.3.1.3 Therapeutic Potential

The surface expression of MSCRAMMs suggest that they interact with the host immune system and therefore could be good vaccine candidates. For ClfA and ClfB this has been exemplified by the presence of antibodies specific for these antigens in human patients with infective endocarditis (Rindi et al., 2006). These naturally-produced antibodies exhibit Fg-binding inhibitory activity and indicate the expression of both of these proteins during human infection (Rindi et al., 2006).
Murine vaccine trials have enhanced the notion that both ClfA and ClfB are good vaccine candidates. Active vaccination with ClfB A-domain reduced levels of murine nasal colonisation with the induced anti-ClfB antibody response sufficient to decolonise the nasal mucosa (Schaffer et al., 2006). Both active and passive immunisation with ClfA A-domain demonstrated decreased virulence in a murine model of septic arthritis with immunisation exhibiting decreased bacterial load within infected joints (Josefsson et al., 2001). Vaccination with ultraviolet-killed L. lactis expressing ClfA provided protection against endocarditis challenge with strain Newman and induced expression of the proinflammatory cytokine IL-17A (Veloso et al., 2015). ClfA has also shown promise in combination vaccines with antibodies raised against a fusion protein of immunogenic regions of ClfA, iron-regulated surface protein B (IsdB), and γ-haemolysin displaying increased opsonisation of the challenge S. aureus strain and decreased bacterial loads in a murine intraperitoneal model (Delfani et al., 2015). Combination vaccines containing ClfA have also provided protection in bloodstream infections in immunocompromised mice potentially more closely reflecting the scenario encountered during particular human infections (Rauch et al., 2014).

Despite these positive murine vaccination experiments, human trials using passive vaccination of ClfA have so far been unsuccessful with no reduction in bacteraemia or late-onset of neonatal sepsis (DeJonge et al., 2007; Weems et al., 2006). Examination of CWA vaccines using rabbit models of infection more closely mimicked the results of human trials and suggested that antibodies directed against bacterial CWA proteins could induce dangerous bacterial aggregates (Spaulding et al., 2014). Future investigations into the development of a S. aureus vaccine may require evaluation in model systems other than the mouse in order to more closely mimic the human S. aureus infection (Salgado-Pabon and Schlievert, 2014).

The development of an effective vaccine can be dependent on the conservation of the vaccine antigens among the bacterial population. The gene variability of both clfA and clfB has been investigated in 224 S. aureus isolates (Murphy et al., 2011). Both genes were present in all isolates except for one strain that contained a truncated version of ClfA and 9 strains that contained truncated versions of ClfB (Murphy et al., 2011).
Point mutations were responsible for the low genetic variation identified between strains with the 2 most diverse ClfA alleles displaying 86% amino acid identity and the 2 most diverse ClfB alleles displaying 94% amino acid identity (Murphy et al., 2011). ClfA and ClfB variants do not display functional diversity with the residues lining the ligand-binding trench conserved in all strains (Murphy et al., 2011). Even though the sequence diversity of ClfA is relatively low, antigenic variation was detected between 2 *S. aureus* strains with decreased antibody affinity for the heterologous versus the homologous strain (Brady et al., 2013). This suggests that a ClfA-targeted vaccine may not provide universal protection.

The low conservation of *sdrE* among *S. aureus* strains suggests that this would not be a viable vaccine candidate (McCarthy and Lindsay, 2010). On the other hand, *sdrC* and *sdrD* are only absent in 10.3% of strains analysed (McCarthy and Lindsay, 2010). The only vaccine currently trialled for the Sdr proteins was a combination vaccine of SdrD, SdrE, IsdA, and IsdB that induced opsonophagocytic antibodies and provided protection in a murine abscess infection model (Stranger-Jones et al., 2006). More work is needed to closely evaluate the promise of SdrD as a vaccine candidate.

### 1.3.2 Fibronectin Binding Protein A and B

#### 1.3.2.1 Ligand-Binding Mechanism

The characteristic feature of FnBPA and FnBPB in comparison to other MSCRAMM proteins is the C-terminal repetitive region consisting of Fn-binding repeats (FnBRs) with FnBPA having 11 and FnBPB having 10 repeats (Flock et al., 1987; Joh et al., 1998; Meenan et al., 2007). These repeats undergo a disordered-to-ordered transition in the presence of Fn to produce stable $\beta$-sheets (House-Pompeo et al., 1996). Each repeat adheres to the N-terminal F1 modules of Fn with high and low affinity repeats characterised (House-Pompeo et al., 1996; Joh et al., 1998; Meenan et al., 2007). Mechanical stress may be able to regulate the binding interaction of FnBRs and Fn suggesting that *S. aureus* can differentiate Fn present in healthy or damaged tissue (Chabria et al., 2010). FnBPs adhere to Fn using a tandem $\beta$-zipper binding interaction with each FnBR contributing a 4th antiparallel strand to the triple stranded $\beta$-sheet of an F1 module (Figure 1.6) (Schwarz-Linek et al., 2003). Crystal structures of FnBRs
in complex with F1 modules demonstrate that each FnBR forms an antiparallel β-strand with 4 sequential F1 modules (Figure 1.6) (Bingham et al., 2008). These complexes are assumed to be relevant during human infection due to the presence of natural antibodies that only adhere to ligand induced epitopes (Meenan et al., 2007). Specifically the antibodies recognise FnBR-F1 complexes but not disordered FnBR (Meenan et al., 2007).

FnBPs also interact with ECM proteins via the N-terminal A-domain. Both FnBPA and FnBPB bind to the Fg γ-chain, α-elastin, and tropoelastin with FnBPB exhibiting higher affinity for all ligands (Burke et al., 2011; Keane et al., 2007b; Roche et al., 2004; Wann et al., 2000). FnBPB also adheres to Fn via the A-domain with a lower affinity than the interaction observed at the FnBR suggesting that the key Fn-binding site in vivo is likely to be FnBR-mediated (Burke et al., 2011). Fg-binding of FnBPB is inhibited in the presence of Ca$^{2+}$ ions but this is not the case for FnBPA (Burke et al., 2011; Wann et al., 2000). Three dimensional modelling demonstrated that the N2N3 subdomains of FnBPA are sufficient for ligand-binding with the identification of a putative latch suggesting that the binding mechanism is DLL mediated (Keane et al., 2007a). In contrast, binding of both FnBPs to tropoelastin and FnBPB to Fn does not require either the binding trench residues in the case of tropoelastin, or the latch sequence in the case of Fn for adherence (Burke et al., 2011; Keane et al., 2007b). The crystal structure of the FnBPA N2N3 subdomains in complex with a Fg γ-chain peptide demonstrated that FnBPA does not require the latch region for binding to any ligand in the N2N3 trench with the presence of the latch enhancing binding to Fg by only 2.5 times (Stemberk et al., 2014). The close proximity of the most N-terminal FnBR to the A-domain Fg-binding site suggests that Fn-binding by the FnBR is likely to prevent Fg-binding (Stemberk et al., 2014) This highlights that Fn-binding is probably the predominant function of FnBPs in vivo (Stemberk et al., 2014).
Figure 1.6. Tandem β-zipper interaction of FnBPs to Fibronectin. Fn-binding by the FnBRs of FnBPs results in a disordered to ordered transition with FnBRs contributing β-strands to the Fn F1 modules. This β-strand conformation allows one FnBR to adhere to multiple F1 modules. Image taken from (Schwarz-Linek et al., 2004).
1.3.2.2 Role in Pathogenesis and Immune Evasion

Comparison of strains isolated from either invasive disease or carriage associate the presence of the fnbA gene to invasive disease with specific FnBR mutations linked to clinical disease (Hos et al., 2015; Lower et al., 2011; Peacock et al., 2002). Atomic force microscopy (AFM) determined that these specific SNPs increase Fn-binding affinity suggesting that high levels of Fn-binding are important features of S. aureus disease (Lower et al., 2011). Fn is a 440 kDa glycoprotein present as a soluble dimer in human blood and as insoluble fibrils as part of the ECM (Pankov and Yamada, 2002). Fn is made up of 3 types of modules, type I (responsible for the interaction with collagen and fibrin), II, and III (responsible for interaction with integrins via the RGD sequence) that are each composed of 2 anti-parallel β-sheets (Pankov and Yamada, 2002). As well as a structural role in the ECM, Fn is also important during wound healing and clot formation (Pankov and Yamada, 2002). Due to this role in clot formation and wound healing, both FnBPs are capable of aggregating human plasma and bind to activated platelets with FnBPA capable of inducing platelet aggregation (Heilmann et al., 2004; Sinha et al., 2000). Interactions with clot fibrin are important during platelet aggregation with FnBPA directly interacting with Factor XIIIa of the coagulation cascade resulting in cross-linking of FnBPA glutamine103 with the α-chain of fibrin (Matsuka et al., 2003; Severina et al., 2006). Multiple pathways promote FnBPA-mediated platelet aggregation with indirect interactions to the αIIbβ3 platelet receptor via both Fn and Fg, and the FcγRIIa platelet receptor via FnBPA specific antibodies (Fitzgerald et al., 2006). This FnBPA-fibrin-platelet interaction is sufficient to induce infective endocarditis when FnBPA is expressed on the surface of L. lactis (Que et al., 2001).

FnBPs also facilitate disease persistence by mediating S. aureus invasion into multiple host cell types including keratinocytes, endothelial cells, epithelial cells, and osteoblasts (Clarke and Foster, 2006). Electron microscopy demonstrated that FnBPs are not essential for S. aureus invasion but deletion of both fnbA and fnbB genes produced a 64.4% decrease in internalisation (Brouillette et al., 2003). The presence of FnBPA on the surface of L. lactis or on the surface of a bead are sufficient for invasion with both the Fg-binding A-domain and FnBR of FnBPA required for
invasion of cardiac valve endothelium (Arrecubieta et al., 2006; Que et al., 2005; Sinha et al., 2000). Invasion occurs via a Fn bridge and interaction with α5β1 integrin that can be blocked by an RGD-containing peptide (Fowler et al., 2000; Sinha et al., 1999). Live cell imaging demonstrated that FnBP-dependent invasion is a slow process requiring α5β1 integrin clustering and actin remodelling that is followed by phagosome formation and internalisation of the bacterium (Schröder et al., 2006). The speed of FnBPA-mediated invasion can be shortened by increasing the number of FnBR present on the bacterial surface either by increasing the number of FnBRs or increasing the expression of FnBPA (Edwards et al., 2010). Manipulating FnBPA to increase the speed of invasion directly correlated with increases in virulence in a murine model of sepsis demonstrating the importance of FnBPs in S. aureus pathogenesis (Edwards et al., 2010).

FnBPs are also implicated in S. aureus persistence by mediating biofilm formation in both ica-dependent and ica-independent manners (O'Neill et al., 2008). FnBP-dependent biofilm was observed in methicillin resistant S. aureus (MRSA) strains and required the A-domain but not the ability to bind Fg (O'Neill et al., 2008). In the CA-MRSA strain LAC, FnBPs are essential for biofilm formation with FnBPs uncharacteristically expressed during stationary phase (McCourt et al., 2014). AFM identified that FnBPs mediate biofilm formation using homophilic Zn$^{2+}$-dependent dimerization of the A-domain providing strong cell-to-cell adhesion (Herman-Bausier et al., 2015). A similar mechanism facilitates SdrC-mediated biofilm formation suggesting that A-domain dimerization may be a common feature of S. aureus MSCRAMMs (Barbu et al., 2014).

### 1.3.2.3 Therapeutic Potential

The disordered nature of the FnBR suggests that this region may not be strongly antigenic and greater focus has been given to the vaccine potential of the A-domain of FnBPA. Residues 110 to 263 of FnBPA are highly immunogenic and protect mice from a lethal S. aureus challenge due to increased opsonophagocytosis of the challenge strain (Zuo et al., 2014). FnBPA has also demonstrated vaccine promise in combination with other antigens with a fusion vaccine providing high antibody titre and protection in an intraperitoneal murine model (Zhou et al., 2006). Combination
vaccination with ClfA provided protection in a murine aortic patch model, and a vaccine combination with ClfA, SdrD and a non-functional SpA provided protection in immunocompromised mice (Arrecubieta et al., 2008; Rauch et al., 2014). However, as is the case for ClfA, some vaccination trials have provided negative results with vaccination of UV-killed L. lactis expressing FnBPA inducing antibodies that enhanced, rather than inhibited, Fg and Fn interactions and did not provide protection against infective endocarditis (Veloso et al., 2015).

The presence of fnbA is highly conserved among the S. aureus population with certain strains not encoding fnbB (McCarthy and Lindsay, 2010). Sequence analysis of FnBPA and FnBPB from multiple S. aureus strains identified 7 isotypes of both FnBPA and FnBPB with the FnBPA isotypes displaying 66% to 76% sequence identity and the FnBPB isotypes displaying 61% to 85% sequence identity (Burke et al., 2010; Loughman et al., 2008). Most of the sequence diversity was located in the N2 and N3 subdomains with variable residues predicted to be surface exposed and not likely to impact upon ligand-binding (Burke et al., 2010; Keane et al., 2007a; Loughman et al., 2008). Antibodies raised against a specific isotype are unable to cross-react demonstrating antigen variation between FnBPs (Burke et al., 2010; Loughman et al., 2008). These data suggest that a FnBP-based vaccine may not provide protection to all S. aureus clinical isolates (Burke et al., 2010; Loughman et al., 2008). Interestingly the FnBP isotypes did not correlate with the core genome phylogeny or with the phylogeny of the other fnb gene, even though fnbA and fnbB are encoded in tandem, suggesting that recombination has contributed to the evolution of each protein individually (Burke et al., 2010; Loughman et al., 2008; McCarthy and Lindsay, 2010).

1.3.3 Collagen Adhesin

The C-terminal repeat domain of the collagen adhesin (Cna) contains B\textsubscript{CNA} repeated domains that have a unique sequence from the B\textsubscript{SDR} domains of the Sdr family (Deivanayagam et al., 2000; Patti et al., 1992). Strain variation is exhibited in the number of B\textsubscript{CNA} repeated domains present with at least one and a maximum of 4 repeats depending on the strain (Gillaspy et al., 1998). The function of the B\textsubscript{CNA} repeated domains is not known but they do not bind to collagen and are not thought to act as a stalk with the presence of a capsule and levels of cna transcription correlated
with collagen binding ability (Gillaspy et al., 1998; Hartford et al., 1999; Snodgrass et al., 1999).

1.3.3.1 Ligand-Binding Mechanism

The N1 and N2 subdomains of Cna are sufficient for collagen binding providing 6 high and 2 low affinity binding sites in collagen (Patti et al., 1993). The crystal structure of the N1 and N2 subdomains display jelly roll domains containing 2 β-sheets of 5 antiparallel β-strands (Figure 1.7) (Symersky et al., 1997). Co-crystals of the N1 and N2 subdomains in complex with collagen confirmed the multiple binding sites of Cna in collagen and highlighted the importance of a long flexible linker between the N1 and N2 subdomains in ligand-binding (Figure 1.7) (Zong et al., 2005). This long flexible linker creates the ligand-binding site with the N2 subdomain binding to 2 strands and the N1 subdomain binding to the third strand of the collagen triple helix (Figure 1.7) (Zong et al., 2005). A collagen hug binding mechanism was proposed whereby the N2 subdomain creates the initial hydrophobic interaction (Figure 1.7) (Zong et al., 2005). Valine172 of the flexible linker then interacts with a proline residue of collagen providing repositioning of the N1 subdomain and wrapping of the linker around collagen (Zong et al., 2005). Locking occurs due to C-terminal extension of the N2 subdomain and β-strand complementation into the N1 subdomain in a manner similar to the DLL binding mechanism (Figure 1.7) (Zong et al., 2005). This binding model highlights that Cna is only capable of binding to monomeric collagen and not collagen fibres suggesting that it provides bacterial adherence at sites of tissue injury (Zong et al., 2005). Structural comparisons of the binding interaction of Cna and the collagen adhesin Ace from Enterococcus faecalis, identified that the length of the inter-domain linker can influence collagen binding affinity (Zong et al., 2005). Specifically, a shorter linker exhibiting increased binding affinity but decreased promiscuity of binding with reduced binding sites in collagen (Ross et al., 2012).
Figure 1.7. Collagen Hug Model of Cna Binding. Collagen binds in the binding trench between the N1 and N2 subdomains created by the flexible linker. Collagen binding produces conformational repositioning of the N1 subdomain that wraps around the collagen. The binding interaction is stabilised by β-strand complementation of the C-terminal N2 subdomain into the N1 subdomain. Image taken from (Foster et al., 2014).
1.3.2.2 Role in Pathogenesis and Immune Evasion

Collagen is one of the most abundant proteins in vertebrates and is a structural scaffold of the ECM and connective tissue made up of a characteristic triple helix structure that is glycine and proline rich (Speziale et al., 2009). There are 28 types of collagen with Type I the most abundant, present in skin, bones, and tendons (Speziale et al., 2009). Cna-deficient *S. aureus* strains demonstrate decreased bacterial loads and reduced immune responses within joints in a murine septic arthritis model (Patti et al., 1994b). The presence of Cna also increased virulence in a murine model of osteomyelitis facilitating the spread of infection within the bone and in a rabbit model of corneal keratitis (Elasri et al., 2002; Rhem et al., 2000). Strains expressing mutated, collagen binding-deficient Cna demonstrated the importance of collagen binding in a septic arthritis model compared to a collagen binding wild type (Xu et al., 2004).

Cna, and other collagen-binding MSCRAMMs, also have a role in immune evasion with direct binding to the complement component C1q via the N1N2 subdomains (Kang et al., 2013). Adherence to C1q inhibited the classical complement pathway by affecting the complex of C1q with C1r and therefore reducing membrane attack complex formation on the surface of *S. aureus* (Kang et al., 2013). This immune evasion mechanism could be relevant in all *S. aureus* infections and highlights the importance of Cna (Kang et al., 2013).

1.3.2.3 Therapeutic Potential

Immunisation of mice with the recombinant A-domain of Cna increased survival in models of sepsis and mastitis (Hu and Guo, 2013; Nilsson et al., 1998). A fusion vaccine containing both Cna and FnBPA produced high opsonophagocytic antibody titre and provided protection in an intraperitoneal murine model (Zhou et al., 2006). However, a DNA vaccine containing *cna* was not able to provide protection in an intraperitoneal murine model even though high Cna specific antibody titres were achieved (Therrien et al., 2007). The use of Cna as a vaccine candidate is hindered by the low conservation of *cna* among *S. aureus* strains but it does have a higher prevalence in strains causing invasive *S. aureus* infections (McCarthy and Lindsay, 2010; Peacock et al., 2002).
1.3.4 Host Specific Binding Interactions of *S. aureus* with Fibrinogen

Analysis of the sequence variation present in host ECM proteins identified that Fn exhibits little variation both within and between host species (McCarthy and Lindsay, 2010). In contrast, Fg exhibits considerable sequence diversity both within host and between host species (McCarthy and Lindsay, 2010). Both the β- and γ-chains of Fg displayed mostly within host variation whereas the Fg α-chain displayed high levels of variation between host species precluding achievement of an accurate alignment of the Fg α-chain (McCarthy and Lindsay, 2010). This sequence variation in Fg is reflected in the ability of Fg-binding MSCRAMMs to interact with Fg from specific hosts reflecting the co-evolution of *S. aureus* with its host. For example, ClfB binds to a Fg repeat only present in the human Fg α-chain (Figure 1.8A) (Walsh et al., 2008). Bbp also exhibits human specificity with residue variation in the 561 to 575 region of Aα Fg preventing adherence to Fg of other host species (Figure 1.8B) (Vazquez et al., 2011). Sequence alignment identified that both canine and feline Fg were the most closely related to human Fg (Figure 1.8B) but binding of recombinant Bbp N2N3 to canine and feline Fg was not observed, and a peptide of canine Aα 561 to 575 was unable to block the binding of Bbp to human Fg (Vazquez et al., 2011). The interaction of SdrG of *S. epidermidis* is also highly host specific (Ponnuraj et al., 2003). SdrG binds to the N-terminal Bβ at the thrombin cleavage site and single site mutations demonstrated that residues Phe 10, Phe 11, Ser 12, Ala 13, Arg14, and Gly 15 are required for SdrG binding (Figure 1.8C) (Davis et al., 2001; Ponnuraj et al., 2003). Sequence alignment of this region in multiple hosts suggests that SdrG has a highly specific host interaction with human Fg (Figure 1.8C) (Ponnuraj et al., 2003).

In contrast, ClfA exhibits equal binding to human, canine, feline, murine, and porcine Fg but reduced binding to bovine Fg with no binding to ovine Fg (Geoghegan et al., 2010). In the same study Fbl of *Staphylococcus lugdunensis*, which binds to the same site within the Fg γ-chain as ClfA, exhibited the same host-specific Fg interaction except that Fbl also demonstrated reduced binding to feline Fg (Geoghegan et al., 2010). The lack of ovine Fg-binding by both of these MSCRAMMs can be attributed to the single residue substitution Q407A within the γ-chain peptide (Figure 1.8D) (Geoghegan et al., 2010).
Figure 1.8. Sequence Alignments of Fibrinogen-Binding Sites. Sequence alignments using Clustal Omega (EMBL-EBI) of (A) ClfB binding site (Fg from other hosts do not contain this α-chain repeat), (B) Bbp binding site, (C) SdrG binding site, and (D) ClfA binding site. Colouring scheme: small, hydrophobic residues (red), acidic residues (blue), basic residues (magenta), hydroxyl, sulphydryl, and amine residues (green).
SpsD of *S. pseudintermedius* also displays similar host specific interactions with the Fg γ-chain with reduced binding to ovine Fg and equivalent binding to human, canine, bovine and porcine Fg (Pietrocola et al., 2013). The host specific binding interaction of FnBPA and FnBPB has not been reported but due to the similarity in Fg-binding site in comparison to ClfA, Fbl, and SpsD, it is likely that they also display host-specific Fg-interactions.

SpsL expressed on the surface of *L. lactis* exhibits a unique host specific binding interaction with Fg with the highest binding displayed to canine, avian, and equine Fg and weaker interactions observed for human and bovine Fg (Bannoehr et al., 2011). This suggests that SpsL promotes specificity of *S. pseudintermedius* for the canine host (Bannoehr et al., 2011). This unique host tropism of SpsL does not correlate with the highly specific nature of an Aα or Bβ interaction or the tropism of the γ Fg interaction described above suggesting that SpsL may adhere to a unique site in Fg.

### 1.4 Molecular Characterisation of *S. pseudintermedius* SpsD and SpsL

#### 1.4.1 SpsD A-domain Ligand Binding

Molecular analysis of SpsD recombinant A-domain truncates, encoded by *S. pseudintermedius* clinical isolate 326, demonstrated that the N2N3 subdomains (residues 167 to 519) are sufficient for adherence to Fg, Fn, CK-10 and the novel ligand α-elastin making SpsD the most promiscuous MSCRAMM characterised to date (Pietrocola et al., 2013). Further characterisation revealed that SpsD N2N3 adheres to the C-terminal 17 residues of the Fg γ-chain, both the N-terminal (N29) and C-terminal (residues 1266 to 1908) fragments of Fn, and the C-terminal glycine-serine-rich Ω loops of CK-10 (Pietrocola et al., 2013). Surface plasmon resonance (SPR) analysis demonstrated that the N2N3 subdomains of SpsD have strong binding affinity to canine Fg (K_D 0.341±0.056 μM) with weaker affinities to recombinant human CK-10 (residues 544 to 563) (K_D 0.840±0.032 μM), α-elastin (K_D 1.020±0.150 μM), and Fn (K_D 2.157±0.702 μM) (Pietrocola et al., 2013). Recombinant proteins lacking the putative locking and latch sequences (residues 499 to 519) identified that this sequence is required for Fg and α-elastin interaction providing strong evidence for a DLL
binding mechanism (Pietrocola et al., 2013). In contrast, Fn and CK-10-binding were not affected by the absence of the putative locking and latching sequences suggesting that these ligands are not bound via a DLL binding mechanism (Pietrocola et al., 2013). In support of this, mutation of a residue predicted to be present within the binding trench (F288A), and known to be essential for ligand-binding in both FnBPB and ClfA, did not affect the binding interaction of SpsD with Fn and CK-10 but binding to both Fg and α-elastin was greatly reduced (Pietrocola et al., 2013). This suggests that SpsD binds to both Fg and α-elastin in an overlapping binding trench but the exact binding site for both Fn and CK-10 is unclear (Pietrocola et al., 2013). Similarly to other Fg γ-chain binding MSCRAMMs, SpsD modulates thrombus formation by inhibiting clot formation and ADP-induced platelet aggregation in vitro (Pietrocola et al., 2013).

1.4.2 SpsD and SpsL Promote *S. pseudintermedius* Internalisation

Recombinant truncates of SpsD region C (residues 520 to 846) and SpsL repeat region (residues 538 to 823), encoded by *S. pseudintermedius* clinical isolate ED99, exhibit high affinity Fn-binding sites with SPR analysis identifying binding affinities in the nM range, $K_D 1.7\pm0.38$ and $0.81\pm0.02$ nM respectively (Pietrocola et al., 2015). The higher binding affinity for region C of SpsD in comparison to the N2N3 subdomains suggests that region C is the primary binding site for Fn within SpsD (Pietrocola et al., 2015). The characterisation of these high affinity Fn-binding sites suggest that both SpsD and SpsL could promote *S. pseudintermedius* internalisation. Multiple *S. pseudintermedius* clinical isolates demonstrate invasion of canine progenitor epidermal keratinocyte (CPEK) cells with invasion of ED99 requiring the presence of Fn (Pietrocola et al., 2015). The expression of either SpsD or SpsL on the surface of *L. lactis* was sufficient to provide bacterial internalisation (Pietrocola et al., 2015). Incubation of bacteria with either N-terminal N29 of Fn, recombinant SpsD C or SpsL FnBR, or antibodies directed against $\alpha_5\beta_1$ integrin inhibited internalisation (Pietrocola et al., 2015). This demonstrates that SpsD and SpsL mediate internalisation using a Fn bridge to the $\alpha_5\beta_1$ integrin (Pietrocola et al., 2015). CPEK actin polymerisation was also required for invasion of ED99 in a Src-dependent manner suggesting an invasion mechanism akin to that reported for *S. aureus* (Pietrocola et al., 2015). Internalisation of ED99 had a detrimental effect upon CPEK cells that became rounded and detached
after 36 h of infection and displayed decreased viability suggesting that \textit{S. pseudintermedius} may produce toxins that kill CPEK cells. (Pietrocola et al., 2015).

1.5 Abscess Formation during \textit{S. aureus} Infection

\textit{S. aureus}-associated sepsis often has a lethal outcome partly due to the development of abscesses in multiple organs (Lowy, 1998). The process of \textit{S. aureus} seeding and abscess development has been well characterised using murine models of bloodstream infection with various bacterial proteins implicated in the 4 stages of abscess formation (Figure 1.9) (Cheng et al., 2009). Much less is known about the development of \textit{S. aureus} skin abscesses with the host response thought to play a more integral role in abscess development (Kobayashi et al., 2015a).

Retro-orbital injection of bacteria directly into the bloodstream promotes seeding of bacteria into organs after only 3 h (Cheng et al., 2009). This initial seeding of organs requires bacterial survival in the bloodstream and therefore bacterial immune evasion (Stage I) (Cheng et al., 2011). A key immune evasion mechanism of \textit{S. aureus} in the bloodstream is bacterial agglutination mediated by ClfA, coagulase (Coa), and vWbp (McAdow et al., 2011; Walker et al., 2013). vWbp aids fibrin blood clot formation by activating factor XIIIa non-proteolytically leading to the cross-linking of Fn to the $\alpha$-chain of fibrin (Thomer et al., 2013).

After bacterial seeding, Stage II involves the influx of immune cells to initiate abscess formation that are visible by histopathology at 48 h post infection (Cheng et al., 2009). Isolated \textit{S. aureus} cells are not identifiable at this stage with bacteria internalised within polymorphonuclear leukocytes (PMNs) (Cheng et al., 2009). Iron acquisition dependent upon IsdA and IsdB is essential for bacterial survival at this stage with SdrD also required due to an unidentified mechanism (Cheng et al., 2009). The requirement of these 3 CWA proteins for abscess formation at Stage II could explain the protective properties of a multicomponent vaccine containing IsdA, IsdB, SdrD, and SdrE in a murine model of abscess formation (Stranger-Jones et al., 2006).
Figure 1.9. Schematic of *S. aureus* Abscess Formation after Bloodstream Infection. Abscess formation occurs in 4 stages. Stage I involves the survival of *S. aureus* in the blood, requiring ClfA, and bacterial seeding of organs. Stage II is the initial abscess development with recruitment of immune cells. This stage requires iron acquisition and SdrD. Stage III is the maturation of the abscess. At this stage bacteria are evident in the centre of the abscess as a community of replicating bacteria encased in a pseudocapsule. Coa and vWbp are essential for this stage. Stage IV is the rupture of the abscess and dissemination of bacteria into the bloodstream. The cycle then repeats. Adapted from (Cheng et al., 2009).
As the abscess develops a central staphylococcal abscess community (SAC) containing replicating \textit{S. aureus} is observed (Stage III) (Cheng et al., 2009). Electron microscopy demonstrates that this SAC is encapsulated by a pseudocapsule of fibrin deposits that separate the SAC from PMNs (Cheng et al., 2009). The pseudocapsule is then surrounded by a layer of necrotic neutrophils, a layer of healthy immune cells, and a second layer of necrotic immune cells separated from the healthy tissue by an eosinophilic rim (Cheng et al., 2009). Using a 3D \textit{in vitro} model, the development of a pseudocapsule along with a second layer termed the outer dense microcolony-associated meshwork is sufficient to act as a barrier against neutrophil phagocytosis (Guggenberger et al., 2012). Both Coa and vWbp are required for this protective encapsulation with deletion of both genes preventing abscess development after bloodstream infection (Cheng et al., 2010). Coa and vWbp are secreted \textit{S. aureus} proteins that interfere with the natural coagulation cascade by interacting with and activating prothrombin as well as directly binding to Fg (Cheng et al., 2010). This activation of prothrombin via the D1 and D2 domains of Coa and vWbp produces conformational changes in prothrombin that create a high affinity interaction with Fg leading to the cleavage of Fg and formation of fibrin (Panizzi et al., 2006). Prothrombin, fibrin, Coa, and vWbp all co-localise in the pseudocapsule and are required for pseudocapsule formation (Cheng et al., 2010). As other Fg-binding proteins of \textit{S. aureus} are not able to promote abscess formation it is presumed that the ability of Coa and vWbp to activate prothrombin is central to their involvement in abscess formation rather than the ability to bind to Fg (Cheng et al., 2010). Antibodies directed against Coa and vWbp are able to block clotting of murine blood as well as providing protection against lethal \textit{S. aureus} challenge in mice demonstrating the importance of these proteins in \textit{S. aureus} pathogenesis (Cheng et al., 2010; McAdow et al., 2012). Both SpA and Emp are also involved in abscess maturation by an unknown mechanism (Cheng et al., 2009).

Stage IV of abscess development requires migration of the abscess to the organ periphery and then abscess rupture providing dissemination of \textit{S. aureus} (Cheng et al., 2009). Secreted proteins, extracellular adhesion proteins (Eap) and ESAT-6-like secretion system (Ess), are required for this dissemination phase and the development
of a persistent *S. aureus* infection that restarts the abscess formation cycle (Burts et al., 2008; Cheng et al., 2009).

### 1.6 Hypothesis

MSCRAMMs provide molecular interactions between bacteria and host species and are implicated in multiple disease processes such as platelet aggregation, immune evasion, and colonisation of the nares (Foster and Hook, 2014). *S. pseudintermedius* encodes MSCRAMMs SpSD and SpSL that adhere to ECM proteins when expressed on the surface of the heterologous host *L. lactis* (Bannoehr et al., 2011) or as recombinant proteins (Pietrocola et al., 2013; Pietrocola et al., 2015). We propose that these proteins are key virulence factors of *S. pseudintermedius* and mediate interactions with Fg, Fn, and CK-10. These ECM interactions could be involved during skin abscess development.
1.7 Project Aims

The aim of this project was to elucidate the function of SpsD and SpsL in *S. pseudintermedius* pathogenesis and their potential as therapeutic targets by:

1. Generating the first gene deletion strains of *S. pseudintermedius* and investigating the ECM-binding properties of SpsD and SpsL in *S. pseudintermedius* strain ED99

2. Evaluating the potential for functional diversity in SpsD and SpsL among the *S. pseudintermedius* population and investigating the potential of SpsD and SpsL as novel therapeutic targets

3. Performing molecular investigation of the host specific binding interaction of SpsL to Fg from different host species

4. Generating a murine skin infection model of *S. pseudintermedius* and investigating the role of SpsD and SpsL in the pathogenesis of *S. pseudintermedius* ED99 in this model
Chapter 2 General Materials and Methods
2.1 Bacterial Culture Conditions

*S. pseudintermedius* and *S. aureus* were routinely cultured in brain heart infusion (BHI) broth (Oxoid) at 37°C with shaking at 200 rpm or plated on tryptone soy agar (TSA) (Oxoid) unless otherwise stated. Where appropriate cultures were supplemented with 10 µg/ml chloramphenicol (Cm) (Sigma-Aldrich), or 1 µg/ml anhydrotetracycline (ATc) (Sigma-Aldrich). All *Escherichia coli* strains were routinely cultured in Luria-Bertani (LB) broth (Melford Laboratories) at 37°C with shaking at 200 rpm or plated on LB agar. Where appropriate *E. coli* cultures were supplemented with 100 µg/ml ampicillin (Amp) (Formedium), 15 µg/ml chloramphenicol (Cm) (Sigma), 400 µg/ml erythromycin (Erm) (Sigma) or 15 µg/ml tetracycline (Tc) (Sigma). *Lactococcus lactis* strains were routinely cultured in M17 broth (Oxoid) supplemented with 0.5% (v/v) glucose, 0.5% (v/v) lactose, and 10 µg/ml erythromycin (Erm) at 30°C without shaking.

2.2 Genomic DNA Extraction

Staphylococcal genomic DNA was routinely extracted from 500 µl of stationary phase culture using the PurElute™ Bacterial Genomic Kit (Edge Biosystems). The manufacturer’s instructions were followed except for the addition of 250 µg/ml lysostaphin (Ambi Products) to the Spheroplast Buffer followed by incubation for 20 min at 37°C rather than 10 min. DNA pellets were suspended in 50 µl Elution Buffer (Qiagen), and DNA was quantified using a NanoDrop™ 1000 (Thermo Scientific).

2.3 Polymerase Chain Reaction

Custom oligonucleotides were purchased from Invitrogen after design using DNASTAR Lasergene® Core Suite 9. High-fidelity PCR reactions included 0.2 µM forward and reverse primers, approximately 100 ng of template DNA, 0.25 mM dNTPs (Promega), 1 x *PfuUltra™* II reaction buffer (Agilent Technologies), 1 U *PfuUltra™* II Fusion HS Polymerase (Agilent Technologies) and dH₂O to a final volume of 50 µl. The thermocycler programme included an initial denaturation at 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 20 s, annealing at varying
temperatures for 20 s, extension at 72°C for 15 s per kb, followed by final extension at 72°C for 3 min, unless otherwise stated.

Low fidelity PCR reactions were performed using 0.625 U GoTaq® DNA polymerase (Promega), 0.2 µM forward and reverse primers, 0.25 mM dNTPs (Promega), 1.5 mM MgCl₂ (Promega), 1 x GoTaq® Flexi Buffer (Promega), and dH₂O to a final volume of 25 µl. The thermocycler programme included, an initial denaturation at 95°C for 3 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at varying temperatures for 30 s, extension at 72°C for 1 min per kb, followed by a final extension at 72°C for 5 min, unless otherwise stated.

For colony PCR reactions, part of a colony was used to inoculate 10 µl of dH₂O and boiled at 95°C for 10 min. After centrifugation, 2 µl of this sample was used as template DNA following the same thermocycler conditions to those described previously.

2.4 DNA Agarose Electrophoresis

PCR products were resolved in 1% (w/v) agarose gels (Melford Laboratories) with 1 x Tris borate EDTA (TBE) buffer containing either 1 x SYBR® Safe DNA gel stain (Invitrogen) or 1 x GelRed™ (Biotium Limited). Electrophoresis was typically carried out at 100 V for 45 to 60 min followed by visualisation using a G:Box (Syngene). Samples were loaded with 1 x BlueJuice™ (Invitrogen) and run alongside a 1 kb DNA ladder (Promega).

2.5 Purification of PCR Products

PCR products were either purified using QIAquick® PCR purification or gel extraction kits (Qiagen), following the manufacturer’s instructions, or by mixing 10 µl of PCR reaction with 0.5 µl 10 x Antarctic Phosphatase Buffer (New England BioLabs), 0.5 µl Exonuclease I (New England BioLabs), 0.5 µl Antarctic Phosphatase (New England BioLabs), and 8.5 µl dH₂O and processing the sample at 37°C for 15 min followed by 80°C for 15 min. DNA was quantified using a NanoDrop™ 1000 (Thermo Scientific).
2.6 Plasmid Extraction and Concentration

Plasmids were routinely extracted using the QIAquick® Spin MiniPrep kit (Qiagen) following the manufacturer’s instructions except for the supplementation of buffer P1 with 100 µg/ml lysostaphin (Ambi Products) when isolating plasmid from staphylococcal isolates, or 100 U/ml mutanolysin and 100 µg/ml lysozyme (Sigma-Aldrich) when isolating plasmid from Lactococcus isolates, followed by incubation at 37°C for 30 min. Low copy number plasmids were concentrated using Pellet Paint® Co-precipitant (Novagen) following the manufacturer’s instructions. Plasmid DNA was quantified using a NanoDrop™ 1000 (Thermo Scientific).

2.7 Restriction Digestion and Ligation

Restriction digestion reactions were formulated as described in the manufacturer’s instructions using restriction enzymes purchased from New England BioLabs. Reactions were incubated for 2 h 30 min at 37°C and heat inactivated if required. When necessary re-ligation was prevented by incubating at 37°C for 30 min with 2 µl Antarctic Phosphatase Reaction Buffer and 5 U of Antarctic Phosphatase (New England BioLabs) and heat inactivated at 65°C for 5 min. Products of digestion were analysed by 1% (w/v) agarose gel and extracted using a QIAquick® Gel Extraction kit (Qiagen) following the manufacturer’s instructions. Ligation reactions were performed using T4 DNA Ligase Reaction Buffer and T4 DNA Ligase (New England BioLabs) following the manufacturer’s instructions with the volume of plasmid and insert determined depending on a 3:1 molar ratio of insert: plasmid. Reactions were made up to 20 µl using nuclease-free dH₂O (Severn Biotech Ltd) and either incubated at room temperature for 30 min or overnight at 16°C. Before transformation the ligation plasmids were purified by dialysis using 0.025 µm filter circular discs (MilliPore) on MilliQ water at room temperature for 20 min.

2.8 Sanger Sequencing

Sanger sequencing was performed by Edinburgh Genomics, University of Edinburgh. Chromatogram data from both strands was analysed for quality using FinchTV and
analysed using SeqMan Pro (DNASTAR Lasergene® Core Suite 9). To ensure accuracy of the sequence data, at least two reads were obtained for each nucleotide of the sequence.

2.9 Preparation of *E.coli* Electro-competent Cells and Electro-transformation

*E. coli* competent cells were produced by inoculating 25 ml of LB broth (Melford Laboratories) with 250 µl of overnight culture followed by incubation at 37°C with shaking at 200 rpm until an OD₆₀₀ of 0.6. Cells were placed on ice for 15 min and centrifuged at 4000 rpm for 10 min at 4°C. Cells were suspended once in an equal volume, once in a half volume, and once in a quarter volume of ice cold 10% (v/v) glycerol (Sigma-Aldrich) before final suspension in 2 ml ice cold 10% (v/v) glycerol. 50 µl aliquots were produced for storage at -80°C. For electro-transformation, appropriate concentrations of plasmid made up to 5 µl in dH₂O were mixed with 50 µl competent cells and transferred to an ice-cooled 0.2 cm gap cuvette (Sigma-Aldrich). Electroporation was carried out at 100 Ω, 25 µF, and 2.5 kV, and 390 µl of LB broth was immediately added and the cells were allowed to recover, for 1 h at 37°C with shaking at 200 rpm, before plating 100 µl onto LB agar supplemented with the appropriate antibiotic followed by incubation at 37°C for at least 15 h.

2.10 Gene Cloning for Recombinant Protein Expression

The gene of interest was amplified from genomic DNA using high fidelity PCR as described in section 2.3. This PCR product was blunt cloned into pSC-B using the StrataClone Blunt PCR Cloning Kit (Agilent Technologies) with selection on LB containing 100 µg/ml Amp and 40 µl of 2% (w/v) X-gal. White colonies were selected and the insert removed from purified plasmid using restriction digestion and ligated as described in sections 2.6 and 2.7. Transformation of the ligated plasmid was performed into either *E. coli* DH5α or StrataClone Gold competent cells (Agilent Technologies) depending on the efficiency rate of the transformation. Colonies were screened for ligated plasmid by colony PCR and electro-transformed, as described in section 2.9,
into the expression hosts of *E. coli* XL-1 Blue (Agilent Technologies) or BL21 DE3 (New England BioLabs) depending on the plasmid being utilised.

2.11 Sequence Ligase Independent Cloning

Sequence ligase independent cloning (SLIC) uses sequence complementation in the cloning primers to produce constructs rather than restriction digestion and ligation (Figure 2.1). Plasmid DNA was linearized using restriction digestion and purified using QIAquick® gel extraction kit (Qiagen) following the manufacturer’s instructions. Plasmid was amplified using Platinum® PCR Supermix (Invitrogen) containing 0.2 µM forward and reverse primers. The thermocycler programme included, an initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at varying temperatures for 30 s, and extension at 68°C for 1 min per kb. The cloning insert is amplified using *PfuUltra™* II Fusion HS Polymerase (Agilent Technologies) as described in section 2.3 and purified before SLIC.

For SLIC, PCR products were incubated with 3 U of T4 DNA Polymerase (New England BioLabs) in a reaction with 4 µl NEBuffer 2 (New England BioLabs), 2 µl 100 mM DTT (Invitrogen), 4 µl (w/v) 2M Urea (Sigma), 0.4 µg BSA (New England BioLabs), and 1 µg of vector or insert DNA made up to 40 µl with dH₂O, at 23°C for 20 min. Addition of 2 µl 500 mM EDTA and heat inactivation at 75°C for 20 min stopped the reaction. 5 µl of the treated vector and 5 µl of the insert were mixed and processed on a Thermocycler with an initial 10 min hold at 65°C before a step-wise gradient of 1°C increments down to 25°C was applied. This 10 µl reaction was concentrated as described in section 2.6 before being electro-transformed into *E. coli* DC10B (Monk et al., 2012) as described in section 2.9.
Figure 2.1 Sequence Ligase Independent Cloning. Both the gene of interest (goi) and plasmid were amplified by PCR. The primers used to amplify the goi contained complementary sequence to the plasmid. After PCR purification, T4 DNA polymerase treatment produced sticky ends of both PCR products. These sticky ends (green boxes) contain complementary sequence and annealed during a step-wise decrease in temperatures from 65°C to 25°C. Transformation into E. coli completed the construct sequence to produce the final construct, plasmid::goi.
2.12 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

0.75 mm spacer plates were used to prepare 10- or 15-well SDS-PAGE gels using Bio-Rad gel casting apparatus. 12% (w/v) resolving gels were made with 3 ml 40% (w/v) acrylamide (Sigma-Aldrich), 2.5 ml 1.5 M Tris-HCl pH 8.8, 100 μl 10% (w/v) sodium-dodecylsulphate (SDS) (Fisher Scientific), 100 μl 10% (w/v) ammonium persulphate (APS) (Sigma-Aldrich), and 10 μl tetramethylethylenediamine (TEMED) (Sigma-Aldrich) made up to 10 ml in dH₂O and sealed with 1 ml isopropanol (Fisher Scientific). 4% stacking gels were made with 1 ml 40% (w/v) Acrylamide, 2.5 ml 0.5 M Tris-HCl pH 6.8, 100 μl 10% (w/v) SDS, 100 μl 10% (w/v) APS, and 10 μl TEMED, made up to 10 ml in dH₂O. This was applied to the 12% resolving gel and the comb inserted. SDS-PAGE was carried out with 1 x running buffer (5 x running buffer containing 15 g Tris Base, 5 g SDS, and 72 g glycine, made up to 1 litre in dH₂O). Protein samples were prepared using 2 x Laemmli Sample Buffer (Sigma-Aldrich) and incubated at 95°C for 5 min. Samples were loaded alongside ColourPlus Protein Marker (New England BioLabs) or PageRuler Prestained Protein Ladder (Thermo Scientific). Electrophoresis was carried out at 150 V for 90 min with gels stained using InstantBlue™ (Expedeon Protein Solutions) and imaged with an Epson scanner.

2.13 Western Blot

After SDS-PAGE, proteins were transferred onto nitrocellulose membrane (Amersham Hybond ECL) using a semi-dry method (BioRad TurboBlot) with extra thick Western blotting filter paper (Fisher Scientific) at 25 V for 30 min in Transfer Buffer (20 mM Tris Base (Fisher Scientific), 154 mM glycine (Sigma-Aldrich), and 20% (v/v) methanol (Fisher Scientific) made up to 1 litre with dH₂O). Membranes were blocked either overnight at 4°C or for more than 2 h at room temperature in 8% (w/v) non-fat dried milk (Fluka Analytical) in PBS. Primary antibody was applied to the blocking buffer and incubated with the membrane for 2 h at room temperature. The blot was washed with 1% milk-PBST (0.1% Tween-20) for 3 x 15 min, before incubation with secondary antibody in 1% milk-PBST for 1 h at room temperature. After washing, blots were visualised using equal volumes of enhanced
chemiluminescence (ECL) solution 1 (1 ml stock luminal, 440 µl p-coumaric acid, 100 mM Tris-Cl pH 8.5, 90 ml dH2O) and solution 2 (64 µl 30% H2O2, 100 mM Tris-Cl pH 8.5, 90 ml dH2O) and exposed using Amersham Hyperfilm ECL (GE Healthcare Life Sciences).

### 2.14 Chicken Antibody Generation and Purification

Recombinant proteins were used to generate chicken antibodies at Moredun Research Centre Edinburgh. Chicken antibodies were employed due to the lack of interaction with staphylococcal protein A. IgY antibodies were purified from egg yolks using the Eggspress IgY Purification Kit (Gallus ImmunoTech), according to the manufacturer’s instructions. As egg yolks were received in a frozen state they were defrosted overnight at 4°C before being incubated in 6 volumes of chilled buffer A at 4°C for at least 20 h.

Further purification of the IgY antibodies was performed using CNBr-activated Sepharose™ 4B (GE Healthcare Life Sciences), following the manufacturer’s instructions, using a gravity flow column (Bio-Rad). PBS was used as a binding agent with the sample applied 3 times to the Sepharose™ 4B to increase binding. The IgY antibody was eluted in 5 ml 100 mM glycine buffer pH 3.0 with immediate addition of Tris-HCl to neutralise the elution. IgY was stored at -20°C or at 4°C with addition of 0.1% (v/v) sodium azide for short-term storage.

### 2.15 Source of Extracellular Matrix Proteins

Bovine, human, and ovine Fg was purchased from Sigma-Aldrich in powder form and solubilised in pre-warmed PBS. Canine Fg was not commercially available and so was purified from whole canine blood as described below. Murine Fg and murine Fn were purchased from Abcam, bovine Fn was purchased from EMD Millipore, and bovine collagen type I was purchased from Life Technologies. All Fg samples were purified to remove contaminating Fn using Gelatin-Sepharose™ 4B (GE Healthcare Life Sciences) as described in section 2.17. Murine CK-10 was purified from a pQE-30 construct (Walsh et al., 2004) as described in section 2.18.
2.16 Purification of Canine Fibrinogen from Whole Blood

Canine Fibrinogen was purified from beagle sodium citrate whole blood (Lampire Biological Products) using a previously described method (Doolittle et al., 1967). 10 ml of blood was centrifuged twice (3000 rpm, 10 min, 4°C) and the plasma recovered. After 15 min incubation on ice, “Fraction I” was precipitated by cautious addition of 0.22 volumes of ice cold 50% (v/v) ethanol solution (Fisher Scientific) buffered to pH 7.1 with 0.055 M trisodium citrate. After 5 min incubation at -3°C in a frozen isopropanol bath, the precipitate (4000 rpm, 10 mins, -3°C) was suspended in 0.5 original volume of ice cold 7% ethanol solution buffered to pH 6.5 with 0.055 M trisodium citrate and incubated at -3°C for 5 min. The precipitate (4000 rpm, 15 min, -3°C) was suspended in 0.25 original volume of 0.055 M trisodium citrate buffer (pH 6.5) at 30°C and incubated on ice for an additional 15 min. The “cold-insoluble material” was precipitated by cautious addition of ice cold 20% (v/v) ethanol to a final concentration of 2% and incubated on ice for a further 5 min. The supernatant (4000 rpm, 10 min, 0°C) was made to a final concentration of 8% (v/v) by gradual addition of 20% (v/v) ethanol and incubated on ice for 5 min. The precipitate (4000 rpm, 10 min, 0°C) was suspended in 0.5 original volume PBS. Fg was purified of contaminating Fn as described below.

2.17 Depletion of Fibronectin from Fibrinogen Samples

Contaminating Fn was removed from all Fg samples using Gelatin-Sepharose™ 4B (GE Healthcare Life Sciences). 1 ml Gelatin-Sepharose™ 4B was applied to a gravity flow column (Bio-Rad) and conditioned with 40 ml of PBS. The Fg sample was applied to the column, collected, and quantified with a NanoDrop™ 1000 (Thermo Scientific) using the extinction coefficient of 15.1 and molecular weight of 340 kDa. Aliquots were stored at -80°C. All Fg samples were visually analysed for purity by SDS-PAGE and the depletion of Fn confirmed by Western blot analysis as described in section 2.13 with 1 µg/ml rabbit anti-Fn IgG and 0.2 µg/ml goat anti-rabbit HRP conjugated IgG.
2.18 Purification of Mouse Cytokeratin-10 using Hybrid Conditions

Recombinant murine CK-10 C-terminus (294-570) (Walsh et al., 2004) was purified from *E. coli* XL-1 Blue cells (Agilent Technologies) from the expression construct pQE-30::mCK-10 (Qiagen). Cell lysate was produced under Invitrogen denaturing conditions. Briefly, the cell pellet was suspended in 40 ml guanidium lysis buffer (6M guanidine hydrochloride, 20 mM sodium phosphate, 500 mM NaCl, pH 7.8) and rocked at room temperature for 30 min. Cells were lysed using the One Shot at 25 psi (Constant Systems) and lysate produced by centrifugation at 5000 rpm for 30 min.

Ni-NTA Agarose (Invitrogen) was applied to a Bio-Rad column and prepared under denaturing conditions, as follows, the agarose was washed with 6 ml dH2O and 12 ml denaturing binding buffer (8M Urea, 20 mM sodium phosphate, 500 mM NaCl, pH 7.8), suspended in the cell lysate, rocked at room temperature for 20 min, and then dripped through the column. The column was washed with 8 ml denaturing binding buffer, 8 ml denaturing wash buffer (8M Urea, 20 mM sodium phosphate, 500 mM NaCl, pH 6.0), 24 ml native wash buffer (50 mM NaH2PO4, 0.5 M NaCl, 30 mM imidazole, pH 8.0), and the protein was eluted in 8 ml native elution buffer (50 mM NaH2PO4, 0.5 M NaCl, 250 mM imidazole, pH 8.0) and analysed by SDS-PAGE.

2.19 Solid Phase Adherence Assays

96-well MaxiSorp® plates (Nunc) were coated overnight at 4°C using 2-fold serial dilutions of Fg, Fn, or CK-10 in PBS using 20 to 0 μg/ml. After washing in PBS, wells were blocked with 100 μl 4% (w/v) non-fat dried milk (Fluka Analytical) for at least 1 h at room temperature. Bacterial strains were cultured to the appropriate OD600 before being washed and re-suspended to an OD600 of 1.0 in PBS. Each well was inoculated with 100 μl of bacteria, in triplicate, and plates were incubated for 2 h at 37°C for Staphylococci or 30°C for *L. lactis* strains. After washing in PBS, the bound bacterial cells were fixed with 100 μl 25% (v/v) formaldehyde (Sigma-Aldrich) and incubated at room temperature for 30 min. After washing in PBS, cells were stained with 50 μl 0.5% (w/v) crystal violet (Sigma-Aldrich) for 3 min, washed with PBS and solubilised in 100 μl 5% (v/v) acetic acid (BDH Lab Supplies) for 10 min. Plates were analysed using a Synergy™ HT plate reader (BioTek) at a wavelength of 590 nm.
2.20 Extracellular and Cell Wall Associated Protein Profiles of *S. pseudintermedius*

Extracellular fractions were obtained by pelleting 20 ml of culture at 4000 rpm for 10 min and concentrating 12 ml of the supernatant using an Amicon® Ultra-15 Centrifugal Filter Unit (Millipore), 10 kDa molecular weight cut-off (MWCO), at 4000 xg for 20 min. Cell wall associated (CWA) protein fractions were obtained by pelleting 20 ml of exponential phase or 5 ml of stationary phase culture, washing in PBS, and suspending in 1 ml lysis buffer (50mM Tris-Hcl, 20mM MgCl₂, pH 7.5, 30% (w/v) raffinose) supplemented with 400 µg/ml lysozyme (Ambi Products) and cOmplete protease inhibitors (Roche Life Sciences). Samples were incubated at 37°C for 20 min and pelleted at 6000 xg for 20 min with the supernatant being retained and stored at -20°C.

2.21 Flow Cytometry

Bacterial surface expression of protein was analysed using flow cytometry for both *S. pseudintermedius* ED99 and *L. lactis* constructs. Bacteria were cultured to the appropriate growth phase, washed and suspended in PBS to an OD₆₀₀ of 0.3. 10 µg/ml of primary antibody was incubated with 500 µl of bacterial cells on ice for at least one h. The cells were washed twice with PBS (16000 g for 5 min) before re-suspending in 500 µl PBS and incubating with 1 µg/ml of F(ab) goat anti-chicken IgG conjugated with FITC (Sigma-Aldrich) or goat anti-mouse IgG conjugated with PE (BD Biosciences) on ice for 45 min in the dark. The cells were washed twice with PBS, suspended in the final 500 µl volume and analysed immediately using FACSCalibur™ (BD Biosciences) and CellQuest™ Pro (BD Biosciences). Data was analysed using FlowJo, and controls included bacteria incubated without antibody or with secondary antibody only.

2.22 Statistical Analysis

Prism 6 (GraphPad) was used to present data with statistical analysis performed using Minitab 16. All data was analysed for normality, using the Anderson-darling test, as
well as equal variance between groups before performing either parametric or nonparametric analyses. Multiple comparisons were performed when appropriate. Solid phase adherence assays and ELISA-type binding assays were analysed at one particular concentration of recombinant protein or ECM protein. For data displaying statistical significance, * represents $p \leq 0.05$, ** represents $p \leq 0.01$, and *** represents $p \leq 0.001$. 
Chapter 3 The Interaction of *S. pseudintermedius* ED99 with Extracellular Matrix Proteins
3.1 Introduction

The ability of bacterial pathogens to adhere to host receptors is essential for bacterial colonization and initiation of disease. Multiple *S. pseudintermedius* clinical isolates have been analysed for interactions with the ECM (Geoghegan et al., 2009; Schmidt et al., 2009). The ability to adhere to proteins such as Fg and Fn varied between strains but implied the existence of CWA proteins capable of mediating interactions with the ECM (Geoghegan et al., 2009; Schmidt et al., 2009). Whole genome sequencing of *S. pseudintermedius* identified a family of 18 CWA proteins with 2, SpsD and SpsL, with the potential to adhere to ECM proteins (Bannoehr et al., 2011; Ben Zakour et al., 2011). Experiments involving expression of SpsD and SpsL on the surface of the heterologous host *L. lactis* demonstrated that both proteins are capable of binding to ECM proteins Fg and Fn, and that SpsD also binds CK-10 (Bannoehr et al., 2011). More detailed analysis of recombinant truncated fragments of SpsD have revealed that the N2N3 subdomains are involved in binding to Fg, Fn, CK-10, and α-elastin and region C is involved in high-affinity Fn-binding (Pietrocola et al., 2013; Pietrocola et al., 2015). High affinity Fn-binding interactions of the repeat region of SpsL have also been molecularly investigated (Pietrocola et al., 2015). However, a demonstration of the function of these proteins in promoting *S. pseudintermedius* adherence has not been carried out to date.

In order to fulfil molecular Koch’s postulates, the effect of deleting putative virulence factors from the bacterial pathogen must be investigated followed by complementation (Falkow, 1988). This approach has been utilised extensively to investigate the function of *S. aureus* CWA proteins in ECM-binding and pathogenesis. However, gene deletions of *S. pseudintermedius* have not yet been created and genetic manipulation of Staphylococcal species can be challenging due to the presence of restriction modification (RM) systems that limit horizontal gene transfer. These RM systems can be avoided in some staphylococcal strains using recently developed genetic tools (Monk et al., 2012). In the current study these genetic tools are applied to produce the first gene deletion mutants of *S. pseudintermedius* allowing the function of SpsD and SpsL in ECM-binding to be investigated.
3.2 Aims

1) Investigate the ECM interaction of *S. pseudintermedius* ED99 and *L. lactis* expressing SpsD and SpsL

2) Use allelic replacement to produce *spsD* and *spsL* deletion mutants of ED99 and repair the ED99 deletion mutants

3) Investigate the ECM-binding of the isogenic mutant strains in comparison to wild type ED99
3.3 Materials and Methods

3.3.1 Bacterial Strains, Plasmids and Primers Employed in the Current Study

Bacterial strains, plasmids, and primers employed in the current study are listed in Tables 3.1, 3.2, and 3.3 respectively. A vector map of pIMAY, the plasmid employed for allelic replacement is shown in Figure 3.1. A derivative of pIMAY, pIMAY-Z that encodes a lacZ gene, was employed for allelic replacement of spsL (Monk et al., 2015).

3.3.2 Genetic Manipulation of pIMAY

Due to the antisense secY mechanism encoded in pIMAY, growth of S. aureus is inhibited in the presence of both ATc (1 µg/ml) and Cm (10 µg/ml). However, this phenomenon was not observed for S. pseudintermedius ED99 containing pIMAY. BLASTn analysis identified variation in the secY gene of S. aureus and S. pseudintermedius suggesting that introduction of a secY gene from ED99 into pIMAY may support inhibition of growth. SLIC (section 2.11) produced this manipulated pIMAY vector as follows: after pIMAY digestion with BglII (New England BioLabs), the initial high-fidelity PCR reactions were carried out at an annealing temperature of 50°C with extension time of 15 s to amplify secY (561 bp), and an extension time of 2 min to amplify pIMAY (5182 bp). Plasmids were generated in E. coli DC10B and then transformed into S. aureus RN4220 and S. pseudintermedius ED99 and growth was analysed on TSA containing both ATc and Cm.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
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<tr>
<td><strong>S. pseudintermedius</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ED99</td>
<td>Canine pyoderma isolate</td>
<td>(Simou, 2005)</td>
</tr>
<tr>
<td>ED99∆spsD</td>
<td>Gene deletion of <em>spsD</em></td>
<td>This study</td>
</tr>
<tr>
<td>ED99∆spsL</td>
<td>Gene deletion of <em>spsL</em></td>
<td>This study</td>
</tr>
<tr>
<td>ED99∆spsL∆spsD</td>
<td>Gene deletion of <em>spsL</em> and <em>spsD</em></td>
<td>This study</td>
</tr>
<tr>
<td>ED99∆spsD Rep</td>
<td>Repaired <em>spsD</em> gene</td>
<td>This study</td>
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<tr>
<td>ED99∆spsL Rep</td>
<td>Repaired <em>spsL</em> gene</td>
<td>This study</td>
</tr>
<tr>
<td><strong>S. aureus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RN4220</td>
<td>ST8, premature stop codons in <em>hsdR</em> and <em>sauUSI</em></td>
<td>(Kreiswirth et al., 1983)</td>
</tr>
<tr>
<td>Newman</td>
<td>Fg-binding positive control</td>
<td>(Duthie and Lorenz, 1952)</td>
</tr>
<tr>
<td>SH1000</td>
<td>Fn-binding positive control</td>
<td>(Horsburgh et al., 2002)</td>
</tr>
<tr>
<td>MSSA 476</td>
<td>Collagen-binding positive control</td>
<td>(Holden et al., 2004)</td>
</tr>
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<td></td>
<td></td>
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<td><em>lacZΔM15, endA, recA</em>-deficient</td>
<td>Stratagene</td>
</tr>
<tr>
<td>DC10B</td>
<td>DH10B background, Δdcm</td>
<td>(Monk et al., 2012)</td>
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<td></td>
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<tr>
<td><em>L. lactis</em> pOri23</td>
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<td>(Que et al., 2000)</td>
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<tr>
<td><em>L. lactis</em> pOri23::<em>spsD</em></td>
<td><em>spsD</em> expressing construct</td>
<td>(Bannoehr et al., 2011)</td>
</tr>
<tr>
<td><em>L. lactis</em> pOri23::<em>spsL</em></td>
<td><em>spsL</em> expressing construct</td>
<td>(Bannoehr et al., 2011)</td>
</tr>
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<td>Plasmid</td>
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</tr>
<tr>
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<td><em>Bam</em>HI, <em>Pst</em>I</td>
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<tr>
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<td><em>Sal</em>I, <em>Pst</em>I</td>
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<td>Vector for blunt end ligation</td>
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<td>Thermosensitive gene replacement plasmid, pVW01ts derivative</td>
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</tr>
<tr>
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<td>pIMAY derivative containing lacZ</td>
<td>-</td>
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<td>spsD deletion plasmid</td>
<td><em>Kpn</em>I, <em>Sac</em>I</td>
</tr>
<tr>
<td>pIMAY-Z::spsL</td>
<td>spsL deletion plasmid</td>
<td>SLIC</td>
</tr>
<tr>
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<td>spsD complementation plasmid</td>
<td>SLIC</td>
</tr>
<tr>
<td>pIMAY::spsL Rep</td>
<td>spsL complementation plasmid</td>
<td>SLIC</td>
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<td>pIMAY::secY ED99</td>
<td>Manipulation of pIMAY</td>
<td>SLIC</td>
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<td>Primer Name</td>
<td>Sequence (5’ to 3’)*</td>
<td>Function</td>
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<tr>
<td>---------------------</td>
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<td><strong>Deletion Constructs</strong></td>
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<td><em>spsD</em> A</td>
<td>CCCGAATTCTAGCCTGAAGCAATTAAGAAGT</td>
<td>pIMAY::<em>spsD</em></td>
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<tr>
<td><em>spsD</em> B</td>
<td>TTGCATGCCCTACACAAATAA</td>
<td>pIMAY::<em>spsD</em></td>
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<tr>
<td><em>spsD</em> C</td>
<td>ttaggcatgcTCTTCAATGAAATAAGCATCA</td>
<td>pIMAY::<em>spsD</em></td>
</tr>
<tr>
<td><em>spsD</em> D</td>
<td>CCCGAATTCCGTACTATCATATAATAGGTGACAC</td>
<td>pIMAY::<em>spsD</em></td>
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<tr>
<td>pIMAY-Z F (kpnI)</td>
<td>GGTACCCAGCTTTGTCCCTTTTAGTAGG</td>
<td>pIMAY::<em>spsL</em></td>
</tr>
<tr>
<td>pIMAY-Z R (SacI)</td>
<td>GAGCTCAATTCCGCCCTATAGTGAGTCG</td>
<td>pIMAY::<em>spsL</em></td>
</tr>
<tr>
<td>pIMAY-Z <em>spsL</em> A (KpnI)</td>
<td>cctcactaaaggaacaaaatggtagC</td>
<td>pIMAY::<em>spsL</em></td>
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<tr>
<td><em>spsL</em> B</td>
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<tr>
<td><em>spsL</em> C</td>
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<tr>
<td>pIMAY-Z <em>spsL</em> D (SacI)</td>
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<tr>
<td>Primer Name</td>
<td>Sequence (5’ to 3’)*</td>
<td>Function</td>
</tr>
<tr>
<td>-------------</td>
<td>----------------------</td>
<td>----------</td>
</tr>
<tr>
<td><strong>Repaired Constructs</strong></td>
<td></td>
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<tr>
<td><em>spsD</em> A</td>
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<td>pIMAY::<em>spsD</em> Rep</td>
</tr>
<tr>
<td><em>spsD</em> B</td>
<td>AATGCTATATATATTTTTATTTGTAATCAC</td>
<td>pIMAY::<em>spsD</em> Rep</td>
</tr>
<tr>
<td><em>spsD</em> C</td>
<td>tgtgattacaataaaatatatatagcattCGAAAGCATAAACTTGGCG</td>
<td>pIMAY::<em>spsD</em> Rep</td>
</tr>
<tr>
<td><em>spsD</em> D</td>
<td>cgaactcactataggcgaattgcgtACTATCATAATATGTTGACAC</td>
<td>pIMAY::<em>spsD</em> Rep</td>
</tr>
<tr>
<td><em>spsL</em> A</td>
<td>ctcactaagggaaacaagctggtacCGGATGCAAATTTTTCGAAT</td>
<td>pIMAY::<em>spsL</em> Rep</td>
</tr>
<tr>
<td><em>spsL</em> B</td>
<td>ATGTTTTTTTTTTTCTTTCTTTTGTAACAC</td>
<td>pIMAY::<em>spsL</em> Rep</td>
</tr>
<tr>
<td><em>spsL</em> C</td>
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</tr>
<tr>
<td><em>spsL</em> D</td>
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<td>pIMAY::<em>spsL</em> Rep</td>
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Table 3.3 continued

<table>
<thead>
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<th>Primer Name</th>
<th>Sequence (5’ to 3’)*</th>
<th>Function</th>
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</thead>
<tbody>
<tr>
<td>pIMAY MCS F</td>
<td>TACATGTCAAGAATAAAACTGCCAAGAC</td>
<td>Transformation Check</td>
</tr>
<tr>
<td>pIMAY MCS R</td>
<td>AATACCTGTGACGGAAGATCCTTCG</td>
<td>Transformation Check</td>
</tr>
<tr>
<td>spsD OUT F</td>
<td>CGCGAAAAAATATTTAATTTACTTGTGG</td>
<td>Integration PCR</td>
</tr>
<tr>
<td>spsD OUT R</td>
<td>GCGCCATCGACAATGAACG</td>
<td>Integration PCR</td>
</tr>
<tr>
<td>spsL OUT F</td>
<td>CATGCAACATAAGGCAGCAT</td>
<td>Integration PCR</td>
</tr>
<tr>
<td>spsL OUT R</td>
<td>TACGTCAAAGTGAAACGTTTGAC</td>
<td>Integration PCR</td>
</tr>
<tr>
<td>Seq spsL Mut F</td>
<td>TTAATTTCAGGGTATGGAGTGAT</td>
<td>ED99ΔspsL Sanger sequencing</td>
</tr>
<tr>
<td>Seq spsL Mut R</td>
<td>AAGGCCATCGTAAACCATAAAG</td>
<td>ED99ΔspsL Sanger sequencing</td>
</tr>
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### Table 3.3 continued

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5’ to 3’)*</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manipulation of pIMAY</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pIMAY secY F</td>
<td>AGATCTCCTCTCGCCTGTCCCC</td>
<td>pIMAY::secY ED99</td>
</tr>
<tr>
<td>pIMAY secY R</td>
<td>CCCATCAAGCTTTATTTAATTATCTCT</td>
<td>pIMAY::secY ED99</td>
</tr>
<tr>
<td>ED99 secY F</td>
<td>ggggacagggcgagaggagtctTGATCTAATGTTTCAACGCTTGTT</td>
<td>pIMAY::secY ED99</td>
</tr>
<tr>
<td>ED99 secY R</td>
<td>agagtataataataagctttagggAAGATGATAATAGAAATACCATTACCA</td>
<td>pIMAY::secY ED99</td>
</tr>
<tr>
<td>Check secY F</td>
<td>GCGCTGCTTCCCTGCTGTTT</td>
<td>Sequencing</td>
</tr>
<tr>
<td>Check secY R</td>
<td>TCTGGGCAGTTTACGGGTTGTA</td>
<td>Sequencing</td>
</tr>
</tbody>
</table>

*Underlined sequences represent restriction digestion sites and lower case sequences represent complementary sequence in allelic replacement primers. Restriction digestion enzymes employed are provided alongside the primer name.
Figure 3.1. pIMAY Vector Map. pIMAY is derived from 3 plasmids with the region taken from each plasmid shown by the coloured arrows. The *E. coli* origin of replication (p15A) and selective chloramphenicol marker (*cat*) are derived from pIMC (blue arrows) (Monk et al., 2008). The temperature sensitive Gram-positive replication genes are derived from pVE6007 (red arrows) (Maguin et al., 1992) and the tetracycline inducible antisense *secY* region are derived from pKORI (black arrows) (Bae and Schneewind, 2006). This plasmid can replicate normally in *E. coli* at 37°C but is highly temperature sensitive in Staphylococci (Monk et al., 2012). pIMAY-Z is a derivative of pIMAY including a *lacZ* gene (Monk et al., 2015). Image created using Savvy and adapted from (Monk et al., 2012).
3.3.2 Construction of Allelic Replacement Plasmids

Allelic replacement was employed to generate gene deletion and repaired strains. Figure 3.2 gives an overview of the allelic replacement method employed. For these deletion strains, flanking regions of *spsD* and *spsL* were amplified using high fidelity polymerase as described in general methods (section 2.3), with an annealing temperature of 52°C (AB region) or 45°C (CD region), and an extension time of 15 s. After PCR purification, as described in general methods (section 2.5), 1 µl of both the AB and CD products were spliced together using an overlapping PCR with an annealing temperature of 50°C and extension time of 20 s. For the repaired strains, the same method was employed except that the whole gene of interest as well 500 bp flanking regions were amplified. The PCR reactions were performed with an annealing temperature of 50°C and extension time of either 15 s (AB region) or 1 min (CD region). The overlapping AD PCR reactions were performed with an annealing temperature of 55°C and extension time of 1 min 10 s.

Cloning of the *spsD* deletion flanking regions involved blunt cloning into pSC-B (StrataClone), restriction digestion with *KpnI* and *SacI* (New England BioLabs), and ligation into pIMAY as described in the general methods (section 2.10). Cloning of the *spsL* deletion flanking regions and both repaired plasmids utilised the SLIC technique outlined in general methods (section 2.11). pIMAY was digested using *KpnI* and *SacI* (New England BioLabs) as described in general methods (section 2.7). Amplification of pIMAY was performed with an annealing temperature of 50°C and extension time of 6 min. *DpnI* (New England BioLabs) digestion of the plasmid PCR reaction removed contaminating plasmid DNA allowing SLIC to be performed. Allelic replacement plasmids were then electro-transformed into *E. coli* DC10B cells with 100 µg/ml Amp to select for positive clones (section 2.9). Allelic replacement plasmids were screened by PCR, restriction digestion, and confirmed by Sanger sequencing as described in the general methods (sections 2.3, 2.7, and 2.8).
3.3.3 Preparation of *S. pseudintermedius* Electro-competent Cells and Electro-transformation

ED99 competent cells were produced using a procedure outlined previously (Monk et al., 2012). Overnight culture was diluted to an OD$_{600}$ of 0.5 in 50 ml pre-warmed TSB (Oxoid) and re-incubated for 30 min at 37°C with shaking at 200 rpm. The cells were placed on ice for 10 min and centrifuged at 3900 xg for 10 min at 4°C. The pellet was washed twice with 50 ml sterile ice-cold milliQ water, suspended once in 10 ml 10% (v/v) ice-cold glycerol (Sigma-Aldrich, UK), and once in 2 ml ice-cold 10% (v/v) glycerol. The final pellet was suspended in 250 µl ice-cold 10% (v/v) glycerol and aliquot into 50 µl volumes for storage at -80°C. For electro-transformation, competent cells were thawed on ice for 5 min and placed at room temperature for 5 min. The cells were centrifuged at 5000 xg for 1 min and suspended in 80 µl 10% (v/v) glycerol supplemented with 500 mM sucrose (Fisher Scientific). 5 µg of plasmid in no more than 5 µl was applied and the mixture transferred to a 0.1 cm gap cuvette (Sigma-Aldrich). Electroporation at 100 Ω, 25 µF, and 2.1 kV. 1 ml of TSB supplemented with 500 mM (w/v) sucrose (Fisher Scientific) was immediately applied to the cells and they were allowed to recover for over 2 h at 28°C with shaking at 200 rpm before plating 100 µl onto TSA supplemented with 10 µg/ml Cm (Sigma-Aldrich). Plates were incubated at permissive temperature, 28°C, for at least 48 h.

3.3.4 Allelic Replacement

For plasmid integration, confirmed transformants were suspended into 200 µl of TSB and diluted 1:10 000. 100 µl of this dilution was plated onto TSA supplemented with 10 µg/ml Cm and incubated at 37°C overnight. 18 large colonies were streaked onto TSA 10 µg/ml Cm and incubated at 37°C overnight. Colonies were screened for integration by high fidelity colony PCR as described in general methods (section 2.3) using the A and D cloning primers and OUT primers (Figure 3.2) with annealing temperature of 57°C and extension time of 1 min.
Figure 3.2. Schematic Representation of Allelic Replacement. (A) 500 bp flanking regions of the gene of interest (goi) were amplified by PCR using primers A to D (Table 3.3). The complementary sequence within primer C of primer B allows overlapping PCR to produce a splicing PCR product of 1 kb. This PCR product is cloned into pIMAY using restriction digestion and ligation. (B) Flanking regions are amplified as stated for (A) except that complementary sequences to pIMAY are also present in primers A and D (Table 3.3). Primers are also employed to PCR amplify pIMAY with SLIC employed to produce the deletion plasmids. (C) Single crossover events, at 37°C, occur due to homologous recombination in 2 potential directions, AB or CD. The direction of crossover is determined by colony PCR with OUT, A, and D primers. Double crossover, at 28°C, allows plasmid excision producing a gene deletion mutant or recreation of the wild type. Deletion of spsD removed 3096 bp from the genome and deletion of spsL removed 2793 bp from the genome. PlasMapper version 2.0 (Dong et al., 2004) was employed to produce the plasmid outline.
Colonies representing putative integrants were inoculated into 10 ml TSB and incubated at 28°C, 200 rpm overnight. The cultures were temperature shifted to 37°C with shaking at 200 rpm for 12 h. This cycle of temperature shifting was repeated for up to 4 additional rounds with dilution of 1:1000 into fresh 10 ml TSB after each temperature shift. To screen for double cross-over events, 100 µl of culture was serially diluted to 10⁻⁶ and 100 µl plated onto TSA followed by incubation at 37°C overnight. Each colony was replica plated onto TSA and TSA containing 10 µg/ml Cm. Colonies with Cm sensitivity were tested for gene loss using colony PCR and then confirmed by PCR with gDNA template using OUT primers, and Sanger sequencing as described in the general methods (sections 2.3 and 2.8).

3.3.6 Validation of Gene Deletion and Repaired Strains

In order to rule out the acquisition of spurious mutations during allelic replacement, the growth phenotype of gene deletion and repaired strains was compared to the wild type using a FLUOstar® Omega (BMG Labtech) plate reader with OD₆₀₀ readings taken every 30 min for at least 12 h. Overnight cultures were diluted to an OD₆₀₀ of 0.05 in 200 µl of BHI per well. Triplicate cultures were analysed in each experiment and the experiment was repeated 3 times. Protein profiles were also analysed at both exponential and stationary phases as described in general methods (section 2.20). Western blot analysis was performed as described in general methods (section 2.13) and employed 1 µg/ml anti-SpsD or anti-SpsL N2N3 IgY and 0.5 µg/ml F(ab’)2 rabbit anti-chicken HRP-conjugated IgG (Bethyl Laboratories).

3.3.7 Solid Phase Adherence Assays

Bacterial adherence to ECM proteins was analysed as described in the general methods (section 2.19). Positive controls employed included S. aureus Newman (Fg-binding), S. aureus SH1000 (Fn-binding), S. aureus 476 (collagen-binding), and where appropriate ED99. Negative controls employed included L. lactis pOri23, PBS, and ED99ΔspsLΔspsD.
3.3.8 Clumping Assay

Bacteria were cultured to the appropriate OD$_{600}$ in BHI, pelleted and washed with PBS before being suspended in PBS to an OD$_{600}$ of 6.0. Two-fold serial dilutions of canine Fg were carried out in 50 µl volume of PBS in a 96 well plate. After addition of 20 µl of bacteria per well the plate was shaken for 5 min and clumping was assessed by eye.
3.4 Results

3.4.1 *S. pseudintermedius* ED99 Exhibits Adherence to Immobilised Extracellular Matrix Proteins

Previously, *S. pseudintermedius* ED99 has been reported to adhere to canine Fg, murine CK-10, and bovine Fn (Bannoehr et al., 2011). The ability of ED99 to adhere to Fg from different host species was not examined and the Fg employed was not purified to remove contaminating ECM molecules such as Fn (Bannoehr et al., 2011). In the current study Fn was depleted from all Fg samples, as described in the general methods (section 2.17), to allow accurate investigation into the binding of ED99 to Fg from different host species without background levels of binding to contaminating ECM proteins (Figure 3.3A). ED99 demonstrated host specific binding to Fg (p<0.0001) (Figure 3.3A) similar to the binding previously reported for *L. lactis* expressing SpsL (Bannoehr et al., 2011). In addition, ED99 exhibited binding to murine CK-10 (Figure 3.3C) and equivalent binding to both bovine and murine Fn (Figure 3.3B). The adherence of ED99 to multiple ECM proteins from mice suggests that murine models may be a feasible approach for investigating the pathogenesis of *S. pseudintermedius*. ED99 does not adhere to bovine collagen type I (Figure 3.3C).

3.4.2 *L. lactis* expressing SpsD or SpsL Exhibit Adherence to Immobilised Extracellular Matrix Proteins

To investigate the potential function of SpsD and SpsL in mediating the ECM protein interactions of ED99, *L. lactis* expressing strains were investigated for adherence to Fn-depleted Fg from different host species (Figure 3.4), bovine and murine Fn, and murine CK-10 (Figure 3.5), after stationary phase of growth. *L. lactis* expressing SpsD exhibited binding to Fg from all host species except for ovine Fg (Figure 3.4A). *L. lactis* expressing SpsL demonstrated host-specific binding to Fg with the binding to canine Fg higher than the binding to Fg from all the other host species examined (p<0.0001) (Figure 3.4B).
Figure 3.3. ED99 Adheres to Fibrinogen, Fibronectin, and Cytokeratin-10. (A) Fg from different host species. (B) Bovine and murine Fn. (C) Bovine collagen Type I and murine recombinant CK-10. Bacterial adherence was analysed at OD$_{600}$ of 0.6. Results are the mean value of triplicate readings ± standard deviation; n=3. *L. lactis* pOri23 and appropriate *S. aureus* strains were employed as controls (data not shown). Each graph is representative of at least 3 independent experiments. Statistical differences in binding to Fg from different host species at 10 µg/ml were analysed by one-way ANOVA and represented by ***p≤0.001.
Figure 3.4. SpsD and SpsL Exhibit Host Specific Fibrinogen Binding When Expressed by *L. lactis*. (A) *L. lactis* expressing SpsD. (B) *L. lactis* expressing SpsL. Bacterial adherence was analysed using stationary overnight cultures. Results are the mean value of 3 independent experiments performed in triplicate ± standard deviation; n=9. *L. lactis* pOri23 was employed as a negative control and ED99 was employed as a positive control (data not shown). Statistical analysis using 2-sample t-tests at 10 µg/ml demonstrated increased binding of *L. lactis* expressing SpsL to canine Fg in comparison to Fg from other host species and is represented by ***p≤0.001.
Figure 3.5. *L. lactis* Expressing SpsD and SpsL Exhibit Fibronectin Binding and SpsD Exhibits Cytokeratin-10 Binding. (A) Bovine and murine Fn. (B) Murine recombinant CK-10. Bacterial adherence was analysed using stationary overnight cultures. Results are the mean value of 3 independent experiments performed in triplicate ± standard deviation; n=9. *L. lactis* pOri23 was employed as a negative control and ED99 was employed as a positive control (data not shown).
Generally *L. lactis* expressing SpsL exhibits lower binding affinity for canine Fg compared to *L. lactis* expressing SpsD. Using this expression system it is unclear whether this is due to differences in binding affinity or in the level of protein expression on the surface of *L. lactis*. *L. lactis* expressing SpsD and SpsL demonstrated comparable binding to both bovine and murine Fn (Figure 3.5A). Furthermore, only *L. lactis* expressing SpsD bound to recombinant murine CK-10 (Figure 3.5B).

### 3.4.3 Generation of *spsD* and *spsL* Deletion Mutants

The previous binding experiments suggested that SpsD and SpsL could mediate the adherence of ED99 to multiple ECM proteins. In order to evaluate the role of SpsD and SpsL in the ECM-binding characteristics of ED99 (Figure 3.3), we constructed the first gene deletion mutants for a *S. pseudintermedius* isolate. Primers were designed to delete the whole of the *spsL* gene with only the signal peptide of the *spsD* gene still encoded in the genome (Table 3.3). From the genome sequence of *S. pseudintermedius* ED99 there are no predicted overlapping reading frames at the *spsL* or *spsD* loci suggesting that the experimental deletions would have no effect on the transcription or translation of secondary proteins. The allelic replacement vector employed has been designed for use with *S. aureus* (Monk et al., 2012). Genetic manipulation of pIMAY to encode ED99 specific antisense secY excision machinery was unsuccessful with ED99::pIMAY capable of growing in the presence of both the pIMAY resistance marker, Cm, and the antisense inducer, ATc (data not shown). The presence of *lacZ* in the genome of ED99 prevented blue-white screening for plasmid excision with the *lacZ*-encoding vector pIMAY-Z (Monk et al., 2015). Identification of mutant strains therefore involved screens of more than 3000 colonies plated after 4 rounds of temperature shifting. Confirmation of gene deletion mutants were performed using OUT primers with ED99Δ*spsD* resulting in a PCR product of 1094 bp and ED99Δ*spsL* resulting in a PCR product of 829 bp (Figure 3.6). A double deletion strain was then produced using the same method by deleting the *spsD* gene from the ED99Δ*spsL* background with the pIMAY::*spsD* plasmid (Figure 3.6). Sanger sequencing confirmed the correct construction of each deletion mutant.
Figure 3.6. PCR Confirmation of Gene Deletion. OUT primers located outside the flanking regions of the gene of interest allow identification of gene deletions (Table 3.3). OUT PCR reactions employed an annealing temperature of 57°C and extension time of 1 min 10 s for spsD and an annealing temperature of 49°C and extension time of 1 min for spsL. No spurious mutations were identified in the flanking regions of either gene by Sanger sequencing.
3.4.4 Validation of the *spsD* and *spsL* Deletion Mutants

Once sequencing analysis had confirmed effective allelic replacement of *spsD* and *spsL* in ED99, the growth characteristics of the deletion mutants were analysed. Each mutant was indistinguishable in growth rate and growth yield from the wild type (Figure 3.7A). To ensure the single gene deletions did not result in pleiotropic effects, or that spurious mutations had not been introduced during the allelic replacement process, extracellular and CWA protein profiles of ED99 and mutant derivative strains were analysed at both exponential and stationary phases (Figure 3.7B). All strains examined had indistinguishable protein profiles (Figure 3.7B). Loss of SpsD and SpsL expression was confirmed by Western blot analysis of CWA protein profiles (Figure 3.8). Western blot analysis indicated that SpsD was expressed maximally at early exponential phase (OD$_{600}$ of 0.2) whereas the expression of SpsL was observed throughout exponential phase with highest expression at OD$_{600}$ of 0.6 (Figure 3.8). These time-points were subsequently employed to examine the ECM-binding activity of ED99Δ*spsD* and ED99Δ*spsL*.

3.4.5 ED99Δ*spsL*Δ*spsD* Exhibits Complete Loss of Binding to Fibrinogen, Fibronectin, and Cytokeratin-10

Solid phase adherence assays were employed to compare the ECM-binding of ED99 wild type and ED99Δ*spsL*Δ*spsD* in different growth phases. ED99Δ*spsL*Δ*spsD* exhibited loss of binding to Fg from all host species, bovine and murine Fn, and recombinant CK-10 at all growth phase time points tested (Figure 3.9). Accordingly, in all future solid phase adherence assays ED99Δ*spsL*Δ*spsD* was employed as a negative control.
Figure 3.7. Deletion Mutants Exhibit Equivalent Growth Phenotype and Protein Profiles to ED99. (A) Growth curve analysis comparing all 3 deletion mutants with ED99 wild type. Growth was analysed in BHI at 37°C with shaking in 96 well plates at OD$_{600}$. Readings were taken every 30 min. Each point represents the mean of triplicate cultures ± standard deviation; n=3. This experiment was performed 3 times with consistent results. (B) Extracellular (XC) and cell wall associated (CWA) protein profiles were analysed at both exponential (E) and stationary (S) growth phases. All deletion mutants were analysed but only the double deletion mutant is represented here.
Figure 3.8. Validation of Deletion Mutants by Western Blot Analysis of SpsD and SpsL Expression. (A) Expression analysis of SpsD (B) Expression analysis of SpsL. CWA protein profiles were produced at either OD$_{600}$ of 0.2 for analysis of SpsD expression or OD$_{600}$ of 0.6 for analysis of SpsL expression. Specific antibodies to SpsD N2N3 or SpsL N2N3 were employed. Data is representative of 2 independent experiments.
Figure 3.9. ED99ΔspsLΔspsD Exhibits Loss of Fibrinogen, Fibronectin, and Cytokeratin-10 Binding. Binding to (A) Fg from different host species, (B) Fn, and (C) murine recombinant CK-10. Bacterial binding is analysed after culture to OD\text{\textsubscript{600}} of 0.6. Results are the mean value of triplicate readings ± standard deviation; n=3. Each graph is representative of at least 3 independent experiments. Statistical analysis using 2-sample t-tests at 10 µg/ml demonstrated decreased binding of ED99ΔspsLΔspsD compared to ED99, *p≤0.05, **p≤0.01, ***p≤0.001.
3.4.6 ED99ΔspsD Exhibits Decreased Binding to Bovine Fibrinogen at Early Exponential Phase

Comparison of the binding interaction of ED99ΔspsD with that of wild type ED99 after culture to OD<sub>600</sub> of 0.2 indicated no attenuation in binding to Fn and CK-10 (Figure 3.10B and C). However, there was decreased binding to bovine (p=0.0013) and murine Fg (p=0.108) but not to canine, human, or ovine Fg (Figure 3.10A). This suggests that SpsD is not the primary ECM-binding protein of ED99 at this growth phase or that a redundancy in ECM-binding exists.

3.4.7 ED99ΔspsL Exhibits Decreased Binding to Fibrinogen and Cytokeratin-10 at Mid Exponential Phase

Comparison of the binding interaction of ED99ΔspsL with that of wild type ED99 after culture to OD<sub>600</sub> of 0.6 exhibited complete loss of Fg and CK-10-binding (Figure 3.11A and C). This was also demonstrated when bacteria were cultured to later growth phases (data not shown). These data indicate that SpsL is the predominant CWA protein of ED99 mediating Fg, and CK-10-binding at this growth phase <i>in vitro</i>, under the conditions tested. ED99ΔspsL also exhibited decreased Fn-binding in comparison to ED99 (p&lt;0.001) but the same ablation of binding observed in the ED99ΔspsLΔspsD double mutant is not observed. This suggests that SpsD is still present on the surface of ED99 at this growth phase and mediates Fn but not Fg or CK-10-binding.
Figure 3.10. ED99ΔspSD Exhibits Reduced Bovine Fibrinogen Binding in Comparison to ED99. Binding to (A) Fg from different host species, (B) Fn and (C) murine recombinant CK-10. Bacterial binding is analysed after culture to OD600 of 0.2. Results are the mean value of triplicate readings ± standard deviation; n=3. Each graph is representative of at least 3 independent experiments. Statistical analysis using 2-sample t-tests at 10 μg/ml demonstrated decreased binding of ED99ΔspSD to bovine Fg and are represented as *p≤0.05.
**Figure 3.11.** ED99ΔspsL Exhibits Reduced Fibrinogen, Fibronectin, and Cytokeratin-10 Binding Compared to ED99. Binding to (A) Fg from different hosts, (B) bovine and murine Fn, and (C) murine CK-10. Adherence is analysed at OD600 of 0.6. Results are the mean value of triplicate readings ± standard deviation; n=3. Each graph is representative of at least 3 independent experiments. Statistical analysis using 2-sample t-tests at 10 µg/ml demonstrated decreased binding of ED99ΔspsL and are represented as *p≤0.05, **p≤0.01, and ***p≤0.001.
3.4.8 Generation of Repaired Strains using Allelic Replacement

In order to verify the specific phenotypic contribution of each protein, complementation of each mutant was carried out. Complementation attempts using expression plasmids designed for *S. aureus* were unsuccessful possibly due to poor promoter recognition. Allele replacement was therefore employed to re-introduce the *spsD* and *spsL* genes into the ED99 chromosome. Synonymous mutations were inserted into the repaired strains using primer B in order to allow differentiation of the repaired strain from the wild type. Allelic replacement of *spsL* was performed as described in section 3.3.4. The final DNA crossover event allowing re-introduction of *spsD* required extensive screening with the optimal excision method being identified as subculturing the integrated strain at 28°C and then PCR screening for clones with an intact gene and replicating plasmid. This PCR screening employed integration primers with an annealing temperature of 57°C and extension time of 1 min. Once a clone containing a re-introduced gene had been identified, pIMAY was excised from the cell by subculturing at 28°C for 5 rounds before replica plating as described in section 3.3.5.

Successfully repaired strains were confirmed by OUT PCR with ED99ΔspsD Rep resulting in a PCR product of 4190 bp and ED99ΔspsL Rep resulting in a PCR product of 3622 bp (Figure 3.12). Identification of the synonymous mutation by Sanger sequencing confirmed the generation of repaired strains. Growth analysis identified no defects in growth rate or yield of the repaired strains (Figure 3.13A) and no alteration in protein profiles (Figure 3.13B and C). Western blot analysis of CWA proteins of the repaired strains confirmed the expression of SpsD and SpsL in cell wall fractions from repaired strains (Figure 3.14).
Figure 3.12. PCR Confirmation of Gene Re-introduction. OUT primers located outside the flanking regions of the gene of interest allow identification of gene insertions (Table 3.3). OUT PCR reactions employed an annealing temperature of 57°C and extension time of 1 min 10 for *spsD* and an annealing temperature of 49°C and extension time of 1 min for *spsL*. The addition of a synonymous mutation into the signal peptide of each repaired gene was confirmed using Sanger sequencing.
Figure 3.13. Growth Phenotype and Protein Profiles are maintained in Deletion and Repaired Strains. (A) Growth was analysed in BHI at 37°C with shaking in 96 well plates using a FLUOstar® Omega (BMG Labtech) plate reader at OD\textsubscript{600}. Readings were taken every 15 min with readings taken at every 30 min plotted here for ease of data presentation. Each point represents the mean of 4 cultures ± standard deviation; n=4. This experiment was performed 3 times with comparable results. (B) Cell wall associated and (C) extracellular protein profiles. Protein profiles were analysed at both exponential and stationary phases of growth as indicated. The repaired strains were compared to the wild type as well as the deletion mutants.
Figure 3.14. Validation of Repaired Strains by Western Blot Analysis of SpsD and SpsL Expression. (A) Expression analysis of SpsD (B) Expression analysis of SpsL. CWA protein profiles were produced at either OD$_{600}$ of 0.2 or 0.6 and analysed for SpsD or SpsL expression using antibodies specific to SpsD N2N3 or SpsL N2N3. Data is representative of CWA protein profiles produced on 2 independent occasions.
3.4.9 Re-introduction of \textit{spsD} and \textit{spsL} Results in Restoration of Fibrinogen Binding

Bacterial adherence assays were employed to compare the ECM-binding properties of ED99\textDelta{spsD}Rep and ED99\textDelta{spsL}Rep in comparison to ED99 and the derived deletion strains. Dose-dependent binding to canine Fg demonstrated restored adherence in both ED99\textDelta{spsD}Rep (Figure 3.15A) and ED99\textDelta{spsL}Rep strains (Figure 3.16A). Binding to other host Fg molecules at 20 \(\mu\)g/ml was also analysed with the repaired strains exhibiting increased Fg-binding in comparison to the appropriate deletion strain (Figure 3.15B and Figure 3.16B). Unexpectedly, in some cases adherence to Fg was slightly increased in comparison to the wild type strain. This is not due to mutations in the \textit{spsD} or \textit{spsL} gene or promoter regions and is likely due to experimental variation of the bacterial adherence assay. These experiments confirm that SpsD and SpsL are key Fg-binding proteins of \textit{S. pseudintermedius} with deletion of these proteins resulting in decreased bacterial adherence to ECM proteins.

3.4.10 ED99 Does Not Clump in the Presence of Soluble Fibrinogen

Fg-binding bacterial proteins have the ability to promote bacterial clumping in the presence of soluble Fg. To investigate if this is the case for SpsL, ED99 and ED99\textDelta{spsL}\textDelta{spsD} were analysed for clumping ability in the presence of soluble canine Fg. ED99 exhibited no clumping phenotype at either exponential (OD\textsubscript{600} of 0.6) or stationary phases of growth (Figure 3.17). \textit{S. aureus} Newman was employed as a positive control and exhibited clumping at both growth phases with a clumping titre of 7.8125 \(\mu\)g/ml. Clumping ability of SpsD was not investigated due to the large culture volumes required to analysis clumping at early-exponential growth phase.
**Figure 3.15. Re-introduction of spsD to ED99ΔspsD Restores Fibrinogen Binding.**

Binding of bacteria cultured to OD$_{600}$ of 0.2 to (A) canine Fg and (B) multiple host Fg. Results are the mean value of 3 independent experiments ± standard deviation; n=9. Statistical analysis using 2-sample t-tests at 20 µg/ml demonstrated increased binding of ED99ΔspsD Rep in comparison to ED99ΔspsD and are represented as *p≤0.05, and ***p≤0.001.
Figure 3.16. Re-introduction of *spsL* to ED99∆spsL Restores Fibrinogen Binding. Binding of bacteria cultured to OD600 of 0.6 to (A) canine Fg and (B) multiple host Fgs. Results are the mean value of 3 independent experiments ± standard deviation; n=9. Statistical analysis using 2-sample t-tests at 20 µg/ml demonstrated increased binding of ED99∆spsL Rep in comparison to ED99∆spsL and are represented as ***p≤0.001.
Figure 3.17. ED99 Does Not Clump in the Presence of Soluble Fibrinogen. (A) Exponential (OD$_{600}$ of 0.6) and (B) stationary phase cultures were examined for the ability to clump in the presence of decreasing concentrations of canine Fg. *S. aureus* Newman was employed as a positive control for clumping and PBS was employed as a negative control. *S. aureus* Newman exhibited a clumping titre of 7.8125 µg/ml.
3.5 Discussion

The importance of investigating the function of bacterial proteins within their natural genetic background is stated by the molecular Koch’s postulates (Falkow, 1988). The deletion of both spsD and spsL in the S. pseudintermedius ED99 genetic background exhibited loss of Fg, Fn, and CK-10-binding (Figure 3.9) suggesting the importance of both proteins in S. pseudintermedius host-pathogen interactions. Re-introduction of spsD and spsL genes confirmed that SpsD and SpsL are key ECM-binding proteins of S. pseudintermedius ED99 in vitro (Figures 3.16 and 3.17). It is noteworthy that L. lactis expressing SpsL does not exhibit CK-10-binding (Figure 3.5) in contrast to its role in promoting CK-10-binding by ED99 (Figure 3.11). This discrepancy suggests that SpsL expression on the surface of L. lactis and ED99 differs. Potentially proteolytic cleavage of SpsL on the surface of L. lactis limits CK-10 but not Fg- or Fn-binding. Conversely CK-10-binding by ED99 may involve a complex of proteins with SpsL not sufficient for CK-10-binding but important in the CK-10-binding complex. Whatever the mechanism for the discrepancy between L. lactis and ED99, this observation highlights the need for studies evaluating the function of specific proteins in their natural genetic background rather than relying on the use of heterologous hosts.

In a collaboration with P. Speziale, the deletion mutants generated in the current study have allowed detailed investigation into S. pseudintermedius invasion of canine keratinocytes in vitro (Pietrocola et al., 2015). Both SpsD and SpsL are capable of promoting Fn-dependent invasion of ED99 with ED99ΔspsLΔspsD exhibiting significantly reduced cellular internalisation (Pietrocola et al., 2015). The Fn-binding assays performed in the current study highlight the redundancy of SpsD and SpsL with deletion of one protein insufficient to eliminate Fn-binding (Figure 3.10 and 3.11). This redundancy suggests that Fn-binding and invasion are important for S. pseudintermedius pathogenesis but this remains to be evaluated in vivo.

The construction of the first deletion mutants in S. pseudintermedius in the current study represents an important milestone. The availability of techniques to genetically manipulate S. pseudintermedius will allow broad new insights into the pathogenesis of S. pseudintermedius. In turn this increased understanding of key virulence factors
involved in canine pyoderma pathogenesis could allow the design of novel therapeutics. However, genetic manipulation is hindered by the presence of RM systems that limit plasmid transfer into *S. pseudintermedius*. Genetic investigation of ED99 identified an incomplete Type I RM system, predicted to be non-functional, as well as Type II and Type IV RM systems (McCarthy et al., 2015; Monk and Foster, 2012). The Type IV RM *SauSI* endonuclease homologue encoded by ED99 is predicted to recognise and degrade cytosine methylation of DNA (Monk and Foster, 2012). The *dcm* gene of *E. coli* mediates cytosine methylation of DNA and so the use of *E. coli* strain DC10B, containing a deletion of *dcm*, as a primary host, facilitated transformation of ED99 (Bhagwat et al., 1986; Monk et al., 2012). The transformation efficiency of ED99 using this system was still relatively low suggesting that the encoded Type II RM system, with a predicted restriction cut site of CTRYAG, is limiting plasmid transfer efficiency (McCarthy et al., 2015). For optimal transformation of ED99, a novel DC10B background *E. coli* strain would be required encoding ED99 specific *hsdM* and *hsdR* genes. This would allow appropriate methylation of pIMAY for optimal ED99 transformation. This method of plasmid artificial modification has been achieved for *S. aureus* with clonal complex-specific type I modification genes cloned into *E. coli* DC10B (Monk et al., 2015).

The development of specialised allelic replacement vectors for *S. pseudintermedius* would also be extremely useful. pIMAY was specifically designed for use in *S. aureus* with *lacZ* employed to identify transformed colonies and the anti-*secY* antisense mechanism allowing efficient excision at the final stages of allele replacement (Bae and Schneewind, 2006; Monk et al., 2015). Neither of these mechanisms were functional for ED99 with insertion of a *S. pseudintermedius* specific *secY* sequence proving ineffective at inducing lethality. This either suggests that the *secY* promoter within pIMAY is non-functional in ED99 or that SecY is not essential for ED99 growth. This hampered the ability to cure ED99 of pIMAY after homologous recombination making the final step of allelic replacement a labour intensive exercise requiring screening of thousands of colonies. The development of expression plasmids allowing complementation of *S. pseudintermedius* would also be highly beneficial to this research field. Attempts were made in the current study to complement the ED99 deletion mutants using expression plasmids designed for *S. aureus*. This was
unsuccessful suggesting that some \textit{S. aureus}-specific promoters may be ineffective in \textit{S. pseudintermedius}. The lack of expression analysis tools for \textit{S. pseudintermedius} meant that gene re-introduction procedures were required. Overall, further studies looking at the function of specific proteins in \textit{S. pseudintermedius} pathogenesis would benefit from a more extensive array of genetic manipulation tools specifically designed for use in this staphylococcal species. The high prevalence of the MRSP ST71 clone and its apparent zoonotic potential may warrant the development of cloning techniques tailored to this clinical isolate (Perreten et al., 2010).

The deletion mutants developed in the current study demonstrate that only 2 surface proteins of \textit{S. pseudintermedius} ED99 facilitate binding to Fg, Fn, and CK-10 under the growth conditions tested (Figure 3.9). This is in contrast to \textit{S. aureus} which has at least 6 surface proteins mediating Fg-binding activity, 3 mediating Fn-binding activity and 2 responsible for CK-10-binding activity (Foster et al., 2014). Redundancy in ED99 is observed with both SpsD and SpsL mediating Fg and CK-10-binding at early-exponential phase and Fn-binding throughout exponential phase. However, the binding phenotype of ED99\textDelta{spsL}\textDelta{spsD} suggests that no other surface protein of ED99 mediates Fg, Fn, and CK-10-binding \textit{in vitro} (Figure 3.9). On the other hand, it is known that expression of CWA proteins of \textit{S. aureus} can vary depending on both the growth conditions and the genetic background (Dreisbach et al., 2010; Stentzel et al., 2014). This is also likely to be the case for \textit{S. pseudintermedius} with the potential that IsdA homologues or other proteins may promote Fg-binding in iron-limited growth conditions as observed in \textit{S. aureus} (Clarke et al., 2004). Adherence to ECM proteins under iron-limiting conditions could be particularly important \textit{in vivo} (Clarke et al., 2004). SpsB, SpsH, SpsJ, and SpsO all share homology to Sdr proteins and so it will be important to characterise the adherence of these proteins to ECM proteins (Bannoehr et al., 2011). Solid phase adherence assays only investigate surface-bound ligand receptors and do not take into account the interaction of secreted bacterial proteins. For example, \textit{S. aureus} secreted coagulase (Coa) and von Willebrand binding protein (vWbp) have been observed to bind to Fg (Thomer et al., 2013). Of note, ED99 encodes a vWbp protein but its Fg-binding capacity has not been examined to date.
The use of Fn-depleted Fg from different host species allowed the host specific binding tropism of ED99 to be investigated in the current study (Figure 3.3). Both ED99 and L. lactis expressing SpsL demonstrated the same host tropism for Fg with canine Fg promoting the highest level of binding. Host-specific Fg-binding has been identified for most S. aureus Fg-binding proteins. In the case of ClfA, and other Fg γ chain binding proteins, loss of ovine Fg-binding has been attributed to a single residue substitution Q407A in the C-terminal γ chain (Geoghegan et al., 2010). The preferential binding to canine Fg suggests that SpsL has a role in the host adaptation of S. pseudintermedius and further investigation into the molecular basis for this host specificity is warranted (Chapter 5).

A detailed expression analysis of ED99ΔspsD and ED99ΔspsL in vitro is performed in Chapter 4. However, validation of the gene deletion mutants in the current study suggests that SpsD is only expressed during early exponential phase (Figure 3.8). This conflicts with the functional role of SpsD in Fn-binding throughout exponential phase (Figure 3.11). Molecular analysis of SpsD using recombinant proteins has identified Fn-binding functions for both the N2N3 subdomains and the C region (Pietrocola et al., 2013; Pietrocola et al., 2015). This suggests that loss of Fg, Fn, and CK-10 binding by cleavage of the N2N3 subdomains of SpsD would still provide Fn-binding of the C region explaining the redundancy of only Fn-binding at mid-exponential growth phase (Figure 3.11). This hypothesis is investigated in more detail in Chapter 4.

The use of allele replacement to verify the function of SpsD and SpsL in host-pathogen interactions of S. pseudintermedius raises questions regarding their roles during colonisation and infection. The expression of SpsD on the surface of L. lactis is sufficient to promote adherence to canine corneocytes and it would be important to investigate the adherence of ED99 deletion mutants to canine corneocytes (Bannoehr et al., 2011). The capacity of both SpsD and SpsL to adhere to murine ECM proteins (Figures 3.4 and 3.5) allow the role of the proteins in the pathogenesis of S. pseudintermedius disease to be investigated using murine models of infection. The development of the first murine infection model of S. pseudintermedius is detailed in Chapter 6. S. pseudintermedius clinical isolates are often characterised for biofilm formation with most isolates displaying high biofilm production (DiCicco et al., 2014;
Gawande et al., 2014; Terry and Neethirajan, 2014). Initial observations suggest that SpsD and SpsL are not required for biofilm formation but more detailed analysis is needed to confirm this (data not shown). Clumping of *S. pseudintermedius* is strain-dependent and analysis performed here identifies that ED99 does not exhibit a clumping phenotype in the presence of soluble canine Fg (Figure 3.17). This suggests that unlike bacterial proteins such as ClfA, SpsL is unable to mediate cell-cell adherence using Fg as a bridge (Ní Eidhin et al., 1998).

In summary, the first gene deletion mutants of *S. pseudintermedius* have demonstrated key functions for SpsD and SpsL in mediating adherence to Fg, Fn, and CK-10. This suggests that SpsD and SpsL could be involved in important host-pathogen interactions underpinning *S. pseudintermedius* pathogenesis. Optimisation of allele replacement and complementation techniques will be invaluable when characterising additional virulence factors of *S. pseudintermedius*. The importance of SpsD and SpsL in mediating internalisation of *S. pseudintermedius* has already been reported with more research needed to fully characterise the multiple functions of these proteins during pathogenesis (Pietrocola et al., 2015).
Chapter 4 Variation of SpS\textsubscript{D} and SpS\textsubscript{L} among \textit{S. pseudintermedius} Natural Populations
4.1 Introduction

The construction of the first gene deletion strains of *S. pseudintermedius* have confirmed the importance of SpsD and SpsL in mediating binding to components of the host ECM. With the rapid emergence and clonal spread of MRSP, therapeutic options for canine pyoderma are becoming increasingly limited (Gold et al., 2013; Perreten et al., 2010). Ideally, rationale vaccine design targeting conserved bacterial factors involved in key host-pathogen interactions would be performed. This approach can only be successful if therapeutic targets are expressed by all strains in *S. pseudintermedius* natural populations and exhibit limited genetic diversity. Sequence analysis is therefore required to examine the distribution of genes encoding virulence factors among the *S. pseudintermedius* population and to investigate the extent of inter-strain genetic diversity. For example, in *S. aureus*, whole genome sequence analysis was used to investigate the distribution of genes encoding surface and immune evasion proteins among 58 diverse strains and identified the extent of genetic diversity of each determinant between strains (McCarthy and Lindsay, 2010). Of the MSCRAMMs analysed, only FnBPA was encoded by all strains with ClfA, ClfB, FnBPB, SdrC, and SdrD encoded by most strains and Cna absent from the majority of strains (McCarthy and Lindsay, 2010). All surface proteins exhibited genetic diversity between strains with FnBPA displaying the highest levels of inter-strain variation (McCarthy and Lindsay, 2010). These data are useful when identifying potential vaccine candidates involved in *S. aureus* host-pathogen interactions.

Genetic analysis of the *S. pseudintermedius* population has demonstrated the presence of *spsL* in all strains analysed with *spsD* present in all strains except ST258 and ST259 (Bannoehr et al., 2011; Diribe et al., 2014; Latronico et al., 2014; McCarthy et al., 2015). Analysis of a small number (n=15) of *S. pseudintermedius* whole genome sequences noted that *spsD* and *spsL* exhibited diversity between strains but detailed genetic analysis was not performed (McCarthy et al., 2015). The potential role of both SpsD and SpsL in *S. pseudintermedius* host-pathogen interaction during canine pyoderma suggests that they could represent novel therapeutic targets and so detailed genetic analysis of these proteins is warranted. Here a range of clinical isolates from our strain collection as well as publically available genome sequences were employed
to investigate the sequence diversity of *spsD* and *spsL* genes and their expression among clinical isolates.

## 4.2 Aims

1) Determine the sequence diversity of *spsD* and *spsL* among *S. pseudintermedius* clinical isolates selected to represent the breadth of species diversity

2) Investigate the molecular processes contributing to the diversification of *spsD* and *spsL*

3) Determine the variation present in the number of repeat domains of both *spsD* and *spsL*

4) Investigate the expression of SpsD and SpsL among clinical isolates
4.3 Materials and Methods

4.3.1 DNA Sequencing of spsD and spsL

The *S. pseudintermedius* strains employed in the current study are detailed in Table 4.1. 21 of these strains were obtained from our isolate collection and genome sequences of the other strains were employed from online resources such as the National Centre for Biotechnology Information (NCBI) and the European Nucleotide Archive (ENA) (Table 4.2).

Genomic DNA was amplified using high-fidelity PCR as described in general methods (section 2.3) using primers listed in Table 4.3. Additional primers employed for Sanger sequencing are also listed in Table 4.3. Annealing temperature of 51°C were used to amplify both *spsD* and *spsL* A-domain sequences with extension times of 45 s for *spsD* and 25 s for *spsL*. After PCR purification (section 2.5), samples were sequenced as described in general methods (section 2.8).

4.3.2 Multilocus Sequence Typing

Multilocus sequence typing (MLST) was performed using primers listed in Table 4.3 specific for genes *ack, fdh, purA*, and *sar*. High fidelity PCR was performed as described in general methods (section 2.3) with an annealing temperature of 52°C and extension time of 10 s. Sequences were analysed in MEGA 5 (Tamura et al., 2011) and the *S. pseudintermedius* MLST database curated by Vincent Perreten (Solyman et al., 2013) to identify sequence types (ST) (Table 4.1).
Table 4.1. *S. pseudintermedius* Strains Employed in the Current Study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sequence Type (ST)</th>
<th>Geographic Origin</th>
<th>Host Origin</th>
<th>Isolation Year</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>94-062394</td>
<td>26</td>
<td>USA</td>
<td>Canine</td>
<td>1995</td>
<td>(Edwards et al., 1997)</td>
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<td>95-072195</td>
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<td>Canine</td>
<td>1995</td>
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<td>326</td>
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<td>690</td>
<td>24</td>
<td>UK</td>
<td>Feline eye</td>
<td>2006</td>
<td>(Bannoehr et al., 2007)</td>
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<td>3279 MRSP</td>
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<td>Canine elbow</td>
<td>2005</td>
<td>(Bannoehr et al., 2007)</td>
</tr>
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<td>3414</td>
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<td>UK</td>
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<td>(Bannoehr et al., 2007)</td>
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<td>Canine eye</td>
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<td>8478</td>
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<td>UK</td>
<td>Canine nares</td>
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<td>Canine</td>
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<td>Strain</td>
<td>Sequence Type (ST)</td>
<td>Geographic Origin</td>
<td>Host Origin</td>
<td>Isolation Year</td>
<td>Reference</td>
</tr>
<tr>
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<td>BH47</td>
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<td>USA</td>
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<td>HT20030646</td>
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<td>M721-99</td>
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<td>N900260</td>
<td>66</td>
<td>France</td>
<td>Human</td>
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<td>(Bes et al., 2002)</td>
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Table 4.2. *S. pseudintermedius* Genomes Employed in the Current Study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sequence Type (ST)</th>
<th>Geographic Origin</th>
<th>Host Origin</th>
<th>Isolation Year</th>
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<td>Canine</td>
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<td>K7</td>
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<td>BNG3</td>
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<td>GL117B</td>
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<td>Germany</td>
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<td>Strain</td>
<td>Sequence Type (ST)</td>
<td>Geographic Origin</td>
<td>Host Origin</td>
<td>Isolation Year</td>
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Table 4.3. Primers Employed in the Current Study

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<td><strong>MLST</strong></td>
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<tr>
<td><em>ack</em> F</td>
<td>CACCACTTCACAACCCAGCAAACT</td>
<td>680</td>
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<tr>
<td><em>ack</em> R</td>
<td>AACCTTCTAATAACGCAGCAGCA</td>
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<tr>
<td><em>fdh</em> F</td>
<td>TGCGATAACAGGATGTGTTT</td>
<td>408</td>
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<tr>
<td><em>fdh</em> R</td>
<td>CTITCTATAGATCAGCGCC</td>
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<tr>
<td><em>purA</em> F</td>
<td>GATTACTTCAAAGGTATGTTT</td>
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<tr>
<td><em>purA</em> R</td>
<td>TCGATAGAGGTAATAGATAAGTC</td>
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<tr>
<td><em>sar</em> F</td>
<td>GGATTTAGTCAGTTCAAATTT</td>
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<tr>
<td><em>sar</em> R</td>
<td>GAACCATTCCGCCCATGAA</td>
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<td><strong>Sequencing</strong></td>
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<td><em>spsD</em> F</td>
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<td><em>spsD</em> R</td>
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<td><em>spsD</em> A-domain F</td>
<td>GCATCATTCTTTATTGGGGACATT</td>
<td>2473</td>
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<tr>
<td><em>spsD</em> A-domain R</td>
<td>AAGAAACCAGCATCGATGACATA</td>
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<tr>
<td><em>spsD</em> N2N3 F</td>
<td>ACCGTGTCCGTACCACGATTA</td>
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<td><em>spsD</em> N2N3 R</td>
<td>CCTTCCCCACTTGCAATTAGA</td>
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<tr>
<td><em>spsD</em> B-domain F</td>
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<td>666</td>
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<tr>
<td><em>spsD</em> B-domain R</td>
<td>CTTTTCTGTTTTCCGCTTTCTCA</td>
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<tr>
<td><em>spsL</em> A-domain F</td>
<td>TCATTGTTGGGGACTCA</td>
<td></td>
</tr>
<tr>
<td><em>spsL</em> A-domain R</td>
<td>TATCAATCAGCATGCTATTT</td>
<td></td>
</tr>
<tr>
<td><em>spsL</em> Mid F</td>
<td>GAGTGCGGAGGAAGTGTCTAA</td>
<td>563</td>
</tr>
<tr>
<td><em>spsL</em> Mid R</td>
<td>ACTTGCAGAAATACGTCTCAC</td>
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</table>
Table 4.3 continued

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5’ to 3’)</th>
<th>PCR Size (bp)</th>
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<tbody>
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<td>AV8024 B-domain F</td>
<td>CAAACCTGTACCGCCGACTTA</td>
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<td>AV8024 N2N3 R</td>
<td>ATAAGTCGGCGTGACAGGTTTG</td>
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<td>HT20030686 N2N3 F</td>
<td>AAAAGCAATGGCACTGGTTATGA</td>
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Number of Repeats

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<td>spsD A-domain F</td>
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<tr>
<td>spsD A-domain R</td>
<td>AAGAAACCAGCATCGATTGACATA</td>
</tr>
<tr>
<td>spsL R Repeats F</td>
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</tr>
<tr>
<td>spsL R Repeats R</td>
<td>GTTGTCGCCGTATCTGGTAG</td>
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4.3.3 Phylogenetic Analysis

Phylogenetic analysis was performed using Clustal W alignment in MEGA 5 and MegaAlign (DNASTAR Lasergene® Core Suite 9). Phylogenetic trees were produced using the Neighbour-Joining method using a 2-Kimura parameter model with 1000 resample by bootstrap. Recombination events were analysed using the Recombination Detection Program (RDP3). Recombination events were accepted if they were predicted by more than three RDP programs (p<0.05).

4.3.4 Structural Modelling

Structural modelling was performed using Phyre² (Kelley et al., 2015) and PyMol (Schrödinger). Sequences were modelled with the crystal structure of ClfA N2N3 (pdb 1N67). Variable residues were coloured in red with specific residues identified to be important for Fg-binding in ClfA coloured orange. The binding motif of SpsD and SpsL were coloured yellow and the latch coloured blue. Specific motifs in spsD were analysed using WebLogo 3.4 (Crooks et al., 2004).

4.3.5 Variability Analysis of Repeat Domains

PCR analysis as described in general methods (section 2.3) was employed to identify the number of B-repeat domains of spsD and the number of fibronectin-binding repeats (FnBR) of spsL using specific primers (Table 4.3). To amplify the A-domain of spsD, specific primers were employed with an annealing temperature of 51°C and extension time of 2 min 30 s (Table 4.3). For spsL the repeat primers were used with an annealing temperature of 50°C and extension time of 1 min 15 s. The length of PCR products obtained was used to infer the number of repeats based on the gene sequence of ED99 spsL. For identification of the number of repeats from whole genome sequences, XSTREAM (Newman and Cooper, 2007) was employed.
4.3.6 Expression Analysis of SpsD and SpsL

Cell wall associated (CWA) and extracellular protein fractions were produced as described in general methods (section 2.20) with samples obtained every hour. Extracellular fractions were concentrated by addition of trichloroacetic acid (TCA) to 10% (v/v) of the equivalent volume of the sample and immediately mixed. The samples were incubated overnight at 4°C and centrifuged at 5,000 rpm for 30 min at 4°C. The pellet was air dried before being suspended in 0.04 volume of Tris-HCl 1.5 M pH 8.8 and stored at -20°C.

Western blot analysis was performed as described in general methods (section 2.13) using 1 µg/ml anti-SpsD and anti-SpsL N2N3 IgY and 0.5 µg/ml F(ab’)2 rabbit anti-chicken-HRP IgG (Bethyl Laboratories). Flow cytometry analysis comparing ED99 and ED99ΔspsLΔspsD was performed as described in general methods (section 2.21) and used 10 µg/ml anti-SpsD and anti-SpsL N2N3 IgY with 1 µg/ml F(ab) goat anti-chicken FITC-labelled IgG. For analysis of SpsD expression, cultures were grown to OD_{600} of 0.2 and for SpsL expression analysis cultures were grown to OD_{600} of 0.6.
4.4 Results

4.4.1 Multilocus Sequence Typing of Clinical Isolates

When examining the genetic diversity within a population it is important to ensure the strains selected are representative of the breadth of population diversity. To that end, a total of 37 *S. pseudintermedius* isolates were examined including 20 clinical isolates from our strain collection and 17 for which whole genome sequences were publically available. Together, they represent 28 STs, were isolated from countries in 3 continents, 3 different host species and were obtained from multiple infection types (Tables 4.1 and 4.2). Of the strains, 4 did not have ST information and so MLST was performed and 3 novel allele combinations were identified, namely: ST493 (strain 326), ST494 (strain LMG22219), and ST495 (strain LMG22220) with the fourth strain, Can6, belonging to ST68 (Table 4.1). Phylogenetic analysis of the MLST concatenated alleles was performed (Figure 4.1). Many of the strains are singly represented on individual branches demonstrating that an evolutionarily diverse set of 37 *S. pseudintermedius* clinical isolates have been selected for this study (Figure 4.1). Four clades can be distinguished from the tree but low bootstrap support is present on the ancestral branches (Figure 4.1). This low bootstrap support could be due to recombination events (although none were detected using RDP3) or could reflect low levels of sequence diversity present in the MLST alleles. None of the strains in the current study clustered using eBURST analysis except those in the same ST (data not shown), confirming the evolutionary diversity of the strains selected for analysis.
Figure 4.1. Evolutionary Diversity among *S. pseudintermedius* Clinical Isolates Employed in the Current Study. Bootstrap consensus neighbour joining tree of concatenated MLST alleles produced in MEGA5. Bootstrap consensus values are provided at the tree nodes with the scale bar indicating genetic distance.
4.4.2 Sequence Analysis of *spsD* and *spsL*

In order to evaluate the genetic diversity of *spsD* and *spsL*, sequence alignments were carried out of the A-domain of *spsL* and the A-domain, C-region, and first B_{SDR} repeat domain of *spsD* among 37 *S. pseudintermedius* clinical isolates using Clustal W. These sequence alignments demonstrated high sequence diversity in *spsD* and low sequence diversity in *spsL*. The 2 most diverse *spsD* alleles were encoded by clinical isolates HH1 (ST71) and HT20030686 (ST49) and shared 76.7% derived amino acid sequence identity. The 2 most diverse *spsL* alleles were encoded by clinical isolates 1726 (ST261) and HT20030686 (ST49) and shared 97.1% derived amino acid sequence identity.

Phylogenetic analysis identified 6 distinct clades from the *spsD* sequence alignment and there was high bootstrap support for the phylogenetic tree (Figure 4.2). Genetic diversity of *spsD* in one clade demonstrated almost identical sequences in 5 distinct STs including ST223, ST68, ST15, ST25, and ST21 (Figure 4.2). Other clades contained fewer strains and long branching demonstrated more diverse *spsD* sequences (Figure 4.2). The phylogeny based on the *spsL* sequence alignment had much lower bootstrap support likely due to the low genetic diversity in *spsL* among the *S. pseudintermedius* population (Figure 4.3). The phylogeny inferred from both *spsD* (Figure 4.2) and *spsL* (Figure 4.3) sequence alignments does not correspond to the MLST-based phylogeny (Figure 4.1) with differential clustering of the clinical isolates. Comparisons of the MLST phylogenetic analysis with that of *spsL* is hampered by the low bootstrap support in the *spsL* phylogenetic tree. The distinct clades identified in the *spsD*-based phylogeny do not correlate with the clades present in the MLST-based phylogeny suggesting that *spsD* is evolving in a manner independent of the rest of the core genome. This suggests that recombination is involved in the evolution of *spsD* in the *S. pseudintermedius* population.
Figure 4.2. Phylogenetic Analysis Exhibiting the Genetic Diversity of \textit{spsD}.
Bootstrap consensus neighbour joining tree of \textit{spsD} sequence alignment produced in MEGA. Bootstrap consensus values are provided at the tree nodes with the scale bar indicating genetic distance.
Figure 4.3. Phylogenetic Analysis Exhibiting the Genetic Diversity of *spsL*. Bootstrap consensus neighbour joining tree of *spsL* sequence alignment produced in MEGA. Bootstrap consensus values are provided at the tree nodes with the scale bar indicating genetic distance.
4.4.3 Examination of the Role of Recombination in the Evolution of \textit{spsD} and \textit{spsL}

In order to investigate if either \textit{spsD} or \textit{spsL} have diversified through recombination, recombination detection analysis was performed using the suite of programs in RDP3. No recombination events were detected in the \textit{spsL} sequence alignment suggesting that recombination had not had a major impact on \textit{spsL} diversity. Recombination analysis of \textit{spsD} identified 6 highly supported recombination events that have resulted in 8 variants of \textit{spsD} (\textit{spsD}1-8) among 32 of the strains examined (Figure 4.4A). Most of the predicted variants appear to have originated from 3 background variants, \textit{spsD}1-3 (Figure 4.4A). The alleles with the lowest level of sequence diversity are \textit{spsD}1 and \textit{spsD}6 displaying 98.4\% derived amino acid identity. Five additional alleles of \textit{spsD} (\textit{spsD}9-13) were also identified (Figure 4.4B) but a larger sequence dataset would be required to predict the parental (donor) strains.

Comparing the identified variants to the \textit{spsD} phylogenetic tree demonstrates clustering of the variants with most strains represented by variants \textit{spsD}2, \textit{spsD}3, \textit{spsD}4, and \textit{spsD}5 (Figure 4.4B). The 5 alleles containing recombination events that could not be accurately defined using RDP3 are present on long branches of the tree highlighting the sequence diversity of these alleles (Figure 4.4B). Examining the distribution of alleles on the MLST phylogenetic tree revealed a lack of correlation between clonal origin and \textit{spsD} allele, consistent with gene recombination events driving the diversification of \textit{spsD} (Figure 4.4C).
Figure 4.4. Recombination has contributed to the Distribution of spsD alleles. (A) Using RDP3, 6 highly supported recombination events were identified in 8 alleles of spsD. (B) Each allele of spsD is presented in comparison to the phylogenetic tree produced from the spsD sequence alignment. (C) Each allele of spsD is presented in comparison to the phylogenetic tree produced from the concatenated MLST allele sequence alignment of 37 S. pseudintermedius clinical isolates.
4.4.4 Modelling of the N2N3 Subdomains of ED99 SpsD and SpsL

The sequence diversity identified in *spsD* and *spsL* from clinical isolates could impact upon ligand-binding if residues essential for function are affected. In order to estimate the structural context of variable residues of the A-domains of SpsD and SpsL, models were produced based on the crystal structure of ClfA N2N3 (Deivanayagam et al., 2002). The amino acid sequence of the N2N3 subdomains of ClfA of *S. aureus* Newman has 29% identity with the N2N3 subdomains of both SpsD and SpsL of *S. pseudintermedius* ED99. Both models predicted a disordered nature of the N1 subdomain with the N2 and N3 subdomains exhibiting classical DEv-IgG-like folds composed of β-strands with an identifiable trench between the two subdomains (Figure 4.5). These models provided residue coordinates for each subdomain, for SpsD, N1 is 37 to 167, N2 is 167 to 322, and N3 is 322 to 520, for SpsL, N1 is 39 to 220, N2 is 220 to 364, and N3 is 364 to 532. Using these coordinates, most of the genetic diversity of *spsD* is confined to the N1, N2 and N3 subdomains with very limited variation within the C-region or B_SDR repeat domain. Only 51.4% of the residues within the N3 subdomain of SpsD are conserved amongst the 37 *S. pseudintermedius* strains examined. However, the model of SpsD N2N3 predicts that most variable residues (coloured red) are exposed on the surface of the protein away from the putative ligand-binding trench (Figure 4.5A). The model of SpsL also demonstrates that the few variable residues within the N2 and N3 subdomains are unlikely to impact upon ligand-binding (Figure 4.5B).

Residues Y255, P335, Y337, and K389 have been identified to be essential for adherence of ClfA to Fg due to their location within the binding trench (Deivanayagam et al., 2002). From the structural models, the corresponding residues in SpsD are R204, N286, F288, and K341 and in SpsL are G232, S318, F320, and K366 (coloured orange) (Figure 4.5). Each of these residues display sequence conservation across all *S. pseudintermedius* isolates examined suggestive of essential roles in the binding mechanism of SpsD and SpsL. In the case of F288 of SpsD the role of this residue in Fg-binding has been demonstrated with site-directed mutagenesis exhibiting loss of Fg-binding (Pietrocola et al., 2013).
Figure 4.5. Structural Modelling Demonstrates Characteristic DEv-IgG Folds. (A) SpsD and (B) SpsL N2N3 models were produced using Phyre² (Kelley et al., 2015) and PyMol. The crystal structure of ClfA N2N3 was used as a modelling template (Protein Data Bank code 1N67). Conserved residues are coloured green and variable residues are coloured red. Residues known to be important for ECM-binding of ClfA are highlighted in orange with the predicted binding motif coloured yellow and the latch coloured blue.
The “dock, lock, and latch” (DLL) binding mechanism utilised by MSCRAMMs to adhere to ligands via the N2 and N3 subdomains requires particular structural motifs including a binding motif (PFSYQKMNGVKFYE in SpsL and SFNLTWDNGVAFYS in SpsD ED99), latch region (NSASGSGELK in SpsL and NNASGEGNDK in SpsD ED99), and the TYTFTDYVD-like motif (VYTFKDYVN in SpsL and RYRFMDYVN in SpsD ED99). Sequence variation in these structural elements could impact upon ligand-binding. These structural elements are conserved in SpsL except for the TYTFTDYVD-like motif of clinical isolate GL117B ST263 that encodes VYTFKGYVN. In contrast, these structural motifs exhibit variation in SpsD from different strains (Figure 4.6). For the binding motif there are 3 distinct sequence variants identified, SFNLTDNGVAFYS, SFNTWKNGVAFYS, and YFNLTWKNGVAFYS, with the initial serine present in all but 2 strains that instead encode a tyrosine (Figure 4.6A). The indicated aspartic acid and lysine residues are present in 19 and 18 strains, respectively, providing opposite charges for this position but maintaining the same hydrophobicity profile (Figure 4.6A). For the latch region there are 2 distinct sequences, NNASGEGNDK, and NKANGEGNDK with 8 isolates of 37 having the second combination that maintains the hydrophilic region (Figure 4.6B). The TYTFTDYVD-like sequence also contains 2 variable residues with 3 distinct combinations, RYRFMDYVN, RYRFTDYVS, and RYRFTDYVN, present in 24, 6, and 7 isolates, respectively (Figure 4.6C). The impact of these mutations has not been investigated but, due to their location within the functional motifs, a role in attenuation of ligand-binding is feasible.

4.4.5 Variation in the Length of Repeat Regions

The extent of variation in length of SpsD and SpsL repeat regions in clinical isolates is unknown. Variation in the number of C-terminal Fn-binding repeats (FnBRs) of SpsL could impact Fn-binding affinity. However, for *S. aureus*, the number of FnBRs is conserved with FnBPA encoding 11 and FnBPB encoding 10 repeats in all strains examined (Meenan et al., 2007). SpsD contains B_{SDR} repeats and proline-rich repeats of unknown function at the C-terminal. Each distinct Sdr protein encodes a fixed number of B_{SDR} repeats between *S. aureus* strains (Josefsson et al., 1998a).
Figure 4.6. SpsD Exhibits Sequence Diversity in Motifs Predicted to be Important for Ligand Binding. The (A) binding motif, (B) latch region, and (C) TYTFTDYVD-like motifs were analysed for sequence diversity in SpsD using WebLogo 3.4 (Crooks et al., 2004). The amino acids are labelled due to their hydrophobicity (hydrophilic residues coloured blue, neutral residues coloured green, and hydrophobic residues coloured black).
PCR analysis was employed here to examine the length of the repeat regions in both spsD and spsL (Figure 4.7). Due to the proximity of the B_SDR and Pro-rich repeats of spsD it was not possible to design primers to investigate the number of Pro-rich repeats in each *S. pseudintermedius* clinical isolate. Instead the number of B_SDR repeats was determined using specific primers to amplify a region of spsD that included the A-domain, C-region, and the B_SDR repeats (Figure 4.7A). This allowed inference of the number of B_SDR repeats in spsD based on the size of the PCR product obtained compared to the ED99 spsD sequence (Figure 4.7A). For the whole genome sequences publically available, only strains HKU10-03, E140, and K7 had assemblies of sufficient quality to identify the number of B_SDR repeats. The distribution of B_SDR repeat variants among isolates is indicated on the spsD phylogenetic tree (Figure 4.8A). Four strains, including ED99, encode only one B_SDR repeat (360 bp). Only one isolate, M721-99, encoded 4 B_SDR repeats with the remainder having either 2 (9 isolates) or 3 (14 isolates). The number of repeats did not correlate with spsD allele phylogeny with intra-ST variation also observed (ST71) (Figure 4.8A). Sequence alignment of the 8 B_SDR repeats publically available displayed 76.2% to 100% derived amino acid sequence identity with the second (and last) B_SDR repeat of K7 representing the most diverse sequence containing a string of 17 unique residues.

For analysis of spsL, primers were designed to only amplify the FnBRs with the size of the PCR product allowing inference of the length of the repeat domain (Figure 4.7B). All isolates employed in the current study could be analysed by comparison with spsL sequences from all publically available whole genome sequences. The distribution of FnBR variants among isolates is indicated on the spsL phylogenetic tree (Figure 4.8B). The number of FnBRs vary from 2 to 8 (222 bp to 888 bp) with the majority of strains encoding more than 4 repeats and the median number of repeats being 6. As for spsD, the number of FnBRs did not correlate with the spsL phylogenetic tree and intra-ST variation in FnBRs was observed (ST71) (Figure 4.8B). Sequence analysis of the FnBRs of ED99 identified four repeats with 100% derived amino acid identity and the two most diverse repeats having 91.9% derived amino acid sequence identity. All of the sequenced strains contain an incomplete FnBR suggesting that degeneration of SpsL FnBRs is common.
Figure 4.7. Repeat Domain Variation is Observed in Both \textit{spsD} and \textit{spsL}. (A) Variation in the number of B_{SDR} repeats of \textit{spsD}. (B) Variation in the number of FnBR of \textit{spsL}. Arrows represent the primer locations employed to identify the repetitive regions of \textit{spsD} and \textit{spsL}. Selected PCR products of varying lengths are displayed with the number of repeats indicated for each strain. SP represents the signal peptide.
Figure 4.8. The Number of Repeats Does Not Correlate with the A-domain Genetic Diversity. (A) The number of B<sub>SDR</sub> repeats of <i>spsD</i>. (B) The number of FnBRs of <i>spsL</i>. PCR or sequence analysis was employed to identify the number of B<sub>SDR</sub> repeats of <i>spsD</i> and the number of FnBRs of <i>spsL</i>. The number of repeats are imposed onto the phylogenetic trees produced from the gene sequence alignments.
4.4.6 Time Course Expression Analysis of SpsD and SpsL of ED99

The generation of \textit{spsD} and \textit{spsL} deletion strains of ED99 allows detailed expression analysis of SpsD and SpsL \textit{in vitro} (Chapter 3). Although \textit{in vitro} expression conditions do not replicate the environment in the host, expression \textit{in vitro} can provide useful information relevant for pathogenesis. With this in mind, time course expression analysis of \textit{S. pseudintermedius} ED99 compared to ED99\textDelta spsD or ED99\textDelta spsL was performed. Culture samples taken every hour were processed to produce CWA and extracellular protein profiles and analysed by Western blot. Separately, flow cytometry was also employed to confirm the expression of SpsD and SpsL on the surface of ED99 compared to ED99\textDelta spsL\textDelta spsD. SpsD was not detected in the cell wall fraction at any time point using Western blot analysis but a truncated immunoreactive fragment was identified in the supernatant fraction (Figure 4.9A). Cell surface expression of SpsD was identified using flow cytometry when cells were analysed at early exponential phase (OD$_{600}$ of 0.2) (Figure 4.9B). SpsL was detected in the cell wall fraction at all time points analysed as well as in the supernatant at later time points (Figure 4.10A). Full length SpsL as well as truncated immunoreactive bands are present in the supernatant. In addition, flow cytometry analysis demonstrated SpsL expression on the surface of ED99 at early- and mid-exponential as well as stationary growth phases with maximal expression at OD$_{600}$ of 0.6 (Figure 4.10B).

Western blot analysis of the cell wall fraction identified a non-specific reactive species of roughly 50 kDa (Figure 4.9A). \textit{S. pseudintermedius} encodes 2 putative SpA orthologues, SpsQ and SpsP, as well as a putative Sbi orthologue, SpsK (Bannoehr et al., 2011). The predicted molecular weight of SpsQ, 51 kDa, suggests that this protein is responsible for the additional reactive species present and that SpsQ is capable of binding to IgY.

Taken together, the data suggest that SpsL is expressed on the surface of ED99 whereas SpsD is mostly present in the supernatant. It is likely that SpsD is cleaved from the cell wall as only truncated versions are present in the supernatant whereas SpsL could be present in the supernatant due to shedding from the cell wall.
Figure 4.9. SpsD is Predominantly Observed in the Supernatant of ED99. (A) Expression of SpsD in CWA and supernatant fractions analysed by Western blot. (B) Flow cytometry analysis of ED99 cultured to OD$_{600}$ of 0.2. All experiments employed anti-SpsD N2N3 IgY antibodies. Experiments were performed at least twice with comparable results. Numbers represent the hour time points at which the samples were taken with stat being a stationary culture.
Figure 4.10. SpsL is Present on the Cell Surface throughout Growth in ED99. (A) Expression of SpsL in CWA and supernatant fractions analysed by Western blot. (B) Flow cytometry analysis of ED99 cultured to OD<sub>600</sub> of 0.6. All experiments employed anti-SpsL N2N3 IgY antibodies. Experiments were performed at least twice with comparable results. Numbers represent the hour time points at which the samples were taken.
4.4.7 Expression Analysis of Clinical Isolates

All of the *S. pseudintermedius* clinical isolates employed in the current study encode *spsD* and *spsL*. However, the presence of a gene does not necessarily equate to protein expression. In order to investigate if the 20 clinical isolates of *S. pseudintermedius* examined from our strain collection express SpsD and SpsL, CWA and supernatant protein profiles were examined at various growth phases using Western blot analysis. From this analysis, stationary phase supernatant samples exhibited the most robust expression profiles (Figure 4.11 and 4.12A) with CWA analysis only demonstrating SpsL expression in certain isolates (Figure 4.13B). Anti-SpsD N2N3 IgY raised against the ED99 SpsD sequence immuno-reacted with 18 out of 20 clinical isolates analysed (Figure 4.11). Anti-SpsL N2N3 IgY raised against the ED99 SpsL sequence immuno-reacted with 11 out of 20 clinical isolates analysed (Figure 4.12).

In order to examine the possibility that sequence diversity could partly explain lack of immunoreactivity we examined the protein expression detected in the context of the phylogenetic trees of the *spsD* and *spsL* gene sequences (Figure 4.13). Clinical isolates not exhibiting expression of SpsD are present on long branches of the *spsD* phylogenetic tree and have low sequence identities compared to ED99 with M721-99 having 83.4% and 94-062394 having 81.9% derived amino acid sequence identity (Figure 4.13A). Of note, strain HT20030686 also encodes *spsD* with a low derived amino acid identity of 81.6%, in comparison to ED99, but supports binding by SpsD-specific antibody (Figure 4.13A). Some correlation of the expression can be observed with the clustering present in the *spsL* phylogenetic tree but, in contrast to *spsD*, there is no link between the expression level and the derived amino acid sequence identity in comparison to ED99 (Figure 4.13B). Due to the use of only one antibody it is not possible to de-lineate a lack of reactivity from the absence of protein expression in the current study. The demonstration of SpsD expression in 90% of the clinical isolates and SpsL expression in only 55% of the clinical isolates does not correlate with the levels of sequence diversity present within these proteins. In order to evaluate the antigenic variation present in SpsD and SpsL, antibodies raised against multiple variants of each protein would be required.
Figure 4.11. Most Clinical Isolates of *S. pseudintermedius* Express SpsD. Western blot analysis of concentrated supernatant fractions from overnight cultures of 20 *S. pseudintermedius* clinical isolates using anti-SpsD N2N3 ED99 IgY and F(ab’2) rabbit anti-chicken HRP-conjugated IgG.
Figure 4.12. Approximately 50% of Tested S. pseudintermedius Clinical Isolates Express SpsL. Western blot analysis of (A) concentrated supernatant fractions from overnight cultures and (B) CWA fractions from mid-exponential cultures of 20 S. pseudintermedius clinical isolates using anti-SpsL N2N3 ED99 IgY and F(ab’)2 rabbit anti-chicken HRP-conjugated IgG.
Figure 4.13. Association of SpsD or SpsL Protein Expression with \textit{spsD} or \textit{spsL} Genetic Diversity in Comparison to ED99. (A) Expression of SpsD. (B) Expression of SpsL. The level of expression is superimposed onto the gene sequence alignment phylogenetic trees. Strain ED99 is highlighted by the red box.


4.5 Discussion

The increasing prevalence of MDR MRSP is severely limiting the therapeutic options for canine pyoderma (Gold et al., 2013). If novel therapeutics are going to be developed it is not only important to identify key virulence factors of *S. pseudintermedius* but also to examine their genetic diversity among the *S. pseudintermedius* natural population. This study has investigated the genetic diversity and expression of SpsD and SpsL among 37 *S. pseudintermedius* clinical isolates in order to assess their potential as therapeutic targets. Sequence alignments have identified high levels of diversity in *spsD* and low levels of sequence diversity in *spsL*, suggesting that SpsL may be a more robust therapeutic target in comparison to SpsD. The low sequence diversity present in SpsL A-domain is analogous to the genetic diversity reported for ClfA and ClfB of *S. aureus* (Murphy et al., 2011). Similar to SpsL, both ClfA and ClfB evolve largely by point mutation resulting in large numbers of allelic variants with 39 reported for ClfA and 47 reported for ClfB, among 124 *S. aureus* strains examined (Murphy et al., 2011). ClfA displays more sequence variation than ClfB with the most diverse alleles exhibiting protein identities of 86% and 94%, respectively (Murphy et al., 2011). Residues key for ligand-binding are highly conserved exhibiting no effect on ligand-binding function (Murphy et al., 2011). From the structural modelling performed in the current study, it is predicted that residues required for ligand-binding of SpsL are also conserved providing functional preservation between strains (Figure 4.5B). Even though ClfA has low levels of sequence diversity, antigenic variation has been reported suggesting that antibodies generated against SpsL may not cross-react with all clinical isolates (Brady et al., 2013).

In contrast, the high sequence diversity of SpsD A-domain is analogous to the genetic diversity reported for FnBPA and FnBPB of *S. aureus* (Burke et al., 2010; Loughman et al., 2008). The 7 FnBPA isotypes display 66% to 76% sequence identity whilst the 7 FnBPB isotypes display 61% to 85% sequence identity (Burke et al., 2010; Loughman et al., 2008). In a similar fashion to SpsD, the N-terminal A-domains exhibit the most sequence diversity and the phylogeny of FnBPs does not correlate with the clonal origin of the strain (Burke et al., 2010; Loughman et al., 2008). The
genetic diversity present in the A-domain did not impact upon FnBPB function but exhibited antigenic variation (Burke et al., 2010). From the structural modelling performed in the current study, some genetic diversity was identified in putative ligand-binding motifs of SpsD potentially providing functional diversity between strains (Figure 4.5A and Figure 4.6). Both FnBPs are predicted to be evolving by recombination but sensitive recombination detection analysis similar to that described here for spsD has not been carried out to date.

Both SpsD and SpsL exhibit variation in the length of their repetitive regions among S. pseudintermedius clinical isolates. Unlike the genetic diversity identified within the N-terminal A-domain, the number of repeats varied intra-clonally (ST71) suggesting that the C-terminal repeat regions are evolving at an increased rate compared to the N-terminal A-domains. In the case of ClfA, variation in the number of SD repeats, varying between 580 bp to 1320 bp, had no impact on the clumping ability of the S. aureus strain (McDevitt and Foster, 1995). In contrast, FnBPs contain fixed numbers of repeats between S. aureus strains (Meenan et al., 2007). This is in striking contrast to the SpsL FnBRs examined in the current study, which demonstrated large variation in the number of FnBRs (Figure 4.8). The molecular details of SpsL Fnb-binding have not been investigated but due to the sequence similarity to FnBP FnBRs it is postulated that a similar tandem β-zipper binding mechanism could be utilised (Schwarz-Linek et al., 2003). This would imply that the length of the FnBRs within a clinical isolate would determine the number of Fn interactions and hence binding affinity (Bingham et al., 2008). With this in mind, the 2 FnBRs present in K7 (ST233), E140 (ST71), and 95-072195 (ST15) may demonstrate decreased avidity for Fn compared to 1726 (ST261) and GL118B (ST2621) that encode 8 FnBRs. In the case of FnBPA, an increase in the number of FnBRs resulted in increased virulence in a murine model of sepsis (Edwards et al., 2010). It has already been reported that clinical isolates of S. pseudintermedius have differing abilities to adhere to Fn (Geoghegan et al., 2009; Schmidt et al., 2009). It would be interesting to determine if the length of SpsL FnBRs correlate with the Fn-binding ability of particular clinical isolates.

Sequence degeneration was observed in SpsL FnBRs, and one SpsD B_SDR repeat of strain K7. This sequence divergence is similar to that reported in repeat domains of
ClfA, SpA, SasG, and Coa of *S. aureus*, which was attributed to intragenic recombination (McDevitt and Foster, 1995; Phonimdaeng et al., 1990; Roche et al., 2003b; Uhlén et al., 1984). The variation in length observed in the B_{SDR} repeats of SpsD is unique as Sdr proteins examined previously do not contain variation in the number of repeats between strains (Josefsson et al., 1998a). In contrast, B_{CNA} repeats of Cna do vary among clinical isolates (1 to 4 repeats), but this has not been demonstrated to affect ligand-binding (Gillaspy et al., 1998). It remains to be tested if variation in the number of B_{SDR} repeats of SpsD would alter its function.

Extensive recombination analysis of the *S. pseudintermedius* population has not been performed but high recombination rates have been suggested due to the ST clustering of this bacteria (Perreten et al., 2010). Recombination events within *S. aureus* are thought to occur at hotspots close to MGEs and within the oriC environ (Everitt et al., 2014). *spsD* is not encoded in such a hotspot but is demonstrated in the current study to be evolving due to recombination (Figure 4.4). On the other hand, *spsL* is situated at the oriC environ and is positioned close to the integration site, orfx, of a novel *S. pseudintermedius ψSCCmec* element, but was not detected to be evolving by recombination in the current study (Perreten et al., 2013). Both SpSd and SpSL are known to be expressed in vivo with antibodies against both proteins present in convalescent sera (Bannoehr et al., 2011). It is possible that interactions with the immune system are contributing to diversifying selective pressure on SpSd and SpSL in vivo suggesting an important role in host-pathogen interactions. If this is the case then both SpSd and SpSL could be viable targets for therapeutic development, if a broadly effective inhibitory compound can be identified.

The time course expression analysis performed in the current study identified that SpSL is expressed on the surface of ED99 throughout growth with potential shedding into the supernatant. Shedding of CWA proteins can occur during normal cell wall turnover, with hydrolases cleaving proteins from the peptidoglycan cell wall, or due to the autolysis of the bacterial cells. In the case of SpA of *S. aureus*, shedding also occurs due to a lack of processing by sortase A with SpA containing an intact signal peptide present in the supernatant (O'Halloran et al., 2015). In contrast, SpSd was identified in the supernatant in a truncated form suggesting proteolytic cleavage. The expression of
SpsD and SpsL on the surface of ED99 has been previously reported using tandem mass spectrometry (Bannoehr et al., 2011). SpsD and SpsL were both associated with the cell wall at early- (OD$_{600}$ of 0.2) and mid- (OD$_{600}$ of 0.5) exponential phases with SpsL also present at late- (OD$_{600}$ of 0.65) exponential phase (Bannoehr et al., 2011). This corresponds well with the observations of the current study but does suggest that SpsD is present on the cell surface longer than observed here. This discrepancy is likely due to the use of a polyclonal antibody generated only against the N2 and N3 subdomains of SpsD with potential that other domains of SpsD remain surface-bound. This correlates with the Fn-binding data presented in Chapter 3, which demonstrated that SpsD is capable of promoting Fn- but not CK-10- or Fg-binding at late-exponential growth phase. This combined data strongly suggests that the A-domain (~54 kDa) of SpsD is actively cleaved from the cell wall early in exponential phase with the Fn-binding C region retained at the cell wall. The cleaved A-domain may retain ligand-binding function but this has not been analysed. The identification of a cleavage product of SpsD (if relevant in vivo) could directly affect therapeutic design with antibodies directed against the A-domain unlikely to result in increased opsonisation of S. pseudintermedius. However, if the cleaved fragment is functional, there may be a neutralisation effect of bound antibody. Only one protease of S. pseudintermedius has been characterised to date (Wladyka et al., 2008). This zinc-dependent metalloprotease (Pst) shares 63% derived amino acid identity with aureolysin of S. aureus and was demonstrated to provide all of the proteolytic activity of the S. pseudintermedius supernatant (Wladyka et al., 2008). There is potential that this protease is mediating the cleavage of SpsD from the cell surface in a similar fashion to the proteolytic cleavage of ClfB by aureolysin during exponential phase growth of S. aureus (McAleese et al., 2001).

Truncated versions of both SpsD and SpsL were also present in the stationary supernatant samples employed to investigate expression of these proteins in S. pseudintermedius clinical isolates. This analysis indicated that clinical isolates do express SpsD and SpsL with SpsD expression detected more frequently than SpsL expression (Figure 4.13). The lack of SpsD or SpsL expression in a clinical isolate might reflect a lack of antibody cross-reactivity. For SpsD there was some association between the degree of sequence diversity in comparison to ED99 and the detection of
expression suggesting the presence of antigenic variation (Figure 4.13A). More detailed analysis is required to investigate the antigenic variation of SpsD and SpsL in order to examine their potential as therapeutic targets. It is important to note that expression of SpsD and SpsL were not observed for the major *S. pseudintermedius* clone currently circulating throughout Europe, MRSP ST71 HH1. For the development of effective therapeutics that target SpsD or SpsL it would be essential to establish the expression of SpsD and SpsL among multiple isolates from this major clone.

In summary, this study highlights the diversity present within *spsD* and *spsL* among the *S. pseudintermedius* population. Such diversity could impact upon therapeutic design due to the potential for antigenic variation. The predicted disordered nature of SpsL FnBRs would hinder antibody generation against this region but the variation in length reported here implies functional diversity. Functional diversity in the SpsD A-domain could also be present due to polymorphisms in the predicted binding region. Crystal structures of both SpsD and SpsL would be highly beneficial in facilitating the design of short inhibitory peptides. The proposed cleavage of SpsD A-domain warrants further investigation and could have implications for the design of novel therapeutics.
Chapter 5 Analysis of the Binding Interaction of SpsL with Canine Fibrinogen
5.1 Introduction

*S. pseudintermedius* has co-evolved with its canine host and is a natural commensal of the canine nares with carriage rates being reported in the range of 46% to 92% among healthy dogs (Bannoehr and Guardabassi, 2012). Infections caused by *S. pseudintermedius* are predominantly reported in dogs and cats with few clinical cases reported in humans even though it is known that dog owners can be transiently colonised with *S. pseudintermedius* (Laarhoven et al., 2011). The basis for the narrow host-tropism of *S. pseudintermedius* is unclear and under-investigated. An array of putative virulence determinants unique to *S. pseudintermedius* have been identified in the genome but their contribution to host-specificity has not been investigated to date (Ben Zakour et al., 2012). The analysis performed in Chapter 3 identified differences in the SpsL-mediated binding of ED99 to multiple host Fg molecules with SpsL displaying highest affinity for canine Fg. Due to the relevance of bacterial-Fg interactions in pathogenesis this host specific interaction may contribute to the host-tropism of *S. pseudintermedius*. If this is the case then a more detailed understanding of the interaction of SpsL with Fg could aid in the development of novel therapeutics and warrants further investigation.

To date MSCRAMMs have not been investigated in detail with regard to their host-specific binding interactions. However, it is known that *S. aureus* ClfA exhibits reduced binding to bovine Fg and no binding to ovine Fg (Geoghegan et al., 2010). The relevance of this host specificity in the pathogenesis of *S. aureus* infection of cows and sheep has not been investigated. Of note, ClfB and Bbp are specialised to adhere to human Fg suggesting a role for these proteins in the adaptation of *S. aureus* to the human host (Vazquez et al., 2011; Walsh et al., 2008).

The molecular interaction of SpsL with Fn has been previously characterised and the focus of the current study is to investigate the protein-protein interaction of SpsL and Fg with the aim of understanding the molecular basis for the host-specific binding interaction (Pietrocola et al., 2015).
5.2 Aims

1) Analyse the affinity of the SpsL fibrinogen interaction

2) Identify the domain of SpsL required for host specific fibrinogen-binding

3) Identify the binding site of SpsL in fibrinogen
5.3 Materials and Methods

5.3.1 Recombinant Protein Expression Plasmids

Strains and plasmids employed in the current study for recombinant protein expression are listed in Table 5.1. Standard cloning techniques (section 2.10) were employed to produce expression constructs using high fidelity PCR at 50°C annealing temperature and 40 s extension time. Multiple cloning site (MCS) PCR was performed at 50°C annealing temperature and 2 min extension time.

5.3.2 Recombinant Protein Expression

Recombinant protein expression plasmids were stored in *E. coli* DH5α at -80°C and transformed into *E. coli* BL21 (section 2.9) prior to induction. Protein expression was induced from *E. coli* BL21 or XL-1 blue cells at OD_{600} of 0.6 to 0.8 with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sigma-Aldrich) for 4 h at 37°C or overnight at 16°C. Cells were harvested at 5000 rpm for 20 min and stored at -20°C. 1 ml samples of the un-induced and induced cultures were analysed for induction of protein expression by SDS-PAGE before progressing with purification.

5.3.3 Native Purification of Recombinant Hexa-Histidine (His₆)-tagged Proteins

Induction pellets were defrosted on ice and suspended in 6 ml binding buffer (10mM imidazole, 20 mM Tris, 500 mM NaCl, pH 8.0) supplemented with cOmplete protease inhibitors (Roche Life Sciences). Bacterial lysis was achieved using the OneShot (Constant Systems) with a single pulse at 25 psi. Lysates were pelleted at 13000 rpm for 20 min before the supernatant was collected and filter sterilised through 0.45 μm filters (Millipore).
<table>
<thead>
<tr>
<th>Strain/Plasmid</th>
<th>Description</th>
<th>Reference</th>
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<td>pQE-30</td>
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<td>Qiagen</td>
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<td>BL21 (DE3)</td>
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<td>Dr Brandon Garcia, unpublished</td>
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<td>This study</td>
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<tr>
<td>XL-1 Blue pQE-30::spsL A+1R</td>
<td>His-tagged SpsL A-domain + 1 repeat (39-582)</td>
<td>This study</td>
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<tr>
<td>TOPP3 pQE-30::spsL R</td>
<td>His-tagged SpsL Repeat domain (538-823)</td>
<td>(Pietrocola et al., 2015)</td>
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<tr>
<td>TOPP3 pQE-30::spsD CB</td>
<td>His-tagged SpsD CB and B-domain (520-846)</td>
<td>(Pietrocola et al., 2015)</td>
</tr>
<tr>
<td>TOPP3 pQE-30::alpha Fg</td>
<td>His-tagged Fg alpha chain</td>
<td>(Vazquez et al., 2011)</td>
</tr>
<tr>
<td>TOPP3 pQE-30::beta Fg</td>
<td>His-tagged Fg beta chain</td>
<td>(Vazquez et al., 2011)</td>
</tr>
<tr>
<td>TOPP3 pQE-30::gamma Fg</td>
<td>His-tagged Fg gamma chain</td>
<td>(Vazquez et al., 2011)</td>
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Table 5.2. Primers Employed to Create Recombinant Protein Expression Plasmids

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'-3')</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>spsLA F</td>
<td>GGATCCATGAAGATGTCACTGAAACAA</td>
<td>pQE-30::spsLA</td>
</tr>
<tr>
<td>spsLA R</td>
<td>CTGCAGCCATCCTATATCAATCACATGGC</td>
<td>pQE-30::spsLA</td>
</tr>
<tr>
<td>spsLA+1R F</td>
<td>GGATCCATGAAGATGTCACTGAAACAA</td>
<td>pQE-30::spsLA+1R</td>
</tr>
<tr>
<td>spsLA+1R R</td>
<td>CTGCAGTGTCTATCTTTCTGTCATTTTC</td>
<td>pQE-30::spsLA+1R</td>
</tr>
<tr>
<td>pQE-30 MCS F</td>
<td>CCCGAAAAGTGCCACCTG</td>
<td>PCR Analysis</td>
</tr>
<tr>
<td>pQE-30 MCS R</td>
<td>GTTCTGAGGTCATTACTGG</td>
<td>PCR Analysis</td>
</tr>
</tbody>
</table>

* Underlined nucleotides identify restriction digestion sites BamHI (forward primers) or PstI (reverse primers).
Native purification was performed using a peristaltic pump P-1 (GE Healthcare) and 1 ml pre-packed HisTrap™ FF crude IMAC columns (GE Healthcare) or the AKTA prime purification system (GE Healthcare) using Ni-NTA Superflow resin (Qiagen). For both systems the column was washed with binding buffer before applying the lysate. The column was washed with further binding buffer before applying elution buffer (500 mM imidazole, 20 mM Tris pH 8.0, 500 mM NaCl). Fractions were analysed by SDS-PAGE.

5.3.4 Recombinant Protein Concentration, Dialysis, and Quantification

Protein Elutions were concentrated using Amicon Ultra-15 Centrifugal Filter Units, 10 kDa molecular weight cut off (MWCO) (Millipore) and dialysed in PBS using either one or 5 ml Float-A-Lyzers, 20 kDa MWCO (Spectrum Labs), according to the manufacturer’s instructions. Quantification after dialysis was performed with a Nanodrop 1000 (Thermo Scientific) using extinction coefficients and molecular weights specific for the recombinant protein being analysed. Aliquots were stored at -20°C.

5.3.5 ELISA-type Binding Assay

96-well MaxiSorp® plates (Nunc) were coated overnight at 4°C with one concentration of either Fg (20 µg/ml) or recombinant protein (10 µM of SpsL truncates and SpsD CB and 1 µM of SpsD truncates) diluted in PBS to 50 µl per well. The plate was washed 3 times with PBS before blocking with at least 100 µl of 4% (w/v) non-fat dried milk (Sigma) in PBS for 1 h at room temperature. After washing, 2-fold serial dilutions of either recombinant protein or ECM protein were applied diluted in PBS to 50 µl per well in triplicate and incubated for 1 h at room temperature. The plate was washed thrice with PBST (0.1% Tween-20) and incubated with antibodies diluted in 1% (w/v) milk-PBST with 50 µl per well and incubated for 1 h at room temperature. Detection of recombinant proteins employed 0.1 µg/ml mouse monoclonal anti-poly-His-HRP conjugated IgG (Alpha Diagnostic International). Detection of Fg employed 0.17 µg/ml rabbit polyclonal anti-canine Fg IgG (Abcam) and 0.2 µg/ml goat
polyclonal anti-rabbit-HRP conjugated IgG (Abcam). After the final antibody incubation the plate was washed five times with PBST and analysed using 50 µl tetramethylbenzidine (TMB) for 5 to 10 min before the reaction was stopped by addition of 50 µl 2 M sulphuric acid per well. The plate was analysed using a Synergy™ HT plate reader (BioTek) at 450 nm wavelength.

5.3.6 Surface Plasmon Resonance

Surface plasmon resonance (SPR) analysis was performed using a BIAcore 3000 (GE Healthcare) (Figure 5.1). Equivalent Fg concentrations were applied to a sensor chip CM5 (GE Healthcare) following the manufacturer’s instructions. Recombinant His-tagged SpsL N2N3 or SpsD N2N3 diluted in PBS were applied over the surface of the chip with interactions observed to multiple Fg molecules simultaneously with data blanked to the fourth reference channel of the CM5 chip. Dose-dependent binding interactions were performed starting with the lowest protein concentration. Each sample was injected for 2 min (20 µl/min) with a 15 min dissociation period between injections. Chemical regeneration of the CM5 chip was not performed. Data analysis employed GraphPad Prism 6 with dissociation constants (K_DSS) determined using steady state values and a one binding hyperbola model.

5.3.7 Canine Fibrinogen Far Western

Cell wall associated (CWA) protein profiles of ED99 and ED99ΔspsL were produced as described in the general methods (section 2.20) and analysed for canine Fg-binding in comparison to recombinant SpsL proteins using a Far Western blot approach. After blotting of the appropriate samples and overnight blocking with 8% (w/v) milk-PBS (Sigma-Aldrich) at 4°C, 0.5 µM of canine Fg was applied to the blot in 4 ml PBS and incubated for 2 h at room temperature. After PBS washing the normal blotting procedure, as described in the general methods (section 2.13), was applied with 0.17 µg/ml rabbit polyclonal anti-canine Fg IgG (Abcam) and 0.2 µg/ml goat polyclonal anti-rabbit-HRP conjugated IgG (Abcam) in 1% (w/v) milk-PBST employed to detect canine Fg-binding.
Figure 5.1. Surface Plasmon Resonance Procedure. Fg was captured onto the CM5 chip chemically. Recombinant His-SpsL N2N3 was injected over the 4 flow cells and association produced changes in light refraction corresponding to increased resonance units (RU). After injection, the ligand dissociation resulted in decreased RU. Regeneration returns the baseline to the original state.
5.3.8 Production of Hybrid *L. lactis* Strains

*L. lactis* expressing hybrid proteins were produced using SLIC as outlined in the general methods (section 2.11). pOri23::*spsD* and pOri23::*spsL* constructs (Bannoehr et al., 2011) were employed as plasmid template for SLIC with an annealing temperature of 53°C and extension time of 8 min. *S. aureus* genomic DNA of strains Newman and MSSA 476 were employed as insert template DNA with an annealing temperature of 50°C and extension time of 15 s. Plasmids and strains employed are listed in Table 5.3. Primers employed are listed in Table 5.4. An overview of the procedure employed to make the constructs is provided in Figure 5.2.

5.3.9 Preparation of *L. lactis* Electro-competent Cells and Electro-transformation

*L. lactis* competent cells were produced using a procedure outlined previously (Holo and Nes, 1989). Briefly, overnight culture of *L. lactis* MG1363 was diluted 1:500 into 5 ml fresh GM17 broth (Oxoid) and cultured to an OD$_{600}$ of 0.6 to 0.8 at 30°C static. The cells were diluted 1:10000 in fresh GM17 and 10 µl used to inoculate 100 ml SGM17 broth (GM17 broth containing 2.6% (w/v) glycine and 6.9% (w/v) sucrose) and incubated at 30°C overnight without shaking. This culture was centrifuged at an OD$_{600}$ of 0.2 to 0.4 and washed in 100 ml 0.5 M sucrose, 10% (v/v) glycerol before resuspension in 750 µl 0.5 M sucrose, 10% (v/v) glycerol followed by aliquoting of 50 µl volumes for -80°C storage. For electro-transformation, competent cells were thawed on ice for 5 min before addition of 0.5 µg plasmid. Electroporated at 200 Ω, 25 µF, and 2.5 kV in ice-cooled 0.1 cm cuvettes. 1 ml of GM17 was immediately applied to the cells and they recovered for over 2 h at 30°C without shaking. 250 µl was plated onto GM17 supplemented with 5 µg/ml Erm (Sigma-Aldrich) and incubated at 30°C for 48 h.
<table>
<thead>
<tr>
<th>Strain/Plasmid</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pOri23::spsD</td>
<td>Plasmid for SLIC</td>
<td>(Bannoehr et al., 2011)</td>
</tr>
<tr>
<td>pOri23::spsL</td>
<td>Plasmid for SLIC</td>
<td>(Bannoehr et al., 2011)</td>
</tr>
<tr>
<td>Newman</td>
<td>ClfA SD Repeats PCR</td>
<td>(Duthie and Lorenz, 1952)</td>
</tr>
<tr>
<td>MSSA 476</td>
<td>Cna A-domain PCR</td>
<td>(Holden et al., 2004)</td>
</tr>
<tr>
<td><em>L. lactis</em> pOri23</td>
<td>Empty expression plasmid</td>
<td>(Que et al., 2000)</td>
</tr>
<tr>
<td><em>L. lactis</em> pOri23::spsD</td>
<td>Full length SpsD expression</td>
<td>(Bannoehr et al., 2011)</td>
</tr>
<tr>
<td><em>L. lactis</em> pOri23::spsL</td>
<td>Full length SpsL expression</td>
<td>(Bannoehr et al., 2011)</td>
</tr>
<tr>
<td><em>L. lactis</em> pOri23::spsD A + SD Repeats</td>
<td>SpsD A + ClfA SD repeats expression</td>
<td>This study</td>
</tr>
<tr>
<td><em>L. lactis</em> pOri23::spsL A + SD Repeats</td>
<td>SpsL A + ClfA SD repeats expression</td>
<td>This study</td>
</tr>
<tr>
<td><em>L. lactis</em> pOri23::cna A + spsD Repeats</td>
<td>Cna A + SpsD repeats expression</td>
<td>This study</td>
</tr>
<tr>
<td><em>L. lactis</em> pOri23::cna A + spsL Repeats</td>
<td>Cna A + SpsL repeats expression</td>
<td>This study</td>
</tr>
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Table 5.4. Primers Employed to Create *L. lactis* Hybrid Constructs

<table>
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<tr>
<th>Primer</th>
<th>Sequence (5'-3')*</th>
<th>Function</th>
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</thead>
<tbody>
<tr>
<td><em>spsD A + SD Repeats A</em></td>
<td>gaggtgttgtgcatagcaggtgaATTAGGCGGAAC</td>
<td>pOri23::*spsD A + SD Repeats</td>
</tr>
<tr>
<td><em>spsD A + SD Repeats B</em></td>
<td>TCACCTGCTATGCAAAACAACCTC</td>
<td>pOri23::*spsD A + SD Repeats</td>
</tr>
<tr>
<td><em>spsD A + SD Repeats C</em></td>
<td>ATCTGATACGCCATTGTGATCTTATC</td>
<td>pOri23::*spsD A + SD Repeats</td>
</tr>
<tr>
<td><em>spsD A + SD Repeats D</em></td>
<td>gatgaagatcacaatggcgtatcagatTCTGACCCAGGTT</td>
<td>pOri23::*spsD A + SD Repeats</td>
</tr>
<tr>
<td><em>spsL A + SD Repeats A</em></td>
<td>cagataccttcgtatccaggtgaATTAGGCGGAAC</td>
<td>pOri23::*spsL A + SD Repeats</td>
</tr>
<tr>
<td><em>spsL A + SD Repeats B</em></td>
<td>TCACCTGTAATACGAAGGTATCTG</td>
<td>pOri23::*spsL A + SD Repeats</td>
</tr>
<tr>
<td><em>spsL A + SD Repeats C</em></td>
<td>ATCTGAGCTATCTTCATAATATCTAC</td>
<td>pOri23::*spsL A + SD Repeats</td>
</tr>
<tr>
<td><em>spsL A + SD Repeats D</em></td>
<td>gtagatattattgaagatcagatTCTGACCCAGGTT</td>
<td>pOri23::*spsL A + SD Repeats</td>
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<tr>
<td><em>cna A + spsD Repeats A</em></td>
<td>gacaagaaccttcagaccctgtggtttTTCAGTATAGTAACC</td>
<td>pOri23::*cna A + spsD Repeats</td>
</tr>
<tr>
<td><em>cna A + spsD Repeats B</em></td>
<td>AAACCAGGTCTTGAAGGTGTCTTTGTC</td>
<td>pOri23::*cna A + spsD Repeats</td>
</tr>
<tr>
<td><em>cna A + spsD Repeats C</em></td>
<td>TATCTCGTAAGCTTCAGCATTATTGGC</td>
<td>pOri23::*cna A + spsD Repeats</td>
</tr>
<tr>
<td><em>cna A + spsD Repeats D</em></td>
<td>gcaataatgctgagcagatTTTCATCAACGAAT</td>
<td>pOri23::*cna A + spsD Repeats</td>
</tr>
<tr>
<td>Primer</td>
<td>Sequence (5'-3')*</td>
<td>Function</td>
</tr>
<tr>
<td>---------------------</td>
<td>-----------------------------------------------</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td><em>cna A + spsL Repeats A</em></td>
<td>tccatctatatcaatcacatgtggtttTTCAGTATTAGTAACC</td>
<td>pOri23::cna A + spsL Repeats</td>
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<tr>
<td><em>cna A + spsL Repeats B</em></td>
<td>AAACCACATGTGATTGATATAGGATGGA</td>
<td>pOri23::cna A + spsL Repeats</td>
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<tr>
<td><em>cna A + spsL Repeats C</em></td>
<td>TATCTCGATTCGCTTGAGCTTCATCAT</td>
<td>pOri23::cna A + spsL Repeats</td>
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<tr>
<td><em>cna A + spsL Repeats D</em></td>
<td>atgatgaagctcaagcgaatcgagataTTTCATCAACGAAT</td>
<td>pOri23::cna A + spsL Repeats</td>
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<td>pOri23 MCS F</td>
<td>AACCCGATTGATGGATTGATTAG</td>
<td>PCR Analysis</td>
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<tr>
<td>pOri23 MCS R</td>
<td>ACCGTATTACGCCCTTTGAGTGAG</td>
<td>PCR Analysis</td>
</tr>
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<td><em>spsD A + SD Repeats Mid F</em></td>
<td>GTGCCATTTCTGACCATAACAAC</td>
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<td><em>spsD A + SD Repeats Start R</em></td>
<td>TTGTCACATCTGTCCGTTTCTTCTA</td>
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<td><em>spsD A + SD Repeats End F</em></td>
<td>GCGAGTGATTCAGACTCAGG</td>
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<tr>
<td><em>cna A + spsD Repeats Start R</em></td>
<td>ACTTGTTCCGCTTCACCTTTTATG</td>
<td>Sequencing</td>
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Table 5.4 continued

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<tr>
<td>cna A + spsL Repeats Start R</td>
<td>CACTACTTTGTCCCGCTTCACT</td>
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<tr>
<td>cna A + spsL Repeats End F</td>
<td>AGAAGAGATCACAGAAAAACCACAA</td>
<td>Sequencing</td>
</tr>
<tr>
<td>cna A + spsL Repeats End R</td>
<td>TTTCCATGATTTCTCTTGCTGAG</td>
<td>Sequencing</td>
</tr>
<tr>
<td>cna A + spsL Repeats R</td>
<td>CTCCAACTGCTGTATTCCATC</td>
<td>Sequencing</td>
</tr>
<tr>
<td>cna A + spsL Repeats Mid F</td>
<td>AAAATGACAAAAATGGCAAGACTA</td>
<td>Sequencing</td>
</tr>
</tbody>
</table>

*Lower case letters represent complementary sequences within the primers.
Figure 5.2. Construction of Plasmids for Hybrid Protein Expression. A and D primers were used to amplify the plasmid backbone including the region required for the hybrid protein, in this example the FnBRs of spsL. B and C primers were used to amplify the A-domain of Cna from S. aureus 476 or the SD repeats of ClfA from S. aureus Newman. After PCR purification as described in the general methods (section 2.5), T4 DNA polymerase treatment produced sticky ends of both PCR products. These sticky ends contained complementary sequence (green boxes) allowing annealing during a step wise decrease in temperatures from 65°C to 25°C. Transformation into E. coli completed the missing sequence to produce the final construct, in this example pOri23::cna A + spsL Repeats.
5.3.10 Growth Curve Analysis of *L. lactis* Strains

Bacterial growth was analysed in 96-well Nunclon™ plates (Thermo Scientific) with 1:100 dilution of overnight cultures into 200 µl GM17 broth at 30°C. Optical density readings were taken every hour using a Synergy™ HT plate reader (BioTek) at 600 nm wavelength absorbance.

5.3.11 Production of Cell Wall Associated Protein Fractions of *L. lactis* Strains

50 ml overnight cultures of *L. lactis* strains were washed twice (5000 rpm, 5 min, 4°C) with 5 ml of 0.9 M ice-cold NaCl and suspended in 2 ml digestion buffer (1.1 M sucrose, 10 mM MgCl₂, 2 mM CaCl₂, 0.1 mg/ml RNAse, 0.05 mg/ml DNAse, 250 U/ml mutanolysin, and 500 µg/ml lysozyme in PBS) supplemented with cOmplete protease inhibitors (Roche Life Sciences). The samples were incubated at 37°C with shaking at 200 rpm for 1 h. The reactions were chilled and centrifuged at 10000 xg for 30 min at 4°C. The supernatant was recovered and stored at either 4°C or -20°C. Extracellular protein fractions of *L. lactis* strains were produced by concentrating culture supernatants using Amicon Ultra-15 Centrifugal Filter Units, 10 kDa MWCO (Millipore). CWA and extracellular protein profiles were analysed by Western blot analysis as described in the general methods (section 2.13) using 1 µg/ml chicken anti-SpsD or anti-SpsL N2N3 and 0.5 µg/ml F(ab’)2 rabbit anti-chicken HRP-conjugated IgG (Bethyl Laboratories) or 0.1 µg/ml mouse anti-Cna N2N3 IgG and 1 µg/ml F(ab) goat anti-mouse HRP-conjugated IgG (Abcam).

5.3.12 Flow Cytometry of *L. lactis* Strains

Surface expression of hybrid proteins was analysed by flow cytometry using the protocol outlined in the general methods (section 2.21). Exponential or stationary phase cultures were analysed for protein expression using 10 µg/ml chicken anti–SpsL N2N3 IgY, 10 µg/ml mouse anti–SpsD N2N3 IgG or 10 µg/ml mouse monoclonal anti-Cna IgG (Visai et al., 2000) with 1 µg/ml of F(ab) goat anti-chicken FITC-
conjugated IgG (Sigma-Aldrich) or goat anti-mouse PE-conjugated IgG (BD Biosciences).

5.3.13 Ligand Affinity Blots of Fibrinogen Chains

Fg samples were diluted in 2 x Laemmli Sample Buffer (Sigma-Aldrich) to a final concentration of 1 µg/ml with 10 µg of each Fg sample loaded per well for SDS-PAGE. Western blot analysis was performed as described in the general methods (section 2.13) with the following exception. After blocking of the membrane the appropriate concentration of recombinant protein (5 µM for SpS L A and 0.25 µM for SpSD A) was incubated with the blot at room temperature for 2 h in PBS. After PBS washing, the normal blotting procedure was applied with antibodies employed to detect recombinant protein binding (0.2 µg/ml mouse monoclonal anti-poly-His-HRP conjugated IgG (Alpha Diagnostics)) in 1% (w/v) milk-PBST for 1 h incubation at room temperature.

5.3.14 Denaturing Purification of Human Fibrinogen Chains

Induction pellets were defrosted on ice and suspended in 4 ml lysis buffer (8M urea, 100 mM monosodium phosphate, 10 mM Tris-HCl, pH 8.0) followed by shaking at room temperature for 1 h. Lysates were produced by pelleting at 10000 xg for 20 min and sterilising the supernatant through 0.45 µm filters (Millipore).

Denaturing purification was performed using Ni-NTA Agarose (Invitrogen) conditioned with 6 ml dH2O and 12 ml denaturing binding buffer (8M urea, 100 mM monosodium phosphate, 10 mM Tris-HCl, pH 8.0) before being suspended in the cell lysate followed by shaking at room temperature for 1 h. The resin was applied to a Bio-Rad column and the lysate allowed to pass through by gravity flow. The column was washed with 3 rounds of 4 ml denaturing wash buffer (8M urea, 100 mM monosodium phosphate, 10 mM Tris-HCl, pH 6.3), and eluted in 4 ml denaturing elution buffer (8M urea, 100 mM monosodium phosphate, 10 mM Tris-HCl, pH 4.5) and analysed by SDS-PAGE. Fg chains were then quantified using a BCA assay (Novagen) following the manufacturer’s instruction.
5.4 Results

5.4.1 Recombinant SpsL Exhibits Poor Fibrinogen Binding

The binding site for Fg in SpsL has not yet been determined. From the modelling analysis performed in Chapter 4 it was predicted that SpsL interacts with Fg using the typical “dock, lock, and latch” binding mechanism, with the likelihood that the N2N3 subdomains would be sufficient for Fg-binding (Ponnuraj et al., 2003). With this in mind, expression constructs of the N2N3 subdomains for both SpsL and SpsD were produced and recombinant proteins purified under native conditions (Figure 5.3). Initial optimisation experiments identified that recombinant SpsL adheres to blocking agents BSA and gelatin (data not shown). Subsequently, milk was used for blocking during ELISA-type binding assays.

In order to evaluate if SpsL N2N3 was sufficient for Fg-binding, ELISA-type binding assays were performed with Fg from different host species coated at a single concentration onto the ELISA plate in PBS. After initial experiments it was observed that greater concentrations of SpsL N2N3 recombinant protein were required to identify a protein interaction compared with SpsD N2N3. Accordingly, experiments employed at least 5 μM of SpsL N2N3 or SpsL A and 0.25 μM of SpsD N2N3 or SpsD A (Figures 5.4 and 5.5). Poor binding to Fg was observed for both SpsL N2N3 and SpsL A with high concentrations of recombinant SpsL required for detection of any Fg-binding (Figure 5.4). This poor Fg-binding could suggest that the recombinant SpsL proteins are not natively folded or that the His6-tag becomes obstructed during ligand-binding preventing detection of SpsL adherence to Fg. In contrast, SpsD N2N3 and SpsD A exhibited robust Fg-binding as previously described (Figure 5.5) (Pietrocola et al., 2013). SpsL A exhibited increased binding to human Fg in comparison to canine Fg (p=0.002) with much greater experimental variation observed in the interaction with human Fg. Importantly, the host specific tropism observed with SpsL expressed by *S. pseudintermedius* or heterologously by *L. lactis* was not replicated in ELISA analysis with recombinant proteins.
Figure 5.3. Recombinant Protein Expression. (A) SDS-PAGE of purified recombinant truncates. (B) Schematic of the SpsD and SpsL truncates. Protein domains include the signal peptide (SP) (red), A-domain (orange), C region (purple), B_{SDR} repeats (green), and repeat domains (blue). All recombinant proteins were His-tagged and purified under native conditions. Predicted molecular weights of each recombinant protein are as follows: SpsL N2N3 (38 kDa), SpsL A (58 kDa), SpsL A+1R (62 kDa), SpsL FnBR (34 kDa), SpsD N2N3 (43 kDa), SpsD A (95 kDa), and SpsD CB (38 kDa).
Figure 5.4. SpsL N2N3 and SpsL A Exhibit Poor Fibrinogen Binding. (A) Binding of SpsL N2N3. (B) Binding of SpsL A. Fg from different host species were coated onto the plate and recombinant SpsL applied after blocking. Mouse anti-His-HRP conjugated antibody was employed to detect Fg-binding at 450 nm. Results are the mean value of 3 independent experiments performed in triplicate ± standard deviation; n=9. Statistical analysis comparing canine and human Fg-binding at 5 µM were performed using 2-sample t-tests and represented by **p≤0.01.
Figure 5.5. SpsD N2N3 and SpsD A Exhibit Robust Fibrinogen Binding. (A) Binding of SpsD N2N3. (B) Binding of SpsD A. Fg from different host species were coated onto the plate and recombinant SpsD applied after blocking. Mouse anti-His-HRP antibody was employed to detect Fg-binding at 450 nm. Results are the mean value of 3 independent experiments performed in triplicate ± standard deviation; n=9. Statistical analysis comparing canine and human Fg-binding at 0.25 µM were performed using 2-sample t-tests.
5.4.2 Surface Plasmon Resonance Analysis of SpsL N2N3 Indicates a Low Affinity Binding Interaction with Fibrinogen

Binding affinity cannot be accurately determined from ELISA analysis and so SPR was utilised to determine the binding affinity for recombinant SpsL N2N3 and SpsD N2N3 to canine, feline, and ovine Fg. The data presented is preliminary as attempts to repeat the experiment were unsuccessful due to the weak nature of the Fg-binding of SpsL N2N3. In the single experiment performed, chemical regeneration of the CM5 chip was not applied to prevent unfolding of the coated Fg molecules. SpsD N2N3 and SpsL N2N3 displayed differential dissociation rates with SpsD dissociating more rapidly and full dissociation of SpsL N2N3 was not achieved at the higher protein concentrations. Increasing the injection time would have improved the accuracy of the predicted dissociation constants with higher concentrations of SpsL N2N3 needed to produce saturation of the steady state analysis curves (Figure 5.8).

Dose-dependent binding interactions of SpsL N2N3 exhibited weak binding affinity to all 3 Fg molecules tested (Figure 5.6). Canine Fg displayed the weakest affinity \( (K_{DSS} \, 7.35\pm0.29 \, \mu M) \), followed by ovine Fg \( (K_{DSS} \, 5.27\pm0.26 \, \mu M) \), with feline Fg displaying the strongest affinity \( (K_{DSS} \, 4.46\pm0.22 \, \mu M) \) (Figure 5.6). Binding affinities of this range have been observed previously with ClfB exhibiting a binding affinity of \( K_D \, 5.25\pm1.5 \, \mu M \) (Mulcahy et al., 2012). However, most Fg-binding MSCRAMMs such as ClfA, \( K_D \, 0.77\pm0.06 \, \mu M \), and FnBPA, \( K_D \, 1.5\pm0.1 \, \mu M \), have much higher binding affinities (Geoghegan et al., 2010; Stemberk et al., 2014).

The interaction of SpsD N2N3 with human and canine Fg analysed by SPR has been reported previously (Pietrocola et al., 2013). However, this is the first analysis of the interaction with feline, and ovine Fg (Figure 5.7). From this preliminary analysis, SpsD exhibits host specific interactions with high affinity binding to canine Fg \( (K_{DSS} \, 1.36\pm0.10 \, \mu M) \) and 5 times weaker affinity binding to feline Fg \( (K_{DSS} \, 7.56\pm1.59 \, \mu M) \) with no binding to ovine Fg (Figure 5.7). These dissociation constants are higher than those reported previously for SpsD binding to canine Fg \( (K_{DSS} \, 0.341\pm0.056 \, \mu M) \) and human Fg \( (K_{DSS} \, 0.360\pm0.032 \, \mu M) \) (Pietrocola et al., 2013).
Figure 5.6. Surface Plasmon Resonance Analysis Indicates a Low Affinity of SpsL N2N3 for Fibrinogen. (A) Canine, (B) feline, and (C) ovine Fg were bound to the CM5 chip and increasing concentrations of recombinant SpsL N2N3 applied as the analyte. Predicted steady state affinity measurements ($K_{DSS}$) are shown. Experiments were performed once.
Figure 5.7 Surface Plasmon Resonance Analysis Indicates a High Affinity of SpsD N2N3 for Fibrinogen. (A) Canine, (B) feline, and (C) ovine Fg were bound to the CM5 chip and increasing concentrations of recombinant SpsD N2N3 applied as the analyte. Predicted steady state affinity measurements ($K_{DSS}$) are shown. Experiments were performed once.
Figure 5.8. Surface Plasmon Resonance Steady State Analysis. Steady state analysis was performed for (A) SpsL N2N3 and (B) SpsD N2N3 using a non-linear fit, one site binding (hyperbola) model with data from one experiment. Steady state analysis allowed affinity measurements ($K_{DSS}$) to be predicted.
5.4.3 Recombinant Repeat Domains of SpsL Do Not Promote Fibrinogen Binding

The SPR analysis is consistent with the ELISA-type binding assay data and indicates that the N2N3 of SpsL does not exhibit high affinity binding interactions with Fg. One possible explanation is that the N2N3 subdomains are not sufficient for high affinity binding and that additional domains of SpsL are required. To test this hypothesis, additional recombinant proteins were examined for Fg-binding by ELISAs including (I) a full-length FnBR region and (II) the A-domain including a single FnBR (Figure 5.3). Ideally a full-length recombinant protein of SpsL would have been analysed for Fg-binding but efforts to produce such an expression plasmid were unsuccessful (data not shown). ELISA analysis was initially performed to evaluate the binding of coated recombinant proteins to canine Fg using polyclonal anti-Fg antibodies (Figure 5.9). In these experiments recombinant SpsD CB was employed as a negative control as the C and B-domains of SpsD do not exhibit Fg-binding. Recombinant SpsD N2N3 and SpsD A were employed as positive controls with increased binding of SpsD A in comparison to SpsD N2N3 (Figure 5.9B). SpsL truncates exhibited differential binding to 20 µg/ml canine Fg (p<0.001) (Figure 5.9A). Multiple comparisons identified that SpsL FnBR exhibited slightly increased adherence to canine Fg in comparison to the negative control (p=0.005) suggesting that the FnBRs of SpsL are capable of mediating Fg-binding. However, SpsL FnBR did not exhibit increased Fg-binding in comparison to SpsLA (p=0.578) suggesting that both domains are capable of mediating low affinity Fg-binding.

To further investigate the role of specific domains of SpsL, ELISA analysis was performed in the opposite orientation using canine or human Fg coated at 20 µg/ml and the recombinant protein analysed for binding using mouse monoclonal anti-His-HRP conjugated antibody (Figure 5.10). Preliminary analysis identified that the SpsL FnBR truncate displayed decreased antibody binding in comparison to the other truncates and had to be excluded from the analysis (data not shown). Consistent with the previous ELISA analysis, SpsD A and SpsD N2N3 exhibited strong interactions with canine Fg as well as equivalent binding to human Fg (Figure 5.10C).
Figure 5.9. Canine Fibrinogen Exhibits Poor Binding to SpsL Recombinant Truncates. (A) SpsL recombinant truncates. (B) SpsD recombinant truncates. Recombinant truncates were coated onto ELISA plates at 10 μM in (A) and 1 μM in (B) and canine Fg-binding detected using rabbit anti-canine Fg IgG and HRP-conjugated goat anti-rabbit IgG. Results are the mean value of 3 independent experiments performed in triplicate ± standard deviation; n=9. Statistical analysis comparing recombinant truncates at 20 μg/ml canine Fg were analysed using one-way ANOVA and represented as ***p≤0.001.
Figure 5.10. SpsL Recombinant Truncates Exhibit Poor Binding to Canine and Human Fibrinogen. (A) SpsL truncates binding canine Fg. (B) SpsL truncates binding human Fg. (C) SpsD truncates binding canine and human Fg. 20 µg/ml canine or human Fg were coated and recombinant truncate binding was detected using mouse anti-His-HRP conjugated IgG. Results are the mean value of 3 independent experiments performed in triplicate ± standard deviation; n=9. Statistical analysis comparing recombinant truncates at 10 µM were analysed using one-way ANOVA and represented as ***p≤0.001.
All SpsL truncates exhibited decreased binding to canine Fg in comparison to the negative control, SpsD CB, at 10 µM (p<0.001) (Figure 5.10A). However, SpsL A (p<0.001) and SpsL A+1R (p=0.009) exhibited increased binding to human Fg in comparison to the negative control (Figure 5.10B). From the binding of ED99 to Fg from different hosts, in comparison to ED99ΔspsL, it would be expected that recombinant SpsL truncates would exhibit increased binding for canine compared to human Fg (Figure 3.11). This is not the case with SpsL A and SpsL A+1R exhibiting binding for human but not canine Fg (Figure 5.10). In comparison to the positive controls, the relative binding of SpsL A and SpsL A+1R is very low and, even though it is higher than the negative control, could be described as a background level of Fg-binding (Figure 5.10).

5.4.4 Investigation of SpsL Fibrinogen Binding using Hybrid Protein Constructs Expressed in L. lactis

The poor Fg-binding observed with recombinant truncates of SpsL conflict with the phenotype of surface-bound SpsL in promoting Fg-binding of S. pseudintermedius or L. lactis expressing SpsL in vitro. To this end, L. lactis constructs were produced that express hybrid proteins allowing investigation of the domains of SpsL required for Fg-binding. To investigate the respective contribution of SpsL A-domain and FnBRs to canine Fg-binding, 4 L. lactis hybrid constructs were produced (Figure 5.11). Two constructs, SpsL A + SD Repeats and SpsD A + SD Repeats, involved replacement of the repeat domains of both SpsL and SpsD with the ClfA SD repeats of S. aureus Newman. The other 2 constructs, Cna A + SpsL Repeats and Cna A + SpsD Repeats, involved replacement of the A-domains of both SpsL and SpsD with the Cna A-domain of S. aureus 476. Fg-binding of both the SD repeats of ClfA and the A-domain of Cna have not been reported (Hartford et al., 1999). PCR and Sanger sequencing confirmed the production of hybrid plasmids in pOri23 (Figure 5.12). Growth analysis identified differential growth of the L. lactis strains with the incorporation of SD repeats decreasing growth and SpsL strains having reduced growth compared to SpsD strains. (Figure 5.12).
Figure 5.11. *L. lactis* Hybrid Constructs Employed in the Current Study. (A) Schematic of SpsL (blue), SpsD (purple), ClfA (orange), and Cna (green). (B) Schematic of the hybrid proteins, using the colour scheme in (A), cloned into pOri23 using SLIC and expressed by *L. lactis*. The signal peptide (red) and LPXTG anchor (black) are shown for each protein.
Figure 5.12. PCR Confirmation and Growth Phenotype Analysis of L. lactis Hybrid Strains. (A) MCS primers and Sanger sequencing confirmed the generation of hybrid strains. (B) Growth curve analysis was performed in GM17 media incubated statically at 30°C. Readings were taken every hour using a Synergy™ HT plate reader (BioTek).
Western blot analysis (Figure 5.13) and flow cytometry (Figure 5.14) were employed to evaluate expression of the hybrid proteins on the surface of *L. lactis*. Expression of SpSD, SpSD A + SD Repeats, SpSL, SpSL A + SD Repeats and Cna A + SpSL Repeats were identified in CWA protein profiles with SpSL A + SD Repeats and Cna A + SpSL Repeats also observed in extracellular protein profiles by Western blot analysis (Figure 5.13). CWA proteins analysed by Western blot analysis were present in truncated forms (Figure 5.13). Flow cytometry identified the expression of SpSD, SpSD A + SD Repeats, SpSL, and SpSL A + SD Repeats on the surface of *L. lactis* but not the expression of Cna A + SpSD Repeats or Cna A + SpSL Repeats (Figure 5.14). This expression analysis suggested that, even though the construct sequences had been verified by Sanger sequencing, hybrid proteins were not always expressed by *L. lactis* at a detectable level. This lack of cell surface expression could suggest that particular hybrid proteins are not effectively exported to the surface or that antibody epitopes are no longer present in the hybrid proteins.

Solid phase adherence assays were performed to investigate the interaction of the hybrid proteins with canine Fg and bovine collagen type I (Figure 5.15). Collagen-binding was not detected for Cna A + SpSD Repeats (Figure 5.15C) consistent with the undetectable levels of expression. Collagen-binding was detected for Cna A + SpSL Repeats that was higher than SpSL A + SD Repeats (p<0.001) but large experimental variation in binding was observed (Figure 5.15C). Cna A + SpSL Repeats exhibited no canine Fg-binding suggesting that the FnBRs of SpSL do not mediate Fg-binding (Figure 5.15B). In contrast to this, SpSL A + SD Repeats also exhibited decreased binding to canine Fg in comparison to SpSL (p<0.001) suggesting that the FnBR of SpSL may contribute to optimal Fg-binding (Figure 5.15B). In the case of SpSD, the reduced binding of SpSD A + SD Repeats (p=0.0062) (Figure 5.15A) can be attributed to differential expression of the hybrid and native proteins as observed using both Western blot (Figure 5.13) and flow cytometry (Figure 5.14). The repeat domain of SpSD is therefore unlikely to contribute to Fg-binding but the lack of Cna A + SpSD Repeats expression prevented confirmation of this.
Figure 5.13. Western Blot Expression Analysis of *L. lactis* Hybrid Strains. (A) CWA protein profiles. (B) Supernatant protein profiles. Western blot analysis of protein samples produced from *L. lactis* hybrid strains using antibodies specific to SpsD N2N3, SpsL N2N3, or Cna (151-398) as well as F(ab’)2 rabbit anti-chicken HRP-conjugated IgG (Bethyl Laboratories) and F(ab) goat anti-mouse HRP-conjugated IgG (Abcam). Data is representative of 3 independent experiments. *S. aureus* 476 and *S. pseudintermedius* ED99 were used as positive controls for Cna, SpsD, or SpsL and *L. lactis* pOri23 was used as a negative control (data not shown). The presence of truncated proteins in the CWA protein profiles are likely due to sample processing rather than cleavage within the cell wall (Hartford et al., 2001).
Figure 5.14. Flow Cytometry Analysis of *L. lactis* Hybrid Strains. Antibodies specific to SpsD N2N3, SpsL N2N3 or Cna (151-398) were employed to evaluate the expression of the hybrid proteins on the surface of *L. lactis*. F(ab) goat anti-chicken FITC-conjugated IgG (Sigma-Aldrich) or goat anti-mouse PE-conjugated IgG (BD Biosciences) were employed as secondary antibodies. Graphs show representative data from at least 3 independent experiments. Controls are the *L. lactis* constructs with just secondary antibody labelling. *S. aureus* 476 and *S. pseudintermedius* ED99 were used as positive controls for Cna, SpsD or SpsL and *L. lactis* pOri23 was used as a negative control (data not shown).
Figure 5.15. Binding of *L. lactis* Hybrid Strains to Canine Fibrinogen and Bovine Collagen Type I. (A) Binding of SpsD strains to canine Fg. (B) Binding of SpsL strains to canine Fg. (C) Binding of the Cna hybrid strains to bovine collagen type I. Results are the mean value of 3 independent experiments performed in triplicate ± standard deviation; n=9. *L. lactis* pOri23 was used as a negative control and *S. aureus* 476 or *S. pseudintermedius* ED99 were used as positive controls. Statistical analysis comparing the binding of strains at 20 µg/ml were analysed using Mann Whitney and represented as **p≤0.01 and ***p≤0.001.
Native SpsL appears to be expressed at relatively equivalent levels to SpsL A + SD Repeats (Figures 5.13 and 5.14). This relatively small difference in expression is unlikely to account for the large reduction in canine Fg-binding suggesting that the FnBR region is required for Fg-binding. Although, Cna A + SpsL Repeats surface expression could not be detected (Figure 5.13), collagen adherence but not canine Fg-binding was observed suggesting that SpsL FnBRs do not mediate Fg-binding (Figure 5.15). These data imply that each domain on its own is insufficient to mediate Fg-binding but the combination of both the A-domain and the FnBR are required for optimal Fg-binding. However, differential levels of expression observed confound robust conclusions being drawn from this experimental approach.

5.4.5 Native SpsL of ED99 Exhibits Binding to Canine Fibrinogen

Attempts to produce recombinant full length SpsL were unsuccessful. Accordingly, in order to compare the binding capacity of SpsL derived from S. pseudintermedius to that of recombinant SpsL A-domain, a Far Western blot approach was employed. The Far Western analysis indicated that recombinant SpsL A exhibits reduced Fg-binding in comparison to SpsL derived from S. pseudintermedius (Figure 5.16). These data imply either that full length SpsL is required for high affinity Fg interactions or that post-translational modifications of SpsL underlie the differential Fg-binding observed. Mass spectrometry analysis of native S. pseudintermedius SpsL extracted from the SDS-PAGE gel resulted in a 42% coverage of full length SpsL and did not identify any post-translational modifications (data not shown). However, it is important to note that glycosylation modifications cannot be detected using this method. The presence of SpsD or protein A homologues (SpsP and SpsQ) produced by ED99 and ED99ΔspsL likely account for the other canine Fg immunoreactive bands observed (Figure 5.16). Further experiments are required to understand the binding interaction of SpsL to Fg and explain the differential binding activity of recombinant SpsL truncates and SpsL expressed by S. pseudintermedius ED99.
Figure 5.16. Native SpsL Extracted from the Cell Surface of *S. pseudintermedius* ED99 Exhibits Canine Fibrinogen Binding. CWA protein profiles of ED99 and ED99ΔspsL and recombinant SpsL A were analysed by SDS-PAGE and Western blot for canine Fg-binding. Canine Fg was applied to the blots, after blocking, and binding was detected using rabbit anti-canine Fg IgG and goat anti-rabbit HRP-conjugated IgG. A control blot is included that was not incubated with canine Fg but was incubated with both antibodies. Data is representative of 4 independent experiments. * represents SpsL (predicted 103 kDa).
5.4.6 SpsL Exhibits Fibrinogen Alpha-Chain Binding

It is essential to identify the binding site of SpsL in Fg in order to understand the host-specific binding tropism of SpsL. Accordingly, a ligand affinity blot approach was utilised to examine the interaction of recombinant SpsL A with the separated chains of both canine and human Fg (Figure 5.17). Recombinant SpsD A was employed as a positive control and a clear interaction with the γ-chain was observed (Figure 5.17A), as previously reported (Pietrocola et al., 2013). Recombinant SpsL A did not exhibit an interaction with any Fg-chain even though much higher concentrations of recombinant SpsL A (5 µM) were employed in comparison to SpsD A (0.25 µM) (Figure 5.17B). This lack of interaction with a particular Fg-chain is likely due to the low Fg-binding affinity of recombinant SpsL A-domain. Accordingly, recombinant chains of human Fg were expressed and purified under denaturing conditions and evaluated for Fg-binding using L. lactis constructs expressing full-length SpsD and SpsL (Figure 5.18) as well as wild type ED99 and ED99∆spsD (Figure 5.19). Of note, the use of equal µg ml⁻¹ concentrations and the differences in molecular weight between human Fg and each recombinant Fg chain mean that unequal molar concentrations are being compared in these adherence assays.

L. lactis expressing SpsD exhibited clear binding to the Fg γ-chain that was reduced in comparison to whole human Fg (p<0.001) and a weaker interaction with the Fg α-chain (Figure 5.18A). This γ-chain interaction was consistent with the ligand affinity blot analysis (Figure 5.17A) and previously published observation (Pietrocola et al., 2013). In contrast, L. lactis expressing SpsL (Figure 5.18B), ED99 and ED99ΔspsD (Figure 5.19) exhibited equivalent binding to the Fg α-chain and whole human Fg. This analysis indicates that SpsL binds to the Fg α-chain in a similar fashion to ClfB and Bbp of S. aureus (Vazquez et al., 2011; Walsh et al., 2008). Sequence analysis demonstrated that the amino acid residues of ClfB identified to interact with the Fg α-chain are not conserved in SpsL with only 33.7% derived amino acid sequence identity observed between SpsL and ClfB A-domains (Ganesh et al., 2011).
Figure 5.17. Ligand Affinity Blot Analysis using Recombinant SpsL A. (A) Recombinant SpsD A. (B) Recombinant SpsL A. (C) Control blot. Far Western analysis of the binding of recombinant SpsD A or SpsL A to separated Fg chains. Mouse anti-His-HRP conjugated IgG was employed to detect recombinant protein interactions with the blot. Recombinant proteins were loaded as controls. Data is representative of at least 3 independent experiments.
Figure 5.18. Fibrinogen Chain Binding of SpsD and SpsL Expressed by \textit{L. lactis}. (A) \textit{L. lactis-spsD} binding. (B) \textit{L. lactis-spsL} binding. Adherence assays comparing the binding of \textit{L. lactis} expressing strains to human Fg and recombinant Fg α-, β-, and γ-chains. Results are the mean value of 3 independent experiments performed in triplicate ± standard deviation; \( n = 9 \). \textit{L. lactis} containing pOri23 was employed as a negative control and \textit{S. aureus} Newman as a positive control (data not shown). Statistical analysis comparing the binding of human Fg with a particular chain at 20 \( \mu \text{g/ml} \) were analysed using 2-sample t-test and represented as ***(p≤0.001).
Figure 5.19. SpsL Encoded by ED99 Adheres to the Alpha-Chain of Fibrinogen.

(A) ED99 binding. (B) ED99ΔspsD and ED99ΔspsL binding. Adherence assays comparing the binding of ED99 and ED99ΔspsL to human Fg and recombinant Fg α-, β-, and γ-chains. Results are the mean value of triplicate readings ± standard deviation; n=3. Each graph is representative of 3 independent experiments. ED99ΔspsL was employed as a negative control and S. aureus Newman as a positive control (data not shown). Statistical analysis comparing the binding of human Fg with a particular chain at 20 µg/ml were analysed using 2-sample t-test.
5.5 Discussion

Previously, Fg-binding proteins of *S. aureus* such as ClfA and FnBPA have been demonstrated to mediate Fg-binding using a minimal binding N2N3 region and a “dock, lock, and latch” binding mechanism (Bowden et al., 2008; Burke et al., 2011; Ganesh et al., 2011; Ganesh et al., 2008; Ponnuraj et al., 2003; Xiang et al., 2012). From the molecular analysis of recombinant SpsD truncates presented in the current study, in addition to previously published work, it is assumed that this is the binding mechanism utilised by SpsD to interact with Fg (Pietrocola et al., 2013). SpsD N2N3 exhibits high affinity binding interactions with the Fg γ-chain and displays a similar host specificity as reported for *S. aureus* ClfA and *S. lugdunensis* Fbl (Ganesh et al., 2008; Geoghegan et al., 2010; Pietrocola et al., 2013). This host specificity is probably due to sequence variation in the C-terminal Fg γ-chain (Figure 1.8D).

In contrast, the data presented here suggest that the binding of SpsL to Fg is more complex than predicted from structural modelling (Figure 4.5). Recombinant truncates of SpsL purified from *E. coli* exhibit low affinity interactions to Fg by ELISA and SPR analysis. Low affinity adherence to Fg could feasibly imply incorrect recombinant truncate folding but the recombinant N2N3 subdomains of other MSCRAMMs are sufficient for Fg-binding. However unlikely, incorrect folding cannot currently be ruled out. Due to the differential expression of proteins on the surface of *L. lactis*, the minimal binding region of SpsL was also not identified using a bacterial expression system. However, full-length SpsL extracted from *S. pseudintermedius* ED99 is capable of mediating Fg-binding as demonstrated using Far Western blot analysis (Figure 5.16). The ability of native SpsL to adhere to Fg provides 2 hypotheses that will be examined in the future; namely, Fg-binding of SpsL requires the full length protein or post-translational modifications. In order to investigate these hypotheses, SpsL truncates will be purified from the supernatant of *S. aureus* RN4220 using the pALC2073 expression vector (Bateman et al., 2001). Initially, the Fg-binding potential of SpsL A-domain purified from *S. aureus* RN4220 will be assessed. If the A-domain is sufficient for Fg-binding then mass spectrometry will be employed to investigate the presence of post-translational modifications that could aid in Fg-binding. If the A-
domain is not sufficient for Fg-binding then further SpsL truncates will be expressed in *S. aureus* RN4220 and compared to the Fg-binding of full length SpsL.

Post translational modification of Sdr proteins, such as ClfA and ClfB, has been reported (Hazenbos et al., 2013; Thomer et al., 2014). Glycosylation of the SD repeats occurs in a two-step process with SdgB attaching the primary N-acetyl-D-glucosamine and SdgA attaching disaccharide modifications to each serine residue of the SD repeats (Hazenbos et al., 2013; Thomer et al., 2014). This glycosylation has not been shown to alter Fg-binding but has been identified to decrease agglutination by a so far unknown mechanism as well as protect Sdr proteins from cleavage by neutrophil Cathepsin G (Hazenbos et al., 2013; Thomer et al., 2014). Post-translational modification of the A-domain of an MSCRAMM has not been reported with recombinant N2N3 fragments examined to date sufficient to promote Fg-binding.

The use of *S. aureus* to express a hybrid protein of ClfA A-domain and Cna B<sub>CNA</sub> repeats demonstrated a lack of Fg-binding, suggesting that manipulating the repeat domains of MSCRAMMs can impact upon ligand-binding (Hartford et al., 1999). Given this finding, it is possible that the FnBRs of SpsL are required for, but not directly mediating, Fg-binding of full length SpsL. Of note, Fg-binding bacterial proteins have been reported to use repeat domains to interact with Fg. For example, FbsA of Group B *Streptococcus* interacts with Fg via 16 residue repeat sequences that mediate platelet aggregation (Pietrocola et al., 2005; Schubert et al., 2002). FbsA does not contain the characteristic IgG folds of an MSCRAMM A-domain and Fg-binding repeats have not been identified in MSCRAMM proteins.

The presence of metal ions could also be affecting the binding affinity of SpsL to Fg. The Fg-binding of both ClfA and ClfB has been shown to be inhibited by the presence of mM concentrations of Ca<sup>2+</sup> and Mn<sup>2+</sup> with the ions binding to EF-hand-like motifs in the A-domain (Ni Eidhin et al., 1998; O’Connell et al., 1998). In contrast, FnBPA and FnBPB do not contain EF-hand-like motifs and their ligand-binding activity is not affected by the presence of Ca<sup>2+</sup>, Mg<sup>2+</sup>, or Mn<sup>2+</sup> (Wann et al., 2000). Similarly, EF-hand-like motifs have not been identified in SpsL suggesting that ions are not involved in the regulation of SpsL ligand-binding but this remains to be analysed.
Many questions still remain as to the binding interaction of SpsL with Fg. The purification of SpsL truncates from *S. aureus* RN4220 supernatant should allow these questions to be answered. Once a minimal binding region is identified this will be used to perform SPR analysis to identify the affinity of SpsL interaction with the Fg α-chain. Crystal structures of SpsD and SpsL N2N3 are being prepared (Ganesh, Texas A&M, Houston, unpublished) and this could provide invaluable insight into the mechanism of Fg-binding utilised by SpsL.

The identification of the Fg α-chain-binding of SpsL (Figure 5.17) is consistent with a previous study showing that *S. pseudintermedius* 326 can bind to the Fg α-chain (Geoghegan et al., 2009). CWA proteins of *S. aureus* with identified interactions to the Fg α-chain include ClfB, Bbp, and the extracellular protein Efb (Palma et al., 1998; Vazquez et al., 2011; Walsh et al., 2008). The A-domains of MSCRAMMs generally contain low sequence identity and this was also observed upon sequence alignment of SpsL, ClfB, and Bbp. BLASTp analysis of SpsL A-domain shows greater amino acid sequence similarity to FnBPA (37%) and ClfA (27%) than to the α-chain binding MSCRAMMs of *S. aureus*, ClfB and Bbp. ClfB binds specifically to repeat 5 of the Fg α-chain of human Fg, a repeat absent from Fg in other host species (Figure 1.8A) (Walsh et al., 2008). In contrast, Bbp binds to residues 561 to 575 of the Fg α-chain of human Fg and is not able to interact with feline, canine, bovine, ovine, murine, or porcine Fg (Vazquez et al., 2011). The residues, 561 to 575, are present in Fg from multiple hosts but the sequence is not conserved (Figure 1.8B). The host specificity of SpsL (Figure 3.11) exhibits a unique tropism compared to the host-specific binding of ClfB and Bbp, suggesting that SpsL binds to a unique site in the Fg α-chain. It is possible that a particular motif is sufficient for SpsL-binding and that this motif is present in both Fg and CK-10 as has been observed for ClfB, but this remains to be investigated (Xiang et al., 2012).

Analysis of *L. lactis* expressing SpsD-binding to recombinant Fg chains suggests that SpsD may have a secondary Fg-binding site in the α-chain in addition to the γ-chain interaction (Figure 5.18). The lack of Fg α-chain binding observed with recombinant SpsD N2N3 suggests that a distinct region of SpsD may be required for this interaction (Pietrocola et al., 2013). It has been suggested that ClfA may have a secondary Fg-
binding site out with the $\gamma$-chain but this has not yet been confirmed (Wann et al., 2000).

In summary, this study suggests that SpsL mediates Fg-binding using a unique binding mechanism. The minimal region of SpsL providing Fg-binding has not been identified using either recombinant truncates or hybrid proteins expressed by *L. lactis*. Truncated derivatives of SpsL expressed in Staphylococci should allow identification of the minimal SpsL peptide required for high affinity host-specific Fg $\alpha$-chain binding.
Chapter 6 Investigation of SpsD and SpsL as Virulence Factors in a Murine Skin Infection Model
6.1 Introduction

The generation of single gene deletion mutants of *S. pseudintermedius* ED99 has identified key roles for these proteins in binding to the ECM *in vitro*. The ability of ED99 to adhere to murine Fg, Fn, and CK-10 suggests that the relevance of these interactions for the pathogenesis of *S. pseudintermedius* can be investigated using *in vivo* murine models of infection. Various other MSCRAMMs have been analysed for their role in pathogenesis using isogenic mutants and murine models. Such pathogenesis work has utilised various murine models many of which involve intravenous inoculation of the wild type and isogenic mutant strain to investigate the development of septic arthritis, endocarditis, or septicaemia (Flick et al., 2013; Josefsson et al., 2001; Kuypers and Proctor, 1989; Palmqvist et al., 2005; Patti et al., 1994b; Que et al., 2005; Rauch et al., 2014). Intravenous inoculation can also be employed to analyse the formation of bacterial abscesses and bacterial persistence in internal organs such as the kidneys (Cheng et al., 2009). The nature of *S. pseudintermedius* infection in dogs prevented us from taking this systemic infection model approach as pyoderma is a far more frequent outcome of *S. pseudintermedius* infection with septicaemia only rarely being reported (Philbey et al., 2013). Murine skin infection models were therefore thought to be more appropriate for the analysis of virulence factors involved in *S. pseudintermedius* pathogenesis.

Skin infection models have been employed previously to analyse the role of CWA proteins of *S. aureus* such as SpA and *S. aureus* surface protein X, in addition to loss of all cell surface proteins in a Sortase A-deficient strain (Kwiecinski et al., 2014; Li et al., 2012; Patel et al., 1987). These infection models involve subcutaneous inoculation of *S. aureus* to produce surface lesions on the back or flank of the mouse with inflammatory responses becoming evident within 24 h and lesions taking about 2 weeks to clear (Malachowa et al., 2013). These surface lesions can be analysed using histopathology to morphologically describe abscess formation and recruitment of neutrophilic granulocytes after infection challenge with the wild type and isogenic mutant strains. These skin infection models are more relevant for analysing the pathogenesis of *S. pseudintermedius* *in vivo* allowing the roles of SpsD and SpsL in the development of skin lesions and abscesses to be investigated.
6.2 Aims

1) Develop a murine skin infection model with *S. pseudintermedius* ED99 wild type

2) Investigate the role of both SpsD and SpsL in abscess formation after infection challenge with ED99ΔspsLΔspsD

3) Analyse the individual contributions of SpsD and SpsL in abscess formation using single mutants of ED99ΔspsD and ED99ΔspsL
6.3 Materials and Methods

6.3.1 Bacterial Inoculum

Inoculum was produced by diluting an overnight culture of *S. pseudintermedius* 1:100 into 50 ml BHI and incubating at 37°C with shaking at 200 rpm until an OD₆₀₀ of approximately 0.6. 10 ml of culture was centrifuged, washed in PBS and suspended to the relevant OD₆₀₀ to provide 10⁷ colony forming units (CFU) per 100 µl in PBS. The exact CFU was determined using serial dilution and 100 µl plated onto TSA. The exact inocula used in each experiment are detailed in Table 6.1.

6.3.2 Murine Skin Infection Model

Animal experiments and associated procedures were conducted according to the requirements of the UK Home Office Animals Scientific Procedures Act, 1986 under project licence 60/4134. In all experiments BALB/cANCrl (Charles River, hereafter abbreviated as BALB/c) female mice aged between 10-12 weeks were used. BALB/c mice were anaesthetised using isoflurane and shaved using electric clippers 24 h pre-inoculation. Bacteria were subcutaneously injected on the back of the BALB/c mouse. 5 mm needle guards were employed to standardise the depth of inoculation after the first experiment. Mice were caged with 6 or less mice per cage with at least 6 mice per experimental group. Monitoring in the morning included weighing the mice, taking a photo of the area inoculated, measuring the length of any lesion along the longest axis using callipers, noting the presence or absence of a lump, as well as identifying any clinical signs of illness using a standardised health assessment protocol (Appendix I). Monitoring in the evening involved visually analysing the mice for signs of general illness. A 20% loss of body weight or signs of illness representing a severe phenotype were employed as criteria for immediate euthanasia. Using a graded evaluation system for the physical and clinical appearance of infected mice, points are given at the monitoring assessment and animals are immediately killed by a schedule 1 method when a certain point threshold is reached (Appendix I).
# Table 6.1. Overview of the Murine Skin Infection Experiments

<table>
<thead>
<tr>
<th>Exp</th>
<th>Time Points (h)</th>
<th>Experimental Groups and Exact Inoculum (CFU / 100 µl)</th>
<th>Samples Collected</th>
<th>Mice per Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>24, 48</td>
<td>ED99 (8.23x10^6) and PBS</td>
<td>H&amp;E slides</td>
<td>6</td>
</tr>
<tr>
<td>I</td>
<td>96</td>
<td>ED99 (7.05x10^6) and PBS</td>
<td>H&amp;E slides</td>
<td>6</td>
</tr>
<tr>
<td>II</td>
<td>48, 72</td>
<td>ED99 (1.52x10^7), ED99ΔspsLΔspsD (1.74x10^7), and PBS</td>
<td>H&amp;E slides</td>
<td>6</td>
</tr>
<tr>
<td>III</td>
<td>24</td>
<td>ED99 (1.36x10^7), ED99ΔspsLΔspsD (1.05x10^7), ED99ΔspsD (1.25x10^7), ED99ΔspsL (1.21x10^7)</td>
<td>H&amp;E slides</td>
<td>10</td>
</tr>
<tr>
<td>III</td>
<td>48, 72</td>
<td>ED99 (9.77x10^6), ED99ΔspsLΔspsD (9.00x10^6), ED99ΔspsD (1.06x10^7), ED99ΔspsL (1.30x10^7)</td>
<td>H&amp;E slides</td>
<td>10</td>
</tr>
<tr>
<td>IV</td>
<td>24</td>
<td>ED99 (7.77x10^6), ED99ΔspsLΔspsD (7.73x10^6), ED99ΔspsD (6.67x10^6), ED99ΔspsL (7.20x10^6)</td>
<td>CFU</td>
<td>10</td>
</tr>
<tr>
<td>IV</td>
<td>48, 72</td>
<td>ED99 (9.40x10^6), ED99ΔspsLΔspsD (8.63x10^6), ED99ΔspsD (9.00x10^6), ED99ΔspsL (8.47x10^6)</td>
<td>CFU</td>
<td>10</td>
</tr>
</tbody>
</table>

Data presented in this thesis are from the experiments outlined in this table but additional experiments were also performed as detailed in the text. Exact inoculum values are indicated. For all infection experiments an inoculation dose of 1x10^7 CFU per mouse was aimed for as this was identified as the optimal inoculum in previously undertaken pilot experiments. Experiments performed over 3 time points were carried out at the same time with more than one inoculum produced to ensure fresh inoculum throughout the injection procedure.
Premature euthanasia was never required with all experimental mice reaching the predefined end point of the experiment. All mice were humanely culled using a schedule 1 procedure at 24, 48, 72, or 96 h post-infection (p.i). Spleens were excised for analysis along with the skin lesion.

6.3.3 Lesion Excision and Histopathology Analysis

Skin lesions were excised and immediately immersed in 10% formal buffered saline (Fisher Scientific). Spleens were also excised and measured for length using a standard ruler and/or weighed. Once fixed, skin lesions were trimmed and histopathology slides produced by the Pathology Department of the Royal Dick School of Veterinary Studies, University of Edinburgh. Histology slides were routinely stained with hematoxylin and eosin (H&E) giving nuclei a blue staining and proteinaceous material a pink staining. Particular slides were also stained using Gram staining to identify Staphylococcal bacteria. Histopathology slides were analysed blind for lesion length along the longest axis using a magnifying glass and callipers as well as disease severity and categorised depending on the type of pathology present. This analysis was independently performed twice before unblinding. All histopathology slides included for analysis contained the following 5 layers of skin: epidermis, dermis, panniculus, panniculus carnosus muscle, and adventitia (Figure 6.3A). Histopathology slides were removed from the analysis if only superficial layers of the skin were present on the slide.

6.3.4 Histopathology Severity Scoring

The severity of pathology present overall on the histopathology slide was scored using the following system: grade 0, no pathology present; grade 1, mild pathology; grade 2, moderate pathology; grade 3, marked pathology; and grade 4, severe pathology. Dr Pip Beard, a veterinary pathologist (The Roslin Institute & R(D)SVS, University of Edinburgh), supervised the development of this severity grading system providing invaluable assistance in the analysis of histopathology slides. All pathology types present within the histopathology slides were severity graded using the same criteria with detailed descriptions of each severity grade given in the text.
6.3.5 Murine Skin Infection Model: Colony Forming Units Enumeration

Mice were inoculated, monitored, and culled as described in section 6.3.2. After lesion excision the area containing the experimental lesion was sliced into equal sections using a scalpel. These skin sections (containing the whole of the skin lesion) were applied to 1 ml PBS in Lysing Matrix D tubes containing 1.4 mm ceramic spheres (MP Bio). After calculating the weight of the skin lesion, homogenisation of the skin samples were performed by pulsing the samples at 4.0 m/sec twice for 20 s per pulse with a min break between pulses. Triplicate serial dilutions were produced per homogenate and 3 dilutions plated per serial dilution series, the dilutions plated varied depending on the time point of the infection. Plates were incubated at 37°C overnight to allow colony counting.

6.3.6 Statistical Analysis

Prism 6 (GraphPad) was employed to present data with statistical analysis performed using Minitab 16. All data was analysed for normality, using the Anderson-Darling test, and equal variance between experimental groups. If these parameters were met then either a 2-sample unpaired t-test or one-way ANOVA analysis was performed depending on the number of groups being analysed. Multiple comparisons were performed when appropriate. If the data were not normally distributed and could not be successfully transformed, then nonparametric analysis was performed such as the Kruskal Wallis or Mann-Whitney U test analysis. For analysis of severity grading, ordinal logistic regression (OLR) was performed with pathology type data being analysed in pairs using Fisher’s exact analysis. For data displaying statistical significance: * represents $p \leq 0.05$, ** represents $p \leq 0.01$, and *** represents $p \leq 0.001$. The data of each experiment and each time point was analysed separately with data between time points not pooled except to analyse the pathology type data. Pooling of data was generally not appropriate because different inocula were employed both within and between experiments (Table 6.1).
6.4 Results

6.4.1 Development of the Murine Skin Infection Model

Pilot studies were performed (experiment I) to develop the first murine infection model of *S. pseudintermedius*. In these pilot studies approximately $1 \times 10^7$ CFU of wild type ED99 strain was subcutaneously injected into the back region of each of 6 mice in 100 µl volume and mice were monitored for periods of 24, 48 or 96 h.p.i to investigate the development of skin lesions. A technical failure in the inoculation meant that one 24 h animal was excluded from the study because it did not receive the full inoculum. Control mice received injections of 100 µl PBS with 3 mice per group for the 24 and 48 h, and 6 mice for the 96 h infection. This mouse model resulted in mild symptoms of generalised illness with body weight displaying slight decreases over the 96 h infection period that were not statistically different from the PBS control group (Figure 6.1A). Some discharge from the eyes could also be noted early after challenge but decreased as the infection progressed. The other most common sign of illness was starrin of the fur on the back of the mouse that again became less common with infection progression. No change in behaviour was noted in the mice while performing this study.

During the monitoring of the mice any lesion present on the surface of the skin was measured using callipers with lesions only observed in the ED99-infected mice (Figure 6.1B). At 24 h p.i lumps were visible on the back or flank of the mouse with lesions becoming more evident at 48 h p.i (Figure 6.2). As the infection progressed the length of the surface lesions appeared to remain relatively constant with lumps tending to become more pronounced indicating discharge of exudate (Figure 6.2). Over an 8 day infection period some lesions appeared to undergo a healing process with the disappearance of the lump but this was not seen in all mice (data not shown). It is assumed that the lesions would eventually become self-limiting and heal as previously noted in *S. aureus* skin infection models (Kennedy et al., 2010).
Figure 6.1. ED99-Infected Mice Exhibit Mild Weight Loss and the Development of Surface Lesions. (A) Percentage change in body weight. (B) Surface lesion length (mm). Monitoring data from experiment I, 96 h p.i, with BALB/c mice inoculated with either 7.05x10^6 CFU of *S. pseudintermedius* ED99 or PBS. Results are the mean value ± standard error of the mean for all groups; n=6. 2-sample unpaired t-test demonstrated no significant loss of body weight but increased surface lesion length in ED99-infected mice compared to PBS control infected mice at 48, 72, and 96 h p.i and are represented as *p≤0.05 and **p≤0.01.
Figure 6.2. Gross Pathology of the Experimental Lesions Developed in ED99-Infected Mice. (A) Representative ED99-infected mouse from experiment I, 96 h infection course. (B) Representative PBS control mouse from experiment I, 96 h. The time the image was taken p.i is given in hours. Redness, swelling, and surface lesions were only detected for the ED99-infected mice.
Histopathology examination of the inoculated area was performed for 29 mice. One animal was removed from analyses due to a technical problem with the sectioning of the skin sample during slide preparation. No histopathology lesions were noted in the mice inoculated with PBS (Figure 6.3A). The changes present in the mice inoculated with *S. pseudintermedius* can be summarised as focal, acute to subacute, suppurative dermatitis, panniculitis and myositis with abscess formation, myofibre degeneration and the presence of intra-lesional bacteria. Formation of an abscess (defined as a focal accumulation of neutrophils (some degenerate), cellular debris and bacteria) was the most important histopathological change identified, and was present in the dermis and/or deeper structures of 11 out of the 17 mice inoculated with *S. pseudintermedius* (Figure 6.3B). In the 11 sections with an abscess present the lesion length was measured (Figure 6.4A). No significant difference was noted in lesion length at different time points p.i. All slides analysed were categorised for severity of histopathological changes (Figure 6.4B). The scoring system employed was: grade 0, no pathology noted; grade 1, a mild increase in the number of neutrophils and lymphocytes present in the panniculus and deeper connective tissue layers; grade 2, formation of an abscess in the skin accompanied by inflammation in surrounding tissues; grade 3, similar to 2 but displaying more extensive pathology with the abscess effacing a larger area of the section. Overlying epidermal ulceration was often noted in these sections; grade 4, severely affected tissue with large abscess formation, epidermal ulceration, and disruption of normal tissue architecture. Severity scoring showed a trend of the more severe pathology present at the latest time point, but this was not statistically significant (Figure 6.4B). There was large mouse to mouse variation in the abscess length and severity scoring, therefore in future experiments a needle guard was employed to standardise the inoculation procedure.

There was no significant difference in the spleen length of ED99-infected mice compared to PBS-infected mice at any time point but the spleen size of ED99-infected mice increased over time (p=0.045) (Figure 6.4C).
Figure 6.3. Subcutaneous Inoculation of Mice with *S. pseudintermedius* ED99 Causes Abscess Formation. (A) Skin from the inoculation site of a mouse administered with PBS. The 5 layers of the skin and a hair follicle are labelled. (B) Skin from the inoculation site of a mouse administered with *S. pseudintermedius* ED99. A large, well circumscribed abscess, composed of a necrotic centre surrounded by a concentric ring of neutrophils and cellular debris, is present in the deeper layers of the skin. A higher magnification image of the periphery of the lesion is shown in (C), showing mild epidermal hyperplasia, increased inflammatory cells in the dermis and panniculus, and disruption of the panniculus carnosus muscle fibres with neutrophils adjacent to the abscess.
Figure 6.4. Histopathology Analysis Demonstrates Abscess Formation of Varying Length and Severity in *S. pseudintermedius* ED99-Infected Mice. (A) Lesion length (mm). (B) Severity grading. (C) Spleen length (mm). Individual values are plotted with mean values represented as a line; n=6 except exclusions of one mouse due to the inoculation at 24 h and one histopathology slide at 96 h. Kruskal-Wallis analysis identified increased spleen length over time in ED99-infected mice, *p<0.05.
6.4.2 Deletion of Both spsD and spsL Reduces Abscess Formation

After this pilot study, experiment II was performed to investigate the role of both SpsD and SpsL in this murine infection model by comparing S. pseudintermedius ED99 and double deletion strain ED99ΔspsLΔspsD-infected mice. To capture the early development within abscess formation it was decided to make comparisons at 48 and 72 h. One animal of the 48 h ED99ΔspsLΔspsD-infected group was excluded from the study because it did not receive the full inoculum upon injection. Gross examination identified that the ED99ΔspsLΔspsD-infected mice did not develop highly pronounced lumps but instead had longer surface lesions compared to the ED99-infected mice (Figure 6.5). This was confirmed though analysis of the surface lesion length taken during daily monitoring with increased surface lesion length in ED99ΔspsLΔspsD-infected mice at both 48 and 72 h p.i (p=0.001). (Figure 6.6B). Larger decreases in body weight were observed in ED99ΔspsLΔspsD-infected mice compared to the ED99-infected mice at 48 h (p=0.010) but not at 24 (p=0.099) or 72 (p=0.055) h p.i suggesting a more severe disease upon deletion of the spsD and spsL genes (Figure 6.6A).

Histopathology examination of the inoculated area was performed for 29 mice in the study. Three animals were removed from analyses due to problems with the slide preparation, two 48 h and one 72 h ED99ΔspsLΔspsD-infected animals. Histopathology analysis of tissue from the mice infected with ED99 revealed pathological changes similar to those in the pilot experiment, predominated by the presence of a focal, well circumscribed abscess (Figure 6.7A). However, in the ED99ΔspsLΔspsD-infected mice focal abscess formation was not identified (Figure 6.7C). The histopathological changes in these sections were instead characterised by poorly delineated, regionally extensive areas of suppurative inflammation of the skin, particularly in the deeper layers, with the inflammatory infiltrate extending laterally between tissue planes (Figure 6.7C). These changes can be summarised as cellulitis. Neutrophils, both viable and degenerate, were the predominant inflammatory cell present in both types of pathology (abscess formation and cellulitis) (Figures 6.7BD).
Figure 6.5. Gross Pathology of the Experimental Lesions Developed in ED99 and ED99ΔspsDΔspsL-Infected Mice 72 h Post-infection. (A) ED99-infected mice. (B) ED99ΔspsLΔspsD-infected mice. Images are of all mice in experiment II at 72 h p.i in which BALB/c mice were subcutaneously infected with 1.52x10^7 CFU of ED99 or 1.74x10^7 CFU of ED99ΔspsLΔspsD. No lesions developed in PBS-infected control mice.
Figure 6.6. ED99ΔspsLΔspsD-Infected Mice Exhibit More Severe Weight Loss and Larger Lesion Lengths than ED99-Infected Mice. (A) Percentage change in body weight. (B) Surface lesion length (mm). Monitoring data from experiment II, 72 h p.i. Results are the mean value ± standard error of the mean for all groups; n=6 for ED99 and ED99ΔspsLΔspsD and n=3 for PBS. Significant differences between ED99 and ED99ΔspsLΔspsD-infected mice were analysed by 2-sample t-test and are represented as **p≤0.01 and ***p≤0.001.
Figure 6.7. Subcutaneous Inoculation of Mice with *S. pseudintermedius ED99ΔspsLΔspsD* Causes Cellulitis. (A) Skin from the inoculation site of a mouse administered with *S. pseudintermedius ED99* displaying a focal abscess. Higher magnification of the abscess (B) reveals it is composed predominantly of degenerate neutrophils admixed with cellular debris. (C) Skin from the inoculation site of a mouse administered with *S. pseudintermedius ED99ΔspsLΔspsD*. The inflammation is horizontally oriented, effaces the panniculus carnosus muscle layer and extends into the adjacent panniculus and adventitia (displaying typical characteristics of cellulitis). Higher magnification of the area of inflammation (D) reveals it is composed predominantly of degenerate neutrophils admixed with cellular debris. Histopathology analysis of PBS control mice exhibited no abnormal pathology. Boxes present in (A) and (C) represent the approximate area of (B) and (D).
No difference in infection outcome was exhibited between the 2 experimental groups at 72 h (p=0.1167), but at 48 h the ED99-infected mice were more likely to display abscess formation while the ED99ΔspsLΔspsD-infected mice were more likely to display cellulitis pathology (p=0.0333) (Figure 6.8A). Combining the data across time points confirmed this decreased abscess formation in ED99ΔspsLΔspsD-infected mice (p=0.0014) (Figure 6.8B).

The severity and extent of the histopathological changes were scored from 0 to 4. Grade 0: minimal pathological changes; grade 1: mild inflammatory changes consisting of a small to moderate number of inflammatory cells (neutrophils, macrophages, mast cells, lymphocytes, plasma cells and eosinophils) scattered through the skin; grade 2, moderate pathological changes that can be characterised as one of 2 patterns, either “focal” with a well-defined abscess (which can be centred at different depths of the skin) with an associated inflammatory infiltrate, or “regionally extensive” with a laterally-oriented, poorly defined accumulation of neutrophils and cell debris extending between the tissue planes (cellulitis); grade 3, marked pathological changes that are similar to grade 2 but more severe and extensive; grade 4, severe pathological changes that are similar to grade 3, but more severe and extensive. No significant differences were identified in the severity of infection between the experimental groups with wide within-group variation observed (Figure 6.9B). In contrast, histopathology assessment of lesion length identified larger lesions in ED99ΔspsLΔspsD-infected mice in comparison to ED99-infected mice at both 48 (p=0.022) and 72 h (p<0.0001) (Figure 6.9A). Larger spleen lengths were identified in ED99ΔspsLΔspsD-infected mice at 48 h (p=0.0156) but comparable spleen lengths were displayed at 72 h p.i (p=0.3889) (Figure 6.9).

This experiment was repeated with similar conclusions being drawn. The lack of statistically significant differences between the experimental groups in terms of severity grade meant that this analysis was not performed in future experiments.
Figure 6.8. ED99ΔspsLΔspsD-Infected Mice Exhibit Altered Infection Outcome in Comparison to ED99-Infected Mice. (A) Outcome of infection analysed at each time point. (B) Combined outcome of infection. The number of histopathology slides displaying each infection outcome is presented; n=12 for ED99 and n=8 for ED99ΔspsLΔspsD. No abscess formation was identified in the ED99ΔspsLΔspsD-infected mice. Fisher’s exact analysis identified different infection outcome between ED99 and ED99ΔspsLΔspsD-infected mice at 48 h p.i and when time points were combined and are represented as *p≤0.05, and **p≤0.01.
### Figure 6.9. ED99ΔspsLΔspsD-Infected Mice Exhibit Longer Histopathology Lesions and Spleens than ED99-Infected Mice but Similar Severity of Infection.

(A) Lesion length (mm). (B) Severity grading (C) Spleen length (mm). Individual values are plotted with the mean represented as a line; variable group sizes are present. Differences between ED99- and ED99ΔspsLΔspsD-infected mice were analysed using 2-sample t-test for lesion length, OLR for severity grades, and Mann Whitney for spleen length and are represented as *p*≤0.05 and **p**≤0.001.
6.4.3 SpsL Promotes Abscess Formation

In order to establish the effect of both SpsD and SpsL on infection outcome experiment III was performed using ED99ΔspsD and ED99ΔspsL single mutant experimental groups, alongside the ED99 wild type and ED99ΔspsLΔspsD experimental groups. Three time points were analysed with mice euthanized at 24, 48, and 72 h with 10 mice per group. Gross examination suggested that ED99ΔspsD-infected mice displayed a well demarcated pathology more similar to the ED99-infected mice while the ED99ΔspsL-infected mice displayed a pathology more similar to the ED99ΔspsLΔspsD-infected mice (Figure 6.10). Differences were observed in the body weight between the 4 experimental groups at 24 h (p=0.012), 48 h (p=0.001), and at 72 h p.i (p<0.001) (Figure 6.11A). Multiple comparisons identified decreased body weight of ED99ΔspsL-infected mice in comparison to ED99-infected mice at 48 h (p=0.0234) and 72 h p.i (p=0.0013) (Figure 6.11A). Increased body weight of ED99ΔspsD-infected mice was exhibited at 48 h p.i in comparison to ED99ΔspsLΔspsD-infected mice (p=0.0323) and at 72 h p.i in comparison to ED99ΔspsL-infected mice (p=0.0119) (Figure 6.11A).

Analysis of the surface lesion length identified differences between the 4 experimental groups at all 3 time points (p<0.0001) (Figure 6.11B). Multiple comparisons identified increased lesion length in ED99ΔspsL-infected mice in comparison to ED99-infected mice at 24 h (p<0.0001), 48 h (p=0.0002), and 72 h p.i (p=0.0004). Decreased surface lesion length was identified in ED99ΔspsD-infected mice in comparison to ED99ΔspsL-infected mice at 24 h (p=0.0007), 48 h (p=0.0116), and 72 h p.i (p=0.0067). Gross examination therefore suggested a more severe pathology in ED99ΔspsL-infected mice with pronounced losses in body weight and larger surface lesions comparable to the observations already made in ED99ΔspsLΔspsD-infected mice.
Figure 6.10. Gross Pathology of the Experimental Lesions Developed in Each Experimental Group. Representative experimental lesions of 4 mice in each experimental group in experiment III, time point 72 h. ED99- and ED99ΔspsD-infected mice display smaller surface lesions with more pronounced lumps while the ED99ΔspsL- and ED99ΔspsLΔspsD-infected mice display longer lesions and lack of distinct lump formation. There is clear variation within each experimental group. BALB/c mice were subcutaneously injected with either 9.77x10⁶ CFU of ED99, 9.00x10⁶ CFU of ED99ΔspsLΔspsD, 1.06x10⁷ CFU of ED99ΔspsD, or 1.30x10⁷ CFU of ED99ΔspsL.
Figure 6.11. ED99ΔspsLΔspsD- and ED99ΔspsL-Infected Mice Exhibit More Severe Weight Loss and Longer Lesions than ED99- and ED99ΔspsD-Infected Mice. (A) Percentage change in body weight. (B) Surface lesion length (mm). Results are the mean value ± standard error of the mean, n=10 mice per group. (C) Histopathology lesion length (mm) with individual values plotted and mean values represented as a line; n is provided in the graph legend. Significant differences between all 4 inoculum were analysed using one-way ANOVA for (A) and (B) and Kruskal-Wallis for (C) and are represented as *p≤0.05 and ***p≤0.001.
Histopathology examination of the inoculated area was performed for 120 mice during the course of the study. Four animals were removed from the analyses due to technical problems with the slide preparation, two 24 h ED99-infected animals, one 48 h and one 72 h ED99ΔspsLΔspsD-infected animals. Assessment of the histopathology slides allowed categorisation into focal abscess, cellulitis, or a third intermediate category (Figure 6.12). This intermediate category was allocated if there was a major area of pathology but it could not be assigned to either the cellulitis or abscess category. Statistical analysis could only be performed between each pair of experimental groups. Analysis of separate time points identified differences in infection outcome between certain groups. For instance at 24 h p.i ED99ΔspsD-infected mice were more likely to develop a focal abscess than ED99ΔspsLΔspsD-infected mice (p=0.0319) and at 48 h p.i ED99ΔspsL-infected mice were more likely to develop cellulitis than ED99-infected mice (p=0.0498). Combining the outcome of infection from all time points identified that ED99-infected mice are more likely to develop an abscess than both ED99ΔspsL- (p=0.0004) and ED99ΔspsLΔspsD-infected mice (p=0.0023) and that ED99ΔspsLΔspsD-infected mice are more likely to develop cellulitis than both ED99ΔspsD- (p=0.0037) and ED99-infected mice (p=0.0023) (Figure 6.12). No difference in histopathology lesion length was observed between the 4 experimental groups at any time point p.i (Figure 6.11C). This experiment comparing 4 bacterial groups was repeated giving the same conclusions as presented here.

6.4.5 Gram Staining Identifies Gram-Positive Bacteria in Both Infection Outcome Pathologies

In order to investigate the presence of bacteria within both the focal abscess and cellulitis pathology, Gram staining of the histopathology slides from experiment II was performed revealing the presence of stained bacteria associated with both pathologies (Figure 6.13). Considerable variation was identified between histopathology slides with the presence of Gram-positive bacteria identified in 40% of the histopathology slides analysed.
Figure 6.12. ED99ΔspsL- and ED99ΔspsLΔspsD-Infected Mice Exhibit a Cellulitis Infection Outcome. Combined data from experiment III is presented as the number of histopathology slides displaying each infection outcome per experimental group. The graph legend details the number of histopathology slides analysed for each experimental group. Occasionally a tissue section would contain features of both the abscess and cellulitis pathology patterns and was classified as intermediate type. Significant differences between individual bacterial groups, represented with brackets, were analysed by Fisher’s exact and are represented as **p≤0.01.
Figure 6.13. Gram-Positive Bacteria are Present in Both Infection Outcome Pathologies. (A) Focal abscess. (B) Cellulitis pathology. Gram staining identified Gram-positive bacteria present in both the focal abscess and the regionally extensive cellulitis pathology. Images are taken from mice in experiment II.
6.4.6 CFU Enumeration of the Murine Skin Infection Model

Gram staining analysis was not sufficient to quantify the number of viable bacteria present within the skin lesions. There could be an association between the different pathologies identified and the bacterial tissue loads. To investigate this hypothesis, experiment III was repeated but instead of analysing the area of skin lesion by histopathology, the lesions were homogenised to determine the number of live bacteria present in the tissue (CFU/mg tissue weight) (Figure 6.13). The monitoring data for experiment IV is not provided but the same disease severity correlations between experimental groups described above were identified in experiment IV.

Statistical analysis of log10 transformed data identified differences in CFU/mg between all 4 experimental groups at both 48 h (p=0.0002), and 72 h (p<0.0001) p.i. Multiple comparisons at 48 h p.i demonstrated decreased bacterial load in ED99-infected mice in comparison to both ED99ΔspsL- (p=0.0052) and ED99ΔspsLΔspsD-infected mice (p=0.0020) (Figure 6.14). Decreased bacterial load was also observed in ED99ΔspsD-infected mice in comparison to both ED99ΔspsL- (p=0.0098) and ED99ΔspsLΔspsD-infected mice (p=0.0044) (Figure 6.14). At 72 h p.i decreased bacterial load in ED99-infected mice was observed in comparison to ED99ΔspsLΔspsD-infected mice (p<0.0001) (Figure 6.14). Increased bacterial load was observed in ED99ΔspsLΔspsD-infected mice in comparison to ED99ΔspsL- infected mice (p=0.0053) and ED99ΔspsD-infected mice (p<0.0001) (Figure 6.14). Increased bacterial load were also observed in ED99ΔspsL-infected mice in comparison to ED99ΔspsD-infected mice (p=0.0039) (Figure 6.14). This indicates that there is increased bacterial survival at 48 and 72 h p.i upon deletion of the spsL gene providing an association between the outcome of infection and bacterial survival.
Figure 6.14. ED99ΔspsL- and ED99ΔspsLΔspsD-Infected Mice Exhibit Increased Bacterial Loads at 48 and 72 Hours Post-Infection. CFU were analysed after homogenisation of the excised skin lesion. The logged data is plotted as CFU per mg of homogenised tissue. Individual values are plotted ± standard deviation with the mean indicated by the line; n=10. Statistical analysis was performed using one-way ANOVA with Tukey’s multiple comparison analysis identifying differences between pairs of experimental groups, signified with the brackets, and represented as *p≤0.05, **p≤0.01, ***p≤0.001.
6.5 Discussion

If novel therapeutics are going to be developed to combat *S. pseudintermedius*-associated canine pyoderma, key virulence factors involved in *S. pseudintermedius* pathogenesis will need to be identified using relevant disease models. Here we developed a *S. pseudintermedius* murine skin infection model with subcutaneous injection of bacteria leading to the formation of skin lesions on the backs of mice. Histopathology analysis identified that ED99 wild type-infected mice developed classical focal abscesses under the skin containing large accumulations of neutrophils and cell debris (Figure 6.3). In mice infected with strains deficient in both *spsD* and *spsL* the production of focal abscesses were rare with a poorly demarcated, regionally extensive inflammatory response occurring instead, characteristic of cellulitis pathology (Figure 6.7C). Further analysis identified that single deletion of *spsL* is sufficient to produce this cellulitis pathology rather than focal abscesses. This strongly implicates SpsL in the formation of skin abscesses by *S. pseudintermedius*. Future experiments are required to confirm this observation by investigating the infection outcome of ED99ΔspsL Rep-infected mice.

Abscess formation and cellulitis are distinct clinical outcomes but both pathologies can present simultaneously (Kobayashi et al., 2015a). Cellulitis is an acute infection that effects both the dermis and subcutaneous layers of the skin with histopathology analysis exhibiting diffuse infiltration of neutrophils (Bailey and Kroshinsky, 2011). Trauma to the skin as well as immunosuppression are the greatest risk factors of cellulitis (Bailey and Kroshinsky, 2011). There is little understanding of how cellulitis develops but low bacterial enumeration from human patient biopsies suggests the disease is primarily driven by the host immune response (Bailey and Kroshinsky, 2011). Cellulitis in dogs is rare but cases mediated by *S. pseudintermedius* have been reported with damage to the lymphatic system during cellulitis promoting the development of bacteraemia and toxic shock (Girard and Higgins, 1999). In rare cases this will progress to necrotising fasciitis with *S. pseudintermedius*-mediated necrotising fasciitis reported previously (Mayer and Rubin, 2012; Weese et al., 2009). In contrast, skin abscesses are focal accumulations of inflammatory cells that encase bacteria (Kobayashi et al., 2015a). Our understanding of the development of skin
abscesses is limited with the host immune response thought to play a major role (Kobayashi et al., 2015a).

The development of cellulitis in this murine model correlated with an increased severity of disease associated with greater decreases in body weight after subcutaneous injection, larger surface lesions (Figure 6.6) and larger lesion length at the point of histopathology analysis (Figure 6.9). The deletion of spsL and therefore the cellulitis pathology was linked to increased bacterial load in the area of skin inflammation at 48 and 72 h p.i (Figure 6.14). No growth defects have been observed in the ED99 deletion strains in vitro that would account for the observed differences in bacterial load (Figure 3.7A). This suggests that the immune response produced during the cellulitis pathology is incapable of clearing the bacterial load as effectively as the immune response produced during abscess formation. Increased severity of murine skin infection mediated by S. aureus is linked to decreased neutrophil recruitment with murine studies investigating the role of the immune response during infection demonstrating that a loss of T- and B-cells leads to a more severe skin infection (Miller et al., 2006; Montgomery et al., 2014). Of note, immune cell recruitment is not impaired in the cellulitis pathology reported in the current study, with large influxes of neutrophils apparent at the infection site (Figure 6.7). It would be interesting to follow the bacterial load over a longer time period to investigate if the infection persists during cellulitis or if both infections would resolve at the same rate. Time limitations have currently prevented this analysis but further studies are planned.

Both abscess formation and cellulitis are not common features of superficial bacterial folliculitis (SBF) or AD, the two most common forms of canine pyoderma-mediated by S. pseudintermedius (Hillier et al., 2014). The role of SpsD and SpsL in the pathogenesis of S. pseudintermedius during SBF and AD has therefore not been examined in the current study. Preferably S. pseudintermedius pathogenesis would be characterised using a canine model of skin infection. This would be ideal for investigating the Fg-binding activity of SpsL, as interactions with murine Fg are weaker than with canine Fg (Figure 3.3A). It is also assumed that other proteins of S. pseudintermedius will be adapted to the canine host even though host specificity of other S. pseudintermedius proteins has not been reported to date. Canine skin infection
models have been investigated by others but so far these models have been unsuccessful (Couto, 2014, University of Lisbon, Portugal, personal communication). An allergic model of atopic dermatitis has been produced in Beagles but this model does not involve the inoculation of bacteria (Kim et al., 2015a). We have also been working to establish a murine epicutaneous model of skin infection such as that already developed for *S. aureus* and employed to study the effect of toxins on the development of atopic dermatitis (Hahn et al., 2009; Hahn and Sohnle, 2013; Kugelberg et al., 2005; Nakamura et al., 2013). This infection model either applies bacteria directly to the surface of the mouse after mild abrasion of the skin using tape stripping or uses δ-toxin to pre-treat the skin before bacterial inoculation to allow a more natural colonisation and infection to occur (Nakamura et al., 2013). Attempts to develop this AD model for *S. pseudintermedius* have so far been unsuccessful with inoculation of $1.33 \times 10^9$ CFU of *S. pseudintermedius* ED99 onto the surface of the skin of BALB/c mice, after tape stripping, producing only a small area of erythema and swelling without development of a lesion or abscess (data not shown). These mice displayed discharge from the eyes 48 h after inoculation that persisted for 2 days suggesting the generation of a systemic inflammatory response. More work is needed to improve this model and any future trials could use C3H/HeN mice that develop more extensive inflammatory host responses compared to other laboratory mouse inbred strains, such as BALB/c mice, and are generally more susceptible to bacterial infection (Bergmann et al., 2013).

The skin infection model developed here is therefore the best *in vivo* model currently available to test the pathogenesis of *S. pseudintermedius* skin infections. The major limitations of this model is the high variability observed within bacterial groups and between experiments with the mice infected with ED99ΔspsL and ED99ΔspsLΔspsD exhibiting increased variability compared to the ED99- and ED99ΔspsD-infected mice. Variability within inoculation groups has also been reported in a staphylococcal rabbit skin infection model investigating differences between high and low virulence *S. aureus* strains (Meulemans et al., 2007). This variation does not alter the conclusion or outcome of this study but it does mean that larger groups of mice are needed ($n \geq 8$) in order to ensure replication of results and to provide enough power for statistical analysis. The variation observed could be due to the method of injection with the inoculum immediately becoming dispersed under the skin. In spite of the variation,
significant differences could be identified between experimental groups with clear changes in the pathology upon deletion of \textit{spsL} alone or both \textit{spsD} and \textit{spsL} genes (Figure 6.12). In human disease, abscess formation can often be accompanied by cellulitis and vice versa but this is not always the case and combinations of cellulitis and abscess formation are not recorded in previous murine skin infection models of \textit{S. aureus} (Kobayashi et al., 2015a). One study has identified that altering the size of the bacterial inoculum can affect the pathology and disease outcome observed with high doses of \textit{S. aureus} leading to loss of abscess formation (Kwiecinski et al., 2014). However, in the current study a consistent size of inoculum was employed across all experiments and so disease outcome cannot be attributed to the size of the bacterial inoculum (Table 6.1).

The implication of one bacterial protein, SpsL, in the development of abscess formation and cellulitis, suggests that bacterial factors rather than the host immune response are driving the development of skin pathologies. The role of specific \textit{S. aureus} virulence factors in the development of abscesses after bloodstream infection are well documented (Cheng et al., 2011). These abscesses contain centres of replicating Staphylococci with secreted proteins Coa and vWbp essential for pseudocapsule formation that shields bacteria from the immune response (Cheng et al., 2010). Such pseudocapsules and inner centres of replicating bacteria were not evident in the skin abscesses developed in this murine model preventing parallels being drawn to these kidney abscesses induced after systemic administration of \textit{S. aureus} bacteria. Instead the abscesses presented here are more akin to abscesses developed in a rabbit model of \textit{S. aureus} skin infection (Kobayashi et al., 2011). These rabbit skin abscesses contained large accumulations of neutrophils and necrotic centres similar to the abscesses observed in the current study (Kobayashi et al., 2011). In the rabbit model of skin infection, the deletion of \textit{α}-type phenol soluble modulins (PSM\textit{α}) and \textit{α}-toxin decreased the size of skin abscesses while deletion of Panton-Valentine leucocidin (PVL) delayed abscess resolution (Kobayashi et al., 2011). Murine models of \textit{S. aureus} skin infections have also identified that toxins have a role during pyoderma with \textit{α}-toxin required for wild type size abscesses and the development of dermonecrosis (Kennedy et al., 2010). Phenol soluble modulins are also required for wild type levels of dermonecrosis with toxins thought to cause direct tissue damage as
well as activating a pro-inflammatory response (Chatterjee et al., 2011; Wang et al., 2007). It is unlikely that SpsL is directly impacting upon the degree of tissue damage in the infection model presented in the current study but this remains to be ruled out.

More work is needed to define the role of SpsL during *S. pseudintermedius* mediated infection of murine skin. Future experiments plan to confirm the essential role of SpsL in abscess formation using the ED99ΔspsL Rep strain as well as investigating the resolution of each disease outcome. The expression of SpsD and SpsL *in vivo* will also be analysed using immunohistochemistry and will be especially important to determine for SpsD considering its limited impact on the pathology observed.

In summary, the murine model of *S. pseudintermedius* skin infection developed here strongly implicates SpsL in the formation of skin abscesses with ED99ΔspsL-infected mice developing cellulitis. This is contradictory to the present view that the development of both skin abscesses and cellulitis are largely host-immune cell mediated. Instead it suggests that bacterial factors are additionally involved in the control of abscess formation and host and bacterial factors might both contribute to the development of abscesses. Future work will need to investigate how SpsL can influence neutrophil recruitment and/or bacterial dissemination.
Chapter 7 General Discussion
Canine pyoderma is one of the most common diseases presented at small animal veterinary clinics. The high clinical relevance of this disease is not reflected by the level of research into the pathogenesis of *S. pseudintermedius*, with little understanding of how this bacteria interacts with its host to produce opportunistic infections. Pathogenesis research to date has focused on using recombinant proteins to investigate the function of specific virulence factors in isolation. Toxins produced by *S. pseudintermedius* have received the most attention with ExpA and ExpB demonstrated to produce canine tissue damage that cause clinical features of AD when injected under the skin of dogs (Futagawa-Saito et al., 2009; Iyori et al., 2011). In contrast, less is known about the role of bacterial surface proteins during *S. pseudintermedius* pathogenesis and their impact on colonisation, invasion and virulence during canine pyoderma. The whole genome sequence of *S. pseudintermedius* ED99 allowed CWA proteins unique to both the SIG and *S. pseudintermedius* to be identified (Bannoehr et al., 2011; Ben Zakour et al., 2011). Of these 18 surface proteins, SpsD and SpsL have been investigated using recombinant proteins and expression in the heterologous host *L. lactis* (Bannoehr et al., 2011; Pietrocola et al., 2013). These studies provide molecular detail of the potential of SpsD and SpsL to mediate adherence to ECM proteins such as Fg and Fn but they do not provide evidence for the role of these proteins in *S. pseudintermedius* pathogenesis.

Molecular Koch’s postulates advise that the classification of a bacterial virulence factor depends upon the ability to delete the gene of interest and show loss of virulence in a relevant model of pathogenesis (Falkow, 1988). In order to complete the postulates this gene must also be re-introduced and a gain of virulence observed (Falkow, 1988). This current study is the first report of the genetic manipulation of *S. pseudintermedius* in an attempt to fulfil molecular Koch’s postulates. Solid phase adherence assays identified that both SpsD and SpsL are required for ECM-binding of *S. pseudintermedius* in vitro. The gene deletion mutants were also analysed using an *in vivo* mouse infection challenge model with SpsL identified as central for the development of skin abscesses in mice. Although the current study provides a proof of principle for genetic manipulation of *S. pseudintermedius*, the development of specialised plasmids and RM-deficient strains are required to increase the efficiency of *S. pseudintermedius* allele replacement.
The importance of using such tools is highlighted in the current study with the identification of a novel binding interaction between SpsL and CK-10 when expressed by *S. pseudintermedius*. In ClfB, CK-10-binding is implicated in the adherence of *S. aureus* to epidermal cells (O’Brien et al., 2002). Previously, it was reported that *L. lactis* expressing SpsL did not promote adherence to canine corneocytes or to CK-10 (Bannoehr et al., 2011; Bannoehr et al., 2012). In contrast, *L. lactis* expressing SpsD adhered to CK-10 and exhibited variable adherence to canine corneocytes (Bannoehr et al., 2011; Bannoehr et al., 2012). ED99 also exhibited corneocyte adherence at both exponential (OD$_{600}$ of 0.6 to 0.8) and stationary phase (Bannoehr et al., 2012). It is possible that SpsL expressed by ED99 mediates adherence to corneocytes by interacting with CK-10. However, another cell surface protein of ED99, SpsO, also mediated adherence to corneocytes and it is possible that SpsD mediated corneocyte adherence by interacting with α-elastin (Bannoehr et al., 2012; Pietrocola et al., 2013).

More work is therefore needed to evaluate the epidermal adherence of ED99. The gene deletion strains produced in the current study would allow the roles of SpsD and SpsL in epidermal adherence of *S. pseudintermedius* ED99 to be fully evaluated.

ClfB uses the same sequence motif to adhere to Fg, CK-10, loricrin, and dermokine (Ganesh et al., 2011; Mulcahy et al., 2012; Xiang et al., 2012). Neither SpsD or SpsL adhere to the same Fg site as ClfB but SpsD adheres to the same glycine-serine rich Ω loops in CK-10 (Pietrocola et al., 2013). It would be interesting to examine the potential adherence of SpsD and SpsL to loricrin given that the interaction of ClfB with loricrin has been demonstrated to be essential for nasal colonisation of *S. aureus* (Mulcahy et al., 2012).

The shared ligands of SpsD and SpsL could suggest that these proteins exhibit functional redundancy but each protein interacts with different sites within Fg with SpsD adhering to the Fg γ-chain (Pietrocola et al., 2013) and SpsL adhering to the Fg α-chain. This could have different functional implications during pathogenesis. For example, recombinant SpsD N2N3 blocks thrombin-mediated fibrin formation and platelet aggregation by adhering to the C-terminal of the Fg γ-chain suggesting that SpsD expressed on the surface of ED99 could mediate platelet aggregation (Pietrocola et al., 2013). This is analogous to the role for ClfA in binding to the C-terminal Fg γ-
chain to mediate platelet aggregation and clumping in the presence of soluble Fg (McDevitt et al., 1994; McDevitt et al., 1997). ClfB Fg α-chain interaction is also capable of mediating bacterial clumping in the presence of soluble Fg (Ní Eidhin et al., 1998). In contrast, the adherence of SpsL to the Fg α-chain did not promote detectable clumping of ED99 in soluble Fg. This may imply that SpsL adheres to the central E-domain of Fg, and the N-terminal of the Fg α-chain, rather than the outer D-domains. SdrG of S. epidermidis adheres to the E-domain of Fg at the thrombin cleavage site of the Fg β-chain and does not stimulate clumping in platelet-poor plasma (Brennan et al., 2009; Davis et al., 2001). SdrG is also incapable of adhering to thrombin cleaved Fg and therefore fibrin (Davis et al., 2001). Preliminary data implies that SpsL is also incapable of adhering to human fibrin strengthening the hypothesis that SpsL adheres to the N-terminal α-chain (data not shown). The Fg α-chain is more diverse than the Fg β- and γ-chains but the α-chain derived FpA exhibits less sequence diversity between species than the β-chain derived FpB (Binnie and Lord, 1993; McCarthy and Lindsay, 2010). The interaction of SdrG to the thrombin cleavage site of FpB is highly host-specific with SdrG binding only to human Fg (Ponnuraj et al., 2003). The reduced sequence diversity of FpA, in comparison to FpB, could explain the broader Fg-binding tropism exhibited by SpsL. We plan to investigate the hypothesis that SpsL adheres to the thrombin cleavage site in the Fg α-chain. If SpsL does adhere to this site then SpsL could inhibit fibrin formation in vivo and prevent wound healing during canine pyoderma.

The different timings of expression of SpsD and SpsL during the growth curve in vitro imply differential regulation and different roles in pathogenesis. This could mean that SpsD and SpsL function at different stages of infection or within different host niches. Cleavage of SpsL on the cell surface of ED99 does not occur but truncated SpsL was identified in clinical isolates suggesting that SpsL expression could be differentially processed or regulated between S. pseudintermedius strains. The time course expression analysis and Fn binding assays performed in the current study suggest that the SpsD A-domain is cleaved from the surface of ED99 during exponential phase. Tandem mass spectrometry analysis of ED99 did not identify SpsD expression after OD600 of 0.5, supporting the conclusion that SpsD expression is limited to exponential growth phase (Bannoehr et al., 2011). This suggests that SpsD is expressed in a similar
manner to ClfB, FnBPA, and FnBPB in vitro (McAleese et al., 2001; McGavin et al., 1997; Mohamed et al., 2000). The transcription of clfB is maximal at mid- to late-exponential phases with the presence of CWA ClfB regulated by aureolysin, which cleaves the N1 subdomain at a SLAVA motif negatively impacting upon Fg-binding (McAleese et al., 2001). FnBPs are also expressed during exponential growth with loss of expression during stationary phase (Mohamed et al., 2000). FnBPs are regulated by the V8 protease via cleavage of FnBPs from the S. aureus surface (McGavin et al., 1997). The expression of proteases has not been extensively studied in S. pseudintermedius but an aureolysin homologue, Pst, identified in the supernatant could mediate the cleavage of SpsD (Wladyka et al., 2008). This cleavage of SpsD does not impact upon Fn-binding suggesting that the C region of SpsD remains intact on the cell surface. It would be interesting to identify if SpsD A-domain cleaved from the cell surface is capable of adhering to ECM proteins, which would suggest SpsD functions as both a secreted and CWA protein. SpA of S. aureus functions as both a CWA and secreted protein with the secreted form contributing to immune evasion in whole blood (O’Halloran et al., 2015). In contrast to SpsD, this secreted form of SpA is not a cleavage product but exists as a full length protein in the supernatant (O’Halloran et al., 2015). The high genetic diversity present in the A-domain of SpsD suggests antigenic variation between S. pseudintermedius clinical isolates. This antigenic variation may be particularly important if the secreted A-domain is functional and targeted by neutralising antibodies generated during canine pyoderma.

A functional, truncated version of SpsD A-domain would be a unique feature for an MSCRAMM. SpsD also contains a unique C region that mediates high affinity Fn-binding (Pietrocola et al., 2015). The A-domain of SpsD is analogous to FnBPB in terms of its high level of strain-dependent sequence diversity, a low affinity Fn-binding site, an α-elastin interaction and a Fg γ-chain interaction (Burke et al., 2010; Burke et al., 2011; Loughman et al., 2008; Pietrocola et al., 2013; Roche et al., 2004; Wann et al., 2000). However, the SpsD A-domain also adheres to CK-10 in a manner similar to ClfB and contains C-terminal B_{SDR} repeats (Josefsson et al., 1998a; Pietrocola et al., 2013; Walsh et al., 2004). In contrast to the Sdr proteins, the number of SpsD B_{SDR} repeats can vary in length between S. pseudintermedius strains more akin to the B_{CNA} repeats of Cna (Gillaspy et al., 1998). These features make SpsD a mosaic
MSCRAMM that is the most promiscuous MSCRAMM characterised to date with the potential that the CK-10 and Fn A-domain interactions are not mediated using a DLL binding mechanism (Pietrocola et al., 2013). With all of these binding ligands it is striking that we did not observe a major role for SpsD in the murine skin infection model employed in the current study. The expression of SpsD in this model was not analysed and potentially SpsD is not being expressed at high levels. Antibodies generated to SpsD have been demonstrated in canine pyoderma patients and so in vivo expression of SpsD is likely during natural infections (Bannoehr et al., 2011). Investigation of ED99ΔspsD in an alternative murine or canine model, displaying clinical features of AD or examining colonisation may allow elucidation of the role of SpsD during S. pseudintermedius carriage or pathogenesis.

To date, we have been unable to identify the minimal Fg-binding region of SpsL suggesting that SpsL has unique Fg-binding properties. The recombinant A-domains of other MSCRAMMs examined to date are sufficient to mediate Fg-binding but this is not the case for SpsL suggesting that the full-length protein, the FnBRs, or post-translational modifications are required. In the case of ClfA, the SD repeats are required as a stalk to expose the ligand-binding A-domain on the bacterial cell surface but the recombinant N2N3 subdomains are sufficient for Fg-binding in vitro (McDevitt and Foster, 1995; McDevitt et al., 1995; Risley et al., 2007). The possible requirement of full-length SpsL to mediate Fg-binding is inconsistent with the observation of truncated versions of SpsL in the supernatant of S. pseudintermedius clinical isolates. Far Western blot analysis will be employed to determine the Fg-binding ability of these truncated SpsL proteins. If full length SpsL is required for Fg-binding then these truncates are likely to be non-functional. Alternatively, post-translational modification of SpsL may be required for Fg-binding. Glycosylation of bacterial proteins is increasingly being recognised with N-acetylglucosaminylation of the SD repeats of the Sdr family in S. aureus promoting agglutination and immune evasion of Sdr proteins (Hazenbos et al., 2013; Thomer et al., 2014). Glycosylation is also important in the nasal colonisation of S. aureus with glycosylation of wall teichoic acid essential for adherence to nasal epithelial cells (Winstel et al., 2015). Future work will focus on determining the Fg-binding capacity of SpsL truncates expressed in S. aureus RN4220.
Whatever the mechanism utilised by SpsL to adhere to Fg is, SpsL is functional on the surface of ED99 and promotes ECM-binding. In a murine model of skin abscess infection this functional SpsL contributes to the development of skin abscesses with ED99ΔspsD-infected mice preferentially developing cellulitis. There has been limited research focussing on the development of *S. aureus* skin abscesses or cellulitis but the host immune response is thought to drive the development of both pathologies (Bailey and Kroshinsky, 2011; Kobayashi et al., 2015a). A murine intradermal infection model demonstrated that *S. aureus* activation of immune receptors such as TLR2, NOD2, and FPR1 induces the expression of IL-1β, which is essential to mediate the neutrophil recruitment required for the generation of intradermal abscesses (Cho et al., 2012). In the current study, large numbers of neutrophils are present in both the abscess and cellulitis pathologies. This suggests that *S. pseudintermedius* is capable of inducing the production of IL-1β and mediating the recruitment of neutrophils to the skin. The neutrophils then either develop an abscess in the case of ED99- or ED99ΔspsD-infected mice or develop cellulitis in the absence of SpsL. It is assumed, in the skin environment, that abscess formation is more beneficial to the host than cellulitis. In this model cellulitis was accompanied by more severe signs of infection and clinically cellulitis is capable of spreading deep into the tissue, causing lymphatic damage that can lead to bacteraemia and sepsis (Bailey and Kroshinsky, 2011). The long-term implications of each pathology have not been explored in this murine model, and will be the focus of future work.

It is unlikely that the role of SpsL in cellular invasion is impacting upon this murine model as bacteria are injected subcutaneously preventing the requirement to invade through the skin barrier (Pietrocola et al., 2015). The ability of SpsL to adhere to ECM proteins could provide a host protein shield to ED99 *in vivo*. Preliminary electron microscopy analysis of ED99 incubated with Fg and Fn suggest that these proteins are capable of coating ED99 with this shielding lost in ED99ΔspsL (data not shown). In *S. aureus*, an extracellular binding protein, Efb, binds to C3 deposited on the surface of *S. aureus* and recruits Fg to the cell surface (Ko et al., 2013). This Fg shields *S. aureus* and blocks opsonophagocytosis as an immune evasion mechanism (Ko et al., 2013). In the present study it is unlikely that shielding of ED99 prevented neutrophil phagocytosis but it could promote bacterial aggregation of ED99. One study
examining the role of clotting of plasma as an immune evasion mechanism of *S. aureus* highlighted the importance of bacterial aggregation in abscess formation (Loof et al., 2015). A low *S. aureus* inoculum injected subcutaneously produced aggregates in the wild type but not in the *coa* mutant (Loof et al., 2015). These aggregates appeared defined and correlated with abscess development (Loof et al., 2015). In the current study, SpsL mediated aggregation of ED99 could influence the formation of abscesses. During histopathology analysis, particular slides contained regions of neutrophil clustering (data not shown). These slides did not contain a developed abscess and were processed from either ED99- or ED99ΔspsD-infected mice 24 h p.i., suggesting a possible requirement of bacterial aggregation for neutrophil clustering and initiation of abscess formation.

We therefore propose a simple model (Figure 7.1) for the function of SpsL in abscess formation whereby SpsL mediate the coating of ED99 in ECM proteins such as Fn and Fg. This shielding produces bacterial aggregation and clustering of neutrophils (Figure 7.1). These neutrophil clusters are sufficient to mediate abscess development. The loss of SpsL eliminates bacterial aggregation and neutrophil clustering leading to a different pathology resembling cellulitis (Figure 7.1). This model suggests that the localisation of bacteria in the skin can influence the development of the host immune response. Electron microscopy of infection abscesses is currently being performed to determine if bacterial aggregation is evident during abscess formation but not during cellulitis. An alternative explanation for the loss of abscess formation upon deletion of *spsL* could be an indirect effect of the gene deletion that impacts upon a bacterial co-factor or ligand-binding complex. Validation of the *spsL* deletion mutant did not suggest any pleiotropic effects of the mutation and so it is unlikely that the role of SpsL in the development of skin abscesses can be attributed to an indirect effect.
Figure 7.1. Model of SpsL-Mediated Abscess Formation. The expression of SpsL promotes shielding of *S. pseudintermedius* ED99 with ECM proteins. This allows bacterial aggregation leading to neutrophil clustering and abscess formation. A loss of SpsL expression prevents bacterial aggregation and therefore neutrophil clustering. The lack of neutrophil clustering promotes cellulitis.
From this murine model it would be assumed that a drug designed to inhibit the function of SpsD or SpsL would not be advantageous to acute infection outcome. Inhibition of SpsD may not provide a change to the acute infection outcome with inhibition of SpsL possibly exacerbating infection outcome. However, longer infection studies are required to establish the effect of the loss of SpsD and SpsL on the persistence of *S. pseudintermedius* skin infection in this murine model. In contrast, SpsD and SpsL are valid vaccine candidates for the purpose of preventing *S. pseudintermedius* infection. The role of SpsD and SpsL in ECM-binding suggests they have roles in colonisation as well as reported functions in cellular invasion that could be essential for the development of skin infections (Pietrocola et al., 2015). Due to the sequence diversity and potential cleavage of SpsD A-domain, the B$_{SDR}$ repeats are maybe the most viable vaccine antigens of SpsD. SpsL A-domain would be a good vaccine antigen candidate with the disordered nature of the FnBRs making them poor vaccine candidates. Both SpsD and SpsL are expressed by clinical isolates and are capable of producing a natural antibody response during canine pyoderma (Bannoehr et al., 2011). In the case of *S. aureus* the success of a vaccine will not only depend upon the ability to produce a functional antibody response but also in the ability of the vaccine to elicit a cellular T-cell mediated response (Fowler and Proctor, 2014). This has been investigated in the case of ClfA vaccination with UEA-1 used in conjunction with ClfA to promote a mucosal T-cell mediated immune response that promoted clearance of systemic *S. aureus* infection in a purely T-cell mediated manner (Misstear et al., 2014). The use of a TLR7 agonist adjuvant in a multicomponent *S. aureus* vaccine also generated a dominant Th1 immune response as well as high antibody titres (Bagnoli et al., 2015). This suggests that careful selection of both the adjuvant and vaccine antigens could be key to the successful development of a *S. pseudintermedius* vaccine.

In conclusion, this study has provided the *S. pseudintermedius* research field with important tools for the genetic manipulation of *S. pseudintermedius* as well as an *in vivo* model of both skin abscess and cellulitis infection. Using these tools we have characterised important functions of SpsD and SpsL in the ECM-binding of *S. pseudintermedius* ED99 and established a role of SpsL in the development of skin abscesses. A novel Fg α-chain binding mechanism of SpsL has been identified that is
continuing to be characterised. SpsD A-domain is predicted to be cleaved from the surface of ED99 with the potential that this could have a functional role during *S. pseudintermedius* infection. Future work will focus on characterising the novel Fg-binding mechanism of SpsL and the function of this protein in abscess development.
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Appendix I Health Assessment Protocol
Health Assessment Sheet: Infected Mice

Group / Experimental treatment:  

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<th>Mouse strain and sex</th>
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Ear marking  

Remarks

1. Examination of the mouse within the cage without physical contact:

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<td>Failure to groom</td>
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<td>Discharge from eyes or nose</td>
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<td>Hunched body</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Change in gait</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2. Skin lesions:

<table>
<thead>
<tr>
<th>Category</th>
<th>AM</th>
<th>PM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lump</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Size (callipers) mm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Redness / discharge</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse scratching</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Photo No.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3. Behaviour:

<table>
<thead>
<tr>
<th>Category</th>
<th>AM</th>
<th>PM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Change in temperament</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isolated, away from others</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Restless</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reluctant to move</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Recumbent</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2. Clinical examination of the mouse:

<table>
<thead>
<tr>
<th>Category</th>
<th>AM</th>
<th>PM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cold to touch</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breathing abnormal</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1. Score

<table>
<thead>
<tr>
<th>Subtotal all categories:</th>
<th>AM</th>
<th>PM</th>
</tr>
</thead>
</table>

Severity grade

Total number of points  

Termination of animal?  

(see criteria below)

Signature of responsible person

Score system

Severity grades:

Criteria for euthanasia

Euthanasia will be performed when any of the following criteria are met:

1) A score of 2 is given to 3 or more parameters (except for "clinical signs" - category 4)

2) Both clinical signs receive a score of 1

3) An overall body weight loss of 20% is observed

4) Skin lesions are greater than 30mm in diameter

5) A total score of > = 5 in any of the categories listed

Signature of responsible person

Score system

Severity grades:

Criteria for euthanasia

Euthanasia will be performed when any of the following criteria are met:

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Signature of responsible person
Appendix II Published Paper