Expression of biofilm associated genes in *Staphylococcus pseudintermedius*

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by
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in
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Abstract

Expression of biofilm associated genes in *Staphylococcus pseudintermedius*

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*Staphylococcus pseudintermedius* is a common commensal organism of canines that has emerged and disseminated internationally as an important pathogen. The biofilm forming ability of this bacterium is increasingly appreciated as a virulence factor, especially in surgical site and implant associated infections, where the formation of biofilms results in persistent, recurrent infections that are often recalcitrant to treatment. This thesis is an investigation into the genetic basis of biofilm formation in *Staphylococcus pseudintermedius*. The initial step was the development and validation of a quantitative PCR test including the identification of suitable reference genes in this species. Subsequently, that test was used to examine the expression of multiple biofilm associated genes responsible for numerous aspect of biofilm development and maturation. Significant variations in expression levels between surfaces were identified, providing potential insights into several clinical situations. This work will form the foundation for future studies examining specific details of biofilm formation and development, as well as studies directly examining clinical formed biofilms.
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A final thanks goes to my family, as well as Meryl Herberts and my resident mates for all their support, encouragement and immeasurable assistance during my residency.
Declaration of Work Performed

I declare that with the exception of the items below, all work reported in this thesis was performed by me.

DNA sequencing where described was performed by Macrogen Inc., Seoul, Republic of Korea
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List of Abbreviations

ASA - American Society of Anesthesiologists
BAG - Biofilm Associated Gene
cDNA - Complementary DNA
Dru - Direct Repeat Unit
ECM - Extracellular Matrix
eDNA - Extracellular DNA
ELISA - Enzyme Linked Immunosorbent Assay
Log - Logarithmic phase growth
MALDI-TOF MS - Matrix Assisted Laser Desorption Ionization Time Of Flight Mass Spectroscopy
MBEC - Minimum Biofilm Eradication Concentration
MIQE - Minimum Information for reporting of qPCR Experiments
MRSA - Methicillin Resistant Staphylococcus Aureus
MRSP - Methicillin Resistant *Staphylococcus pseudintermedius*
MSCRAMM - Microbial Surface Component Recognizing Adhesive Matrix Molecule
MSSA - Methicillin Susceptible Staphylococcus Aureus
MSSP - Methicillin Susceptible *Staphylococcus pseudintermedius*
PBS - Phosphate Buffered Saline
PDS - Polysdioxanone
PIA - Polysaccharide Intracellular Adhesin
PMMA - Polymethylmethacrylate
PS - Polystyrene
qPCR - Quantitative Polymerase Chain Reaction

RIN - RNA Integrity Number

SCCmec - Staphylococcal Chromosomal Cassette containing the Methicillin resistance gene mecA

Si - Silicone

SS - Stainless Steel

SSI - Surgical Site Infection

ST - Sequence Type

Stat - Stationary phase growth

Ti - Titanium

VBNC - Viable But Non-Culturable
1. Chapter 1 – Literature Review

Surgical site infections

Definition of surgical site infection

Surgical site infections (SSIs) are an inherent risk of any surgical procedure. There are multiple underlying reasons and predisposing factors that lead to their development; fundamentally it is the interaction between the host (patient), the infectious organism, and the environment that dictates if a SSI will develop. SSIs also cover a spectrum of clinical conditions, resulting in anything from mild peri-incisional erythema and inflammation, to large pockets of purulent material surrounding implants or in body cavities with significant risk of developing into systemic sepsis. Guidelines have been developed to standardize the diagnosis and classification of SSIs, aiding in the reporting and study of SSIs. While these guidelines have been developed primarily for use in the field of human healthcare, they are widely applied to veterinary medicine.
<table>
<thead>
<tr>
<th>Surgical site infection category</th>
<th>Classifying features</th>
</tr>
</thead>
</table>
| **Superficial SSI**              | SSI occurring within 30 days of surgery  
• Infection involving only skin of subcutaneous tissues  
• At least one of the following:  
  - Purulent drainage (±laboratory confirmation)  
  - Organism isolated from fluid or tissue  
  - Pain, swelling, redness or heat AND incision reopened by surgeon UNLESS culture negative |
| **Deep SSI**                     | SSI occurring within 30 days of surgery, or within 1 year if an implant is present and infection related to that surgery or implant  
• Infection affecting the deep tissue (muscles, fascia) of the incision  
• At least one of the following  
  - Purulent drainage from deep incision, but not from organ or space  
  - Abscess or other evidence of infection on direct exam, histopathology, on imaging, or at the time or re-operation  
  - Spontaneous dehiscence of deeper incision, OR deliberate opening by a surgeon based on fever, localized pain or tenderness, UNLESS site is culture negative |
| **Organ or Space SSI**           | SSI occurring within 30 days of surgery, or within 1 year if an implant is present and infection related to that surgery or implant  
• Infection involves any part of the anatomy (organ or space) that was opened or manipulated  
• At least one of the following  
  - Purulent discharge from the organ or space  
  - Organism isolated from aseptically obtained sample from the tissue, organ or space  
  - Abscess or other evidence of infection on direct exam, histopathology, on imaging, or at the time or re-operation |

Table 1.1: Surgical site infection classification (from human medicine)\(^9\).

Surgical site infections can result in significant morbidity and mortality; prolonged and increased pain, delayed healing, inferior cosmesis and the need for prolonged medical care (revision surgeries, prolonged hospitalization, implant removal) and associated costs\(^1,7-9\). Veterinary pathogens are also increasingly being recognized as affecting people, especially infections of medical implants, and pets with SSIs may represent a zoonotic risk\(^10,11\). There are also less tangible effects; frustration for both the care provider and
owners, with concurrent disruption of the owner pet bond and changes in the perception of the level of care provided.

**Etiology of surgical site infections**

While SSIs are a ubiquitous risk with any surgery, there has been significant work to help identify predisposing factors for SSI, and to identify ways to prevent them in both human and veterinary patients\(^{12,13}\). Many of the factors that predispose to SSIs are difficult to modify. Subsequently the main interventions that have been effective in the reduction of SSI rates have been the realization of the need for sterile surgical instruments, general peri-operative sterility\(^{14}\), and more recently, the use of peri-operative antibiotics\(^{15-17}\). Generally, the reduction of pathogen loads in wound has resulted in significant reductions in SSI rates\(^{18}\).

Understanding the predisposing factors for SSI can help guide timing for surgery, planning of peri-operative antimicrobial prophylaxis, and discussions of expectations with owners. In some cases, some of those factors can be resolved or managed in order to help reduce SSI risk, especially in cases of elective surgery.

Surgical procedures can be classified according to the expected degree of bacterial contamination, which directly relates to the risk of developing a surgical site infection\(^{6,19}\). Table 2 shows the criteria for classification of surgical procedures, as well as the reported range of SSIs for surgical procedures within that category.
<table>
<thead>
<tr>
<th>Wound classification</th>
<th>Category descriptions</th>
<th>Range of infection rates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clean</td>
<td>• Non-inflamed surgical wound</td>
<td>2.0-4.8%</td>
</tr>
<tr>
<td></td>
<td>• No entry of genitourinary, respiratory or gastrointestinal tract</td>
<td></td>
</tr>
<tr>
<td>Clean-contaminated</td>
<td>• Controlled entry of genitourinary, respiratory or gastrointestinal tract without expected contamination • Clean wound with drain placed</td>
<td>3.5 – 5%</td>
</tr>
<tr>
<td>Contaminated</td>
<td>• As clean-contaminated with unexpected spillage or contamination</td>
<td>4.6 – 12%</td>
</tr>
<tr>
<td></td>
<td>• Operation on atraumatic wounds without purulent discharge</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Major breaks in aseptic technique</td>
<td></td>
</tr>
<tr>
<td>Dirty</td>
<td>• Operations on traumatic wounds with purulent discharge</td>
<td>6.7 – 18.1%</td>
</tr>
<tr>
<td></td>
<td>• Gross contamination from hollow viscus (e.g. gastrointestinal content contamination)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Wounds grossly contaminated before surgery</td>
<td></td>
</tr>
</tbody>
</table>

Table 1.2: Surgical procedure classification scheme and associated SSI rates\(^{20}\).

Many other risk factors have been identified for the development of SSIs in veterinary patients; similar to those seen in human medicine\(^1\). Some are inherent characteristics of the patient; overweight animals are at increased risk on infection, as are males\(^1\). It is thought that the androgenic hormones may result in a balance of inflammatory and anti-inflammatory mediators that makes infection more likely\(^8\). Several endocrinopathies have been implicated in increasing SSI risk\(^{21}\); hyperadrenocorticism and diabetes mellitus both increase risk of infection through promotion of bacterial growth or immunosuppression. Hypothyroidism has been reported as a risk factor in veterinary medicine, but has not been in human medicine; the pathophysiology of this relationship is unknown\(^{22}\). Anesthetic risk category as ranked using the American Society of
Anesthesiologists (ASA) classification scheme is also reported as a risk factor for SSI, which likely relates to the overall physiological state and health of the patient\textsuperscript{1}.

Numerous other local factors have also been identified, starting from before surgery, through surgery, and into the post-operative and recovery period. While administration of antimicrobials immediately peri-operatively has decreased SSI rates, prolonged or inappropriately timed pre-operative administration has increased those rates, potentially due to induction of or selection for resistance organisms, or inadequate concentration of antimicrobial at the wound at the subsequent time of surgery\textsuperscript{23}. Clipping of hair is a necessity in most situations in veterinary patients, as contamination of a wound with hair increases SSI risk. Clipping hair too far in advance however increases SSI rates\textsuperscript{24}, likely due to physical irritation of the skin and subsequent breakdown of host defenses\textsuperscript{21,25}.

At the time of surgery, choice of induction agents plays a role, with the lipid present in propofol suggested as a growth medium for bacteria\textsuperscript{26}. Increased length of surgery and of general anesthesia allows more time for contamination of the wound, and increases infection risk\textsuperscript{27}. The number of people present in surgery, as well as movement of people in and out of the surgical theatre also increases surgical risk, likely through similar mechanisms\textsuperscript{1}. Surgical closure with staples has shown increased risk compared to skin sutures, for unknown reasons\textsuperscript{28}. Drains placed at surgery increase SSI rate, likely either because they are a route for ascending infection, or because surgical procedures where drains are required are more often inherently ‘dirty’. Recovery of patients in the ICU also increases SSI risk, likely owing either to a relationship to systemically compromised patients, or due to the increased prevalence of multi resistant pathogens in those settings,
and increased patient handling resulting in more chances for contamination of surgical
sites\(^1\).

While elective orthopedic surgeries are generally considered clean procedures, with
an expected risk of infection around 5%, certain of these procedures (most notably the
tibial plateau leveling osteotomy in canine patients) appear to have a higher than expected
incidence of infection\(^{28-30}\). Additionally, surgical site infections can be complicated by the
presence of implants, which can to provide a safe haven for bacteria (often in the form of
biofilms\(^{29,31,32}\)), resulting in recurrence of infection following cessation of antimicrobial
treatment. Implants can also provide a location for bacteria to lodge and develop infection,
sometimes significant periods post-operatively. The source of these infections may be
long-delayed recrudescence of an indolent infection, or may represent \textit{de novo} infections
developed from hematogenous seeding of bacteria onto the implant\(^{33}\).

Increased interest in the prevention and treatment of SSIs in veterinary medicine is
stemming from increased prevalence of multi-drug resistance bacterial infections\(^{34-37}\).
Additionally there is increasing risk of development of SSIs in a patient population that is
increasingly affected by other co-morbidities, and with the provision of increasingly
complex surgical procedures\(^8\). Realization of the potential for a ‘post-antibiotic era’\(^{38}\) in the
face of rapid emergence and dissemination of highly resistant organisms is edging towards
reality in human medicine\(^{39}\), and will follow in veterinary medicine.

Implant-associated SSIs are of special interest due to the increased frequency of
surgically implanted medical devices such as bone plates and total joint prosthesis\(^{40}\), and
the potentially devastating complications of deep SSIs in those cases\(^{41}\). The ongoing use of
routine intravenous catheters\(^{42}\) and a myriad of other permanent and semi-permanent
implants (e.g. vascular access ports, central catheters, stents, pacemakers)\textsuperscript{43,44} in human and veterinary medicine, and the importance of biofilms in infections of, and sourced from those implants provides additional impetus for research in this field.

\textbf{Staphylococcus pseudintermedius}

\textit{Staphylococcus} spp. are ubiquitous in veterinary patients, both as part of the commensal microbiota of many animals, and as opportunistic pathogens\textsuperscript{45}. They are of particular concern with respect to SSI because of frequently identified virulence factors, most notably the ability to form biofilm. They are also frequently present as commensal organisms giving them opportunity to cause infection, and are well adapted to developing and transferring resistance to many commonly used antimicrobials.

\textbf{General biological aspects}

As mentioned, staphylococcal infection are relatively common in veterinary medicine, and for the first half of the 20\textsuperscript{th} century, most were classified as belonging to the species \textit{Staphylococcus aureus}. In 1970, several other species, including \textit{S. intermedius} were distinctly identified from a number of veterinary infections\textsuperscript{46}. More recently, \textit{S. pseudintermedius} was identified as a distinct species from \textit{S. intermedius}, and has since been realized as the predominant species present in canine and feline commensal populations and infections\textsuperscript{47}. It has also been identified as a human and zoonotic pathogen\textsuperscript{48,49}.

\textbf{Methicillin resistance in \textit{Staphylococcus pseudintermedius}}

While extensive use of antimicrobials has significantly reduced morbidity and mortality due to these pathogens, is has also resulted in the development and spread of antimicrobial resistance in this species\textsuperscript{34,50}. Methicillin resistance is used as an indicator as
in human staphylococci as an indicator of multi-drug resistance, and methicillin resistant *S. pseudintermedius* (MRSP) is now a major cause of external (e.g. ear, skin) and surgical site infections in dogs. MRSP infections are subsequently of increasing concern, as their frequent resistance to many antimicrobials significantly limits treatment options. This increasing antimicrobial resistance is likely a major factor in the emergence of this pathogen.

**Sequence type distributions**

*Staphylococcus pseudintermedius* is present worldwide, though there seem to be some regional variation in the specific strains present. The first major study examined strains isolated from North America and Europe, and while thirteen different sequence types (STs) were identified, ST71 and ST68 were the most frequent, representing 56 and 13% of the samples respectively, with ST71 predominating in Europe and ST68 in North America; other studies have found similar results. Interestingly, skin infections resulted from a broad range of types, whereas all surgical site infections were appreciated as ST71; though this may be an observational anomaly as opposed to a true difference in pathogenicity. Studies have shown other regional variations, as well as confirming the presence of specific canine strains in human infections.

**Staphylococcal chromosomal cassette carrying methicillin resistance**

The staphylococcal chromosomal cassette containing the methicillin resistance gene *mecA (SCCmec)* has also been examined in *S. pseudintermedius* because of its association with multidrug resistance and the emergence of MRSP. The cassette contains the methicillin resistance gene itself, as well as other associated genes responsible for movement of the cassette, and regulation of those activities. The cassette is ubiquitous.
across species of *Staphylococcus*, and many types are reported (at least eleven major types at present)\textsuperscript{67,68}. Similar variation in geographic distribution for SCCmec types as for sequence types have been reported for *S. pseudintermedius*. The SCCmec chromosomal cassette also contains a cluster of repeated 40bp sequences called direct repeat units (dru); analysis of the specific sequence of these clusters (yielding a dru type) is increasingly used as a method to distinguish between otherwise phenotypically identical MRSP strains. Since the sequences are present in the SCCmec cassette, this type of analysis cannot be performed on methicillin susceptible *S. pseudintermedius* (MSSP) strains\textsuperscript{69}, however as the prevalence of multidrug and methicillin resistant *S. pseudintermedius* is increasing, research has been focused more on those strains. Dru types have shown applicability in differentiating between members within a sequence type, enabling more specific tracking of strains during outbreaks\textsuperscript{70,71}. This typing method is being used with increasing frequency in the analysis of MRSP\textsuperscript{72,73}.

**Staphylococcal biofilms**

**Definition and overview of biofilms**

The most common concept of bacteria is the planktonic form; individual bacteria either on a surface or in a fluid. Biofilms are much more complex; they are often polymicrobial aggregations of bacteria encased within a complex polymeric extracellular matrix (ECM) composed of mixtures of polysaccharide, protein and nucleic acids produced by the organisms in the biofilm\textsuperscript{31,74}. Furthermore, the bacteria are thought to be in an altered metabolic state allowing for the production and maintenance of the biofilm, and are generally resistance to antimicrobial therapy\textsuperscript{31}. There also appear to be some aspects of intracellular communication between organisms in the biofilm; coordinating production
and maturation of the biofilm\textsuperscript{75}, regulating expression of virulence factors\textsuperscript{76,77}, and triggering release of encased organisms back to the planktonic state\textsuperscript{55,78}.

Biofilm formation is generally discussed in several stages but in reality, there is likely significant overlap between them, and all processes are likely occurring simultaneously, depending on the external influences on the biofilm. Generally, there must be an initial adhesion to a surface, followed by production of ECM and maturation of the biofilm, then release of planktonic organisms. Increasingly there is complex interplay appreciated between these stages, the organisms present, and the effects of the extracellular environment\textsuperscript{66,79}, however traditionally, these processes have been discussed individually as the adhesion or attachment, maturation and detachment phases (Figure \textbf{1.1})\textsuperscript{55}.

\begin{center}
\includegraphics[width=\textwidth]{biofilm_diagram.png}
\end{center}

Figure 1.1: Traditional growth progression of staphylococcal biofilms. Adapted from Otto et al. (2013)\textsuperscript{55}. 

10
Understanding of the triggers that incite biofilm formation is developing but is still poorly understood, and is likely multifactorial. The known cues are consistent with biofilm being a protective mechanisms for the bacteria; reduced nutrient availability, including specifically limitations in iron and oxygen trigger biofilm initiation. Understanding these triggers, and the resultant metabolic pathways may provide therapeutic targets for biofilm treatment.

The biofilm state is a broad classification, and encompasses organisms in the early stages of adherence, through to mature polymicrobial communities in thick extracellular polymeric matrices. Biofilms display temporal variability, and their composition and character changes over time in response to external triggers, and signaling within the biofilm. This variability has been documented in the external physical nature of the biofilm, progressing from a thin monolayer of bacteria through to a complex three-dimensional structure. It is likely that there is significant concurrent variation in gene expression. There has also been microstructure documented in biofilms, with small channels, and regional distribution of specific components of the biofilm; similarly localized variation in gene expression within the embedded bacteria has been identified.

The literature with regards to *S. pseudintermedius* biofilms specifically is limited; there are less than a dozen papers examining that specific topic. There is a significantly broader knowledge base available for *Staphylococcus* generally, most of it relates to methicillin susceptible and resistant *S. aureus* (MSSA and MRSA) and *S. epidermidis*, likely owing to its major role as a human pathogen. Much of this information likely carries over to MRSP and MSSP, however there are numerous documented differences in mechanisms.
and behavior of biofilms not only between staphylococcal species but even between strains of a single species, so direct application of information from methicillin resistant and susceptible S. aureus to correlated questions in MRSP and MSSP must be done with care.

**General concepts of bacterial adherence**

Adherence of bacteria to surface is vital as the initiating event in biofilm formation. There are numerous factors known to affect adherence, ranging from aspects of the surface, growth conditions and specifics of the bacterial strain. It is a significantly complex process starting with initial non-specific interactions\(^\text{86}\), moving progressively and concurrently through stages facilitated by participation of specific adhesion molecules\(^\text{87}\) and development of a complex extracellular matrix resulting in a comparatively robust attachment\(^\text{88}\).

**Adhesion**

*Surface characteristics modulating adherence*

The mechanism of initial adherence of bacteria forming biofilms is dependent on the nature of the surface. Inherent characteristics such as the homogeneity and roughness of the surface, the material and inherent surface energy, microscale variations in composition (especially in alloys), inherent charge on the surface, and the presence of any adsorbed molecules will all significantly affect the interactions of bacteria with those surfaces\(^\text{32,89,90}\). There is also evidence that the environment in which adherence is occurring, or pre-treatment of the surface may also modify adhesion interaction of bacteria\(^\text{91}\). *In vitro* experiments have demonstrated significant variation in the amount of biofilm formed on various surfaces depending on pre-treatment with serum; non-specific binding of proteins in the serum to the surface alters the available attachment sites for bacteria\(^\text{92}\). Additionally
human plasma has been demonstrated to increase expression of proteins mediating adherence, and the presence of plasma also appears to result in biofilms with increased antimicrobial resistance\textsuperscript{93}. These factors are both important clinically; the exact inflammatory milieu present at a surgical site may significantly affect bacterial adherence, and modification of that environment at the time of implantation may provide a way to reduce biofilm formation.

With regards to \textit{in vitro} studies, experimental conditions must be carefully planned, as there is strong evidence that essentially any experimental condition (culture medium, growth surface) can significantly affect the amount, and likely also the specific characteristics of the biofilm that forms\textsuperscript{92,94}. Even physical characteristics of growth conditions are important to consider; stirred versus unstirred conditions are likely important as well, as experiments examining biofilm morphology under variable shear conditions during growth show that those variables are also important in modulating biofilm formation\textsuperscript{90,95}.

There may also be ‘conditioning’ of the bacteria that occurs with growth. It is already known that multiple passages of bacteria through culture media can affect expression of virulence factors\textsuperscript{96}. Passage through biofilm states also seems to increase the fitness of a given organism for growth as biofilm, and reduce its fitness for planktonic growth. The corollary also appears true, planktonic organisms may have a reduced ability to form biofilm compared to samples more immediately isolated from biofilms\textsuperscript{97}.

\textit{Non-specific adhesion interactions}

The initial event involving the bacteria in the formation of a biofilm is adherence of planktonic organisms to a surface, either biological (as in a wound) \textsuperscript{91,98} or synthetic (as in
biofilms on implants)\textsuperscript{99}. The initial adhesion is reversible and, electrostatic, hydrophobic and ionic forces may all play a role\textsuperscript{86,100}. Many of these interactions will be significantly altered by the environment in which those interactions are occurring. pH changes will result in changes in protein conformation and charge, altering their subsequent interactions with bacterial surface proteins, as well as interactions of proteins free in solution with the surface\textsuperscript{101}. Ionic species present in solution are also likely to affect those interactions; high ionic strength solutions can disrupt ionic and hydrogen-bonding interactions, and some specific ions are vital for certain protein-protein bonds (e.g. zinc).

Specific bacterial components mediating adherence

There are also numerous adhesins that can be produced by bacteria. Some of these mediate these early, reversible attachments, while other are much more specific, and produce a stronger bond. Collectively, these molecules are the molecular surface components recognizing adhesive matrix molecules (MSCRAMMs)\textsuperscript{87}. These are receptor proteins covalently attached to the bacterial cell surface (in \textit{Staphylococcus}, the peptidoglycan cell wall) that recognize and bind to specific extracellular moieties\textsuperscript{105}. Numerous MSCRAMMs have been reported in various staphylococcal species, ranging from non-specific autolysins (\textit{atl}) that serve a dual function in adhesion as well as degradation of cell wall during replication\textsuperscript{106,107}, and molecules with specific targets such as fibrinogen\textsuperscript{108}, fibronectin\textsuperscript{109}, collagen\textsuperscript{87} and elastin\textsuperscript{105}. There are numerous MSCRAMMs reported in \textit{Staphylococcus}; Table 1.3 shows both the genes examined in this study, as well as other genes reported in \textit{Staphylococcus} spp. generally. Naming of the MCRAMMs can be deceiving, often structural or sequence homologues will be named differently when
independently identified in multiple species, and many MSCRAMMs are named for a single ligand, when they bind multiple ligands in vivo.

<table>
<thead>
<tr>
<th>Gene or gene family</th>
<th>Abbreviation</th>
<th>Ligand or function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bifunctional autolysin</td>
<td><em>atlA, atlE</em></td>
<td>Adhesive (vitronectin) and peptidoglycan hydrolase</td>
</tr>
<tr>
<td>Elastin binding protein</td>
<td><em>ebpS</em></td>
<td>Elastin</td>
</tr>
<tr>
<td>Fibronectin binding protein</td>
<td><em>finbA, finbB</em></td>
<td>Fibronectin</td>
</tr>
<tr>
<td>Fibrinogen binding protein</td>
<td><em>fib</em></td>
<td>Fibrinogen</td>
</tr>
<tr>
<td>Fibrinogen and fibronectin binding protein</td>
<td><em>fbe, spsE</em> (in <em>S. pseudintermedius</em>)</td>
<td>Fibronectin and fibrinogen</td>
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<td>Extracellular fibrinogen binding protein</td>
<td><em>ecb</em></td>
<td>Fibrinogen</td>
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<tr>
<td>Collagen binding protein, or collagen adhesion protein</td>
<td><em>cna</em></td>
<td>Collagen</td>
</tr>
<tr>
<td>Clumping factors A and B</td>
<td><em>clfA, clfB</em></td>
<td>Complement regulator protein</td>
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<td><em>bbp family</em></td>
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<td><em>sdr family</em></td>
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<td><em>eno</em></td>
<td>Laminins</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> surface protein</td>
<td><em>sasG</em></td>
<td>Squamous epithelium</td>
</tr>
<tr>
<td>ECM binding protein</td>
<td><em>Embp</em></td>
<td>Fibronectin</td>
</tr>
</tbody>
</table>

Table 1.3 Known MSCRAMMs in *Staphylococcus*, genes in bold have been identified in *S. pseudintermedius*.

Maturation of biofilms and the extracellular matrix

The next classical phase of biofilm development is maturation. This stage primarily relates to the production of the extracellular matrix, but also includes changes in the metabolism of the bacteria as they become embedded in that matrix. This stage can overlap with the adherence phase, in that the components of the ECM help the biofilm adhere to the underlying surface. There is also significant overlap with the subsequent detachment phase, primarily in terms of regulation, which will be discussed later.

The extracellular matrix is produced by the bacteria forming the biofilm, part of the definition of a biofilm. It is a complex mixture of polysaccharides, often with significant
proteinaceous and nucleic acid components\textsuperscript{88,113}. While much of this material is secreted by the cells forming the biofilm, there is also a portion of the mixture resulting from autolysis of bacterial cells forming the biofilm\textsuperscript{113}. The proportions of each component forming a biofilm may also change with other genes present in the organism; MRSA biofilms have been documented as containing significantly more extracellular DNA (eDNA) and protein that those formed by MSSA\textsuperscript{114}. Some Staphylococcal biofilms are almost entirely proteinaceous, despite the formative organisms containing the genes necessary for formation of the most common extracellular polysaccharide matrix polysaccharide intracellular adhesin (PIA)\textsuperscript{115}. The physical structure of the biofilm and the ECM has been shown to be significantly variable, and there is evidence of significant spatial variation in gene expression, phenotype, ECM makeup and concentrations of nutrients and waste products within biofilms\textsuperscript{116}. Multiple factors including shear forces during formation, as well as nutrient limitation has resulted in documented changes in the gross appearance of the biofilm, ranging from relatively flat plaques adherent to the surface through to mushroom like growths with significant three dimensional structure\textsuperscript{81,88}. There is also evidence of microstructure within the biofilm. The presence of small channels within the matrix, as well as small cystic structures, and focal areas of bacteria in the planktonic form has been documented in various biofilm samples. The role of these microstructures is not fully elucidated. They likely allow communication within sections of the biofilm, as well as between the ‘core’ of the biofilm and the external environment. Focal areas of planktonic organisms may represent portions of the biofilm approaching detachment\textsuperscript{89,117}. It has also been hypothesized that the ECM structure observed experimentally may simply be a remnant after bacterial exodus.\textsuperscript{118}
The most heavily examined component of ECM in Staphylococcus is PIA, which is produced through the combined actions of the genes in the icaADCB operon. There are also ica independent biofilm formation mechanisms.

The nucleic acids are the least examined components of the ECM; it is likely that this eDNA has multiple roles as a structural component, an aid to initial adherence to surfaces, and as a mechanisms of intracellular information transfer.

In addition to holding the bacteria onto the surface, the matrix also helps to prevent phagocytosis of the embedded bacteria and provides a physical barrier to diffusion or harmful molecules (such as antimicrobials) and nutrients into the biofilm. There is however some evidence that the biofilm ECM does not always function as a non-specific diffusion barrier. Studies have documented no diffusion impairment to certain antibiotics to which the biofilms under examination still show increased resistance. These disparities in observations may relate to the specific growth conditions, ultrastructural characteristics (such as microchannels) and the exact composition of the ECM. For example, MSSA biofilms generally are rich in PIA, while MRSA biofilms often are much richer in protein. Diffusion of antimicrobials into a biofilm depends on the specific antibiotic examined, and likely also depends on the type of biofilm ECM present.

Maturation in terms of gene expression has been proposed in multiple studies, especially as a mechanism of increased antimicrobial resistance. Maturation resulting in spatial variation in gene expression is also well reported. Generally there is thought to be an overall downregulation of metabolic activity in biofilm embedded organisms, there have also been identified increases in antimicrobial resistance genes and other virulence
factors associated with biofilm state transformation\textsuperscript{127,128}. There are however only rare reports of alteration of gene expression in biofilms of \textit{S. pseudintermedius}\textsuperscript{129}.

**Detachment of bacteria from biofilms**

The last traditional stage of biofilm development is detachment, where bacteria embedded from the biofilm are detached, either as planktonic organisms, or as small clusters of bacteria still associated with ECM, allowing them to move to other locations and set up new infections or biofilms\textsuperscript{79}.

Increasing research in the examination of structural aspect of staphylococcal biofilms is identifying progressive complexity in all stages of growth. The overlap between maturation and detachment is becoming blurred, as intermediate stages of biofilms microstructure leading to release of planktonic bacteria are identified\textsuperscript{118}. Detachment ultimately results from progressive maturation of a biofilm resulting in formation of pockets of planktonic bacteria that are subsequently released to the environment, or progressive changes in the ECM resulting in portions of the biofilm from which planktonic bacteria are subsequently released. More research has examined the role of the accessory gene regulator (\textit{agr}) system as a major controller of these stages\textsuperscript{100,130,131}. More recent studies are identifying focal areas of differentiated structure and gene expression leading to bacterial release through \textit{agr} independent growth stages defined as ‘multiplication’ and ‘exodus’, indicating that there are likely multiple mechanisms that effect detachment\textsuperscript{118}.

**Regulatory genes in staphylococcal biofilms**

Overriding all of these stages and important in all aspect of biofilm formation from initial adhesion through to detachment is the control of these changes. While traditionally bacteria exist as single, independent organisms, there is significant evidence of
communication and differentiation of cells within bacterial biofilms, and ultimately coordinated behavior of those bacteria in response to their surroundings in a fashion potentially greater than just direct individual response to external stimulus\textsuperscript{55}. The systems that help coordinate and modify behavior of organism in a biofilm are generally referred to as quorum sensing systems, even though research is progressively indicating roles beyond just the ‘sensing’ of other nearby organisms. These control mechanisms have been implicated in every stage of biofilm development. It is likely that the control of biofilm behavior and intracellular communication is markedly more complex that initially thought, with multiple interplaying regulatory pathways responsible for biofilm behavior\textsuperscript{78}.

There is evidence of direct regulation of some genes by external environmental conditions. There are also several specific quorum sensing genes, including the accessory gene regulator (\textit{agr})\textsuperscript{75}, \textit{lux}\textsuperscript{132}, and staphylococcal accessory regulator (\textit{sar}) operons\textsuperscript{100}. Several of the genes previously described including the \textit{icaADCB}\textsuperscript{133} and \textit{SCCmec}\textsuperscript{66} operons also contain internal regulatory genes. The expression and function of these genes has been somewhat examined in other staphylococcal species, however the interplay of these regulatory genes, and their function if any in \textit{S. pseudintermedius} has yet to be examined.

**Clinical relevance of biofilms**

**Mechanisms of resistance to treatment and the host immune system**

While the mechanisms of the increased resistance to antimicrobials seen in biofilm embedded organisms is still incompletely understood, it has been demonstrated in multiple studies\textsuperscript{74,125}. The use of multiple antimicrobials, and combinations of antimicrobials have been tested against biofilms, as organisms in biofilm form are often highly resistant to therapy. While some medications are effective at ‘regular’ MICs, biofilms are resistant to
some antimicrobials at up to 1000x the MIC\textsuperscript{134}. Some antimicrobials are synergistic; fosfomycin and clarithromycin have showed synergistic effects\textsuperscript{135}, and there has been much examination of combinations including rifampin and daptomycin, owing to their frequent utilization against staphylococcal infections\textsuperscript{136}. Penetration of various antimicrobials into staphylococcal biofilms has also been investigated; diffusion of oxacillin, cefotaxamine and vancomycin was reduced through ECM, whereas amikacin and fluoroquinolones were not\textsuperscript{126}. Methicillin has also been investigated in multiple studies, owing to the relative importance of methicillin resistance in biofilm infections.\textsuperscript{94,114} Certain antibiotics at subinhibitory concentrations have been demonstrated to increase biofilm formation and also to induce upregulation of some biofilm associated genes\textsuperscript{137}. Likely there are multiple characteristics of biofilms and the embedded organisms that afford this increased resistance. Ultimately, it is the resistance to treatment and ability of biofilms to become senescent that is clinically relevant, resulting in recalcitrant and recrudescent infections.

The extracellular matrix likely plays a major role in resistance of biofilms to therapy, and one of the major mechanisms is as a physical barrier. The ECM provides both a diffusion barrier to limit exposure of organisms in the ECM to some antimicrobials, and also to smaller components of host defenses (antibodies, complement). The polymeric structure also provides physical protection from dehydration and physical removal, and the large bulk provided by the ECM frustrates phagocytosis by host cellular defenses\textsuperscript{104}. Other poorly understood components of the ECM including proteins, signaling molecules and nucleic acids which likely play secondary roles\textsuperscript{88}. Protein components may have enzymatic activity to help protect against antimicrobials or other bacteria, may participate in the extracellular digestion of exogenous molecules to provide nutrient to the cells in the
biofilm, or may directly be signaling molecules. Nucleic acids likely also participate in transfer of information such as antimicrobial resistance genes in additional to their structural role\textsuperscript{88}.

Secondly, bacteria embedded in biofilm undergo significant changes in metabolism, generally resulting in reduced cellular activity. Most antimicrobials target cellular machinery that is active during various phases of replication or other cell activity; protein synthesis, DNA replication, cell wall production\textsuperscript{81,138}, so senescent cells would be expected to resistant to these antimicrobials, much in the same way bacteriostatic antimicrobials can antagonize drugs traditionally considered bactericidal. While this theory has been proposed, it remains to be conclusively demonstrated. The stage of bacterial growth described as the viable but non-culturable (VBNC) state has been known for decades, but has only recently been implicated in biofilms\textsuperscript{139}, and the resistance of biofilms to antimicrobial therapy may relate to this metabolic state\textsuperscript{127}. Not surprisingly, VBNC bacteria are garnering increased investigation as a likely cause of the frequent reports of clinically diagnosed SSIs associated with an implant that respond to antimicrobial therapy, but from which no microorganisms can be cultured. Interestingly, exposure to antibiotics may induce transformation of biofilm embedded organisms into the VBNC state (which notably has yet to be documented in \textit{S. pseudintermedius})\textsuperscript{140}. Again, a large component of the inherent resistance of the VBNC state is the overall downregulation of expression and activity of various constitutive enzymatic activities that would otherwise be antimicrobial targets.

Lastly, there is likely a direct effect from the presence and expression of specific antimicrobial resistance genes. While the mechanism by which these genes would confer
resistance is not expected to be any different than in the planktonic state, it is unknown if expression of these genes is changed in biofilm embedded S. pseudintermedius.

**Clinical situations involving biofilms**

Biofilms have been identified in numerous clinical situations, and the clinical relevance of biofilms and biofilm associated infections has likely been previously underestimated. Specifically, biofilms have been identified in numerous locations including on the cornea, in wounds, in cases of osteomyelitis and fractures, implants and catheters. They have been implicated in recrudescent and chronic infections in many of these situations. Further understanding of biofilms in general, and in each of these specific situations is important in understanding the etiology of, and directing therapy of these infections.

The presence of biofilms developed from infections of surgical implants is of particular concern, due to the recalcitrant and recurrent infections that develop in those cases. Implant removal is ultimately required in the majority of cases in order to obtain control over those infections, and prevent recurrence.

**Prevention and treatment of biofilms**

Limited work has been done specifically in S. pseudintermedius with regards to treatment of biofilm associated infections, and disruption of biofilms. Initial studies examined the resistance of biofilms to treatment, and developed the concept of minimum biofilm eradication concentration (MBEC), the minimum concentration of an antimicrobial needed to eradicate an established infection, which is contrasted to the minimum concentrations needed to kill all organisms in the planktonic phase, or prevent any ongoing growth (minimum bactericidal and bacteriostatic concentrations).
Other studies have examined the synergistic effects of various antimicrobials in the treatment and eradication of biofilm infections. Fosfomycin has shown promise as a comparatively effective antimicrobial in the treatment of biofilms\textsuperscript{135}. Additionally, there has been interest in identifying and utilizing other methods to disrupt biofilms and expose the embedded organisms in order to eliminate biofilm associated infections. One specific molecule of interest is DispersinB\textsuperscript{145}, an enzyme produced by \textit{Aggregatibacter actinomycetemcomitans} that breaks down PIA, one of the major polysaccharide component of the ECM. The enzyme appears to be helpful in removal of MRSP biofilms; its use significantly increased yield of biofilm when used to harvest biofilms grown \textit{in vitro}\textsuperscript{129}.

Modification of surfaces in an attempt to prevent biofilm formation has been examined in other veterinary pathogens; chlorhexidine\textsuperscript{43} and silver (personal communication, A. Ogilvie 2014) have both been examined as coating on urinary catheters to prevent biofilm formation and subsequent urinary tract infection with variable success. Otherwise there is a significant paucity of research with respect to veterinary applications.

Generally, research towards the prevention of biofilms has been ongoing the longest in the prevention biofilms in industrial settings; biofilms are important problems in many industrial settings (paper production, food processing), in the marine transport industry, and numerous other situation such as heat exchange and cooling piping in power plants and other industries\textsuperscript{146}. Bio-fouling, which in many situation initiates as the formation of bacterial biofilms, result in flow obstruction and frictional inefficiencies adding billions in extra costs to these industries worldwide. Unfortunately, many of the anti-biofouling coating developed for those industries are unsuitable for use in the medical field, due to the
significant biotoxicity of the coatings that can be used in those settings (such as organotin and heavy metal based compounds)\textsuperscript{147}.

There is however increasing and rapidly accelerating research in the development of biocompatible and sufficiently non-toxic coatings to prevent biofilm formation in clinically relevant situations such as on catheters and surgical implants. There is also significant research ongoing towards developing techniques for the \textit{in vivo} eradication of biofilms, to allow \textit{in situ} treatment of biofilm affected and infected implants, eliminating the cost and morbidity associated with removal or replacement. Pubmed searches for “implant biofilm prevention” and “implant biofilm treatment” respectively garner 160 and 420 results, with over 85\% of the resulting articles published in the last decade.

In terms of preventing colonization and formation of biofilms, ongoing research generally focuses on surface modification including modification of physical surface characteristics (polishing, micro and nano scale texturing)\textsuperscript{89}, deposition of a surface coating that prevents adhesion\textsuperscript{148}, or coating of the surface with antimicrobial or anti-adhesive molecules that may also elute into the peri-implant region\textsuperscript{43,149}.

Implant material was one of the first factors affecting implant infection to be examined. Titanium and stainless steel are the most commonly used materials for the internal fixation of fractures. It has been documented that titanium has higher biocompatibility than stainless steel\textsuperscript{150}; soft tissues tend to adhere to titanium implants, while a fibrous capsule and small amounts of fluid accumulate around stainless steel. It is thought that these characteristics mean stainless steel implants are more prone to developing infections, a finding that has been confirmed in clinical studies\textsuperscript{151}. Subsequently, a myriad other surface types and modifications have come under study.
The rationale for attacking biofilms even before they form is encapsulated by the concept of the ‘race for the surface’\textsuperscript{152}. Any surface placed in a biological situation will become coated with protein, nucleic acid, host cells and bacterial cells (if they are present). If bacterial adhesion can be prevented or delayed, it provides the chance for antimicrobials, the host immune system, or a combination of the two to eliminate those organisms before they form resistant biofilms\textsuperscript{90}. It also allows time for the host tissues to adhere to the implants, which can further prevent infection. Thus, the overall trend of research it to improve biocompatibility of implants, while limiting bacterial adhesion; an ideal surface will provide both of these functions, and will have additional physical benefits in terms of appropriate strength and wear resistance for good function as an implant (Figure 1.2). To that end, a wide array of surface treatments has been examined, approaching the problem from a variety of directions, many of which overlap with proposed mechanisms for biofilm treatment (Table 1.4). Despite the difficulty in treating developed biofilm based infections, there is still significant ongoing research towards their treatment and disruption, owing to their persistent prevalence and occurrence in both veterinary and human medicine. Again, a wide variety of approached have been taken to this problem; unfortunately a single ideal solution is still elusive (Table 1.4).
Figure 1.2: Ideal implant surface character combinations, from Gallo et al. (2014)\textsuperscript{153}.
<table>
<thead>
<tr>
<th>Category</th>
<th>Subcategory</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prevention of</td>
<td>Chemical surface modification</td>
<td>Polymers (e.g. Teflon)</td>
</tr>
<tr>
<td>adhesion or</td>
<td></td>
<td>Super-hydrophobic surfaces</td>
</tr>
<tr>
<td>absorption</td>
<td>Physical modification</td>
<td>Nano-patterned surfaces</td>
</tr>
<tr>
<td></td>
<td>Biological modification</td>
<td>Pre-absorption (albumin, cationic peptides)</td>
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<tr>
<td></td>
<td></td>
<td>Pre-colonization with non-pathogenic organisms</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bacterial signaling (quorum quenching)</td>
</tr>
<tr>
<td>Bactericidal or</td>
<td>Organic</td>
<td>Antibiotics</td>
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<td>anti-biofilm</td>
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<td>Chitosan and derivatives</td>
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<tr>
<td>action</td>
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<td>Antimicrobial peptides</td>
</tr>
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<td></td>
<td></td>
<td>Immune signaling (interleukins)</td>
</tr>
<tr>
<td></td>
<td>Inorganic</td>
<td>Silver</td>
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<tr>
<td></td>
<td></td>
<td>Titanium oxides</td>
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<tr>
<td></td>
<td></td>
<td>Exotic alloys</td>
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<tr>
<td></td>
<td></td>
<td>Other bactericidal ions (copper, zinc, selenium)</td>
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<tr>
<td></td>
<td></td>
<td>Photoactivated antimicrobial agents</td>
</tr>
<tr>
<td></td>
<td>Biological</td>
<td>ECM disruption (DispersinB, lysostaphin, proteinase K)</td>
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<td></td>
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<td>Bacteriophage</td>
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<td>Anti-staphylococcal vaccination</td>
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<td>Host immunomodulation</td>
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<tr>
<td></td>
<td>Physical</td>
<td>Induced electrical current</td>
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<tr>
<td></td>
<td></td>
<td>Shockwave</td>
</tr>
<tr>
<td></td>
<td>Other</td>
<td>Nanocontainers linked to sensors</td>
</tr>
</tbody>
</table>

Table 1.4: Summary of research into biofilm prevention and disruption technology\textsuperscript{153-156}.

**Clinically relevant growth conditions**

Limited previous research has shown variation in biofilm properties dependent on growth conditions, including surface type, culture media and numerous other specific culture conditions\textsuperscript{92}.

In terms of clinical research, materials used for orthopedic implants are of primary importance, as infection of these implants most often requires removal of the implants, which can be a devastating consequence in some situations such as total joint replacement.
surgeries. The primary materials used are stainless steel (most commonly the alloy 316L), and titanium. Secondarily, polymethylmethacrylate (PMMA) is used to cement many types of implants in place. Increasingly, catheter materials, and polymeric materials in general, are also becoming of increased interest. Increased use of peripheral catheters, as well as advanced vascular access catheters for procedures such as hemodialysis are resulting in prolonged catheter placement, and increased infection risk. Further, increasing prevalence of permanent implants that rely heavily on silicone type polymers including ureteral and urethral stents and bypass implants, as well as implants to allow long term, minimally invasive access to the thoracic cavity is increasing the risk that infections, specifically biofilm based infections will start to play an important role in these situation. There are well known risks of infection with certain catheter types and situations, most notable urinary catheters; biofilms have been identified in these situations, and understanding those biofilms may help prevent their occurrence.

Certain types of suture material have been definitively identified as an infection risk, and biofilms have been identified of samples of suture from clinical cases. The presence of foreign material generally is also known to be a risk factor for development of a surgical site infection. While the exact mechanisms for these observations is unknown, it is likely that biofilm formation on suture surfaces is at least a contributing biofactor. Biofilm research on *S. pseudintermedius* has thus far substantively only been performed *in vitro*, with most experimentation having been performed on polystyrene and glass surfaces. Since quantitative biofilm formation has already been shown to vary by surface, and recent studies are confirming that surface type can significantly affect biofilm response to antimicrobials, it is likely that other aspect of biofilm character as also affected by
surface, so understanding the specifics of biofilm on the surfaces where most research has thus been performed is also warranted.

The dependence of biofilm properties on growth conditions has important implications for clinical applications of *in vitro* research. While some work has tried to examine biofilms under more clinically relevant conditions, experimental conditions are still likely different from clinical situations. Composition of growth media used *in vitro* are significantly different from those around an implant or catheter. Situations where biofilms form on vascular implants, or on heart valves, result in the biofilms forming under significant shear stress compared to the static conditions under which most *in vitro* testing is performed. Similarly, the ‘growth medium’ for clinical biofilms formed in urine or serum bathing an implant will be markedly different than the semi synthetic growth media used in bench top research. The impact of these factors with respect to the characteristics of the biofilms that result is unknown, so any *in vitro* experiment should try to approximate *in vivo* conditions as closely as possible to help ensure applicability of experimental results to clinical situations. Comparisons of *in vivo* biofilms to those formed under experimental conditions may help determine what factors are most important, and help guide development of applicable *in vitro* models.

**Gene expression analysis**

**Methods of quantification and quantitative polymerase chain reaction**

Expression of various genes can be measured in a numerous ways; each with various advantages and downsides. Direct measurement of protein transcripts can be performed relatively easily, and the various methods available can be extremely sensitive and specific. Western blot analysis, MALDI-TOF MS measurement, ELISA assays and many
other tests are valuable, but each have significant limitations in the amount of sample required for reasonable analysis, or in successful quantification of small amounts.

Examination of the DNA encoding the targets of interest can be performed, however for most chromosomal targets, variation in expression is effected by altering transcription levels of a single gene, so there would be no change in the DNA during changes in expression.

Persistent desires to sensitively examine variation in gene expression in small samples have led to the development of new techniques. Instead of directly analyzing proteins, transcripts produced from a gene can now be quantified. The central dogma of molecular biology describes the primary flow of information from DNA to RNA to protein, and while there can be post-transcription alteration in translation levels (more often in eukaryotic organisms), generally it is held that the amount of RNA produced in a prokaryotic organisms such as MRSP can be used as a proxy for protein expression.

Various molecular biology techniques allow for the detection and quantification of small amounts of nucleic acid. The polymerase chain reaction (PCR) is the mainstay of these techniques, allowing exponential duplication of DNA; this technique however cannot be used on RNA samples due to the lack of RNA dependent RNA polymerase, or mixed function RNA/DNA dependent DNA polymerases. Reverse transcription however allows DNA to be produced from the RNA isolated from any specimen of interest; then the polymerase chain reaction is used to amplify the DNA to a level where it can be accurately quantified. In the traditional sense, this method could be used to confirm the expression or lack of expression of a gene, but is limited in providing useful quantification.
Quantitative PCR (qPCR) was developed as a way to quantify RNA and DNA amounts. For RNA, a reverse transcription step must be performed first to yield complementary DNA (cDNA). Subsequently, PCR is performed on the sample, duplicating the DNA present with each cycle. A fluorescent dye is added to the reaction, that fluoresces only when bound to double stranded DNA. The fluorescent signal is measured after each cycle, and the number of cycles required to reach a measurement threshold of fluorescence is then directly related to the amount of DNA or RNA present initially. These methods can be extremely sensitive and specific, and have supplanted most other methods of gene expression analysis\textsuperscript{161,162}. There are however multiple steps in the analysis that can result in significant error if not properly performed; guidelines have been produced to help standardize qPCR methodology, and ensure reporting of only appropriately performed experiments\textsuperscript{163,164}. Research has been performed to identify at which step variability is introduced during qPCR analysis of biofilms, and shows that variability between harvests of biofilm is the most critical step to perform multiple times to ensure representative results are obtained.\textsuperscript{165}

PCR and qPCR do not operate in an exact world however, and while mechanistically the amount of DNA should double with each amplification step, this is not always true, especially when reaction conditions are determined to allow overall best function of the PCR reactions for multiple genes. Thus, preliminary testing is required to determine the PCR efficiency of any genes to be use (how much does the DNA amplify with each cycle).

**Reference genes**

qPCR is not a direct quantification technique. Since it is extremely difficult to ensure complete transfer of material between each step in the process, and to require that the
same amount of material is used for each quantification, qPCR uses a relative quantification. This significantly simplifies many experimental aspects; however results in a reliance on adequate reference genes. Reference genes (previously referred to as ‘housekeeping genes’; this terminology is outdated and avoided\textsuperscript{163}) are genes for which expression does not change over the range of conditions to be examined (constitutive expression). This allows expression of genes of interest to be measured relative to those reference genes. For any experiment, measurement of the expression levels of the genes of interest is performed in addition to the reference genes identified. Taking into account the PCR efficiency of all of the genes, and relating to the reference genes, a relative expression level can be determined for each gene of interest. Unfortunately, many early experiments assumed constitutive expression of potential reference genes, resulting in significant error. More recent work has underlined the need to confirm the constitutive expression of reference genes, and has recommended that multiple reference genes be used\textsuperscript{163,164}.

For qPCR experiments, both reference and target genes must be identified and validated. Such genes were previously identified in other \textit{Staphylococcus} spp. as previously discussed, and many homologues were identifiable in \textit{S. pseudintermedius}\textsuperscript{129}. The presence of those genes must still be confirmed in any strains to be studied, and the specific sequence of those genes confirmed. Furthermore, primers have to be developed that target those genes, and the molecular behaviour of the primers and genes confirmed: specificity of the primers, optimum PCR amplification conditions and PCR efficiency all have to be measured\textsuperscript{163}.
Minimum information for quantitative polymerase chain reaction experiments

As mentioned, qPCR is a complex procedure, with multiple steps and requirements that must be observed in order to produce reliable, quality data. The most important factors to be observed have been discussed already, there are myriad other details that must be considered in performing the experiments, and reported in order to allow others to repeat and evaluate any qPCR experiment. To help promote the performance of quality qPCR experiments, and adequate reporting of results, guidelines have been produced and widely accepted that detail the requirements for qPCR performance and reporting; the minimum information for reporting of qPCR experiments (MIQE) guidelines\textsuperscript{163,164}. These guidelines cover the spectrum of information for experiments, ranging from all aspect of methodology through to data analysis, and are separated into essential and desired data across a range of categories (to allow reproduction of results). Examples of some of the information considered essential and desired is provided in Table 1.5.
Infections have been a persistent problem in medicine, and even with the advent of antimicrobial therapy, the problem remains. Biofilms have likely been a persistent component, however their importance has only recently been recognized and appreciated.

The identity and role of several genes responsible for bacterial adhesion, biofilm formation, antimicrobial resistance and gene regulation in biofilms has been examined in
some *Staphylococcus* spp., but evaluation of veterinary specific strains has yet to be reported. Furthermore, gross examination of the quantitative production of biofilm by some *Staphylococcus* spp. has identified significant effects from surface type and culture conditions, but the mechanisms remain cryptic.

Quantitative PCR has emerged as the predominant method to evaluate gene expression in a range of situations and species. The method is extremely sensitive and specific, but requires care in its application. Guidelines have been produced to guide the proper performance and reporting of these experiments, allowing a broader application of this powerful technique.

**Objectives**

The objectives of this study were:

1) Develop and validate a qPCR assay for use in *S. pseudintermedius*, including identification of appropriate reference genes.

2) Identify and validate primers for multiple biofilm associated genes in *S. pseudintermedius*, and measure expression of those genes in the biofilm state relative to planktonic growth phases.

3) Compare expression of multiple biofilm associated genes across multiple clinically relevant surfaces.

4) Compare expression of multiple biofilm associated genes across multiple clinical isolates of *S. pseudintermedius*, on multiple clinically relevant surfaces.
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53. Ruscher, C. *et al.* Prevalence of Methicillin-resistant Staphylococcus


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<td>124</td>
<td>Molin, S. &amp; Tolker-Nielsen, T. Gene transfer occurs with enhanced efficiency in biofilms and induces enhanced stabilisation of the biofilm structure.</td>
<td>Current opinion in biotechnology</td>
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<td>125</td>
<td>Patel, R. Biofilms and Antimicrobial Resistance.</td>
<td>Clinical Orthopaedics and Related Research &amp;NA</td>
<td>41</td>
<td>47</td>
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<tr>
<td>132</td>
<td>Xu, L. et al. Role of the luxS quorum-sensing system in biofilm formation and virulence of Staphylococcus epidermidis.</td>
<td>Infection and Immunity</td>
<td>74</td>
<td>488–496</td>
</tr>
<tr>
<td>137</td>
<td>Wang, Q. et al. Enhancement of Biofilm Formation by Subinhibitory Concentrations</td>
<td></td>
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</tbody>
</table>
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Abstract

Background
Quantitative PCR is rapidly becoming the standard method for analyzing gene expression in a wide variety of biological samples however it can suffer from significant error if stably expressed reference genes are not identified on which to base the analysis. Suitable reference genes for qPCR experiments on *Staphylococcus pseudintermedius* have yet to be identified.

Results
Three reference genes in *S. pseudintermedius* were identified and validated from a set of eight potential genes (*proC*, *gyrB*, *rplD*, *rho*, *rpoA*, *ftsZ*, *recA*, *sodA*). Two strains of *S. pseudintermedius* were used, and primer specificity and efficiency were confirmed and measured. Ranking of the genes with respect to expression stability revealed *gyrB*, *rho* and *recA* as the best reference genes. This combination was used to quantify expression of a single biofilm associated gene, *icaA*, in logarithmic, stationary and biofilm growth phases, revealing that expression was significantly upregulated in the biofilm growth phase in both strains.

Conclusion
Three reference genes, *gyrB*, *rho* and *recA*, were identified and validated for use as reference genes for quantitative PCR experiments in *S. pseudintermedius*. Also, the biofilm associated gene *icaA* was shown to be significantly upregulated in biofilm samples, consistent with its role in biofilm production.

Background

*Staphylococcus pseudintermedius* is a common commensal organism of canines, but is also one of the most common causes of opportunistic infections\textsuperscript{1-3}. 
Recently, methicillin resistant *S. pseudintermedius* (MRSP) has emerged and disseminated internationally\textsuperscript{4,5}, with 2 major sequence types (ST68 and ST71) representing the variety of clinical infections in most regions\textsuperscript{3}. One area of concern is the ability of this bacterium to produce biofilm, something that might be an important virulence factor and complicate elimination of infections\textsuperscript{6,7}. Expression of genes pertaining to initial bacterial surface adherence and intercellular adhesion following biofilm formation, such as microbial surface components recognizing adhesive matrix molecule (MSCRAMMs) which mediate cellular adhesion, and the intracellular adhesion (*icaADBC*) operon\textsuperscript{8}, reported to be at least partially responsible for biofilm formation, likely affect the *in vivo* behavior of this organism, including resistance to therapy\textsuperscript{9,10}. While there is significant postulation regarding these factors, understanding of the expression of antimicrobial resistance and biofilm associated genes in *S. pseudintermedius* and the subsequent clinical implications is still poor.

Quantitative real-time PCR (qPCR) is increasingly employed to quantify gene expression. While it can be very sensitive and specific, there are numerous pitfalls in its application that can easily result in misleading and incorrect conclusions. One of the most frequent errors is a failure to confirm the constitutive expression of the reference genes used to measure the relative expression of genes of interest. Normalization of results in qPCR is vital to limit variability introduced by experimental conditions, sample preparation and analysis, and is one of the main underlying tenets of qPCR analysis. Selection of inappropriate reference genes can result in grossly incorrect conclusions owing to the miscalculation of gene
expression. These and other reasons have prompted the development of minimum information for publication of qPCR experiments (MIQE) guidelines to ensure integrity, consistency and transparency of qPCR experiments, including standards for all aspects of experimental design, analysis and reporting\(^\text{11}\).

Current recommendations suggest a minimum of three reference genes (ideally with M values below 1 for heterogenous samples), and the inclusion of additional genes as necessary to obtain a pairwise variation value < 0.15\(^\text{12}\). This requires specific validation of candidate reference genes in the bacterium (and ideally strains) to be studied, something that is lacking for \textit{S. pseudintermedius}. While multiple qPCR studies have been performed in various \textit{Staphylococcus} spp\(^\text{13,14}\), study and validation of reference genes in \textit{S. pseudintermedius} is lacking, and it cannot be assumed that data from other staphylococci apply to this species.

The objectives of this study were to evaluate several potential reference genes in \textit{S. pseudintermedius}, to identify the optimum gene or gene combinations for future qPCR expression studies and to evaluate expression of ica\textit{A} using validated reference genes.

**Results and Discussion**

**RNA isolation**

The modified protocol afforded good to excellent yields (mean yield 53.5 ± 23.4 µg, range 16.9 – 92.2 µg, concentration 639 ± 94 µg/µL), as well as good RNA purity and integrity (RNA Integrity Number mean 9.1 ± 0.4) (Table 2.1), though there was evidence of some mild contamination from purification reagents (260/230 < 2). qPCR was performed on all RNA preparations to confirm
DNA elimination; quantification cycle numbers were at least 20 cycles lower for RNA samples than equivalent amounts of DNA.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Concentration (ng/µL)</th>
<th>260/280</th>
<th>260/230</th>
<th>Total RNA recovery (µg)</th>
<th>RIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>A42 log A</td>
<td>616.2</td>
<td>2.15</td>
<td>1.09</td>
<td>61.6</td>
<td>9.6</td>
</tr>
<tr>
<td>A42 log B</td>
<td>771.4</td>
<td>2.15</td>
<td>2.27</td>
<td>77.1</td>
<td>n.t.</td>
</tr>
<tr>
<td>A42 log C</td>
<td>805.5</td>
<td>2.15</td>
<td>2.27</td>
<td>80.5</td>
<td>9.0</td>
</tr>
<tr>
<td>A54 log A</td>
<td>823.1</td>
<td>2.14</td>
<td>2.20</td>
<td>82.3</td>
<td>9.4</td>
</tr>
<tr>
<td>A54 log B</td>
<td>919.4</td>
<td>2.13</td>
<td>1.67</td>
<td>91.9</td>
<td>n.t.</td>
</tr>
<tr>
<td>A54 log C</td>
<td>921.6</td>
<td>2.14</td>
<td>2.03</td>
<td>92.2</td>
<td>9.4</td>
</tr>
<tr>
<td>A42 stat A</td>
<td>535.4</td>
<td>2.10</td>
<td>2.00</td>
<td>53.5</td>
<td>9.2</td>
</tr>
<tr>
<td>A42 stat B</td>
<td>449.4</td>
<td>2.02</td>
<td>1.95</td>
<td>44.9</td>
<td>n.t.</td>
</tr>
<tr>
<td>A42 stat C</td>
<td>493.0</td>
<td>2.05</td>
<td>1.66</td>
<td>49.3</td>
<td>8.8</td>
</tr>
<tr>
<td>A54 stat A</td>
<td>403.3</td>
<td>2.05</td>
<td>2.05</td>
<td>40.3</td>
<td>8.5</td>
</tr>
<tr>
<td>A54 stat B</td>
<td>630.1</td>
<td>2.13</td>
<td>1.89</td>
<td>63.0</td>
<td>n.t.</td>
</tr>
<tr>
<td>A54 stat C</td>
<td>397.5</td>
<td>2.05</td>
<td>1.89</td>
<td>39.7</td>
<td>8.8</td>
</tr>
<tr>
<td>A42 BF A</td>
<td>732.4</td>
<td>2.12</td>
<td>2.17</td>
<td>36.6</td>
<td>9.4</td>
</tr>
<tr>
<td>A42 BF B</td>
<td>738.6</td>
<td>2.11</td>
<td>2.18</td>
<td>36.9</td>
<td>n.t.</td>
</tr>
<tr>
<td>A42 BF C</td>
<td>887.3</td>
<td>2.12</td>
<td>2.19</td>
<td>44.4</td>
<td>8.4</td>
</tr>
<tr>
<td>A54 BF A</td>
<td>606.9</td>
<td>2.11</td>
<td>1.97</td>
<td>30.3</td>
<td>9.5</td>
</tr>
<tr>
<td>A54 BF B</td>
<td>337.9</td>
<td>2.06</td>
<td>1.74</td>
<td>16.9</td>
<td>n.t.</td>
</tr>
<tr>
<td>A54 BF C</td>
<td>421.9</td>
<td>2.03</td>
<td>1.99</td>
<td>21.1</td>
<td>8.9</td>
</tr>
</tbody>
</table>

(n.t: not tested, log: logarithmic, stat: stationary, BF: biofilm samples)

Table 2.1 - RNA quality and recovery.

The modified protocol for RNA and DNA extraction, namely the inclusion of lysostaphin (and Dispersin B for biofilm samples) in the initial lysis buffer resulted in substantial and reproducible increases in RNA and DNA yield during harvesting (results not shown). Dispersin B catalyzes the hydrolysis of polysaccharide intercellular adhesin (PIA), a major constituent of the extracellular matrix of *Staphylococcus spp*. Biofilms. Incorporating this enzyme in the solution to recover the biofilm from a surface, and in the lysis buffer is thought to increase recovery by enzymatically degrading the extracellular matrix of biofilm, releasing adherent
bacteria for recovery, and exposing them to the lysis solution. The ability of this simple method to yield an adequate quantity and quality of DNA from biofilm-embedded bacteria was an important finding and will facilitate future studies of gene expression in biofilms.

**Amplification specificity and determination of PCR efficiency of reference genes**

Quantification cycle (Cq) for each reaction was plotted against the log of DNA concentration, and the slopes of the curves were used to calculate the PCR efficiency values, which ranged from 1.79 to 1.87, with excellent regression coefficients (Table 2.2). Despite the range in melting points of the qPCR products (Table 2.2), all primers amplified well under the conditions listed. Melt curve analysis showed a single melt curve for each target gene, and DNA agarose gel electrophoresis revealed a single peak for each product. Sequencing of the qPCR products matched the sequence of the desired target in all cases.

<table>
<thead>
<tr>
<th>Candidate reference genes</th>
<th>Slope of the curve</th>
<th>r² on the slope</th>
<th>PCR efficiency</th>
<th>Melting temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>proC</td>
<td>-3.78</td>
<td>0.997</td>
<td>1.84</td>
<td>80.0</td>
</tr>
<tr>
<td>gyrB</td>
<td>-3.85</td>
<td>0.998</td>
<td>1.82</td>
<td>79.5</td>
</tr>
<tr>
<td>rplD</td>
<td>-3.86</td>
<td>0.998</td>
<td>1.82</td>
<td>75.5</td>
</tr>
<tr>
<td>rho</td>
<td>-3.68</td>
<td>0.997</td>
<td>1.87</td>
<td>81.0</td>
</tr>
<tr>
<td>rpoA</td>
<td>-3.89</td>
<td>0.996</td>
<td>1.81</td>
<td>76.5</td>
</tr>
<tr>
<td>ftsZ</td>
<td>-3.85</td>
<td>0.998</td>
<td>1.82</td>
<td>79.0</td>
</tr>
<tr>
<td>recA</td>
<td>-3.83</td>
<td>0.998</td>
<td>1.82</td>
<td>81.0</td>
</tr>
<tr>
<td>sodA</td>
<td>-3.97</td>
<td>0.996</td>
<td>1.79</td>
<td>79.5</td>
</tr>
</tbody>
</table>

Table 2.2 - Reference and target gene efficiency determination and amplicon
Comparison of qPCR reaction products

Melt curve analyses and gel electrophoresis were also performed on and compared between samples recovered from qPCR reactions performed on cDNA prepared using random hexamers, single gene specific primer and combination gene specific primer reverse transcript reactions; there were no differences in any of the products obtained from those obtained from qPCR or genomic DNA.

Stability assessment and validation of reference genes

Relative expression levels (quantification cycle numbers) were entered into the Microsoft Excel (Microsoft Canada, Mississauga, ON) visual basic application geNorm\textsuperscript{12}, which calculated stability values (M values) for each gene (Table 2.3). GeNorm was also used to calculate normalization factors using combinations of the most stably expressed genes, and calculated the pairwise variation between these factors to identify the optimum number of reference genes to use (Table 2.4). The three genes with the lowest individual M values were used in combination (rho, recA, gyrB) for subsequent expression analysis.

<table>
<thead>
<tr>
<th>Candidate Reference Genes</th>
<th>geNorm M value</th>
</tr>
</thead>
<tbody>
<tr>
<td>proC</td>
<td>0.701</td>
</tr>
<tr>
<td>gyrB</td>
<td>0.604</td>
</tr>
<tr>
<td>rplD</td>
<td>0.769</td>
</tr>
<tr>
<td>rho</td>
<td>0.595</td>
</tr>
<tr>
<td>rpoA</td>
<td>0.663</td>
</tr>
<tr>
<td>ftsZ</td>
<td>0.848</td>
</tr>
<tr>
<td>recA</td>
<td>0.599</td>
</tr>
<tr>
<td>sodA</td>
<td>1.150</td>
</tr>
</tbody>
</table>

Table 2.3 - geNorm gene stability (M) values
Number of genes used & Pairwise variation of normalization factors \\
2 vs 3 & 0.119 \\
3 vs 4 & 0.096 \\
4 vs 5 & 0.082 \\
5 vs 6 & 0.112 \\
6 vs 7 & 0.107 \\
7 vs 8 & 0.135 \\

Table 2.4 – Pairwise variation in normalization factor for combinations of reference genes

While stability of expression of reference genes is important, reference genes ideally also have a high PCR efficiency. The primers we identified had efficiency ranging from 1.79 to 1.84 (with a value of 2.0 representing 100% efficiency), and while the final three genes used for normalization did not have the highest efficiencies, the values are accounted for during normalization of qPCR expression data.

With respect to the identification of reference genes, these results are specifically only applicable to the two strains studied under the three growth phases sampled. However, the two strains that were studied comprised the two main international MRSP clones3, suggesting that these genes will be suitable for broad studies of MRSP gene expression. The excellent stability of expression of these genes over the broad range of conditions studied also indicate that these targets will likely function very well as reference genes over a generally broad range of conditions.
**icaA expression**

Expression of *icaA* was significantly higher in the biofilm compared to logarithmic and stationary phases (p = 0.0093 (A42), p < 0.0001 (A54), (Table 2.1). Individual Tukey’s post-hoc p-values for individual comparisons were for logarithmic vs biofilm p = 0.015 (A42); p = 0.0001 (A54) and for stationary vs biofilm p = 0.015 (A42); p = 0.0002 (A54). There was no difference in expression level between logarithmic and stationary phases for either strain, individual Tukey’s post-hoc p > 0.9999 (A42), p = 0.8258 (A54)

![Graph](image)

Figure 2.1 - Relative expression of *icaA* in logarithmic, stationary and biofilm growth phases
These findings are unsurprising given the role of this gene in formation of polysaccharide intracellular adhesin, but it is noteworthy that such a profound alteration in expression was detectable. Further investigation of expression of this gene under other conditions and on a variety of surfaces, as well as studies of expression of other biofilm associated genes such as MSCRAMMs.

**Conclusion**

Proper development of qPCR assays, including reference gene assessment, is a critical quality control step in the application of this tool. This study has identified a group of *S. pseudintermedius* reference genes, and used those reference genes to demonstrate a significant expression change in a biofilm associated gene. This information provides a vital background for the performance of gene expression studies in this increasingly important veterinary pathogen.

**Methods and Materials**

**Bacterial strains and culture conditions**

Two canine *S. pseudintermedius* strains were chosen, representing the two main international MRSP clones, sequence type (ST) 68 (strain A42) and ST71 (strain A54). These two strains were isolated from clinical infections, and were both previously classified as moderate biofilm formers using a polystyrene plate assay (unpublished data). Isolates were grown in tryptic soy broth supplemented with 1% (w/v) dextrose for all growth conditions. Samples were isolated as single colonies from streak plates prepared from -80°C freezer stocks on Columbia blood agar (Sheep blood), and incubated aerobically in a shaker at 37°C. Logarithmic phase growth was defined in a preliminary study (data not presented) as >1 and <6.
hours of growth, and OD\textsubscript{600} >0.5 and <2.0; stationary phase growth was collected at >12 (12-24) hours, and OD\textsubscript{600} >2.2. For logarithmic and stationary samples, 10\textsuperscript{9} cells were harvested, centrifuged (13,000 x g for 30 seconds) to pellet the cells, then immediately processed to recover RNA as described below. Biofilm samples were produced by incubating glass Erlenmeyer flasks unshaken at 37\textdegree C for >48 (48-54) hours, at which time a visible film was present on the flask surface. The media was removed, and the flasks were washed twice with phosphate buffered saline (PBS, pH 7.4). Biofilm was harvested by incubating the flask with 10 ug/mL Dispersin B (Kane Biotech, Winnipeg, MB) a biofilm degrading enzyme isolated from \textit{Aggregatibacter actinomycetemcomitans}, in PBS, shaking the flask for five minutes at room temperature (the visible film dispersed producing a turbid solution), and then pelleting by centrifugation (13,000 x g for 30 seconds) the cells present in the solution. The pellet of cells was immediately processed to recover RNA as described below.

\textbf{Primer design and determination of PCR efficiency}

Eight candidate reference genes (Table 2.5) were evaluated using the validation software geNorm\textsuperscript{12}. These genes had been examined in \textit{S. epidermidis} and \textit{S. aureus} in previous studies\textsuperscript{13,14}, and analogous sequences were identified in \textit{S. pseudintermedius} for evaluation in this study. Primers were designed using a combination of GeneRunner software version 3.05 (Hasting Software, Inc.) and the National Centre for Biotechnology Information online primer designing tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) using gene sequences for all
<table>
<thead>
<tr>
<th>Candidate Reference Genes</th>
<th>Function</th>
<th>Primer Sequence (5’-3’)</th>
<th>Expected size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>proC</em></td>
<td>Pyrrolidine-5-carboxylate reductase</td>
<td><em>proC</em>-F gcgcgaatacaaatgcgacag&lt;br&gt;<em>proC</em>-R aaaaatgcagggccactttcc</td>
<td>180</td>
</tr>
<tr>
<td><em>gyrB</em></td>
<td>DNA gyrase B subunit</td>
<td><em>gyrB</em>-F gcgtccgttattgaagcc&lt;br&gt;<em>gyrB</em>-R aaccctcaactggcaacctgc</td>
<td>240</td>
</tr>
<tr>
<td><em>rplD</em></td>
<td>50S ribosomal protein L4</td>
<td><em>rplD</em>-F gcctaagaataaggttcg&lt;br&gt;<em>rplD</em>-R cctctcgtgttgtgtgttg</td>
<td>237</td>
</tr>
<tr>
<td><em>rho</em></td>
<td>Transcription termination factor Rho</td>
<td><em>rho</em>-F caggttaaatagttggtaatttg&lt;br&gt;<em>rho</em>-R cctgtctcgtatatcttcttttg</td>
<td>215</td>
</tr>
<tr>
<td><em>rpoA</em></td>
<td>DNA-directed RNA polymerase sigma factor</td>
<td><em>rpoA</em>-F ctatcatcattacagggtgc&lt;br&gt;<em>rpoA</em>-R caaaatttcaacatcaactgtgc</td>
<td>231</td>
</tr>
<tr>
<td><em>ftsZ</em></td>
<td>Cell division protein ftsZ</td>
<td><em>ftsZ</em>-F gtccactcatttcgaaggt&lt;br&gt;<em>ftsZ</em>-R catattgttttaacggtcagc</td>
<td>254</td>
</tr>
<tr>
<td><em>recA</em></td>
<td>Recombinase A</td>
<td><em>recA</em>-F gcctaggtttagatattgataac&lt;br&gt;<em>recA</em>-R ggtgcgaagatgattacgc</td>
<td>228</td>
</tr>
<tr>
<td><em>sodA</em></td>
<td>Superoxide dismutase</td>
<td><em>sodA</em>-F cgacaaacctgacaggtacc&lt;br&gt;<em>sodA</em>-R caacagccagcccaacc</td>
<td>227</td>
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</table>

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Candidate Genes</th>
<th>Function</th>
<th>Primer Sequence (5’-3’)</th>
<th>Expected size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>icaA</em></td>
<td>N-acetylglucosaminyl-transferase</td>
<td><em>icaA</em>-F tggccacacctttgtgccacc&lt;br&gt;<em>icaA</em>-R taggcgtgtaggctgtaggg</td>
<td>178</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.5 - Candidate reference and target genes, primers and amplicon sizes.

Primers for *icaA* were available from a previous study\textsuperscript{15} and were validated for use in qPCR as described for the potential reference gene primers.

Pooled chromosomal DNA from the two strains under investigation (mixture of equal concentrations from each strain) was used to generate dilution series for PCR efficiency calculations and for amplicon melting point assessment. For efficiency determination, a six fold dilution series was prepared in triplicate. Twenty µL reactions containing from 100 ng to 0.1 pg of total DNA were prepared in 96 well plates (Bio-Rad) using the LightCycler 480 SYBR Green I Master qPCR
reaction mixture (Roche Applied Science, Indianapolis, IN), following the manufacturer's instructions. Primer concentration was 0.5 µM for each primer in the final reaction. A Bio-Rad C1000 thermal cycler with a CFX96 Real-Time System and Bio-Rad CFX Manager 2.0 software (Bio-Rad Life Sciences, Mississauga, ON) was used to run the following optimized thermocycling parameters: denaturation at 95 °C for 10 minutes followed by 50 cycles of 10 seconds at 95 °C, 30 seconds at 62 °C, 30 seconds at 72 °C, then a melting curve analysis running from 65 to 95 °C with steps and measurements every 0.5 °C. The thermocycler software calculated quantification cycle (Cq), efficiency and regression coefficients from the recovered data. Amplicon identity and primer specificity was confirmed by sequencing of the PCR products (fluorescent capillary Sanger method, Macrogen, Seoul, Korea), melt curve analysis and gel electrophoresis. qPCR reactions were tested over a range of primer concentrations and annealing temperatures and times to determine optimum reaction conditions.

**DNA isolation**

Cells were collected from a logarithmic phase sample as described above, and processed using the High Pure PCR Template Preparation Kit (Roche Applied Science, Indianapolis, IN) following the manufacturer’s instructions with the exception that lysostaphin (Sigma-Aldrich, Oakville, ON) was added at 0.1 mg/mL in the initial lysis step. Concentration and purity at 260/280 nm was measured using a Nanodrop ND-100 spectrophotometer (Nanodrop Technologies Inc., Wilmington, DE), and the samples were qualitatively examined for shearing by DNA gel electrophoresis.
RNA isolation

Each pellet of cells was resuspended in 2 mL PBS (pH 7.4), centrifuged to a pellet and the supernatant discarded. The pelleted cells were subsequently processed using the Qiagen RNeasy Mini kit (Qiagen, Germantown, MD), following the manufacturer’s instructions, with the following changes. The cells were initially resuspended in tris-EDTA buffer (pH 7.5) containing 15 mg/ml lysozyme (Sigma-Aldrich), 2 mg/mL proteinase K (Sigma-Aldrich), 25 µg/mL Dispersin B (Kane Biotech Inc., Winnipeg, MB) and 0.1 mg/mL lysostaphin (Sigma-Aldrich). All optional steps for additional washes or spins during the procedure were performed. Logarithmic and stationary phase samples were eluted from the purification column with two volumes of 50 µL sterile nuclease free water, biofilm samples were eluted with one volume of 50 µL (volumes chosen so as to obtain final RNA concentrations above 400 ng/µL). All samples were treated with the DNAfree DNase kit (Ambion, Austin, TX) as per the manufacturer’s instructions using a total of 2 units per reaction, added in two aliquots. A Nanodrop ND-100 spectrophotometer (Nanodrop Technologies Inc.) was used to measure RNA concentration and purity at 260/280 nm. Three samples of each RNA sample were measured and the measured concentrations were averaged. Representative samples were submitted for Bioanalyzer analysis (Agilent Technologies, Santa Clara, CA). Real-time PCR was performed using the efficiency determination protocol on RNA samples to confirm the absence of DNA using gyrB primers. RNA samples were stored at -20 to -80 ºC until further use.
Reverse transcription / cDNA preparation

Two µg of total RNA was used in each 20 µL gene specific reverse transcription reaction using the Omniscript RT PCR kit (Qiagen), using 10 µM of each primer, 0.5 mM dNTP, 1 U reverse transcription enzyme, provided buffer diluted to 1x and the remainder as water. A mixture of forward and reverse primers for all nine genes was used. Reactions were incubated at 42°C for 60 minutes. Reverse transcription products were purified using the QIAquick PCR purification kit (Qiagen) as per the manufacturer’s instructions, eluted with 100 µL Qiagen EB buffer, and stored at -20°C until further use.

qPCR for gene stability and icaA

The same protocol for efficiency testing was used to examine stability of potential reference gene and icaA expression, with the only change being that only 30 cycles were performed. In triplicate, cDNA produced from all three growth phases were subjected to qPCR (as described for the efficiency evaluation) for each candidate gene. In each 20 µL reaction, 5 µL of purified RT reaction product (equivalent to 100 ng of RNA prior to reverse transcription) was used. No template (negative; water) and DNA (positive; 100 ng DNA) controls were included in each run. No amplification was identified in negative controls. Intra-plate normalization was performed using the measured level of icaA in the positive control on each plate.

icaA expression, statistical analysis

Expression of icaA was compared between the three growth phases for the two strains using the combination of three reference genes. Normalization factors,
calibration factors and relative expression was calculated as per Hellemans et al\textsuperscript{16}.

A one-way ANOVA with Tukey’s post-hoc test was performed to compare expression between growth phases. P-values < 0.05 were considered significant.

**Acknowledgements**

The authors wish to thank Joyce Rousseau for her tireless effort and support in the laboratory.

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References


3. Chapter 3 - Biofilm associated gene expression in 

*Staphylococcus pseudintermedius* on a variety of implant 

materials.

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Abstract

Objective: To evaluate the expression of biofilm associated genes in Staphylococcus pseudintermedius on multiple clinically relevant surfaces.

Study design: In vitro experimental study

Sample population: Two strains of methicillin resistant S. pseudintermedius isolated from clinical infections representing the most common international isolates, and 8 clinically relevant surfaces: polymethylmethacrylate, stainless steel, titanium, latex, silicone, polydioxanone, polystyrene and glass.

Methods: A quantitative polymerase chain reaction (qPCR) assay for expression of genes related to biofilm initial adhesion, formation/maturation, antimicrobial resistance and intracellular communication was developed and validated. S. pseudintermedius biofilms were grown on the 8 clinically relevant surfaces, and samples of logarithmic and stationary growth phases were also collected. The qPCR assay was used to measure gene expression in those samples.

Results: Significant differences in gene expression were identified both between surfaces, and between strains for most gene/strain/surface combinations studied. Expression of genes responsible for production of extracellular matrix were increased in biofilms. Expression of genes responsible for initial adhesion and intracellular communication was markedly variable. Antimicrobial resistance gene expression was increased on multiple surfaces, most notably on stainless steel and titanium.

Conclusions: A method for the evaluation of expression of multiple biofilm associated genes in S. pseudintermedius was successfully developed and applied to
the study of biofilms on multiple surfaces. Significant differences between surfaces were identified. These data have implications for future clinical research in that variations in expression of these genes have a bearing on further understanding the development and treatment of implant associated biofilm infections.

**Introduction**

*Staphylococcus pseudintermedius* is one of the leading causes of opportunistic infections in canines,¹-³ with methicillin resistant strains (methicillin resistant *S. pseudintermedius*, MRSP) resulting in increasingly difficult to treat infections worldwide.⁴,⁵

Many of these infections are likely complicated by the presence of biofilm, which is increasingly appreciated as a virulence factor in this bacterium.⁶-⁸,⁹ Traditionally bacteria have only been appreciated in the planktonic form, which is defined as bacteria free floating in a fluid suspension. This life form is contrasted to biofilms, which are a community of microorganisms embedded in an extracellular matrix adhered to a biological or non-biological surface.¹⁰ Biofilm formation occurs through multiple orchestrated stages; the timeframe for these stages is poorly described.¹¹ Initial adherence to surfaces is mediated by any number of microbial surface components recognizing adhesive matrix molecules (MSCRAMMs).⁶,¹² Once adhered, other genes mediate formation of the extracellular matrix; one of the most studied is the intracellular adhesin operon (*icaADCB*) which is responsible for the formation of polysaccharide intracellular adhesin (PIA).¹³ Regulation of subsequent detachment of planktonic organisms from the biofilm, and of gene expression generally in the biofilm state is regulated by multiple genes, including those in the accessory gene regulation (*agr*) family.¹⁴,¹⁵
Biofilms have emerged as a topic of clinic research due to their association with increased resistance to antimicrobials, and their implication in recrudescent and refractory infections. This is likely afforded through multiple mechanisms, including the physical characteristics of the biofilm, downregulation of antimicrobial targets in the biofilm state and upregulation of antimicrobial resistance genes such as the methicillin resistance gene, mecA.

Previous studies have shown significant variation in *Staphylococcus epidermidis* and *S. pseudintermedius* biofilm formation on different inorganic and organic surfaces. Despite this, the majority of *in vitro* testing and evaluation of MRSP biofilms is still performed on polystyrene surfaces. Clinically, however, a multitude of other surfaces are relevant including materials comprising orthopedic implants, catheters, drains, suture materials and bone cement. Understanding the behavior of biofilms on these surfaces will be important in developing therapies to prevent and treat biofilm associated infections.

Quantitative reverse transcript polymerase chain reaction (qPCR) is emerging as the primary method of examining gene expression in a variety of different environments. While qPCR is a powerful tool for probing gene expression, there are significant potential pitfalls in its application. The minimum information for quantitative PCR experiment (MIQE) guidelines were produced to help standardize the performance of these experiments. One component of these is the use of validated reference genes. A recent study has identified suitable reference genes for use in gene expression quantification in *S. pseudintermedius*, and identified significant upregulation of a gene (*icaA*) associated
with biofilm formation in MRSP biofilm samples. These preliminary studies have provided the foundation for qPCR gene expression studies in this species.

The objectives of this study were to examine the expression of several genes associated with *S. pseudintermedius* biofilms including MSCRAMMs, members of the ica and agr groups, and the mecA gene in 2 strains of MRSP over a range of clinically relevant surfaces. We hypothesized that there would be significant variation in gene expression between surfaces.

**Materials and methods**

Two MRSP strains were chosen, representing the two main international MRSP sequence types (STs), ST68 (strain A42) and ST71 (strain A54). These two strains were previously isolated from clinical infections in dogs, and were both previously classified as moderate biofilm formers using a polystyrene plate assay. Single colonies of pure growth on Columbia blood agar were obtained. These were inoculated into tryptic soy broth supplemented with 1% (w/v) dextrose for all growth conditions. Broth was incubated aerobically in a shaker at 37°C. Logarithmic and stationary growth phases defined previously were grown and harvested (10⁹ cells) by centrifugation (13,000 x g for 30 seconds), then immediately processed to recover RNA as described below.

Biofilm samples were produced by incubating glass Erlenmeyer flasks unshaken at 37°C for >48 (48-54) hours after inoculation of 2 mL of mid logarithmic phase growth (OD₆₆₀ between 0.8 and 1.2) into 100 mL of growth medium. Samples of each surface representing 40-100 cm² of surface area to be tested were added to the flasks in a sterile fashion immediately prior to inoculation. Surface area for each sample was calculated using rough geometric approximations of the materials used (e.g. cylinders, spheres).
Samples tested included stainless steel (316L, 2.0 mm veterinary cuttable plates, 2.0 mm self tapping screws and 1.0 mm cerclage wire, Synthes, Ontario, Canada), titanium (2.0 mm veterinary cuttable plates, 2.0 mm self tapping screws, Synthes), silicone (wound evacuator, BARD medical, Covington, Georgia, USA), polystyrene (MBEC Biofilm Inoculator Polystyrene Plate, Innovotech, Alberta, Canada), polymethylmethacrylate PALACOS R bone cement, Heraeus Medical, Mississauga, Ontario, Canada, prepared aseptically as per manufacturers instructions), Latex (10 French Latex Foley catheter, Teleflex Medical Canada, Ontario, Canada), polydioxanone (PDS II, Ethicon, Somerville, New Jersey, USA). Materials available sterile as provided for clinical use (catheters, suture, drains) were used as provided from their sterile packaging. Implants were cleaned using a standard clinical protocol of an enzymatic soap followed by multiple rinses, then steam autoclaved before use. Polymethylmethacrylate was mixed as per manufacturers instructions under sterile condition, and formed into beads using a clinical bead mold. After the growth period, for samples of biofilm on glass, the culture medium was removed, and the flasks were washed twice with 10 mL phosphate buffered saline (PBS, pH 7.4). For all other materials, the medium was removed, and the material washed twice with 10 mL PBS. The material was then placed in a sterile Erlenmeyer flask, and washed again with PBS (10 ml). Biofilm was subsequently harvested by incubating the surfaces with 10 µg/mL Dispersin B (Kane Biotech, Winnipeg, MB) a biofilm degrading enzyme isolated from *Aggregatibacter actinomycetemcomitans*, in 10 mL PBS, shaking the flask for five minutes at room temperature, and then pelleting by centrifugation (13,000 x g for 30 seconds) the material released. The pellet of cells was immediately processed to recover RNA as described below.
DNA isolation

Cells were collected from a logarithmic phase sample as described above, and processed using the High Pure PCR Template Preparation Kit (Roche Applied Science, Indianapolis, IN) following the manufacturer’s instructions with the exception that lysostaphin (Sigma-Aldrich, Oakville, ON) was added at 0.1 mg/mL in the initial lysis step. Concentration and purity at 260/280 nm was measured using a Nanodrop ND-100 spectrophotometer (Nanodrop Technologies Inc., Wilmington, DE), and the samples were qualitatively examined for shearing by DNA gel electrophoresis.

Primer design and determination of PCR efficiency

Primers available for reference genes and for icaA from a previous study were used. Primers for icaB, icaC, icaD, spsE, ebpS, mecA, atl, agrA and agrB had been examined in S. epidermidis and S. aureus in previous studies, and analogous sequences were identified in S. pseudintermedius for evaluation in this study. Primers were designed (Table 3.1) using a combination of GeneRunner software version 3.05 (Hasting Software, Inc.) and the National Centre for Biotechnology Information online primer designing tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) using gene sequences for two strains of S. pseudintermedius (strains ED99 and HKU10-03) available from Genbank (http://www.ncbi.nlm.nih.gov/genbank/). Primers for mecA were generated using all available sequences for penicillin binding protein 2a from S. pseudintermedius.
Table 3.1: Reference and target gene primer information

Pooled chromosomal DNA from the two strains under investigation (mixture of equal concentrations from each strain) was used to generate dilution series for PCR efficiency calculations and for amplicon melting point assessment. For efficiency determination, a six fold dilution series was prepared in triplicate. Twenty µL reactions containing from $10^{-13}$ to $10^{-7}$ g of total DNA were prepared in 96 well plates (Bio-Rad) using the LightCycler 480 SYBR Green I Master qPCR reaction mixture (Roche Applied Science, Indianapolis, IN), following the manufacturer’s instructions. Primer concentration was 0.5 µM for each primer in the final reaction. A Bio-Rad C1000 thermal cycler with a CFX96 Real-Time System and Bio-Rad CFX Manager 2.0 software (Bio-Rad Life Sciences, Mississauga, ON) was used to run the following optimized thermocycling parameters: denaturation at 95 °C for 10 minutes followed by 50 cycles of 10 seconds at 95 °C, 30 seconds at 62 °C, 30 seconds at 72 °C, then a melting curve.
analysis running from 65 to 95 °C with steps and measurements every 0.5 °C. The thermocycler software calculated quantification cycle (Cq), efficiency and regression coefficients from the recovered data. Amplicon identity and primer specificity were confirmed by sequencing of the PCR products (fluorescent capillary Sanger method, Macrogen, Seoul, Korea), melt curve analysis and gel electrophoresis. qPCR reactions were tested over a range of primer concentrations, annealing temperatures and times to determine optimum reaction conditions.

**RNA isolation**

Each pellet of cells was resuspended in 2 mL PBS (pH 7.4), centrifuged to a pellet and the supernatant discarded. The pelleted cells were subsequently processed using the Qiagen RNeasy Mini kit (Qiagen, Germantown, MD), following the manufacturer’s instructions, with the following changes. The cells were initially resuspended in tris-EDTA buffer (pH 7.5) containing 15 mg/ml lysozyme (Sigma-Aldrich), 2 mg/mL proteinase K (Sigma-Aldrich), 25 µg/mL Dispersin B (Kane Biotech Inc., Winnipeg, MB) and 0.1 mg/mL lysostaphin (Sigma-Aldrich). Samples were incubated at 37 °C for 45 minutes, then processing continued as per manufacturers instructions. All optional steps for additional washes or spins during the procedure were performed. Logarithmic and stationary phase samples were eluted from the purification column with two volumes of 50 µL sterile nuclease free water, biofilm samples were eluted with one volume of 50 µL. All samples were treated with the DNAfree DNAsPe kit (Ambion, Austin, TX) after elution from the RNA purification column as per the manufacturer’s instructions using a total of 2 units per reaction, added in two aliquots. A Nanodrop ND-100 spectrophotometer (Nanodrop Technologies Inc.) was used to measure RNA
concentration and purity at 260/280 nm. Three samples of each RNA sample were measured and the readings averaged. Representative samples were submitted for Bioanalyzer analysis (Agilent Technologies, Santa Clara, CA). Real-time PCR was performed using the efficiency determination protocol on RNA samples to confirm the absence of DNA using gyrB primers. RNA samples were stored at -20 to -80 °C until further use.

Reverse transcription / complementary DNA (cDNA) preparation

Two µg of total RNA was used in each 20 µL gene specific reverse transcription reaction using the Omniscript RT PCR kit (Qiagen) using 10 µM of each primer, 0.5 mM dNTP, 1 U reverse transcription enzyme, provided buffer diluted to 1x and the remainder as water. A mixture of forward and reverse primers for all twelve genes was used. Reactions were performed at 42°C for 60 minutes. Reverse transcription products were purified using the QIAquick PCR purification kit (Qiagen) as per the manufacturer’s instructions, eluted with 100 µL Qiagen EB buffer, and stored at -20 °C until further use.

qPCR for gene stability and icaA

In triplicate, cDNA produced from logarithmic and stationary growth phases, and from biofilm samples from all 8 surfaces was subjected to qPCR (as described for the efficiency evaluation) for all 12 genes. In each 20 µL reaction, 5 µL of purified RT reaction product (equivalent to 100 ng of RNA prior to reverse transcription) was used. No template (negative; water) and DNA (positive; 100 ng DNA) controls were included in each run.
Calculation of relative gene expression and statistical analysis

Expression of the nine genes of interest was compared between the two growth phases and the eight biofilm samples for the two strains using the combination of three reference genes. Normalization factors, calibration factors, and relative expression was calculated as per Hellemans et al.\textsuperscript{30} including inter-plate normalization using all 12 genes on the positive controls on each plate. One way ANOVA was performed for each gene; when significant differences were identified, Tukey’s test was performed to identify specific comparisons that were statistically significant. P-values less than 0.05 were considered significant.

Results

For tables 2-5, materials tested are represented by the following abbreviations: stainless steel (SS), titanium (Ti), polydioxanone (PDS), polystyrene (PS), polymethylmethacrylate (PMMA) and silicone (Si). Stationary growth phase samples are indicated by ‘stat’, and logarithmic phase growth samples by ‘log’. Statistically relevant groups as indicated by Tukey’s pairwise comparisons are indicated by superscript. Within each row of a table, any samples the same letter present (e.g. A, AB, ABC) are not significantly different. Where no differences were identified by ANOVA, no superscript letters are given. P values for all the comparisons are provided in the additional online information.

Expression of genes responsible for ECM production were increased in biofilms on glass compared to both planktonic growth phases examined (Table 3.2). Between surfaces, expression was notably higher on glass. While only quantitative, the relative rankings of expression levels were roughly the same between the two strains studied,
with the notable difference being where expression in PMMA biofilms ranked relative to the other surfaces. For all cases except icaA in strain A54, expression of these genes was lowest in the stationary phase.

Table 3.2 - Relative gene expression levels of ica genes in multiple MRSP strains on multiple surfaces.

There were no consistent differences identified in expression of MSCRAMMs between surfaces for the 2 strains (Table 3.3). spsE expression was increased in biofilms on latex for both strains. For ebpS and atl, no expression differences were identified for strain A42. With strain A54 for those two genes, the stationary and logarithmic phases had the highest expression level of those genes, respectively. There were no other consistent variations in expression identified.
Table 3.3 - Relative gene expression levels for MSCRAMM genes in multiple MRSP strains on multiple surfaces.

Table 3.4 shows the measured relative expression for the quorum sensing genes studied. With regards to *agrA* in A42, there were no significant differences identified. *agrB* in that strains was notably upregulated in the logarithmic growth phase. In strain A54, *agrA* was significantly upregulated in the logarithmic phase compared to several surfaces (PDS, Latex, PS and Si). Conversely for *agrB*, logarithmic phase and latex surface growth had the lowest expression levels, with glass having the highest level.

Table 3.4 - Relative gene expression levels for quorum sensing genes in multiple MRSP strains on multiple surfaces.

Expression of the antimicrobial resistance gene *mecA* was poorly correlated between strains (Table 3.5). In strain A42, expression was lower in most biofilms.
compared to planktonic samples. Conversely, expression was lowest in logarithmic growth for strain A54 compared to most biofilms.

Table 3.5 - Relative gene expression levels for mecA in multiple MRSP strains on multiple surfaces.

The following information is available for this article online: Significant surface comparisons. Table of p-values for all significant comparisons between surfaces. This material is available in appendix 1.

Discussion

The significant variation in expression of biofilm associated genes identified in this study may have important future clinical implication in the prevention and treatment of *S. pseudintermedius* biofilm associated infections. Understanding expression of the genes that play a role in the formation of these biofilms may allow prevention of their formation or discovery of novel treatment regimes with targeted therapy.

The increased expression of genes responsible for the formation of PIA in biofilms identified in this study allays the important role of ECM in biofilm based infections. Selecting materials for catheters and implants that do not promote ECM formation may help reduce the prevalence of recurrent and resistance infections in those situations. While no significant differences were identified specifically between surfaces, the power of this study was limited and some of the numerical differences that were present might be significant given a larger sample size. Of particular clinical interest for further study would be assessment of potential differences in expression of ECM genes.
between stainless steel and titanium. For instance, based on the results of this study, a sample size of only 5 would be required to give an 80% chance of identifying a difference in expression of icaC between stainless steel and titanium. Further investigation of these differences may provide a mechanistic understanding of the difference seen in infection rates and biofilm formation between stainless steel and titanium implants.\textsuperscript{19,31}

Potentially of greater interest is the identification of differences in expression levels of MSCRAMMs between surfaces. Specifically for spsE, expression was notably upregulated in latex biofilms. These variations in expression may indicate that expression of these genes are induced by the presence of their respective specific substrates (or potentially analogues); latex may have a suitable binding site for the spsE ligand, and so may induce its upregulation; however, this is entirely speculative. Clinically, targeted therapy to block specific adhesion mediators may help prevent initial adhesion and biofilm formation. Understanding of the mediators responsible for initial adhesion on specific substrates is a vital first step towards that goal. Further examination of any temporal variability on expression of these genes should also be pursued; expression of these genes may be transient only around the time of initial adhesion.

Bacteria within biofilms are known to be more resistant to most antimicrobials compared to their planktonic counterparts, with concentrations of antibiotics required to eradicate or significantly inhibit the biofilm-embedded bacteria often tens or hundreds of times that required to inhibit planktonic organisms.\textsuperscript{32} This is an important clinical consideration, as these concentrations can be impossible to reach \textit{in vivo}, limiting treatment options for biofilm infections. Multiple mechanisms for this resistance are
postulated; however, few have been definitively proven. Our results indicate that upregulation of mecA in strain A54 may account for increased resistance in biofilm. The same however was not identified for the other strain that was examined. The reasons for the differences in expression of the antimicrobial gene examined in this study remain unclear; it may be that different mechanisms account for increased antimicrobial resistance between the 2 strains, or that there are other undetermined influences on expression of this gene. mecA resides on a large chromosomal cassette, and these two STs harbor different SCCmec types (A42 SCCmec V and A54 SCCmec II/III), so further identification and investigation of regulatory genes found on those SCCmec types would likely provide additional insight to this data. The upregulation of expression of this gene in biofilms also raises concern that expression of other resistance genes could be similarly increased in biofilm, something that warrants further study.

Regulation of many biofilm genes is thought to be controlled in several ways, including the lux and agr systems. Targeting these systems for therapy may allow a single therapeutic target to affect multiple aspects of biofilm formation. In Staphylococcus aureus, agr deletion mutants had increased production of biofilm. It would follow that upregulation of this inhibitory gene would be anticipated as bacteria become encased within ECM, and production of that matrix is tapered. This was appreciated for both genes; for agrA with strain A54, and for agrB with strain A42. There was also lower expression of agrB appreciated in strain A54 in logarithmic growth compared to biofilm phase on several surfaces. This is again consistent with the potential downregulation of this gene during initial biofilm production, with increased expression as stable biofilm has been formed.
The most important finding of this research is that there are significant differences in expression of almost all of the genes studied when compared between different surfaces. This has significant implications for future in vitro research work on biofilms in MRSP and highlights the importance of using clinically relevant substrates for growth of biofilms for in vitro study.

Further study is required to fully understand the variation in gene expression identified here. Additional numbers of strains will need to be examined to further identify and confirm trends in expression. This may be elucidated by comparison to other strain information such as biofilm competency and ECM composition and physical character. Examination of temporal variation in expression is also warranted. It is likely that there is some variation during the phases of biofilm formation. Identification of genes that specifically necessary for initial adherence, or for detachment from biofilm back to the planktonic phase, may provide therapeutic targets to prevent initial biofilm formation, or to prevent recrudescence of infection from quiescent biofilms. Examination of samples of clinical biofilms will also be an important step in understanding the etiology of these infections.

Given that there were significant surface effects on gene expression, and based on previous studies that identified differences in biofilms when exposed to various biological substances such as serum, further investigation of the effects of growth medium on biofilm formation, gene expression, and the interaction of those with surface type is warranted as well.

The major limitation of this study is the in vitro nature. Despite the use of more clinically relevant surfaces, this study still relied on a simplistic in vitro model as
compared to the situation in vivo. Biofilms are often polymicrobial communities, and clinically relevant biofilms form in the presence of a complex milieu of biological influences (e.g. serum proteins, inflammatory cytokines and mediators). This study examined biofilms formed by a single organism in culture media under strictly controlled conditions. The complex interactions in vivo of polymicrobial communities and the external biological environment with the surface where a biofilm is forming remain poorly examined. Additionally, while prokaryotic organisms generally have no post-transcriptional modification and there is usually good correlation between RNA levels and protein expression, qPCR assumes that gene expression directly relates to functional levels of the gene product, an assumption that has not been definitively shown in staphylococcal biofilms. Furthermore, while the activity of the genes studied in this work has not been directly examined in this species, the role of analogous genes in other staphylococci has been examined and related to biofilm formation and regulation.

This initial screening study confirmed that there is significant dependence of biofilm gene expression on surface type. This has implications for future work in showing that any in vitro study will need to take surface type into account when modeling an in vivo situation. Many of the results obtained are consistent with clinical observations of resistance to therapy in clinical infections where biofilms are likely present.

Acknowledgment

The authors thank Joyce Rousseau for her tireless assistance in the laboratory.

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4. Chapter 4 – General discussion

*Staphylococcus pseudintermedius* is an important veterinary pathogen and its clinical significant is progressively increasing. Infections caused by this bacterium can be difficult to treat, in part because of its tendency to develop resistance to antimicrobials and to form biofilm. Further understanding of the development of biofilms is a vital step towards preventing and treating these clinically important infections.

Detailed examination of the individual steps in biofilm formation and maturation over time is vital to the understanding of these complex structures. There are multiple genes responsible for each step of biofilm formation, including those responsible for initial adhesion of bacteria to surfaces, production of the extracellular matrix, and specific antimicrobial resistance mechanisms. There are also complex regulatory systems in place to guide biofilm formation and maturation, subsequent release of organisms to form new infections, and antimicrobial resistance expression. Molecular studies of biofilm formation, and other aspects of staphylococcal virulence could provide profound insight into this pathogen, and quantitative PCR (qPCR) is a powerful tool for evaluation of expression of genes such as these. Accurate qPCR data is dependent on identification and validation of suitable stable reference genes, something that had not been described for *S. pseudintermedius*. Thus, the first step in these investigations was the development, optimization and validation of a qPCR assay in *S. pseudintermedius*, including assessment of expression of a single biofilm associated gene (BAG), *icaA*. This included development of methods to successfully grow and harvest MRSP biofilms,
and to collect RNA in adequate quantity and quality from those biofilms. Next, multiple putative reference genes were identified from previously recovered genomes of *S. pseudintermedius*, and primers developed to target them. Probes for *icaA* were similarly developed. The experimental details of the qPCR assay were refined, then the necessary preliminary work performed to validate the qPCR assay. Once the validated qPCR study had been developed, it was used to examine the expression of *icaA* in 2 strains of MRSP, identifying significant increases in expression of that gene in biofilms compared to planktonic phases, consistent with its role in production of biofilm extracellular matrix. This study was a critical component of future work in this thesis, and will provide important data for others to perform qPCR studies of this pathogen, since studies performed without validated reference genes are of questionable relevance.

Successful validation of stable reference genes led to subsequent study of several additional BAGs, including genes responsible for initial adhesion, biofilm maturation, antimicrobial resistance and gene regulation. One problem with the *in vitro* study of biofilms is the complex nature of biofilm formation and questions about the ability of studies to replication the *in vivo* clinical situation. One major aspect is the use of polystyrene plates for biofilm assessment, since that material is not present in patients or most patient care materials (e.g. surgical implants, suture, catheters). Therefore, methods to grow and harvest biofilms were extended to a number of clinically and experimentally important surfaces, including polystyrene, polymethylmethacrylate, polydioxanone, latex, silicone, stainless steel and titanium. These new methods, in addition to those described in chapter 2 were used to
examine expression of 9 BAGs on 10 different surfaces, in addition to the logarithmic and stationary planktonic phases, again using 2 clinically isolated strains of MRSP. While the upregulation of icaA seen in chapter 2 was not confirmed, logarithmic phase planktonic growth did have significantly lower expression of that gene than any of the biofilms studied. There were few consistent differences identified in MSCRAMM gene expression; spsE expression was increased in biofilms formed on latex in both strains. Otherwise, ebpS and atl both had increased expression in the logarithmic and stationary phase growth of strain A54. Examination of expression of regulatory genes revealed agrB to be upregulated in the logarithmic phase of A42. Conversely that gene had the lowest expression level in the logarithmic phase growth of A54. Otherwise in that species agrA was significantly upregulated in the logarithmic phase. Expression of the antimicrobial resistance gene mecA was also different between the species, with the lowest levels of expression identified in the biofilms of strain A42, whereas with strain A54, expression was lowest in the logarithmic phase. Generally it was notable that expression of the ica gene family and the mecA was higher in titanium than stainless steel for both strains. Expression variation in biofilms grown on titanium may explain clinical differences in prevalence of biofilms on that surface, and other difference seen in clinical behavior of that material. Furthermore, strong suggestions of potential differences in expression of BAGs in biofilms formed on stainless steel and titanium is an excellent indication to specifically examine those materials in more detail; focusing on variations in temporal variations in BAG expression, as well as overall level of BAG expression.
This data confirms that there is significant variation in biofilm character based on substrate, and underlines the importance of being as close as possible to clinical conditions with any *in vitro* study. Furthermore, these results allay potential variation between biofilms between surfaces that may help to explain certain clinical observations relating to implant associated infections. Additional experimental work was performed with a goal towards examining expression of several BAGs in biofilm on stainless steel and titanium using a broader range of clinical isolates of MRSP, as described in appendix 2. The work confirmed the presence of the previously examined BAGs in 12 additional strains of MRSP. In order to facilitate the large number of analyses required for that screening study, Procedural changes allowing for the rapid processing of multiple samples were made. Unfortunately, despite efforts to ensure appropriate purification of samples between steps, these changes resulted in amplification of non-specific targets during qPCR in several situations, likely due to contamination between steps with reverse transcription and PCR primers. This precluded analysis of several of the genes entirely, and for multiple individual samples. Within the samples where analysis was feasible, no significant differences were identified between surfaces for each strain, nor between strains for any gene.

The work described in chapter 3 and appendix 2 was primarily limited by small numbers of samples for each situation, and subsequently a low power to identify differences. Despite that, significant differences were identified in many situations. While there were indications of differences in several situations relevant to specific clinical questions, many of those results were not statistically significant. For
examples, mechanistic reasons for differences in infection rates and infection behavior between stainless steel and titanium have been elusive. Analysis of the results of this work show that while there were no significant differences identified in expression level of genes responsible for ECM formation (with 3 samples), a power calculation based on those results show that a samples size of 5 would have an 80% chance of identifying a difference at a confidence level of 0.05 (for an individual comparison).

Examination of the results of this work in light of specific clinical questions could help to guide further examinations. Focusing on specific comparisons instead of screening a broad range of samples, and subsequently having increased numbers of samples for each comparison may help identify mechanistic explanations for clinical observations and other experimental results, and would be the most immediate next step to refine and further the specific observations made in this study.

Additionally, no attempts were made to examine temporal variation in BAG expression. In other species, variation in gene expression has been documented, both spatially within the complex three dimensional structures that biofilm forms, and temporally during development and maturation of biofilms. Specific understanding of spatial and temporal variation in the multiple steps of biofilm formation in MRSP remains unexplored. Examination of any variation of this nature stands to be of interest in all aspect of biofilm development. For example, examination of early biofilms (within hours of formation) may reveal upregulation of MSCRAMMs responsible for initial adhesion. Expression of the ica family of genes may also have a peak during biofilms formation as the biofilm develops to a fully
‘mature’ form. Expression of the regulatory genes is likely to shed light on any
temporal variations thus identified. Understanding these specific is an important
first step towards developing therapeutics to block biofilm formation or
development at those specific steps.

As evidenced by the results in appendix 2, future work must take exact
experimental methodology into account. These results highlight the sensitivity of
qPCR analysis. Future studies should either use the protocols as described in
chapter 3; alternatively use of the vacuum manifold for sample purification could be
pursued. Methodological optimization would however need to be performed to
identify if and when contamination is occurring, and what modifications are
required to eliminate those problems.

Lastly, this was an exclusively in vitro study. The use of clinically relevant
surfaces is an important step towards in vivo applicability, but the use of semi
synthetic growth media, and lack of investigation of clinically relevant growth
conditions such as in the presence of serum may still limit the direct applicability of
these study results. Inclusion in future in vitro studies of canine serum in the
growth media, or pre-treatment of surfaces with serum or blood may provide a
more clinically relevant result.

The results of this study have identified multiple differences in biofilm gene
expressions that have specific bearing on clinical questions and observations. These
results will help to guide future targeted comparisons related to specific clinical
questions, and will provide guidelines for the scope and nature of those
examinations. More generally, this study has developed and validated a gene
expression assay for use in MRSP, providing a strong basis for future examinations of BAG expression in that species. This assay will be a critical tool in the performance of those investigations in the future.
### 5. Appendix 1 - Supplemetnal Information for Chapter 3

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</table>

Supplemental* information* for Chapter 3
Appendix 1 - p values for all significant comparisons between surfaces for various biofilm associated genes.

<table>
<thead>
<tr>
<th></th>
<th>Log vs. P</th>
<th>Log vs. Latex</th>
<th>Log vs. SS</th>
<th>Glass vs. P</th>
<th>Glass vs. Latex</th>
<th>Glass vs. SS</th>
<th>A54agrA</th>
<th>A54epS</th>
<th>A54spE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log</td>
<td>0.0017</td>
<td>0.0003</td>
<td>0.0046</td>
<td>Stat</td>
<td>0.0065</td>
<td>Glass</td>
<td>0.0133</td>
<td>0.0373</td>
<td>0.0436</td>
</tr>
<tr>
<td>P</td>
<td>0.032</td>
<td>0.0006</td>
<td>0.0113</td>
<td>Latex</td>
<td>0.0436</td>
<td>Glass</td>
<td>0.0043</td>
<td>0.004</td>
<td>0.0101</td>
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<tr>
<td>PMMA</td>
<td>0.017</td>
<td>0.0373</td>
<td>0.0045</td>
<td>Stat</td>
<td>0.0157</td>
<td>Latex</td>
<td>0.0048</td>
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<td>0.004</td>
</tr>
<tr>
<td>Ti</td>
<td>0.015</td>
<td>0.0373</td>
<td>0.0045</td>
<td>Latex</td>
<td>0.0048</td>
<td>Latex</td>
<td>0.0043</td>
<td>0.004</td>
<td>0.004</td>
</tr>
<tr>
<td>Glass</td>
<td>0.003</td>
<td>0.0043</td>
<td>0.045</td>
<td>Stat</td>
<td>0.0255</td>
<td>Latex</td>
<td>0.0025</td>
<td>0.004</td>
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<tr>
<td>A54agrA</td>
<td>0.032</td>
<td>0.0043</td>
<td>0.0101</td>
<td>Latex</td>
<td>0.0048</td>
<td>Latex</td>
<td>0.0043</td>
<td>0.004</td>
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<tr>
<td>A54epS</td>
<td>0.0373</td>
<td>0.0043</td>
<td>0.0101</td>
<td>Latex</td>
<td>0.0048</td>
<td>Latex</td>
<td>0.0043</td>
<td>0.004</td>
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<tr>
<td>A54spE</td>
<td>0.0436</td>
<td>0.0043</td>
<td>0.0101</td>
<td>Latex</td>
<td>0.0048</td>
<td>Latex</td>
<td>0.0043</td>
<td>0.004</td>
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6. Appendix 2 – Additional screening of biofilm gene expression on stainless steel and titanium

Introduction

An additional 12 strains of MRSP were screened for the 12 genes examined in Chapter 3, and the same methodology as used in that chapter was subsequently used to measure expression of those genes in biofilms harvested from stainless steel and titanium, as well as in the logarithmic and stationary growth phases.

Materials and methods

Materials and method used were identical to those described in Chapter 3, aside from the change that during the preparation of the RNA, and cleanup of the cDNA after reverse transcription, a vacuum manifold was used to pull solutions through the purification column, instead of centrifugation, as described as an option in the manufacturers instructions.

The strains used are shown in Table 6.1 below.
<table>
<thead>
<tr>
<th>Strain ID</th>
<th>Biofilm forming ability (OD\textsubscript{570})</th>
<th>Sequence type</th>
<th>DRU type</th>
</tr>
</thead>
<tbody>
<tr>
<td>A12</td>
<td>3.24 (strong)</td>
<td>111</td>
<td>dt10h</td>
</tr>
<tr>
<td>A42</td>
<td>1.18 (moderate)</td>
<td>68</td>
<td>dt11a</td>
</tr>
<tr>
<td>A54</td>
<td>0.71 (moderate)</td>
<td>71</td>
<td>dt8f</td>
</tr>
<tr>
<td>KB32</td>
<td>0.49 (weak)</td>
<td>71</td>
<td>dt9a</td>
</tr>
<tr>
<td>KB31</td>
<td>0.39 (weak)</td>
<td>71</td>
<td>dt9a</td>
</tr>
<tr>
<td>KB110</td>
<td>0.40 (weak)</td>
<td>68</td>
<td>dt10h</td>
</tr>
<tr>
<td>KB113</td>
<td>1.73 (strong)</td>
<td>68</td>
<td>dt10h</td>
</tr>
<tr>
<td>KB235</td>
<td>0.49 (weak)</td>
<td>71</td>
<td>dt9a</td>
</tr>
<tr>
<td>KB258</td>
<td>0.47 (weak)</td>
<td>71</td>
<td>dt9a</td>
</tr>
<tr>
<td>KB282</td>
<td>0.27 (weak)</td>
<td>68</td>
<td>dt10h</td>
</tr>
<tr>
<td>SP77</td>
<td>0.53 (weak)</td>
<td>68</td>
<td>dt11a</td>
</tr>
<tr>
<td>P457</td>
<td>0.72 (moderate)</td>
<td>71</td>
<td>dt9a</td>
</tr>
</tbody>
</table>

Table 6.1 - \textit{S. pseudintermedius} strains used.

One way ANOVA was performed to compare expression across strains tested, within each surface / growth condition group when more than 3 conditions were being compared; when significant differences were identified, Tukey’s test was performed to identify group comparisons that were statistically significant.

Student’s t-test was used to compare expression levels when only 2 conditions were being compared.

**Results**

All 12 genes were identified in the chromosomal DNA of each of the 12 strains used.

Bioanalyzer analysis was performed on 45 / 144 RNA samples; the mean RIN measured was 8.2 (interquartile range 7.8 – 9.1).

Melt curve analysis of the qPCR runs revealed multiple samples with evidence of amplification of multiple targets. Melt curve analysis and gel electrophoresis of a representative run is shown in Figure 6.1 and Figure 6.2.
Figure 6.1 - DNA electrophoresis of qPCR products. Upper row, sample demonstrating non-specific amplification, 100bp ladder at right. Lower row, sample demonstrating single product amplification, two 100 bp ladders at right. Samples in order from left to right, gryB, rho, recA, icaA, icaC, icaD, spsE, ebpS, mecA, atl, agrB, agrA.

Figure 6.2 - Melt curve analysis of qPCR reaction shown also in lane 7 from left, upper row, figure 6.1.

Samples that had clear evidence of non-specific amplification (multiple peaks on melt curve analysis) were excluded from subsequent analysis. This restriction precluded the analysis of and several strain / condition / gene combinations, as well
as the genes icaC, icaD, mecA in all circumstances. While the expression of these genes could not be measured; the positive controls on each plate for the four genes still amplified as expected in all instances, and the results from those positive controls were used for inter plate normalization. Relative expression values for all samples measured are shown in Table 6.2 (all measurements replicate triplicate measurements). Blank spaces correspond to situations where contamination precluded analysis. For all samples measured, triplicate measurements were performed. Among the samples where expression was measured, it was compared between each strain for each gene / surface combination, and also compared for each surface for each gene / strain combination. There were no significant differences identified in the expression levels measured between strains for any of the genes examined. P values for all comparisons performed are shown in Table 6.3.
<table>
<thead>
<tr>
<th></th>
<th>icaA</th>
<th>spsE</th>
<th>ebpS</th>
<th>atl</th>
<th>agrB</th>
<th>agrA</th>
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<td>A12 log</td>
<td>4.4 ± 1.5</td>
<td>1.9 ± 0.4</td>
<td>1.5 ± 0.3</td>
<td>2.8 ± 0.6</td>
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<td>A12 SS</td>
<td>3.8 ± 1.5</td>
<td>1.3 ± 0.3</td>
<td>2.6 ± 0.6</td>
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<tr>
<td>A12 Ti</td>
<td>5.4 ± 10.1</td>
<td>1.9 ± 2.8</td>
<td>3.5 ± 5.0</td>
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<td></td>
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<tr>
<td>A42 log</td>
<td>1.9 ± 3.2</td>
<td>1.5 ± 1.7</td>
<td>1.7 ± 1.8</td>
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<tr>
<td>A42 stat</td>
<td>2.6 ± 1.5</td>
<td>4.3 ± 1.9</td>
<td>3.1 ± 1.5</td>
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<td>A42 SS</td>
<td>5.1 ± 9.8</td>
<td>2.0 ± 3.8</td>
<td>2.9 ± 4.4</td>
<td>1.8 ± 3.1</td>
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<td>A54 stat</td>
<td>3.6 ± 4.0</td>
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<td>1.7 ± 1.5</td>
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<td>A54 SS</td>
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<td>1.2 ± 0.6</td>
<td>1.8 ± 1.1</td>
<td>1.5 ± 0.7</td>
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<tr>
<td>A54 Ti</td>
<td>4.0 ± 2.5</td>
<td>2.0 ± 1.3</td>
<td>1.5 ± 0.9</td>
<td>2.7 ± 1.6</td>
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<tr>
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<td>2.3 ± 2.9</td>
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<td>1.5 ± 1.0</td>
<td>3.2 ± 2.5</td>
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<tr>
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<td>1.2 ± 0.3</td>
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<td>1.7 ± 0.6</td>
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<tr>
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<td>1.5 ± 2.6</td>
<td>2.4 ± 4.0</td>
<td>1.9 ± 3.2</td>
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<td>7.3 ± 5.8</td>
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<td>2.4 ± 1.8</td>
<td>1.8 ± 1.9</td>
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<td>3.4 ± 2.4</td>
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<td>2.3 ± 5.0</td>
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<td>2.1 ± 1.5</td>
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<td>1.4 ± 0.9</td>
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<td>5.8 ± 6.2</td>
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<td>3.4 ± 3.4</td>
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<tr>
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<td>4.3 ± 9.2</td>
<td>4.1 ± 9.7</td>
<td></td>
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<td></td>
</tr>
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<td>2.1 ± 4.0</td>
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<td>1.3 ± 1.4</td>
<td>2.2 ± 2.8</td>
<td>1.9 ± 1.3</td>
<td>1.6 ± 1.5</td>
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<tr>
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<td>2.4 ± 2.3</td>
<td>2.1 ± 0.9</td>
<td>1.4 ± 0.6</td>
<td>2.3 ± 1.4</td>
<td>2.3 ± 0.6</td>
<td>1.7 ± 0.8</td>
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<td>2.8 ± 2.7</td>
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<td>4.9 ± 4.3</td>
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<td>2.3 ± 2.2</td>
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</tr>
<tr>
<td>SP77 Ti</td>
<td>4.3 ± 9.2</td>
<td>4.1 ± 9.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P457 log</td>
<td>1.5 ± 1.3</td>
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<td>1.2 ± 0.8</td>
<td>1.1 ± 0.7</td>
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</tr>
<tr>
<td>P457 SS</td>
<td>2.6 ± 1.3</td>
<td>1.7 ± 0.5</td>
<td>1.3 ± 0.4</td>
<td>1.8 ± 0.9</td>
<td>1.1 ± 0.2</td>
<td>1.3 ± 0.3</td>
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<td>P457 Ti</td>
<td>3.6 ± 2.5</td>
<td>1.8 ± 1.2</td>
<td>1.2 ± 0.9</td>
<td>2.3 ± 1.7</td>
<td>1.2 ± 0.7</td>
<td>1.2 ± 0.8</td>
</tr>
<tr>
<td>P457 Ti</td>
<td>3.9 ± 3.3</td>
<td>1.9 ± 1.6</td>
<td>1.3 ± 1.0</td>
<td>2.7 ± 2.0</td>
<td>1.0 ± 0.8</td>
<td></td>
</tr>
</tbody>
</table>

Table 6.2 - Relative expression levels and standard deviation for all analyzed samples. Blank spaces correspond to samples where nonspecific amplification precluded analysis.
Table 6.3 - P values for identified variation in expression between strains.

<table>
<thead>
<tr>
<th></th>
<th>Log</th>
<th>Stat</th>
<th>SS</th>
<th>Ti</th>
</tr>
</thead>
<tbody>
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<td>icaA</td>
<td>0.717</td>
<td>0.242</td>
<td>0.964</td>
<td>1.000</td>
</tr>
<tr>
<td>spsE</td>
<td>1.000</td>
<td>0.970</td>
<td>0.971</td>
<td>0.995</td>
</tr>
<tr>
<td>ebpS</td>
<td>1.000</td>
<td>0.999</td>
<td>0.240</td>
<td>0.992</td>
</tr>
<tr>
<td>atl</td>
<td>0.999</td>
<td>0.706</td>
<td>0.967</td>
<td>0.999</td>
</tr>
<tr>
<td>agrB</td>
<td>0.589</td>
<td>0.888</td>
<td>0.796</td>
<td>0.797</td>
</tr>
<tr>
<td>agrA</td>
<td>0.934</td>
<td>0.934</td>
<td>0.934</td>
<td>0.528</td>
</tr>
</tbody>
</table>

Table 6.4 - P values for identified variation in expression between surfaces. Blank spaces correspond to situations where expression was measured on less than 2 strain / surface combinations.

While the details of processing for each sample were not recorded, subjectively there was significant variation in the time taken for samples and wash buffers to elute through the columns used for purification, especially during RNA purification.
Discussion

No significant differences in expression levels were identified between strains for each growth stage / gene combination, nor between any surfaces for each strain / gene combination.

It is likely that the use of the vacuum manifold instead of centrifugation resulted in contamination of the cDNA with the full complement of primers as used in the RT reactions, and potentially DNA contamination of the RNA preparations. While this contamination may have been minor, the sensitivity of PCR means even minor contamination can result in significant problems. In both possible situations, the contamination would result in non-specific amplification as demonstrated by these results. This contamination may have resulted from differences in interaction time between the solutions and the purification filters, or hold-over of solutions between steps due to incomplete clearing of solutions by use of the vacuum manifold versus centrifugation.

A future study with the goal of comparing the samples produced through centrifugation and using the vacuum would elucidate this problem. Otherwise, modifying the experimental protocol (e.g. by including additional rinse / wash steps) to help reduce contamination may ameliorate this problem for future studies.