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THE STAPHYLOCOCCUS PSEUDINTERMEDIUS ADHESIN SPSD CONTAINS A CENTRAL FIBRONECTIN-BINDING DOMAIN

Andrea S. Bordt

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A

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by

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Houston, Texas

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DEDICATION

I dedicate this thesis to my many fathers, mothers, sisters, and brothers – scientific, biological, and adopted. Many people have had a significant impact on my life and scientific career and have shown me what it means to be an ethical researcher, to struggle through, to endure, to trust, and to discover. I am fortunate to have a network of people who have supported and encouraged me: Mama C and Papa Doc who have stood by me through it all, my amazing Mom, my Tabernacle and Mission24 church families, Suz, Naomi, Tammy, Kimmy, Dindi, Naters, Trevor, Leslee, Matty, Mama Lynn, Rose Ann, Lance and Andrea, Janichka and John, the Lopez Clan, Caná, Srishtee, Xiaowen, Brooke, Sabitha, Danielle, Emanuel, José, Brandon, Lida, Twaillah, Olivia, and Navella. Those I wish to particularly honor include Dr. Wendy Perryman, Dr. Hal Reed, Dr. John Korstad, Dr. Lowell Sever, Dr. David Marshak, Dr. Tom Goka, Dr. Eric Brown, and Dr. Tommy Douglas.

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THE *STAPHYLOCOCCUS PSEUDINTERMEDIUS* ADHESIN SPSD CONTAINS A CENTRAL FIBRONECTIN-BINDING DOMAIN

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Staphylococcus pseudintermedius is a Gram-positive bacterium significant because of its ability to cause costly and difficult to treat veterinary infections worldwide. It exhibits several similarities to *Staphylococcus aureus*, however, very little is known about its surface adhesins. Surface adhesins in *S. aureus* are significant contributors to pathogenesis. *S. pseudintermedius* encodes the surface protein SpsD, which contains characteristics of the microbial surface components recognizing adhesive matrix molecules family and confers attachment of the heterologous host *Lactococcus lactis* to fibronectin. This work has identified a centrally-located fibronectin binding domain in SpsD which binds the 30 kDa N-terminal domain of fibronectin with high affinity. The data indicate that a tandem β-zipper mechanism of binding may be taking place, and warrants further study into SpsD's role in overall colonization of the host.

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ABBREVIATIONS

MSCRAMMs: microbial surface components recognizing adhesive matrix molecules

ECM: extracellular matrix

FnBD: the fibronectin-binding central region of SpsD (strain ED99): *spsD*$_{1585\text{-}2100}$, SpsD$_{529\text{-}700}$

NTD: the 30 kDa N-terminal domain of fibronectin, composed of the first five F1 modules

GBD: the 45 kDa gelatin-binding domain of fibronectin

CBD: the 120 kDa cell-binding domain of fibronectin

Hep-2: the C-terminal 40 kDa heparin-binding domain of fibronectin

SPR: surface plasmon resonance

CK10: cytokeratin 10

ID_{50}: infectious dose 50%; number of bacteria needed to produce infection in 50% of a population

LD_{50}: lethal dose 50%; number of bacterial needed to be lethal to 50% of a population

Chapter 1: Background and Significance

Staphylococcus pseudintermedius

Staphylococcus pseudintermedius is a Gram-positive bacterium known for producing costly veterinary infections worldwide, particularly in dogs. Infections commonly include surgical wound infections and pyoderma in atopic dogs. Although comparatively rare, when humans do become infected the resulting disease is generally severe (1). Until 2007, it was believed that the microorganism responsible for many of these infections was *Staphylococcus intermedius* (2, 3). *S. pseudintermedius* was first described as a unique species by Devriese and others (4). Veterinary bacterial testing is complicated by the presence of the *S. aureus*-like veterinary pathogens *Staphylococcus intermedius* (5) and *Staphylococcus hyicus* (4, 6), among others. For this reason, additional diagnostic tests must be performed to adequately determine bacterial identifications (4). Isolates from four different host species (lung tissue from a cat in 1999, skin lesion from a horse in 1999, ear lesion from a dog in 2001, and liver tissue from a parrot in 2003) demonstrated similar electrophoretic patterns using tRNA intergenic length polymorphism analysis (4). Definitive evidence of the presence of a distinct new species came from 16S rRNA sequencing, which showed 100% similarity between the four strains (4). This new strain was classified as a member of the *S. intermedius* group as defined by Takahashi et al. (1999) and named *S. pseudintermedius* – false *intermedius* (4).

S. pseudintermedius is a commensal organism of healthy dogs (1, 7). It colonizes the nose and perianal area of healthy dogs and has been found on cellular phones in a veterinary clinic (8), indicating its ability to contaminate the

environment of colonized/infected animals. Importantly, antibiotic-resistant strains are increasingly isolated worldwide (1).

Surface adhesins of *S. pseudintermedius*

It is common for pathogenic bacteria to express components on their cell surface that aid in initial adherence and colonization through interactions with host tissues. *S. pseudintermedius* is no exception. Surface adhesins often contribute to bacterial pathogenesis and have been sought after for vaccine components or therapeutic targets. *S. pseudintermedius* is able to adhere to dog corneocytes – terminally-differentiated keratinocytes, which have lost their nuclei (9). *S. pseudintermedius* is also able to adhere to several host extracellular matrix (ECM) components (7, 10, 11). ECM components are often targeted by microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) and are of particular importance in opportunistic pathogens. Studying MSCRAMM:ECM interactions indicates that prevention of colonization can lead to decreased infection rates. The information gained from MSCRAMM:ECM interactions has lead to the design of small molecule inhibitors and vaccines to target these interactions.

S. pseudintermedius strain ED99 is predicted to encode three surface proteins with the hallmark characteristics of MSCRAMMs: N-terminal signal sequence, A domain containing repeated IgG-like folded domains, B repeats, and LPXTG anchoring motif (12, 13). The N-terminal signal sequence is necessary for directing the immature protein to the Sec secretory pathway (14, 15) and the LPXTG motif

followed by a hydrophobic domain and a largely positive tail (14, 16) are used for anchoring. The LPXTG motif is used by the enzyme Sortase to covalently anchor the mature protein to the cell wall peptidoglycan in *S. aureus* (13). The A-domain and B repeats of many *S. aureus* MSCRAMMs are involved in colonization and/or pathogenesis through interactions with host ligands. For instance, the IgG-like folds of the A domain of SdrG interact with the host protein fibrinogen using a dock-lock-latch mechanism (17), while the B repeats of FnBPA are known to bind the host protein fibronectin via the extended tandem beta-zipper mechanism (18), which in turn increases *S. aureus* infection potential.

Currently, there is a lack of knowledge about the structure and function of *S. pseudintermedius* adhesins. Based on sequence analysis of the available strains, *S. pseudintermedius* encodes three putative MSCRAMMs: SpsD, SpsL, and SpsO (7). These are characterized as potential MSCRAMMs based on their genetic structure, which predicts that they contain the hallmarks of MSCRAMMs. Like some previously characterized MSCRAMMs, strain ED99 SpsD and SpsO confer adherence to canine corneocytes when heterologously expressed on the surface of *Lactotoccus lactis* subspecies *cremoris* strain MG1363 (11). Additionally, SpsD and SpsL expressed on the surface of *L. lactis* confer attachment to fibrinogen from multiple species, bovine fibronectin, and mouse cytokeratin 10 (12). SpsD and SpsL-specific IgGs have been detected in sera from dogs with bacterial pyoderma (12), suggesting that these proteins are expressed during *S. pseudintermedius*

infection. Taken together, these data suggest an important role for these putative MSCRAMMs in *S. pseudintermedius* pathogenesis and warrant their further study.

Fibronectin

The host glycoprotein fibronectin is a dimer of two 250 kDa monomers and is present in two major forms: a soluble protein found in circulation, and an insoluble protein found in the extracellular matrix (19). Fibronectin is a binding partner for both host and microbial proteins (19). Its domain organization is depicted in Figure 1 (19). The 30 kDa N-terminal domain (NTD) is comprised of the first five F1 modules (^{1}F1-^{5}F1) and is a common target for bacterial adhesins, in addition to its ability to bind the host proteins heparin and fibrin (19). The 120 kDa cell binding domain (CBD) is also a target for bacterial adhesins, including *Streptococcus pyogenes* Protein H and *Porphyromonas gingivalis* Fim, and plays an important role in host immunity (19) (20) (21).

Fibronectin-targeting bacterial surface proteins

A number of bacterial proteins target fibronectin, with consequences including bacterial attachment to host matrices and cells, invasion of host cells via a fibronectin bridge, and various disruptions of the host immune responses (12, 22). These bacterial proteins target various fibronectin domains and even specific modules. Relevant to the current topic, adhesins from *S. aureus*, *Streptococcus pyogenes*, and *Streptococcus dysgalactaie* interact with fibronectin primarily at the

30 kDa NTD (Table 1, 19). These facts make it clear that binding to fibronectin is an important strategy employed by bacterial pathogens and warrants further study.

Table 1. Several bacterial adhesins target the fibronectin NTD.

Organism	Adhesin	Region Targeted	
		NTD	GBD
S. aureus	FnBPA	X	
	FnBPB	X	
S. pyogenes	PrtF1/SfB1	X	X
S. dysgalactiae	FnBB	X	

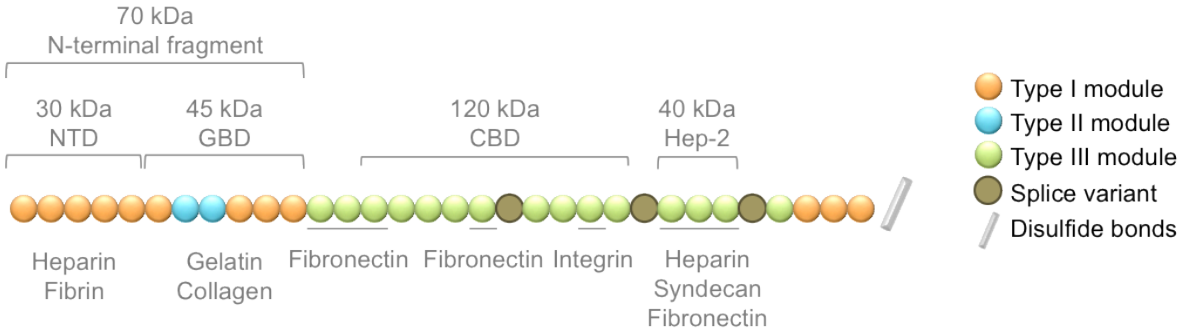


Figure 1. Domain organization of fibronectin. Fibronectin is a modular protein, composed of three types of modules (Type I, orange; Type II, blue; Type III, green). Fibronectin can be proteolytically cleaved into several fragments: an N-terminal 70 kDa fragment containing both the 30 kDa and 45 kDa N-terminal domain (NTD) and gelatin-binding domain (GBD), respectively, a 120 kDa proteolytic fragment containing the cell-binding domain (CBD) and a 40 kDa heparin-binding domain (Hep-2). Schematic adapted from (19), Figure 1, with permission,

Tandem β-zipper binding mechanism

Several microbial surface proteins target the 30 kDa NTD region of fibronectin. For example, *S. pyogenes*, and *S. aureus* surfaces present the proteins SfbI and FnBPA, respectively. These proteins contain loosely conserved sequences which have been experimentally determined to bind the NTD (18). The degree of conservation among bacterial NTD-targeting sequences varies. The mechanism of binding utilized by these proteins is the extended tandem β-zipper, in which the intrinsically-disordered fibronectin-binding portion of the bacterial protein forms an anti-parallel β-strand along the E strand of the fibronectin F1 module triple-stranded β-sheet (18, 23). FnBPA repeats are approximately 40 amino acids in length and interact with the fibronectin F1 modules of the NTD. This binding mechanism is known as the tandem β-zipper mechanism (18).

The current impetus for research into *S. pseudintermedius* is vaccine development based on information gleaned from studying other bacterial surface proteins. The goal of the current research is to determine the regions within SpsD and fibronectin that mediate their interaction.

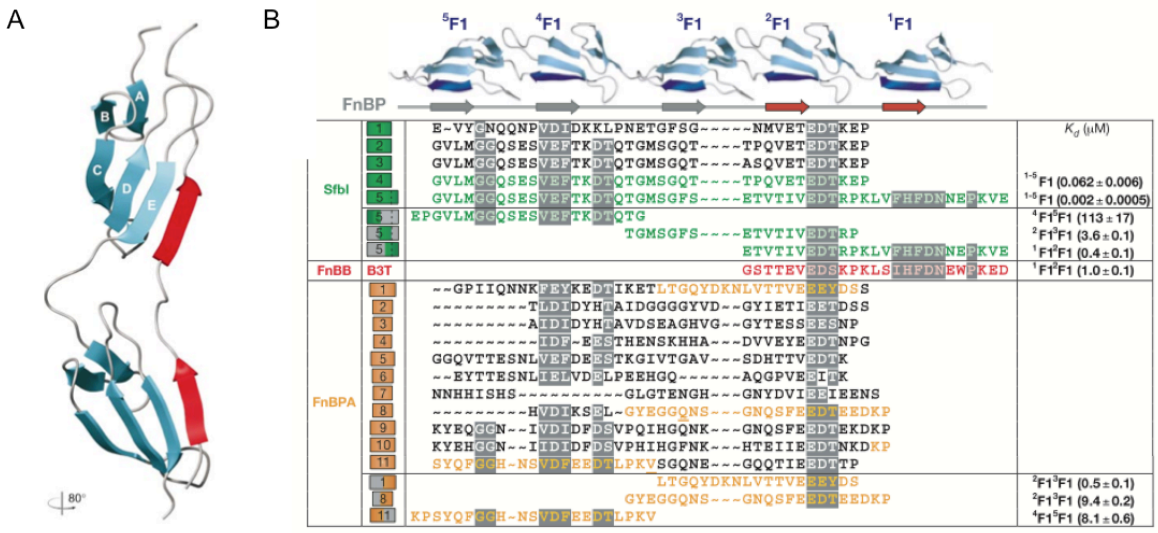


Figure 2. Tandem β-zipper binding mechanism. A. Ribbon diagram of fibronectin modules F1 modules (blue) and the *S. dysgalactiae* protein B3 (red) forming an anti-parallel bond with the E strands of the F1 modules. **B.** Fibronectin modules ^{1}F1-^{5}F1 (blue) drawn with fibronectin-binding protein (grey). β-strands for which three-dimensional data is available are shown in red. Bacterial protein sequences experimentally demonstrated to bind the fibronectin NTD and binding K_d values are shown. Reprinted by permission from Macmillan Publishers Ltd: [NATURE] (Schwarz-Linek and others (18), Figures 2 and 3), copyright (2003).

Chapter 2: Methods and Results

METHODS

Bioinformatics

The *S. pseudintermedius* strain ED99 signal sequence boundary was predicted by the signal sequence predictor SignalP 4.0 server (24). Protein secondary structure predictions were computed by the PHYRE (25), and alignments were made using the ClustalW2 server (26). National Library of Medicine National Center for Biotechnology Information BLAST and GenBank services were also utilized (27).

Cloning

Traditional cloning methods were used to make the constructs used in this study. For construction of pABspsD1585-2100, the *S. pseudintermedius* ED99 *spsD* sequence inserted into pOri23 was used as template (generous gift from Dr. Ross Fitzgerald). PCR conditions for generating $spsD_{1585\text{-}2100}$ were as follows: 2 µM forward primer – 5'-gcaggatccggcaacctggaactctagaggagaca-3', 2 µM reverse primer – 5'- gcagaattcctatttcggcttctcaacgat-3', 1X Phusion® HF Buffer (New England Biolabs, Inc., #B0518S), 0.2 µM each dNTP (New England Biolabs, Inc., #N0447S), 34.25 ng template DNA, and 0.01 U Phusion® High-Fidelity DNA Polymerase (New England Biolabs, Inc., #M0530S) in 50 µL water. Cycling conditions were as follows: initial denaturation at 98 °C for 30 sec; 40 cycles of denaturation at 98 °C for 30 sec, extension at 59 °C for 30 sec, and annealing at 72 °C for 30 sec; final extension at 70 °C for 10 min, and hold at 25 °C. PCR product was cleaned using the DNA Clean & Concentrator™-5 kit (Zymo Research, #D4004), and run on a 1% agarose gel in 1X TAE for size confirmation. Insert was

double digested overnight at 37 °C with 1X NEBuffer 4 (New England Biolabs, Inc., #B7004S), 1X BSA, 1 U each BamHI-HF® and EcoRI (New England Biolabs, Inc., #R3136S and #R0101S, respectively), and 9 µL *spsD*$_{1585\text{-}2100}$ in 20 µL of water and again cleaned using the DNA Clean & Concentrator^{TM}-5 kit. Concurrently, pGEX-5X-1 (GE Healthcare, code #28-9545-53) was double-digested, cleaned, and dephosphorylated using Antarctic Phosphatase (New England Biolabs, Inc., #M0289S) as directed. The ligation reaction was carried out for 4 h at room temperature with the following: 1X Rapid Ligation Buffer (Promega, #C6711), 0.5 µL vector, 1.5 µL insert, 1 µL T4 DNA Ligase (New England Biolabs, Inc., #M0202S). Plasmids were transformed into competent *E. coli* TG-1 cells at 42 °C for 45 sec and plated onto Luria Burtani (Sigma-Aldrich) agar with 0.1 mg/mL ampicillin (USB) at 37 °C overnight. After plasmid extraction using the QIAprep Spin Miniprep Kit (Qiagen, #27106), correct sequence was confirmed by GENEWIZ (South Plainfield, NJ). pABspsD1585-1845 and pABspsD1846-2100 were generated in much the same way. Primers for generating *spsD*$_{1585\text{-}1845}$ were: forward – 5'-gcaggatccggcaacctggaactctagaggagaca-3' and reverse – 5'-gaattcctacgtatcttcgtcgtattcaacgacatca-3'. Primers for generating *spsD*$_{1846\text{-}2100}$ were: forward – 5'-ggatccacaactggcatgttaacaggtgc-3' and reverse – 5'-gcagaattcctatttcggcttctcaacgat-3'. PCR reactions were carried out using Phusion® High-Fidelity DNA Polymerase following manufacturers' instructions. *spsD*$_{1585\text{-}1846}$ was sequentially digested with BamHI and NotI (New England Biolabs, Inc., #R0189S) for 1 h at 37 °C each, cleaned, and ligated into dephosphorylated, BamHI and NotI-digested pGEX-5X-1 vector. *spsD*$_{1846\text{-}2100}$ was sequentially

digested with BamHI and EcoRI for 1 h at 37 °C each, cleaned, and ligated into dephosphorylated, BamHI and EcoRI-digested pGEX-4T-1 (GE Healthcare, code #28-9545-49) vector. Plasmids were transformed into competent XL-1 Blue cells and plated as above. Correct sequences were confirmed by GENEWIZ. Clones with correct sequences were made into frozen stocks by the addition of sterile glycerol and stored at ⁻80 °C.

Protein purification.

The recombinant FnBD (GST-SpsD$_{529\text{-}700}$) was purified over glutathione beads, following the manufacturer's instructions (GE Healthcare). Briefly, cells from freezer stocks were inoculated into Luria Bertani Broth + 0.1 mg/mL ampicillin and grown at 37°C with shaking overnight. These primary cultures were then inoculated into 1L of LB + 0.1 mg/mL ampicillin at a dilution of 1:20, incubated an additional 3h, then transferred to 18°C, and induced overnight with isopropylthio-β-galactoside. Cells were pelleted, resuspended in PBS and frozen. To purify, cells were mechanically lysed and proteins were purified over glutathione beads. Eluted proteins were dialyzed against PBS and quantified spectroscopically.

ELISA assay

For completion of Enzyme-linked immunosorbent assay-like assay, wells of Immulon^{TM} 4HBX plates were coated with 1ug/well human full-length fibronectin (F0895, Sigma), N-terminal 70kDa domain (F0287, Sigma), 30kDa N-terminal domain (F9911, Sigma), 40kDa gelatin-binding domain (F0162, Sigma), 120kDa

cell-binding domain (F1904, Millipore), Hep-2 domain (F1903, Millipore), or BSA V (Sigma-Aldrich, #A9647-100g), sealed, overnight at 4°C. Plates were washed with PBS, incubated with SpsD constructs or GST-FnBPB$_{N2N3}$ for 1h at room temperature, washed, and GST fusion proteins were detected using an HRP-conjugated anti-GST secondary antibody.

Bacterial attachment assay

Human fibronectin (see above) was coated in a concentration of 1 ug/100uL onto Immulon 4HBX plates overnight at 4 °C in PBS. Plates were washed, blocked in 5% BSA in PBS for 1 h at 37 °C, washed, and bacteria (O.D.$_{600}$ = 1) were introduced. Following a 1 h incubation at room temperature, unbound bacteria were washed away, remaining cells were fixed with 4% formaldehyde-buffered solution (Formalde-Fresh, Fisher, #SF93-4) for 1 h at room temperature, stained with 0.5% crystal violet for 5 min at room temperature, and stain was eluted with 5% acetic acid in water for 10 min at room temperature. Absorbance was read at 590nm on a SpectramaxM5 reader.

Inhibition assay

Inhibition assays were carried out similarly to crystal violet attachment assays, except for a second binding step. For the SpsD FnBD inhibition assay, recombinant FnBD was added to immobilized full-length fibronectin at increasing concentrations for 30 min at room temperature, then *L. lactis* expressing full-length SpsD (O.D.$_{600}$ = 1 final concentration) was added and incubated for an additional 30 min before

washing. For the fibronectin NTD inhibition assay, *L. lactis* expressing SpsD was pre-incubated for 30 min at room temperature with NTD before addition to the immobilized full-length fibronectin. Assays were then continued in the same manner as the bacterial attachment assays, described above.

Surface plasmon resonance.

Surface plasmon resonance analyses were carried out using a Biacore 3000 (GE Healthcare/Biacore) at 25 °C following the general methods described by Ross and others (28). Briefly, recombinant FnBD was captured on the chip using an anti-GST monoclonal antibody and fibronectin was flowed over the chip at various serial dilutions. For analysis, baseline was subtracted and kinetics parameters calculated as described (28).

RESULTS

Bannoehr and others (12) first reported the existence of several putative surface anchored proteins of *S. pseudintermedius* strain ED99. Three of these (SpsD, SpsL, and SpsO) were predicted to be MSCRAMMs. SpsD heterologously expressed on the surface of *L. lactis* conferred attachment to immobilized fibronectin and other host extracellular matrix components (12). This finding is notable, because attachment is a precursor to colonization and infection. Since it is known that *S. pseudintermedius* can colonize and infect its hosts, there is a need to understand the mechanism of interaction for vaccine and therapeutic development.

To determine the region within fibronectin targeted by SpsD, an attachment assay was used. SpsD was heterologously expressed on the surface of *L. lactis* and its ability to confer attachment to a panel of fibronectin proteolytic fragments was assessed (Figure 3). The results demonstrate that *L. lactis* expressing SpsD adhered to full-length fibronectin, the N-terminal 70 kDa fragment, and the 30 kDa N-terminal domain (NTD). Each of these contains the 30 kDa NTD, indicating that SpsD targeted the 30 kDa NTD for binding.

Next, it was important to identify the region within SpsD responsible for binding fibronectin. Schwarz-Linek and others (18) demonstrated experimentally that certain sequences within the bacterial adhesins FnBPA (*Staphylococcus aureus*) and Sfb1 (*Streptococcus pyogenes*) bind the fibronectin NTD using the extended tandem β-zipper mechanism. There is a conserved E-D/E-T/S/Y motif in FnBPA for ^{2}F1 and D/E-T/S motif for ^{4}F1 interactions (18, 22, 29). Alignments between the *S. pseudintermedius* strain ED99 SpsD primary amino acid sequence and the NTD-binding sequences from FnBPA and Sfb1 indicated the presence of a region of similarity stretching from residues 529-700 of SpsD, provisionally named the fibronectin-binding domain (FnBD; Figure 4A, blue bar; Figure 4B, blue). Within this domain lie two discreet regions of similarity: residues 529-579 and residues 682-700 (Figure 4A, grey bars).

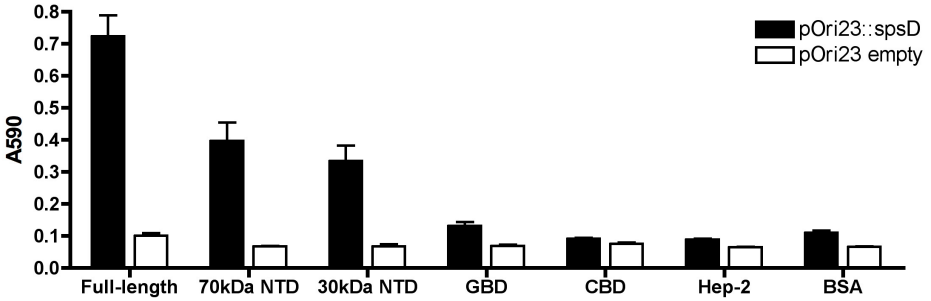
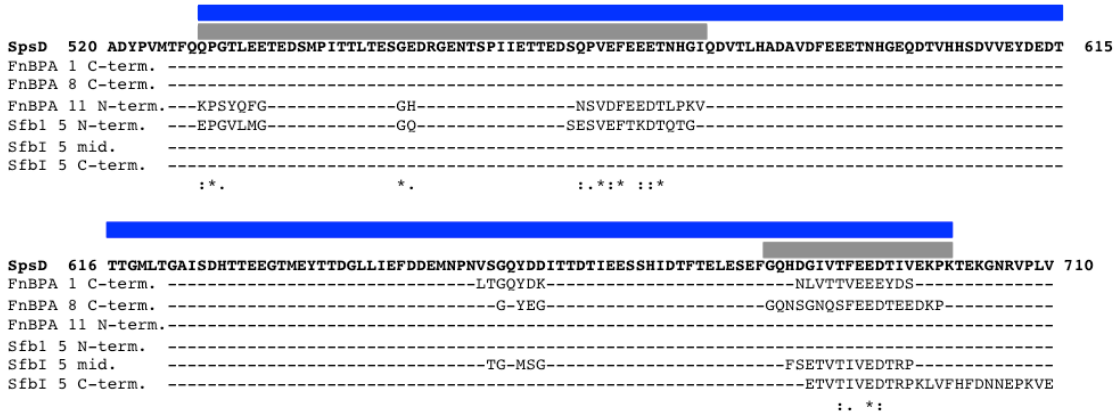


Figure 3. Heterologous expression of SpsD on the surface of *L. lactis* confers attachment to the N-terminal portion. Full-length fibronectin and proteolytic fragments were immobilized and the ability of *L. lactis* expressing full-length SpsD to adhere to fibronectin was assessed using a crystal violet attachment assay. *L. lactis* containing the empty vector and immobilized BSA were included as negative controls.

A



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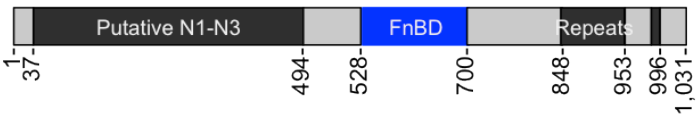

Figure 4. The *S. pseudintermedius* strain ED99 FnBD, SpsD$_{529-700}$. A. Compilation of ClustalW2 alignments between full-length SpsD and several fibronectin NTD-binding sequences from the *S. aureus* protein FnBPA and the *S. pyogenes* protein Sfb1. The FnBD is denoted by a blue bar, while the discreet regions of similarity (residues 529-579 and 682-700) are indicated by grey bars. An asterisk (*) indicates residues with full identity, a colon (:) indicates conservation of strongly similar residues, and a period (.) indicates conservation of weakly similar residues. **B.** Schematic of *S. pseudintermedius* strain ED99 SpsD including the FnBD (blue).

Since it is known that the bacterial protein regions involved in the tandem beta-zipper mechanism are intrinsically disordered, it was important to investigate the secondary structure of the FnBD. The sequence for *S. pseudintermedius* strain ED99 SpsD FnBD was submitted to the PHYRE2 protein fold recognition server, and the results indicate that it too is predicted to have an intrinsically-disordered secondary structure (Figure 5).

In order to confirm the presence and fibronectin-binding properties of the SpsD FnBD, traditional genetic cloning methods were used to generate pABspsD1585-2100 (Figure 6). The recombinant GST fusion protein was expressed and purified (Figure 7A) and was found to bind preferentially to the fibronectin NTD by both ELISA (Figure 7B) and surface plasmon resonance (Figure 7C and 7D). An ELISA-type assay demonstrated that the recombinant FnBD was able to bind to the three fibronectin fragments containing the NTD (A). Surface plasmon resonance demonstrated that the immobilized recombinant FnBD:fibronectin interaction was high affinity, particularly with the NTD (K_D^{app} = 0.51nM). Surface plasmon resonance using Biacore also allows one to investigate the stoichiometry of interactions. The stoichiometry evaluation of the interaction suggested a 2:1 binding stoichiometry (data not shown).

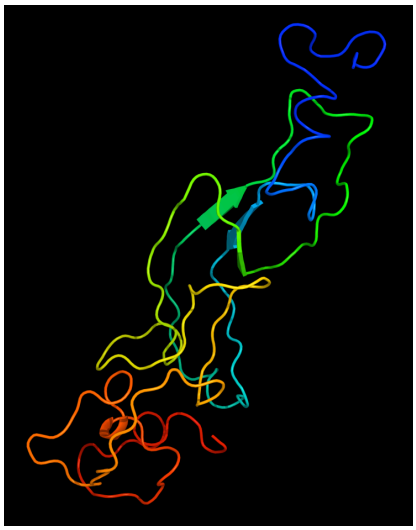

Figure 5. The *S. pseudintermedius* strain ED99 SpsD FnBD is predicted to be intrinsically disordered. The primary amino acid sequence of the FnBD was submitted to the PHYRE2 server for secondary structure analysis.

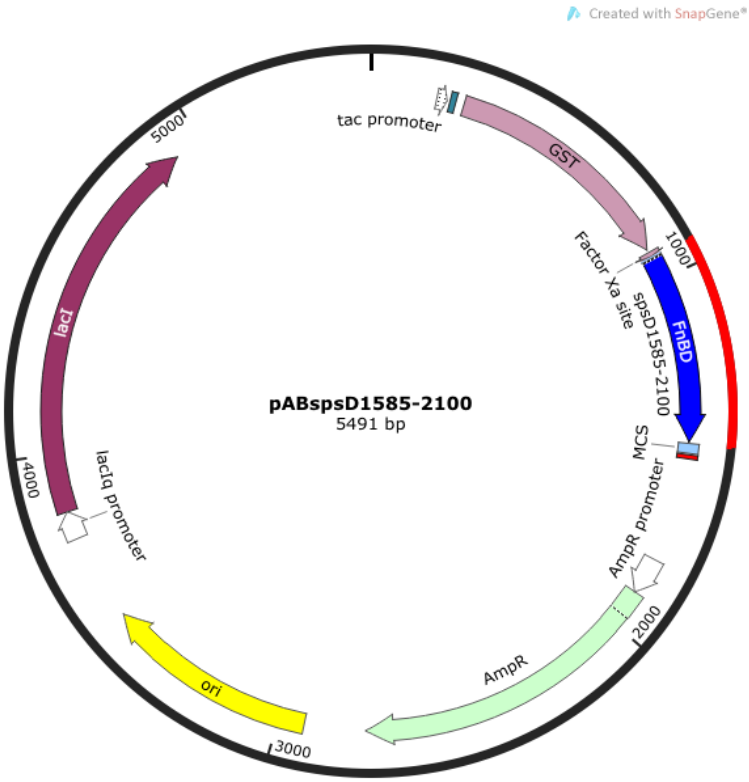

Figure 6. pABspsD1585-2100. *S. pseudintermedius* strain ED99 FnBD (spsD1585-2100) inserted into pGEX-5X-1. The plasmid contains a tac promoter, Factor Xa site, and AmpR gene. Created with SnapGene (www.snapgene.com).

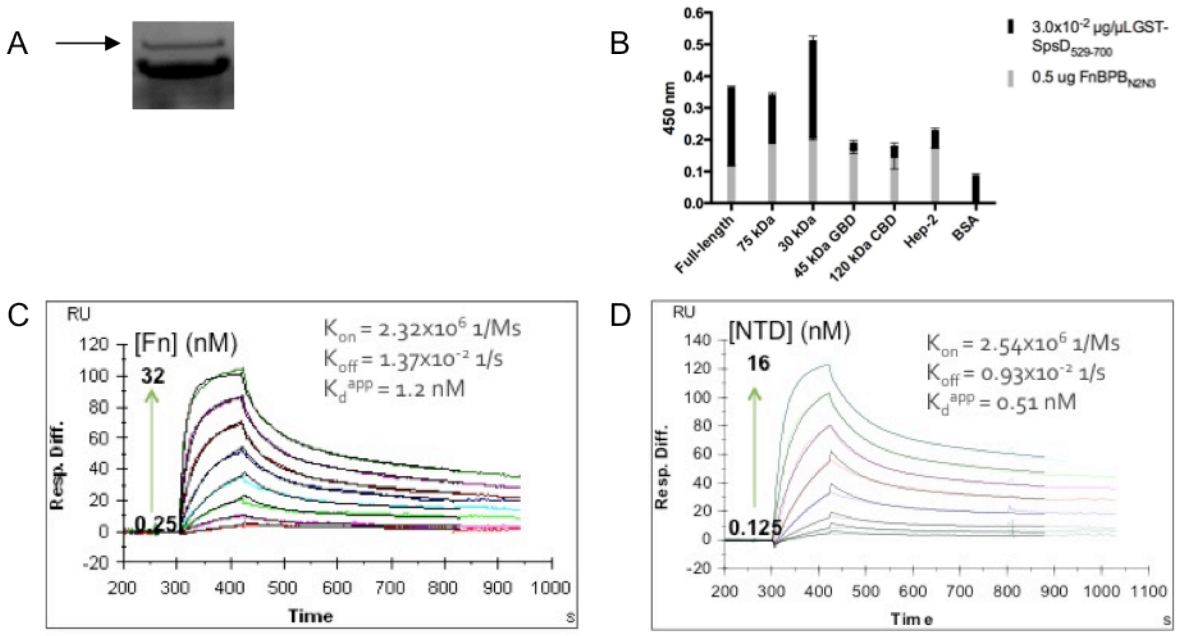

Figure 7. The *S pseudintermedius* recombinant FnBD binds the fibronectin NTD with high affinity. A. 12% SDS-PAGE of purified recombinant FnBD, arrow marks ~55 kDa, and the predicted size of the GST fusion protein is 45.8 kDa. **B.** The ability of the recombinant FnBD to bind fibronectin was assessed using an ELISA-type assay. Fibronectin full-length and proteolytic digests were immobilized onto the plate and recombinant FnBD was added. The non-fibronectin-binding N2N3 region of the *S. aureus* adhesin FnBPB and coated BSA were used as negative controls. **C.** Surface plasmon resonance was used to assess the binding kinetics of the recombinant FnBD to full-length fibronectin and the fibronectin NTD (**D**) interactions. Recombinant FnBD was captured on the chip and fibronectin was flowed over the chip at increasing concentrations.

To further investigate the specificity of the FnBD:fibronectin NTD binding, bacterial inhibition assays using full-length SpsD expressed on the surface of *L. lactis* were carried out. To determine whether the FnBD of SpsD and the NTD of fibronectin are necessary for this interaction, full-length fibronectin was immobilized on a polystrene plate. As the concentration of recombinant FnBD increased, the availability of binding sites for full-length SpsD on the surface of *L. lactis* was decreased, leading to a decrease in attachment (Figure 8A). The same effect was observed when the fibronectin NTD was used as an inhibitor (Figure 8B). Taken together, these results indicate that the FnBD and the 30 kDa fibronectin NTD are region within each protein which are important for mediating their binding.

Within the FnBD two possible fibronectin binding regions were predicted based on primary amino acid sequence similarity to FnBPA and SfbI: $SpsD_{529\text{-}579}$ and $SpsD_{682\text{-}700}$. To investigate this, two constructs were generated, each encompassing roughly one half of the FnBD (GST-$SpsD_{529\text{-}615}$ and GST-$SpsD_{616\text{-}700}$; Figure 9A), and were submitted to surface plasmon resonance analysis. Surface plasmon resonance is a powerful tool for investigating molecular interactions. The basic principle relies on the immobilization of one binding partner onto a chip and gathering data from their interaction. The results are displayed in a sensorgram, in which the upward curve describes association (on) and the downward curve, dissociation (off). The data are then fit using one of several binding models. A simple 1:1 binding may be described by the equation A + B $\leftrightarrow$ AB, in which k_{a1} describes association and k_{d1} describes dissociation. A more complex model of

binding occurs when there is a conformational change upon binding. Such a binding may be described by the equation A + B↔AB↔ABx, where k_{a2} describes the tendency to form this conformation and k_{d2} describes the dissociation to form AB. The value for k_{a2}/k_{d2}, then, describes the tendency for the resulting product to undergo a conformational change during binding. The molar binding ration (*N*) indicates the stoichiometry of the equation. The results of surface plasmon resonance analysis of the SpsD constructs indicate that each half of the FnBD binds to fibronectin NTD with high affinity (K_D^{app} = 0.17-0.84 nM) and thus contains a fibronectin NTD-binding site (Figure 9B; Table 2, *N* values).

$$A + B \underset{k_{d1}}{\overset{k_{a1}}{\rightleftharpoons}} AB \underset{k_{d2}}{\overset{k_{a2}}{\rightleftharpoons}} ABx$$

Construct	k_{a1} (1/Ms; x10^6)	k_{d1} (1/s; x10^{-3})	K_D k_{d1}/k_{a1} (nM)	k_{a2} (1/s; x10^{-3})	k_{d2} (1/s; x10^{-3})	k_{a2}/k_{d2}	K_D^{app} (nM)	*N*
GST-SpsD$_{529-700}$	2.54	9.32	3.67	5.29	0.86	6.15	0.51	1.87
GST-SpsD$_{529-615}$	1.65	8.81	5.34	6.67	1.25	5.34	0.84	0.79
GST-SpsD$_{616-700}$	1.73	5.74	3.32	10.8	0.59	18.31	0.17	1.32

Table 2. The binding of SpsD recombinant constructs to fibronectin. Recombinant SpsD FnBD (blue), SpsD$_{529-615}$ (green), and SpsD$_{616-700}$ (red) bind the fibronectin NTD with high affinity (K_D^{app}). Recombinant FnBD is predicted to bind fibronectin NTD with a stoichiometry of 2:1 (*N* = 1.87). As predicted, each half of the FnBD is able to bind to approximately one fibronectin NTD (*N* = 0.79, SpsD$_{529-615}$; *N* = 1.32, SpsD$_{616-700}$).

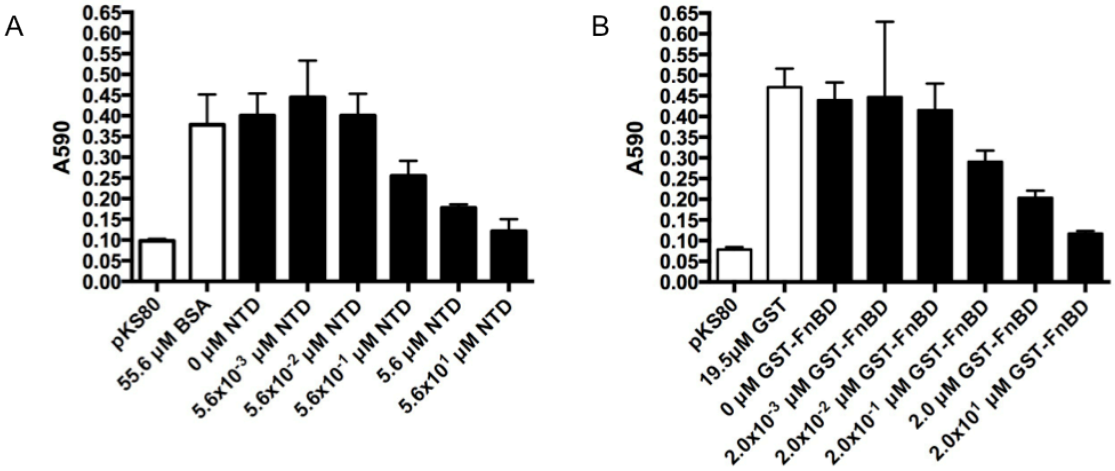

Figure 8. The SpsD fibronectin-binding domain or the 30 kDa fibronectin NTD inhibit binding of *L. lactis* expressing SpsD to fibronectin-coated surfaces. An adaptation of the crystal violet attachment assay was used to determine whether the FnBD:fibronectin interaction could be inhibited by either soluble recombinant FnBD or the 30 kDa fibronectin NTD. Full-length fibronectin was coated on the plate and increasing concentrations of recombinant FnBD (**A**) or the 30 kDa fibronectin NTD (**B**) were added to the coated fibronectin or *L. lactis* expressing full-length SpsD, respectively. *L.lactis* containing empty pKS80 vector, coated BSA, and recombinant GST were used as negative controls.

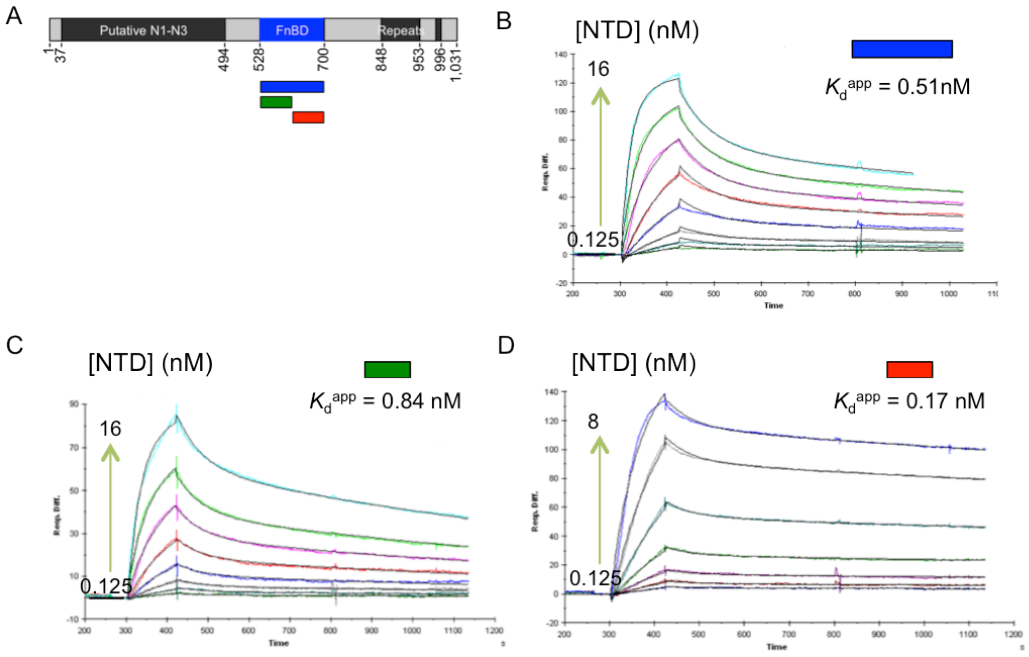

Figure 9. The *S. pseudintermedius* FnBD contains N- and C-terminal fibronectin-binding regions. A. Schematic of SpsD. Three GST fusion recombinant proteins were made: FnBD (**B**, GST-SpsD$_{529\text{-}700}$, blue), GST-SpsD$_{529\text{-}615}$ (**C**, green), and GST-SpsD$_{616\text{-}700}$ (**D**, red). Binding kinetics of all three constructs were tested (purified protein for recombinant FnBD and bacterial lysates for GST-SpsD$_{529\text{-}615}$ and GST-SpsD$_{616\text{-}700}$) using surface plasmon resonance and were shown to bind with high affinities using a two-step binding model in which a conformational change occurs upon binding (black lines; all y-axes in units of RU).

Taken together, these results indicate the presence of a centrally-located fibronectin-binding domain within *S pseudintermedius* strain ED99 SpsD which binds the NTD of fibronectin with high affinity. The predicted intrinsically-disordered nature of the FnBD, along with the calculated stoichiometry of two NTDs bound per every one FnBD, suggest that the binding mechanism may be that of the tandem β-zipper adopted by several other fibronectin NTD-targeting bacterial adhesins.

Chapter 3: Discussion and Conclusion

The results of this study indicate that full-length SpsD, a putative MSCRAMM of *S. pseudintermedius*, binds the extracellular matrix component fibronectin. Full-length SpsD on the surface of *L. lactis* was found to confer attachment to human fibronectin N-terminal fragments containing the 30 kDa NTD (Figure 3). This result indicated that SpsD targets the NTD of fibronectin for binding. Efforts were then taken to determine the minimal binding region within SpsD responsible for this fibronectin interaction. SpsD's primary amino acid similarity with other NTD-targeting bacterial surface proteins suggested the presence of a centrally-located fibronectin-binding domain, FnBD. This FnBD was further predicted to contain two general areas of similarity, at the N- and a C-terminal ends. Also, like several other NTD-targeting adhesins, this central putative fibronectin-binding domain (FnBD) was predicted to be intrinsically disordered, suggesting that it may use the tandem β-zipper mechanism of binding. Subsequently, a recombinant GST fusion protein of the SpsD FnBD was generated and demonstrated to bind fragments of human fibronectin containing the 30 kDa NTD. This binding pattern was reproduced using surface plasmon resonance, and the binding was shown to be of high affinity, with a predicted stoichiometry of two NTDs for every FnBD. Further analysis into this interaction using inhibition assays demonstrated that both recombinant FnBD and the fibronectin NTD were able to reduce attachment of *L. lactis* expressing full-length SpsD to immobilized full-length fibronectin, suggesting that the SpsD FnBD and the fibronectin NTD are the regions primarily responsible for mediating their interaction. The SpsD FnBD was predicted to contain two possible fibronectin NTD-binding sites based on primary amino acid sequence similarity to other NTD-

targeting bacterial adhesins, and surface plasmon resonance stoichiometry data concurred. Therefore, two GST-tagged recombinant constructs were generated, each encompassing approximately half of the FnBD. These constructs were submitted to surface plasmon resonance and the results demonstrated that each bound one fibronectin NTD molecule, confirming the 2:1 predicted stoichiometry. Taken together, this data indicates that SpsD targets the NTD of fibronectin by utilizing at least 2 high affinity ligand-binding sites within its FnBD. These conclusions add to the knowledge of how *S. pseudintermedius* may colonize the host, but also raises a number of questions that may warrant initiation of several areas of focus.

S. pseudintermedius is a pathogen which exhibits species specificity. Experiments with SpsD expressed on the surface of *L. lactis* have shown that SpsD attaches to canine, bovine, human, equine, and avian fibrinogen (12). Although the experiments presented here were completed with human fibronectin, it is likely that comparable results would be observed with canine fibronectin, given the fact that SpsD appears to attach equally well to human and dog fibrinogen at a concentration of 40 µg/mL (12), and that an alignment between human and canine fibronectin NTD sequences demonstrated that the sequences differ in only one residue (ClustalW2 alignment, data not shown; P83 canine and S93 human fibronectin sequences, located in the region between the ^{1}F1 and ^{2}F1 modules).

Heterologous expression is allows one to tease out contributions attributable to individual proteins by expressing them alone on the surface of an unrelated host. Heterologous expression studies also allow one to observe redundancies in naturally-occurring proteins. Although these studies are artificial, they do provide important information regarding characteristics of individual proteins. For example, Mulcahy and others (30) demonstrated that the *S. aureus* MSCRAMM ClfB was able to confer attachment of *L. lactis* expressing ClfB to the nares of mice, thereby demonstrating its role in nasal colonization. These data may have been difficult to obtain without the use of ClfB *L. lactis* heterologous expression since *S. aureus* is known to express many surface adhesins, complicating the assessment of the roles of individual adhesins. Therefore, heterologous expression was used to evaluate various SpsD mutants and ligand-binding domains to better identify portions involved in contributing to pathogenesis. Results of experiments using SpsD heterologously expressed on the surface of *L. lactis* demonstrated that SpsD targets the fibronectin NTD and that this interaction may be inhibited by using both recombinant FnBD and fibronectin NTD assays.

Biofilm formation is another common virulence strategy used by bacterial pathogens. *S. pseudintermedius* biofilm formation is documented and of particular clinical interest due to its involvement in surgical site infections (31, 32). Though frequently observed, *S. pseudintermedius* biofilms are incompletely characterized and no definitive preventive or treatment has been identified. Singh and others (33) found that 96% of *S. pseudintermedius* clinical strains from dogs (121 methicillin-

resistant and 19 methicillin-sensitive) were either strong or moderate biofilm producers. DiCicco and others (31) found clarithromycin to be ineffective at eradicating *S. pseudintermedius* biofilms. Promisingly, Turk and others (32) and Song and others (34) have found DispersinB and manuka essential oil, respectively, to be promising biofilm treatments. The roles of SpsD and other *S. pseudintermedius* adhesins and surface proteins in biofilms could be examined using mutant strains deficient or defective for SpsD and other proteins.

S. aureus has been shown to induce invasion of endothelial cells through the use of a fibronectin bridge mediated by fibronectin binding proteins (22, 35) in which fibronectin's interaction with β1 integrins facilitated non-phagocytic cell uptake (19, 35). This is advantageous for pathogens, providing access to an intracellular niche useful for escape from the immune system. There are currently no reports of intracellular *S. pseudintermedius*, so an important question to investigate would be to determine whether SpsD and/or other putative *S. pseudintermedius* MSCRAMMs mediate endothelial and/or other cell internalization. Data described here indicates that *S. pseudintermedius* could use a fibronectin bridge for invasion.

A definitive binding mechanism for SpsD and fibronectin has yet to be described completely. Data here indicate that a tandem β-zipper mechanism of binding between the SpsD FnBD and fibronectin NTD is likely, but more experiments are needed before this can be confirmed. Circular dichroism, useful for determining secondary structure, could be used on His-tagged FnBD protein and related

fragments, and these constructs could also be subjected to NMR or co-crystallization for both secondary structure determination and binding characterization in the presence of fibronectin NTD. Structural and binding information gleaned from these types of assays are the gold-standard for determining amino acid residue contacts and configurations.

Studies using animal models may provide information about SpsD immunogenicity. Delayed-type hypersensitivity assays provide information about T cell responses, and immunoglobulin analysis (by ELISA, for example) gives one a picture of B cell responses. Another useful metric that can be examined using animal models is pathogenicity. Investigations into most effective dose and route for reproducing the natural disease, as well as infectious (ID_{50}) and lethal (LD_{50}) doses, may also be performed. The use of animal models for investigating bacterial surface adhesins like SpsD is aided greatly by the fact that *L. lactis* may be introduced without harm to the host at the correct dose. The allows one to heterologously express a protein or protein fragment of interest on the surface of *L. lactis* for use in models to examine colonization and infection. The Fitzgerald laboratory (The University of Edinburgh, UK) has generated a strain of *S. pseudintermedius* ED99 defective for *spsD*. This strain would be useful in functional assays, cell attachment assays, and animal models. Although *S. pseudintermedius* is primarily a canine pathogen, it has been shown to bind to ECM proteins from several species. Since mouse models are typically used for animal studies, the differences between mouse and dog/human fibronectin need to be explored and the ability of SpsD and other

putative *S. pseudintermedius* MSCRAMMs to bind to mouse ECM proteins needs to be confirmed. The use of animal models would also allow for the determination of the vaccine potential of SpsD or any of its components. The proof of concept for investigating SpsD and other *S. pseudintermedius* adhesins for inclusion in vaccines comes from the successful use of *S. aureus* ClfB administered systemically or intranasally in reducing colonization and the ability of α-ClfB monoclonal antibody to reduce nasal colonization (36).

As *S. pseudintermedius* emerges as an important player in the veterinary arena, an important focus will continue to be broadly identifying and characterizing all putative *S. pseudintermedius* MSCRAMMs to understand how this opportunistic pathogen interacts with its hosts. It will be important to determine the domain organization of these proteins, which will allow for the investigation of the roles different domains play, and for the comparison and contrasting of putative *S. pseudintermedius* MSCRAMMs with other known bacterial proteins. Chimeric proteins may then be made in which regions of interest are switched in and out, making custom proteins to test the properties of specific regions of *S. pseudintermedius*.

S. pseudintermedius strain ED99 SpsD also appears to encode an interesting repeat region, comprised of a series of five nearly identical repeated sequences bracketed by a conserved PQP motif (Figure 10). This is noteworthy, because the PQP tripeptide is known to be recognized by specific antibodies and to be present

in bacterial proteins, *B. parapertussis* pertactin, for example (37, 38). Pertactin is thought to function as an adhesin and immunogen. Beyond the scope of this research, *S. pseudintermedius* strain ED99 SpsL also appears to contain interesting repeats. Rather than a PQP tripeptide, SpsL repeats are punctuated by QGPQ regions. These are of interest because a Basic Local Alignment Search Tool (BLAST) search for QGPQ returns result including: Vpr protein of HIV I, Envelope 2 protein of Hepatitis C, Glutenin, and the T-cell receptor V beta chain.

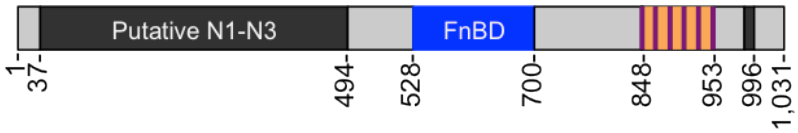

Figure 10. Schematic of *S. pseudintermedius* strain ED99 SpsD including proposed repeats. The repeat region ranges from residue 849 to 956 and includes five repeated sequences (orange) and six PQP tripeptides (purple).

This study has identified a central fibronectin-binding domain within an *S. pseudintermedius* surface adhesin which may be used for colonization and/or invasion via the host protein fibronectin. Discoveries of this kind are vital for the development of therapeutics such as vaccines, small molecule inhibitors, and other treatments. This study provides important insights into a surface protein of an important and ubiquitous worldwide veterinary pathogen.

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VITA

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