Molecular population and colonisation factor analysis of the *Staphylococcus intermedius* group

Jeanette Bannoehr

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Declaration

The research presented in this thesis is entirely my own work, except where otherwise stated. No part of this thesis has been submitted in any other application for a degree or professional qualification.

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Content

List of commonly used abbreviations

Abbreviation	Full name
α	alpha
aa	amino acid(s)
AD	atopic dermatitis
AFLP	amplified fragment length polymorphism
agr	accessory gene regulator
AIP	autoinducing peptide
APS	ammonium persulphate
~	approximately
β	beta
BHI	Brain Heart Infusion
bp	base pairs
BSA	bovine serum albumin
CA-MRSA	community-acquired methicillin-resistant *Staphylococcus aureus*
CC	clonal complex
CFU	colony-forming units
CK10	cytokeratin 10
CK14	cytokeratin 14
ClfA	clumping factor A
ClfB	clumping factor B
Cna	collagen-binding protein
CPEK	canine epidermal keratinocyte progenitors
CWA	cell wall-anchored
dH_2O	distilled water
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
ECM	extracellular matrix
E. coli	*Escherichia coli*
EE	exudative epidermitis
ELISA	Enzyme-Linked ImmunoSorbent Assay
Fg	fibrinogen

Abbreviation	Full name
Fn	fibronectin
FnbpA	fibronectin-binding protein A
FnbpB	fibronectin-binding protein B
γ	gamma
g	gram
GM17	M17 medium supplemented with 0.5% (weight in volume) glucose
h	hour(s)
HA-MRSA	hospital-acquired MRSA
HRP	horseradish peroxidase
IgG	Immunoglobulin G
IL	interleukin
IsdA	Iron-regulated surface determinant protein A
ITS-PCR	intergenic ribosomal DNA spacer polymorphism-polymerase chain reaction
kb	kilobase pairs
kDa	Kilodalton
LB	Luria-Bertani
L. lactis	*Lactococcus lactis*
M	Molar
mg	milligram (10^{-3} gram)
min	minute(s)
ml	millilitre (10^{-3} litre)
MLST	multilocus sequence typing
mM	millimolar (10^{-3} molar)
MRSA	methicillin-resistant *Staphylococcus aureus*
MRSI	methicillin-resistant *Staphylococcus intermedius*
MRSP	methicillin-resistant *Staphylococcus pseudintermedius*
MSCRAMM(s)	*microbial surface components recognizing adhesive matrix molecules*
MSSA	methicillin-susceptible *Staphylococcus aureus*
MSSP	methicillin-susceptible *Staphylococcus pseudintermedius*

Abbreviation	Full name
OD_{600}	optical density at 600 nm
ORF	open reading frame
oriC	origin of replication
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
PVL	Panton-Valentine leukocidin
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
S.	*Staphylococcus*
sar	staphylococcal accessory regulator
SCC*mec*	staphylococcal cassette chromosome *mec*
Sdr	serine-aspartate dipeptide repeat
SdrC	serine-aspartate dipeptide repeat-containing protein C
SdrD	serine-aspartate dipeptide repeat-containing protein D
SdrE	serine-aspartate dipeptide-containing repeat E
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Ser-Asp	serine-aspartate
SIG	*Staphylococcus intermedius* group
SpA	staphylococcal Protein A
Sps	*Staphylococcus pseudintermedius* surface protein
ssp.	subspecies
SSSS	Staphylococcal Scalded Skin Syndrom
ST	sequence type
TBE	Tris-borate/EDTA
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	Trishydroxymethylaminomethane
TSS	toxic shock syndrome
µg	microgram (10^{-6} gram)

Abbreviation	Full name
µl	microlitre (10^{-6} litre)
µM	micromolar (10^{-6} molar)
V	volt
v/v	volume in volume
w/v	weight in volume

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Abstract

The Gram-positive bacterium *Staphylococcus intermedius* is regarded as the major cause of canine pyoderma, a common skin infection of dogs. However, despite its clinical importance, the population genetic structure of *S. intermedius* is poorly understood. The current study examined the population genetic structure of *S. intermedius* using a multilocus DNA sequencing approach. A collection of 99 isolates phenotypically identified as *S. intermedius* and originating from a broad array of animal hosts in several different countries was investigated. Phylogenetic analysis indicated that the isolates belonged to three distinct species including *S. intermedius, Staphylococcus pseudintermedius*, and *Staphylococcus delphini*, together referred to as the *S. intermedius* group (SIG). Importantly, it was discovered that all canine isolates investigated belonged to the *S. pseudintermedius* phylotype and it was concluded that *S. pseudintermedius*, not *S. intermedius*, is the common cause of canine pyoderma. Further, it was revealed that *S. delphini* is more clinically important than was previously thought. The allelic variation of *agrD*, which encodes the autoinducing peptide (AIP) of the *agr* quorum sensing system in staphylococci, was determined for all isolates. Four AIP variants were identified, including three which were present in all three species, suggesting that a common quorum sensing capacity has been conserved despite species differentiation in very different host niches. Considerable clonal diversity was revealed within the *S. pseudintermedius* species, including several methicillin-resistant clones which have evolved by recent acquisition of the *mecA* gene. Using the sequence diversity identified, a simple diagnostic test was developed based on a PCR-RFLP approach to discriminate *S. pseudintermedius* from *S. intermedius* and *S. delphini*.

Having established that *S. pseudintermedius* is the common canine pyoderma pathogen, this study aimed to investigate key host-pathogen interactions involved in colonisation of its canine host. Bioinformatic analysis of the whole genome sequence of a clinical isolate of *S. pseudintermedius* (strain ED99) revealed 17 genes encoding predicted LPXTG-containing cell wall-anchored (CWA) surface proteins. A diverse collection of *S. pseudintermedius* isolates and closely related staphylococcal species

was screened for the presence of the genes encoding the novel CWA proteins. The majority of genes were widely distributed among the isolates examined, with nine genes being exclusive to *S. pseudintermedius* and eight being also present in other members of the SIG. In Gram-positive bacteria, a family of CWA proteins called *microbial surface components recognising adhesive matrix molecules* (MSCRAMMs) mediates bacterial adherence to extracellular matrix proteins of the host. Three of the 17 predicted novel CWA proteins, designated SpsD, SpsL and SpsO, were selected for further characterisation of their role in host-pathogen interactions and were cloned and expressed on the surface of the surrogate host *Lactococcus lactis.* Solid phase adherence assays employing host extracellular matrix proteins and canine corneocytes were performed to identify host receptors for the putative MSCRAMMs. *L. lactis* expressing SpsD demonstrated binding to fibronectin, fibrinogen, and cytokeratin 10, SpsL mediated binding of *L. lactis* to fibronectin and canine fibrinogen, and SpsD and SpsO both mediated *L. lactis* adherence to canine corneocytes. Additionally, a cell culture assay using a commercially available canine epidermal cell line was developed and the adherence of *S. pseudintermedius* ED99 and the *L. lactis* constructs to the cell line was tested. *S. pseudintermedius* ED99, but none of the MSCRAMM-expressing *L. lactis* strains, adhered to the canine epidermal cells *in vitro*, suggesting that receptors for *S. pseudintermedius* adherence which are present in *ex vivo* corneocytes are not present in undifferentiated canine epidermal cell line preparations.

Taken together, the present study provides broad new insights into the classification and evolution of the SIG, and the molecular interaction of *S. pseudintermedius* with its canine host.

Chapter 1

General Introduction

1.1 Introduction to staphylococci

The name *Staphylococcus* originates from the Greek term *staphylé* which can be translated as 'a bunch of grapes', and refers to the tendency of the bacterial cells to assemble in clusters (van Belkum *et al.*, 2009). *Staphylococcus aureus*, the best characterised member of the genus *Staphylococcus*, was first described in 1880 by a Scottish surgeon, Sir Alexander Ogston (van Belkum *et al.*, 2009).

Staphylococci are Gram-positive, catalase-positive, facultatively anaerobic, non-motile bacteria that colonise the skin and mucous membranes of mammalian and avian hosts as commensals (Murray *et al.*, 2002). Staphylococci can be divided into coagulase-positive species (CPS) such as *S. aureus* and *Staphylococcus intermedius* and coagulase-negative species (CNS) such as *Staphylococcus epidermidis* and *Staphylococcus schleiferi* (Murray *et al.*, 2002). To date, over 40 different staphylococcal species have been identified (Kwok and Chow, 2003), but only a small number of these are associated with clinical disease.

1.2 Clinical relevance of *Staphylococcus* species

Although normally commensal, several staphylococcal species can cause a variety of diseases in humans and animals. *S. aureus* is of major importance as a human pathogen causing a wide spectrum of infections of the skin, including pustules, boils, impetigo, abscesses, wound infections, conjunctivitis, and food-poisoning (Murray *et al.*, 2002). Patients who suffer from atopic dermatitis (AD), a chronic inflammatory cutaneous disease of allergic origin, are predisposed to *S. aureus* infection (Baker, 2006). Moreover, *S. aureus* may initiate severe deep infections such as septicaemia, endocarditis, pneumonia, osteomyelitis, septic arthritis, and toxic shock syndrome (Murray *et al.*, 2002; Wisplinghoff *et al.*, 2004).

Coagulase-negative staphylococci have become increasingly recognised as being important in causing human disease (von Eiff *et al.*, 2002; Wisplinghoff *et al.*, 2004). For example, *S. epidermidis* may cause bacteraemia, endocarditis, surgical wound infection, urinary tract infection and opportunistic infection of medical devices, such

as catheter-related infection (Sewell *et al.*, 1982; Murray *et al.*, 2002; von Eiff *et al.*, 2002). *Staphylococcus saprophyticus* is an important cause of urinary tract infection, especially of cystitis in young women (von Eiff *et al.*, 2002; Raz *et al.*, 2005). *Staphylococcus capitis* causes bacteraemia and endocarditis, often after colonisation of implanted medical devices (Sandoe *et al.*, 1999; Cone *et al.*, 2005). *Staphylococcus haemolyticus* infections have included bacteraemia, endocarditis, urinary tract and wound infections (Murray *et al.*, 2002), while *Staphylococcus schleiferi* and *Staphylococcus lugdunensis* have been associated with endocarditis, catheter-associated and urinary tract infections, osteomyelitis, septic arthritis, and wound infections (von Eiff *et al.*, 2002).

Several staphylococcal species are also important as pathogens of livestock and companion animals. For example, *S. aureus* is a major cause of mastitis in ruminants (Brouillette and Malouin, 2005; Barkema *et al.*, 2006; Melchior *et al.*, 2006), of skeletal infections and septicaemia in broiler chickens (Smith and Mac Namee, 2001), and of staphylococcosis in rabbits (Hermans *et al.*, 2003). Rabbit staphylococcosis may present as mastitis, subcutaneous abscesses, pododermatitis and septicaemia and is a serious epidemic and economic problem in rabbitries (Hermans *et al.*, 2003). *Staphylococcus hyicus* is another staphylococcal species of economic importance which causes exudative epidermitis (EE), a generalised cutaneous infection with greasy exudation, exfoliation, and vesicle formation in young pigs (Sato *et al.*, 1999; Werckenthin *et al.*, 2001). In companion animals, *S. intermedius* is the major cause of canine superficial pyoderma and is also associated with canine otitis externa and feline pyoderma (Werckenthin *et al.*, 2001; Gross *et al.*, 2005; Oliveira *et al.*, 2008).

1.2.1 Staphylococcal antimicrobial resistance

In human medicine, bacterial antimicrobial resistance is on the increase and methicillin-resistant *S. aureus* (MRSA) are a global health problem (Woodford, 2005; van Belkum *et al.*, 2009). For a long time, MRSA were limited to nosocomial environments (hospital-acquired MRSA, HA-MRSA), but now community-acquired MRSA (CA-MRSA) are on the increase, causing severe infections in otherwise

healthy individuals (Chambers, 2001; van Belkum *et al.*, 2009). Resistance to methicillin is encoded by the *mecA* gene which is located on staphylococcal cassette chromosome (SCC) elements which have been acquired by *S. aureus* strains through horizontal gene transfer (Enright *et al.*, 2000; Fitzgerald *et al.*, 2001; Hanssen and Ericson Sollid, 2006). The antimicrobial commonly used for the treatment of MRSA infection in humans is vancomycin. However, the first clinical MRSA isolates which are also vancomycin resistant were reported in 2002 (Srinivasan *et al.*).

The anterior nares are the major carrier site for *S. aureus* in humans, and between 10% and 35% of healthy individuals in the population are persistent carriers, 20% to 75% are intermittent carriers, and 5% to 50% are non-carriers (Kluytmans and Wertheim, 2005). *S. aureus* nasal colonisation is a multifaceted process which is still not fully understood, but seems to be influenced by the expression of bacterial virulence factors, the condition of the host (e.g. underlying disease), and by environmental factors such as the hospital or overpopulated areas (van Belkum *et al.*, 2009). Nasal carriage is a clear risk factor for *S. aureus* infection in hospitalised patients, especially since studies have shown that colonising and infecting isolates often share identical genotypes (von Eiff *et al.*, 2001). Mupirocin nasal ointment is the treatment of choice for decolonisation of the nares (Kluytmans and Wertheim, 2005; Muller *et al.*, 2005; Wertheim *et al.*, 2005), but *S. aureus* resistance to mupirocin is on the increase (Upton *et al.*, 2003).

Similar to human medicine, antimicrobial resistance of animal pathogens has emerged in the veterinary field. MRSA transmission between humans and pets has been reported on various occasions (Lloyd, 2007; Faires *et al.*, 2009) and the veterinary pathogen *S. intermedius* has been shown to be resistant to numerous antibacterial agents with some strains being multiresistant to three or more classes (Werckenthin *et al.*, 2001; Guardabassi *et al.*, 2004a; Ganiere *et al.*, 2005; Morris *et al.*, 2006b). Surveillance of *S. intermedius* with regard to antibacterial resistance has become more important over recent years and several reports describe increasing resistance in different European countries (Guardabassi *et al.*, 2004b; Lloyd, 2007). Even though sensitivity to newer antibacterial classes such as fluoroquinolones is

still high (Werckenthin *et al.*, 2001; Waller, 2005), resistance to enrofloxacin, marbofloxacin, and ciprofloxacin has been detected (Lloyd *et al.*, 1999). A study among dog and pigeon isolates from Japan reported that *S. intermedius* resistance to several classes of antibacterials was significantly higher in isolates of canine, compared to pigeon origin, but all isolates were susceptible to oxacillin (Futagawa-Saito *et al.*, 2007). Overall, *S. intermedius* resistance to β-lactam antibiotics was considered low at the beginning of the 21st century (Lloyd, 2007), but the first cases of methicillin-resistant *S. intermedius* (MRSI) had already been detected in the USA (Kania *et al.*, 2004; Waller, 2005), and a retrospective study on clinical isolates obtained from dogs in the USA between 2001 and 2005 reported a clear increase of multi-drug resistance in *S. intermedius* over this time period with 93% of the multi-drug resistant isolates from 2005 resistant to oxacillin (Jones *et al.*, 2007). In 2007, clinical cases of MRSI in dogs and a cat were first described in Europe and isolates were not only resistant to β-lactam antibiotics, but to all classes of antimicrobials commonly used in small animal practice (Loeffler *et al.*, 2007). MRSI could represent a reservoir of antimicrobial resistance which could be transferred to humans. For example, using pulsed-field gel electrophoresis (PFGE) analysis, *S. intermedius* isolates from 12 dogs suffering from deep pyoderma were compared with *S. intermedius* isolates colonising the dogs' owners, and six of the owners were carriers of the same strain as their dogs (Guardabassi *et al.*, 2004a). Furthermore, all strains tested were resistant to at least two antimicrobials, including penicillin, tetracycline, and chloramphenicol (Guardabassi *et al.*, 2004a). In summary, these reports indicate the emergence of bacterial antimicrobial resistance in veterinary medicine, and the need for appropriate screening methods and selective antimicrobial use.

1.2.2 Canine skin and keratinocyte migration and differentiation-anatomical and physiological features

Mammalian skin consists of epidermis, dermis (including the adnexae), and subcutis. The epidermis is a stratified squamous epithelium that has several cell layers comprising (from inner to outer) the stratum basale, stratum spinosum, stratum granulosum and stratum corneum (Scott *et al.*, 2001b). An additional layer, the

stratum lucidum, is present in some mammalian species at some sites, e.g. in canine footpads (Scott *et al.*, 2001b). Keratinocytes are the major cell type in the epidermis, representing approximately 85% of the total cell population; other major cell types include 3% to 8% Langerhans' cells (the function of which is antigen presentation), approximately 5% melanocytes (pigment production), and approximately 2% Merkel's cells (mechanoreceptors) (Suter *et al.*, 1997; Scott *et al.*, 2001b).

Keratinocytes undergo a precisely regulated process of differentiation as they migrate from the least differentiated basal layers to the upper layers of the skin (Suter *et al.*, 1997). Differentiation finally results in apoptosis of the epidermal cells, visible by dissolution of the nucleus and other organelles, and formation of the cornified envelope (Suter *et al.*, 1997). The cornified envelope is the insoluble outer membrane of the stratum corneum consisting of protein substrates such as loricrin which are highly cross-linked by transglutaminases (Suter *et al.*, 1997). The stratum corneum itself is a mechanically highly resistant, impermeable layer of terminal differentiated, fully keratinised keratinocytes (corneocytes) and intercellular lipids (Suter *et al.*, 1997). The epidermal cell renewal time (from stratum basale to stratum granulosum) is approximately 22 days for adult Beagle dogs (Baker *et al.*, 1973; Kwochka and Rademakers, 1989).

Keratinocyte proliferation and differentiation are distinct processes influenced by several factors produced by keratinocytes and other cutaneous cells (Suter *et al.*, 1997). For example, epidermal growth factor (EGF), transforming growth factor α (TGF-α) and interleukin-6 (IL-6) stimulate keratinocyte growth, whereas transforming growth factor β (TGF-β) and vitamin D3 inhibit proliferation (Suter *et al.*, 1997). In addition, vitamin D3 stimulates differentiation (Suter *et al.*, 1997). *In vitro* studies of human keratinocytes have shown that increased extracellular calcium concentrations induce differentiation by elevating the intracellular free calcium concentration (Sharpe *et al.*, 1989). However, the proliferation and terminal differentiation of keratinocytes is a multifaceted process that is still not fully understood. For canine keratinocytes in particular, the information available is very limited. Recently, a secreted protein, canine keratinocyte differentiation-associated

protein (cKdap), which has 87% amino acid identity with human keratinocyte differentiation-associated protein (hKdap), has been detected in canine granular keratinocytes (Yagihara *et al.*, 2006). Human Kdap is expressed in more differentiated, granular keratinocytes and is thought to be involved in the formation of the cornified envelope and desquamation (Tsuchida *et al.*, 2004).

Epidermal keratinocytes change their protein expression pattern as they migrate through the different epidermal layers. In particular, the expression of intercellular and cell matrix adhesion molecules is specific to the location of the keratinocyte (Jensen and Wheelock, 1996). Suter *et al.* (1990) have shown that antigen display on the cell surface of canine keratinocytes differs depending on the stage of differentiation. A substantial part of the intracellular cytoskeleton of epithelial cells is formed by keratins which are composed of epithelium-specific subunits (Suter *et al.*, 1997). Keratin chains or subunits are either acidic (type I) or basic (type II) and form different heterodimers, each consisting of one acidic and one basic chain (Suter *et al.*, 1997). One well-recognised difference between basal cells and suprabasal cells of the canine epidermis is that the former expresses cytokeratin 14 and the latter cytokeratin 10 (CK10) (Walter, 2001).

Epidermal keratinocytes produce an array of carbohydrates depending on their stage of differentiation. Sugars play an important role in cell-to-cell communication, being recognised by ubiquitous complex proteins called lectins (Lloyd *et al.*, 2007). Lectins are produced by host cells, but also by bacterial pathogens, and may promote bacterial adherence through interactions with carbohydrates displayed on the host cell surface (Lloyd *et al.*, 2007). In fact, adherence inhibition studies have demonstrated that polyalkylglycosides reduce *S. intermedius* adherence to canine corneocytes, unlike monosaccharides and fructoologosaccharides which have no inhibitory effect (McEwan *et al.*, 2006b).

Keratinocytes are involved in skin immunity and can promote an inflammatory response by secreting several cytokines and by displaying cytokine receptors on their cell surfaces (Lloyd *et al.*, 2007). In inflammation of the skin and wound healing,

human keratinocytes can be activated by infiltrating neutrophils and can produce antibacterial peptides, such as human cationic antimicrobial protein 18 (hCAP-18) (Borregaard *et al.*, 2005).

Canine skin has several specific features compared to other mammalian species, which may facilitate bacterial colonisation and infection. These include a thinner stratum corneum, a higher pH (between 5.5 and 8.6) and the absence of lipid follicular plugs at the entrance to the hair infundibulum (Scott *et al.*, 2001a; Patel and Forsythe, 2008).

1.2.3 Classification of the canine cutaneous bacterial flora

In canine dermatology, bacterial microorganisms present on the skin surface and hairs are descriptively categorised as residents, transients, and nomads (Saijonmaa-Koulumies and Lloyd, 1996; Scott *et al.*, 2001a; Patel and Forsythe, 2008).

Resident bacteria inhabit the skin as commensals, re-populate the host after removal and prevent colonisation by pathogenic bacteria (Saijonmaa-Koulumies and Lloyd, 1996; Scott *et al.*, 2001a). *Micrococcus* species, coagulase-negative staphylococci, *α*-haemolytic streptococci, *Propionibacterium acnes*, and *Acinetobacter* species are a few examples of bacteria considered normal residents of the surface of dog skin (Scott *et al.*, 2001a). The classification of *S. intermedius* in the context of canine dermatology has been controversial in the past (see section 1.2.4.1 below), but it should now be considered as a resident of the canine skin (Patel and Forsythe, 2008).

Transient bacteria do not naturally live and multiply on the skin, but they can become established for short periods and may be involved in pathogenic processes as secondary invaders (Scott *et al.*, 2001a; Patel and Forsythe, 2008). Bacteria such as *Escherichia coli*, *Proteus mirabilis*, *Corynebacterium* species, *Bacillus* species, and *Pseudomonas* species are examples of transient bacteria of dog skin (Scott *et al.*, 2001a).

Nomads are not normal residents of the skin, but they can establish short term colonisation and proliferation in a suitable cutaneous microenvironment and may cause disease depending on numerous factors such as a reduced host immune defence or otherwise altered surroundings (Saijonmaa-Koulumies and Lloyd, 1996; Patel and Forsythe, 2008).

1.2.4 Clinical aspects of *S. intermedius*-host interactions

1.2.4.1 *S. intermedius* in healthy dogs

Previously, opinions varied as to whether *S. intermedius* should be regarded as one of the resident or transient organisms on dog skin (Saijonmaa-Koulumies and Lloyd, 1996). In addition, it was also described as a cutaneous nomad, referring to its main locations on the hair, nose, and anus, from where it disseminates to the skin surface, colonising areas where the microenvironment is altered (Lloyd *et al.*, 1991). In recent years, the resident status of *S. intermedius* has become well accepted, and it is now considered a resident and opportunistic pathogen on canine skin and mucous membranes (Patel and Forsythe, 2008). In healthy dogs, *S. intermedius* colonises the skin, hair follicles/coat, and particularly the mucocutaneous sites such as the nose, mouth, and anus (Devriese and De Pelsmaecker, 1987; Cox *et al.*, 1988; Allaker *et al.*, 1992a; Allaker *et al.*, 1992b). *S. intermedius* colonises the skin soon after birth as demonstrated by Saijonmaa-Koulumies and Lloyd (2002b) who isolated the species from all 18 puppies examined within eight hours postnatum, probably by transfer from the dam.

1.2.4.2 Classification of canine bacterial skin infections

The term ‘pyoderma’ describes a pyogenic dermatitis which can be caused by infectious or non-infectious mechanisms (such as inflammation or neoplasia) (http://www.merckvetmanual.com, accessed on 30/08/09). However, the term ‘pyoderma’ commonly refers to a bacterial skin infection and this definition is applied henceforth in this text. Canine pyoderma is usually divided according to the depth of infection into surface, superficial, and deep categories (Scott *et al.*, 2001a).

Surface pyoderma is limited to the skin surface and is characterised by superficial erosions. Examples are intertrigo and acute moist dermatitis (Patel and Forsythe, 2008). Surface pyoderma is regarded as bacterial colonisation, which is usually managed with hygienic measures and topical antibacterials (Scott *et al.*, 2001a). Recently, a 'bacterial overgrowth syndrome' (BOG) has been described. It is a superficial skin condition caused by hyperproliferation of *S. intermedius*, which may affect local areas, larger regions or can be generalised over the skin and mucosal surfaces (Pin, 2008). The main clinical signs include pruritus and offensive odour; skin lesions may present as erythema, lichenification, hyperpigmentation, excoriations, and alopecia, whereas papules, pustules, epidermal collarettes, and crusts are lacking (Pin *et al.*, 2006; Pin, 2008). A combination of topical and systemic antibacterial therapy is appropriate in most cases as the bacterial overgrowth might affect broader skin areas than indicated by the cutaneous lesions suggesting that BOG might be a generalised condition (Pin *et al.*, 2006; Pin, 2008). Further, dogs suffering from BOG often have an underlying allergic skin condition (Pin *et al.*, 2006; Pin, 2008).

Superficial pyodermas include the epidermis and follicular epithelium; infections are most commonly follicular (superficial or deep folliculitis), although non-follicular intra- or subcorneal pustules (often termed impetigo) may also occur (Scott *et al.*, 2001a). The main physical feature is papular pyoderma, with or without often transient follicular pustules which have a variably inflamed erythematous base and are filled with white or yellow purulent material (Gross *et al.*, 2005). There are several forms of superficial pyoderma, including juvenile impetigo and folliculitis and short-haired dog pyoderma, both of which tend to affect young dogs, and superficial spreading pyoderma which is age independent (Scott *et al.*, 2001a; Gross *et al.*, 2005). If pustules are present, the existence of a central protruding hair in the pustules is strongly suggestive of bacterial folliculitis (Gross *et al.*, 2005). Follicular pustules rupture easily and develop into crusted papules, which may develop collarettes (superficial spreading pyoderma, synonym exfoliative pyoderma) (Gross *et al.*, 2005). It has been suggested that superficial spreading pyoderma may result from staphylococcal exfoliative toxin and superantigen activity (Hendricks *et al.*,

2002; Terauchi *et al.*, 2003b; Gross *et al.*, 2005). The other physical features of superficial pyoderma are variable, but pruritus is common and alopecia, erythema, and hyperpigmentation may occur (Gross *et al.*, 2005). Successful management of superficial pyoderma usually requires antibacterial shampoos in combination with systemic antibacterial therapy in all but the mildest cases (Scott *et al.*, 2001a; Patel and Forsythe, 2008). Immunomodulatory therapy is sometimes used in recalcitrant cases (Scott *et al.*, 2001a; Patel and Forsythe, 2008).

Deep pyoderma is a cutaneous bacterial infection which involves structures beyond the hair follicle, i.e. the dermis and subcutis surrounding the hair follicle (furunculosis), and is generally characterised by suppuration, sinus formation, severe pain, and pruritus (Scott *et al.*, 2001a). Deep pyoderma is commonly subclassified into a number of types, sometimes by cause (e.g. pyotraumatic folliculitis), but more commonly by site (e.g. muzzle and chin folliculitis and furunculosis, pododermatitis, and generalised deep pyoderma) (Scott *et al.*, 2001a). A combination of systemic antibacterials and topical treatment are required to control the infection (Scott *et al.*, 2001a; Patel and Forsythe, 2008).

1.2.4.3 Prevalence and treatment of canine pyoderma in veterinary practice

Dogs presented with skin problems to veterinary surgeons are commonly diagnosed with superficial pyoderma, and *S. intermedius* is the primary pathogen isolated from these cases (Medleau *et al.*, 1986; Scott *et al.*, 2001a; Gross *et al.*, 2005). Indeed, superficial bacterial pyoderma is the most frequent skin disease of dogs after flea allergy dermatitis, which it may also accompany (Gross *et al.*, 2005). Staphylococcal infections in dogs are generally triggered by an underlying condition which needs to be identified and treated along with treatment of the bacterial infection in order to reduce the possibility of relapse (Scott *et al.*, 2001a; Patel and Forsythe, 2008). Canine pyoderma has clear predilection site predispositions (the groin and axillae) (Scott *et al.*, 2001a; Patel and Forsythe, 2008) but the reasons for this are unknown, and *S. intermedius* does not adhere more avidly to corneocytes of the axillae and groin compared with other sites (Forsythe *et al.*, 2002). The most important differential diagnoses of superficial pyoderma to be considered are demodicosis,

dermatophytosis, and pemphigus foliaceus (Patel and Forsythe, 2008). Microscopic examination of pustule contents, hair, skin scrapings, bacterial and dermatophyte culture, and histopathological examination of skin biopsies are the common diagnostic tests used to make a definitive diagnosis (Gross *et al.*, 2005). Canine cutaneous staphylococcal infections may be secondary to predisposing factors, such as anatomical defects, poor grooming, or other diseases such as allergies (flea allergy dermatitis, AD, cutaneous adverse reactions to foodstuffs), non-allergic-related parasitism (demodicosis), or endocrinopathies resulting in immunosuppression (hypothyroidism, hyperadrenocorticism, diabetes mellitus) (Peikes *et al.*, 2001; Gross *et al.*, 2005; Colombo *et al.*, 2007). *S. intermedius* is also the main organism isolated from canine deep pyoderma and canine otitis externa (Gross *et al.*, 2005; Oliveira *et al.*, 2008).

Canine staphylococcal pyoderma in veterinary practice is treated with various systemic antibacterial agents such as the lincosamides (clindamycin, lincomycin), trimethoprim-potentiated sulfonamides, β-lactam antibiotics like clavulanic acid-potentiated amoxicillin and cephalosporins (e.g. cefalexin), and the fluoroquinolones (e.g. enrofloxacin, marbofloxacin) (Dillon, 1983; Frank and Kunkle, 1993; Scott *et al.*, 1998; Carlotti *et al.*, 1999; Littlewood *et al.*, 1999; Ganiere *et al.*, 2001; Horspool *et al.*, 2004). Supplementing systemic therapy, shampoos containing the antibacterial agents benzoyl peroxide, ethyl lactate, or chlorhexidine may be applied topically (Kwochka and Kowalski, 1991; Lloyd and Lamport, 1999; de Jaham, 2003). However, it is crucial in the management of canine pyoderma to identify possible underlying causes.

For the treatment of canine idiopathic recurrent pyoderma (IRP), immunomodulatory approaches, such as the use of bacterins have been developed (Foster, 2004). In the USA, a commercial bacterin derived from *S. aureus* (Staphage Lysate, SPL®, Delmont Laboratories, USA) and a preparation of *Propionibacterium acnes* (ImmunoRegulin®, Neogen Corporation, USA) are available for the treatment of IRP. Staphage Lysate was shown to increase the antibacterial response of dogs with superficial IRP significantly in a double-blind, placebo-controlled study (DeBoer *et*

al., 1990). A recent study in the UK has investigated the effect of an autogenous vaccine in the treatment of IRP (Curtis *et al.*, 2006). A bacterin was produced for each dog by culturing a *S. intermedius* isolate from each animal, and was injected subcutaneously concurrent to antibacterial treatment (Curtis *et al.*, 2006). The bacterin-receiving group showed a significantly better treatment response after a 10-week period compared to the control group (Curtis *et al.*, 2006). Immunomodulatory preparations may enhance cell-mediated immunity, but the exact mechanisms are unknown (Scott *et al.*, 2001a), and the potential of staphylococcal bacterins in the treatment of IRP needs to be investigated further (Curtis *et al.*, 2006). The *S. intermedius* autogenous vaccine is currently the only product licensed for canine use in the UK (Horvath and Neuber, 2007).

S. intermedius does not typically colonise humans but transmission between humans and their pets has been reported occasionally (Guardabassi *et al.*, 2004a), and some authors classify it as a true zoonotic pathogen (Tanner *et al.*, 2000). Importantly, *S. intermedius* has been isolated from dog bite wound infections of humans, and several cases of serious invasive infections have been reported (Talan *et al.*, 1989; Pottumarthy *et al.*, 2004).

1.2.4.4 *S. intermedius* adherence to canine corneocytes

Adherence, 'the specific attachment of microorganisms to epithelial cells' (Lloyd *et al.*, 2007), is a necessary requirement for bacterial colonisation and infection, and several studies have investigated the potential of *S. intermedius* to adhere to corneocytes from dogs and other hosts. Some data indicate that *S. intermedius* binds to corneocytes in a canine host-specific manner (Simou *et al.*, 2005b). However, another study reported that *S. intermedius* did not show any adherence preference for canine, compared with human corneocytes (Woolley *et al.*, 2008), whereas the quantitative adherence of *S. intermedius* to feline corneocytes was reported to be reduced by comparison with canine and human corneocytes (Lu and McEwan, 2007; Woolley *et al.*, 2008). Furthermore, staphylococcal adherence to canine corneocytes appears to have a predilection for certain dog breeds such as Boxers and Bull terriers (Forsythe *et al.*, 2002).

S. intermedius isolates from canine pyoderma lesions and isolates from healthy canine carriers do not significantly differ in their adherence to corneocytes from healthy dogs (Saijonmaa-Koulumies and Lloyd, 2002a) suggesting that host factors or bacterial factors other than adhesins promote infection. When comparing *S. intermedius* isolates from pyoderma pustules with isolates from macroscopically non-affected sites of the same dog, Pinchbeck *et al.* (2006) demonstrated that the majority of isolates was indistinguishable by PFGE, supporting the status of *S. intermedius* as an opportunistic pathogen.

The mechanism by which *S. intermedius* binds to canine corneocytes is unknown, but it is most likely a specific receptor-ligand interaction, as *S. intermedius* binds to corneocytes in a sigmoid dose-responsive fashion (Forsythe *et al.*, 2002), and incubation of *S. intermedius* cells with trypsin results in a dose-dependent loss of staphylococcal adherence (Simou *et al.*, 2005a).

The binding of bacteria to epidermal cells is likely to vary depending on the stage of keratinocyte differentiation, as the surface protein expression profile alters throughout differentiation. *S. aureus* adherence to human keratinocytes increases with the level of differentiation (Bibel *et al.*, 1982). Cho *et al.* (2001) noted that bacterial binding was mainly located at the stratum corneum in cryostat sections prepared from skin biopsies taken from atopic and non-atopic humans. However, a study which examined *S. aureus* adherence in a mouse keratinocyte model indicated that binding is strain-dependent, with some *S. aureus* strains binding better to differentiated keratinocytes, while others lacked any difference in binding affinity for cells at varying differentiation stages (Miyake *et al.*, 1990).

In addition to the use of corneocytes *ex vivo*, keratinocyte primary culture has been used for bacterial adherence studies. For *S. aureus*, mouse epidermal cells have been cultured from newborn CD-1 mice by a trypsin flotation procedure (Miyake *et al.*, 1990). In addition, biopsies from human skin have been used to generate keratinocyte primary cultures (Cho *et al.*, 2001). Canine skin primary cultures (and

subcultures from primary cells) have been established as a model to study the pathogenesis of dermatological diseases (Wilkinson *et al.*, 1987; Kohler *et al.*, 2001; Baumer and Kietzmann, 2007). Serra *et al.* (2007) have developed a canine skin equivalent with primary cells from abdominal skin biopsies, whereby keratinocytes were seeded onto a collagen gel bio-matrix (a mixture of type I collagen and canine fibroblasts). After 14 days of growth, four distinct cell layers comparable to canine epidermis *in vivo* could be distinguished including a cornified layer (Serra *et al.*, 2007). Recently, a canine keratinocyte cell line derived from the epidermis of an adult Beagle has become available commercially (canine epidermal keratinocyte progenitors, CPEK, CELLn-TEC Advanced Cell Systems, Switzerland). The phenotype of this cell line resembles that of basal keratinocytes, but differentiation can be induced (Yagihara *et al.*, 2009).

1.2.4.5 Canine atopic dermatitis (AD) and staphylococcal adherence

Canine AD is similar to human AD and is characterised by a type I hypersensitivity reaction (predominantly IgE antibody-associated) to environmental allergens, such as house dust mites, plant pollens, and moulds (Mc Ewan, 2000; Olivry *et al.*, 2001). The predisposition to develop AD is genetic and the main physical feature is cutaneous pruritus (Olivry *et al.*, 2001). Recently, an 'atopic-like dermatitis' (ALD) condition has been distinguished from the classic definition of AD (Halliwell, 2006; Olivry, 2008). In classic AD, IgE antibodies are detectable whereas dogs suffering from ALD show physical signs but IgE antibodies cannot be demonstrated (Halliwell, 2006; Olivry, 2008). In human medicine, the terms 'intrinsic type of AD' and 'atopiform dermatitis' (AFD) have been suggested for AD patients without allergen-specific IgE (Schmid-Grendelmeier *et al.*, 2001; Brenninkmeijer *et al.*, 2008).

Several bacterial adherence studies have focused on the comparison between normal and atopic canine skin, and found that *S. intermedius* is more prevalent on atopic compared with healthy dogs (Fazakerley *et al.*, 2009), and that *S. intermedius* adheres more avidly to corneocytes of atopic dogs, whether sampled from an inflamed area or not, compared with corneocytes of healthy animals (McEwan, 2000;

McEwan *et al.*, 2005; Simou *et al.*, 2005c; Mc Ewan *et al.*, 2006a). It is possible that AD alters the availability of cutaneous receptors and/or extracellular matrix proteins involved in staphylococcal adherence, facilitating bacterial binding (Simou *et al.*, 2005c; McEwan *et al.*, 2006a). In humans with AD, *S. aureus* colonisation is increased and the bacteria can be isolated from a high proportion of lesions (Gong 2006). It has been shown *in vitro* that adherence of *S. aureus* strains expressing the fibrinogen- and fibronectin-binding proteins Fibronectin binding protein A (FnbpA), Fibronectin binding protein B (FnbpB), Clumping factor A (ClfA), and Clumping factor B (ClfB) to skin biopsies derived from patients with AD is enhanced compared to mutant strains lacking these surface proteins, indicating a role in binding to atopic skin cells (Cho *et al.*, 2001).

1.3 Pathogenesis of staphylococcal disease

The pathogenesis of staphylococcal disease is best characterised in *S. aureus*, whereas little is known about *S. intermedius*. Because of the similarities in phenotype and disease properties between *S. aureus* and *S. intermedius*, it is informative to summarise the current knowledge about *S. aureus* pathogenesis before reviewing our understanding of *S. intermedius* biology. Therefore, virulence factors of *S. aureus* are summarised first in this section with the main focus on *S. aureus* cell wall-anchored (CWA) surface proteins. The second part discusses *S. intermedius* virulence factors identified to date.

1.3.1 Virulence factors of *S. aureus*

1.3.1.1 Enzymes

S. aureus secretes a variety of extracellular enzymes, such as coagulase, protease, hyaluronidase, lipase, nuclease, fibrinolysin, and penicillinase, which facilitate survival and colonisation in the host (Murray *et al.*, 2002; Foster, 2005).

1.3.1.2 Biofilm formation

Biofilms, i.e. multilayered cell clusters that protect the bacteria from the host's immune system and from antimicrobial agents, are formed by a variety of

staphylococcal species. Biofilms are a major problem in medical device-related infection in humans, with *S. epidermidis* and *S. aureus* being the two major pathogens isolated from these cases (Gotz, 2002; Otto, 2008). Biofilm formation consists of three stages including initial attachment, maturation, and detachment (Otto, 2008). The initial attachment is often mediated by staphylococcal surface proteins, such as ClfA, adhering to host extracellular matrix components; maturation involves intercellular aggregation caused by the polysaccharide intercellular adhesin (PIA), teichoic acids (TAs), and proteins such as the accumulation-associated protein (Aap) (Otto, 2008). PIA, TAs, and Aap provide the main structure of 'the extracellular matrix of biofilm-forming staphylococci' (traditionally referred to as 'slime') (Otto, 2008). The typical structure of a mature biofilm has been descriptively termed as 'towers' or 'mushrooms' intersected by 'fluid-filled channels' allowing nutrients to reach deeper biofilm layers (Otto, 2008). Finally, cell detachment in biofilms is important for the dissemination of bacterial cells to new sites and is promoted by surfactant-like phenol-soluble modulin (PSM) peptides, mechanical flow forces (i.e. in blood vessels), and downregulation of biofilm-promoting bacterial factors (Gotz, 2002; Otto, 2008).

1.3.1.3 Capsule

Extracellular capsular polysaccharides are produced by a wide number of invasive bacterial species, and protect the bacteria from phagocytosis by neutrophils, facilitating bacterial survival during infection (O'Riordan and Lee, 2004; Foster, 2005). Most human *S. aureus* strains produce a polysaccharide capsule, and at least 11 putative capsular serotypes have been identified (O'Riordan and Lee, 2004). Earlier studies revealed that the majority (~70%) of clinical and commensal isolates belong to capsular polysaccharide type 5 or type 8 (CP5 and CP8) (Arbeit *et al.*, 1984; Hochkeppel *et al.*, 1987).

1.3.1.4 Lipoteichoic acid and peptidoglycan

Lipoteichoic acid (LTA) and peptidoglycan (PepG) are important components of the Gram-positive bacterial cell wall, and function as both structural elements and as virulence factors (Murray *et al.*, 2002). Gram-positive organisms do not produce

lipopolysaccharides (LPS) or endotoxin which contribute to septic shock caused by Gram-negative bacteria (Thiemermann, 2002). However, studies have shown that Gram-positive bacteria, such as *S. aureus,* can cause septic shock and multiple organ failure in animal models, and also activate the release of proinflammatory cytokines in human blood *in vitro*, where LTA and PepG function synergistically (De Kimpe *et al.*, 1995; Wang *et al.*, 2000). The mechanism of synergism between LTA and PepG to cause septic shock and organ failure and their actual importance as inducers of an inflammatory response in humans *in vivo* have not yet been clarified (Thiemermann, 2002).

1.3.1.5 Cytotoxins

S. aureus produces a number of cytotoxins, such as α-haemolysin (Hla), β-haemolysin, δ-haemolysin (Hlb), γ-haemolysin (Hlg), leukocidin (LukF and LukS), and Panton-Valentine leukocidin (PVL), which target an array of host cells (Menestrina *et al.*, 2003; Kaneko and Kamio, 2004). For example, Hla lyses a variety of host cells from different species, including human leukocytes and erythrocytes and has an especially high affinity for rabbit erythrocytes (Bhakdi and Tranum-Jensen, 1991; Schmitt *et al.*, 1999). β-haemolysin causes hot-cold haemolysis of sheep erythrocytes (Haque and Baldwin, 1969) and is lethal for rabbits when intravenously applied in large doses (Gow and Robinson, 1969). It was also shown to be leukocidal for guinea pig macrophages (Chesbro *et al.*, 1965), has sphingomyelinase activity, and disrupts the host cell membrane (Gaskin *et al.*, 1997). δ-haemolysin lyses rabbit erythrocytes and is an ion-channel forming peptide that functions synergistically with β-haemolysin (Kerr *et al.*, 1995; Donvito *et al.*, 1997). However, its haemolytic activity seems to be distinct from its channel forming ability (Kerr *et al.*, 1995). Hlg is cytolytic towards erythrocytes from humans and other mammalian hosts, whereas LukF/LukS only lyse rabbit, but not human erythrocytes (Kaneko and Kamio, 2004). LukF/LukS and PVL have a cytolytic effect on leukocytes (Kaneko and Kamio, 2004).

Hla, Hlg, Luk, and PVL are pore-forming toxins. Hla is a one-component mushroom-shaped protein whereas Hlg, Luk and PVL are composed of two distinct

proteins which act together to form the active pore (Menestrina *et al.*, 2003; Kaneko and Kamio, 2004). The pore-forming toxins damage the host cell by creating a pore in the host cell membrane, which interferes with the osmotic balance and finally leads to lethal swelling of nucleated cells and osmotic lysis of erythrocytes (Schmitt *et al.*, 1999; Menestrina *et al.*, 2003; Kaneko and Kamio, 2004).

PVL has recently been the focus of intense research efforts and some authors have assigned PVL a prominent role in the pathogenesis of *S. aureus* infection especially in the development of skin abscesses and furuncles, necrotising pneumonia, and community-acquired MRSA infection (Labandeira-Rey *et al.*, 2007; van Belkum *et al.*, 2009). For example, Labandeira-Rey *et al.* (2007) reported that PVL was sufficient to cause necrotising pneumonia in mice and also noted that PVL expression interfered with global gene regulators leading to alteration of expression profiles of other virulence factors. However, a different study compared a *S. aureus* wildtype isolate belonging to the PFGE USA300 type, associated most commonly with CA-MRSA infection in the USA, to its *pvl* isogenic mutant in its potential to cause skin infection and pneumonia in mice, and concluded that there was no difference in pathogenic potential between the two strains (Bubeck Wardenburg *et al.*, 2008). Furthermore, it was noted recently that a point mutation in the accessory gene regulator, *agr*, of the *S. aureus* strain used by Labandeira-Rey *et al.* (2007) caused decreased *agr* activity, and may be responsible for the differences observed in virulence phenotypes by the different research groups (Villaruz *et al.*, 2009). Reparation of the mutation resulted in a phenotype that did not reproduce the same pathologic effects on mice that were reported previously (Villaruz *et al.*, 2009). Overall, the reports about the involvement of PVL in *S. aureus* pathogenesis remain controversial, and further studies are required to elucidate its involvement in CA-MRSA infection (van Belkum *et al.*, 2009).

1.3.1.6 Exfoliative Toxins

S. aureus produces three isoforms of exfoliative toxin, designated ETA, ETB, and ETD (Yamaguchi *et al.*, 2002; Plano, 2004; Nishifuji *et al.*, 2008). These toxins are encoded by mobile genetic elements in a minority of *S. aureus* strains which are

associated with the medical conditions Staphylococcal Scalded Skin Syndrom (SSSS) and Bullous Impetigo (BI) (Ladhani, 2003; Plano, 2004). SSSS usually affects infants and young children, and is a generalised blistering skin disorder (Ladhani, 2003). The bacteria usually colonise a distal site in the patient, e.g. the nose or a wound, and the toxins circulate in the bloodstream (Ladhani, 2003). If the patient lacks protective antibodies, the toxins reach the epidermis and cause characteristic lesions (Ladhani, 2003). The localised form of exfoliative toxinoses, BI, develops if *S. aureus* penetrates the protective barrier of the skin and exfoliative toxins, which produce local blisters, are released (Ladhani, 2003). Normally, SSSS and BI are treated with antibiotics and go into remission quickly (Ladhani, 2003). However, SSSS may occur in adults with underlying disease and can be lethal (Ladhani, 2003).

The exfoliative toxins bind to a desmosomal glycoprotein, desmoglein-1, in the epidermis and disrupt intercellular adhesion leading to blister formation (Ladhani, 2003). ETA, ETB, and ETD have been identified as serine-proteases, which specifically cleave a single peptide bond in the extracellular region of desmoglein-1 resulting in loss of intercellular adhesion of the keratinocytes in the superficial epidermal layers of humans and mice (Ladhani, 2003; Plano, 2004; Nishifuji *et al.*, 2008). In a similar manner, the exfoliative toxins ExhA, ExhB, ExhC, and ExhD of *S. hyicus* are able to cleave porcine desmoglein-1 and cause exfoliation of the porcine skin (Nishifuji *et al.*, 2008).

1.3.1.7 Staphylococcal superantigens

S. aureus produces a family of toxins that have both superantigen and emetic activity in the host (pyrogenic toxin superantigens, PTSAgs) (Dinges *et al.*, 2000). Members of the PTSAg group include staphylococcal enterotoxins and the toxic shock syndrome toxin-1 (TSST-1) (Dinges *et al.*, 2000).

1.3.1.7.1 Superantigen activity

PTSAgs interact with the adaptive immune system to stimulate non-specific T-cell proliferation (Balaban and Rasooly, 2000). Normally, antigens are processed and

presented to specific T-cells via the major histocompatibility complex (MHC), class I and II, which is displayed on the surface of antigen-presenting cells (APC) and the antigen-MHC-complex is recognised by the T-cell antigen receptor (TCR) (Balaban and Rasooly, 2000). The TCR consists of either α- and β-, or δ- and γ-chains with a variable region in the β-chain (Vβ) (Balaban and Rasooly, 2000). Each superantigen interacts with a subset of the variable Vβ part of TCRs, resulting in T-cell activation in a non-specific and potent manner (Balaban and Rasooly, 2000; Foster, 2005). The high level of T-cell activation and subsequent cytokine production leads to septic shock and toxic shock syndrome (TSS) (Foster, 2005). Furthermore, the antibody response to specific antigens may fail due to incorrect T-cell activation resulting in immunosuppression in the host (Foster, 2005).

1.3.1.7.2 Enterotoxins

Staphylococcal enterotoxins (SEs) are a subfamily of secreted superantigens causing staphylococcal food poisoning when absorbed with contaminated food (Le Loir *et al.*, 2003). After a short incubation time (2 h to 6 h) the consumption of preformed SEs leads to gastroenteritis including nausea, vomiting, diarrhoea, and abdominal pain (Balaban and Rasooly, 2000). The symptoms are normally self-limiting and decline after 24 h (Balaban and Rasooly, 2000). However, only a very small quantity of SE is necessary to cause symptoms, and the toxins are very thermostable and resist proteolytic enzymes in the digestive tract, such as pepsin or trypsin (Le Loir *et al.*, 2003).

Enterotoxins are grouped into SEs with confirmed emetic activity and enterotoxin-like toxins (SEls) that have not been proven to induce vomitus (Fraser and Proft, 2008). At least 20 different SE or SEl types have been identified in *S. aureus* to date, including the SEs A-E, G-J, and TSST-1, and the SEls K-R, U, U2, and V (Fraser and Proft, 2008). The sequence homology of SEs varies considerably, ranging from 15% between SEB and SEK to 90% amino acid identity between SEA and SEE (Fraser and Proft, 2008), but all SEs share the same predicted protein structure, consisting of two domains with a β-barrel motif and a β-grasp motif which are separated by a shallow cavity (Balaban and Rasooly, 2000; Le Loir *et al.*, 2003). The

TCR binding site is most likely to be located in the shallow cavity, whereas the emetic activity is localised on a distinct region in the protein which has not yet been identified (Balaban and Rasooly, 2000; Foster, 2005). The most common toxin involved in staphylococcal food poisoning is SEA, followed by SED (Balaban and Rasooly, 2000). Most of the SEs are encoded on mobile genetic elements and some are regulated by the *agr* system (Le Loir *et al.*, 2003).

1.3.1.7.3 Toxic Shock Syndrome Toxin-1 (TSST-1)

TSST-1 causes the symptoms of TSS, an acute illness with high fever, hypotension, diffuse erythematous rash, desquamation of the skin, involvement of at least three organ systems, and potential lethal outcome (Dinges *et al.*, 2000). A major epidemic of TSS in young women occurred in the 1980s associated with menstruation, tampon-use, and the isolation of *S. aureus* from vaginal and cervical sites (Dinges *et al.*, 2000). Subsequently, TSS has also been observed in patients secondary to childbirth, wound-infection, influenza, and AIDS (Dinges *et al.*, 2000).

TSST-1 strongly activates T-cells and releases cytokines, especially tumor necrosis factor alpha (TNF-α) and other vasoactive mediators, that cause severe hypotension (Dinges *et al.*, 2000). TSST-1 may also bind directly to endothelial receptors enhancing hypotension (Dinges *et al.*, 2000). Moreover, TSST-1 has been shown to increase sensitivity to endotoxin when injected in rabbits (Schlievert, 1982). This hypersensitivity effect may be caused by TSST-1 cytotoxicity to hepatocytes and subsequently reduced hepatic clearance of circulating toxins (Dinges *et al.*, 2000).

TSST-1 is encoded by a family of staphylococcal pathogenicity islands (SaPIs), and is expressed as a precursor protein which becomes activated after cleavage of a signal sequence (Dinges *et al.*, 2000). The TSST-1 protein structure is similar to the SE protein structure and consists of an A domain composed of a central α-helix surrounded by a five-strand β-sheet and a B domain of five β-strands which form a barrel motif (Dinges *et al.*, 2000).

1.3.1.7.4 Superantigen-like proteins

Screening of *S. aureus* genomes revealed a group of genes encoding for proteins that share two conserved amino acid motifs located at the N- and C-terminus, respectively, with superantigens, but do not stimulate T-cell proliferation and are not able to bind MHC class II molecules (Fraser and Proft, 2008). These superantigen-like proteins, SSLs, interfere with the host innate immune response and several of the 14 SSLs encoded on *S. aureus* genomes have been characterised (Fraser and Proft, 2008). For example, SSL-7 interacts with Immunoglobulin A, IgA, by blocking the IgA leukocyte receptor, enhancing bacterial survival on mucosal membranes, whereas SSL-5 and SSL-11 inhibit adherence of neutrophils (Fraser and Proft, 2008).

1.3.1.8 Chemotaxis inhibitory protein of staphylococci

Chemotaxis inhibitory protein of staphylococci (CHIPS) is a secreted protein, which protects *S. aureus* from the host immune response by interfering with neutrophil chemotaxis and extravasation to the site of inflammation (de Haas *et al.*, 2004; Foster, 2005). When bacteria colonise a site in the host, chemoattractants such as bacterial formylated peptides and the complement system components C3a and C5a are released and attract neutrophils to fight the infection (Foster, 2005). The CHIPS protein can bind to the formyl peptide receptor (FPR) and the C5a receptor (C5aR) on the neutrophil surface and block neutrophil activity (de Haas *et al.*, 2004; Foster, 2005). Another protein interacting with neutrophil activation is the extracellular adherence protein (Eap), which may act synergistically with CHIPS (Foster, 2005).

1.3.1.9 *S. aureus* adherence-mediating surface proteins

S. aureus was traditionally considered to be an extracellular pathogen (Finlay and Cossart, 1997), but more recent studies have characterised the role of *S. aureus* as an intracellular invader (Lowy, 2000; Reilly *et al.*, 2000; Ellington *et al.*, 2003; Sinha and Herrmann, 2005). The microorganism is now considered to be facultatively intracellular (Sinha and Herrmann, 2005). The initial adherence of *S. aureus* to host tissue is a crucial step in infection (Patti *et al.*, 1994a; Foster and Hook, 1998). An array of surface proteins are made by *S. aureus* and function as adhesins in the

infection process by mediating binding to the extracellular matrix (ECM) and serum components in the host (Hauck and Ohlsen, 2006). The ECM is a complex network of proteins and carbohydrates which fills the intercellular space in host tissue (Lodish *et al.*, 2000). It embeds the cells, supports cell-coherence and creates an optimal environment for cell growth and differentiation, for instance by storing important hormones (Lodish *et al.*, 2000).

Two major groups of adhesive proteins can be distinguished in Gram-positive bacteria. Firstly, proteins which are not covalently anchored to the bacterial cell wall and become released in the culture supernatant, named SERAMs (*secretable expanded repertoire adhesive molecules*) (Chavakis *et al.*, 2005; Hauck and Ohlsen, 2006). Secondly, the MSCRAMM family (*microbial surface components recognizing adhesive matrix molecules*) includes proteins which remain linked to the bacterial surface (Foster and Hook, 1998; Hauck and Ohlsen, 2006).

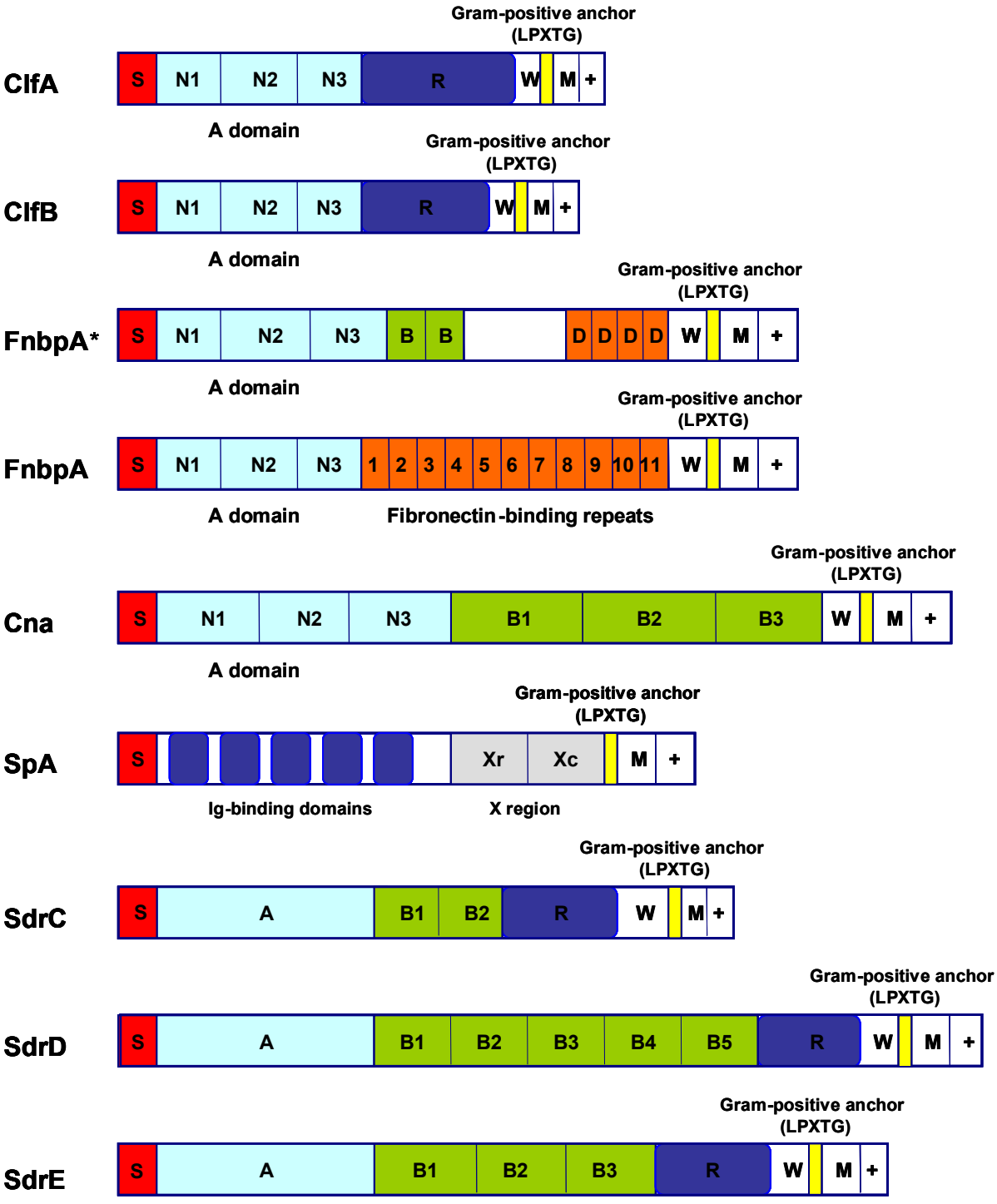
1.3.1.9.1 Secretable expanded repertoire adhesive molecules (SERAMs)

SERAMs are predominantly involved in endothelium infection (Chavakis *et al.*, 2005). For example, the extracellular adherence protein (Eap) is expressed by *S. aureus* during the late exponential phase (Harraghy *et al.*, 2003), and is controlled by the regulatory systems *agr* and staphylococcal accessory regulator (*sar*) (Dunman *et al.*, 2001). Analysis of *S. aureus* strain FDA574 revealed that Eap consists of 689 aa, including a 110 aa sequence which is repeated six times (Jonsson *et al.*, 1995). Eap has been shown to enhance *S. aureus* adherence to epithelial cells and to fibroblasts, and to support bacterial internalisation by these eukaryotic cells (Hussain *et al.*, 2002; Kreikemeyer *et al.*, 2002). Eap binds to several host plasma proteins, such as fibrinogen (Fg), fibronectin (Fn), and prothrombin (Palma *et al.*, 1999). Further, it acts as an anti-inflammatory factor on endothelial cells through interference with the recruitment of neutrophils leading to inflammation by binding to intercellular adhesion molecule 1 (ICAM-1) which is crucial for leukocyte binding (Chavakis *et al.*, 2002). The ~70 kDa Eap protein is expressed by the majority of *S. aureus* clinical isolates and may play a role in the pathogenesis of chronic rather than acute infections (Chavakis *et al.*, 2002; Lee *et al.*, 2002; Harraghy *et al.*, 2003).

Other examples of SERAMs include extracellular fibrinogen binding protein (Efb), which interacts with the Fg α-chain and the complement factor C3b; extracellular matrix binding protein (Emp), which binds to Fg, Fn, and vitronectin; von Willebrand factor binding protein (vWbp); and coagulase (Coa), which interacts with Fg and prothrombin (Hauck and Ohlsen, 2006).

1.3.1.9.2 Microbial surface components recognising adhesive matrix molecules (MSCRAMMs)

MSCRAMMs of *S. aureus* share common features in their primary protein structure (Figure 1.1). The amino-terminal possesses a ~40 aa signal sequence which is necessary for Sec-dependent protein secretion (Foster and Hook, 1998). The signal sequence is followed by a unique, non-repetitive sequence (A domain) responsible for ligand-binding in most MSCRAMMs (Patti *et al.*, 1994a; Foster and Hook, 1998). The C-terminus contains a wall-spanning region (W-region) and a hydrophobic membrane-spanning domain (M-region) followed by positively charged residues (Foster and Hook, 1998). A characteristic of MSCRAMMs is the presence of a conserved LPXTG motif which anchors the protein to the cell wall (Patti *et al.*, 1994a; Foster and Hook, 1998). The motif is cleaved between threonine and glycine residues by a transpeptidase called sortase (Foster and Hook, 1998). Subsequently, the carboxyl group of threonine is covalently linked to an amino group of a branched peptide in the peptidoglycan layer of the cell wall (Ton-That *et al.*, 1997). Hence, the surface protein is anchored to the cell wall and can only be released by a murolytic enzyme such as the glycyl-glycine endopeptidase lysostaphin (Foster and Hook, 1998). A subgroup of MSCRAMMs shares the presence of a domain mainly consisting of serine-aspartate (Ser-Asp) dipeptide repeats (McDevitt *et al.*, 1994; Foster and Hook, 1998). This region is called the R domain and is situated between the wall-spanning region W and the A domain in the protein structure (Figure 1.1) (Foster and Hook, 1998). Its presence enables the A domain to fully interact in ligand binding by acting as a molecular stalk (Hartford *et al.*, 1997). Surface proteins which contain the Ser-Asp dipeptide region can be classified as the MSCRAMM Sdr-family (Foster and Hook, 1998).


Gram-positive anchor (LPXTG)
ClfA
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A domain
Gram-positive anchor (LPXTG)
ClfB
S
N1
N2
N3
R
W
M
+
A domain
Gram-positive anchor (LPXTG)
FnbpA*
S
N1
N2
N3
B
B
D
D
D
D
W
M
+
A domain
Gram-positive anchor (LPXTG)
FnbpA
S
N1
N2
N3
1
2
3
4
5
6
7
8
9
10
11
W
M
+
A domain
Fibronectin-binding repeats
Gram-positive anchor (LPXTG)
Cna
S
N1
N2
N3
B1
B2
B3
W
M
+
A domain
Gram-positive anchor (LPXTG)
SpA
S
Xr
Xc
M
+
Ig-binding domains
X region
Gram-positive anchor (LPXTG)
SdrC
S
A
B1
B2
R
W
M
+
Gram-positive anchor (LPXTG)
SdrD
S
A
B1
B2
B3
B4
B5
R
W
M
+
Gram-positive anchor (LPXTG)
SdrE
S
A
B1
B2
B3
R
W
M
+

Figure 1.1. Schematic diagram of the domain structure of several MSCRAMMs of *S. aureus*. ClfA, clumping factor A; ClfB, clumping factor B; FnbpA, fibronectin-binding protein A (the asterisk indicates the traditional organisation structure, the new structure is given underneath); Cna, collagen-binding protein; SpA, *S. aureus* Protein A; SdrC, SdrD, SdrE, Ser-Asp dipeptide (SD) repeat-containing proteins C, D, and E; 'S' corresponds to the signal sequence (red); 'A', 'B', and 'D' stand for A, B, and D domains (light blue, green, and orange, respectively); 'R' corresponds to the Ser-Asp dipeptide repeats (dark blue); N1, N2, and N3 subdomains of the A domain are indicated; 'W' represents the wall-spanning region; 'M' represents the membrane-spanning region; '+' stands for positively charged residues at the C-terminal; Fn-binding repeats of FnbpA (orange), Ig-binding domains (dark blue), and X region of SpA (grey) are labelled; 'Xr' signifies an octapeptide repeat; 'Xc' signifies a non-repeated region; 'LPXTG' specifies the positions of the LPXTG-motif in the protein sequence (yellow). [Adapted from Foster and Hook, 1998; Keane *et al.*, 2007b.]

Examination of the completed genome sequences of *S. aureus* has resulted in the identification of over 20 putative MSCRAMM genes (Foster and Hook, 1998; Roche *et al.*, 2003; Hauck and Ohlsen, 2006). For example, four different Fg-binding proteins (ClfA, ClfB, FnbpA, FnbpB) have been characterised to date. Further examples of MSCRAMMs include Collagen binding protein (Cna), Elastin binding protein (EbpS), staphylococcal Protein A (SpA), Bone sialoprotein binding protein (Bbp), and the Sdr-proteins SdrC, SdrD, and SdrE (Foster and Hook, 1998; Hauck and Ohlsen, 2006). The function of these proteins and their role in pathogenesis is the subject of intensive research efforts.

1.3.1.9.2.1 Clumping Factor A (ClfA)

One of the best characterised MSCRAMMs of *S. aureus* is ClfA. ClfA can bind to soluble and immobilised Fg and fibrin (Table 1.1) (McDevitt *et al.*, 1994) causing cell clumping and tissue adherence, and is an important virulence factor, especially in staphylococcal adhesion to implanted medical devices (Cheung and Fischetti, 1990), polymeric biomaterials (Vaudaux *et al.*, 1995), and wound-associated infections (Villavicencio and Wall, 1996). Furthermore, ClfA has been shown to play a role in the pathogenesis of staphylococcal septic arthritis (Josefsson *et al.*, 2001) and infective endocarditis (Moreillon *et al.*, 1995; Stutzmann Meier *et al.*, 2001). Additionally, binding to soluble Fg in plasma assists *S. aureus* to escape from opsonophagocytosis by neutrophils (Higgins *et al.*, 2006).

ClfA has a typical MSCRAMM structure, including an R domain (Figure 1.1) which varies in size in different strains and a conserved A domain (McDevitt and Foster, 1995). The A domain contains a ~330 aa sequence required for ligand binding (McDevitt and Foster, 1995). The C-terminal part of the A domain (residues 500-559) has been found to be important for ClfA function, and antibodies specific for this region are able to reduce Fg binding (Hartford *et al.*, 2001). Substitution of the adjacent residues Glu_{526} and Val_{527} results in an almost total loss of Fg binding (Hartford *et al.*, 2001). Furthermore, the residues Pro_{336} and Tyr_{338} may be crucial in ClfA binding to Fg (Deivanayagam *et al.*, 2002) and the expression of Pro_{336} and Tyr_{338} substitution mutants fails to mediate adherence to Fg compared to the

Table 1.1. Specified MSCRAMMs of *S. aureus* and their ligands.

MSCRAMM[a]	Ligand[b]						
	Fg	**Fn**	**En**	**T-En**	**Cn**	**CK10**	**vWF**
ClfA	+						
ClfB	+					+	
FnbpA	+	+	+	+			
FnbpB	+	+	+				
Cna					+		
Ebps			+	+			
SpA							+

[a]ClfA, clumping factor A; ClfB, clumping factor B; FnbpA, fibronectin-binding protein A; FnbpB, fibronectin-binding protein B; Cna, collagen-binding protein; Ebps, elastin-binding protein of *S. aureus*; SpA, staphylococcal Protein A.

[b]Fg, fibrinogen; Fn, fibronectin; En, elastin; T-En, tropoelastin; Cn, collagen; CK10, cytokeratin 10; vWF, von Willebrand factor; '+' indicates adherence.

wildtype ClfA in *Lactococcus lactis* (Loughman *et al.*, 2005). ClfA mutants, either lacking Pro_{336} and Tyr_{338} or carrying amino acid substitutions in these positions demonstrated reduced virulence in mouse infection models of septic arthritis and sepsis (Josefsson *et al.*, 2008).

Deivanayagam *et al.* (2002) have solved the crystal structure of the minimum truncate of ClfA which supports Fg-binding activity. The authors identified a hydrophobic pocket within the A domain which may represent the ligand-binding site (Deivanayagam *et al.*, 2002). Interestingly, the Val_{527} residue was found to be situated within the pocket (Deivanayagam *et al.*, 2002). Analysis of the crystal structure revealed three subdomains within the A domain (N1, N2, and N3) (Deivanayagam *et al.*, 2002). Whereas N1 maybe sensitive to protease cleavage, N2 and N3 describe a region of ~320 residues which form the Fg-binding site (Deivanayagam *et al.*, 2002; Rivera *et al.*, 2007).

The mechanism of ClfA binding to Fg may be similar to the 'dock, lock, and latch' mechanism which was proposed for SdrG, a Fg-binding surface protein of *S. epidermidis*, and a member of the Sdr-family (Ponnuraj *et al.*, 2003; Bowden *et al.*, 2008). This model is thought to function as follows: The C-terminus of the Fg molecule initially 'docks' into the hydrophobic pocket formed by N2 and N3 and subsequently a structural rearrangement from an open to a closed conformation of SdrG takes place which 'locks and latches' the Fg onto the bacterial protein (Ponnuraj *et al.*, 2003). Interestingly, ClfA does not require an open conformation and binds Fg with higher affinity in a stabilised closed conformation (Ganesh *et al.*, 2008). Therefore, the binding mechanism of ClfA has been described as a 'latch and dock' variant of the 'dock, lock, and latch' mechanism of SdrG (Ganesh *et al.*, 2008).

The Ser-Asp dipeptide region (R region) of ClfA appears to be essential for the A domain to bind Fg (Hartford *et al.*, 1997). Deletion of the R region results in an approximately 500-fold reduction in the cell clumping titre of ClfA (Hartford *et al.*, 1997). However, the length of the R region can be truncated to 80 amino acids without a decrease in the clumping titre (Hartford *et al.*, 1997). Hartford *et al.* (1997)

suggested that the R region may be important for spanning the peptidoglycan of the cell wall and for positioning the A domain at the right distance from the cell surface for optimal Fg-binding (Hartford *et al.*, 1997). This hypothesis is supported by the observation that the R region of ClfA maintains functional surface expression of the collagen-binding protein Cna in a chimeric protein (Hartford *et al.*, 1999).

The presence of certain metal cations has an influence on the Fg-binding capacity of ClfA. For example, calcium (Ca^{2+}) and manganese (Mn^{2+}) are able to inhibit binding of Fg to the A domain whereas magnesium (Mg^{2+}) and monovalent cations do not have an inhibitory effect (O'Connell *et al.*, 1998). O'Connell *et al.* (1998) predicted that ClfA has a binding site for Ca^{2+} and Mn^{2+} which, when occupied, leads to a conformational change in ClfA resulting in a Fg-binding defect. It has been proposed that this inhibitory site is situated within an EF-hand motif of the A domain (O'Connell *et al.*, 1998). However, the display of the ClfA N2-N3 domain crystal structure does not indicate the presence of a classical EF-hand (a β-turn followed by a β-strand) (Deivanayagam *et al.*, 2002). Nevertheless, three metal-ion binding sites are revealed and their organisation and function remain to be characterised (Deivanayagam *et al.*, 2002). The N2 and N3 subdomains are folded in a similar way and each resembles a constant Immunoglobulin G-fold (C-type IgG-fold) with two additional β-strands (Deivanayagam *et al.*, 2002). The C-type IgG-fold consists of ABED strands on the front and GFC strands on the back of the fold whereas two additional β-strands are located between the D and E strands of the N2 and N3 domains of ClfA, termed D′ and D″ (Figure 1.2) (Deivanayagam *et al.*, 2002). The novel IgG-fold identified in ClfA was termed DEv-IgG variant and based on secondary structure predictions, was proposed to be present in other Fg-binding MSCRAMMs, namely ClfB, FnbpA, and FnbpB (Deivanayagam *et al.*, 2002).

Fg, an abundant blood plasma protein, consists of two sets of α-, β-, and γ-polypeptide chains, joined together through disulfide bridges (Figure 1.3) (Mosesson, 2005). The blood enzyme thrombin cleaves Fg to form fibrin, and initiates fibrin polymerisation (Mosesson, 2005). Fg and fibrin are important in blood-clotting, wound healing, and cellular and matrix interactions (Mosesson, 2005). The C-

terminus of the Fg γ-chain has been identified as the binding site for ClfA (Figure 1.3) (McDevitt *et al.*, 1997). Interestingly, this binding site also interacts with the integrin $\alpha_{IIb}\beta_3$ receptor on the surface of human platelets (Hawiger *et al.*, 1982; Kloczewiak *et al.*, 1984; McDevitt *et al.*, 1997). The result of Fg binding to the integrin receptor is platelet aggregation and platelet-fibrin thrombi formation (Farrell *et al.*, 1992; Rooney *et al.*, 1996). Even though ClfA and the integrin $\alpha_{IIb}\beta_3$ receptor target the same Fg segment, they interact with different residues and ClfA binding can be inhibited with synthesised mutated peptides of the Fg binding site that do not interfere with the binding activity of the integrin receptor (Ganesh *et al.*, 2008).

As discussed, ClfA is an important virulence factor in *S. aureus* infection, and it is a potential immunotherapeutic target for prevention and treatment of *S. aureus* infection. For example, recombinant ClfA has been used successfully to protect mice against septic arthritis, and passive immunisation with ClfA antibodies has been shown to prevent sepsis-induced death in an animal model (Josefsson *et al.*, 2001). Furthermore, a murine monoclonal antibody (mAb 12-9), specific for ClfA, reduced *S. aureus* binding to Fg and protected mice from sepsis-induced death (Hall *et al.*, 2003). A humanised version of mAb 12-9 (Aurexis®) protected rabbits challenged with an MRSA strain against bacteraemia (Patti, 2004). Aurexis® has been tested in clinical trials with healthy individuals and in patients with documented *S. aureus* bacteraemia, and might become a valuable adjunctive therapeutic in the treatment of *S. aureus* infection in the future (Weems *et al.*, 2006). However, a recent study employing mouse models of *S. aureus* bacteraemia and wound infection concluded that *S. aureus* expression of ClfA *in vivo* was strain- and time-dependent and therefore ClfA was unlikely to be sufficiently protective in a single-antigen-based vaccine (Nanra *et al.*, 2009).

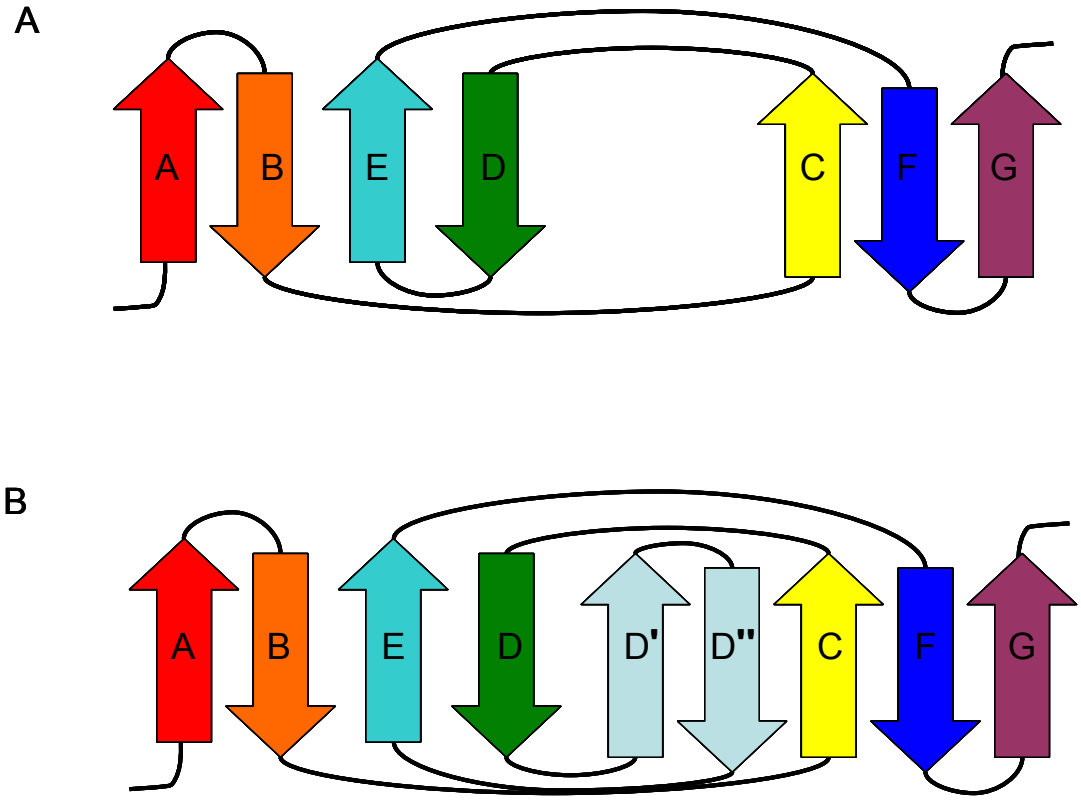


Figure 1.2. Schematic diagram of the (A) constant IgG-fold and the (B) IgG-fold variant of ClfA, designated DEv-IgG variant. The constant IgG-fold consists of strands A (red), B (orange), C (yellow), D (green), E (turquoise), F (dark blue), and G (purple). The DEv-IgG variant has two additional β-strands, D` and D`` (light blue), located between the D and E strands of the N2 and N3 domains of ClfA. [Adapted from Deivanayagam *et al.*, 2002.]

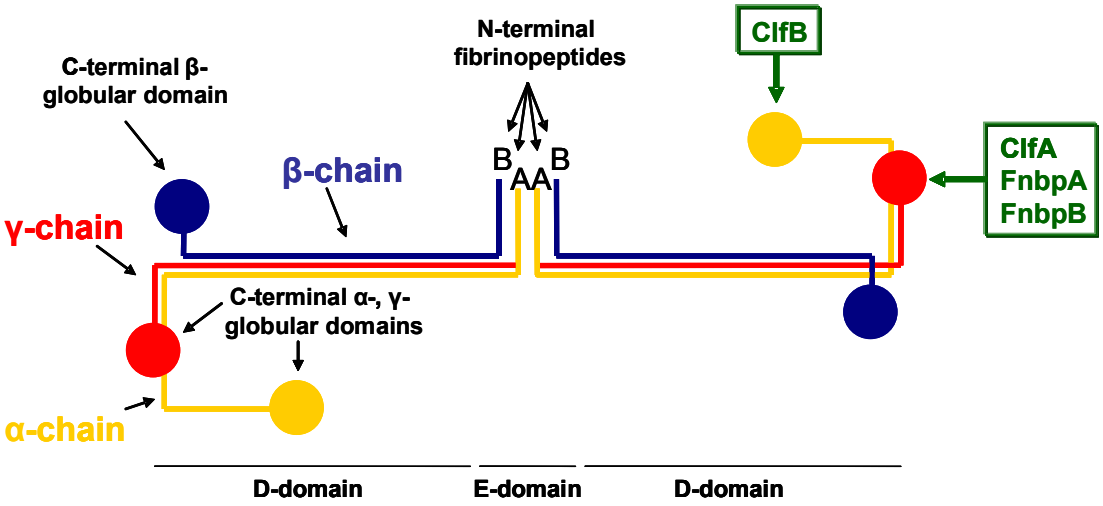


Figure 1.3. Schematic diagram of the structure of fibrinogen (Fg). The Fg molecule consists of two D domains and a central E domain containing pairs of Aα-, Bβ-, and γ-peptide chains with N-terminal fibrinopeptides and C-terminal globular domains. The α-, β-, and γ-chains are linked by disulfide bonds. The binding regions for the *S. aureus* MSCRAMMs ClfA, FnbpA, FnbpB, and ClfB are indicated. [Adapted from Rivera *et al.*, 2007.]

1.3.1.9.2.2 Clumping Factor B (ClfB)

ClfB of *S. aureus* also has the ability to clump bacteria in a solution of Fg and to mediate bacterial adherence to immobilised Fg (Table 1.1) and *ex vivo* medical devices (Ni Eidhin *et al.*, 1998). Unlike ClfA, ClfB is only expressed in the exponential phase of bacterial growth in strain Newman, and is absent in the stationary phase (Ni Eidhin *et al.*, 1998). Apparently, transcription ceases towards the stationary phase and the ClfB expressed on the cell surface is susceptible to cleavage by aureolysin, a metalloprotease (McAleese *et al.*, 2001).

Even though both clumping factors are similar in their structural organisation (Figure 1.1) and function, the *clfA* and *clfB* genes are distinct (Foster and Hook, 1998). Comparing the two proteins reveals 41% identity for the signal sequence, 47% for the wall-anchoring region, but only 26% for the A domain (Ni Eidhin *et al.*, 1998). Moreover, ClfB binds to the Aα-chain rather than to the γ-chain of human Fg (Figure 1.3) (Walsh *et al.*, 2008). The ClfB binding site has been located to residues 316 to 328 of the αC domain of the Aα-chain which also plays an important role in haemostasis and wound healing (Walsh *et al.*, 2008). Like ClfA, ClfB interaction with Fg can be inhibited by the divalent cations Ca^{2+} and Mn^{2+} (Ni Eidhin *et al.*, 1998). The R region is located at a similar site in ClfA and ClfB (Figure 1.1) (Hartford *et al.*, 1997), and ClfB has an abbreviated EF-hand motif in its A domain (Ni Eidhin *et al.*, 1998). Similar to ClfA, the A domain of ClfB is responsible for ligand-binding (Walsh *et al.*, 2004). The A domain consists of three subdomains N1, N2, and N3 (Perkins *et al.*, 2001), and for the binding process, the 'dock, lock, and latch' model has been suggested as described for ClfA (Ponnuraj *et al.*, 2003; Walsh *et al.*, 2008).

It has been suggested that ClfA and ClfB function synergistically to mediate bacterial attachment especially in certain environments such as the blood stream or cardiac valves (Ni Eidhin *et al.*, 1998). However, ClfB has been reported to have a limited role in an experimental endocarditis animal model (Entenza *et al.*, 2000).

The most consistent site for the isolation of *S. aureus* from human carriers are the anterior nares (Kluytmans and Wertheim, 2005). ClfB has been demonstrated to play an important role in nasal colonisation of *S. aureus* by mediating adherence to desquamated nasal epithelial cells (O'Brien *et al.*, 2002b). Specifically, ClfB interacts with CK10 (Table 1.1) expressed on the surface of epithelial cells (O'Brien *et al.*, 2002b). Keratins are structural proteins which can be classified into type I and type II (Fuchs, 1995). Type I keratins involve the subtype CK10 and tend to be smaller and more acidic than type II keratins (Fuchs, 1995). The CK10 molecule contains three different domains, including a head-, central α-helical rod-, and tail region (Figure 1.4) (Walsh *et al.*, 2004). The A domain of ClfB interacts with the C-terminal tail domains of CK10 (Walsh *et al.*, 2004).

Fg and CK10 both interact with the A domain of ClfB, and it has been demonstrated that ClfB mutants with amino acid substitutions in the putative latching sequence are defective in their ability to bind to CK10 and to Fg, suggesting that they share the same binding site and that they may bind with a similar mechanism (Walsh *et al.*, 2008).

Since ClfB is an important virulence factor in nasal colonisation, its immunotherapeutic potential has been investigated. Mice immunised with a recombinant vaccine of the A domain of ClfB have reduced levels of nasal colonisation compared to a control group (Schaffer *et al.*, 2006). The same effect was observed after passive immunisation with a monoclonal antibody against ClfB (Schaffer *et al.*, 2006). In a human colonisation model, the anterior nares of 16 healthy human volunteers were inoculated experimentally with *S. aureus* expressing ClfB and a *S. aureus* mutant deficient in ClfB expression (Wertheim *et al.*, 2008). The *S. aureus* strain lacking ClfB was eradicated from the human nares significantly faster than the wildtype strain (Wertheim *et al.*, 2008).

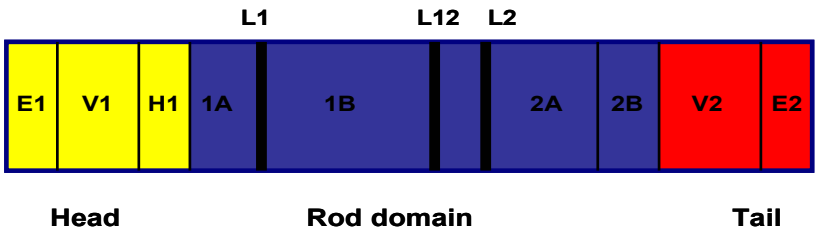


Figure 1.4. Schematic diagram of the structure of cytokeratin 10 (CK10). The CK10 molecule consists of a central helical rod domain (blue), and non-helical N-terminal and C-terminal regions, termed head (yellow) and tail (red). The rod domain contains helical subdomains (1A, 1B, 2A, 2B), which are divided by non-helical linker regions (L1, L12, L2). The N-terminal head domain consists of subdomains E1 (serine- and glycine rich), V1 (a quasi-repeat region), and H1 (conserved in type I keratins). The C-terminal tail region is composed of subdomains V2 (a quasi-repeat region and variable in size), and a conserved E2 domain. [Adapted from Walsh *et al.*, 2004.]

1.3.1.9.2.3 The fibronectin-binding MSCRAMMs FnbpA and FnbpB

S. aureus expresses two Fn-binding proteins, FnbpA and FnbpB, on the cell surface (Flock *et al.*, 1987; Signas *et al.*, 1989; Jonsson *et al.*, 1991). Both Fnbps are expressed during the exponential phase of bacterial growth (Saravia-Otten *et al.*, 1997). They are able to bind to Fn (Greene *et al.*, 1995), Fg (Wann *et al.*, 2000), elastin (Roche *et al.*, 2004), and FnbpA has been recently shown to interact with tropoelastin (Keane *et al.*, 2007a) (Table 1.1).

The role of FnbpA and FnbpB in pathogenesis is multifaceted. They mediate adherence to the endothelium and consequently promote internalisation of the bacteria by endothelial cells (Peacock *et al.*, 1999). This step may be crucial in the infection process of deeper tissues (Peacock *et al.*, 1999). Furthermore, their impact on the pathogenesis of endocarditis has been investigated. Whereas older studies are contradictory (Kuypers and Proctor, 1989; Flock *et al.*, 1996), more recent studies indicate that Fnbps act as major virulence factors in infective endocarditis (IE), possibly in conjunction with ClfA (Que *et al.*, 2001; Que *et al.*, 2005). Fnbps may play an important role in IE by causing platelet activation, resulting in bacteria-platelet aggregation and thrombi on cardiac valves (Fitzgerald *et al.*, 2006). Furthermore, FnbpA and FnbpB, but not ClfA, mediate adherence of *L. lactis* to human cultured endothelial cells, supporting their putative role in the pathogenesis of IE (Heying *et al.*, 2007). FnbpA and FnbpB, in addition to ClfA, mediate adherence to polyurethane membranes of ventricular assist devices *in vitro* and in a mouse model, when expressed individually on the surface of *L. lactis*, suggesting a major contribution in *S. aureus* infection of medical devices (Arrecubieta *et al.*, 2006).

Fnbps may be important in causing orthopaedic infections (Nair *et al.*, 2000). The prevalence of *fnbA* and *fnbB* genes in *S. aureus* strains responsible for orthopaedic infections is extremely high (98.4% to 99.5%) (Arciola *et al.*, 2005). Moreover, *S. aureus* is a major pathogen of bone, causing septic arthritis and osteomyelitis, and mutants lacking the *fnb* genes lose their ability to be internalised by osteoblasts (Ahmed *et al.*, 2001) suggesting an important role in virulence. Fnbps also mediate adherence of *S. aureus* in animal mastitis. *In vitro*, these MSCRAMMs are

fundamental for the infection of bovine mammary gland cells (Lammers *et al.*, 1999) and *in vivo*, they promote staphylococcal colonisation of murine mammary glands (Brouillette *et al.*, 2003). Additionally, Fnbp-negative mutants show reduced binding to human AD skin biopsies *ex vivo* indicating a role in the colonisation of patients suffering from AD (Cho *et al.*, 2001).

FnbpA and FnbpB have a typical MSCRAMM structure (Figure 1.1) (Signas *et al.*, 1989; Jonsson *et al.*, 1991). The *fnbA* and *fnbB* genes are homologous in large regions of their sequences, and the FnbpA and FnbpB A domains share 45% sequence identity (Jonsson *et al.*, 1991; Roche *et al.*, 2004). However, FnbpA contains two B repeat regions (B1 and B2) located next to the C-terminal side of the A domain, which are not found in FnbpB (Signas *et al.*, 1989).

The traditional structural organisation of FnbpA and FnbpB includes three complete and one partial D repeat domain (D1, D2, D3, D4) and early studies reported that each repeat functions as a Fn-binding region (Signas *et al.*, 1989). Interestingly, the D domains lack a folded secondary structure, but undergo a conformational change into a more ordered structure upon ligand-binding (House-Pompeo *et al.*, 1996; Foster and Hook, 1998). However, it was reported previously that FnbpA has a second Fn-binding site outside the D repeats (Joh *et al.*, 1998; Williams *et al.*, 2002) and more recent work has shown that FnbpA contains 11 putative Fn-binding domains (FnbpA-1 to FnbpA-11) which bind Fn through a tandem β-zipper and are located within the A to D regions (Figure 1.1) (Schwarz-Linek *et al.*, 2003; Schwarz-Linek *et al.*, 2006). The Fn-binding domains consist of Fn-binding repeats, FnBRs, each containing short homologous motifs (Schwarz-Linek *et al.*, 2003) and it was suggested that the motifs within each FnBR have to be arranged in a particular order to be functional (Schwarz-Linek *et al.*, 2006). Fn is a glycoprotein that is present in blood plasma and other extracellular fluids, in connective tissues, and on many cell surfaces (Mosesson and Amrani, 1980). Fn mainly consists of Fn type 1, 2, and 3 (F1, F2, and F3) modules (Pankov and Yamada, 2002), and the bacterial binding region is located at the N-terminal domain of Fn (NTD) and contains five F1 modules (Figure 1.5) (Schwarz-Linek *et al.*, 2003; Schwarz-Linek *et al.*, 2006).

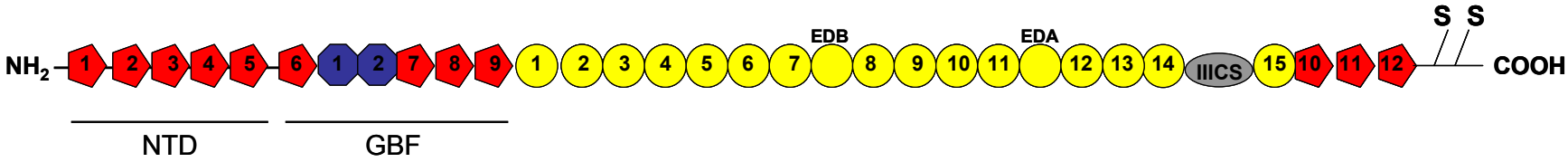


Figure 1.5. Schematic diagram of the structure of fibronectin (Fn). Fn mainly consists of type I (F1, red), type II (F2, blue), and type III (F3, yellow) modules (termed Fn repeats). The Fn monomer contains 12 F1-, 2 F2-, and 15-17 F3-modules and normally forms a dimer with another Fn molecule via disulfide bonds at the C-terminus. The Fn molecule is encoded by a single gene, but alternative splicing of a pre-mRNA results in different variants, alternatively spliced regions are indicated (EDB, extra domain B; EDA, extra domain A; IIICS, type III connecting segment). The N-terminal domain (NTD), consisting of type I modules (^{1}F1-^{5}F1), interacts with Fibronectin-binding protein A, FnbpA, of *S. aureus*. GBF, gelatin-binding fragment. [Adapted from Pankov *et al.*, 2002; Schwarz-Linek *et al.*, 2004.]

The three-dimensional structure of the tandem β-zipper binding model was first revealed for the synthetic peptide B3, derived from the B3 repeat of FnBB, a Fn-binding surface protein of *Streptococcus dysgalactiae*, in complex with two of the Fn type 1 modules (^{1}F1 ^{2}F1) (Schwarz-Linek *et al.*, 2001; Schwarz-Linek *et al.*, 2003). The F1 modules share a consensus fold of 'a double-stranded antiparallel β-sheet', 'folded over a triple-stranded antiparallel β-sheet' (Schwarz-Linek *et al.*, 2004), and the B3 peptide creates a fourth antiparallel strand to the triple-strand antiparallel β-sheet, leading to the tandem β-zipper formation (Schwarz-Linek *et al.*, 2003).

The number of FnBRs may be strain-dependent, but the majority of *S. aureus* isolates carry *fnbA* and *fnbB* genes encoding for 11 FnBRs in FnbpA and 10 in FnbpB, respectively (Schwarz-Linek *et al.*, 2006). Based on the interaction of B3 from *S. dysgalactiae* with ^{1}F1 and ^{2}F1, it was noted that the N-terminal half of the B3 peptide binding to the ^{2}F1 module was only about a quarter of the typical FnBR in length and it was concluded that each FnBR may interact with four F1 modules in the NTD of Fn (Schwarz-Linek *et al.*, 2006). Therefore, a functional FnBR was defined as 'a linear array of specific ^{n}F1-binding segments, arranged in the correct order to bind 1:1 to the NTD of Fn in an anti-parallel fashion' (Schwarz-Linek *et al.*, 2006). Recently, the crystal structures of two FnBRs, FnbpA-1 and FnbpA-5, in complex with Fn F1 modules were revealed and it was confirmed that each FnBR segment interacts with four F1 modules (Bingham *et al.*, 2008).

Fn interacts with heparin, fibrin, collagen, and cell-surface structures in regulating cell adhesion (Mosesson and Amrani, 1980). Cell adhesion of Fn is mediated via the integrin $(\alpha_5)\beta_1$, present on the surface of many mammalian cells including epithelial and endothelial cells (Sinha *et al.*, 1999). This interaction may serve as a bridge to the Fnbps of *S. aureus* allowing cellular invasion by the bacteria (Dziewanowska *et al.*, 1999; Sinha *et al.*, 1999; Fowler *et al.*, 2000; Sinha *et al.*, 2000). In fact, Fnbps are sufficient and required to allow *S. aureus* invasion of the host cell (Sinha *et al.*, 2000; Sinha and Herrmann, 2005). Furthermore, a recent study reported that the presence of at least one FnBR module is sufficient to mediate *L. lactis* adherence to human endothelial cells *in vitro* (Heying *et al.*, 2009).

The Fg-binding site of FnbpA and FnbpB is situated within the A domain, which binds to the γ-chain of Fg (Figure 1.3) (Wann *et al.*, 2000). Roche *et al.* (2004) have demonstrated that FnbpA and FnbpB mediate elastin-binding through their A domains. Elastin is a hydrophobic polymer, consisting of covalently cross-linked precursor-molecules called tropoelastin (Rodgers and Weiss, 2005). Elastin is a component of the ECM that confers resilience and elasticity to body tissues such as the lung, aorta, and the skin (Rodgers and Weiss, 2005). Therefore, the binding of Fnbps to elastin may promote staphylococcal infection of these tissues (Roche *et al.*, 2004). Additionally, binding of FnbpA to the elastin precursor tropoelastin was reported recently suggesting that FnbpA may interfere with elastogenesis during infection (Keane *et al.*, 2007a).

Fg and elastin interact with the same region of the A domain of FnbpA, and the 'dock, lock, and latch' binding mechanism was suggested for both extracellular matrix proteins based on structure modelling and functional analysis (Keane *et al.*, 2007b). The A domain of FnbpA (in particular the N2 and N3 subdomains) is highly divergent and seven different isotypes were identified by DNA hybridisation of the *fnbA* gene among an array of *S. aureus* strains representing common MLST sequence types (Loughman *et al.*, 2008). Of note, the region containing the putative ligand-binding trench was conserved and each A domain isotype was able to bind to Fg and elastin (Loughman *et al.*, 2008). Further, sequence comparison of the Fn-binding regions of FnbpA based on published *S. aureus* genomes revealed that FnBRs (namely FnbpA-2 to FnbpA-11) were conserved, indicating that the sequence variation of FnbpA is largely restricted to the N2-N3 subdomains (Loughman *et al.*, 2008).

The A domains of ClfA, FnbpA, and SdrG of *S. epidermidis* all interact with Fg and share a highly conserved secondary structure despite only ~20% amino acid identity (Rivera *et al.*, 2007). Resolving the crystal structures of the Fg-binding domains of ClfA and SdrG revealed the presence of two similar variants of the IgG-like fold located in the N2-N3 subdomains of both proteins (Deivanayagam *et al.*, 2002; Ponnuraj *et al.*, 2003). The structure of the ligand-binding N2-N3 subdomains of

FnbpA is predicted to be similar to ClfA based on molecular modelling supporting the hypothesis that ClfA, FnbpA, and SdrG may bind Fg in a similar fashion (Deivanayagam *et al.*, 2002; Rivera *et al.*, 2007). Furthermore, FnbpA and ClfA may share the same critical residues for Fg-binding (Keane *et al.*, 2007b).

1.3.1.9.2.4 The collagen-binding MSCRAMM Cna

S. aureus expresses at least one MSCRAMM that can bind to collagen (collagen-binding protein, Cna) (Table 1.1) (Switalski *et al.*, 1989; Patti *et al.*, 1992). Collagen is a major component of the ECM of connective tissues and the interstitial tissue of parenchymal organs mediating stability and structure to tissues and organs (Gelse *et al.*, 2003). At least 26 distinct genes encoding for different collagen types have been identified but all collagen types share a characteristic triple helix consisting of three polypeptide chains (Gelse *et al.*, 2003). Type I collagen is a major component of tendons, skin, ligaments, cornea, and of the organic mass of bone, and is the best characterised of the collagens (Gelse *et al.*, 2003).

Cna is an important virulence factor in the pathogenesis of *S. aureus* bone and joint infections. In a murine animal model, more than 70% of mice infected with a Cna-positive strain showed symptoms of septic arthritis compared to only 27% challenged with a Cna-negative strain (Patti *et al.*, 1994b). However, in a study investigating the prevalence of *cna* in strains isolated from orthopaedic infections, only 46% of the strains were positive for *cna* (Arciola *et al.*, 2005). The presence of Cna does not seem to be essential for *S. aureus* to infect joints and bones but helps to establish and maintain infection (Elasri *et al.*, 2002; Xu *et al.*, 2004). In poultry, vaccination with a recombinant fragment of Cna was protective against septic death, and reduced bone colonisation (Smeltzer and Gillaspy, 2000). However, a Cna-based DNA vaccine was not protective against *S. aureus* in a mouse model of intra-peritoneal infection even though an antibody response to the vaccine was induced (Therrien *et al.*, 2007). A different study investigated the antigenic potential of Cna in combination with ClfA, FnbpA, and the SERAM protein Efb in a multiple plasmid DNA vaccine approach in a mastitis infection model (Castagliuolo *et al.*, 2006). Polyvalent serum from mice after exposure to the DNA vaccine was reported to significantly reduce *S.*

aureus adherence to bovine mammary gland epithelial cells *in vitro*, and immunised mice were protected significantly against *S. aureus* challenge in a mastitis model (Castagliuolo *et al.*, 2006).

Cna is important in the formation of thrombi in the bloodstream, since it mediates adherence of *S. aureus* to exposed collagen on the subendothelium (Mascari and Ross, 2003). Similar, Cna serves as a virulence factor in keratitis, enhancing suppurative inflammation in a rabbit model (Rhem *et al.*, 2000). Surprisingly, Cna does not appear to contribute to the increased binding of *S. aureus* to atopic skin (Cho *et al.*, 2001).

Cna has the typical structure of an MSCRAMM (Figure 1.1). Cna possesses B domains between the A domain and the wall-spanning region which each consist of 187 aa, and are repeated between one and four times, depending on the strain (Foster and Hook, 1998). The function of the B repeats is unknown but lack of the B domains does not alter the collagen-binding activity of Cna (Hartford *et al.*, 1999). It has been suggested that the B repeats may help to present the A domain away from the bacterial surface (Deivanayagam *et al.*, 2000) because the capsule of heavily encapsulated strains can mask and inhibit Cna function, if the protein is expressed too close to the cell surface (Gillaspy *et al.*, 1998).

The crystal structure of the collagen-binding subdomain of Cna within the A domain has been solved and consists of two anti-parallel β-sheets and two short α-helices (Symersky *et al.*, 1997). One of the β-sheets has a 'binding trench' which can hold a collagen triple helix (Symersky *et al.*, 1997; Rich *et al.*, 1999). The A domain can bind to different collagen types, and the collagen triple helices have multiple binding sites for the A domain of Cna (Foster and Hook, 1998). The complete A domain shows a higher affinity to collagen than the subdomain (Foster and Hook, 1998). Therefore, a novel binding mechanism has been suggested which is termed the 'Collagen Hug' model (Zong *et al.*, 2005). The authors divide the A domain into three subdomains N1, N2, and N3, in a similar fashion to ClfA and ClfB (Zong *et al.*, 2005). They found that the collagen-binding affinity of the two subdomains N1 and

N2 is even higher than the affinity of the whole A domain (Zong *et al.*, 2005). In their model, N1 and N2 work together to enclose and 'hug' the collagen triple helix (Zong *et al.*, 2005).

1.3.1.9.2.5 The elastin-binding MSCRAMM EbpS

In addition to the Fnbps, *S. aureus* expresses another elastin-interacting MSCRAMM, the elastin-binding protein of *S. aureus*, EbpS (Table 1.1) (Park *et al.*, 1991; Park *et al.*, 1996). EbpS is an atypical member of the MSCRAMM family, as it is deficient in an N-terminal signal sequence and an LPXTG motif (Downer *et al.*, 2002). Furthermore, EbpS is an integral membrane protein, rather than a CWA surface protein, which contains three hydrophobic domains H1, H2, and H3 (Figure 1.6) (Downer *et al.*, 2002). Only the N-terminus of the protein is surface exposed and involved in ligand-binding (Downer *et al.*, 2002). The elastin-binding domain has been assigned to a 21 aa region within the N-terminus of EbpS (Park *et al.*, 1999). The EbpS binding site of tropoelastin has been located within the N-terminal region, and is distinct from the binding site for elastin receptors on mammalian cells (Park *et al.*, 1991).

EbpS was originally identified in strain Cowan (Park *et al.*, 1991; Park *et al.*, 1996), but the *ebps* gene is present in all 25 *S. aureus* strains examined in a prevalence study of known adhesin genes (Smeltzer *et al.*, 1997). The function of EbpS has not been clarified yet but may enable *S. aureus* to specifically colonise elastin-rich tissues such as lungs or the aorta (Downer *et al.*, 2002).

1.3.1.9.2.6 Protein A (SpA)

S. aureus Protein A (SpA) is one of the best characterised surface proteins of Gram-positive bacteria. Originally, it was noteworthy for its ability to bind to Immunoglobulin G (IgG) and it was the first immunoglobulin-binding protein to be discovered, that was not part of the mammalian immune system (Forsgren and Sjoquist, 1966). SpA contains four or five ligand-binding domains, depending on the

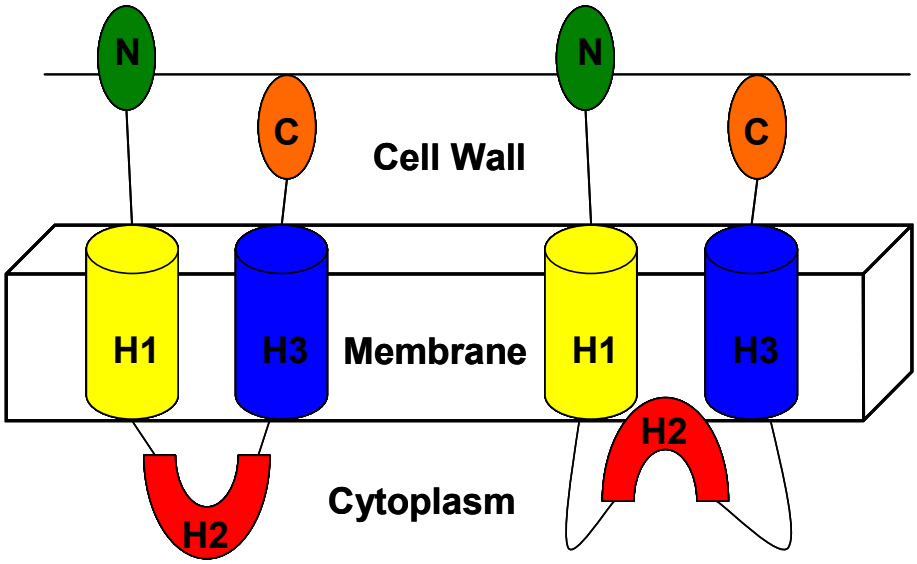


Figure 1.6. Schematic diagram demonstrating two possible EbpS (elastin-binding protein of *S. aureus*) conformations. EbpS is an integral membrane protein; N indicates the surface-exposed N-terminal (green); C indicates the C-terminal (orange). H1 (yellow), H2 (red), and H3 (blue) are hydrophobic domains with H1 and H3 being transmembrane domains and H2 being in one of the two shown orientations. [Adapted from Downer *et al.*, 2002.]

S. aureus strain, which are exposed on the cell surface (Uhlen *et al.*, 1984; Navarre and Schneewind, 1999; Foster, 2005). Each domain binds to the Fc-region of IgG (Navarre and Schneewind, 1999; Foster, 2005) leading to occupation of the Fc-region of the immunoglobulin and lack of recognition by neutrophil Fc receptors (Foster, 2005). The anti-phagocytic effect of SpA may contribute to host immune avoidance (Foster, 2005). SpA is typically expressed in the exponential phase of growth, since expression is regulated by bacterial regulation systems, including *agr* and *sar* (Cheung *et al.*, 1997; Gao and Stewart, 2004).

SpA has the typical structure of an MSCRAMM (Figure 1.1), but due to the lack of a specific ligand was not considered an MSCRAMM until the late 1990s (Foster and Hook, 1998). The discovery that SpA binds von Willebrand factor finally classified it as a member of the MSCRAMM family (Table 1.1) (Hartleib *et al.*, 2000). Von Willebrand factor (vWF), a multimeric glycoprotein, is present in blood plasma, platelets, endothelial cells, and the subendothelial matrix of the vessel wall (Ruggeri and Ware, 1993). After endovascular trauma, vWF mediates platelet adhesion and aggregation to the injured tissue (Ruggeri and Ware, 1993). This interaction results in the formation of platelet thrombi and in haemostasis (Ruggeri and Ware, 1993). SpA binds to soluble vWF and also supports *S. aureus* adhesion to vWF-adsorbed surfaces (Herrmann *et al.*, 1997; Hartleib *et al.*, 2000) and so is likely to have an impact on the pathogenesis of intravascular *S. aureus* disease (Herrmann *et al.*, 1997; Foster and Hook, 1998; Hartleib *et al.*, 2000). SpA of *S. aureus* strain Newman was shown to increase the severity of septic arthritis and septic death in an animal model (Palmqvist *et al.*, 2002). SpA was not able to induce platelet aggregation independently when expressed in *L. lactis* but may enhance aggregation when displayed on the staphylococcal cell surface as *S. aureus* double-mutants lacking *clfA* and *spa* demonstrated reduced ability to activate platelet aggregation compared to wildtype and *clfA* single-mutant strains (O'Brien *et al.*, 2002a).

A specific feature of Protein A is the presence of the X region, a highly variable area consisting of a repeat region (X_r) and a non-repetitive C-terminal domain (X_c) (Figure 1.1) (Guss *et al.*, 1984, Uhlen *et al.*, 1984). The X_r region is encoded by a

variable number of 24 bp repeats with conserved flanking regions, and the nucleotide sequence variation of the hypervariable X region provides the basis for a well-used typing approach for *S. aureus* isolates (*spa* typing) (Shopsin *et al.*, 1999; Koreen *et al.*, 2004).

1.3.1.9.2.7 The serine-aspartate dipeptide repeat (Sdr)-family of *S. aureus*

Staphylococcal surface proteins which contain the Ser-Asp dipeptide repeat region (R region) form a subgroup within MSCRAMM proteins known as the Sdr-family (Foster and Hook, 1998). Members of the Sdr-family identified in *S. aureus* to date are ClfA (McDevitt *et al.*, 1994), ClfB (Ni Eidhin *et al.*, 1998), the Ser-Asp dipeptide (SD) repeat-containing proteins SdrC, SdrD, SdrE (Josefsson *et al.*, 1998a), and a bone sialoprotein-binding protein (Bbp) (Tung *et al.*, 2000).

ClfA and ClfB are the prototypes of the Sdr-family. *S. aureus* expresses SdrC, SdrD, and SdrE which have the same structural organisation as ClfA and ClfB, but contain two to five additional B repeats (Figure 1.1) (Foster and Hook, 1998, Josefsson *et al.*, 1998a). The B repeats are located between the A domain and the R region and each include a putative EF-hand for high affinity Ca^{2+} binding (Josefsson *et al.*, 1998a; Josefsson *et al.*, 1998b). The binding of Ca^{2+} appears to be essential for the structural integrity of the B repeats as they collapse when Ca^{2+} is removed (Josefsson *et al.*, 1998b). However, the function of the B domains has not been identified yet (Josefsson *et al.*, 1998a; Josefsson *et al.*, 1998b).

The A domains of the Sdr-members are less than 30% identical to each other, suggesting a common origin but a distinct function (Foster and Hook, 1998, Josefsson *et al.*, 1998a). SdrE has been demonstrated to stimulate human platelet aggregation when expressed in *L. lactis* (O'Brien *et al.*, 2002a) but a ligand for SdrE has not been identified yet. *S. aureus* strain Newman mutants lacking SdrC and SdrD colonise the nares of mice to a similar extent as the wildtype strain (Schaffer *et al.*, 2006). However, it has been reported recently that adherence to human desquamated epithelial cells of *S. aureus* strain Newman mutants deficient in either SdrC or SdrD expression was reduced compared to the wildtype (Corrigan *et al.*, 2009). Further,

expression of SdrC and SdrD in *L. lactis* mediated lactococcal binding to human desquamated epithelial cells suggesting that SdrC and SdrD may have an important function in *S. aureus* adherence to the human nares (Corrigan *et al.*, 2009). In fact, Corrigan *et al.* (2009) demonstrated that a *S. aureus* strain Newman derivative, lacking ClfB, SdrC, SdrD, and the iron regulated surface determinant protein A (IsdA), was completely defective in adherence to human desquamated epithelial cells. The *S. aureus* surface protein IsdA binds to proteins of the cornified envelope, such as involucrin, loricrin, and CK10 (Clarke *et al.*, 2009) and mediates binding to nasal cells *in vitro* (Corrigan *et al.*, 2009).

A previous study investigated the distribution of the *sdr* genes among 497 invasive and non-invasive *S. aureus* strains (Sabat *et al.*, 2006). At least one *sdr* gene (*sdrC)* was shown to be present in all strains (Sabat *et al.*, 2006). Another study examined *S. aureus* isolates for a number of virulence factor genes and found the *sdrC* gene to be present in all isolates, whereas *sdrD* and *sdrE* were absent in 56% and 52%, respectively, of the carriage and invasive isolates investigated (Peacock *et al.*, 2002).

Another member of the Sdr-family of *S. aureus* is the bone sialoprotein-binding protein (Bbp) (Tung *et al.*, 2000). Bbp has been shown to interact with bone sialoprotein, a glycoprotein that is part of the bone and dental ECM (Yacoub *et al.*, 1994). The binding site for *S. aureus* within the bone sialoprotein has been identified as a nonapeptide sequence at the N-terminus of the protein (Ryden *et al.*, 1997). In comparison with other members of the Sdr-family, Bbp is most similar to SdrE with 76% identity in the A domain and 95% to 96% identity in the B region, suggesting that they may be allelic variants of the same protein (Tung *et al.*, 2000; Sabat *et al.*, 2006).

1.3.1.9.2.8 Serine-aspartate dipeptide repeat (Sdr)-proteins of other Staphylococci

Sdr-proteins have been identified in a number of other *Staphylococcus* species. For example, *S. epidermidis*, a common cause of foreign-body and medical device-related infections, contains genes encoding SdrF, SdrG, and SdrH (McCrea *et al.*,

2000). SdrF mediates adherence to type I collagen and to explanted transcutaneous drivelines from ventricular assist devices, when expressed on the surface of *L. lactis*, indicating an important role in the pathogenesis of medical device-related infections (Arrecubieta *et al.*, 2007; Arrecubieta *et al.*, 2009). SdrF has the typical MSCRAMM structure including four B repeats, located between the A domain and the Sdr region, similar to *S. aureus* SdrC, SdrD, and SdrE (McCrea *et al.*, 2000). Of note, the SdrF binding site to collagen is located at the B domain, and each of the four B repeats is able to bind to collagen independently (Arrecubieta *et al.*, 2007). A putative ligand for the A domain of SdrF has not been identified to date. SdrG of *S. epidermidis* interacts with the Bβ-chain of Fg through its A domain (Davis *et al.*, 2001) and was the first MSCRAMM for which the 'dock, lock, and latch' binding mechanism was suggested (Ponnuraj *et al.*, 2003; Bowden *et al.*, 2008). SdrH has a unique structure with a 277 residue long region (termed C-region) located at the C-terminal side of the Sdr region, and lacks an LPXTG-anchor motif (McCrea *et al.*, 2000). No function or ligand has been assigned to SdrH to date.

S. capitis, an important pathogen of very-low-birth-weight infants, expresses SdrX, an Sdr-protein which has the ability to bind to human collagen type VI (Liu *et al.*, 2004), and *S. saprophyticus*, involved in urinary tract infections in young women, produces SdrI, a collagen-binding Sdr-protein (Sakinc *et al.*, 2006).

1.3.2 Virulence factors of *S. intermedius*

Similar to *S. aureus*, *S. intermedius* produces a variety of virulence factors including enzymes such as coagulase, protease, and thermonuclease, surface proteins such as clumping factor and Protein A, and toxins such as cytotoxins, exfoliative toxin, and enterotoxins (Hajek, 1976; Raus and Love, 1983; Terauchi *et al.*, 2003b; Futagawa-Saito *et al.*, 2004a; Futagawa-Saito *et al.*, 2004b; Futagawa-Saito *et al.*, 2006). In addition, *S. intermedius* has also been shown to form biofilms (Futagawa-Saito *et al.*, 2006). However, our knowledge about the pathogenesis of *S. intermedius* is very limited and most virulence factors have not been characterised in detail.

1.3.2.1 *S. intermedius* cytotoxins

S. intermedius produces α-haemolysin and β-haemolysin and causes haemolysis of rabbit erythrocytes and hot-cold haemolysis of sheep erythrocytes (Hajek, 1976; Dziewanowska *et al.*, 1996; Futagawa-Saito *et al.*, 2006). As for *S. aureus*, the β-haemolysin has sphingomyelinase activity with a high affinity to sphingomyelin (Dziewanowska *et al.*, 1996). Similar to PVL in *S. aureus*, *S. intermedius* also produces a bicomponent leukotoxin, Luk-I, which is encoded by two co-transcribed genes, *lukS* and *lukF* (Prevost *et al.*, 1995; Futagawa-Saito *et al.*, 2004a). Luk-I has been shown to be leukotoxic for polymorphonuclear cells but only slightly haemolytic for rabbit red blood cells (Prevost *et al.*, 1995). Futagawa-Saito *et al.* (2004a) have compared the prevalence of the *luk-I* locus in canine and pigeon strains and interestingly, while all canine isolates were positive for *lukS* and *lukF* and were highly cytotoxic for rabbit leukocytes, all pigeon strains were positive for *lukS*, but not for *lukF*, and showed only low cytotoxicity.

1.3.2.2 *S. intermedius* exfoliative toxin

S. intermedius exfoliative toxin (SIET) is a protein of 330 aa, including a 42 aa signal peptide and has an approximate molecular weight of 30 kDa (Terauchi *et al.*, 2003a). SIET has been shown to have a rounding effect on cultured epithelial cells based on culture filtrates of 60 *S. intermedius* strains from canine pyoderma cases (Terauchi *et al.*, 2003b). SIET was further tested in animal models by subcutaneous injection and an exfoliative effect was observed in one-day-old chickens, hamsters, and dogs, but not in rats and mice (Terauchi *et al.*, 2003a; Terauchi *et al.*, 2003b). The dogs injected with SIET developed clinical signs, such as erythema, exfoliation, and crusting, which are similar to symptoms seen in canine pyoderma, human SSSS, and porcine EE (Terauchi *et al.*, 2003b). Investigation of the prevalence of the *siet* gene by PCR screening of 45 *S. intermedius* isolates from dogs detected the *siet* gene in 21 of the isolates, which were predominantly from skin/wound infections and otitis externa (Lautz *et al.*, 2006).

1.3.2.3 *S. intermedius* superantigens

S. intermedius has been demonstrated to produce proteins which cross-react with antibodies raised against the staphylococcal superantigens SEA, SEB, SEC, SED, and TSST-1 (Hendricks *et al.*, 2002). Edwards *et al.* (1997) have identified a canine type C enterotoxin (SEC_{canine}) from canine pyoderma isolates, which is distinct from other SEs, but shares their ability to induce vomitus and T-cell proliferation. The prevalence of sec_{canine} in *S. intermedius* populations is variable. Whereas Becker *et al.* (2001) identified 31 of a total of 247 canine *S. intermedius* isolates positive for the sec_{canine} gene, Futagawa-Saito *et al.* (2004b) screened 106 pigeon and canine strains and found only a single sec_{canine}-positive strain. Instead, Futagawa-Saito *et al.* (2004b) identified a novel enterotoxin-related gene, *se-int*, which was detected in all 44 canine strains tested by this group, but has not been further characterised. *S. intermedius* was reported to be responsible for a food poisoning outbreak in the USA (Khambaty *et al.*, 1994) but to date, the enterotoxigenic potential of *S. intermedius* has not been fully clarified. Furthermore, staphylococcal superantigens SEA and SEB have been shown to increase T-cell blastogenesis in peripheral canine blood mononuclear cells *in vitro*, but the relevance of this in the pathogenesis of canine pyoderma remains unclear (Hendricks *et al.*, 2002).

1.3.3 Quorum sensing in Staphylococci

Bacteria have intra- and extracellular signalling systems, which allow cell-to-cell communication (quorum sensing), monitoring of the presence of other bacteria, and adaptation of gene expression to changes in population density (Camilli and Bassler, 2006). For quorum sensing, bacteria release small molecules, called autoinducers, which are detected by other bacteria and the producing organism itself, and once a certain threshold of autoinducer concentration has been reached a signal transduction cascade in the cells is initiated leading to alteration of gene expression in the whole population (Camilli and Bassler, 2006).

1.3.3.1 Global regulation of virulence factors in *S. aureus*

In *S. aureus*, the expression of virulence factors varies according to growth phase and growth conditions; the *S. aureus* virulon is regulated by global regulatory systems, such as *agr* and *sarA* (Novick and Geisinger, 2008). The *agr* regulator is part of a complex regulatory network which becomes activated in a population-density dependent manner in mid-exponential phase leading to upregulation of secreted exoproteins and downregulation of CWA surface proteins (Novick and Geisinger, 2008). Several *S. aureus* virulence factors are upregulated by the *agr* system, including cytotoxins, capsular polysaccharides 5 and 8, the majority of superantigens, and many enzymes (Novick, 2003; Bronner *et al.*, 2004). Conversely, several surface proteins, such as SpA, FnbpA, and FnbpB are downregulated (Novick, 2003; Bronner *et al.*, 2004). The virulence of *S. aureus* mutants lacking either *agr*, *sarA*, or both regulatory systems is considerably reduced compared to the isogenic wildtype strain (George and Muir, 2007).

1.3.3.1.1 The accessory gene regulator, *agr*

The *agr* regulator is a two-component system, consisting of a transmembrane receptor, sensing an extracellular signal, and an intracellular effector, causing a signal response. The ~3 kb *agr* locus consists of two divergent transcription units, RNA II and RNA III, with two distinct promoters, P2 and P3. The RNA II transcription unit, driven by P2, contains four distinct genes, *agrB*, *agrD*, *agrC,* and *agrA,* encoding for the two-component system, AgrC and AgrA, and its autoinducing ligand (Figure 1.7). The autoinducing peptide, AIP, is encoded as a propeptide by *agrD* and processed to its active form through proteolytic digestion by AgrB, an anchored membrane protein. The mature AIP consists of seven to nine amino acid long peptides which are secreted into the extracellular environment. Subsequently, AIP binds to the cell surface of the secreting-, and other bacterial cells via a transmembrane receptor, AgrC. The binding of AIP leads to a conformational change in the cytoplasmic histidine kinase domain at the C-terminus of AgrC, resulting in the phosphorylation of a cytoplasmic protein, AgrA. The conformational change induced in AgrA enables it to bind to the *agr* promoters P2 and P3, leading to

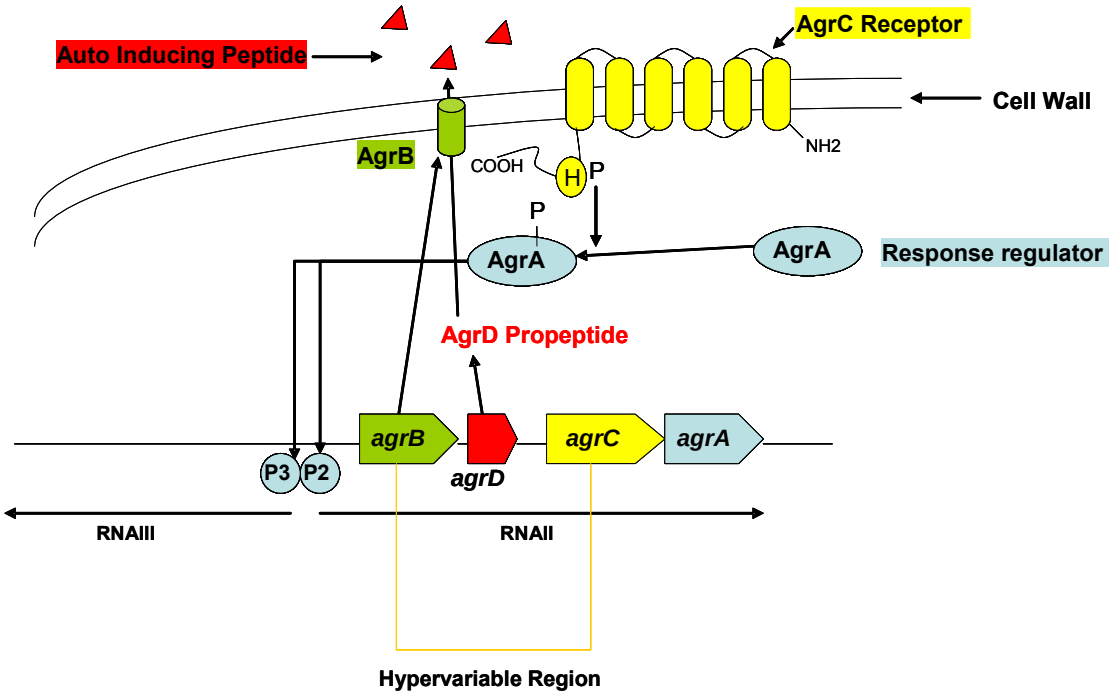


Figure 1.7. Schematic diagram of the global accessory gene regulator (*agr*) of *S. aureus*. The *agr* locus consists of two divergent operons P2 and P3 (blue) with two transcripts RNA II and RNA III. The P2 operon contains four distinct genes: *agrD* (red) encodes the propeptide of the auto inducing peptide (AIP; red); *agrC* (yellow) encodes the transmembrane receptor for AIP; *agrB* (green) encodes for AgrB (green) which processes the AIP propeptide; *agrA* (blue) encodes for the response regulator AgrA (blue) which upregulates transcription of the two promoters P2 and P3. The hypervariable region within the *agr* locus is indicated. [Adapted from Dufour *et al.*, 2002.]

promoter activation. (This section is based on reviews of the *agr* global regulatory system by Novick (2003) and Bronner *et al.* (2004)).

The RNA III transcript, driven by P3, encodes for a regulatory RNA molecule, RNA III, and also contains a single gene, *hld*, encoding for δ-haemolysin. The RNA III molecule consists of 514 nucleotides, organised in a complex secondary structure containing 14 β-hairpins and three long-range interactions. The RNA III molecule is the effector of the *agr* operon which initiates upregulation of the transcription of many genes encoding extracellular proteins, and downregulation of many surface protein genes by direct interaction or by interference with transcription regulators, such as SarT and SarS (Novick, 2003; Bronner *et al.*, 2004)

1.3.3.1.2 The staphylococcal accessory regulator, *sarA*

The *sarA* locus encodes for the SarA protein, a DNA binding protein which regulates exoprotein and surface protein expression by two different pathways. Firstly, SarA, among other regulatory genes, interacts directly with *agr* through binding to the P2 and P3 promoter regions of the *agr* locus, increasing RNA II and RNA III transcription (*agr*-dependent pathway) (Novick and Geisinger, 2008). SarA maintains basal expression of *agr* until the threshold for autogenous activation is reached (George and Muir, 2007). Secondly, SarA can bind directly to conserved regions ('Sar boxes') within the promoter regions of genes encoding several exo-, and surface proteins, altering transcription (*agr*-independent pathway) (Bronner *et al.*, 2004). For example, SarA upregulates *fnbpA*, *fnbpB*, *hla*, *hld*, and *hlg*, but downregulates *spa* and *cna* gene expression (Bronner *et al.*, 2004).

The *sarA* operon consists of three overlapping transcripts, sarP1, sarP3, and sarP2, regulated by three distinct promoters, P1, P3, and P2. SarA expression is auto-activated by the presence of the SarA protein and varies in a growth phase-dependent manner with a peak during late exponential phase. The SarA protein is a monomer consisting of four α-helices, a short β-hairpin, and a long C-terminal loop. The protein binds to a conserved A/T-rich motif in the promoter regions of target genes as a homodimer. Several genes with high homology to SarA have been identified in

S. aureus, encoding for regulatory proteins which are termed the SarA protein family, including SarR, SarS, SarT, SarU and Rot ('repressor of toxin'). The SarA homologues are involved in the regulation of virulence factors and may also inhibit or activate each others transcription. (This section is based on reviews on the SarA protein family by Bronner *et al.* (2004) and Cheung *et al.* (2004)).

1.3.3.1.3 Genetic diversity of the accessory gene regulator (*agr*) locus in *S. aureus*

The *agr* locus in *S. aureus* is polymorphic and contains a hypervariable region consisting of *agrD* and flanking regions in *agrB* and *agrC* (Figure 1.7) whereas *agrA*, the N-terminal encoding region of *agrB*, and the C-terminal encoding region of *agrC* are highly conserved (Dufour *et al.*, 2002; Novick, 2003). The hypervariability results in expression of different AIPs and four different *agr* subtypes (I – IV) have been identified in *S. aureus* to date (Jarraud *et al.*, 2000). AIP activation of its associated receptor is highly sequence specific and AIP from one subtype can inhibit the *agr* activity of another subtype encoded either by different *S. aureus* strains or by other staphylococcal species (Ji *et al.*, 1997; Otto, 2001; Otto *et al.*, 2001; Bronner *et al.*, 2004; Novick and Geisinger, 2008). The *agr* subtypes I, II, and III are thought to have evolved from a common ancestor whereas subtype IV is believed to have derived from subtype I (Novick and Geisinger, 2008). Since the *agr* locus is a multi-gene system, gene divergence must have arisen simultaneously for genes of each subtype to maintain functionality (Novick and Geisinger, 2008). One strategy to preserve function during mutational events is to broaden specificity, and it has been reported that replacement of critical residues of AgrC type I resulted in reduced specificity and inhibition sensitivity (Novick and Geisinger, 2008).

1.3.3.1.4 Association of accessory gene regulator (*agr*) types and *S. aureus* disease

The importance of *agr* in pathogenesis has been demonstrated in various animal models, but its relevance in human infection *in vivo* has not been fully elucidated (Novick, 2003; Novick and Geisinger, 2008; Traber *et al.*, 2008). Traber *et al.* (2008) demonstrated that *agr*-negative mutants can arise during infection, and that non-

functional *agr*-mutants are involved in infection of humans. Furthermore, the investigation of staphylococcal pneumonia isolates from humans revealed that approximately 20% of isolates were *agr*-negative, supporting their role in causing disease (Novick and Geisinger, 2008). However, these strains were associated with less severe patient outcome indicating a milder form of disease (Novick and Geisinger, 2008). This could be because staphylococcal quorum sensing is important in biofilm formation, and an active *agr* system leads to the detachment of bacterial cells *in vitro*, which may allow the invasion of new infection sites (Boles and Horswill, 2008). Several *agr* types of *S. aureus* have been associated with certain human diseases, including *agr* type III with menstrual TSS and PVL-induced necrotising pneumonia, *agr* type IV with exfoliatin-production, and *agr* type II with reduced susceptibility to vancomycin (Novick, 2003).

1.3.3.1.5 The accessory gene regulator (*agr*) system in *S. intermedius*

Examination of the diversity of the *agr* global regulatory locus has resulted in important insights into the population genetics and virulence of staphylococci. The *agr* locus was first identified in *S. aureus* (Jarraud *et al.*, 2000; Otto, 2001) and an *agr* locus has been found in all *Staphylococcus* species examined thereafter including *S. epidermidis* and *S. intermedius* (Dufour *et al.*, 2002). The *agr* locus of a strain of *S. intermedius* was previously sequenced and consists of 3,436 bp with five open reading frames (ORFs), *agrB*, *agrD*, *agrC*, *agrA*, and *hld*, encoding for the classic two-component regulatory system with AgrD being transformed to AIP by AgrB (Sung *et al.*, 2006). The PCR amplification and sequencing of *agrD* of 20 canine *S. intermedius* isolates of the same geographic origin have revealed genetic variation in the putative AIPs encoded by *agrD*, resulting in three *agr* groups encoding three different putative AIPs (Sung *et al.*, 2006). Of note, all staphylococcal *agr* systems identified to date encode for a cysteine, resulting in a thiolactone ring, in their AIP, whereas the AIP of *S. intermedius* contains a serine, resulting in a cyclic lactone, with auto-activating and cross-inhibiting properties (Ji *et al.*, 2005). However, *S. intermedius* interacts with a cysteine variant of its own AgrD, whereas *S. aureus* does not respond to a serine variant of its AgrD, suggesting that the *S. intermedius* serine

variant might have evolved due to a single point mutation from a cysteine-encoding precursor (Novick and Geisinger, 2008).

1.4 Population genetics of *S. aureus* and *S. intermedius*

1.4.1 Typing methods for bacterial isolates

Traditional methods for differentiation of bacteria into subgroups have been based on differences in phenotype, such as serotype, phage-type, and antibiotic resistance (Feil and Enright, 2004). Other commonly used molecular-based methods for the genomic analysis of *S. aureus* and other staphylococcal species include PFGE, binary typing based on randomly amplified polymorphic DNA in a polymerase chain reaction (RAPD-PCR), and amplified fragment length polymorphism (AFLP) (Khambaty *et al.*, 1994; van Leeuwen *et al.*, 1999; Zadoks *et al.*, 2000; Grundmann *et al.*, 2002; van Leeuwen *et al.*, 2005). These methods are useful for discrimination of closely related strains, but have less value in the analysis of population genetics and evolution (Feil and Enright, 2004), and some authors suggest that they will soon be abandoned as 'old-fashioned banding pattern-based technologies' (van Belkum *et al.*, 2009).

Other methods for the analysis of long-term evolutionary events have been developed, which focus on the comparison of slowly evolving genes (Feil and Enright, 2004). The most important techniques are multilocus enzyme electrophoresis (MLEE) and multilocus sequence typing (MLST) (Feil and Enright, 2004). MLEE analyses the differences in the electrophoretic behaviour of up to 25 gene products of metabolic genes simultaneously, resulting in indirect inference of the allelic variation at each metabolic gene (Feil and Enright, 2004). MLST analyses variation at slowly evolving genes at the genetic level (Feil and Enright, 2004). This typing method is based on the comparison of the DNA sequence of ~500 bp fragments within seven housekeeping genes, resulting in an allelic profile or sequence type (ST) of the analysed bacterial strain (Feil and Enright, 2004). An online MLST database (http://www.mlst.net/) has been established to enable researchers to trace and analyse the distribution of bacterial clones, especially with

regard to virulent or antibiotic-resistant isolates (Feil and Enright, 2004). In order to compare a large number of samples, another online tool called eBURST (http://eburst.mlst.net/) has been introduced, which divides bacterial isolates into clonal complexes (CCs) on the basis of their allelic profiles (Feil *et al.*, 2004). Every member of a CC is 100% genetically identical at six or seven housekeeping loci with at least one other isolate of the same CC (Feil *et al.*, 2004).

Another commonly used typing method for isolates from *S. aureus* outbreaks is staphylococcal protein A (*spa*) typing, based on sequence variation of region X of the *spa* locus (Frenay *et al.*, 1996). Region X contains from 1 to 15 repeats of 24 bp each, and the high sequence diversity is a result of deletions and duplications of the repeats, as well as single nucleotide polymorphisms (Shopsin *et al.*, 1999; Deurenberg and Stobberingh, 2008; van Belkum *et al.*, 2009). The *spa* typing data obtained from laboratories worldwide is collected and publicly available on the *spa*-server (http://spaserver.ridom.de). In August 2009, the database contained 5641 *spa* types from over 95,000 isolates from 69 countries (http://spaserver2.ridom.de/index.shtml, accessed on 27/08/09). Additionally, the algorithm based upon repeat pattern (BURP) was introduced, allowing the allocation of CCs based on *spa* typing data (*spa*-CC), indicating the possible use of this typing method for long term evolutionary analysis in addition to short term surveillance (Mellmann *et al.*, 2007). However, the discriminatory power of this method as a single typing approach for analysis of long term events has been questioned (Deurenberg and Stobberingh, 2008).

The *mecA* gene of *S. aureus*, conferring resistance to methicillin and other β-lactam antibiotics, is located on the staphylococcal cassette chromosome *mec* (SCC*mec*), a mobile genetic element which is also used for molecular typing (Deurenberg and Stobberingh, 2008). To date, seven major SCC*mec* types (I to VII) are identified, based on the encoded recombinase, plus a variety of subtypes depending on the presence of insertion sequences (IS), transposons, and plasmids (Ben Zakour *et al.*, 2008; Deurenberg and Stobberingh, 2008). There are several multiplex PCR approaches for SCC*mec* typing, amplifying different regions of SCC*mec* and varying

in their discriminatory power (Deurenberg and Stobberingh, 2008). In order to clearly delineate each of the 46 major and minor SCC*mec* variants identified to date, diagnostic microarrays may be a more reliable alternative to PCR-based typing in the future (Deurenberg and Stobberingh, 2008).

1.4.2 Population genetic analysis of *S. aureus*

A number of population genetic studies have revealed interesting traits of staphylococcal populations with regard to pathogenicity, host-specificity, and genomic diversity. For example, comparison of *S. aureus* isolates from human and bovine sources has revealed that most *S. aureus* clones are host-specific, i.e. infect only a single host species and transmission between species is a rare event (Fitzgerald *et al.*, 1997; Zadoks *et al.*, 2000). The analysis of whole *S. aureus* genomes provides further insights into *S. aureus* host-adaptation and co-evolution with specific hosts, such as the identification of allelic variation in genes encoding for colonisation factors (Ben Zakour *et al.*, 2008). However, cross-colonisation and -infection of *S. aureus* may occasionally occur and some strains may have the potential to colonise a variety of host species (Simoons-Smit *et al.*, 2000; van Belkum *et al.*, 2009). Furthermore, some reports suggest that *S. aureus* shows a tissue-, rather than a host-specificity (Zadoks *et al.*, 2002; Vautor *et al.*, 2003; van Leeuwen *et al.*, 2005). Van Leeuwen *et al.* (2005) found that pets and zoo animals in close contact with humans were often colonised with *S. aureus* clones of the same AFLP cluster as their owners and zoo keepers, whereas mastitis isolates from livestock ruminants belonged to a distinct AFLP cluster, implying tissue-specificity. However, a study comparing *S. aureus* isolates from the nares and udders of cows, sheep, and goats by PFGE reported that the majority of ovine and caprine isolates clustered together, separate from most bovine isolates, independently of body site of isolation and geographic origin, suggesting host-, and not tissue-specificity (Alves *et al.*, 2009).

S. aureus has a highly clonal population structure and genetic studies of large collections of carrier and invasive human isolates reported that a correlation between genotype and disease potential is lacking, indicating that any genotype can

potentially cause severe disease (Feil *et al.*, 2003; van Belkum *et al.*, 2009). Lindsay *et al.* (2006) analysed a collection of community-acquired invasive and nasal carriage *S. aureus* isolates from the same geographic origin employing microarrays based on genome sequences of seven *S. aureus* strains. The authors were unable to associate invasiveness with clonal lineages and/or gene presence suggesting that factors other than bacterial gene content alone contribute to *S. aureus* pathogenesis (Lindsay *et al.*, 2006). Population genetic studies have investigated the origin of methicillin-sensitive *S. aureus* (MSSA) and MRSA strains (Enright *et al.*, 2000; Fitzgerald *et al.*, 2001). MRSA strains are closely related to MSSA strains, including some which share identical allelic profiles, indicating that MRSA have most likely evolved from epidemic successful MSSA ancestors by acquiring the *mecA* gene through horizontal gene transfer (Enright *et al.*, 2000; Fitzgerald *et al.*, 2001). Based on MLST typing, five major CCs were identified among *S. aureus* MRSA isolates (CC5, CC8, CC22, CC30, CC45), which were geographically widespread and shared with MSSA isolates (Enright *et al.*, 2002), indicating that they have developed independently several times from different ancestors, rather than from one common origin (Fitzgerald *et al.*, 2001; Enright *et al.*, 2002; van Belkum *et al.*, 2009).

MRSA strains have been isolated from farm animal species, including cattle, pigs, and chickens (Lee, 2003), and interspecies transmission has been reported for MRSA and MSSA strains (Cefai *et al.*, 1994; Simoons-Smit *et al.*, 2000; Manian, 2003; Morris *et al.*, 2006a; Morris *et al.*, 2006b). Furthermore, a screening of veterinary staff, hospitalised dogs, and environmental sites in a UK animal hospital identified 28 MRSA isolates out of 300 in total with a majority of these isolates being closely related or indistinguishable to epidemic MRSA (EMRSA)-15 (CC22), a major EMRSA strain in the UK (Loeffler *et al.*, 2005). Similarly, a study using *spa* typing and PFGE among isolates from canine, feline, equine, and human origin in the UK and Ireland demonstrated that small animal and human isolates clustered together in CC22 whereas the equine isolates belonged to a distinct clonal lineage (CC8) (Moodley *et al.*, 2006). These data provide evidence that MRSA strains have also entered animal hospitals (Loeffler *et al.*, 2005; Moodley *et al.*, 2006). Recently, an MRSA clone which is not related to human-associated MRSA clones identified to

date has emerged among pigs in the Netherlands (Deurenberg and Stobberingh, 2008; van Belkum *et al.*, 2009). This clone belongs to the common MRSA ST398 and can cross-infect humans in close contact with pigs, and has been frequently isolated in Dutch hospitals (Deurenberg and Stobberingh, 2008). In 2009, MRSA ST398 was isolated from two horses presented to the Royal Veterinary College in London, which was the first report of MRSA ST398 in animals in the UK (Loeffler *et al.*, 2009).

1.4.3 Population genetic analysis of *S. intermedius*

Our understanding of the diversity within natural populations of *S. intermedius* is very limited. *S. intermedius* was first described by Hajek *et al.* (1976) who noted a remarkable variation in phenotypes of *S. intermedius* isolates from various hosts based on biotype differences, such as mannitol fermentation and clumping factor production. More recent studies have used genomic typing approaches, such as ribotyping, PFGE, and 16S-23S intergenic ribosomal DNA spacer polymorphism analysis (ITS-PCR) for population genetic studies of *S. intermedius* (Khambaty *et al.*, 1994; Hesselbarth and Schwarz, 1995; Mendoza *et al.*, 1998; Aarestrup, 2001; Bes *et al.*, 2002; Wakita *et al.*, 2002; Pinchbeck *et al.*, 2006).

Wakita *et al.* (2002) analysed pigeon, dog, fox, mink, and horse isolates via phage typing and PFGE, and concluded that this combination was a useful tool for epidemiological investigations of *S. intermedius* from various hosts. Pinchbeck *et al.* (2006) used PFGE to investigate genetic relatedness of *S. intermedius* isolated from 40 dogs with superficial pyoderma. Interestingly, the authors found no identical PFGE patterns between samples from different dogs, but in individual dogs, samples isolated from several pustules and from carrier sites were typically identical (Pinchbeck *et al.*, 2006). However, Hesselbarth *et al.* (1994) analysed *S. intermedius* isolates from canine pyoderma patients and healthy canine carriers, and reported that PFGE patterns from isolates originating from healthy and diseased animals were comparable, indicating that similar *S. intermedius* strains may colonise and cause disease. In a different context, PFGE has been used to identify *S. intermedius* as the

responsible pathogen in a food poisoning outbreak in the USA and all cases could be traced back to a single strain (Khambaty *et al.*, 1994).

Ribotyping studies of *S. intermedius* isolates from various host species (dog, pigeon, horse, mink) have revealed three major clusters among the strains tested, and all canine strains belonged to the same cluster, whereas the other isolates varied widely in their ribotypes (Hesselbarth and Schwarz, 1995). The authors suggested that pigeons and horses might be transient carriers of *S. intermedius* which may transmit the bacteria to other species, whereas dogs may be more specific hosts (Hesselbarth and Schwarz, 1995). Chesneau *et al.* (2000) used ribotyping for pigeon and canine isolates and discovered that they belonged to two different clusters. These findings were supported by a study which analysed the ribotypes of *S. intermedius* isolates from various hosts of the canoidea superfamily (dog, skunk, weasel, racoon, red panda, bear) (Aarestrup, 2001). Aarestrup (2001) found that *S. intermedius* possesses remarkable genetic diversity, but all isolates of the same host origin belonged to an identical ribotype cluster, indicating host-specificity or co-evolution between the host species and *S. intermedius*.

Another approach used for *S. intermedius* population genetic analysis is ITS-PCR, a method which determines and compares the length of the intergenic spacer region between the 16S and 23S rRNA genes of different isolates (Mendoza *et al.*, 1998; Bes *et al.*, 2002). This has been suggested as a rapid method to identify isolates on the species level (Mendoza *et al.*, 1998). Bes *et al.* (2002) slightly modified the method and analysed 57 *S. intermedius* isolates from various hosts (human, camel, dog, pigeon, horse, mink) via ITS-PCR typing. Twelve different ITS-PCR types were found with a heterogenous distribution of the pigeon, horse, and mink strains, but with the majority of human and canine strains belonging to the same two ITS-PCR types, suggesting frequent transmission between humans and dogs, and supporting the theory of Hesselbarth and Schwarz (1995) that the other species might be transient carriers (Bes *et al.*, 2002).

1.5 Hypothesis of the project

Bacterial pyoderma is the second most common skin disease in dogs, and the majority of cases are associated with *S. intermedius* infection (Medleau *et al.*, 1986; Scott *et al.*, 2001a; Gross *et al.*, 2005). Remarkable divergence among the *S. intermedius* population has been noted by several authors (Hajek, 1976; Hesselbarth and Schwarz, 1995; Chesneau *et al.*, 2000; Aarestrup, 2001; Bes *et al.*, 2002) and more than one species or subspecies may exist among isolates previously classified as *S. intermedius*. Further, *S. intermedius* has been shown to adhere to canine corneocytes (McEwan *et al.*, 2005; Simou *et al.*, 2005b; Simou *et al.*, 2005c), but the adherence mechanism has not yet been characterised. Staphylococcal surface proteins, termed MSCRAMMs, mediate adherence of the bacteria to host ECM proteins, and it is likely that *S. intermedius* encodes for an array of putative MSCRAMMs which may act as colonisation factors of canine skin.

1.6 Aims of the project

The aims of this study are:

(a) To investigate the population genetic structure of *S. intermedius* natural populations by employing a DNA multilocus sequencing approach.

(b) To identify and examine the distribution of genes encoding putative CWA proteins in the SIG by:
   i. Bioinformatic identification of putative CWA protein-encoding genes in a whole genome sequence of a clinical isolate of *S. pseudintermedius*.
   ii. Investigation of the presence and distribution of the putative CWA protein-encoding genes in a diverse collection of *S. pseudintermedius* isolates and other closely related species.

(c) To characterise putative MSCRAMM-host interactions by examining selected CWA proteins in their ability to mediate adherence to host ECM proteins, canine corneocytes, and a canine epidermal cell line.

Chapter 2

General Materials and Methods

2.1 Bacterial culture conditions and media

Staphylococcal strains were grown on tryptone soya agar (TSA) (Oxoid, UK), or in tryptone soya broth (TSB) (Oxoid, UK), or brain heart infusion broth (BHI) (Oxoid, UK). *E. coli* strains were grown on Luria-Bertani (LB) agar (Miller, Formedium^{TM}, UK) or in LB broth (Melford Laboratories, UK). For isolation of single colonies, bacteria were grown overnight on TSA or LB agar at 37°C. Broth cultures were grown either to stationary phase for approximately 16 h or to exponential phase of growth (OD_{600} 0.6 to 0.8 unless stated otherwise) at 37°C with constant shaking at 200 rpm. *Lactococcus lactis* strains were grown on GM17 agar (M17 medium (Oxoid, UK) supplemented with granulated agar (Melford Laboratories, UK) and 0.5% glucose) at 30°C for up to 24 h for single colony isolation, and in GM17 broth (M17 medium (Oxoid, UK) supplemented with 0.5% glucose) statically at 30°C for stationary (16 h) or exponential (OD_{600} 0.6 to 0.8) phase of growth. Antibiotics were added as appropriate and as stated in each chapter. Bacterial strains and isolates were stored in appropriate liquid growth media containing 20% (v/v) glycerol at -70°C.

2.2 Oligonucleotides

Oligonucleotides employed in this study were synthesised commercially by Sigma-Genosys, UK or Invitrogen, UK.

2.3 DNA isolation, purification, and analysis

2.3.1 Genomic DNA extraction

Overnight broth cultures of staphylococci (1 ml) were centrifuged at 5000 rpm for 5 min to obtain bacterial pellets. Genomic DNA extraction was carried out with the Bacterial Genomic DNA Purification Kit (Edge Biosystems, USA) according to the manufacturer's instructions. Prior to incubation at 37°C for 10 min, 125 µg/ml lysostaphin (AMBI products, USA) was added. Genomic DNA pellets were resuspended in 100 µl of 1x Tris-EDTA (TE) buffer (10 mM Tris, 1 mM EDTA, pH 8) (Sigma-Aldrich, UK). The integrity of the genomic DNA samples was determined by agarose gel electrophoresis.

2.3.2 Purification of PCR products

PCR products were purified using the QIAquick PCR purification kit (Qiagen, UK) according to the manufacturer's instructions unless stated otherwise.

2.3.3 Gel extraction and purification of DNA fragments

DNA fragments were excised from agarose gels without UV light exposure by first separating the lane containing the DNA ladder from the gel after staining, and marking the position of ladder DNA fragments with a sterile pipette tip under UV light before rejoining with the rest of the gel. By reference to the ladder, DNA fragments of the appropriate size could be cut from the gel with a sterile scalpel without UV exposure. Gel purification of DNA fragments of interest was carried out using the QIAquick Gel Extraction Kit (Qiagen, UK) according to the manufacturer's instructions.

2.3.4 Plasmid DNA isolation and purification

Overnight broth cultures were centrifuged at 5000 rpm for 5 min and plasmid was purified using the QIAprep Spin Miniprep Kit (Qiagen, UK) according to the manufacturer's instructions. For plasmid DNA isolation from *L. lactis* cultures, 100 U/ml mutanolysin (Sigma-Aldrich, UK) and 100 µg/ml lysozyme (Sigma-Aldrich, UK) were added to buffer P1.

2.3.5 Restriction endonuclease digestion of DNA

Genomic DNA and plasmid DNA was digested with restriction endonucleases (New England Biolabs, UK) and appropriate buffers (New England Biolabs, UK) according to the manufacturer's instructions. The total reaction volume was typically 40 µl for staphylococcal DNA and 20 µl for diagnostic digestion of plasmid DNA. A typical digestion reaction consisted of 1-2 µl restriction endonuclease (New England Biolabs, UK), 1x appropriate buffer (New England Biolabs, UK) with or without addition of 1x Bovine Serum Albumin (BSA) (New England Biolabs, UK) according to the manufacturer's recommendations, ~5 µg of genomic DNA and ~50 ng of plasmid DNA respectively. Genomic DNA was digested for 16 h and plasmid DNA

for 2 h unless stated otherwise and incubation temperatures were chosen as recommended by the manufacturer (New England Biolabs, UK).

2.3.6 DNA agarose gel electrophoresis

DNA was analysed in 0.7% to 2% (w/v) agarose (Melford Laboratories, UK) gels containing either 0.5 µg/ml ethidium bromide (Sigma-Aldrich, UK) or 1x SYBR® Safe DNA gel stain (Invitrogen, UK) in 1x Tris-borate EDTA (TBE) buffer (Sigma-Aldrich, UK). Electrophoresis was carried out at 80 to 100 V for 60 to 90 min and the size of DNA fragments was compared to standard molecular weight ladders (100 bp DNA ladder or 1 kb DNA ladder, Promega, USA). The DNA samples were mixed with 1x final concentration gel loading dye (BlueJuice^{TM}, 10x, Invitrogen, UK, or Blue/Orange Loading Dye, 6x, Promega, USA) prior to running. For visualisation, the ChemiImager^{TM}, 4000i v4.04 low light imaging system was used (Alpha Innotech Corporation, USA).

2.3.7 DNA quantification

DNA quantification was performed by spectrophotometric measurement of the absorption at a wavelength of 260 nm using a NanoDrop ND-1000 (Thermo Scientific, USA; http://www.nanodrop.com).

2.3.8 DNA sequencing

Sequencing reactions were carried out at SBS Sequencing Service (King's Building, The University of Edinburgh, UK) using 5 µl purified PCR-amplified DNA (approximately 50 to 90 ng) plus 1 µl sequencing primer (3.2 pmole/µl) with the BigDye^{TM} Terminator v3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, UK) according to the manufacturer's instructions. Each sequencing reaction included 25 cycles with denaturation for 30 s at 95°C, annealing for 20 s at 50°C and extension for 4 min at 60°C. The nucleotide sequence was determined with a 3730 DNA Analyzer (Applied Biosystems, UK).

2.3.9 DNA sequence analysis

DNA sequences were assembled using BioEdit Sequence Alignment Editor Software (http://www.mbio.ncsu.edu/BioEdit/bioedit) and the Staden package (Staden, 1996). Nucleotide and amino acid sequences were aligned using AlignX in Vector NTI Advance^{TM}10 (Invitrogen, UK).

2.4 Protein analysis

2.4.1 Protein quantification

Proteins were quantified using spectrophotometric measurement of absorbance at a wavelength of 280 nm with general reference setting (NanoDrop ND-1000, Thermo Scientific, USA; http://www.nanodrop.com).

2.4.2 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE gels consisted of 10% or 12% acrylamide resolving gels and 4% acrylamide stacking gels. Resolving gels contained 10% or 12% (v/v) acrylamide (40% solution, Sigma-Aldrich, UK), 375 mM Tris-HCl, pH 8.8, 0.1% (w/v) SDS (Fisher Scientific, UK), 0.1% (w/v) ammonium persulfate (APS) (Sigma-Aldrich, UK), and 0.1% (v/v) Tetramethylethylenediamine (TEMED) (Sigma-Aldrich, UK). Stacking gels contained 4% (v/v) acrylamide (40% solution, Sigma-Aldrich, UK), 125 mM Tris-HCl, pH 6.8, 0.1% (w/v) SDS (Fisher Scientific, UK), 0.1% (w/v) APS (Sigma-Aldrich, UK), and 0.1% (v/v) TEMED (Sigma-Aldrich, UK). Protein samples were prepared in sample buffer (Laemmli, Sigma-Aldrich, UK) as appropriate and subjected to SDS-PAGE in 1x Tris-glycine running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS (w/v), pH 8.3) using protein electrophoresis apparatus from Bio-Rad (Bio-Rad Laboratories, UK) at 100 to 150 V for 90 to 120 min as required. Polyacrylamide gels were either used for Western blot analysis or stained with Colloidal Coomassie Blue Stain (Severn Biotech Ltd., UK) overnight and destained in distilled water for 12 to 24 h.

2.4.3 Western blotting

Protein samples were subjected to SDS-PAGE as described in section 2.4.2 and transferred onto nitrocellulose membranes (Amersham Hybond™ ECL™, GE Healthcare, UK) in transfer buffer (20 mM Trismethylamine, 154 mM glycine, 20% (v/v) methanol) at 60 V for 90 min using protein electrophoresis apparatus from Bio-Rad (Bio-Rad Laboratories, UK). After transfer, nitrocellulose membranes were blocked with 8% (w/v) skim milk (Skim milk powder, Fluka, Sigma-Aldrich, UK) overnight at 4°C, followed by 3 washes for 15 min each in washing buffer (0.05% (v/v) TWEEN® 20 (Sigma-Aldrich, UK), 1% (w/v) skim milk (Skim milk powder, Fluka, Sigma-Aldrich, UK) in PBS) with gentle shaking at room temperature. Primary antibody was added to the appropriate dilution and blots were incubated with antibody for 2 h at room temperature. After 3 washes for 15 min each, secondary antibody was added to the appropriate dilution and the blots were incubated for 1 h at room temperature. The washing step was repeated as before (3 washes, 15 min each) and blots were incubated with equal volumes (3 ml each) of ECL solution 1 (2.5 mM luminol (Fluka, Sigma-Aldrich, UK), 0.4 mM p-coumaric acid (Sigma-Aldrich, UK), 100 mM Tris-HCl, pH 8.5) and ECL solution 2 (0.02% (v/v) H_2O_2, 100 mM Tris-HCl, pH 8.5) for 5 min at room temperature unless stated otherwise. For signal detection, membranes were wrapped in cling film and exposed to chemiluminescence film (Hyperfilm™, Amersham Biosciences, GE Healthcare, UK) within a Hypercassette™ (Amersham Biosciences, GE Healthcare, UK) for an appropriate duration, usually less than 1 min, depending on the strength of signal. The Hyperfilm™ was developed in an x-ray processor (Optimax, PROTEC Medizintechnik GmbH & Co. KG, Germany) with x-ray developer solution (Champion Photochemistry, UK) and x-ray fixer solution (Photosol Ltd., UK).

Chapter 3

Population genetic structure of the '*Staphylococcus intermedius* group'

3.1 Introduction

S. intermedius has traditionally been considered the major cause of canine pyoderma (Medleau *et al.*, 1986; Devriese and De Pelsmaecker, 1987; Cox *et al.*, 1988; Allaker *et al.*, 1992a; Allaker *et al.*, 1992b; Werckenthin *et al.*, 2001; Saijonmaa-Koulumies and Lloyd, 2002b). However, a high level of genotypic and phenotypic diversity has been observed by several investigators (Hajek, 1976; Meyer and Schleifer, 1978; Hesselbarth and Schwarz, 1995; Chesneau *et al.*, 2000; Aarestrup, 2001), suggesting that more than one species may exist. The host-specific occurrence of more than one species or subspecies among the *S. intermedius* population has been speculated and the existence of a '*S. intermedius* group' (SIG) was suggested (Chesneau *et al.*, 2000; Sasaki *et al.*, 2007b). However, our understanding of the population genetic structure of *S. intermedius* is very limited.

In 2005, Devriese *et al.* described *Staphylococcus pseudintermedius*, a new species isolated from a dog, a cat, a horse, and a parrot. Based on 16s rRNA sequencing, *S. pseudintermedius* was closely related to *S. intermedius* and to *Staphylococcus delphini* (Devriese *et al.*, 2005). *S. delphini* was first isolated from purulent skin lesions of dolphins in 1988 (Varaldo *et al.*) and has rarely been reported since (Bjorland *et al.*, 2005).

3.2 Aims and Strategy

To elucidate the population genetic structure of *S. intermedius*, and the closely related *S. pseudintermedius* and *S. delphini* by:

1) Employing a DNA multilocus sequencing approach to investigate the genetic diversity of bacterial isolates from broad host and geographic origin which were phenotypically identified as *S. intermedius*.

2) Examination of the allelic variation of *agrD*, which encodes the autoinducing peptide (AIP) of the *agr* quorum-sensing system.

3) Analysis of the clonal diversity of methicillin resistant *S. pseudintermedius* (MRSP).

4) Development of a simple molecular diagnostic test to discriminate *S. pseudintermedius* from *S. intermedius* and *S. delphini* using a PCR-restriction fragment length polymorphism (RFLP) approach.

3.3 Materials and Methods

3.3.1 Bacterial strains used for DNA multilocus sequence analysis

In total, 105 bacterial isolates were examined in this study including 99 which were previously identified as *S. intermedius* in different laboratories in the USA, Canada, Japan, UK (including Scotland and England), France, Belgium, Czech Republic, Germany, and Sweden from an array of diseased and healthy animal species including dogs, humans, cats, horses, camels, and pigeons (Table 3.1). Identification was carried out with standard phenotypic tests which varied slightly depending on the laboratory but included production of coagulase and anaerobic acid from mannitol, sucrose and D-Trehalose, and lack of hyaluronidase production, lack of growth on P-agar containing acriflavin, and the production of β-galactosidase when grown in OPNG broth according to the original description of the species by Hajek (1976). In addition, the type strain of *S. delphini* ATCC 49171 (Varaldo *et al.*, 1988a), four strains of the recently described *S. pseudintermedius* species including the type strain LMG 22219 (Devriese *et al.*, 2005), the type strains of *Staphylococcus schleiferi* sub. *schleiferi* ATCC 43808 (Freney *et al.*, 1988), *Staphylococcus schleiferi* sub. *coagulans* ATCC 49545 (Igimi *et al.*, 1990), *Staphylococcus lutrae* DSM 10244 (Foster *et al.*, 1997), *S. hyicus* ATCC 11249 (Devriese *et al.*, 1978), *Staphylococcus chromogenes* ATCC 43764 (Devriese *et al.*, 1978), and *Staphylococcus felis* ATCC 49168 (Igimi *et al.*, 1989) were included in the study (Table 3.1).

S. lutrae, *S. schleiferi* ssp. *coagulans*, *S. hyicus*, and *S. chromogenes* were a gift from G. Foster; *S. pseudintermedius* strains were kindly provided by F. Haesebrouck and E. De Graef, and *S. intermedius* isolates by U. Andersson, M. Bes, G. Bohach, A. Chow, J. Freney, K. Futagawa-Saito, J. Harris, L. Hume, D. Lloyd, A. Loeffler, and S. Weese.

Table 3.1. Staphylococcal strains and isolates used for DNA multi locus sequence analysis with host and geographic origin, sequence type (ST), *agr* type, and *mecA* status.

Isolate[a]	Host origin	Geographic origin	Reference	ST	*agr* type	*mecA*[b]
ED99	Dog, pustule	UK	(Simou *et al.*, 2005b)	2	III	-
M 1351/03	Dog, skin biopsy	UK	This study	3	II	-
M 407/03	Dog, skin biopsy	UK	This study	4	IV	-
M 707/99	Dog, ear	UK	This study	2	III	-
M1332/03	Dog, wound	UK	This study	5	II	-
M 695/99	Dog, ear	UK	This study	6	III	-
M 741/99	Dog, skin	UK	This study	7	IV	-
M 1337/03	Dog, vagina	UK	This study	8	II	-
M 1333/03	Dog, ear	UK	This study	9	II	-
M 717/99	Dog, skin	UK	This study	10	III	-
M 721/99	Dog, pustule	UK	This study	11	IV	-
HT20030674	Camel	France	(Bes *et al.*, 2002)	12	IV	-
HT20030676	Camel	France	(Bes *et al.*, 2002)	12	IV	-
HT20030677	Camel	France	(Bes *et al.*, 2002)	13	I	-
HT20030679	Camel	France	(Bes *et al.*, 2002)	14	IV	-

Isolate[a]	Host origin	Geographic origin	Reference	ST	*agr* type	*mecA*[b]
HT20030680	Camel	France	(Bes *et al.*, 2002)	12	IV	-
HT20030683	Dog	France	(Bes *et al.*, 2002)	15	II	-
HT20030684	Dog	France	(Bes *et al.*, 2002)	16	III	-
HT20030685	Dog	France	(Bes *et al.*, 2002)	17	IV	-
HT20030686	Dog	France	(Bes *et al.*, 2002)	18	II	-
N900260	Human	France	(Bes *et al.*, 2002)	19	II	-
N910201	Human	France	(Bes *et al.*, 2002)	20	III	-
N940276	Human	France	(Bes *et al.*, 2002)	21	IV	-
N940453	Human	France	(Bes *et al.*, 2002)	22	II	-
LY19990344	Human	France	(Bes *et al.*, 2002)	23	IV	-
AV 8001	Dog	Japan	(Futagawa-Saito *et al.*, 2004a)	24	II	-
AV 8002	Dog	Japan	(Futagawa-Saito *et al.*, 2004a)	25	II	-
AV 8027	Dog	Japan	(Futagawa-Saito *et al.*, 2004a)	24	II	-

Isolate[a]	Host origin	Geographic origin	Reference	ST	*agr* type	*mecA*[b]
AV 8012	Dog	Japan	(Futagawa-Saito *et al.*, 2004a)	26	I	-
AV 8010	Dog	Japan	(Futagawa-Saito *et al.*, 2004a)	27	II	-
AV 8024	Dog	Japan	(Futagawa-Saito *et al.*, 2004a)	28	III	-
AV 8033	Dog	Japan	(Futagawa-Saito *et al.*, 2004a)	29	III	-
AV 8047	Pigeon	Japan	(Futagawa-Saito *et al.*, 2004a)	30	II	-
AV 8051	Pigeon	Japan	(Futagawa-Saito *et al.*, 2004a)	31	II	-
AV 8081	Pigeon	Japan	(Futagawa-Saito *et al.*, 2004a)	1	I	-
AV 8063	Pigeon	Japan	(Futagawa-Saito *et al.*, 2004a)	32	I	-
AV 8061	Pigeon	Japan	(Futagawa-Saito *et al.*, 2004a)	1	I	-
96-022396	Feline	USA	(Edwards *et al.*, 1997)	33	IV	-
93-071493	Dog	USA	(Edwards *et al.*, 1997)	34	III	-

Isolate[a]	Host origin	Geographic origin	Reference	ST	*agr* type	*mecA*[b]
94-060294	Dog	USA	(Edwards *et al.*, 1997)	35	IV	-
94-062394	Dog	USA	(Edwards *et al.*, 1997)	36	I	-
94-072594	Dog	USA	(Edwards *et al.*, 1997)	37	III	-
95-011195	Dog	USA	(Edwards *et al.*, 1997)	38	I	-
95-062195	Dog	USA	(Edwards *et al.*, 1997)	39	II	-
95-062295	Dog	USA	(Edwards *et al.*, 1997)	2	III	-
95-072195	Dog	USA	(Edwards *et al.*, 1997)	39	II	-
95-072495	Dog	USA	(Edwards *et al.*, 1997)	39	II	-
96-022296	Dog	USA	(Edwards *et al.*, 1997)	40	III	-
96-030796	Dog	USA	(Edwards *et al.*, 1997)	41	II	-
96-032996	Dog	USA	(Edwards *et al.*, 1997)	42	IV	-
96-041096	Dog	USA	(Edwards *et al.*, 1997)	43	I	-
M449/06	Dog, ear	UK	This study	19	II	-
M543/06	Dog, thigh lump	UK	This study	44	IV	-

Isolate[a]	Host origin	Geographic origin	Reference	ST	*agr* type	*mecA*[b]
M575/06	Dog, skin	UK	This study	24	II	-
M629/06	Dog, uterus	UK	This study	45	IV	-
M657/06	Dog, skin	UK	This study	46	IV	-
8193	Dog, nasal swab	UK	This study	39	II	-
7357	Dog, cerebro-spinal fluid	UK	This study	5	II	-
8016	Dog, eye	UK	This study	47	I	-
9106	Horse, nasal swab	UK	This study	49	I	-
8485	Horse, nasal swab	UK	This study	50	II	-
8086	Horse, trachea	UK	This study	48	II	-
8185	Dog, eye	UK	This study	51	I	-
388	Dog, face	UK	This study	52	IV	-
9075	Dog, hock	UK	This study	53	I	-
9162	Dog, ear	UK	This study	54	III	-
690	Feline, eye	UK	This study	55	I	-
8239	Dog, urine	UK	This study	56	III	-
8429	Dog, cruciate repair	UK	This study	45	IV	-
8750	Dog, eye	UK	This study	45	IV	-

Isolate[a]	Host origin	Geographic origin	Reference	ST	*agr* type	*mecA*[b]
8478	Dog, nasal swab	UK	This study	57	I	-
3708	Dog, skin	UK	This study	58	III	-
4229	Dog, skin	UK	This study	59	IV	-
2431	Dog, eye	UK	This study	60	III	-
3279	Dog, elbow	UK	This study	29	III	+
9318	Dog, urine	UK	This study	61	III	-
500	Dog, joint	UK	This study	5	II	-
3414	Cat, urine	UK	This study	5	II	-
8639	Dog, ear	UK	This study	62	III	-
BH04	Dog	Canada	This study	66	II	-
BH34	Feline, carrier	Canada	This study	67	II	-
M06-12	Dog, pyoderma	USA	This study	68	IV	+
M06-13	Dog, pyoderma	USA	This study	68	IV	+
Can5	Dog, pyoderma	USA	This study	68	IV	+
Can6	Dog, pyoderma	USA	This study	68	IV	+
Can10	Human, carrier	USA	This study	70	IV	+
Gi1	Dog, pyoderma	Germany	(Loeffler *et al.*, 2007)	71	III	+

Isolate[a]	Host origin	Geographic origin	Reference	ST	*agr* type	*mecA*[b]
13	Dog	Sweden	This study	69	III	+
HH1	Dog, pyoderma	Germany	(Loeffler *et al.*, 2007)	71	III	+
HH3	Dog, otitis	Germany	(Loeffler *et al.*, 2007)	71	III	+
HH4	Feline, pyoderma	Germany	(Loeffler *et al.*, 2007)	71	III	+
BH01	Dog	Canada	This study	72	IV	-
BH47	Dog, carrier	Canada	This study	45	IV	-
BD19698-06	Dog, post-op infection	Sweden	This study	71	III	+
6437-06	Dog, post-op infection	Sweden	This study	71	III	+
7577-06	Dog, tumour	Sweden	This study	71	III	+
bkt001587-07	Dog, post-op infection	Sweden	This study	71	III	+
196-07	Dog, polyuria, polydipsia	Sweden	This study	71	III	+
S. intermedius NCTC 11048^T	Pigeon	Czech Republic	(Hajek, 1976)	1	I	-
S. pseudinter.[c] LMG 22219^T	Feline, lung	Belgium	(Devriese *et al.*, 2005)	63	IV	-
S. pseudinter.[c] LMG 22220	Horse, skin	Belgium	(Devriese *et al.*, 2005)	64	I	-
S. pseudinter.[c] LMG 22221	Dog, otitis	Belgium	(Devriese *et al.*, 2005)	5	II	-

Isolate[a]	Host origin	Geographic origin	Reference	ST	*agr* type	*mecA*[b]
S. pseudinter.[c] LMG 22222	Parrot, liver	Belgium	(Devriese *et al.*, 2005)	5	II	-
S. delphini ATCC 49171^T	Dolphin	Italy	(Varaldo *et al.*, 1988b)	65	II	-
S. chromogenes ATCC 43764^T	Pig	UK	(Devriese *et al.*, 1978)	ND	ND	ND
S. felis ATCC 49168^T	Cat	Japan	(Igimi *et al.*, 1989)	ND	ND	ND
S. hyicus ATCC 11249^T	Pig	Denmark	(Devriese *et al.*, 1978)	ND	ND	ND
S. lutrae ATCC 700373^T	Otter	UK	(Foster *et al.*, 1997)	ND	ND	ND
S. schleiferi ssp. *coagulans* ATCC 49545^T	Dog	Japan	(Igimi *et al.*, 1990)	ND	ND	ND
S. schleiferi ssp. *schleiferi* ATCC 43808^T	Human	France	(Freney *et al.*, 1988)	ND	ND	ND

^{T}Species type strain; [a]Isolates all previously identified as *S. intermedius* except where stated; [b]+, present; -, absent; [c]abbreviated from *S. pseudintermedius*; ND, not done.

3.3.2 Gene loci selected for DNA sequence analysis

Five loci, the 16S rRNA, *cpn60* (*hsp60*), *tuf*, *pta*, and *agrD* genes were selected for DNA sequence analysis. 16S rRNA, *cpn60*, and *tuf* have been used previously in single locus approaches for differentiating staphylococcal species (Goh *et al.*, 1997; Kwok *et al.*, 1999; Kwok and Chow, 2003; Devriese *et al.*, 2005), and *cpn60*, *tuf*, and *pta* have been incorporated into multilocus sequence typing schemes for differentiating strains of several different bacterial species (Feil *et al.*, 2003; Bartual *et al.*, 2005; Zadoks *et al.*, 2005). Furthermore, the allelic variation of *agrD* which encodes the AIP of the *agr* quorum-sensing system was investigated.

3.3.3 PCR amplification of gene fragments for DNA sequence analysis

Oligonucleotide primers were designed for 16S rRNA, *cpn60*, and *agrD* genes based on gene sequences available for *S. intermedius* in GenBank (http://www.ncbi.nlm.nih.gov/Genbank; accession numbers Z26897, AF019773, AY965912, and AF346723), and *pta* primers were designed based on unpublished *S. intermedius pta* nucleotide sequence. Oligonucleotide sequences specific for the *tuf* and *mecA* genes were designed in previous studies (Jonas *et al.*, 2002; Sakai *et al.*, 2004). Oligonucleotide sequences and predicted PCR product sizes for specified gene fragments are shown in Table 3.2. Genomic DNA of the bacterial isolates was extracted as described under general Materials and Methods, and PCR reactions for 16s RNA, *tuf*, *cpn60*, *pta,* and *mecA* genes consisted of 0.2 µM each primer, 0.025 u/µl taq polymerase (Promega, USA), 1.5 mM $MgCl_2$ (Promega, USA), 0.2 mM dNTPs (Promega, USA), and 1 µl genomic DNA template in 96-well PCR microplates (Axygen, Thistle Scientific, UK). The thermocycler conditions for 16s RNA, *tuf*, *cpn60*, and *pta* genes included an initial denaturation for 2 min at 95°C, followed by 30 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 55°C, and an extension for 1 min at 72°C, and a final extension for 7 min at 72°C. The *mecA* thermocycler conditions consisted of an initial denaturation for 5 min at 95°C, followed by 30 cycles of denaturation for 45 s at 95°C, annealing for 45 s at 52°C, and an extension for 1 min at 72°C, and a final extension for 5 min at 72°C. For *agrD* amplification**,** reactions included 0.5 µM each primer, 0.02 u/µl $Vent_R$® DNA Polymerase (New England Biolabs, UK), 1x ThermoPol Reaction Buffer (New

Table 3.2. Oligonucleotides designed for PCR amplification for DNA sequence analysis of partial 16S rRNA, *tuf*, *cpn60*, *pta*, *agrD,* and *mecA* genes.

Name[a]	Sequence (5′- 3′)	Expected size of PCR product (bp)
16S rRNA-F	CCTCTTCGGAGGACAAAGTGA	
16S rRNA-R	GACCCGGGAACGTATTCACC	370
tuf-F	CAATGCCACAAACTCG	
tuf-R	GCTTCAGCGTAGTCTA	500
cpn60-F	GCGACTGTACTTGCACAAGCA	
cpn60-R	AACTGCAACCGCTGTAAATG	550
pta-F	GTGCGTATCGTATTACCAGAAGG	
pta-R	GCAGAACCTTTTGTTGAGAAGC	570
agrD-F	GGGGTATTATTACAATCATTC	
agrD-R	CTGATGCGAAAATAAAGGATTG	300
agrD-R [b]	CTCATGACTATTGCATGCATCG	300
mecA-F	GTAGAAATGACTGAACGTCCGATAA	
mecA-R	CCAATTCCACATTGTTTCGGTCTAA	310

[a]F, forward primer, R, reverse primer; [b]Pigeon isolates only.

England Biolabs, UK), 0.2 mM dNTPs (Promega, USA), and 1 µl genomic DNA template. Thermocycler conditions included an initial denaturation for 2 min at 94°C, followed by 35 cycles of denaturation for 15 s at 94°C, annealing for 30 s at 45°C, and extension for 1 min at 72°C, followed by a final extension for 7 min at 72°C (Sung *et al.*, 2006). PCR products were purified by incubation with Exonuclease I and Antarctic Phosphatase (ExoAP) (New England Biolabs, UK) at 37ºC for 15 min, followed by inactivation at 80°C for 15 min.

3.3.4 DNA sequencing

Sequencing reactions were carried out as described in general Materials and Methods. For 16S rRNA, *cpn60*, *tuf,* and *pta* genes, forward and reverse sequencing reactions were carried out with independently-amplified PCR products to rule out the possibility of *Taq* polymerase generated errors. For *agrD*, forward and reverse sequencing reactions were carried out from a single PCR product generated with Vent® DNA polymerase (New England Biolabs, UK), which contains proof-reading activity and a much lower predicted error rate than *Taq* polymerase. The *cpn60* sequences for *S. delphini* ATCC 49171 and *S. schleiferi* ATCC 43808 (accession numbers AF053571 and AF033622), and the *agrD* sequence for *S. intermedius* NCTC 11048 (accession number AF346723) were obtained from the NCBI GenBank nucleotide database (http://www.ncbi.nlm.nih.gov/Genbank). Further, sequences of 16S rRNA, *tuf*, *cpn60*, and *pta* genes for *S. haemolyticus* JCSC1435, *S. saprophyticus* ATCC 15305, *S. aureus* strain Newman, and *S. epidermidis* RP62A were obtained from the genome projects available at the NCBI database (http://www.ncbi.nlm.nih.gov/; accession numbers NC_007168, NC_007350, NC_009641, and NC_002976, respectively).

3.3.5 DNA sequence and molecular evolutionary analysis

DNA sequence assembly and alignment were performed as described in general Materials and Methods. Phylogenetic analyses were carried out with MEGA v.3.1 software (Kumar *et al.*, 2004). The neighbour-joining method was applied to construct phylogenetic trees using the Kimura 2-parameter model, while the degree of statistical support for the nodes on the minimum evolution tree was evaluated by

examining their percent recovery in 1,000 resample trees by the bootstrap test. For estimating events of recombination, RDP software, which includes the programs GENECONV, BOOTSCAN, MAXIMUM χ^2, CHIMAERA, and SISTER SCANNING, was used (Martin *et al.*, 2005). The index of association standardised (I_A^S) between the different gene loci was calculated using LIAN program (version 3.1, Department of Biotechnology and Bioinformatics University of Applied Sciences Weihenstephan, Germany; http://adenine.biz.fh-weihenstephan.de/lian_3.1/). The value of the index of association (I_A^S) would be expected to be zero for linkage equilibrium when recombination events occur frequently. If the I_A^S value differs significantly from zero ($P < 0.05$), recombination should be rare.

3.3.6 eBURST analysis

Predicted lines of evolutionary descent and clonal complexes in the collection of isolates were identified using the eBURST algorithm (http://eburst.mlst.net). Sequence types (STs) were included in the same group if they shared four of the five gene loci with at least one other ST within the group. Subgroups were defined by the existence of at least three single-locus variants.

3.3.7 Nucleotide sequence accession numbers

The DNA sequences generated in this study were deposited in GenBank (http://www.ncbi.nlm.nih.gov/Genbank), under accession numbers EU157195 to EU157715.

3.3.8 PCR amplification of the partial *pta* gene for restriction endonuclease digestion

Oligonucleotide primers were designed specific for PCR amplification of a 320 bp fragment of the *pta* gene (*pta*-F, AAAGACAAACTTTCAGGTAA; *pta*-R, GCATAAACAAGCATTGTACCG), based on unpublished sequence data of *S. pseudintermedius* strain ED99. Bacterial genomic DNA was extracted as described in general Materials and Methods. PCR amplification was carried out in a 50 µl reaction volume with 0.2 µM of each primer, 0.2 mM dNTPs (Promega, USA), 1.5

mM $MgCl_2$ (Promega, USA), 0.025 u/µl *Taq* DNA polymerase (Promega, USA), and 50 ng DNA template, in 1x reaction buffer (Promega, USA). Thermocycling conditions included a 95°C incubation step for 2 min, followed by 30 cycles of 95°C for 1 min, 53°C for 1 min, and 72°C for 1 min, and a final incubation of 72°C for 7 min. PCR products were resolved in 2% (w/v) agarose by electrophoresis.

3.3.9 Restriction digestion with *Mbo*I endonuclease

25 µl of the PCR reactions were incubated with 5 U of *Mbo*I restriction endonuclease (New England Biolabs, UK) in 1x appropriate digestion buffer (New England Biolabs, UK) for 2 h at 37°C, and the digestion products were resolved in 2% (w/v) agarose by electrophoresis.

3.4 Results

3.4.1 Multilocus sequence analysis of *S. intermedius*

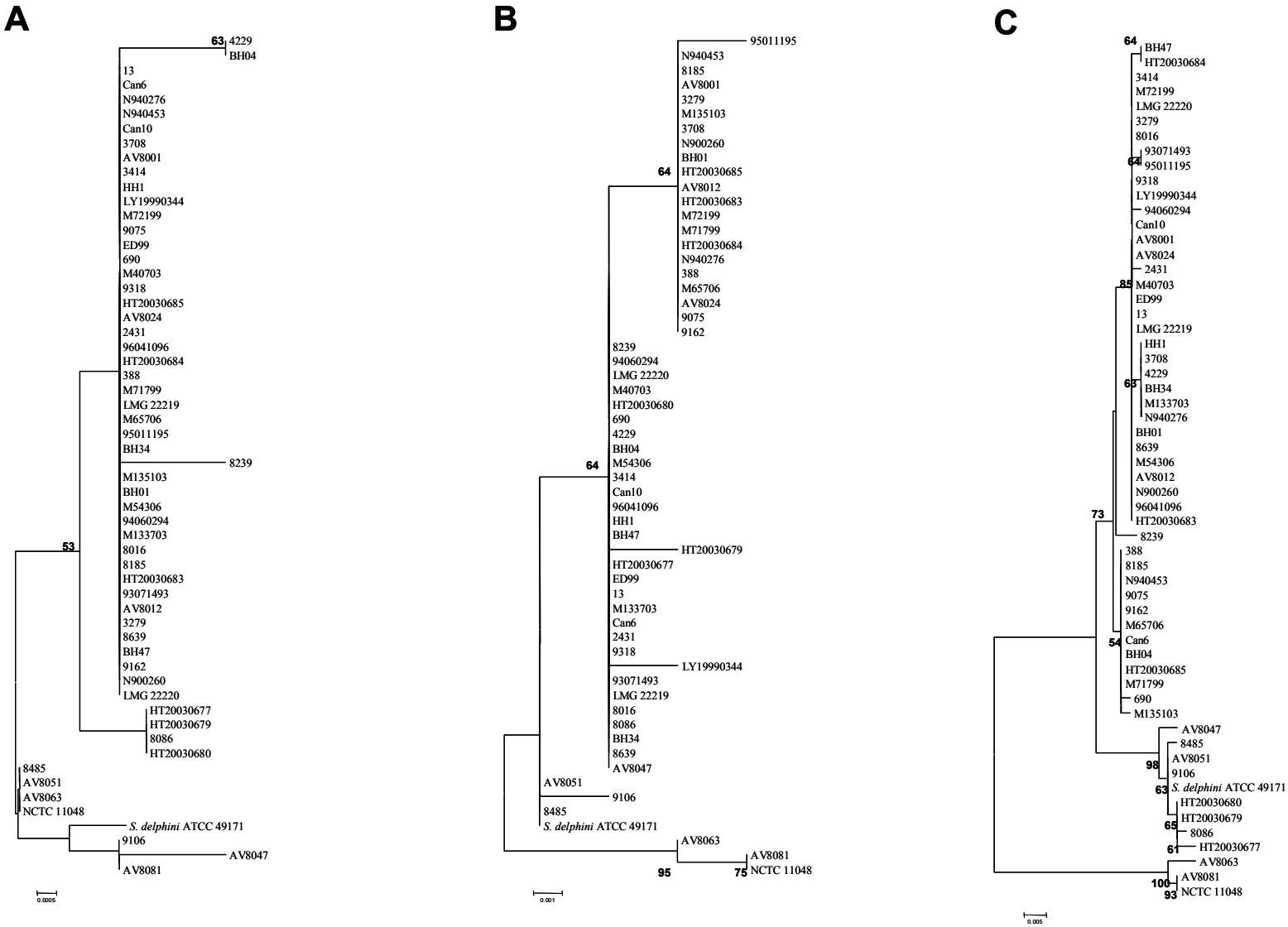
Overall, 192,482 bp representing 521 sequences from 105 isolates including 99 isolates phenotypically identified as *S. intermedius*, 4 isolates of *S. pseudintermedius,* and the type strains of *S. delphini* and *S. schleiferi* sub. *schleiferi* were generated. 16S rRNA gene sequences contained 1.37% variable sites and 7 alleles, *tuf* sequences contained 2.4% and 9 alleles, *cpn60* contained 12.76% and 28 alleles, *pta* contained 10.98% and 18 alleles, and *agrD* contained 13.04% polymorphic nucleotide sites and 9 alleles (Table 3.3). One 16S rRNA allele was shared between *S. pseudintermedius* and *S. delphini*, and one by *S. delphini* and *S. intermedius*. *S. pseudintermedius* and *S. delphini* shared one allele for the *tuf* gene and one for *agrD*. Furthermore, 16S rRNA gene sequence analysis revealed the existence of five ambiguous nucleotide sites characterised by a double peak for a single site in the sequencing trace for eight isolates. A mixed or contaminated template was ruled out by re-purification of the strain to single colony before genomic DNA isolation, repeated PCR and sequencing. These data are consistent with the existence of intra-genomic 16S rRNA gene polymorphisms which have been previously observed (Coenye and Vandamme, 2003). Phylogenetic reconstructions were carried out using concatenated sequences which included both possible 16S rRNA alleles to determine if they influenced tree topology, but no significant difference in topology was observed (data not shown). The least common allelic alternative was selected for each strain and used in phylogenetic analysis and assignment of sequence types.

3.4.2 *S. intermedius* isolates differentiate into three different phylotypes

Neighbour-joining trees were constructed with MEGA3.1 using the Kimura 2-parameter model, combined with 1,000 resample trees by the bootstrap test using sequences for each individual locus (Figure 3.1). Trees constructed with sequences from each housekeeping locus appeared to be generally in good agreement, indicating a robust phylogenetic signal (Figure 3.1). In contrast, the topology of the

Table 3.3. Nucleotide sequence variation for the SIG and for the *S. pseudintermedius, S. delphini,* and *S. intermedius* phylotypes.

Gene locus	Group	No. strains	Total length sequence (bp)	No. variable sites (%)	No. singleton variable sites (%)	No. alleles
16s rRNA	SIG	104	366	5 (1.37)	2 (0.55)	7
	S. pseudintermedius	89		2 (0.55)	1 (0.27)	3
	S. delphini	11		3 (0.82)	1 (0.27)	5
	S. intermedius	4		1 (0.27)	0 (0.00)	1
tuf	SIG	104	417	10 (2.40)	4 (0.96)	9
	S. pseudintermedius	89		3 (0.72)	2 (0.48)	4
	S. delphini	11		3 (0.72)	2 (0.48)	4
	S. intermedius	4		1 (0.24)	1 (0.24)	2
cpn60	SIG	104	431	55 (12.76)	9 (2.10)	28
	S. pseudintermedius	89		14 (3.25)	3 (0.70)	20
	S. delphini	11		19 (4.41)	8 (1.86)	7
	S. intermedius	4		0 (0.00)	0 (0.00)	1
pta	SIG	104	492	54 (10.98)	11 (2.24)	18
	S. pseudintermedius	89		12 (2.44)	6 (1.22)	10
	S. delphini	11		8 (1.63)	7 (1.42)	6
	S. intermedius	4		4 (0.81)	4 (0.81)	2
agrD	SIG	104	138	18 (13.04)	4 (2.90)	9
	S. pseudintermedius	89		6 (4.35)	0 (0.00)	4
	S. delphini	11		11 (7.97)	5 (3.62)	5
	S. intermedius	4		0 (0.00)	0 (0.00)	1


A
63
4229
BH04
13
Can6
N940276
N940453
Can10
3708
AV8001
3414
HH1
LY19990344
M72199
9075
ED99
690
M40703
9318
HT20030685
AV8024
2431
96041096
HT20030684
388
M71799
LMG 22219
M65706
95011195
BH34
8239
M135103
BH01
M54306
94060294
M133703
53
8016
8185
HT20030683
93071493
AV8012
3279
8639
BH47
9162
N900260
LMG 22220
HT20030677
HT20030679
8086
HT20030680
8485
AV8051
AV8063
NCTC 11048
S. delphini ATCC 49171
9106
AV8047
AV8081
0.0005
B
95011195
N940453
8185
AV8001
3279
M135103
3708
N900260
BH01
64
HT20030685
AV8012
HT20030683
M72199
M71799
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AV8024
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AV8047
AV8051
9106
8485
S. delphini ATCC 49171
AV8063
AV8081
95
75
NCTC 11048
0.001
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64
BH47
HT20030684
3414
M72199
LMG 22220
3279
8016
64
93071493
95011195
9318
LY19990344
94060294
Can10
AV8001
AV8024
2431
85
M40703
ED99
13
LMG 22219
HH1
3708
63
4229
BH34
M133703
N940276
BH01
8639
M54306
AV8012
N900260
96041096
HT20030683
73
8239
388
8185
N940453
9075
9162
M65706
54
Can6
BH04
HT20030685
M71799
690
M135103
AV8047
8485
AV8051
98
9106
63
S. delphini ATCC 49171
HT20030680
HT20030679
65
8086
61
HT20030677
AV8063
100
AV8081
93
NCTC 11048
0.005

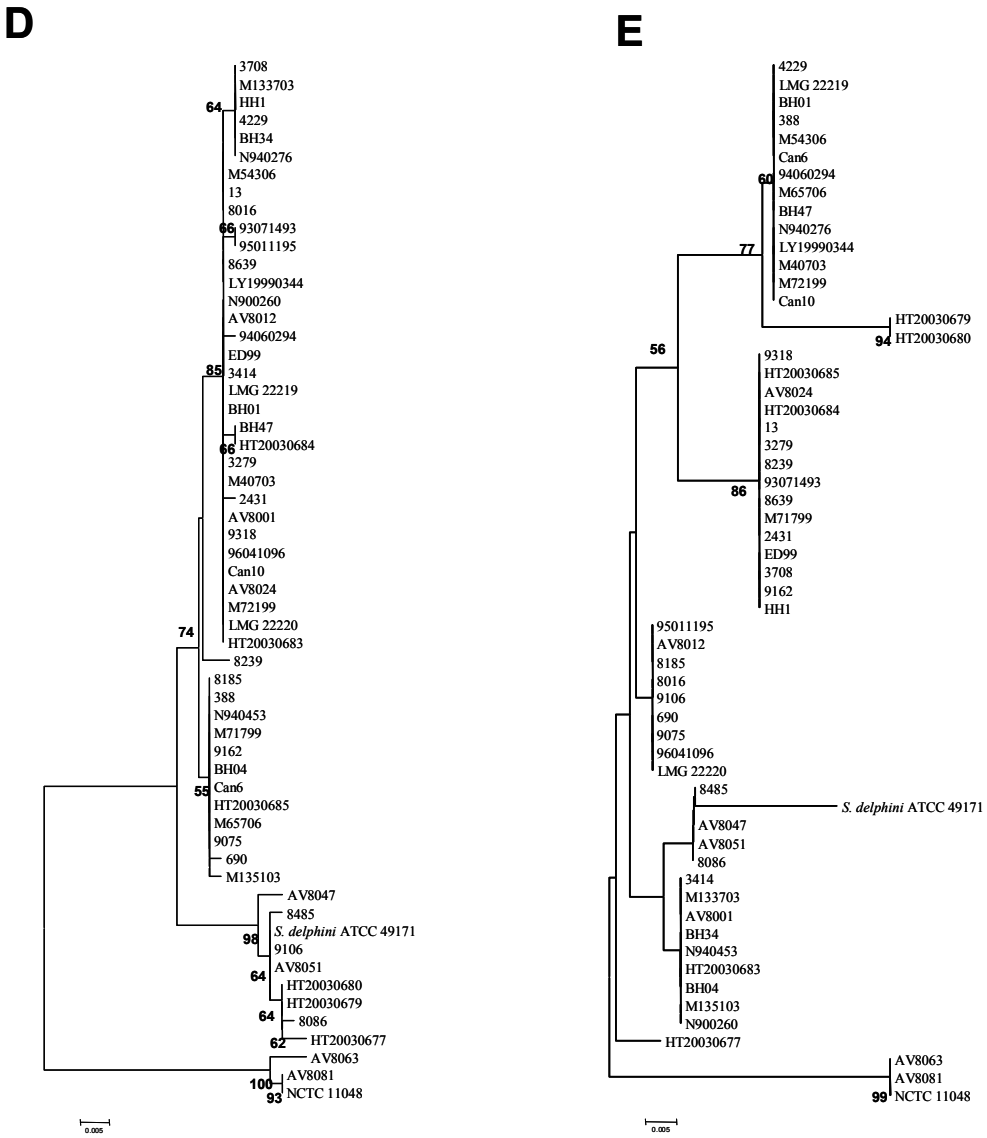


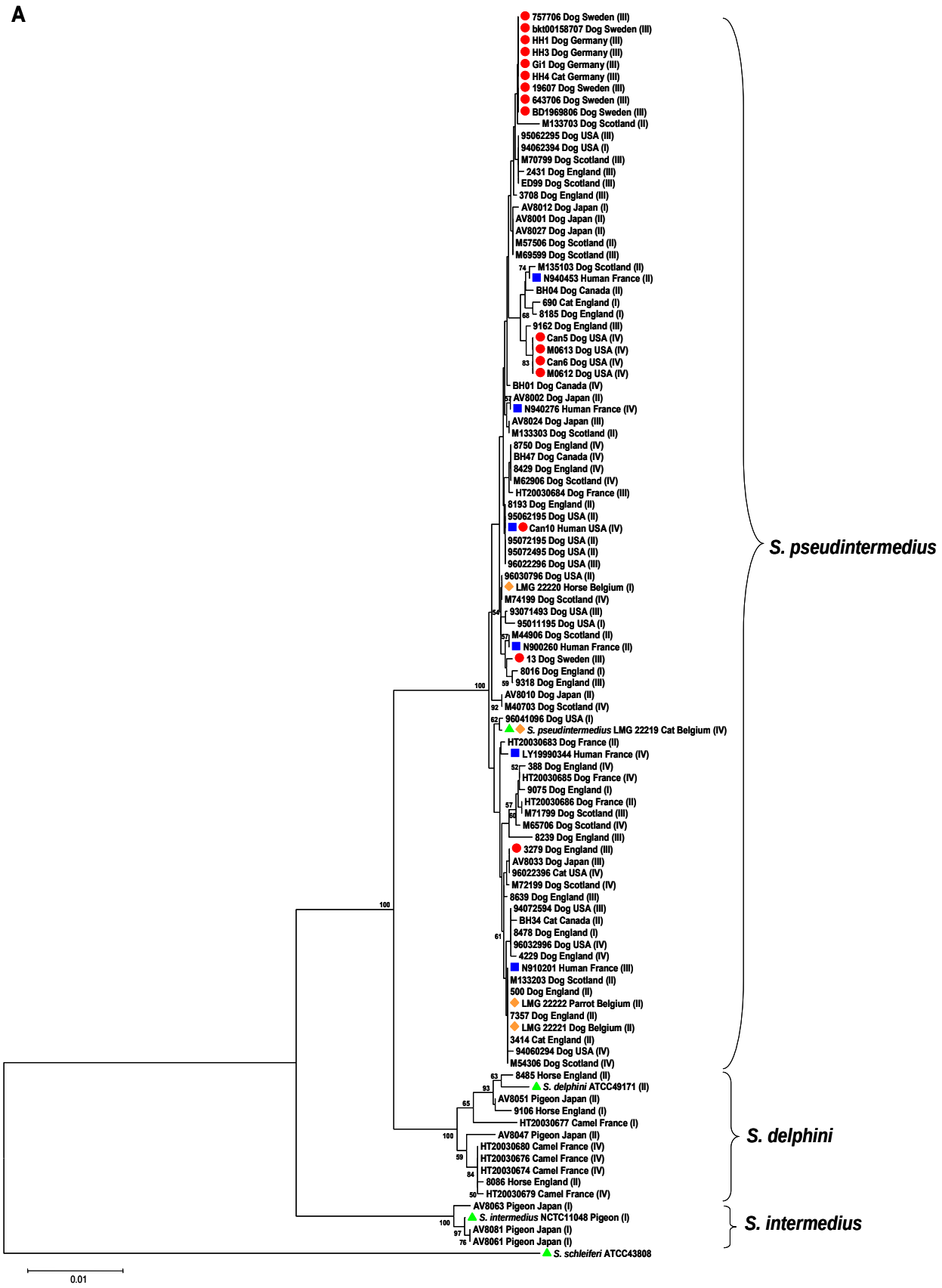
Figure 3.1. Phylogenetic trees constructed with the neighbour-joining method with 1000 bootstrap replicates of single loci including (A) 16S rRNA gene, (B) *tuf*, (C) *cpn60*, (D) *pta*, and (E) *agrD* sequences. A single representative isolate of each genotype identified was included. Bootstrap values over 50% are indicated.

tree generated with *agrD* nucleotide sequence was very different from trees generated with the other gene sequences (Figure 3.1), and *agrD* sequences were not included in the concatenated sequence analysis (Figure 3.2). Phylogenetic analysis based on the concatenated sequence data indicates the existence of 3 major phylotypes with strong confidence (bootstrap values 100% for each node) (Figure 3.2). For comparison, phylogenetic tree reconstruction was also carried out using the Minimum Evolution and UPGMA approaches and each resulted in a topology that was cognate with the NJ trees (data not shown).

Taken together, these data imply the existence of at least three closely-related, but distinct species (*S. intermedius*, *S. pseudintermedius*, and *S. delphini*) among isolates which were phenotypically identified as *S. intermedius*, and which together could be described as the '*S. intermedius* group' (SIG).

3.4.3 The SIG belong to a larger phylogenetic group of animal-associated staphylococcal species

Several staphylococcal species other than the SIG are associated with skin colonisation and/or disease in different animal hosts including *S. schleiferi* ssp. *schleiferi*, *S. schleiferi* ssp. *coagulans* (canine otitis externa and canine pyoderma) (May *et al.*, 2005), *S. lutrae* (isolated from mammary gland, lymphnode, liver, and spleen of deceased otters, potential otter pathogen) (Foster *et al.*, 1997), *S. hyicus* (exudative epidermitis in piglets) (Werckenthin *et al.*, 2001), *S. chromogenes* (bovine mastitis) (Birgersson *et al.*, 1992), and *S. felis* (otitis externa, abscesses, and wounds in cats) (Igimi *et al.*, 1989). A previous study based on single locus sequencing (*cpn60*) has indicated that the SIG may be closely related to the other staphylococcal animal skin pathogens (Kwok and Chow, 2003). In order to investigate this further, the multilocus sequencing approach developed in the current study was applied, and a neighbour-joining tree based on concatenated sequences was constructed with MEGA3.1 using the Kimura 2-parameter model and 1,000 replicate trees by the bootstrap test (Figure 3.3). Further, representative other clinically important species associated with human infections were examined including *S. haemolyticus*


A
757706 Dog Sweden (III)
bkt00158707 Dog Sweden (III)
HH1 Dog Germany (III)
HH3 Dog Germany (III)
Gi1 Dog Germany (III)
HH4 Cat Germany (III)
19607 Dog Sweden (III)
643706 Dog Sweden (III)
BD1969806 Dog Sweden (III)
M133703 Dog Scotland (II)
95062295 Dog USA (III)
94062394 Dog USA (I)
M70799 Dog Scotland (III)
2431 Dog England (III)
ED99 Dog Scotland (III)
3708 Dog England (III)
AV8012 Dog Japan (I)
AV8001 Dog Japan (II)
AV8027 Dog Japan (II)
M57506 Dog Scotland (II)
M69599 Dog Scotland (III)
M135103 Dog Scotland (II)
N940453 Human France (II)
BH04 Dog Canada (II)
690 Cat England (I)
8185 Dog England (I)
9162 Dog England (III)
Can5 Dog USA (IV)
M0613 Dog USA (IV)
Can6 Dog USA (IV)
M0612 Dog USA (IV)
BH01 Dog Canada (IV)
AV8002 Dog Japan (II)
N940276 Human France (IV)
AV8024 Dog Japan (III)
M133303 Dog Scotland (II)
8750 Dog England (IV)
BH47 Dog Canada (IV)
8429 Dog England (IV)
M62906 Dog Scotland (IV)
HT20030684 Dog France (III)
8193 Dog England (II)
95062195 Dog USA (II)
Can10 Human USA (IV)
95072195 Dog USA (II)
95072495 Dog USA (II)
96022296 Dog USA (III)
96030796 Dog USA (II)
LMG 22220 Horse Belgium (I)
M74199 Dog Scotland (IV)
93071493 Dog USA (III)
95011195 Dog USA (I)
M44906 Dog Scotland (II)
N900260 Human France (II)
13 Dog Sweden (III)
8016 Dog England (I)
9318 Dog England (III)
AV8010 Dog Japan (II)
M40703 Dog Scotland (IV)
96041096 Dog USA (I)
S. pseudintermedius LMG 22219 Cat Belgium (IV)
HT20030683 Dog France (II)
LY19990344 Human France (IV)
388 Dog England (IV)
HT20030685 Dog France (IV)
9075 Dog England (I)
HT20030686 Dog France (II)
M71799 Dog Scotland (III)
M65706 Dog Scotland (IV)
8239 Dog England (III)
3279 Dog England (III)
AV8033 Dog Japan (III)
96022396 Cat USA (IV)
M72199 Dog Scotland (IV)
8639 Dog England (III)
94072594 Dog USA (III)
BH34 Cat Canada (II)
8478 Dog England (I)
96032996 Dog USA (IV)
4229 Dog England (IV)
N910201 Human France (III)
M133203 Dog Scotland (II)
500 Dog England (II)
LMG 22222 Parrot Belgium (II)
7357 Dog England (II)
LMG 22221 Dog Belgium (II)
3414 Cat England (II)
94060294 Dog USA (IV)
M54306 Dog Scotland (IV)
8485 Horse England (II)
S. delphini ATCC49171 (II)
AV8051 Pigeon Japan (II)
9106 Horse England (I)
HT20030677 Camel France (I)
AV8047 Pigeon Japan (II)
HT20030680 Camel France (IV)
HT20030676 Camel France (IV)
HT20030674 Camel France (IV)
8086 Horse England (II)
HT20030679 Camel France (IV)
AV8063 Pigeon Japan (I)
S. intermedius NCTC11048 Pigeon (I)
AV8081 Pigeon Japan (I)
AV8061 Pigeon Japan (I)
S. schleiferi ATCC43808
0.01
S. pseudintermedius
S. delphini
S. intermedius

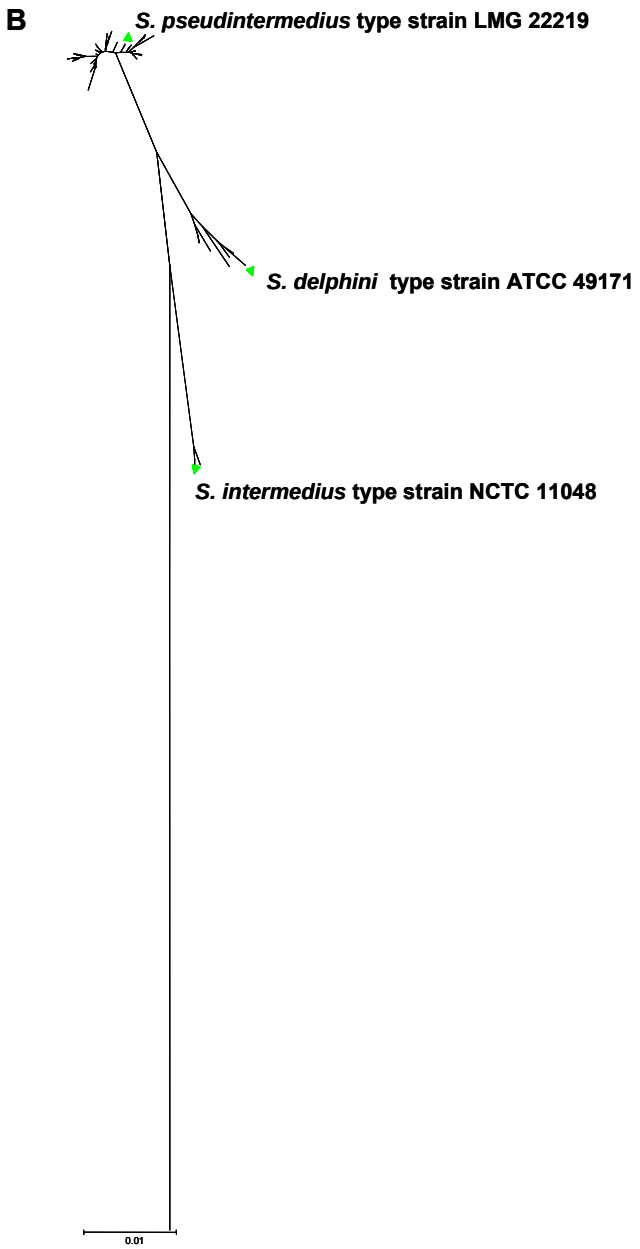


Figure 3.2. Phylogenetic tree constructed with the neighbour-joining method with 1000 bootstrap replicates of concatenated 16S rRNA gene, *tuf*, *cpn60*, and *pta* sequences. (A) Branch-style tree indicating host and geographic origin of all isolates. Methicillin-resistant, *mecA*-positive isolates (red dots), human isolates (blue squares), previously identified *S. pseudintermedius* isolates (yellow diamonds), and species type isolates (green triangles) are indicated along with *agr* type in roman numerals in parenthesis. Bootstrap values over 50% are indicated. (B) Radiating tree indicating the three major phylotypes with species type strains indicated by green triangles.

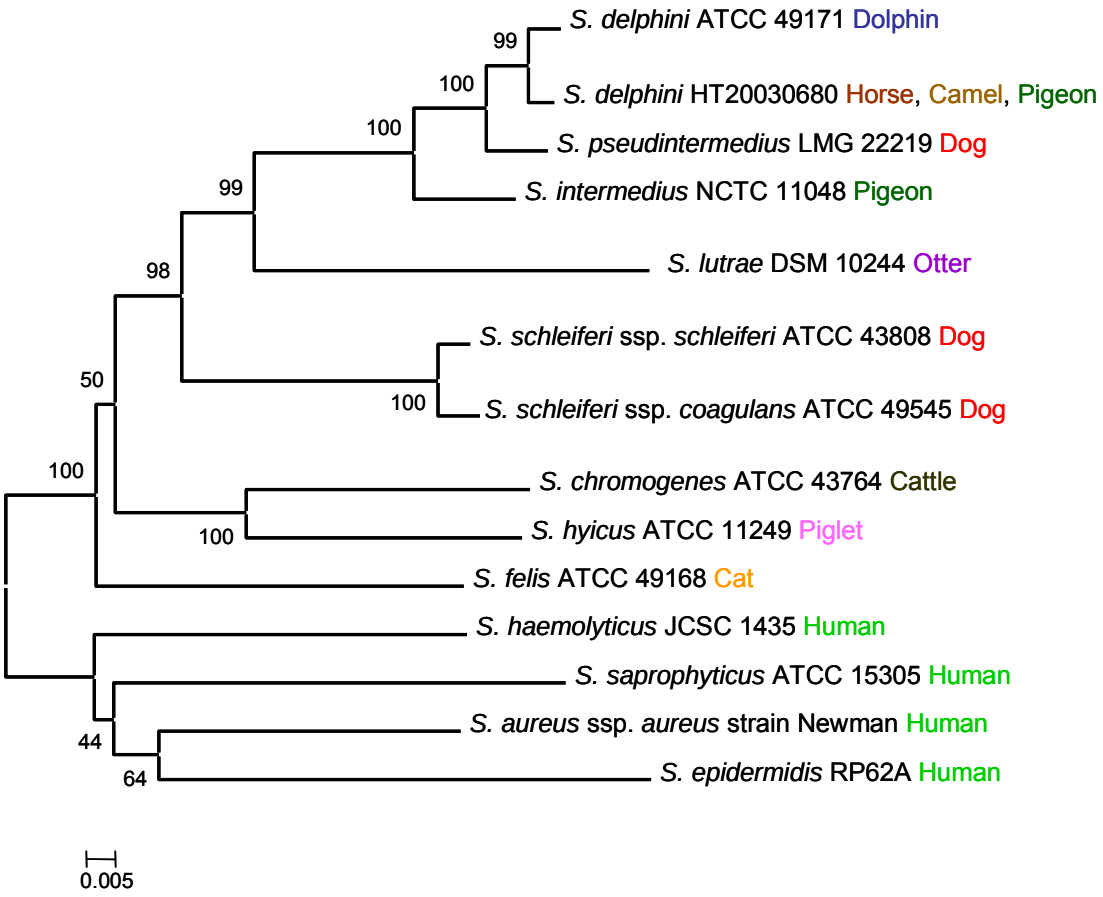


Figure 3.3. Phylogenetic tree showing the relatedness of staphylococcal species associated with skin disease in different animal hosts compared to clinically important staphylococcal species of humans. The phylogenetic tree was constructed with the neighbour-joining method with 1000 bootstrap replicates of concatenated 16S rRNA gene, *tuf*, *cpn60*, and *pta* sequences; bootstrap values are indicated. The host association of each species is indicated. *S. delphini* HT20030680 was included as a representative of a *S. delphini* subgroup identified in the current study, which was distinct from the the *S. delphini* type strain.

JCSC1435, *S. saprophyticus* ATCC 15305, *S. aureus* strain Newman, and *S. epidermidis* RP62A (Figure 3.3). The current study demonstrated that animal skin-associated staphylococcal species are more closely-related to each other than to the human disease-associated species (Figure 3.3), and the SIG belong to a subgroup which is most closely-related to *S. lutrae*. Overall, these data based on multiple gene loci are in good agreement with the findings of Kwok and Chow (2003).

3.4.4 Phylogenetic analysis reveals that *S. pseudintermedius*, and not *S. intermedius*, is the common cause of canine pyoderma

The type strain of *S. intermedius* (NCTC 11048) belongs to a small phylogenetically distinct group of pigeon isolates, which is not representative of the majority of isolates phenotypically identified as *S. intermedius*. All canine isolates (n=75) belong to a single phylotype, which includes the four isolates previously identified as the novel species *S. pseudintermedius* (Devriese *et al.*, 2005) (Figure 3.2). The *S. pseudintermedius* isolates were distributed among the canine isolates in the phylogenetic tree, including two strains, *S. pseudintermedius* LMG 22221 and *S. pseudintermedius* LMG 22222, which were genetically indistinguishable from canine isolates, and belong to one of the most populous nodes (Figure 3.2). These data indicate that the recently described *S. pseudintermedius*, and not *S. intermedius*, is the common cause of canine pyoderma.

The third major phylotype was represented by isolates from horse, camel, and pigeon hosts, and included the *S. delphini* type strain ATCC 49171, isolated from a dolphin (Varaldo *et al.*, 1988), suggesting that *S. delphini* may be commonly misidentified as *S. intermedius* (Figure 3.2).

3.4.5 *S. pseudintermedius* has a largely clonal population structure

To further investigate the population structure of the whole SIG, and of *S. pseudintermedius* and *S. delphini*, the degree of linkage disequilibrium was calculated within the whole SIG and within the *S. pseudintermedius* and *S. delphini* species (Table 3.4). The value of the index of association standardised (I_A^S) for the SIG as a whole was 0.1510 (P<0.001), and for the *S. pseudintermedius* and *S. delphini* species

Table 3.4. Analysis of linkage disequilibrium by calculation of I_A^S for the SIG, and for the *S. pseudintermedius* and *S. delphini* phylotypes.

Group selection	I_A^S	*P* value[a]	
		Parametric	**Monte Carlo (100 iterations)**
SIG	0.151	0.0000	0.001
S. pseudintermedius	0.0877	0.0000	0.001
S. delphini	0.3699	0.0000	0.001
S. intermedius[b]	ND	ND	ND

[a]The value of the index of association (I_A^S) would be expected to be zero for linkage equilibrium when recombination events occur frequently. If the I_A^S (P<0.05) value differs significantly from zero, recombination should be rare.

[b]The small number of isolates of the *S. intermedius* phylotype precluded carrying out this analysis. ND, not done.

it was 0.0877 (P<0.001) and 0.3699 (P<0.001) respectively, indicating that there is linkage disequilibrium within each group tested (Table 3.4). In addition, the RDP2 program was used to search for evidence of recombination among the selected gene loci (Martin *et al.*, 2005). For each locus, none of the recombination detection methods GENECONV, BOOTSCAN, MAXIMUM χ^2, CHIMAERA, and SISTER SCANNING detected any evidence for recombinant sequences among the isolates examined. Taken together, these data suggest a largely clonal population structure for the SIG as a whole, and for the *S. pseudintermedius* and *S. delphini* species.

3.4.6 Successful clones of *S. pseudintermedius* are identified on different continents

The number of alleles at each of the five gene loci (16S rRNA, *cpn60*, *tuf*, *pta*, and *agrD*) was identified and unique complements of alleles were defined as sequence types (ST) (Table 3.1). *S. pseudintermedius* is characterised by the existence of 61 different STs among 89 isolates examined, indicating considerable clonal diversity within the species (Table 3.1). The limited number of *S. pseudintermedius* isolates from human and non-canine animal sources commonly shared identical or closely-related genotypes with *S. pseudintermedius* canine carrier strains, consistent with zoonotic transfer from dogs (Figure 3.2). Identical or closely-related *S. pseudintermedius* STs were found in different continents, indicating broad geographic dissemination of successful clones (Figure 3.2).

3.4.7 Four predicted AIP variants were detected among the SIG strains investigated, including a novel subtype

DNA sequencing of the *agrD* locus revealed the presence of four predicted AIP peptide variants among the strains examined, including a novel fourth variant that has not been described previously (designated type IV) and is specific to 27% of all strains (Table 3.5). All staphylococcal species examined to date have been shown to encode AIP peptides which are unique to that species. In contrast, the three closely related species examined in the current study had AIP peptide variants in common, suggesting the existence of a conserved *agr* quorum-sensing system. All four AIP variants (I-IV) were encoded among the *S. pseudintermedius* isolates, AIP variants I,

Table 3.5. Amino acid sequences of the predicted *agrD*-encoded AIP peptides identified among the strains examined.

***agr* type (AIP)**	**Amino acid sequence**	**Number of strains (%)**
I	RIPTSTGFF	16 (15%)
II	RIPISTGFF	31 (30%)
III	KIPTSTGFF	29 (28%)
IV[a]	KYPTSTGFF	28 (27%)

[a]Novel AIP variant identified in this study.

II, and IV were identified among the *S. delphini* group of isolates, and the *S. intermedius* type strain and related pigeon isolates all encoded AIP type I (Table 3.5).

3.4.8 Assortive recombination has contributed to the distribution of *agr* alleles

A lack of clonal association of *agrD* alleles was observed, and several isolates shared an identical genotype by 16S rRNA gene, *tuf*, *cpn60*, and *pta* loci, but encoded different AIP variants (Figure 3.2). Further, the *agrD* gene tree topology was markedly different from the other gene trees (Figure 3.1). These data and the lack of recombination breakpoints identified within *agrD* sequences using the RDP2 suite of programs suggest that assortive (whole gene) recombination may have contributed to the distribution of *agrD* alleles. There was no identifiable association between *agr* type and host, clinical, or geographic origin.

3.4.9 Sixteen of the *S. pseudintermedius* strains examined encode the *mecA* gene, conferring methicillin-resistance

Presence of the *mecA* gene in 16 of 105 isolates was detected by PCR (Table 3.1). Sequencing of the *mecA* gene in representative isolates revealed a high degree of homology with *mecA* of *S. aureus* origin (95% to 100%; data not shown). These strains had previously been identified as MRSI in the centres where they were isolated and had oxacillin broth microdilution (MIC) levels of >256 µg/ml for all strains bar one (strain 13, a canine isolate from Sweden), which had an MIC of 0.75 µg/ml. All strains previously identified as MRSI belonged to the *S. pseudintermedius* phylotype and are therefore re-classified as methicillin-resistant *S. pseudintermedius* (MRSP). The *mecA* sequencing and MIC determination work was performed by A. Moodley, University of Copenhagen, Denmark.

3.4.10 Methicillin-resistant *S. pseudintermedius* (MRSP) have evolved by multiple *mecA* gene acquisitions by different clones

MRSP genotypes are distributed widely across the *S. pseudintermedius* phylogenetic tree, including one isolate (3279) which is genetically indistinguishable from methicillin-sensitive isolates (ST29), suggesting horizontal transfer of the *mecA* gene

(Figure 3.2). The existence of multiple MRSP clones is supported by eBURST analysis, which shows that MRSP isolates belong to five distinct STs (ST29, ST68, ST69, ST70, ST71). The five STs differed at two to four of the five loci examined, strongly suggesting that they do not share a very recent ancestor (Figure 3.4). Taken together, these data suggest that the *mecA* gene has been acquired multiple times by different *S. pseudintermedius* strains. Of note, 9 out of 10 MRSP isolates from five different centres in Sweden and Germany belong to ST71, indicating that a common clone may predominate in North and Central Europe (Table 3.1). There was no sharing of STs among European and USA MRSP isolates (Table 3.1).

3.4.11 Development of a novel diagnostic test for the identification of *S. pseudintermedius*, using a simple PCR-restriction fragment length polymorphism (RFLP) approach

S. intermedius can be differentiated from the other two species biochemically, but *S. pseudintermedius* cannot be distinguished from *S. delphini* with biochemical tests (Sasaki *et al.*, 2007b). Therefore, a simple diagnostic test for the identification of *S. pseudintermedius* would be useful. Sequence analysis of the *pta* locus revealed the presence of an *Mbo*I restriction site in all *S. pseudintermedius* isolates, which was absent in all *S. intermedius* and *S. delphini* isolates examined. Based on this discovery, a PCR-restriction fragment length polymorphism (RFLP) diagnostic test for the identification of *S. pseudintermedius* was designed. The efficacy of the technique was tested by the analysis of 20 *S. pseudintermedius*, 11 *S. delphini*, and 4 *S. intermedius* isolates, which represented the breadth of diversity identified within the SIG including the type strains (Table 3.6). In addition, representative isolates of *S. aureus*, *S. schleiferi* ssp. *coagulans*, and *S. schleiferi* ssp. *schleiferi* were included (Table 3.6). A *pta* PCR product of 320 bp was successfully amplified from all strains examined. *S. pseudintermedius* PCR products all contained a single *Mbo*I restriction site, resulting in two restriction fragments of 213 bp and 107 bp, respectively (Figure 3.5). In contrast, SIG species *S. delphini* and *S. intermedius*, and the *S. schleiferi* strains did not contain an *Mbo*I restriction site (Figure 3.5). *S. aureus* isolates contained a unique *Mbo*I site, resulting in restriction fragments of 156 bp and 164 bp,

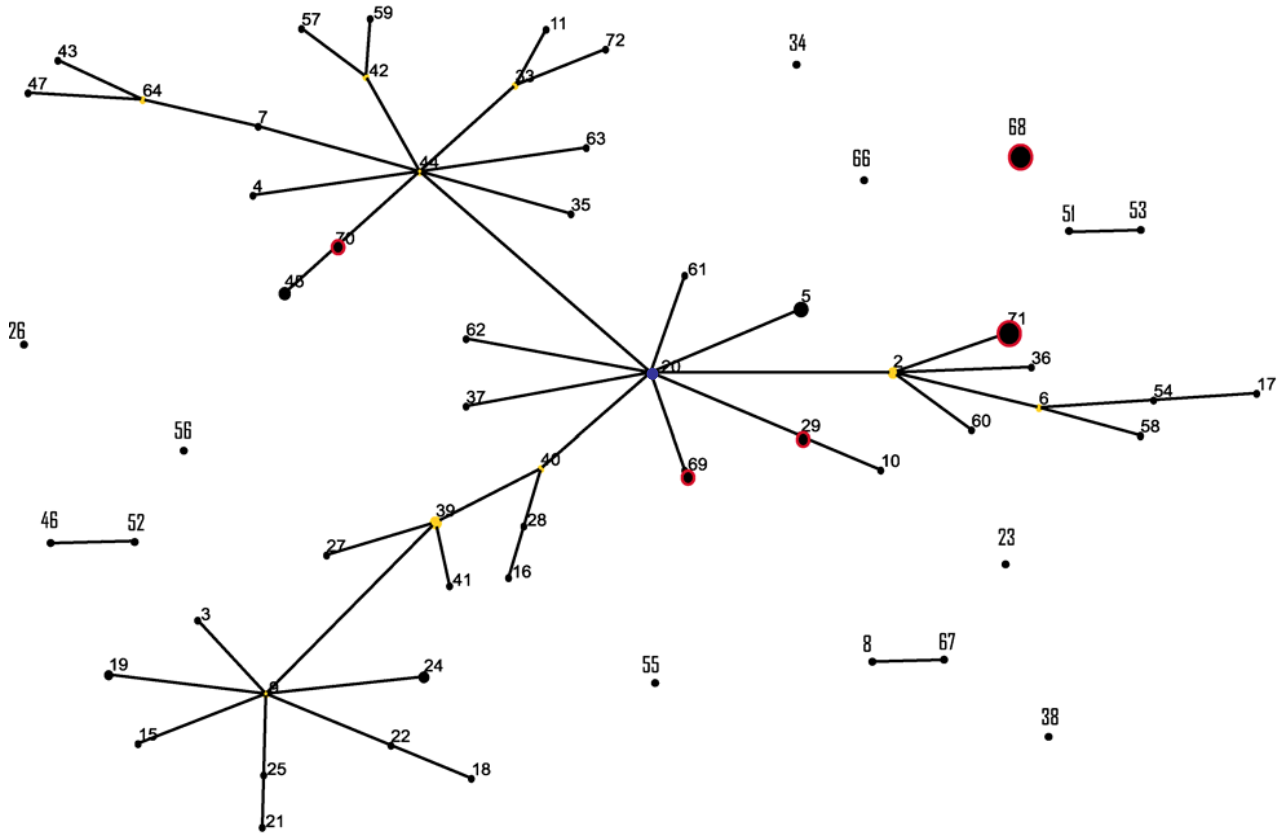


Figure 3.4. Schematic diagram of the clonal relatedness of *S. pseudintermedius* STs predicted by eBURST analysis. Each ST is represented by a black dot, the size of which is proportional to the number of isolates of that ST. Blue and yellow dots denote the predicted group founder and subgroup founders, respectively. Single locus variants are linked by lines. STs which include MRSP strains are indicated by a red circle. STs which are not linked by branches to other STs do not share at least 4 identical alleles with any other ST.

Table 3.6. Staphylococcal isolates and strains included in the PCR-RFLP diagnostic test.

Name	Species ID	Host origin	*Mbo*I restriction[a]
ED99	*S. pseudintermedius*	Dog	+
AV8001	*S. pseudintermedius*	Dog	+
95072195	*S. pseudintermedius*	Dog	+
94062394	*S. pseudintermedius*	Dog	+
N900260	*S. pseudintermedius*	Human	+
HT20030686	*S. pseudintermedius*	Dog	+
M72199	*S. pseudintermedius*	Dog	+
3279	*S. pseudintermedius*	Dog	+
3414	*S. pseudintermedius*	Cat	+
9075	*S. pseudintermedius*	Dog	+
690	*S. pseudintermedius*	Cat	+
8478	*S. pseudintermedius*	Dog	+
HH1	*S. pseudintermedius*	Dog	+
Can6	*S. pseudintermedius*	Dog	+
Can10	*S. pseudintermedius*	Human	+
BH47	*S. pseudintermedius*	Dog	+
AV8024	*S. pseudintermedius*	Dog	+
8016	*S. pseudintermedius*	Dog	+
LMG22219	*S. pseudintermedius*	Cat	+
LMG22220	*S. pseudintermedius*	Horse	+

Name	Species ID	Host origin	*Mbo*I restriction[a]
ATCC49171	*S. delphini*	Dolphin	-
HT20030680	*S. delphini*	Camel	-
9106	*S. delphini*	Horse	-
8485	*S. delphini*	Horse	-
AV8051	*S. delphini*	Pigeon	-
HT20030677	*S. delphini*	Camel	-
AV8047	*S. delphini*	Pigeon	-
HT20030676	*S. delphini*	Camel	-
HT20030674	*S. delphini*	Camel	-
8086	*S. delphini*	Horse	-
HT20030679	*S. delphini*	Camel	-
AV8061	*S. intermedius*	Pigeon	-
NCTC11048	*S. intermedius*	Pigeon	-
AV8063	*S. intermedius*	Pigeon	-
AV8063	*S. intermedius*	Pigeon	-
ATCC43808	*S. schleiferi* subsp. *schleiferi*	Human	-
CCUG37248	*S. schleiferi* subsp. *coagulans*	Dog	-
Newman	*S. aureus*	Human	+
N315	*S. aureus*	Human	+

[a]+, present; -, absent.

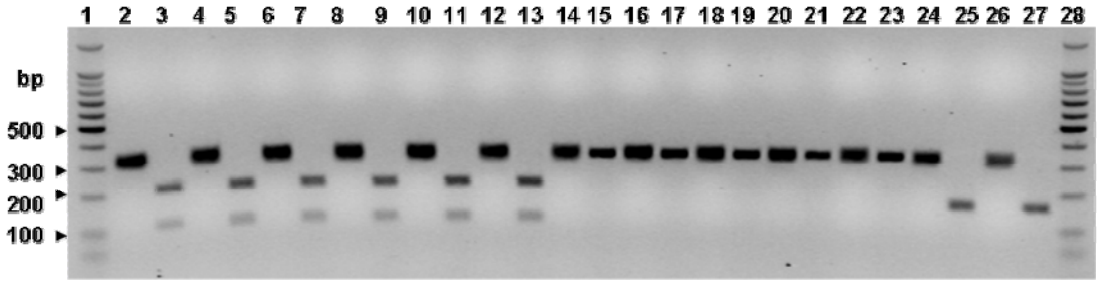


Figure 3.5. Agarose gel electrophoresis of *Mbo*I-restriction digested *pta* PCR products. Lane 1, 100 bp ladder; Lane 2, *S. pseudintermedius* ED99; Lane 3, *S. pseudintermedius* ED99, *MboI*-digested; Lane 4, *S. pseudintermedius* HH1; Lane 5, *S. pseudintermedius* HH1, *Mbo*I-digested; Lane 6, *S. pseudintermedius* Can6; Lane 7, *S. pseudintermedius* Can6, *Mbo*I-digested; Lane 8, *S. pseudintermedius* Can10; Lane 9, *S. pseudintermedius* Can10, *Mbo*I-digested; Lane 10, *S. pseudintermedius* LMG22219; Lane 11, *S. pseudintermedius* LMG22219, *Mbo*I–digested; Lane 12, *S. pseudintermedius* 3414; Lane 13, *S. pseudintermedius* 3414, *Mbo*I–digested; Lane 14, *S. delphini* ATCC49171; Lane 15, *S. delphini* ATCC49171, *Mbo*I–digested; Lane 16, *S. delphini* 9106; Lane 17, *S. delphini* 9106, *Mbo*I–digested; Lane 18, *S. delphini* HT20030680; Lane 19, *S. delphini* HT20030680, *Mbo*I–digested; Lane 20, *S. intermedius* NCTC11048; Lane 21, *S. intermedius* NCTC11048, *Mbo*I–digested; Lane 22, *S. intermedius* AV8061; Lane 23, *S. intermedius* AV8061, *Mbo*I–digested; Lane 24, *S. aureus* Newman; Lane 25, *S. aureus* Newman, *Mbo*I–digested; Lane 26, *S. aureus* N315; Lane 27, *S. aureus* N315, *Mbo*I–digested; Lane 28, 100 bp ladder.

which appeared as a single band after agarose electrophoresis, and was readily distinguishable from the *S. pseudintermedius* restriction fragment profile (Figure 3.5).

3.5 Discussion

The coagulase positive species *S. intermedius* was first described by Hajek *et al.* (1976), based on isolates from pigeons, dogs, minks, and horses. The type strain, NCTC 11048 (also known as ATCC 29663, CCM 5739, or LMG 13351), isolated from a pigeon, has typically been used to represent *S. intermedius* in species differentiation studies (Goh *et al.*, 1997; Kwok *et al.*, 1999; Kwok and Chow, 2003; Devriese *et al.*, 2005). The data from the current study indicate that isolates phenotypically identified as *S. intermedius* are differentiated into three distinct species, *S. intermedius*, the recently described *S. pseudintermedius* (Devriese *et al.*, 2005), and *S. delphini*, which are together referred to as the SIG. The data indicate further, that the *S. intermedius* type strain of pigeon origin represents a distinct taxon that is not representative of the majority of isolates commonly identified as *S. intermedius*. In parallel to the current study, Sasaki *et al.* (2007b) carried out a study of phenotypically identified *S. intermedius* isolates in Japan based on DNA sequences of *sodA* and *hsp60* genes. A population genetic structure consistent with the current study was revealed among the Japanese isolates, and a re-classification of the species was suggested (Sasaki *et al.*, 2007b). Further, interspecies multilocus DNA sequencing analysis was performed on staphylococcal species associated with animal skin infection in addition to species of human clinical importance. The SIG form a distinct group, but are closely related to other animal-skin associated staphylococcal species consistent with the findings of Kwok and Chow (2003).

Over the past 33 years, *S. intermedius* has been considered the common cause of pyoderma in dogs (Medleau *et al.*, 1986; Devriese and De Pelsmaecker, 1987; Cox *et al.*, 1988; Allaker *et al.*, 1992a; Allaker *et al.*, 1992b; Werckenthin *et al.*, 2001). In the current study, all canine isolates examined are classified as *S. pseudintermedius*. These data suggest that the newly described *S. pseudintermedius* species, and not *S.*

intermedius, is the common cause of canine pyoderma. Therefore, canine pyoderma isolates described as *S. intermedius* in previous studies should be re-classified, and are referred to as *S. pseudintermedius* in the remainder of this text. *S. pseudintermedius* is represented by over 60 different STs identified among the 89 isolates examined, revealing considerable clonal diversity within the species. Identical and closely related STs were identified in several countries on different continents, indicating global dissemination of the most successful clones. The clonal diversity and broad geographic distribution of *S. pseudintermedius* suggests that it has co-evolved with its canine host for a long time in evolutionary terms, and possibly since the evolution of dogs between 40 and 50 million years ago (Wayne, 1993). STs of *S. pseudintermedius* isolates of human origin are identical or closely related to isolates of dogs, suggesting zoonotic transmission from the canine host. Indeed, transmission of *S. pseudintermedius* has been shown to occur frequently between dogs affected by deep pyoderma and their owners (Guardabassi *et al.*, 2004a). The third major phylotype found among the isolates previously identified as *S. intermedius* was associated with several different host species including horse, camel, and pigeon, and was phylogenetically aligned with the *S. delphini* type strain isolated from a dolphin (Varaldo *et al.*, 1988b). A previous DNA-DNA hybridisation analysis confirmed that the *S. delphini* type strain represented a distinct species compared to the recently described *S. pseudintermedius* and the *S. intermedius* type strain (Devriese *et al.*, 2005). In summary, these data suggest that *S. delphini* is commonly misidentified as *S. intermedius*, and may be more clinically important than was previously thought. In fact, very few studies have reported the identification of *S. delphini* strains since the original description of the species, but one study in Norway isolated *S. delphini* from a case of bovine mastitis, extending the broad host range observed in the current study (Bjorland *et al.*, 2005). Of note, Sasaki *et al.* (2007b) indicated that more than one species may exist among isolates genetically allied with *S. delphini*.

The *agr* system is conserved throughout the staphylococci, but has diverged along lines that appear to parallel speciation within the genus (Dufour *et al.*, 2002; Wright *et al.*, 2005). In order to further investigate the diversity of SIG populations, the

agrD locus, which encodes the AIP (Dufour *et al.*, 2002; Ji *et al.*, 2005; Wright *et al.*, 2005; Sung *et al.*, 2006), was examined in the current study. Sung *et al.* (2006) identified three *agrD* alleles corresponding to *agr* specificity groups encoding different AIP variants among 20 strains of *S. pseudintermedius* isolated from dogs in a single veterinary hospital in the United Kingdom, and demonstrated biological activity for two of them. Here, the same three predicted AIPs were identified, in addition to a novel fourth AIP, which was encoded by approximately 27% of strains. The AIP variants may correspond to distinct *agr* interference groups (Dufour *et al.*, 2002; Wright *et al.*, 2005; Sung *et al.*, 2006). Of note, all of the predicted AIP variants of the SIG contain a serine in place of the conserved cysteine found in the AIPs of the other staphylococcal species analysed to date, resulting in a lactone, rather than a thiolactone ring (Ji *et al.*, 2005). All previously examined staphylococcal species encode AIPs that are unique to each species (Dufour *et al.*, 2002). However, the present study revealed that *agr* alleles were shared among the three closely related species of the SIG, indicating that a common quorum sensing capacity has been maintained, despite species differentiation in distinct ecological niches. A previous study of *agr* evolution in *S. aureus* reported that genotypes were not associated with more than one *agr* type, indicating that *agr* radiation preceded clonal diversification and that recombination has played a very limited role in the distribution of *agr* diversity (Wright *et al.*, 2005). The study concluded that the species was phylogenetically structured according to *agr* group (Wright *et al.*, 2005). Robinson *et al.* (2005) provided evidence that the *S. aureus* species could be divided into two subgroups that both contain multiple clonal complexes and *agr* groups. The authors proposed that recombination events had resulted in the sharing of *agr* groups between the two subspecies, but concluded that recombination of *agr* has not occurred very frequently within *S. aureus* populations, as *agr* and clone tree topologies within the two subspecies were in agreement (Robinson *et al.*, 2005). In contrast, the association of different *agr* alleles with strains of *S. pseudintermedius* of identical genotype identified in the current study suggests that assortive recombination has frequently contributed to the distribution of *agr* alleles among *S. pseudintermedius* populations. The markedly different topology of the phylogenetic tree constructed with *agrD* sequences compared to trees based on the other four gene

loci is consistent with this theory. Overall, *S. pseudintermedius* has a largely clonal population structure, but recombination appears to have played an important role in the distribution of *agr* alleles within the *S. pseudintermedius* species, and the SIG as a whole. The sharing of *agr* alleles between different species of staphylococci has not been previously observed. This discovery indicates that *agr* differentiation has not occurred strictly along lines that parallel speciation. The lack of an association between *agr* type and SIG species, host, disease, clinical, or geographic origin identified in the current study leads to the question of what selective pressure is driving *agr* diversification. The basis for *agr* diversity and the importance of its biological activity in the SIG remain to be elucidated.

Antimicrobial resistance of staphylococcal animal pathogens, including methicillin-resistance, has been increasingly reported over recent years (Guardabassi *et al.*, 2004b; Kania *et al.*, 2004; Lloyd, 2007; Sasaki *et al.*, 2007a), demonstrating the need for antimicrobial surveillance in the veterinary field. The *mecA* positive SIG strains identified in the current study are all classified as methicillin-resistant *S. pseudintermedius* (MRSP), and have evolved by multiple acquisitions of the *mecA* gene by different *S. pseudintermedius* clones. Of note, a common clone was identified among isolates from five different centers in Sweden and Germany, indicating the existence of a widespread successful clone in Northern and Central Europe. MRSP clones were not shared between Europe and North America, indicating geographic restriction, and probably reflecting the very recent emergence of methicillin-resistant strains (Lloyd, 2007).

S. intermedius can be differentiated from the other two species biochemically, but *S. pseudintermedius* cannot be distinguished from *S. delphini* with biochemical tests (Sasaki *et al.*, 2007b). Moreover, the multilocus DNA sequencing approach developed in the current study is too laborious to be applied in routine diagnostic laboratories for species identification. Therefore, a simple, fast, and reliable diagnostic test for the identification of *S. pseudintermedius* was developed. The PCR-RFLP assay is based on a single *Mbo*I restriction site in the *pta* gene of *S. pseudintermedius*, which is absent in *S. intermedius* and *S. delphini*. The PCR-RFLP

assay is an effective approach to *S. pseudintermedius* identification, and the reproducibility of the method was tested by independent investigators on 112 coagulase-positive staphylococcal field isolates, including 86 of canine origin (Baron *et al.*, 2004), in a diagnostic laboratory in Italy (data not shown). Of the 86 canine isolates, 85 (98.8%) were identified as *S. pseudintermedius* using the PCR-RFLP approach (Bannoehr *et al.*, 2009), consistent with the findings of this study that *S. pseudintermedius* is a common commensal of dogs, and the major canine pyoderma pathogen. The remaining isolate resulted in a *pta* restriction profile which indicated a *S. aureus* identity (Bannoehr *et al.*, 2009).

In summary, the present study revealed the population genetic structure of the SIG, and identified *S. pseudintermedius* as the common canine pyoderma pathogen. Further, important insights into SIG evolution, especially with regard to antimicrobial resistance and regulation of virulence, are provided. The current study represents a much-needed framework for antimicrobial surveillance of *S. pseudintermedius*, and for studies into the molecular basis of disease pathogenesis.

Chapter 4

Genome-wide identification and analysis of novel cell wall-anchored proteins encoded by *Staphylococcus pseudintermedius* ED99

4.1 Introduction

The whole genome sequence of a canine pyoderma isolate of *S. pseudintermedius* (strain ED99) has been determined recently in our laboratory, providing a powerful resource to study bacterial evolution and pathogenesis. The first completed staphylococcal genome sequences, of the related *S. aureus* MRSA strains N315 (methicillin-resistant) and Mu50 (vancomycin-resistant), were published in 2001 (Kuroda *et al.*). To date, over 20 staphylococcal genomes are available from the NCBI database (http://www.ncbi.nlm.nih.gov/; accessed September 10th, 2009), with the number increasing rapidly. Among staphylococcal species, the shared genus-specific core genome encodes for approximately 50% of proteins of an individual species (Becker *et al.*, 2007). The region downstream of the origin of replication (*oriC)* in staphylococcal genomes, known as the '*oriC* environ', is conserved among strains of the same species, but has very limited synteny between different species (Takeuchi *et al.*, 2005). The *oriC* environ encodes for many species-specific proteins including several virulence factors and is therefore thought to contribute to staphylococcal speciation and niche-adaptation (Takeuchi *et al.*, 2005).

For *S. aureus* genomes, a nomenclature which differentiates three distinct genome regions has been proposed, consisting of a core genome (genes conserved in all *S. aureus* strains), core variable (CV) genome (genes shared among strains with the same evolutionary background), and accessory genome, consisting largely of mobile genetic elements (MGE), which can be transferred among strains by lateral gene transfer (Lindsay *et al.*, 2006; Ben Zakour *et al.*, 2008). Staphylococcal surface-associated proteins involved in host-pathogen interactions, such as MSCRAMMs, are generally encoded by the core or CV genome (Lindsay *et al.*, 2006; Ben Zakour *et al.*, 2008). All staphylococcal species sequenced to date possess their own unique MSCRAMM repertoire, which might contribute to host or niche specialisation of the bacterial species to its particular environment (Ben Zakour *et al.*, 2008).

4.2 Aims and Strategy

To identify putative cell wall-anchored (CWA) surface proteins of *S. pseudintermedius* ED99 and to investigate their distribution among diverse *S. pseudintermedius* strains and closely related staphylococcal species by:

1) Bioinformatic analysis of the whole genome sequence of *S. pseudintermedius* ED99 to identify genes encoding putative LPXTG-containing CWA surface proteins. Further examination of the CWA proteins for the presence of characteristic MSCRAMM features such as a signal sequence, repeat region, and Ig-like fold using bioinformatic tools.

2) Screening of a diverse collection of *S. pseudintermedius* strains and of closely related staphylococcal species for the presence of the genes encoding putative CWA proteins by Southern blot and PCR amplification.

4.3 Materials and Methods

4.3.1 Bacterial strains used in this study

S. pseudintermedius strain ED99 is a clinical isolate from a canine pyoderma case presented at the Hospital for Small Animals, Royal (Dick) School of Veterinary Studies, The University of Edinburgh, UK. The whole genome sequence of ED99 was previously determined in our laboratory by Nouri L. Ben Zakour.

The staphylococcal strains examined for the distribution of genes encoding the putative CWA proteins are represented in a phylogenetic tree in Figure 4.1. Additionally, *S. aureus* A/G21/06/02, isolated from a seal in the UK, was included as negative control, and *S. pseudintermedius* ED99 was employed as positive control. *S. lutrae*, *S. schleiferi* ssp. *coagulans*, *S. hyicus*, *S. chromogenes*, and *S. aureus* A/G21/06/02 were a gift from Geoff Foster, Scottish Agricultural College, UK. All other strains are described in Chapter 3, Table 3.1.

4.3.2 Genome-wide screen for genes encoding cell wall-anchored proteins

The *S. pseudintermedius* strain ED99 draft genome was interrogated for homologous sequences using position specific iterative basic local alignment search tool (PSI-BLAST), available from the National Center for Biotechnology Information (NCBI), USA (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and for the presence of a LPX[TSA][GANS] motif pattern by pattern hit initiated basic local alignment search tool (PHI-BLAST), available from NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Signal sequences were predicted by employing the SignalP server (http://www.cbs.dtu.dk/services/SignalP/), provided by the Center for Biological Sequence Analysis (CBS), Technical University of Denmark.

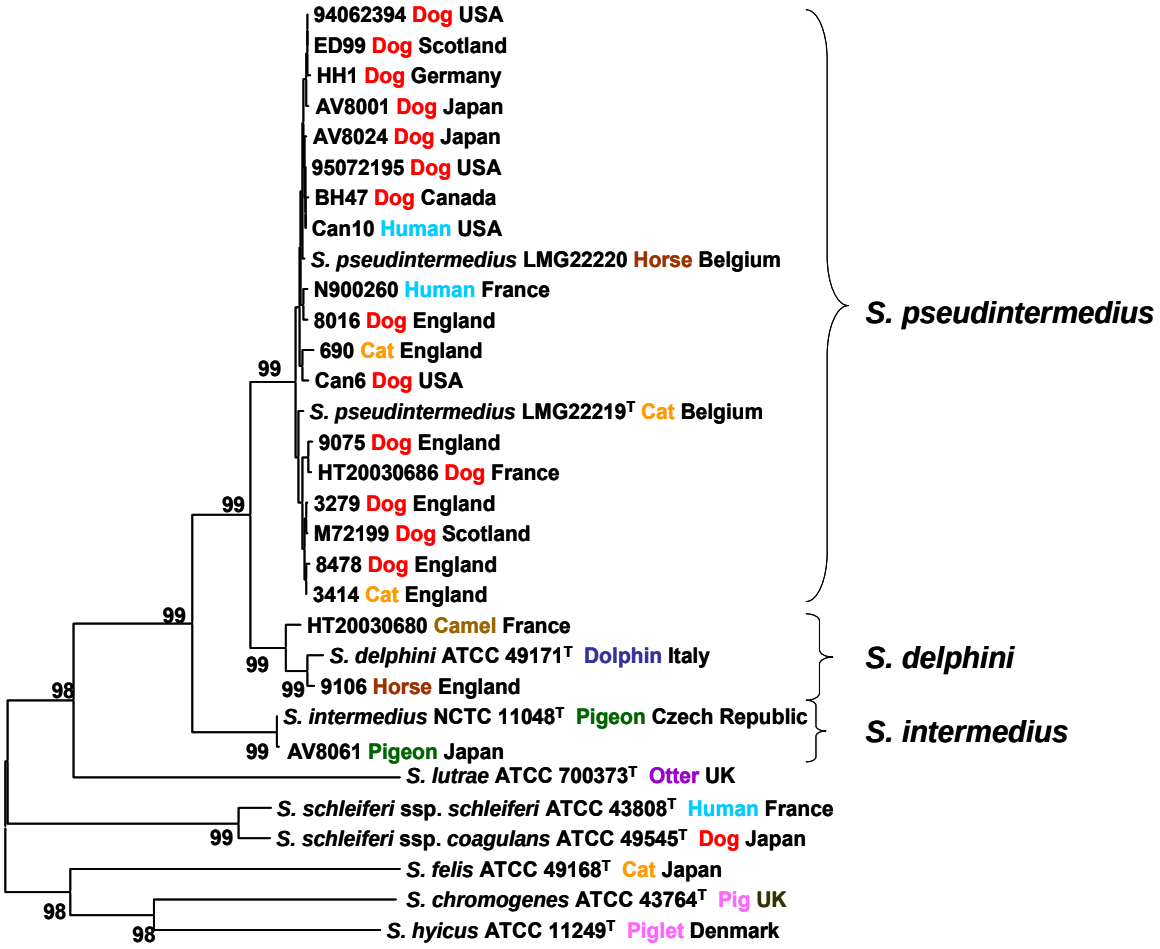


Figure 4.1. Phylogenetic tree representing the staphylococcal strains included in Southern blot and PCR screen for the presence of putative CWA encoding genes identified in *S. pseudintermedius* ED99. The host and geographic origin of each strain is indicated. The phylogenetic tree was constructed with the neighbour-joining method with 1000 bootstrap replicates of concatenated 16S rRNA gene, *tuf*, *cpn60*, and *pta* sequences; bootstrap values are indicated. ^{T}species type strain.

4.3.3 *In silico* structural analysis of cell wall-anchored proteins

The predicted CWA proteins were searched for functional domains using EMBL-EBI InterProScan (http://www.ebi.ac.uk/interpro). Structural analysis was carried out with the PHYRE (Protein Homology/analogY Recognition Engine) fold recognition server, available from the Structural Bioinformatics Group, Imperial College London, UK (http://www.sbg.bio.ic.ac.uk/phyre/). Repeat sequences were predicted by generating nucleic acid dot plots, using software available from Colorado State University, USA (http://www.vivo.colostate.edu/molkit/dnadot/), applying tandem repeats finder software from Boston University, USA (http://tandem.bu.edu/trf/trf.html), and variable sequence tandem repeats extraction and architecture modelling (XSTREAM), available from the University of California, USA (http://jimcooperlab.mcdb.ucsb.edu/xstream/). Sequence alignments and pair-wise sequence comparisons were generated with ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2). Amino acid composition and molecular weight predictions were generated using ProtParam on the ExPASy Proteomics Server (http://www.expasy.ch/tools/protparam.html).

4.3.4 Southern blot analysis

Genomic DNA of 32 staphylococcal strains (Figure 4.1) was extracted and digested as described in general Materials and Methods, using *Hind*III restriction endonucleases (New England Biolabs, UK). Oligonucleotides were designed to amplify ~500 bp products specific for each gene encoding putative CWA proteins (Table 4.1). 50 µl PCR reactions for generating probes contained 1 µl of a 1:10 dilution of genomic DNA template from *S. pseudintermedius* ED99, 0.5 µM forward primer, 0.5 µM reverse primer, 1x reaction buffer (Promega, USA), 1 mM $MgCl_2$ (Promega, USA), 0.2 mM dNTPs (Promega, USA) and 0.025 u/µl *Taq* polymerase (Promega, Madison, WI, USA). The thermocycler programme for PCR amplification of the probes included an initial denaturation step of 94°C for 4 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min, followed by a final extension step at 72°C for 10 min. PCR products were resolved by electrophoresis in a 1% (w/v) agarose gel. Restriction-digested genomic DNA was resolved in a 0.8% (w/v) agarose gel at 80 V for 2.5 h.

Table 4.1. Oligonucleotides for PCR amplification of Southern blot probes.

Name [a, b]	Sequence (5′- 3′)
spsA-F	TTGGTAGTGGACTGCAAATGCC
spsA-R	GGTCTTTCAGTGCGCTCACATC
spsB-F	GCCTTGATTTCGGTGTCAGACA
spsB-R	GCTGCCACCTAGAACGATTGC
spsC-F	CACGCCACTCGGTTGGTTCA
spsC-R	TGGAATGCGCCATAGTACCAAG
spsD-F	TGGTCAAGGAGAATTGCCACA
spsD-R	TTTTCACCACGATCCTCACCA
spsE-F	AACGCGTCAAACAACGTGCAA
spsE-R	CGCCATCAGTCGAAACGTAGGA
spsF-F	AGTGGAAGCAACAGTTGAACGC
spsF-R	TGGACCTACTTGGCTACCACCA
spsG-F	AACACAGCTGACAAAGCAACCG
spsG-R	CGATGTTTGCAGTTTGGCATTT
spsH-F	GATCAAAGGGCACACATGGATG
spsH-R	CCGTTATCAGTGGCAGGTGGT
spsI-F	GCAAACAAAAAAGCACCTGCAC
spsI-R	AAGATTCGCCTGCAATGTCGTA
spsJ-F	GTACGTCGGTTTCATTGAGCCA
spsJ-R	TTGACACTGATGCCGAAGCAC
spsK-F	ATTTCACAAGGGAACGCACATG
spsK-R	TGAGCCGCACGTCTATTCTGAA
spsL-F	ATGGCAAGCAAAATTTGACTGC
spsL-R	GTGTCAGCTTGCGCATCATATG
spsM-F	GGTGCTAAAGCCTACGATGCG
spsM-R	CCTCAACAATACTGCCCGATGA
spsN-F	GTGGCAAGGTCGTTATGACGGG
spsN-R	TGCCCTGATCCTTGATGTTTTGT
spsO-F	GGTAGTGTATCAGTGCTAATAGGAGCC
spsO-R	TTGACAAATCAGTAGCTGATGCATC

[a]F, forward primer; R, reverse primer; [b]*sps*, *Staphylococcus pseudintermedius* surface protein.

The agarose gel was depurinated in 250 mM HCl for 10 min, washed in distilled H_2O, then transferred to denaturation solution (1.5 M NaCl, 0.5 M NaOH) for 1 h. The gel was washed briefly in distilled H_2O and immersed in neutralisation solution (1.5 M NaCl, 0.5 M Tris-HCl, pH 7.5) for 1 h. All steps were carried out at room temperature with gentle agitation. The DNA was transferred onto positively charged nylon membrane (Roche Diagnostics GmbH, Germany) by capillary action with 10x SSC (0.15 M Na_3Citrate, 1.5 M NaCl, pH 7.0), using the method of Southern (Southern, 1975).

After transfer, membrane and gel were removed together and the positions of the DNA ladder were indicated on the membrane with a pencil. The gel was discarded after exposure to UV light to ensure that the DNA had been completely transferred. The membrane was rinsed in distilled H_2O for 1 min and the DNA was cross-linked onto the membrane by UV exposure for 30 s on each side of the membrane. DNA probes were labelled using the ECL™ Direct Nucleic Acid Labelling and Detection System (Amersham Biosciences, GE Healthcare, UK) according to the manufacturer's instructions. In brief, the DNA membrane was pre-hybridised with 25 ml gold hybridisation buffer containing 5% (w/v) blocking buffer and 0.5 M NaCl for 1 h at 42°C. 100 ng DNA probe template was denatured by boiling for 5 min, immediately chilled on ice, and 10 µl labelling reagent and 10 µl glutaraldehyde solution were added. After incubation at 37°C for 10 min, the labelled probe was added to the hybridisation buffer and hybridisation was carried out overnight at 42°C in a rotating hybridisation oven (Jencons Scientific Inc., USA). After hybridisation, 100 ml 5x SSC (75 mM Na_3Citrate, 750 mM NaCl, pH 7.0) was added to the blot, rotating for 5 min while the temperature of the oven was increased to 55°C. Washes were carried out with high stringency primary wash buffer, containing 0.4% (w/v) SDS and 0.1x SSC (1.5 mM Na_3Citrate, 15 mM NaCl, pH 7.0) for 10 min, followed by two washes for 5 min each, and two further washes with 2x SSC secondary wash buffer (30 mM Na_3Citrate, 300 mM NaCl, pH 7.0) for 5 min each. For signal detection, 2.5 ml of detection reagent 1 was mixed with 2.5 ml of detection reagent 2 and applied to the blot. After 1 min incubation at room temperature, excess solution

was removed, and the blot was wrapped in cling film. Membranes were exposed to chemiluminescence film (Hyperfilm^{TM}, Amersham Biosciences, GE Healthcare, UK) within a Hypercassette^{TM} (Amersham Biosciences, GE Healthcare, UK) for 10 s to 30 min, depending on the strength of signal. The Hyperfilm^{TM} was developed in an x-ray processor (Optimax, PROTEC Medizintechnik GmbH & Co. KG, Germany) with x-ray developer solution (Champion Photochemistry, UK) and x-ray fixer solution (Photosol Ltd., UK).

4.3.5 PCR amplification of *spsP* and *spsQ* gene fragments

Oligonucleotides were designed for partial amplification of *spsP* (~300 bp product) and *spsQ* (~600 bp product) based on the *S. pseudintermedius* ED99 genome sequence (Table 4.2). 50 µl PCR reactions consisted of 1 µl of a 1:10 dilution of genomic DNA template, 0.3 µM of the appropriate forward and reverse primer, 1x reaction buffer (Promega, USA), 0.2 mM dNTP's (Promega, USA), 1 mM $MgCl_2$ (Promega, USA) and 0.025 u/µl *Taq* polymerase (Promega, USA). The thermocycler programme included an initial denaturation step at 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 53°C for 1 min and extension at 72°C for 1 min, followed by a final extension step at 72°C for 7 min. PCR products were visualised on 1.2% (w/v) agarose gels.

Table 4.2. Oligonucleotides designed for PCR amplification of regions specific for the two predicted *S. aureus* Protein A (SpA) orthologues in *S. pseudintermedius* ED99.

Name[a]	Sequence (5′- 3′)
spsP-F	CAGGAGGACTAGGGTAATGTTCC
spsP-R	GCAAAACTTGGCGTGTTTACAAG
spsQ-F	CCGCTCTATTTTTAGGTTAATC
spsQ-R	GCGCTTCATCGAAACTTGGCGCAGG

[a]F, forward primer; R, reverse primer.

4.4 Results

4.4.1 Identification of genes encoding 17 putative cell wall-anchored proteins in the *S. pseudintermedius* ED99 genome

The initial search for putative CWA proteins identified 34 sequences that fulfilled at least one of the search criteria (homology to characterised MSCRAMMs in the database, predicted LPXTG motif or variant near the C terminus, predicted signal peptide at the N terminus). After gap closure and combination of incomplete sequences, a total of 17 ORFs encoding putative CWA proteins with a predicted minimum length of approximately 250 amino acids was determined. The 17 predicted CWA proteins were designated 'Sps' for '*Staphylococcus pseudintermedius* surface proteins', followed by a capital letter (SpsA to SpsQ). Their position in the *S. pseudintermedius* ED99 genome is indicated in Figure 4.2. Of note, 8 genes encoding putative CWA proteins are located near the *oriC* environ (Figure 4.2). Homology searches in the database resulted in sequence identities with known staphylococcal proteins ranging from ~30% to ~80% (Table 4.3). Signal sequences, necessary for Sec-dependent protein secretion (Foster and Hook, 1998), were predicted for 14 putative Sps proteins, consisting of 29 aa for SpsC and SpsK, 33 aa for SpsN, SpsP, and SpsQ, 36 aa for SpsD, 37 for SpsG, 38 aa for SpsA, SpsB, and SpsL, 39 aa for SpsH, 44 aa for SpsO, and 48 aa for SpsF and SpsM. No signal sequence was predicted for SpsE, SpsI, and SpsJ (Figure 4.3).

4.4.2 Six *S. pseudintermedius* cell wall-anchored proteins contain predicted tandem repeat regions

The presence of repeat regions is a major feature of staphylococcal MSCRAMMs, such as the Sdr-family, which contain a serine-aspartate dipeptide region (Foster and Hook, 1998). Accordingly, the 17 putative CWA proteins were investigated for the presence of tandem repeat regions, employing specialised programmes as described in the Materials and Methods section. Tandemly repeated regions at the C-terminus were identified for SpsD, SpsF, SpsI, SpsJ, SpsL, and SpsO (Figure 4.3). The repeat region of SpsD consists of five highly conserved repeats of 21 residues with a

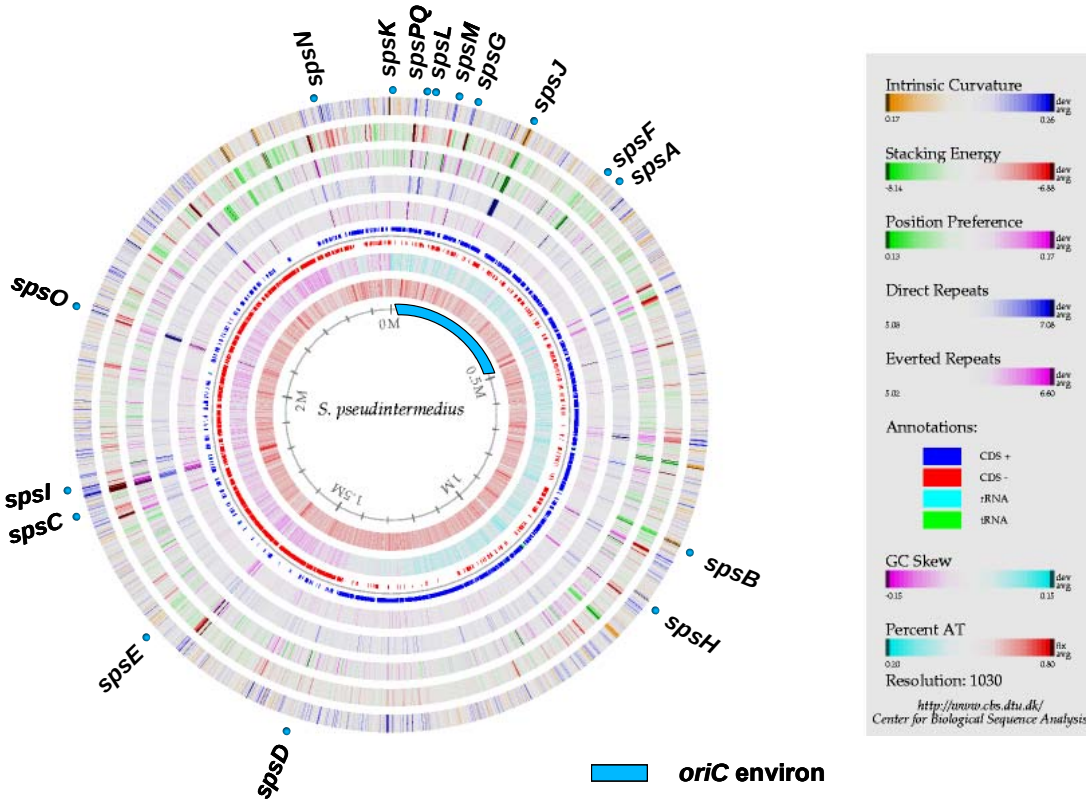
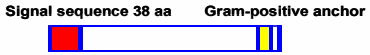
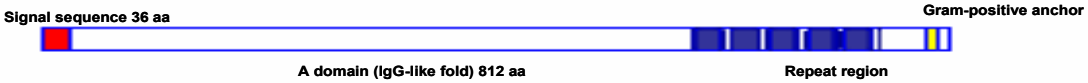
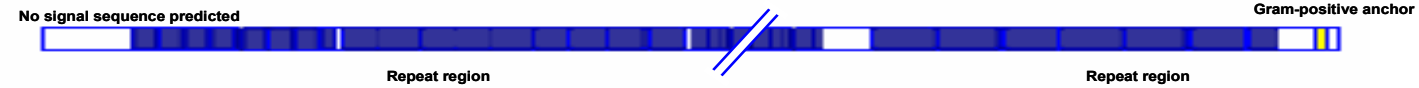


Figure 4.2. Genomic location of the 17 genes encoding putative CWA proteins in *S. pseudintermedius* strain ED99. Eight genes are situated in the *oriC* environ, indicated in blue, and nine are located in the core genome. *sps*, *S. pseudintermedius* surface protein.

Table 4.3. Best BLAST (closest homologue) analysis of the 17 predicted *S. pseudintermedius* cell wall-anchored proteins.

Putative CWA protein	Best Hit (BLAST)	Identity (%)	Similarity (%)
SpsA	LPXTG cell wall surface anchor family protein of *S. aureus* COL	31.2	56.9
SpsB	RodA, a rod shape determining protein of *S. epidermidis* ATCC 12228	69.7	87.8
SpsC	bifunctional autolysin precursor of *S. epidermidis* ATCC 12228	50.7	65.9
SpsD	Fnbp protein homolog of *S. aureus* Mu50	40.7	59.1
SpsE	Fibrinogen binding protein of *S. epidermidis* ATCC 12228	78.6	90.1
SpsF	hypothetical protein, similar to the putative cell surface adhesin SdrF of *S. haemolyticus* JCSC1435	52.8	69.3
SpsG	hypothetical protein, cell wall surface anchor family of *Streptococcus pneumoniae* D39	47.7	63.6
SpsH	Sdr-repeat family protein SdrH, *S. aureus* USA300	36.0	53.1
SpsI	serine-aspartate rich, fibrinogen-binding, bone sialoprotein-binding protein *S. epidermidis* ATCC 12228	37.3	55.5
SpsJ	precursor of a serine-rich adhesin for platelets of *S. haemolyticus* JCSC1435	52.2	61.2
SpsK	IgG-binding protein of *S. aureus* COL	50.4	71.1
SpsL	Fnbp protein homolog of *S. aureus* Mu50	33.4	51.7
SpsM	hypothetical protein, similar to the putative cell surface adhesin SdrF, *S. haemolyticus* JCSC1435	44.4	61.7
SpsN	probable exported protein of *S. aureus* RF122	38.0	60.0
SpsO	serine-aspartate repeat-containing protein C precursor of *Staphylococcus warneri* L37603	50.0	68.0
SpsP	LPXTG-motif cell wall anchor domain of *S. aureus* JH9	60.6	74.3
SpsQ	IgG-binding protein A precursor of *S. aureus* MRSA252	57.0	71.7

Putative CWA protein	Predicted length (amino acids)	Schematic figure
SpsA	619	
SpsB	242	
SpsC	1388	
SpsD	1031	
SpsE	567	
SpsF	1895	
SpsG	1566	
SpsH	453	
SpsI	1144	
SpsJ	3502	

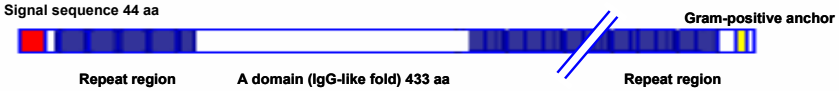
Putative CWA protein	Predicted length (amino acids)	Schematic figure
SpsK	496	
SpsL	930	
SpsM	1440	
SpsN	650	
SpsO	1846	
SpsP	347	
SpsQ	462	

Figure 4.3. Schematic representation of 17 putative CWA proteins predicted in the *S. pseudintermedius* ED99 genome. Sps = *S. pseudintermedius* surface protein. The predicted length in amino acids of each protein is indicated; typical MSCRAMM features are shown as appropriate with signal sequence (red), A domain with IgG-like folds, repeat regions (blue), and cell wall-anchoring LPX[TSA][GANS] motif pattern (yellow). For SpsP and SpsQ (*S. aureus* SpA orthologues), the repeated IgG-binding domains (blue) and X regions (grey) are also indicated.

pair-wise identity of 90% to 100%, and a truncated repeat of three residues at the C-terminal; SpsF contains nine repeats of 86 residues each, with 72% to 100% sequence identity in pair-wise alignments, and a truncated repeat of 65 residues at the C-terminus; SpsI has two different sets of C-terminal repeats, the first consisting of three repeats of 112 residues, which are 77% to 96% identical in any pair-wise alignment, and are predicted to belong to the B-repeat superfamily, sharing 52% overall identity and 67% similarity with the B repeats of SdrD of *S. aureus* strain COL, followed by a truncated C-terminal repeat of 16 residues. The second repeat region of SpsI contains five highly conserved repeats of 14 aa (pair-wise identity 100%), and a conserved truncated repeat of nine residues with one amino acid substitution; SpsJ contains six 80 residue long repeat motifs, followed by a truncated repeat of 35 aa. The repeats share pair-wise identities from 65% to 92% and are well conserved at the C-terminus with decreasing homology at the N-terminal region. SpsL contains seven highly conserved (91% to 100% pair-wise identity) repeats of 37 residues, and one truncated repeat of 20 aa. SpsO contains short repeat motifs of 12 residues which are repeated 96 times and share the consensus motif ESESLSESQS[IV]S (91% and 100% pair-wise sequence identity, respectively), and one truncated repeat of six residues at the C-terminus in which leucine, conserved in all other repeats, is substituted by lysine (ESESKS). None of the predicted repeats belonging to the sdr-family, but the repeat motifs of SpsO are serine-rich, containing 578 serine residues (49.9% of the amino acid composition in the repeat region). Of note, SpsF, SpsJ, and SpsO have a second predicted repeat region at the N-terminus (Figure 4.3). The SpsF repeat contains nine repeated motifs of 15 aa each, which share between 73% and 100% pair-wise identity. The predicted repeat region for SpsJ consist of three different sets of repeats with, starting at the N-terminal, seven short highly conserved repeated motifs of 18 to 20 residues (pair-wise identity 85% to 100%, ‘repeat 1’), and a conserved truncated repeat of nine residues, followed by nine repeated sequences (‘repeat 2’, pair-wise identity ranging from 80% to 100%) of 46 aa, and a third repeat region (‘repeat 3’) of 12 to 20 residues which are repeated 61 times and less conserved (pair-wise identity ranging from 42% to 100%). The 896 aa long ‘repeat 3’ region of SpsJ contains mainly serine (447 residues, 49.9%), threonine (163 residues, 18.2%), and aspartate (113 residues, 12.6%) residues,

resulting in the conserved motifs ‘SDSD’ and ‘STS’. The N-terminal repeats of SpsO are 26 residues long and repeated four times with a pair-wise identity of 76% to 96%, and a truncated repeat of ten residues.

4.4.3 SpsD, SpsI, SpsL, and SpsO have two predicted IgG-like folds

In staphylococcal MSCRAMMs, β-sheet-rich IgG-like folds of the A domain have been shown to be important for ligand-binding (Ponnuraj *et al.*, 2003). Therefore, the putative CWA proteins identified in the *S. pseudintermedius* ED99 genome were examined for predicted IgG-like folds. Based on InterProScan and PHYRE predictions, two IgG-like folds each were identified within the non-repeated A domains of SpsD, SpsI, SpsL, and SpsO (Figure 4.3), dividing the A domains into N1, N2, and N3 subdomains. The predicted locations of the IgG-like folds are residues 167 to 320 (N2) and 322 to 519 (N3) for SpsD, residues 202 to 351 (N2) and 347 to 512 (N3) for SpsI, residues 220 to 363 (N2) and 364 to 531 (N3) for SpsL, and residues 339 to 492 (N2) and 487 to 659 (N3) for SpsO. However, SpsI does not contain a predicted signal sequence and its expression on the cell surface is questionable. Accordingly, it was excluded from further analysis.

4.4.4 The putative cell wall-anchored proteins SpsD, SpsL, and SpsO have several typical MSCRAMM features

Of the 17 putative CWA proteins of *S. pseudintermedius* ED99, SpsD, SpsL, and SpsO contained each of the MSCRAMM features screened for, including a signal sequence at the N-terminus, followed by a non-repeated A domain with two IgG-like folds, dividing the A domain into N1, N2, and N3 subdomains, a tandemly repeated domain at the C-terminus (and at the N-terminus for SpsO), and a C-terminal LPXTG-anchor motif. The main characteristics of SpsD, SpsL, and SpsO are summarised in Table 4.4. Of interest, a TYTFTDYVD motif or variant, important for the ‘dock, lock, and latch’ ligand-binding mechanism (Ponnuraj *et al.*, 2003), was found in SpsD, SpsL, and SpsO, and putative latching sequences were also identified (Table 4.4). Further, putative Fn-binding motifs with weak homology to FnbpA-10 of FnbpA in *S. aureus* (Schwarz-Linek *et al.*, 2003) were detected in the repeat

Table 4.4. Characteristics of the predicted CWA proteins SpsD, SpsL, and SpsO of *S. pseudintermedius* ED99.

	Amino acids	MW (kDa)[a]	Signal peptide	LPXTG motif	Ig-like fold (position)[b]	TYTFTDYVD-like motif (position)[b]	Putative latching sequence (position)[b]	Repeat region (position)	Copy number repeats
SpsD	1031	115	36 aa	LPDTG	167-320 aa 322-519 aa	RYRFMDYVN (267-275 aa)	NNASGEG (491-497 aa)	867-959 aa	5
SpsL	930	103	38 aa	LPKTG	220-363 aa 364-531 aa	VYTFKDYVN (298-306 aa)	NSASGSG (502-508 aa)	543-818 aa	7
SpsO	1846	198	44 aa	LPNTG	339-492 aa 487-659 aa	TYTFTDYVD (424-432 aa)	DKSTALG (635-641 aa)	661-1800 aa 97-216 aa[c]	96 4[c]

aa, amino acids; [a]MW, predicted approximate molecular weight in kDa (kilo dalton); [b]within the A domain; [c]N-terminal repeats.

region of SpsL (24% identity in pair-wise alignments for SpsL1-SpsL6, and 21% for SpsL-7) (Figure 4.4). No homology to Fn-binding motifs of FnbpA was detected in the repeat regions of SpsD and SpsO. Of note, the genes encoding for SpsD, SpsL, and SpsO in the *S. pseudintermedius* ED99 genome are situated in different genomic contexts. While *spsD* is located in a well-conserved region of the core genome, *spsL* is part of the *oriC* environ (Takeuchi *et al.*, 2005) (Figure 4.2). The *spsO* gene appears to be species-specific as it is not present in the genomes of other staphylococcal species (Figure 4.5). The region contains two putative transposases, suggesting that the locus may have been acquired by horizontal gene transfer (Figure 4.5).

4.4.5 *S. pseudintermedius* ED99 encodes for two putative *S. aureus* SpA orthologues

Interestingly, *S. pseudintermedius* ED99 has two predicted orthologues of *S. aureus* SpA (SpsP and SpsQ). The *spsP* and *spsQ* genes have 42% and 62% nucleotide identity, respectively, with *spa* of *S. aureus* strain 8325-4 (GenBank accession number J01786) in pair-wise alignments, and the predicted amino acid sequences are 56% identical for SpsP and 53% identical for SpsQ, respectively, with SpA of *S. aureus* strain 8325-4. SpsP and SpsQ are encoded adjacent to each other in the genomic *oriC* environ (Figure 4.2), with *spsP* (1044 bp) positioned directly upstream of *spsQ* (1389 bp) (Figure 4.6). The 2 genes share 70% nucleotide identity and an overall amino acid identity of 73%, and both contain a predicted N-terminal signal sequence of 33 aa, followed by a repeat region consisting of three and four predicted IgG-binding domains in SpsP and SpsQ, respectively (Figure 4.3). Each IgG-binding domain of SpsP comprises of 55 aa which are 78% to 89% identical to each other, whereas the four IgG-binding domains of SpsQ vary from 59 aa for the first and second, 55 aa for the third, and 52 aa for the fourth domain, and they share between 59% to 86% protein sequence identity in any pair-wise alignment. The repeated IgG-binding domains are well conserved between SpsP and SpsQ, with 67% to 90% sequence identity in pair-wise alignments. On the C-terminal side of the IgG-binding domains, SpsP and SpsQ contain a predicted X region, consisting of a 77 aa long repeat sequence (Xr), and a constant region (Xc) for SpsQ (Figure 4.3).

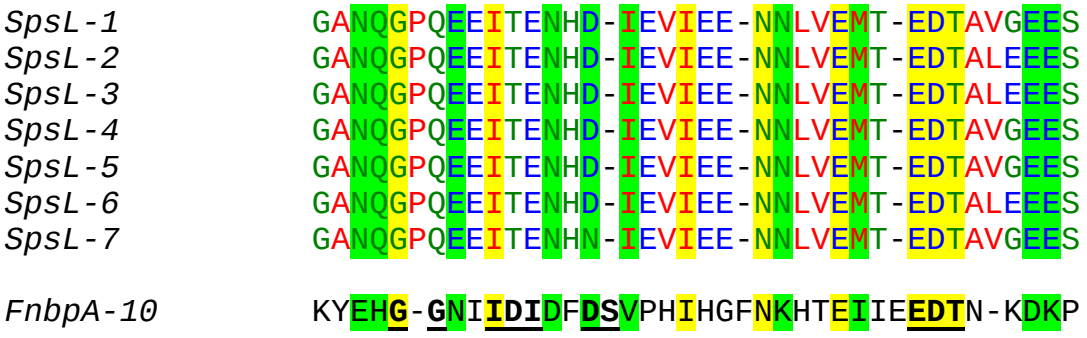


Figure 4.4. Sequence alignment of the seven predicted C-terminal repeats of SpsL (SpsL1-SpsL7), and part of the repeat region of fibronectin-binding protein A (FnbpA) of *S. aureus*, containing putative fibronectin-binding motifs (FnbpA-10). Putative binding motifs are in bold and underlined. Identical residues are highlighted in yellow, conserved substitutions are highlighted in green. Colour code for amino acids of SpsL1 to SpsL7 according to the ClustalW2 program: red, small and hydrophobic (AVFPMILW); blue, acidic (DE); magenta, basic (RK); green, hydroxyl, amine, basic (STYHCNGQ).

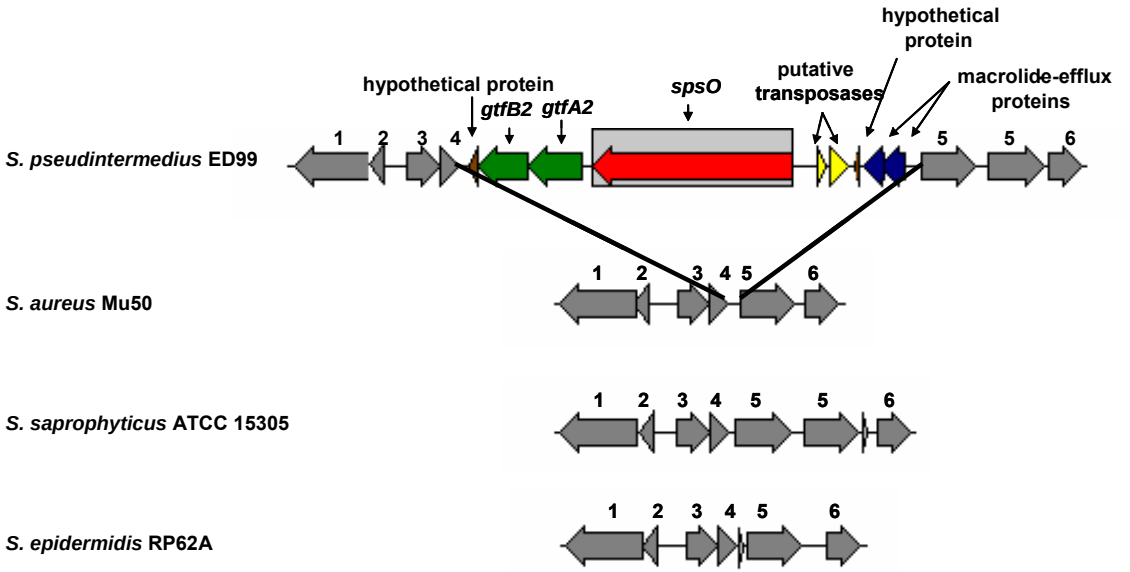


Figure 4.5. Genomic context of the gene encoding the putative CWA protein SpsO in *S. pseudintermedius* ED99. The *spsO* gene (indicated by a red arrow in a grey box) is part of a region of difference, which is not present in the otherwise conserved genome region of *S. aureus* Mu50, *S. saprophyticus* ATCC 15305, and *S. epidermidis* RP62A. The numbered grey arrows indicate the conserved region, with same numbers corresponding to conserved genes in the different staphylococcal species. Besides *spsO*, the region of difference contains two genes encoding for hypothetical proteins (brown arrows), two genes encoding for a putative accessory Sec system (glycosyltransferase GtfB2 and glycosylation protein GtfA2, green arrows), two putative transposases (yellow arrows), and two genes encoding for putative macrolide-efflux proteins (blue arrows). The predicted accessory Sec system gene products have 58% (GtfB2) and 77% (GtfA2) similarity to proteins encoded by *S. saprophyticus* ssp. *saprophyticus* ATCC 15305.

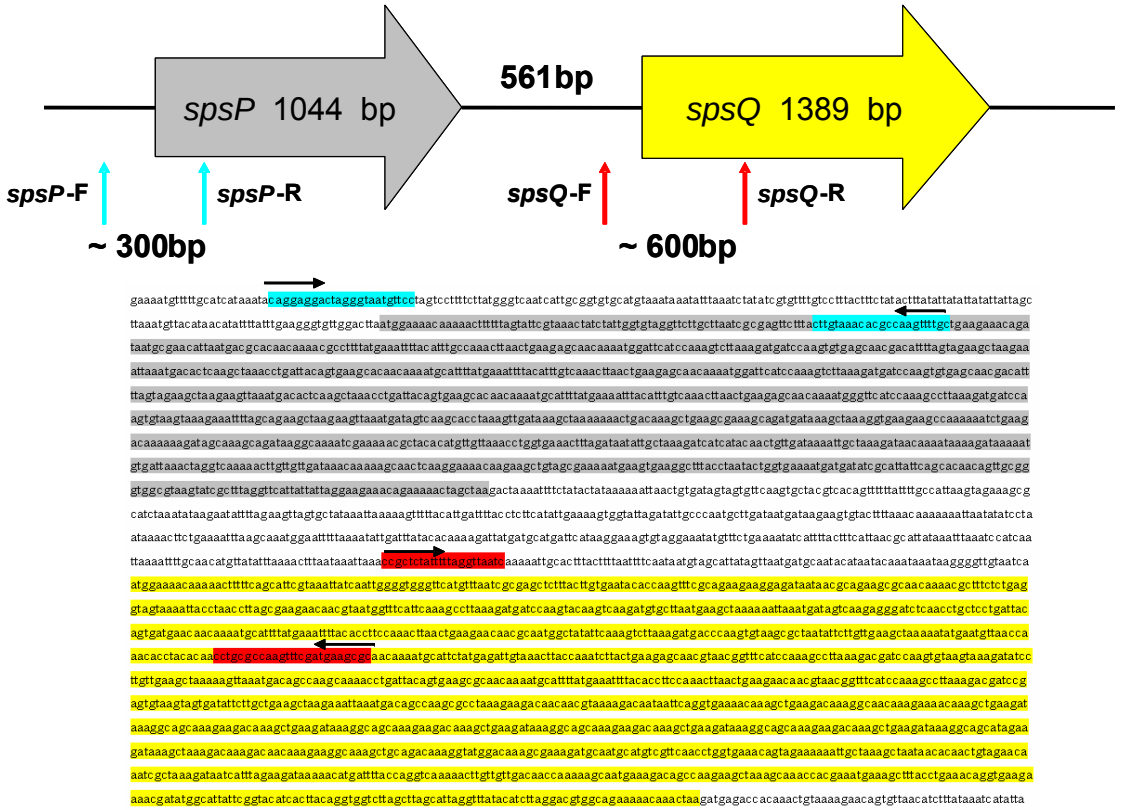


Figure 4.6. Schematic interpretation and DNA sequence of the two *spa*-orthologues *spsP* and *spsQ* encoded by *S. pseudintermedius* ED99. The *spsP* (grey arrow and sequence highlighted in grey) and *spsQ* (yellow arrow, sequences highlighted in yellow) genes are located 561 bp apart, and both contain repeat regions encoding for IgG-binding domains. The location of primers for screening of *spsP* and *spsQ* homologues in other staphylococci by PCR amplification is indicated in light blue for *spsP* and red for *spsQ*. The predicted PCR product sizes are ~300 bp for *spsP* and ~600 bp for *spsQ*.

4.4.6 Distribution of the 17 genes encoding putative cell wall-anchored proteins among the *S. intermedius* group

In order to investigate the distribution of the 17 genes encoding putative CWA proteins identified in the *S. pseudintermedius* ED99 genome among other members of the SIG and closely related staphylococcal species, Southern blot analysis and PCR amplification were performed. A total of 20 *S. pseudintermedius* strains representing the breadth of diversity within the species, representatives of the closely related *S. delphini* and *S. intermedius* species, and other staphylococcal species associated with animal hosts (Figure 4.1) were screened for the presence of the putative CWA encoding genes by Southern blot analysis (*spsA* to *spsO*). For the *S. aureus spa* orthologues *spsP* and *spsQ*, PCR amplification was employed, as the genes share 70% nucleotide identity which precluded design of gene-specific probes for Southern blot analysis. For similar reasons, the primers designed for PCR amplification were located upstream of *spsP* (*spsP*-F), in the non-repeated region of *spsP* (*spsP*-R), in the unique region between *spsP* and *spsQ* (*spsQ*-F), and in a region unique for *spsQ* (*spsQ*-R) (Figure 4.6).

Of the 17 genes examined, 13 were found in all *S. pseudintermedius* strains investigated. The remaining 4 (*spsF*, *spsO*, and the *S. aureus spa* orthologues *spsP* and *spsQ*) were present in 11, 6, 7, and 11 of the 20 *S. pseudintermedius* strains, respectively. Furthermore, 8 of the 17 genes were detected in *S. delphini* and 6 in *S. intermedius*, and 9 genes were exclusive to *S. pseudintermedius*. None of the genes encoding putative CWA proteins was detected in the non-SIG staphylococcal species examined. Representative Southern blots are shown in Figure 4.7, and the results are summarised in Figure 4.8. Of note, it cannot be excluded that DNA sequence variation in PCR primer annealing sites for *spsP* and *spsQ* could contribute to the lack of gene detection for some strains.

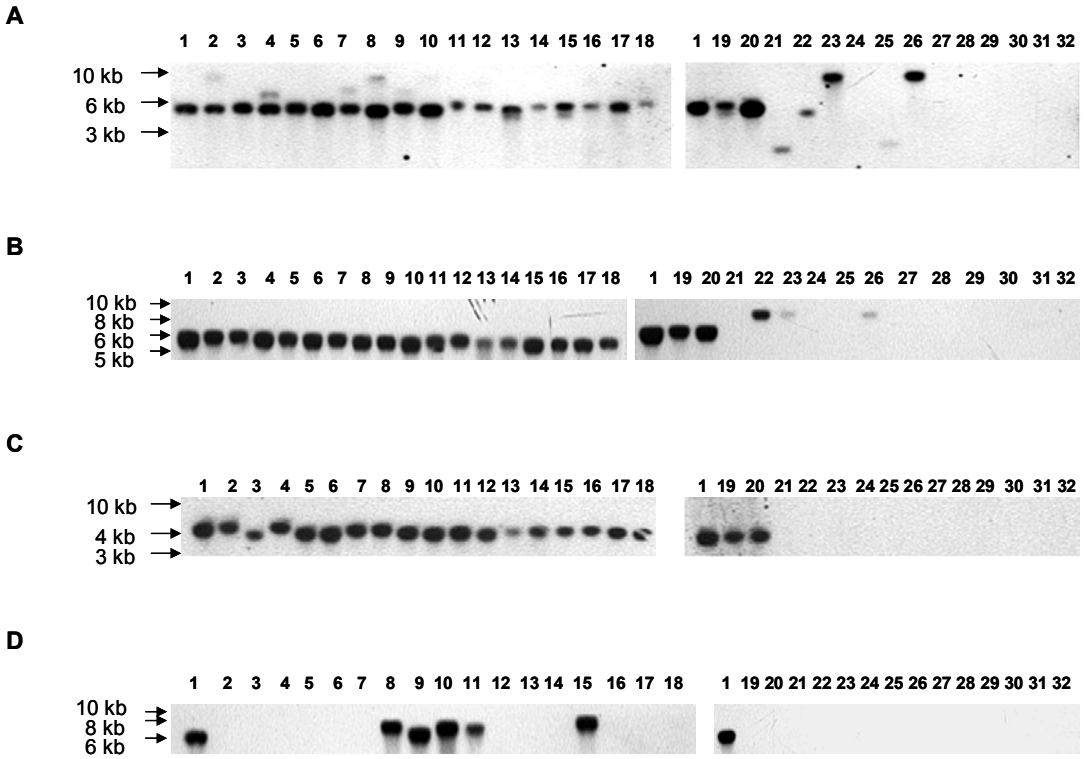


Figure 4.7. Southern blot analysis of the distribution of selected genes encoding putative CWA proteins among 25 members of the SIG (20 *S. pseudintermedius*, 3 *S. delphini*, 2 *S. intermedius* strains) and 7 other staphylococcal species. The numbers above each blot correspond to the staphylococcal strains shown in Figure 4.1 as follows: (1) ED99; (2) AV 8001; (3) 95-072195; (4) 94-062394; (5) N900260; (6) HT20030686; (7) M 721/99; (8) 3279; (9) 3414; (10) 9075; (11) 690; (12) 8478; (13) HH1; (14) Can6; (15) Can10; (16) BH47; (17) AV 8024; (18) 8016; (19) LMG 22219; (20) LMG 22220; (21) AV 8061; (22) HT20030680; (23) 9106; (24) A/G21/06/02 (*S. aureus*, employed as negative control, not included in Figure 4.1); (25) NCTC 11048; (26) ATCC 49171; (27) ATCC 43808; (28) ATCC 49545; (29) ATCC 11249; (30) ATCC 49168; (31) ATCC 43764; (32) ATCC 700373. Genomic DNA was digested using *Hind*III restriction endonuclease and representative blots include: (A) *spsA*, present in all 25 members of the SIG; (B) *spsG*, present in *S. pseudintermedius* and *S. delphini*; (C) *spsL*, present in *S. pseudintermedius* only; (D) *spsO*, present in some, but not in all *S. pseudintermedius* strains investigated. None of the putative CWA protein encoding genes was detected in any of the other staphylococci species (numbers 24 and 27-32). DNA size marker is indicated.

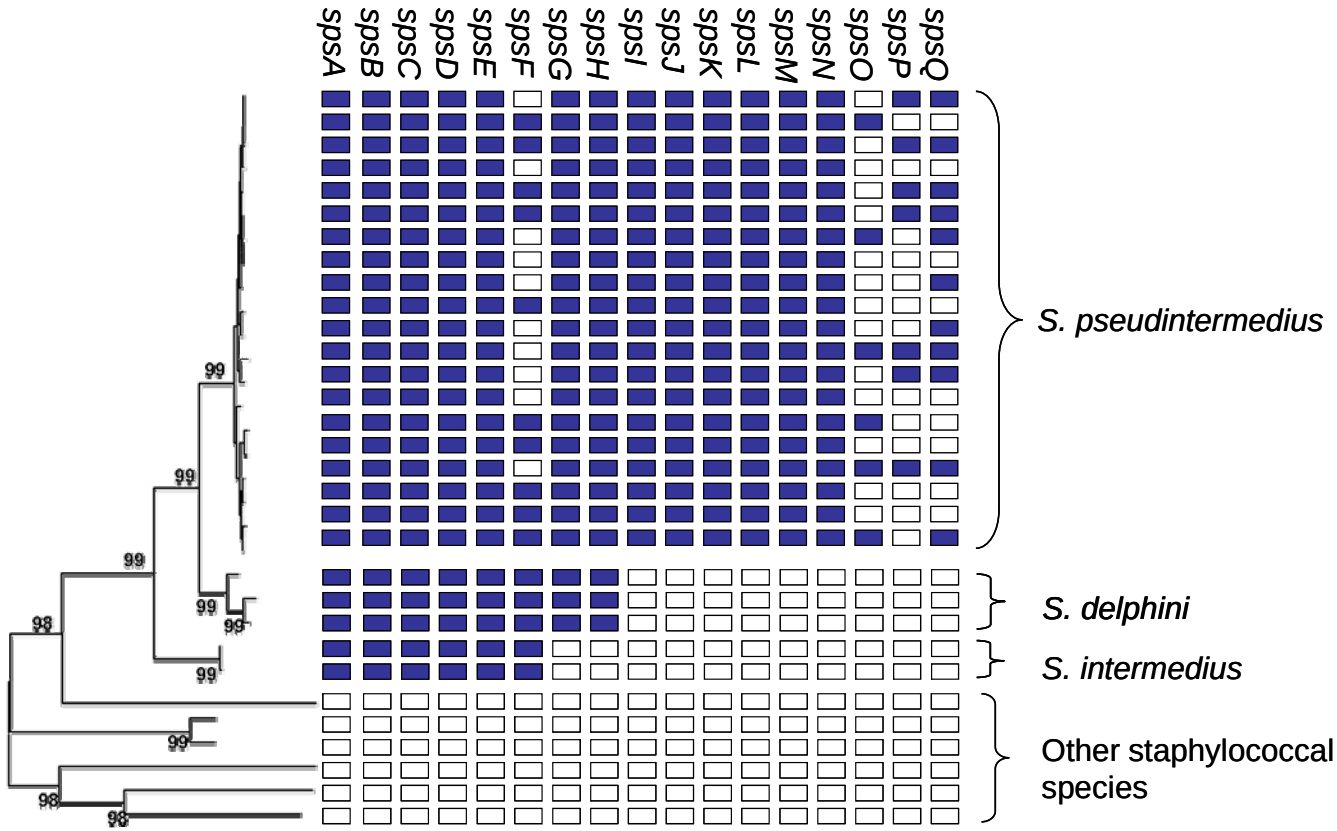


Figure 4.8. Distribution of the genes encoding putative CWA proteins among 20 *S. pseudintermedius* strains, representatives of the closely related *S. delphini* and *S. intermedius*, and other staphylococcal species associated with animal skin disease. The diversity of strains is indicated by a phylogenetic tree; a blue rectangle denotes presence of a gene and an empty rectangle its absence based on Southern blot analysis (for *spsA* to *spsO*) or PCR amplification (for *spsP* and *spsQ*).

4.5 Discussion

In silico analysis of the *S. pseudintermedius* strain ED99 genome revealed the presence of 17 genes encoding putative CWA proteins. The number of predicted CWA proteins encoded by Gram-positive bacteria varies substantially. For example, eight were predicted for the dental pathogen *Streptococcus mutans*, in comparison to 41 for *Enterococcus faecalis*, an opportunistic pathogen causing serious nosocomial human infections (Ponnuraj *et al.*, 2003). Another typical feature of CWA proteins is the presence of an approximately 40 aa signal sequence, which facilitates secretion across the cell membrane (Foster and Hook, 1998). In *S. pseudintermedius* ED99, 14 putative CWA proteins contain a predicted signal sequence, ranging from 29 to 48 residues. In contrast, SpsF, SpsI, and SpsJ lack a predicted signal sequence, suggesting either that the derived proteins are not transported across the cell membrane, or that an alternative, unknown secretion mechanism is involved.

For most MSCRAMMs, the signal sequence is followed by a non-repeated A domain of approximately 500 aa residues, which is usually involved in ligand-binding (Clarke and Foster, 2006). The A domain can be divided into three subdomains (N1, N2, N3), with N1 being sensitive to protease-cleavage, and N2/N3 comprising the ligand-binding site, which often contains IgG-like folds (Deivanayagam *et al.*, 2002; Ponnuraj *et al.*, 2003). In Gram-positive cocci, the number of predicted CWA proteins containing IgG-like folds was reported to vary between three (*S. mutans*) and nine (*E. faecalis*, *S. aureus*) (Ponnuraj *et al.*, 2003). For *S. pseudintermedius* ED99, IgG-like fold-containing non-repetitive A domains were predicted for four putative CWA proteins, including SpsD (notably long, 812 aa), SpsI (635 aa, but no signal sequence), SpsL (504 aa), and SpsO (433 aa). Of note, SpsO contains an N-terminal tandemly repeated region between the signal sequence and the unique A domain. Similarly, N-terminal repeats are predicted for SpsF and SpsJ, and N-terminal repeats have been described previously in SdrI, a collagen-binding protein of *S. saprophyticus* and a member of the Sdr-family of CWA proteins (Sakinc *et al.*, 2006). SdrI contains 21 N-terminal repeats of 12 residues each, but their function is unknown (Sakinc *et al.*, 2006).

In many MSCRAMMs, tandemly repeated regions exist on the C-terminal side of the A domain which, in the case of members of the Sdr-family, are composed of Ser-Asp dipeptide repeats (Foster and Hook, 1998; Clarke and Foster, 2006). Some MSCRAMMs, e.g. *S. aureus* Cna and the *S. aureus* Sdr-family members SdrC, SdrD, and SdrE, contain β-sheet rich B repeats (Foster and Hook, 1998; Clarke and Foster, 2006). Nine of the 17 putative *S. pseudintermedius* CWA proteins contain a C-terminal tandem repeat region, ranging from the longest repeated sequence of 112 aa with the lowest copy number of three for SpsI, to the shortest repeated motif of 12 aa with the highest copy number of 96 for SpsO. The repeats within each predicted Sps protein are well conserved with pair-wise identities ranging from 65% to 100%. Homology to B repeats of known MSCRAMMs was only detected for SpsI, which shares 52% sequence identity with the B repeats of SdrD of *S. aureus*. The function of the B repeats is not understood (Clarke and Foster, 2006), but some authors suggest that they may function as a stalk to project the A domain away from the bacterial surface in a similar fashion to Ser-Asp repeat regions (Bowden *et al.*, 2005). For the predicted CWA proteins of *S. pseudintermedius* ED99, none of the tandem repeats contain Ser-Asp dipeptide repeats. However, two predicted proteins, SpsJ and SpsO, contain serine-rich domains, resulting in SX dipeptide repeats. Similar repeats have been described by Roche *et al.* (2003), who screened publicly available *S. aureus* genomes for novel CWA proteins, and reported SX dipeptide repeats for '*S. aureus* surface protein A' (SasA). Interestingly, strain-dependent variation in the number of repeats for SasA was detected (Roche *et al.*, 2003), but it is currently unknown if similar variation exists for SpsJ and SpsO among different *S. pseudintermedius* strains. The predicted *S. pseudintermedius* CWA protein SpsJ has an unusual predicted primary structure, which includes four different repeat regions, spanning 1976 of the total 3503 residues (56.4%). Three of the repeats are located at the N-terminus, followed by an 800 aa residue non-repetitive sequence, and a further 523 aa long repeat region at the C-terminus. SpsJ has no predicted functional domains, and the relevance of its remarkable structural organisation remains unclear.

Geoghegan *et al.* (2009) reported the presence of a 180 kDa SD repeat-containing protein in *S. pseudintermedius* based on Western immunoblotting with antibodies against the sdr-region of *S. aureus* ClfA. In a similar way, the authors detected a 180

kDa protein which reacted with anti-B repeat antibody of *S. aureus* SdrD and human Fg and concluded that a Fg-binding, SD repeat-, and B repeat-containing protein may exist in *S. pseudintermedius* (Geoghegan *et al.*, 2009). Further, a 6.2 kb DNA fragment was detected by Southern blot, hybridising with the SD repeat-encoding region of *S. aureus* ClfA (Geoghegan *et al.*, 2009). Additional CWA proteins, including B repeat and SD repeat-containing proteins, which are not present in *S. pseudintermedius* ED99, may exist in other strains. However, the methods used by Geoghegan *et al.* (2009) are based on sequence homology, and it is possible that SX repeats, rather than SD repeats were detected. The predicted molecular weights of the serine-rich SpsJ (353 kDa) and SpsO (198 kDa) do not correspond to the protein size (180 kDa) described by Geoghegan *et al.* (2009). However, protein degradation may have contributed to their SDS-PAGE based size prediction. In the current study, only SpsI (predicted molecular weight 124 kDa) contained a region homologous to the B repeats of *S. aureus* SdrD, but it lacked a predicted signal sequence.

Another noteworthy discovery in the *S. pseudintermedius* ED99 genome is the existence of two genes encoding *S. aureus spa* homologues, which are located in close proximity to each other on the genome. SpA of *S. aureus* binds to von Willebrand factor, a protein mediating platelet adhesion and aggregation to damaged vascular endothelium, and also plays an important role in evasion of the host immune response by binding to the Fc fragment of IgG, interfering with phagocytosis by neutrophils (Navarre and Schneewind, 1999; Hartleib *et al.*, 2000; Foster, 2005). *S. aureus* SpA binds IgG via repetitive IgG-binding domains located in the A domain, and the number of domains varies between four and five depending on the strain (Uhlen *et al.*, 1984; Navarre and Schneewind, 1999; Foster, 2005). In *S. pseudintermedius* ED99, SpsP and SpsQ have three and four predicted IgG-binding domains, respectively, which share 67% to 90% pair-wise identity. The biological reason for encoding two SpA-like proteins on the genome is unclear, but it is suggestive of an important function. The *spa*-like genes are located in the '*OriC* environ', a variable region known to contain species-specific genes, and it may be speculated that the two *spa*-orthologues are the result of an ancient intra-genomic duplication event. Of note, Moodley *et al.* (2009) employed the repeat region (Xr) of

the X region of *spsQ* to develop a *spa* typing method for *S. pseudintermedius* isolates, and discovered variation in the number (between 6 and 10) of the 30 bp long tandem repeats among the 31 isolates examined.

The current study investigated the presence and distribution of the genes encoding the predicted CWA proteins in a collection of SIG isolates and representatives of other closely related staphylococcal species associated with animal hosts. Of note, none of the genes were detected in non-SIG staphylococcal species, indicating that the SIG have a unique complement of CWA proteins. Three main categories were identified among the 17 genes examined. Firstly, genes which were present in all members of the SIG, suggesting an ancient origin and/or a conserved function. Of note, one gene, *spsF*, was detected in a subgroup of the *S. pseudintermedius* strains, but also in *S. delphini* and *S. intermedius*, which suggests that it was subject to deletion in one or more *S. pseudintermedius* clonal lineages. Similarly, *spsG* and *spsH* were present in *S. pseudintermedius* and *S. delphini*, but not in *S. intermedius*, suggesting that they were deleted from *S. intermedius*, or acquired by the ancestral lineage of *S. pseudintermedius* and *S. delphini*. The second category comprises genes which were specific for *S. pseudintermedius*, indicating a role in canine-specific interactions, and the third category includes three genes (*spsO*, *spsP*, and *spsQ*) which were exclusive to *S. pseudintermedius,* but were only detected in some of the strains investigated, suggesting acquisition by horizontal gene transfer which may confer strain-dependent functions such as increased virulence. In support of this hypothesis, *spsO*, detected in six *S. pseudintermedius* strains, is located in proximity to two putative transposases, consistent with lateral gene acquisition.

The *in silico* identification of 17 putative CWA proteins in *S. pseudintermedius* ED99 raises questions about the expression of these proteins and their role in colonisation and disease. Surface proteome analysis of early-, mid-, and late exponential phase *S. pseudintermedius* ED99 was performed by Nouri L. Ben Zakour of our laboratory in collaboration with the Moredun Research Institute, Penicuik, Scotland, UK, using liquid chromatography-electrospray ionisation-tandem mass spectrometry (LC-ESI-MS-MS) (data not shown). Six of the 17 putative CWA proteins predicted in the *S. pseudintermedius* ED99 genome were detected on the

bacterial surface, including SpsD, SpsK, SpsL, SpsN, SpsO, and SpsQ. The putative CWA proteins SpsL, SpsN, and SpsQ were identified in all three phases of growth, SpsK was lacking in early-, SpsO in mid-, and SpsD in late exponential phase. The 11 undetected CWA proteins might not have been expressed under the conditions tested, or the expression level might have been below the detection threshold of the LC-ESI-MS-MS method used.

In summary, genome-wide analysis of *S. pseudintermedius* ED99 revealed the presence of 17 genes encoding putative CWA proteins based on typical MSCRAMM features. These data represent an important first step in understanding the critical host-pathogen interactions involved in the pathogenesis of canine pyoderma. All MSCRAMM characteristics examined were identified in SpsD, SpsL, and SpsO. Based on *in silico* analysis and *in vitro* expression data, SpsD, SpsL, and SpsO were selected for functional characterisation as described in Chapter 5 and Chapter 6.

Chapter 5

Adherence of selected putative MSCRAMMs of *Staphylococcus pseudintermedius* ED99 to host ECM proteins

5. 1 Introduction

CWA proteins mediating interactions with host ECM proteins are made by many Gram-positive bacteria including the staphylococci. In particular, *S. aureus* encodes 21 putative MSCRAMMs (Roche *et al.*, 2003), many of which have been characterised, including several which have been demonstrated to bind to multiple ECM proteins. For example, ClfB, FnbpA, and FnbpB all bind to Fg (McDevitt *et al.*, 1994; Ni Eidhin *et al.*, 1998; Wann *et al.*, 2000), FnbpA and FnbpB also interact with Fn (Greene *et al.*, 1995) and elastin (Roche *et al.*, 2004), and ClfB adheres to human desquamated nasal epithelial cells, through an interaction with type I CK10 (O'Brien *et al.*, 2002b; Walsh *et al.*, 2004).

The interaction of *S. pseudintermedius* with the ECM is poorly characterised, but it was previously reported that some strains adhere to Fn, vitronectin, laminin, and collagen type I (Cree and Noble, 1995). Further, during the current study, Professor Tim Foster and colleagues (University of Dublin, Trinity College, Ireland) demonstrated binding of *S. pseudintermedius* to Fg, Fn, CK10, and elastin (Geoghegan *et al.*, 2009).

The characterisation of bacterial MSCRAMMs and the identification of host receptors are important for our understanding of how MSCRAMMs contribute to disease pathogenesis. Importantly, MSCRAMMs are often involved in the early stages of infection and colonisation and represent potential novel therapeutic targets.

5.2 Aims and Strategy

To investigate the role of MSCRAMMs in the adherence of *S. pseudintermedius* to the ECM by:

1) Examination of the ability of *S. pseudintermedius* strain ED99 to bind to the ECM proteins Fn, Fg, and CK10 using solid phase adherence assays.

2) Characterisation of the role of three putative MSCRAMMs (SpsD, SpsL, and SpsO), encoded by *S. pseudintermedius* ED99, in adherence to ECM proteins when expressed on the surface of *L. lactis*.

3) Investigation of the expression of the putative MSCRAMMs SpsD, SpsL, and SpsO during pyoderma infection by detection of specific antibodies in sera from dogs with bacterial pyoderma by Western blot analysis.

4) Investigation into the interaction of recombinant MSCRAMM A domains with Fg chains by Western ligand blot analysis.

5.3 Materials and Methods

5.3.1 Bacterial strains used in this study

Bacterial strains used in this study are listed in Table 5.1. *S. pseudintermedius* strain ED99 was isolated from a canine pyoderma case presented at the Hospital for Small Animals, The Royal (Dick) School of Veterinary Studies, The University of Edinburgh, UK. *S. aureus* strain SH1000 (Horsburgh *et al.*, 2002) was used as a positive control as it has been previously shown to adhere to Fn, Fg, and CK10 in exponential phase of growth (Geoghegan *et al.*, 2009). *S. aureus* strain Newman Δ*clfA* is deficient in ClfA expression (McDevitt *et al.*, 1994), and does not express functional FnbpA or FnbpB (Grundmeier *et al.*, 2004). Accordingly, *S. aureus* strain Newman Δ*clfA* can be used as a negative control for binding to Fn or Fg when grown to stationary phase of growth.

In *L. lactis* adherence studies, *L. lactis* expressing FnbpA, encoded by plasmid pKS80, was used as a positive control for binding to Fg and Fn (Massey *et al.*, 2001). The strain was kindly provided by M. Hook and S. Prabhakaran (Texas A & M University, USA). For Gateway® cloning, electrocompetent *E. coli* DH10B and BL-21 cells were used for transformation, which were a kind gift from J. Haas and E. Fossum, The University of Edinburgh, UK. *L. lactis* constructs expressing individual surface proteins of *S. pseudintermedius* ED99 were generated using *L. lactis* subspecies *cremoris* strain MG1363, kindly provided by P. Moreillon, University of Lausanne, Switzerland.

5.3.2 Bacterial growth curve of *S. pseudintermedius* strain ED99

In order to generate a bacterial growth curve for *S. pseudintermedius* strain ED99, 25 ml of BHI broth was inoculated with 125 µl of overnight broth culture. Bacterial growth at 37°C with shaking at 200 rpm was monitored by spectrophotometric measurement of the absorbance at a wavelength of 600 nm (OD_{600}) every 30 min for 12 h. *S. aureus* strain Newman was included as a control strain, and the experiment was performed in triplicate by inoculation of BHI with cultures originating from three single colonies for each staphylococcal species.

Table 5.1. Bacterial strains used in this study.

Name	Main features	Reference
S. pseudintermedius strain ED99	Canine pyoderma isolate.	(Simou *et al*., 2005b)
S. aureus strain SH1000	Strong adherence to Fn, Fg, and CK10 in exponential phase of growth.	(Horsburgh *et al.*, 2002)
S. aureus strain Newman Δ*clfA*	Deficient in ClfA, no adherence to Fn or Fg in stationary phase of growth.	(McDevitt *et al.*, 1994)
L. lactis expressing FnbpA	Expression of *S. aureus* FnbpA on the cell surface.	(Massey *et al.*, 2001)
L. lactis subspecies *cremoris* strain MG1363	Non-pathogenic, plasmid-free.	(Gasson, 1983)

5.3.3 Production of chemically competent *E. coli* DH5α cells

E. coli DH5α overnight culture (1 ml) was transferred into 100 ml fresh pre-warmed LB broth and grown to an OD_{600} of 0.5 at 37°C with shaking at 200 rpm. The bacterial culture was transferred into 50 ml pre-chilled Falcon tubes (BD Falcon™ Conical Tubes, Scientific Laboratory Supplies, UK) and chilled on ice for 30 min. Bacterial cells were centrifuged at 4000 rpm for 8 min at 4°C and pellets resuspended in 20 ml ice-cold TFB 1 (30 mM potassium acetate, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 100 mM KCl, 15 % (v/v) glycerol, pH 5.8). Samples were chilled on ice for 10 min and centrifuged at 4000 rpm for 8 min at 4°C. Pellets were resuspended in 4 ml ice-cold TFB 2 (10 mM MOPS, 75 mM $CaCl_2$, 10 mM KCl, 15 % (v/v) glycerol, pH 6.5) and 200 µl aliquots were stored at -80°C until further use.

5.3.4 Production of electrocompetent *L. lactis* cells

100 µl of *L. lactis* strain MG1363 overnight culture was transferred into 5 ml fresh GM17 broth and grown to an OD_{600} of 0.6 to 0.8. The exponential phase culture was diluted 1:10, 1:100, 1:1000 and 1:10000 in fresh GM17 and 10 µl of each dilution was added to 100 ml SGM17 broth in medical flat bottles and incubated at 30°C overnight. 400 ml SGM17 broth contained 14.9 g M17 broth (Oxoid, UK), 14 g glycine, 27.4 g sucrose and 2 g glucose in dH_2O. *L. lactis* overnight cultures with an OD_{600} between 0.2 to 0.4 were selected and centrifuged at 4000 rpm for 10 min. Pellets were washed in 100 ml 0.5 M sucrose mixed with 10% (v/v) glycerol and centrifuged at 4000 rpm for 10 min. Pellets were resuspended in 750 µl 0.5 M sucrose, 10% (v/v) glycerol and 50 µl aliquots were stored at -80°C until needed.

5.3.5 Cloning of selected genes encoding putative MSCRAMMs of *S. pseudintermedius* ED99 into *L. lactis* MG1363

5.3.5.1 PCR amplification of genes of interest

Oligonucleotides were designed for PCR amplification of the full-length *spsD*, *spsL*, and *spsO* genes and either *Pst*I, *Sal*I, or *Bam*HI specific restriction sites were inserted on both sides of the DNA fragments (Table 5.2). 50 µl PCR reactions contained 2 µl (approximately 100 ng) genomic DNA template, 0.25 µM forward primer, 0.25 µM

Table 5.2. Oligonucleotides used for amplification of full-length *spsD*, *spsL,* and *spsO* by PCR.

Name[a]	Sequence (5′- 3′)[b]	Restriction endonuclease
spsD-F	CATGGGATCCAGAGAAGAAGAGGGA	*Bam*HI
spsD-R	GCACCTGCAGGGGTTTTTTAATGTG	*Pst*I
spsL-F	GTTATGAGTTAGTCGACAGGGAGTT	*Sal*I
spsL-R	GAAACTGCAGATATATTGACCAC	*Pst*I
spsO-F	GTAGATGGATCCATATTTTGGAGGT	*Bam*HI
spsO-R	CAGCTGCAGACACCTTTAAAAATAT	*Pst*I

[a]F, forward primer; R, reverse primer; [b]inserted restriction sites are underlined.

reverse primer, 1x *PfuUltra*™ II reaction buffer (Stratagene, USA), 0.25 mM dNTP's (Promega, USA) and 1 µl *PfuUltra*™ II Fusion HS DNA polymerase (Stratagene, USA). The thermocycler programme included an initial denaturation step at 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 20 s, annealing at 50°C for 20 s and extension at 72°C for 2 min, followed by a final extension step at 72°C for 3 min. PCR products were visualised on 0.8% (w/v) agarose gels, gel extracted under avoidance of UV light exposure and purified using QIAquick Gel Extraction Kit (Qiagen, UK) according to the manufacturer's instructions.

5.3.5.2 Intermediate cloning of genes of interest using a commercial cloning kit

Purified DNA fragments were cloned into the StrataClone™ Blunt PCR cloning vector pSC-B (Stratagene, USA) using the StrataClone Ultra™ Blunt PCR Cloning Kit (Stratagene, USA) according to the manufacturer's instructions. Each cloning reaction consisted of 3 µl Strataclone Buffer Blunt (Stratagene, USA), 2 µl purified PCR product and 1 µl Strataclone™ Blunt Vector Mix (Stratagene, USA). StrataClone™ SoloPack® competent cells (Stratagene, USA) were transformed according to the manufacturer's instructions and colonies selected using blue-white screening on LB-ampicillin (100 µg/ml)-X-gal plates. White colonies were transferred into 5 ml LB-ampicillin (100 µg/ml) broth and grown overnight at 37°C with shaking at 200 rpm. Plasmid was isolated using QIAprep Spin Miniprep Kit (Qiagen, UK) according to the manufacturer's instructions. Purified plasmids were digested using appropriate restriction endonucleases (New England Biolabs, UK), and diagnostic digests were analysed on 0.8% (w/v) agarose gels.

5.3.5.3 Cloning of putative MSCRAMM genes into the lactococcal plasmid pOri23

For generating DNA constructs, the *E. coli* - *L. lactis* shuttle vector pOri23 (kindly provided by P. Moreillon, University of Lausanne, Switzerland) was used. The pOri23 vector carries the *ermAM* gene for erythromycin resistance, the high-copy-number *oriCol*E1 replicon for autonomous replication in *E. coli* and the constitutive lactococcal promoter *P23* (Que *et al.*, 2000). The multiple cloning site of pOri23

consists of restriction sites for endonucleases *Pst*I, *Sal*I and *Bam*HI (Que *et al.*, 2000).

StrataClone™ plasmids containing the DNA inserts of interest and the *E. coli* - *L. lactis* shuttle vector pOri23 were each digested in a 100 µl total reaction volume containing 10 µl plasmid (approximately 2.5 µg), 20 units appropriate restriction endonucleases (New England Biolabs, UK), and suitable buffers (New England Biolabs, UK) according to the manufacturer's instructions. Restriction digestions were performed at 37°C for 16 h. The restriction fragments to be cloned were extracted from 0.8% (w/v) agarose gels without UV exposure as described in the general Material and Methods and purified using QIAquick Gel Extraction Kit (Qiagen, UK) according to the manufacturer's instructions. DNA inserts and restriction-digested pOri23 plasmid were quantified using spectrophotometry (NanoDrop ND-1000, Thermo Scientific, USA) and ligation reactions were carried out with a plasmid to insert ratio of 1:3 in a 10 µl total ligation reaction volume, consisting of 1 µl vector (approximately 10 ng), 400 units T4 DNA ligase (New England Biolabs, UK), 1x T4 DNA ligase reaction buffer (New England Biolabs, UK), x µl DNA insert (depending on DNA concentration), and x µl sterile water (depending on the volume of DNA insert). Ligations were incubated at 16°C for 16 h.

5.3.5.4 Transformation of *E. coli* DH5α cells

One 200 µl aliquot of chemically competent *E. coli* DH5α cells was defrosted on ice, mixed with 10 µl ligation reaction and kept on ice for 30 min. Cells were heat shocked for 45 s at 42°C and placed on ice for 2 min. 350 µl SOC (28.1 g/l SOB broth (ForMedium™, UK), 3.6 g/l glucose) was added and cells were incubated for 1 h at 37°C in a shaking incubator at 200 rpm. 100 µl of cell suspension per plate was spread onto LB plates containing 500 µg/ml erythromycin (Fisher Scientific, USA) and incubated overnight at 37°C. Colonies were transferred into 5 ml LB-erythromycin (500 µg/ml) broth, and grown overnight at 37°C with shaking at 200 rpm. Plasmids were isolated from overnight cultures using QIAprep Spin Miniprep Kit (Qiagen, UK). Purified plasmids were digested with the appropriate restriction

endonucleases (New England Biolabs, UK), and digests were analysed on 0.8% (w/v) agarose gels to confirm the presence of an insert of the predicted size. Purified pOri23 plasmids containing the DNA inserts of interest were sequenced by primer walking to confirm that no sequence errors had been introduced during PCR amplification.

5.3.5.5 Transformation of electrocompetent *L. lactis* cells

One 50 µl aliquot of electrocompetent *L. lactis* cells was thawed on ice and 2 µl (~20 ng) pOri23 plasmid carrying the DNA insert of interest was added. Electroporation cuvettes (Sigma-Aldrich, UK) were pre-chilled and *L. lactis* cells plus plasmid were transferred into the cuvettes. Electroporation was performed at standard settings (25 µF, 2.5 kV, 200 Ohm) and 1 ml GM17 was added immediately. Cells were incubated at 30°C in a static incubator for 2 h prior to spreading 250 µl of cell suspension per plate onto GM17 plates containing 5 µg/ml erythromycin. Plates were incubated overnight at 30°C.

For screening of *L. lactis* transformants, plasmid was isolated using the Qiagen MiniPrep Kit (Qiagen, UK) with addition of 100 U/ml mutanolysin (Sigma-Aldrich, UK) and 100 µg/ml lysozyme (Sigma-Aldrich, UK) to buffer P1. Diagnostic digests of purified plasmids were carried out with appropriate restriction enzymes and analysed on 0.8% (w/v) agarose gels.

Additionally, colony PCR was performed for pOri23 carrying *spsD* and *spsO* using gene-specific oligonucleotides (Table 5.3). *L. lactis* colonies were resuspended in 10 µl 10% (v/v) IGEPAL (Sigma-Aldrich, UK) and incubated for 10 min at 95°C in a thermocycler machine. 40 µl master mix containing 0.3 µM forward primer, 0.3 µM reverse primer, 0.2 mM dNTP's (Promega, USA), 1 x reaction buffer (Promega, USA), 1.5 mM $MgCl_2$ (Promega, USA) and 0.025 u/µl taq polymerase (Promega, USA) was added. The thermocycler programme included an initial denaturation step at 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 1 min, followed by a final extension step at 72°C for 7 min. PCR products were visualised on 0.8% (w/v) agarose gels.

Table 5.3. Oligonucleotides designed for colony PCR of *L. lactis* transformants.

Name[a]	Sequence (5′- 3′)
spsD-F2	GCCCTGACAACCCTCGTTCCT
spsD-R2	GATGGTTCTGCTACGGGTGCAGC
spsO-F2	CGACCAAAGTACCAATAAATGAAAAC
spsO-R2	GGTAAAGTAAAATCAAAGGAAGC

[a]F, forward primer; R, reverse primer.

5.3.6 Preparation of cell wall-associated protein fractions of *L. lactis* constructs and *S. pseudintermedius* ED99

Subcultures of overnight cultures of either *L. lactis* (1.5 ml in 150 ml fresh GM17 broth) or *S. pseudintermedius* (250 µl in 50 ml fresh BHI broth) were grown to exponential phase of growth (OD_{600} 0.6 to 0.8). Cultures were centrifuged at 5000 x g for 10 min at 4°C and cells were washed in 5 ml of 0.9 M ice-cold NaCl. Cells were then centrifuged at 5000 x g for 5 min at 4°C and the washing step was repeated. Bacterial pellets were resuspended in 2 ml of digestion buffer (1.1 M sucrose, 10 mM MgCl2, 2 mM CaCl2, EDTA-free protease inhibitor (Roche Diagnostics GmbH, Germany), 0.1 mg/ml RNAse (Sigma-Aldrich, UK), 0.05 mg/ml DNAse (Sigma-Aldrich, UK) in PBS, supplemented with 250 U/ml mutanolysin (Sigma-Aldrich, UK) and 500 µg/ml lysozyme (Sigma-Aldrich, UK) for *L. lactis*, or 200 µg/ml lysostaphin (Ambicin L, Ambi Products LLC, USA) for *S. pseudintermedius*). Bacterial cells were incubated in digestion buffer in a shaking incubator at 120 rpm at 37°C for 1 h followed by chilling and centrifugation at 10,000 x g for 30 min at 4°C. Supernatants were recovered and dialysed against PBS overnight at 4°C using Spectra/Por® regenerated cellulose dialysis membranes with a molecular weight exclusion of 8,000 Daltons (Spectrum Laboratories Inc., USA). Samples were concentrated by centrifugation in Vivaspin 2 centrifuge tubes with 30,000 Daltons molecular weight exclusion (Viva Science Ltd., UK) at 3200 x g for 10 min.

5.3.7 Western blot analysis of *L. lactis* constructs

Samples were dissolved in 1x Laemmli sample buffer (Sigma-Aldrich, UK), boiled for 10 min and resolved by SDS-PAGE in 10% polyacrylamide gels by standard procedures, and Western blot analysis was carried out as described in the general Materials and Methods. Three canine sera samples from pyoderma cases (obtained from patients at the Hospital for Small Animals, The Royal (Dick) School of Veterinary Studies, The University of Edinburgh) were pooled and used as primary antibody in a 1:1000 dilution. HRP-conjugated sheep anti-dog antibody was used as a secondary antibody in a 1:5000 dilution (Bethyl Laboratories Inc., USA).

5.3.8 Extracellular matrix proteins used in this study

Bacterial adherence assays were carried out using human and canine Fg (Sigma-Aldrich, UK) and bovine Fn (Calbiochem, EMD Biosciences, USA). In *L. lactis* adherence studies, human, canine, feline, and bovine Fg (Sigma-Aldrich, USA), and human Fn were tested (kindly provided by S. Prabhakaran, Texas A & M University, USA). CK10 was expressed and purified from a pQE30 expression clone, a gift from T. Foster, Trinity College Dublin, Ireland, which encoded the C-terminus (residues 294 to 570) of mouse CK10 (rMCK10 294-570). Recombinant protein expression in *E. coli* XL-1 blue cells (Stratagene, UK) was induced with IPTG (0.5 mM) and protein purified under hybrid conditions according to the manufacturer's instructions using the Ni-NTA bench top purification system (Invitrogen, UK). The CK10 purification was performed by R. Cartwright, The University of Edinburgh, UK.

5.3.9 Solid phase assays of the adherence of *S. pseudintermedius* ED99 and *L. lactis* constructs to extracellular matrix proteins

96-well microtiter plates (MaxiSorp, Nunc™, Thermo Fisher Scientific, Denmark) were coated with 1 μg of ECM proteins or BSA (Sigma-Aldrich, UK) in 50 μl PBS per well overnight at 4°C. For experiments with recombinant CK10, carbonate buffer (15 mM Na_2HCO_3, 35 mM $NaHCO_3$, pH 9.6) was used instead of PBS. Wells were washed three times with PBS, blocked with 2% (w/v) BSA in PBS for 1 h at room temperature and washed a further three times with PBS.

Staphylococcal bacterial cultures were grown either to exponential (OD_{600} of 0.5) or stationary phases of growth, centrifuged at 4000 rpm for 5 min, washed once with PBS and the cells resuspended in PBS to a final OD_{600} of 1. Microtiter plates coated with ECM proteins were incubated with 100 μl per well of bacterial suspension for 2 h at 37°C.

L. lactis bacterial cultures were grown statically at 30°C overnight, centrifuged at 3000 rpm for 5 min, washed once with PBS and the cells resuspended in PBS to a final OD_{600} of 1. Microtiter plates coated with ECM proteins were incubated with

100 µl per well of bacterial suspension for 2 h at 30°C. Plates coated with CK10 were incubated with bacterial suspension for 2 h at 37°C.

Following bacterial incubation, microtiter plates were washed three times with PBS and cells were fixed with 100 µl of 25% (v/v) formaldehyde solution per well for 30 min at room temperature. After three PBS washes, plates were stained with 0.5% (w/v) crystal violet (Sigma-Aldrich, UK) for 3 min and washed 5 times in PBS. Wells were incubated with 5% (v/v) acetic acid for 10 min (100 µl per well) and the absorbance measured at 590 nm. Each assay was carried out in triplicate and each experiment was repeated at least once independently.

5.3.10 Gateway® cloning and expression of the gene region encoding the A domain of selected putative MSCRAMMs

To generate recombinant proteins of the A domains of the putative MSCRAMMs SpsD and SpsL, Gateway® recombination cloning technology was used (Invitrogen, UK).

5.3.10.1 PCR amplification of region of interest

Oligonucleotides were designed for PCR amplification of the region encoding the A domain of *spsD* and *spsL* which included Gateway® compatible upstream and downstream nucleotide extensions (Table 5.4). Gateway® PCR amplification was carried out in two consecutive amplification steps. The first PCR (PCR1) amplified the gene-specific sequences and added part of the Gateway® recombination sides, the second PCR (PCR2) generated complete recombination sides using universal Gateway® primers (AttB1 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCT-3'; AttB2 5'-GGGGACCACTTTGTACAAGAAAGCTGGGT-3'). 50 µl PCR reactions consisted of 2 µl (approximately 100 ng) genomic DNA template for PCR1, 10 µl template (PCR product of PCR1) for PCR2, 0.25 µM of the appropriate gene-specific forward and reverse primer (PCR1), 0.2 pM of the universal Gateway® forward (AttB1) and reverse primer (AttB2) (PCR2), 1x *PfuUltra*™ II reaction buffer (Stratagene, USA), 0.25 mM dNTP's (Promega, USA) and 1 µl *PfuUltra*™ II Fusion HS DNA polymerase (Stratagene, USA). The thermocycler programme for PCR1

Table 5.4. Oligonucleotides designed for PCR amplification of the region encoding the A domain of *spsD* and *spsL*.

Name[a, b]	Sequence (5′- 3′)[c]
spsD-region A-F	TCAGAAGTGAGCGCAACAACAC
spsD-region A-R	ATTCCAGTTTTCTAACGGGAAGAAACCAGC
spsL-region A-F	AATGAAGATGTCACTGAAACAACTGGGAG
spsL-region A-R	CCATCCTATATCAATCACATGGC

[a]F, forward primer; R, reverse primer; [b]*sps*, *Staphylococcus pseudintermedius* surface protein; [c]Gateway® specific nucleotides were added on each side of the DNA fragments (forward extension 5′-AAAAAGCAGGCTCCGCCATG-3′; reverse extension 5′-AGAAAGCTGGGTC-3′).

and PCR2 included an initial denaturation step at 95°C for 2 min, followed by 15 cycles of denaturation at 95°C for 20 s, annealing at 50°C for 20 s and extension at 72°C for 90 s, followed by a final extension step at 72°C for 3 min. PCR products were visualised on 0.8% (w/v) agarose gels and purified using the QIAquick PCR purification Kit (Qiagen, UK) according to the manufacturer's instructions.

5.3.10.2 Generating an entry clone in *E. coli* DH10B cells

For generating a Gateway® entry clone, BP reactions were carried out in a 5 µl total reaction volume consisting of 1 µl pDONR^{TM} 207 Gateway® entry vector (approximately 150 ng) conferring gentamicin resistance (Invitrogen, UK), 1 µl BP clonase (Invitrogen, UK) and 3 µl of purified PCR product. BP reactions were incubated at room temperature for at least 4 h.

One 50 µl aliquot of electrocompetent *E. coli* DH10B cells was thawed on ice and mixed with 2 µl BP reaction. The mixture was transferred into pre-chilled electroporation cuvettes (Sigma-Aldrich, UK) and supplemented with 100 µl 10% (v/v) glycerol. Electroporation was performed at standard settings (25 µF, 2.5 kV, 200 Ohm) and 500 µl LB broth was added immediately. Cells were allowed to recover at 37°C in a shaking incubator at 200 rpm for 1 h prior to spreading 250 µl of cell suspension onto LB plates containing 15 µg/ml gentamicin (Sigma-Aldrich, UK), followed by incubation overnight at 37°C.

Plasmids were isolated from transformant colonies using QIAprep Spin Miniprep Kit (Qiagen, UK), digested with *Ban*II restriction endonuclease (New England Biolabs, UK) and resolved in 0.8% (w/v) agarose gels. Plasmids with the expected restriction digestion pattern were sequenced by primer walking to confirm that no errors had been introduced during PCR amplification.

5.3.10.3 Creation of an expression clone in *E. coli* DH10B cells

For creating a Gateway® expression clone, LR reactions were carried out in a 5 µl total reaction volume consisting of 1 µl entry clone, 2 µl destination vector (approximately 300 ng), 1 µl LR clonase (Invitrogen, UK) and 1 µl MQ water. LR

reactions were incubated at room temperature for at least 4 h. The destination vector (petG-K-NHis) was based on a pET expression vector conferring kanamycin resistance and encoding a 6xHis tag at the N-terminus (the petG-K-NHis vector was a gift from J. Haas and E. Fossum, The University of Edinburgh, UK). *E. coli* DH10B cells were transformed and colonies were screened as described in section 5.3.10.2 except that LB plates containing 50 µg/ml kanamycin (Melford Laboratories, UK) were employed and diagnostic digestions were carried out with *Hind*III and *Xba*I restriction endonucleases (New England Biolabs, UK).

5.3.10.4 Transformation of *E. coli* BL-21 cells

One 30 µl aliquot of electrocompetent *E. coli* BL-21 cells was thawed on ice and mixed with 2 µl of expression vector carrying the gene region of interest. Cells were transformed as described in section 5.3.10.2 with double-selective LB plates containing 50 µg/ml kanamycin (Melford Laboratories, UK) and 150 µg/ml chloramphenicol (Sigma-Aldrich, UK). Diagnostic digestions were performed as described in section 5.3.10.3.

5.3.10.5 Protein expression of Gateway® constructs

Protein expression of Gateway® constructs was induced by adding 0.5 mM IPTG (Sigma-Aldrich, UK) to 50 ml exponential phase bacterial culture, followed by incubation for 4 h at 37°C in a shaking incubator at 200 rpm. 1 ml of induced culture was centrifuged at 14,000 rpm in a table top centrifuge (Sigma-Aldrich, UK) for 1 min, pellets were resuspended in 1x Laemmli sample buffer (Sigma-Aldrich, UK), boiled for 5 min and resolved by SDS-PAGE in 10% polyacrylamide gels according to standard procedures (as described in general Materials and Methods). Western blot analysis was carried out by standard procedures as described in general Materials and Methods with mouse anti-His antibody (Zymed®, Invitrogen, UK) as primary, and peroxidase-rabbit anti-mouse IgG (Zymed®, Invitrogen, UK) as secondary antibody.

5.3.11 Protein purification of Gateway® recombinant proteins

For the purification of Gateway® recombinant protein, 4 l of *E. coli* BL-21 exponential phase liquid cultures were induced by adding 0.2 mM IPTG (Sigma-

Aldrich, USA) for 4 h at 37°C in a shaking incubator at 225 rpm. Bacterial cultures were centrifuged at 4000 rpm for 10 min, pellets were resuspended in 20 ml of nickel column buffer A (20 mM $NaPO_4$, 0.5 M NaCl), and bacterial cells were lysed using a French Press (SLM Aminco, SLM Instruments, Inc., USA). Samples were ultracentrifuged at 40000 rpm, 4°C for 20 min and filtered using a 0.45 µm filter device (Millipore, USA). Proteins were purified by applying the samples to Nickel columns (Nickel HisTrap HP, GE Healthcare, USA) attached to a FPLC machine (ÄKTA, GE Healthcare, USA) using nickel column buffer A and buffer B (20 mM $NaPO_4$, 0.5 M NaCl, 0.5 M Imidazole). Samples were further purified using a Q Sepharose column (HiTrap™ 5 ml QHP, GE Healthcare, USA) attached to a FPLC machine (ÄKTA, GE Healthcare, USA) with Q sepharose column buffer A (1 mM EDTA in PBS, pH=7.4) and Q sepharose column buffer B (1 mM EDTA, 1 M NaCl in PBS, pH=7.4). Samples were extensively dialysed against PBS at 4°C after each column (Fisherbrand dialysis tubing, molecular weight cut off 12,000 – 14,000 Daltons, Fisherbrand, USA). Each step of the purification process was monitored by mixing aliquots of the protein samples with reducing sample buffer (50 mM Tris/HCl, 2% β-mercaptoethanol, 2% sodium dodecyl sulphate, 0.03% bromophenol blue, 10% glycerol, pH 6.8), boiling for 10 min, subjecting the samples to SDS-PAGE on 10% polyacrylamide gels and performing Western blot analysis according to standard procedures as described in general Materials and Methods, using mouse anti-His primary antibody (GE Healthcare Life Sciences, USA), followed by incubation with secondary antibody (goat anti-mouse IgG-AP conjugate, Bio-Rad Life Science, USA).

5.3.12 Western ligand blots

Fg solutions of human, canine, feline, and bovine origin (Sigma-Aldrich, USA) were diluted 1:1 in reducing sample buffer (Sigma-Aldrich, USA) to a final concentration of 1 µg/µl, boiled for 5 min and subjected to SDS-PAGE on 10% polyacrylamide gels (10 µg Fg per lane) according to standard procedures as described in general Materials and Methods. Western blot transfer was carried out by standard procedures, blots were blocked with 5% (w/v) non-fat dried milk powder (Skim milk powder, Sigma-Aldrich, USA) in PBS (Sigma-Aldrich, USA) overnight at 4°C and

incubated with 10 μg/ml of purified recombinant protein in 1% milk for 1h at room temperature. Primary antibody (mouse anti-His antibody from mouse ascites fluid, GE Healthcare Life Sciences, USA) was added in a 1:3000 dilution for 1h at room temperature, followed by incubation with secondary antibody (goat anti-mouse IgG-AP conjugate, Bio-Rad Life Science, USA) in a 1:3000 dilution for 1h at room temperature. Three washes in TBST buffer (0.14 M NaCl, 2.7 mM KCl, 25 mM Tris Base, 0.05% (v/v) Tween-20, pH 7.5) for 5 min each were carried out after each step. Blots were developed by adding 1 ml of 1-step NBT / BCIP developer (Thermo Fisher Scientific, USA).

5.4 Results

5.4.1 Bacterial growth curve of *S. pseudintermedius* strain ED99

As the growth profile of *S. pseudintermedius* ED99 in nutrient broth has not been previously determined, a bacterial growth curve was generated for *S. pseudintermedius* strain ED99 in order to identify time points for further experiments, and *S. aureus* strain Newman was included as a control strain (Figure 5.1). Bacterial growth of three independently inoculated broth cultures was monitored over a 12 h period, and based on the results (Figure 5.1) an OD_{600} of 0.5 was chosen for subsequent exponential phase *S. pseudintermedius* experiments.

5.4.2 *S. pseudintermedius* ED99 adheres to fibronectin, fibrinogen, and cytokeratin 10

S. pseudintermedius ED99 grown to exponential (OD_{600}=0.5) and stationary phases of growth was tested for adherence to bovine Fn, human and canine Fg, and recombinant mouse CK10 in solid phase assays.

In exponential phase of growth, *S. pseudintermedius* ED99 demonstrated adherence to bovine Fn, human and canine Fg, and mouse CK10 (Figure 5.2). When grown to stationary phase of growth, *S. pseudintermedius* ED99 adhered to bovine Fn, but to a lesser degree than in exponential phase (Figure 5.2). Furthermore, adherence of *S. pseudintermedius* ED99 in stationary phase of growth to human and canine Fg was only slightly higher than the adherence of the negative control *S. aureus* Newman Δ*clfA* (Figure 5.2). *S. pseudintermedius* ED99 grown to stationary phase lacked the ability to adhere to CK10 (Figure 5.2). The differences of adherence of *S. pseudintermedius* ED99 in exponential and stationary phases of growth suggests that adherence-mediating surface proteins are expressed in a growth phase-dependent manner.

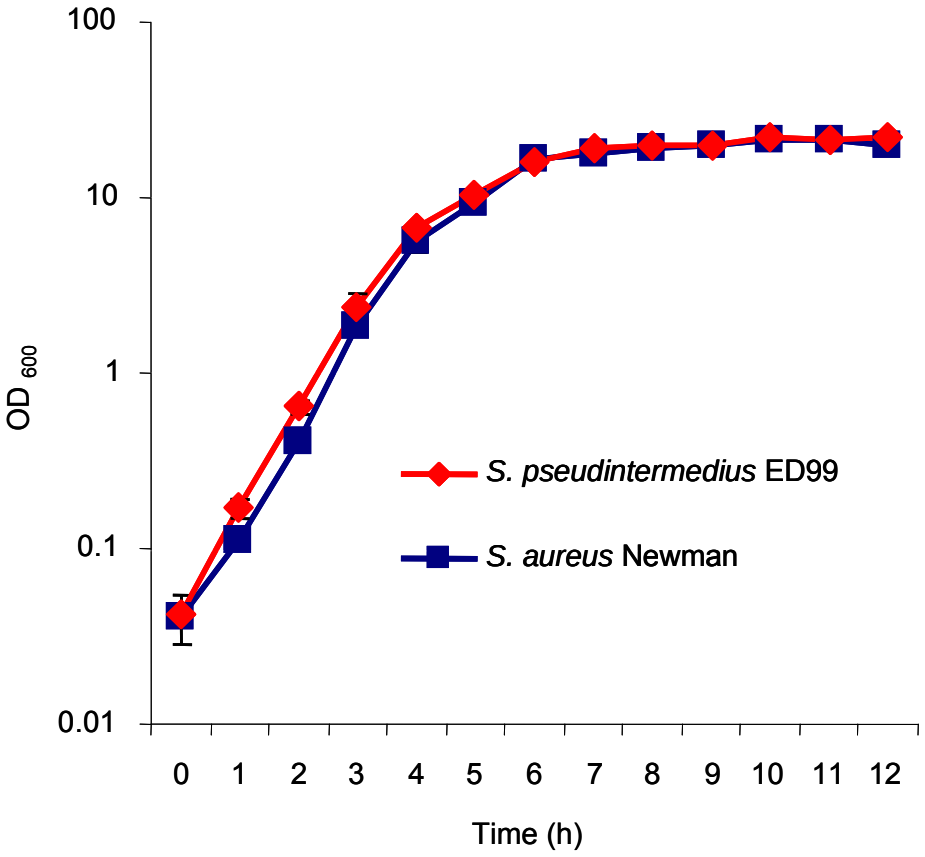


Figure 5.1. Bacterial growth curves of *S. pseudintermedius* strain ED99 and *S. aureus* strain Newman. Time points represent mean values of triplicate experiments, error bars indicate standard deviation.

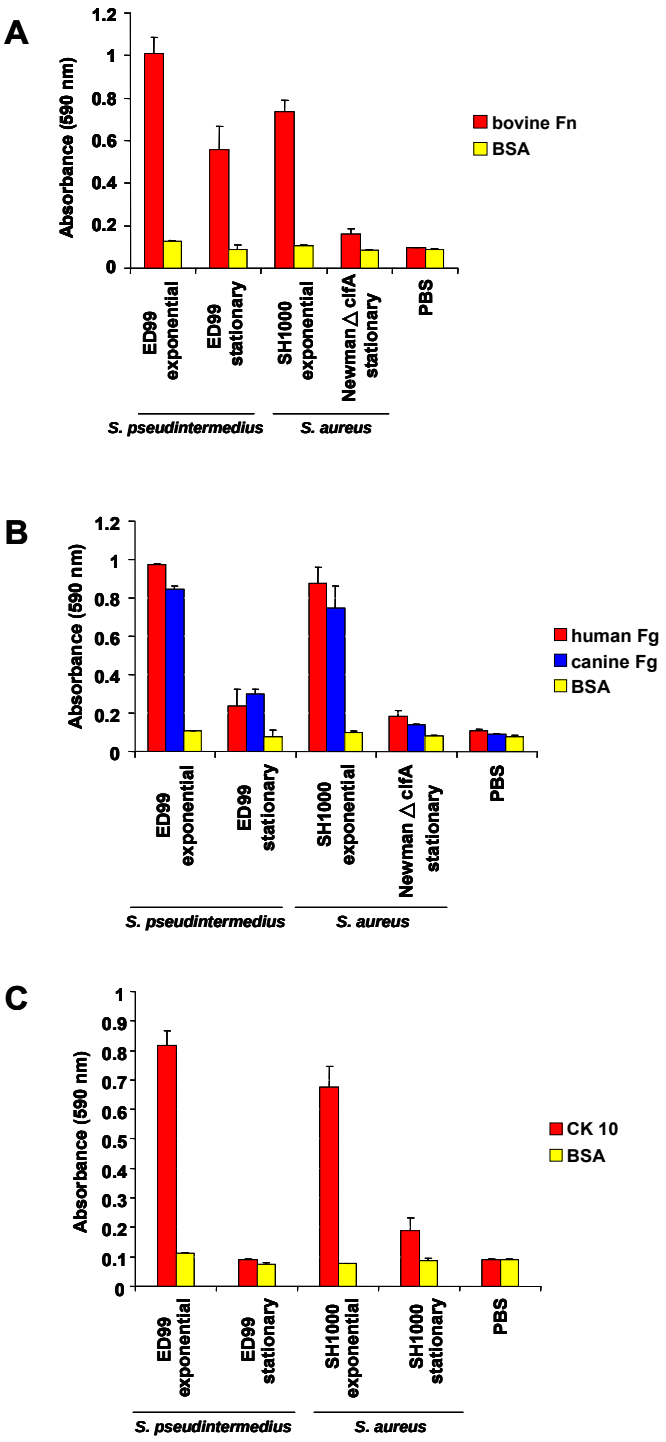


Figure 5.2. Adherence of *S. pseudintermedius* ED99 to (A) Fn; (B) Fg; and (C) CK10. Plates were coated with 1 µg of ECM protein or 1 µg of BSA per well as appropriate. Absorbance was measured at 590 nm and results are expressed as mean values of triplicate samples. Error bars indicate standard deviation. *S. aureus* strain SH1000 (Horsburgh *et al.*, 2002), *S. aureus* strain Newman deficient in ClfA production (McDevitt *et al.*, 1994), and PBS were included as controls.

5.4.3 Cloning and expression of SpsD, SpsL, and SpsO in *L. lactis*

In order to examine the role of putative selected MSCRAMMs independently on the bacterial cell surface, the full-length *spsD* (3096 bp), *spsL* (2793 bp), and *spsO* (5538 bp) genes were cloned into *L. lactis* using the shuttle vector pOri23 (Que *et al.*, 2000). Positive clones were identified by restriction digestion of purified pOri23 plasmids from single colonies of transformed *L. lactis* cells (data not shown). The pOri23 construct inserts were verified by DNA sequencing for *spsL* and *spsD*. For *spsO*, DNA sequence was generated for approximately 3000 bp of the total length of 5538 bp. A segment of the repeat region corresponding to ~2500 bp could not be determined due to the existence of identical tandem repeats which did not allow directed sequencing. As a negative control for subsequent MSCRAMM characterisation studies, *L. lactis* was transformed with the empty vector pOri23, confirmed by restriction digestion analysis. The predicted molecular weights were 115 kDa for SpsD, 103 kDa for SpsL, and 198 kDa for SpsO.

5.4.4 *L. lactis* expressing SpsD and SpsL demonstrated seroreactivity with canine sera from pyoderma cases

The antibody response to SpsD, SpsL, and SpsO *in vivo* was investigated by Western blot analysis employing pooled canine sera from staphylococcal pyoderma cases (n=3). The pyoderma was clinically manifested at the time of blood sampling and the dogs were also diagnosed with AD (Neuber *et al.*, 2008). Cell wall-associated protein fractions of the *L. lactis* constructs and of *S. pseudintermedius* ED99 were subjected to SDS-PAGE, transferred to nitrocellulose membrane and incubated with pooled canine sera from three pyoderma cases as described in Materials and Methods. An array of immunoreactive bands was detected for *S. pseudintermedius* ED99, ranging from 24 kDa to 102 kDa in molecular weight (Figure 5.3). For *L. lactis* expressing SpsD and *L. lactis* expressing SpsL, multiple seroreactive bands in the range of 38 kDa to 225 kDa for SpsD, and 38 kDa and 52 kDa for SpsL were detected, which were absent in the protein fractions of *L. lactis* carrying pOri23 alone (Figure 5.3). In contrast, *L. lactis* expressing SpsO did not demonstrate seroreactivity with sera from dogs diagnosed with pyoderma (Figure 5.3).

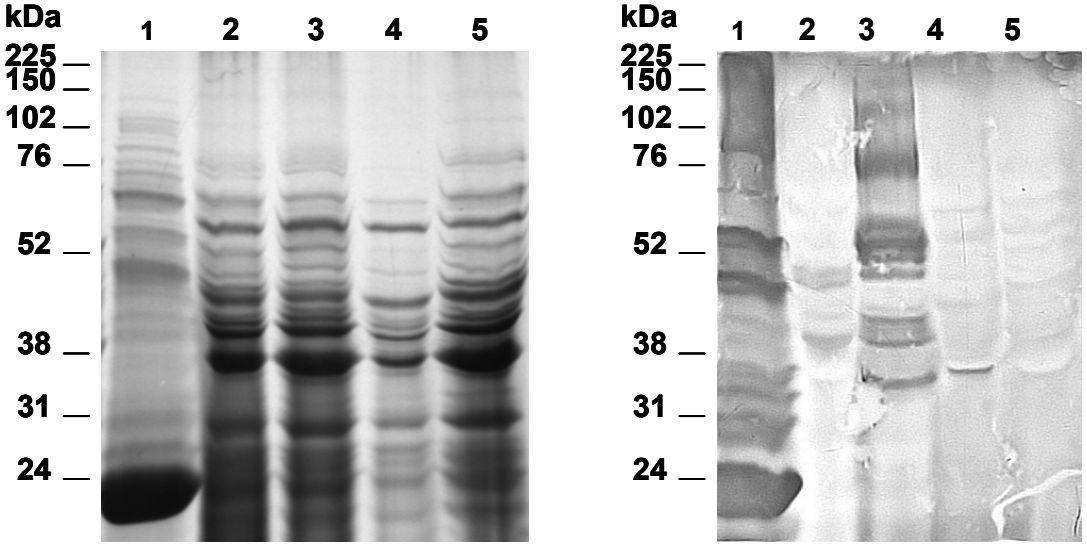


Figure 5.3. Western blot analysis of cell wall-associated proteins of *S. pseudintermedius* ED99 and *L. lactis* expressing SpsD, SpsL, and SpsO with sera from dogs diagnosed with pyoderma. (A) SDS PAGE analysis and (B) Western blot analysis of protein fractions from *S. pseudintermedius* ED99 in exponential phase of growth (lane 1); *L. lactis* expressing SpsL (lane 2); *L. lactis* expressing SpsD (lane 3); *L. lactis* expressing SpsO (lane 4); and *L. lactis* with pOri23 alone (lane 5).

5.4.5 Adherence of *L. lactis* constructs to extracellular matrix proteins

L. lactis expressing SpsO, SpsD, SpsL, and *L. lactis* carrying the vector pOri23 alone were tested for their ability to adhere to human Fn, human, canine, feline, and bovine Fg, and to recombinant mouse CK10 in solid phase assays.

5.4.5.1 The putative MSCRAMMs SpsD and SpsL mediate binding of *L. lactis* to fibronectin

L. lactis expressing SpsD and SpsL demonstrated adherence to human Fn, whereas *L. lactis* expressing SpsO demonstrated increased binding to Fn, but also to BSA, suggestive of a non-specific interaction (Figure 5.4).

5.4.5.2 The putative MSCRAMMs SpsD and SpsL mediate binding of *L. lactis* to fibrinogen, and SpsL demonstrates canine host-specificity

L. lactis expressing SpsD strongly adhered to Fg from several animal sources (Figure 5.5). In contrast, *L. lactis* expressing SpsL adhered to canine and feline Fg only, and did not bind to human and bovine Fg (Figure 5.5), indicating a host-specific interaction. *L. lactis* expressing SpsO did not bind to Fg from any source compared to *L. lactis* with the pOri23 vector alone (Figure 5.5).

5.4.5.3 The putative MSCRAMM SpsD mediates binding of *L. lactis* to cytokeratin 10

L. lactis expressing SpsD demonstrated strong adherence to CK10, whereas *L. lactis* expressing SpsO and SpsL did not show increased binding compared to *L. lactis* with the vector pOri23 alone (Figure 5.6).

5.4.6 Expression of recombinant A domains of SpsD and SpsL

In order to examine the binding activity of SpsD and SpsL with Fg in Western ligand blots, the gene regions encoding for the full-length A domain of SpsD (2436 bp) and SpsL (1512 bp) were cloned into *E. coli*, using Gateway® recombination cloning technology. Positive entry and expression clones were identified by restriction digestion of purified plasmids from single colonies of transformed *E. coli* cells and

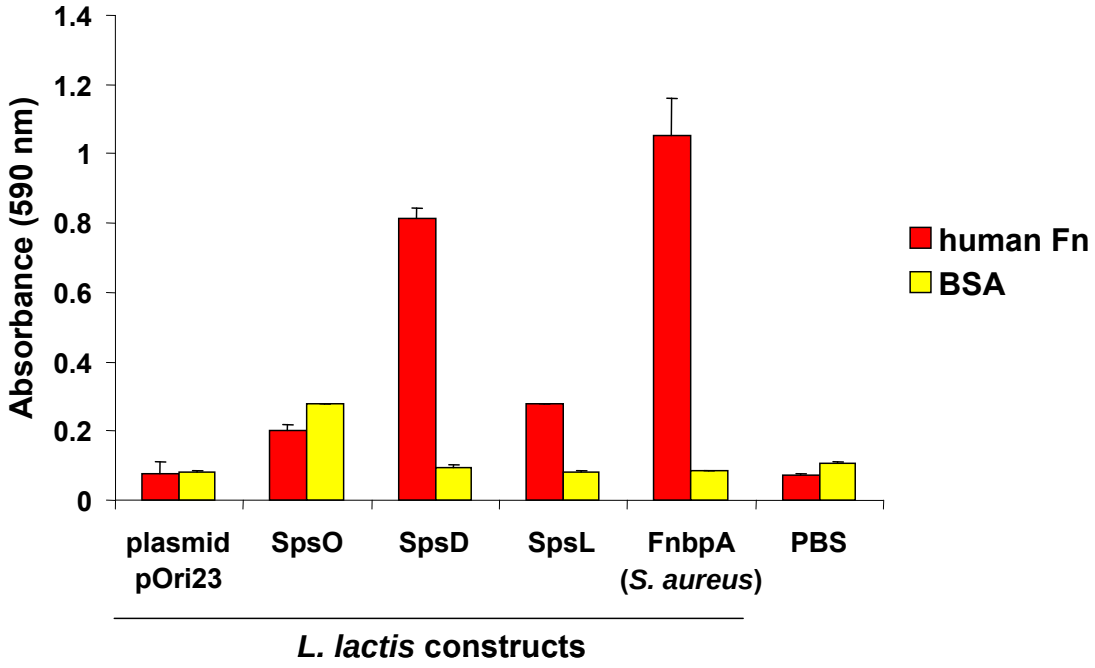


Figure 5.4. Adherence of *L. lactis* expressing specified MSCRAMMs to human fibronectin (Fn). Plates were coated with 1 µg of human Fn or 1 µg of BSA per well. Absorbance was measured at 590 nm and results are expressed as mean values of triplicate samples. Error bars indicate standard deviation. *L. lactis* expressing FnbpA from *S. aureus* and PBS were included as controls.

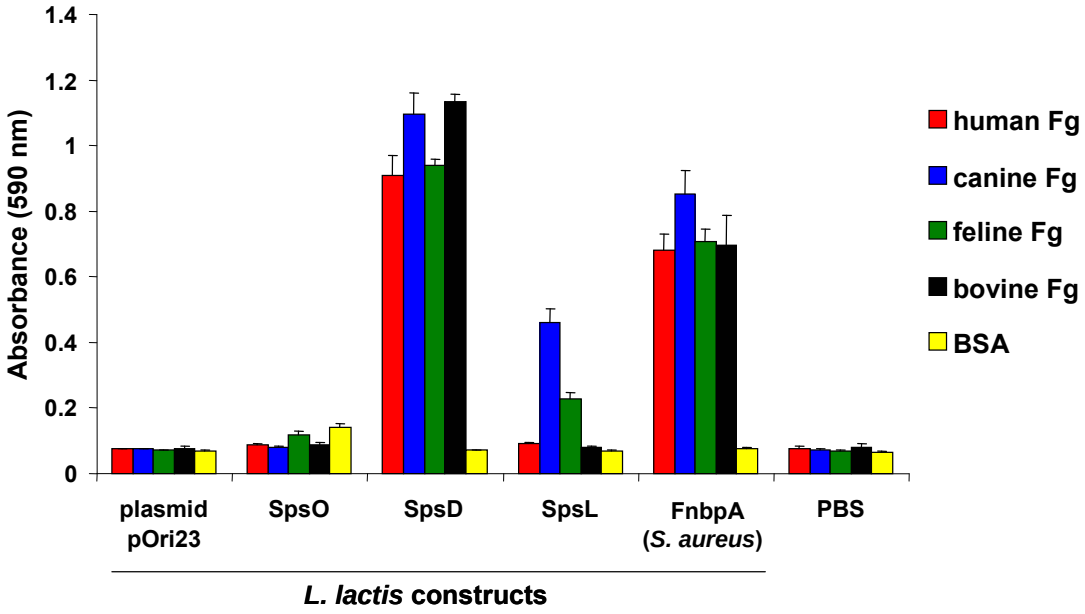


Figure 5.5. Adherence of *L. lactis* expressing specified MSCRAMMs to fibrinogen (Fg) from different animal sources. Plates were coated with 1 µg of Fg or 1 µg of BSA per well. Absorbance was measured at 590 nm and results are expressed as mean values of triplicate samples. Error bars indicate standard deviation. *L. lactis* expressing FnbpA from *S. aureus* and PBS were included as controls.

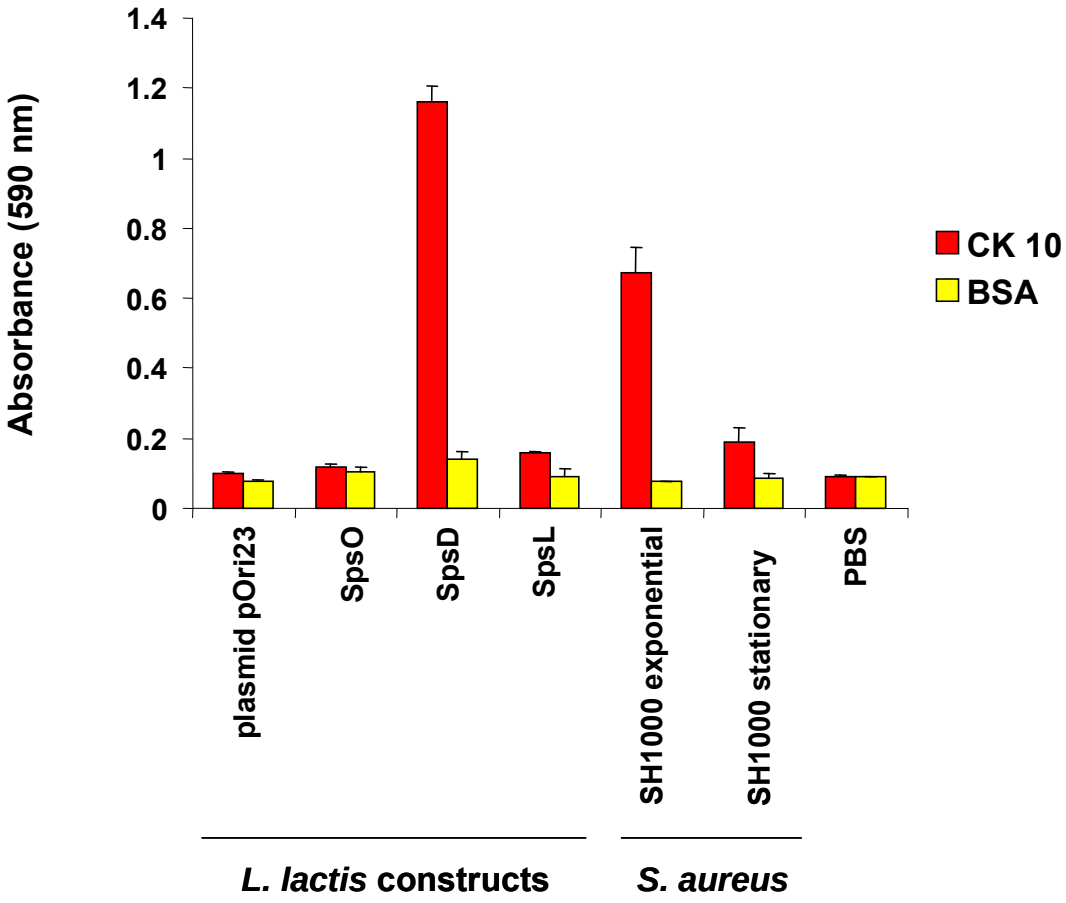


Figure 5.6. Adherence of *L. lactis* expressing specified MSCRAMMs to cytokeratin 10 (CK10). Plates were coated with 1 µg of recombinant CK10 or 1 µg of BSA per well. Absorbance was measured at 590 nm and results are expressed as mean values of triplicate samples. Error bars indicate standard deviation. *S. aureus* strain SH1000 in exponential and stationary phases of growth and PBS were included as controls.

inserts were verified by DNA sequencing (data not shown). Recombinant proteins were expressed by IPTG induction and proteins were purified as described in the Materials and Methods section. The predicted molecular weights were approximately 100 kDa for the A domain of SpsD and 65 kDa for the A domain of SpsL. Protein purification resulted in a single band of ~80 kDa and a total amount of approximately 1.5 mg of protein for the A domain of SpsD, and a single band of ~85 kDa and approximately 0.9 mg of protein for the A domain of SpsL (Figure 5.7).

5.4.7 The A domain of SpsD binds to the fibrinogen γ-chain, and the A domain of SpsL interacts specifically with canine fibrinogen

In order to identify the chain of Fg involved in binding with SpsD and SpsL, ligand affinity blots were performed. Fg of human, canine, feline, and bovine origin was separated into Aα-, Bβ-, and γ-chains by SDS-PAGE (Figure 5.8A), transferred to nitrocellulose membrane and incubated with purified His-tagged recombinant A domain protein for SpsD (rSpsD 55-866), and SpsL (rSpsL 39-542), respectively. The rSpsD recombinant protein bound specifically to the γ-chain of human, canine, feline, and bovine Fg, but not to the Aα and Bβ chains (Figure 5.8B). The rSpsL recombinant protein bound strongly to all three Fg chains of canine origin, and to the α-chain of human Fg (Figure 5.8C).

5.4.8 Sequence diversity of fibrinogen, fibronectin, and cytokeratin 10 from different hosts

The observation of a host-specific interaction of SpsL with Fg is suggestive of sequence variation between the Fg molecules from different host species. Accordingly, the variation of the Aα-, Bβ-, and γ-chains of Fg, in addition to Fn and CK10, was investigated with publically available sequence data. Sequence data emerging from the canine genome project has been released into the public domain since 2004, but only short fragments of the Fg-chains are currently available (http://www.uniprot.org/uniprot, last accessed on September 15th, 2009). For example, the 443 aa long fragment of the canine α-chain (accession number Q28243) represents the central coiled coiled region at the N-terminus. This fragment has 68% sequence identity with feline (accession number O97640), 58% with human

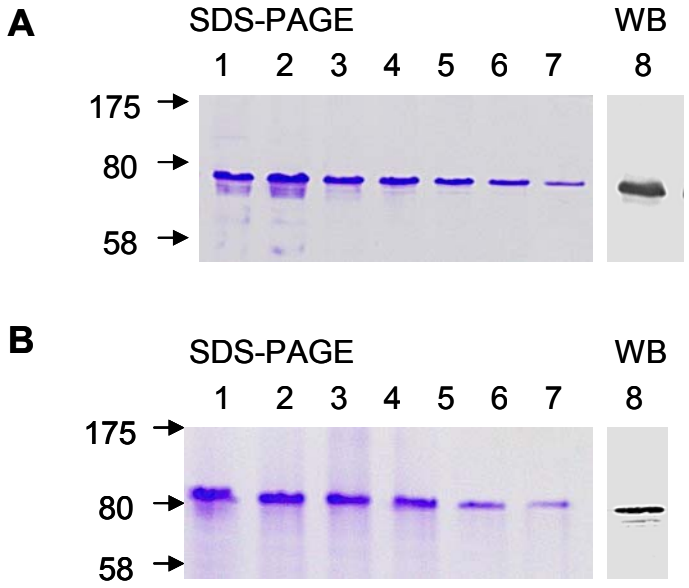


Figure 5.7. Purified recombinant protein of the A domain of (A) SpsD and (B) SpsL. Samples were analysed by SDS-PAGE after purification, and fractions containing the purified A domain were pooled and analysed by Western blot with mouse anti-His primary antibody and goat anti-mouse secondary antibody as described in Materials and Methods. Lanes 1 to 7 indicate different protein fractions containing the purified A domain, lane 8 indicates the pooled sample. Protein size marker is indicated; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; WB, Western blot.

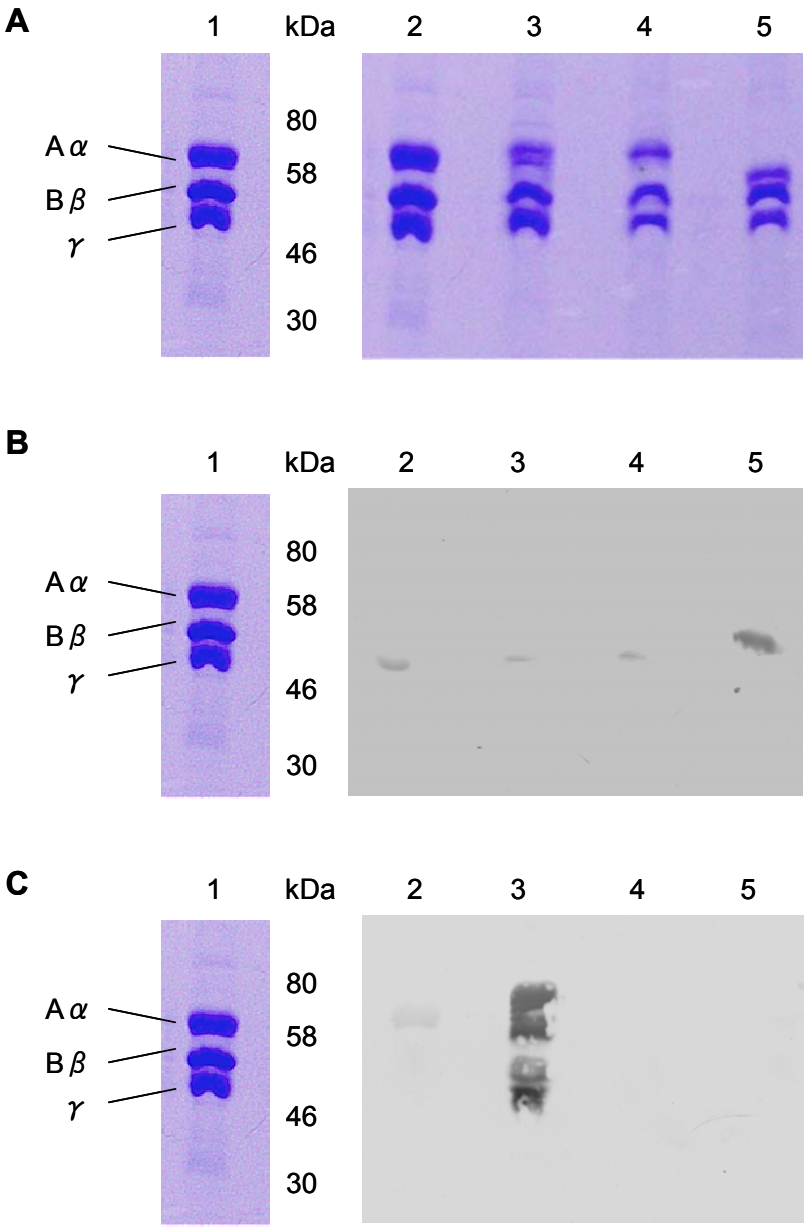


Figure 5.8. Western ligand blots of Fg with purified recombinant proteins of the A domains of SpsD and SpsL. Fg of human (lane 2), canine (lane 3), feline (lane 4), and bovine (lane 5) origin was separated into Aα-, Bβ-, and γ-chains by SDS-PAGE and stained with either (A) Coomassie blue or transferred onto nitrocellulose membrane and probed with recombinant A domain protein for (B) SpsD or (C) SpsL. The different Fg fractions based on human Fg (Coomassie stain, Lane 1) and protein size marker are indicated. The experiment was repeated independently with identical results (data not shown).

(accession number P02671), and 52% with bovine (accession number P02672) N-terminal α-chains, respectively, which includes the thrombin cleavage site and fibrinopeptide A (Figure 5.9). In *S. aureus*, ClfB binds to the Fg α-chain, but the interaction is located at the C-terminus (Walsh *et al.*, 2008). However, SdrG of *S. epidermitis* binds to the N-terminus of the Bβ-chain at the thrombin cleavage site (Davis *et al.*, 2001). The canine Fg Bβ-chain available in the public domain comprises only 31 aa (accession number P02677), which are located at the N-terminus and include the thrombin cleavage site and fibrinopeptide B. The sequence has low homology in ClustalW pair-wise alignments with the N-terminus of feline (50%, accession number P14469), human (45%, accession number P02675), and bovine (45%, accession number P02676) Bβ-chains. The canine Fg γ-chain sequence available consists of only 24 aa (accession number P12800), located in the N-terminal region. The sequence shares 95% identity with the human and bovine N-terminal γ-chains (residues 27 to 50, accession number P02679, and residues 25 to 48, accession number P12799, respectively), but only 12% in pair-wise alignment with the N-terminal fragment of the feline γ-chain available in the database (30 aa, accession number O46475). In *S. aureus*, the MSCRAMMs ClfA, FnbpA, and FnbpB interact with the Fg γ-chain, but the binding site is located at the C-terminus (McDevitt *et al.*, 1997; Wann *et al.*, 2000).

This study used Fn of human origin only, as canine Fn was not available. In order to determine the similarity between human and canine Fn, a search in the UniProtKB database (http://www.uniprot.org/uniprot, last accessed on September 15th, 2009) was performed and revealed that only a fragment of the canine Fn molecule is currently available in the public domain. The 522 aa long sequence (accession number Q28275) includes three F1 modules and four F3 modules, and represents the C-terminal region of canine Fn. A homology search against human Fn (accession number P02751-15) shows 94% identity in the C-terminal region, but the overall homology between human and canine Fn molecules remains unknown. However, the binding site for FnbpA of *S. aureus* is located at the five F1 modules of the N-terminal (NTD) of Fn (Schwarz-Linek *et al.*, 2003; Schwarz-Linek *et al.*, 2006), and

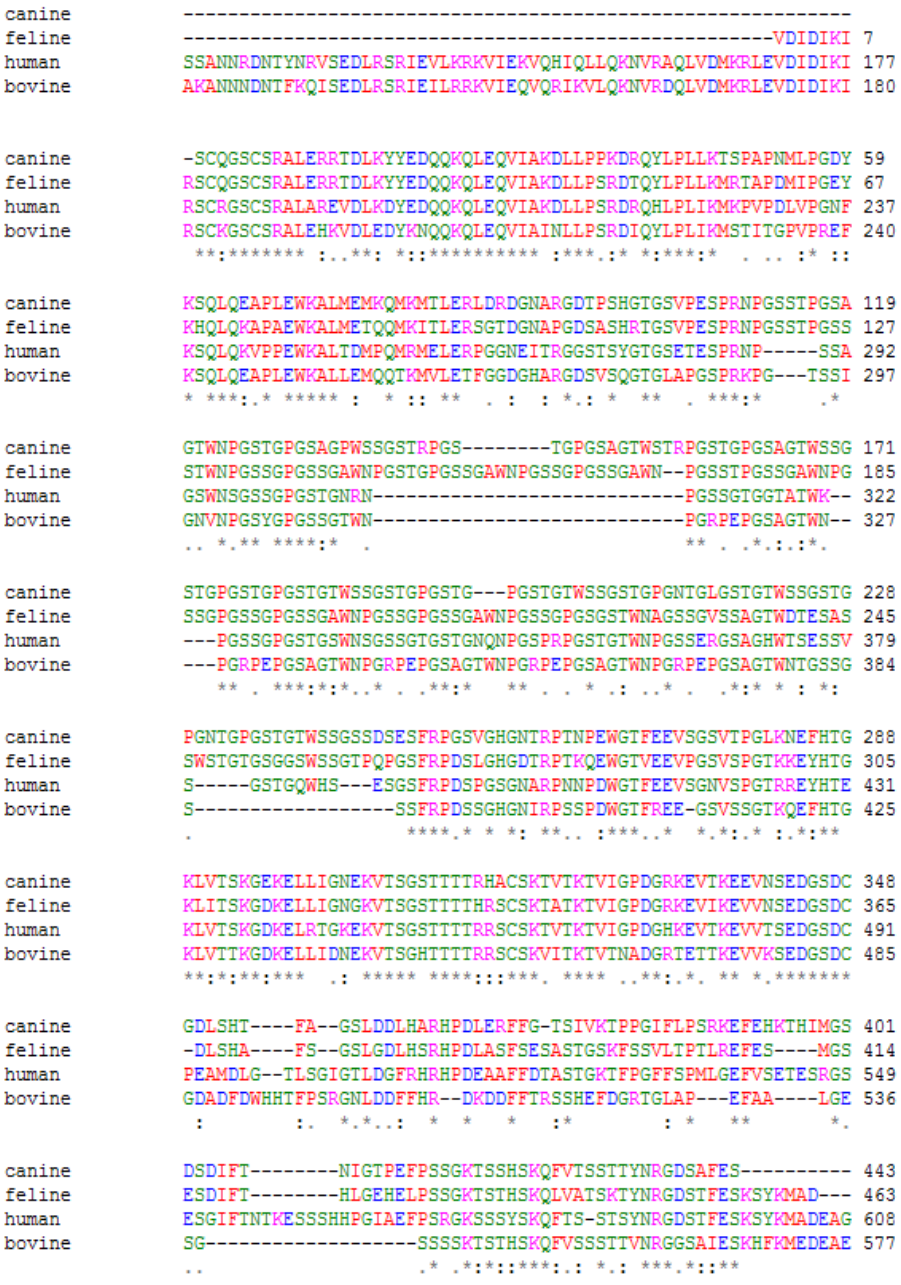


Figure 5.9. Multiple sequence alignment of the fibrinogen α-chains of canine, feline, human, and bovine origin. The sequence length available in the database varied (canine 443 aa, feline 463 aa, human 866 aa, bovine 615 aa). Colour code for amino acids and consensus symbols according to the ClustalW2 program: red, small and hydrophobic (AVFPMILW); blue, acidic (DE); magenta, basic (RK); green, hydroxyl, amine, basic (STYHCNGQ); '*', identical residues in all sequences; ':', conserved substitutions; '.', semi-conserved substitutions.

a previous study indicates that *S. pseudintermedius* strain 326 also interacts with the N-terminal region (Geoghegan *et al.*, 2009).

The present study employed the C-terminal region of mouse CK10. Mouse, human, and canine CK10 sequences are available in the public domain (http://www.uniprot.org/uniprot, last accessed on September 15th, 2009; accession numbers P02535, P13645, Q6EIZO, respectively). Mouse CK10 (length 570 aa) shares 84% sequence identity with canine CK10 (568 aa), and 80% identity with human CK10 (584 aa), whereas canine and human CK10 is 89% identical.

Taken together, these sequence comparison data indicate considerable ECM protein diversity which would influence bacterial colonisation and host-pathogen interactions in different host species.

5.5 Discussion

The current study demonstrated adherence of *S. pseudintermedius* ED99 to the ECM proteins Fg, Fn, and CK10. For *S. aureus*, the ability to bind to host ECM proteins is recognised as an important step in establishing colonisation and subsequent infection of the host (Foster and Hook, 1998; Clarke and Foster, 2006). The pathogenesis of canine pyoderma is poorly understood, but it is likely that MSCRAMMs are involved in the initial adherence of *S. pseudintermedius* to canine skin. The binding of *S. pseudintermedius* to canine corneocytes has been demonstrated in numerous studies (McEwan, 2000; Forsythe *et al.*, 2002; Saijonmaa-Koulumies and Lloyd, 2002a; McEwan *et al.*, 2005; Simou *et al.*, 2005b; Simou *et al.*, 2005c; McEwan *et al.*, 2006a; Lu and McEwan, 2007; Woolley *et al.*, 2008), but only very few studies have investigated the specific interaction with ECM proteins. The binding of *S. pseudintermedius* to Fg, Fn, CK10, elastin, vitronectin, laminin, and collagen type I has been reported, and adherence varied between strains, and was influenced by the bacterial growth phase (Cree and Noble, 1995; Geoghegan *et al.*, 2009). The degree of adherence of *S. pseudintermedius* ED99 to Fg, Fn, and CK10 observed in the present study was increased in exponential phase compared to stationary phase of

growth, and ED99 grown to stationary phase did not adhere to CK10. Geoghegan *et al.* (2009) investigated *S. pseudintermedius* strain 326, a canine pyoderma isolate from England, and reported binding differences between exponential and stationary phase of bacterial growth consistent with the current study. However, strain 326 did adhere to CK10 in stationary phase, but to a lower degree than in exponential phase of growth (Geoghegan *et al.*, 2009). These data indicate strain-dependent variation in ligand-binding repertoires. Also, the CK10 used by Geoghegan *et al.* (2009) was of human origin, whereas the C-terminal region of mouse CK10 was employed in the present study. Accordingly, it is possible that variation in CK10 sequence may have contributed to the differences in findings. Surface protein expression is growth phase dependent in *S. aureus*, and many MSCRAMMs are negatively regulated by the *agr* system resulting in upregulation during exponential phase and downregulation during stationary phase of growth (Novick, 2003; Bronner *et al.*, 2004). Of note, *S. aureus* strain Newman expresses the Fg- and CK10-binding MSCRAMM ClfB only in exponential phase of growth, and transcription ceases towards stationary phase, independent of the *agr* system (Ni Eidhin *et al.*, 1998; McAleese *et al.*, 2001). The decrease in adherence of *S. pseudintermedius* in stationary phase of growth compared to exponential phase suggests that similar regulatory mechanisms such as *agr* may be involved. *S. pseudintermedius* ED99 surface proteome analysis performed in our laboratory demonstrated the expression of SpsD, SpsL, and SpsO, among other Sps proteins, and the expression of SpsD and SpsO was growth phase-dependent (Dr. Nouri L. Ben Zakour, personal communication).

Numerous *S. aureus* MSCRAMMs and their ligands have been characterised, and their role in pathogenesis and as potential vaccine candidates has been investigated in several animal models (Josefsson *et al.*, 2001; Hall *et al.*, 2003; Patti, 2004; Arrecubieta *et al.*, 2006; Castagliuolo *et al.*, 2006; Schaffer *et al.*, 2006; Nanra *et al.*, 2009). Additionally, human colonisation models indicated a role for ClfB in *S. aureus* colonisation (Wertheim *et al.*, 2008). However, the bacterial factors involved in *S. pseudintermedius* colonisation of the canine skin have not been identified yet. The present study investigated three putative MSCRAMMs of *S. pseudintermedius*, SpsD, SpsL, and SpsO, in their ability to mediate binding to ECM proteins

independently when expressed in *L. lactis* as a surrogate host. Cloning and expression of putative staphylococcal MSCRAMMs in the non-pathogenic *L. lactis* has been commonly used for investigation of interactions with ECM ligands in solid phase assays, because it facilitates the examination of individual staphylococcal proteins expressed on the lactococcal cell surface without the interference of other staphylococcal proteins which may have related functions (Que *et al.*, 2001; O'Brien *et al.*, 2002b; Walsh *et al.*, 2004; Arrecubieta *et al.*, 2007). The current study identified ligands for the putative *S. pseudintermedius* MSCRAMMs SpsD and SpsL by expression in *L. lactis*. SpsD mediated adherence to Fg of human, canine, feline, and bovine origin, suggesting that the binding site for SpsD in Fg is conserved in all species tested. In contrast, SpsL mediated adherence specifically to canine and feline Fg but not to human or bovine Fg. Dogs are the primary host of *S. pseudintermedius*, and the interaction of SpsL with canine Fg may represent an example of host-specialisation by the bacteria. Further, the current study investigated the interaction of the recombinant A domains of SpsD and SpsL with individual Fg chains. In *S. aureus* and *S. epidermidis*, MSCRAMM adherence to Fg is mediated via the A domain (McDevitt *et al.*, 1995; Ni Eidhin *et al.*, 1998; Wann *et al.*, 2000; Davis *et al.*, 2001). The present study demonstrated that the A domains of SpsD and SpsL of *S. pseudintermedius* ED99 are capable of binding to Fg. The purified recombinant A domain of SpsD interacted with the γ-chains of human, canine, feline, and bovine Fg. For MSCRAMMs of *S. aureus* binding to Fg, 'dock, lock, and latch' binding mechanisms similar to SdrG of *S. epidermidis* have been suggested (Ponnuraj *et al.*, 2003; Keane *et al.*, 2007b; Bowden *et al.*, 2008; Ganesh *et al.*, 2008; Walsh *et al.*, 2008), and the interaction has been located at the Fg γ- chain for ClfA and FnbpA, to the α-chain for ClfB, and to the βB-fibrinopeptide for SdrG (McDevitt *et al.*, 1997; Wann *et al.*, 2000; Davis *et al.*, 2001; Walsh *et al.*, 2008). SpsD interacted with Fg γ-chains, and a variant of the TYTFTDYVD motif and putative latching sequence have been identified (Chapter 4), suggesting that SpsD may bind to Fg with a similar mechanism. Adherence of *S. pseudintermedius* to the Fg α-chain was reported previously, but β- and γ-chains were not tested (Geoghegan *et al.*, 2009). Therefore, *S. pseudintermedius* may interact with at least two of the Fg chains via different surface proteins. The purified A domain of SpsL reacted strongly with all three

canine Fg chains, weakly with the human α-chain, and not at all with the feline or bovine Fg, supporting the host-specific interaction of *L. lactis* expressing SpsL in the immobilised Fg assays. *S. aureus* ClfB was previously reported to bind to the α- and β-chain of human Fg based on Western ligand blotting (Ni Eidhin *et al.*, 1998), but Walsh *et al.* (2008) demonstrated that ClfB interacts exclusively with the C-terminus of the α-chain of human Fg. Therefore, further investigation is necessary to specify the binding site within canine Fg for the A domain of SpsL. However, the limited Fg sequence data available may suggest that the Aα- and Bβ-chains are less conserved between different host species than the γ-chains, allowing speculation that a host-specific interaction with the N-terminals of the Aα- or Bβ-chains may exist.

SpsD interacted with the γ-chains of all species. However, it has to be emphasised that the data are preliminary, and that further studies are required to properly characterise the interaction of SpsD and SpsL with Fg. SpsD and SpsL mediated adherence of *L. lactis* to human Fn, but the interaction of SpsL was relatively weak. The Fn employed in the current study was of human origin, due to unavailability of canine Fn. The sequence homology between human and canine Fn is unknown to date, but considering the host-specific binding of SpsL to Fg, it would be interesting to determine if a similar phenomenon exists with Fn.

The current study tested the adherence of *L. lactis* expressing SpsD, SpsL, and SpsO to CK10, and binding activity was demonstrated for *L. lactis* expressing SpsD. CK10 is present in the upper layers of the epidermis and is produced by differentiated keratinocytes (Suter *et al.*, 1997), and contains three different domains, including a head-, central α-helical rod-, and tail region (Walsh *et al.*, 2004). The recombinant CK10 used in this study was of murine origin and comprised C-terminal residues 294 to 570, which includes the C-terminal subdomains of the rod region and the tail region (Walsh *et al.*, 2004). In *S. aureus*, ClfB binds to the tail region of CK10, to human desquamated nasal epithelial cells (O'Brien *et al.*, 2002b; Walsh *et al.*, 2004), and contributes to colonisation of the human nares (Wertheim *et al.*, 2008; Corrigan *et al.*, 2009). It is tempting to speculate that *S. pseudintermedius* surface proteins,

such as SpsD, which mediate adherence to CK10 may be involved in colonisation of canine skin.

The present study investigated the antigenic potential of the *L. lactis* constructs by employing canine sera of three pyoderma cases. An antibody response was detected for *L. lactis* expressing SpsD and *L. lactis* expressing SpsL, with multiple immunoreactive bands. It is likely that the surface proteins were partly degraded in the preparation of the cell wall-associated protein fractions, resulting in cross-reactive proteins of smaller size. For *L. lactis* expressing SpsO, no specific cross-reactive proteins were detected, suggesting that the canine sera contained antibodies against SpsD and SpsL, but not SpsO. These data indicate that SpsD and SpsL are expressed by *S. pseudintermedius* during colonisation or disease *in vivo*. The lack of antibodies against SpsO is consistent with the findings in the previous chapter that SpsO is not present in all *S. pseudintermedius* isolates, suggesting that some canine patients are infected with strains that do not produce SpsO. The canine immune response to staphylococcal skin infection is not well understood. Some studies have examined the humoral immune response to cell wall-associated fractions of *S. pseudintermedius*, for example Shearer and Day (1997) investigated differences in serum IgG and anti-staphylococcal IgG concentrations of dogs with different skin conditions compared to healthy dogs in ELISA assays. Serum IgG in atopic dogs with superficial pyoderma was significantly elevated compared to healthy dogs, but serum IgG of non-atopic dogs with flea bite dermatitis and superficial pyoderma was not (Shearer and Day, 1997). Further, anti-staphylococcal IgG was measured, and IgG levels were significantly elevated in dogs with pyoderma compared to healthy dogs (Shearer and Day, 1997). Neuber *et al.* (2008) compared IgG responses of dogs with skin disease, including nine pyoderma cases, compared to healthy dogs by immunoblotting of six *S. pseudintermedius* pyoderma isolates with the dogs' sera. The detected IgG response of dogs with and without pyoderma to the six *S. pseudintermedius* isolates was similar, and the molecular weights of the immunoreactive bands were between 20 kDa and 198 kDa (Neuber *et al.*, 2008), including bands at the approximate sizes of SpsD (115 kDa), SpsL (103 kDa), and SpsO (198 kDa). The data from the current study indicate that an antibody response

is generated towards SpsD and SpsL during infection, suggesting that they may have antigenic potential *in vivo*. The characterisation of SpsD and SpsL as MSCRAMMs with affinity for ECM proteins indicates an important role in canine host-pathogen interactions. Taken together, these data suggest that SpsD and SpsL may represent novel therapeutic targets.

Chapter 6

Adherence of selected putative MSCRAMMs of *Staphylococcus pseudintermedius* ED99 to canine keratinocytes

6.1 Introduction

Bacterial adherence to host tissue is a crucial step in colonisation and subsequent infection (Beachey, 1981). Several studies have investigated the potential of *S. pseudintermedius* to adhere to canine corneocytes using *ex vivo* corneocyte adherence assays (McEwan, 2000; Forsythe *et al.*, 2002; Saijonmaa-Koulumies and Lloyd, 2002a; McEwan *et al.*, 2005; Simou *et al.*, 2005b; Simou *et al.*, 2005c; McEwan *et al.*, 2006a; Lu and McEwan, 2007; Woolley *et al.*, 2008).

A number of different approaches have been used to collect corneocytes for bacterial adherence studies. For example, canine corneocytes have been collected using double-sided adhesive tape (Forsythe *et al.*, 2002; Saijonmaa-Koulumies and Lloyd, 2002a; Simou *et al.*, 2005b; Simou *et al.*, 2005c), manual dislodgement (McEwan *et al.*, 2005), a 'scrub cup technique' (McEwan *et al.*, 2006a), or by applying adhesive discs to the inner aspect of the dog's pinnae (Lu and McEwan, 2007; Woolley *et al.*, 2008).

For quantification of bacterial adherence, investigators either used computerised image analysis (Forsythe *et al.*, 2002; Simou *et al.*, 2005b; Simou *et al.*, 2005c; Woolley *et al.*, 2008) or counted bacteria visually per random microscopic field (Saijonmaa-Koulumies and Lloyd, 2002a) or per 100 consecutive corneocytes (McEwan, 2000; McEwan *et al.*, 2005; McEwan *et al.*, 2006a). Lu and McEwan (2007) directly compared visual and computerised counting and reported that there was good agreement between the two methods.

The adherence of *S. pseudintermedius* to canine corneocytes increases with longer incubation times, higher temperature and higher bacterial concentrations (Saijonmaa-Koulumies and Lloyd, 2002a; Lu and McEwan, 2007). The formation of bacterial aggregates ('microcolonies') also increases with higher bacterial concentrations and is a recognised problem for adherence quantification, which has been overcome by calculating percentage adherence area rather than obtaining numerical counts (Forsythe *et al.*, 2002), by counting each microcolony as one unit (Saijonmaa-

Koulumies and Lloyd, 2002a), or by reducing the bacterial concentration to eliminate clumping (Lu and McEwan, 2007).

The collection of *ex vivo* corneocytes is dependent upon the availability of appropriate canine donors, is time consuming, and requires the cooperation of the dog (handling issues). Furthermore, the sampling of live animals introduces additional variables into the assay because of known variation in adherence to corneocytes from different breeds (Forsythe *et al.*, 2002) and variations in sampling technique employed by different investigators. In addition, using this technique, the reverse side of the corneocyte to that normally exposed to the environment has to be used for testing the interaction with bacteria.

To address some of the limitations of using *ex vivo* corneocytes, keratinocyte primary cultures have also been used in the investigation of bacterial adherence. For *S. aureus*, mouse epidermal cells (Miyake *et al.*, 1990) and biopsies from human skin (Cho *et al.*, 2001) have been cultured and canine skin primary cultures (and subcultures from the primary cells, respectively) have been established (Wilkinson *et al.*, 1987; Kohler *et al.*, 2001; Baumer and Kietzmann, 2007; Serra *et al.*, 2007). Similar to corneocyte sampling, primary cultures require access to suitable cell donors which have to be either anaesthetised or euthanased. Therefore, the use of an epidermal cell line, with the capability to differentiate, would be potentially markedly advantageous to increase objectivity and facilitate bacterial binding assays.

One such cell line, derived from epidermal keratinocyte progenitors of an adult Beagle dog, has recently become available commercially (CPEK cells, CELLn-TEC Advanced Cell Systems, Switzerland). The phenotype of this cell line resembles that of basal keratinocytes rather than differentiated corneocytes as determined by the expression of α_6-integrin and CK14 and the lack of CK10 expression (Shibata *et al.*, 2008). The adhesion molecule α_6-integrin is a subunit of the α_6-/β_4-integrin complex which is only expressed on the epidermal-dermal junction of basal keratinocytes (Sonnenberg *et al.*, 1991; Yamazoe *et al.*, 2007). Additionally, CPEK cells express the proliferation marker Proliferating Cell Nuclear Antigen (PCNA), indicating

proliferating basal keratinocytes (Shibata *et al.*, 2008), and an array of cytokines and chemokines (Maeda *et al.*, 2009). Recently, Yagihara *et al.* (2009) induced differentiation of CPEK cells by seeding cells in high density and allowing continuous growth for 18 days.

6.2 Aims and Strategy

To investigate the role of three putative MSCRAMMs (SpsD, SpsL, SpsO) encoded by *S. pseudintermedius* strain ED99 in binding to canine corneocytes by:

1) Examination of the ability of SpsD, SpsL, and SpsO to mediate adherence to *ex vivo* canine corneocytes by expression of each MSCRAMM independently on the cell surface of the heterologous host *L. lactis*.

2) Development of a novel adherence assay utilising the commercial canine epidermal cell line CPEK for bacterial adherence studies and employing the assay to examine the adherence of *S. pseudintermedius* ED99 and the respective roles of MSCRAMMs SpsD, SpsL, and SpsO in adherence to CPEK cells.

6.3 Materials and methods

6.3.1 Bacterial strains used in this study

For adherence studies to *ex vivo* canine corneocytes, *S. pseudintermedius* strain ED99, *L. lactis* strain MG1363, *L. lactis* expressing SpsD, SpsL, SpsO, and *L. lactis* carrying the pOri23 plasmid alone, generated as described in Chapter 5, were used. For adherence studies to CPEK cells, *S. pseudintermedius* ED99, *Staphylococcus hominis* ATCC 27844 (species type strain), and the *L. lactis* constructs were tested.

6.3.2 Canine corneocyte adherence assay

6.3.2.1 Dogs

For preliminary experiments to confirm adherence of *S. pseudintermedius* ED99 and non-adherence of *L. lactis*, corneocytes were obtained from a seven-year-old male neutered Border collie cross-breed with no history or physical signs of systemic or cutaneous disease. Corneocytes for the *L. lactis* adherence study were obtained from five dogs of different breeds (one Labrador retriever, two Border collies, and two cross-breeds). Three dogs were ovariohysterectomised females and two were entire males. The median age was seven years (range one to twelve years) (Table 6.1). The dogs showed no abnormalities on general physical examination and had no history or physical signs of skin disease at the time of corneocyte collection. All dogs were privately owned by staff or students of the Royal (Dick) School of Veterinary Studies, The University of Edinburgh. None of the dogs had received topical or systemic drug treatments for at least three weeks prior to the day of corneocyte collection.

6.3.2.2 Collection of corneocytes

Samples were taken from the ventral abdomen and inner thigh. If necessary, sample sites were clipped with Oster clippers (Oster Cryotech, USA) using a number 40 blade. For collection of corneocytes, the method described by Forsythe *et al.* (2002) was used. Briefly, the area was cleaned of surface debris and commensal bacteria by applying four strips of single sided adhesive tape (Cellux, Henkel Consumer

Table 6.1. Details of the dogs sampled for the corneocyte adherence study.

Dog	Breed	Age (years)	Gender
1	Jack Russell terrier cross	12	FN
2	Border collie	7	FN
3	Labrador retriever	5	ME
4	Whippet cross	1	FN
5	Border collie	7	ME

FN, ovariohysterectomised female; ME, male entire

Adhesives, UK), using each strip once. To collect corneocytes, double-sided, clear, adhesive wig tape (Tropical Tape Super Grip, USA) was mounted onto a microscope slide in 1 cm² pieces and applied to the same area of skin surface 10 times with gentle force. Slides were investigated by microscopic examination and only slides with at least 75% corneocyte coverage were used in the study.

6.3.2.3 Corneocyte adherence assay

The corneocyte slides were positioned in moisture chambers (Nunc™, Thermo Fisher Scientific, Denmark) as described by Forsythe *et al.* (2002). The moisture chambers consisted of 30 cm x 30 cm plastic trays with lids and were prepared by lining the trays with moistened paper towels. *S. pseudintermedius* ED99 stationary or exponential (OD_{600} of 0.5) phase cultures and *L. lactis* exponential phase cultures (OD_{600} 0.6 to 0.8) were centrifuged at 4000 rpm for 5 min, washed with PBS and resuspended in PBS to a final OD_{600} of 0.5. The moisture chambers were placed in a static incubator and 250 µl of bacterial suspension was added to each 1 cm² of tape, forming a meniscus on the tape. Slides incubated with 250 µl of sterile PBS were included as a control. The slides were incubated at 37°C for 90 min and washed in PBS. Each slide was stained with 0.5% (w/v) crystal violet (Sigma-Aldrich, UK) for 90 s before rinsing off with PBS. The slides were air-dried and a drop of immersion oil (Cargille Laboratories Inc., USA) and a cover slip (Scientific Laboratory Supplies, UK) were added before microscopic quantification. All slides were prepared in duplicate on the same day and incubated at the same time. Prior to incubation with bacterial suspensions or PBS, each slide was labelled with a letter code to allow identification after the microscopic analysis. The identification code on each slide was hidden by a third party for subsequent image acquisition so that the investigator was blinded to the origin of the slide.

6.3.2.4 Quantification of adherent bacteria

For quantification of adherent bacteria, computerised image analysis was used as described previously by Forsythe *et al.* (2002) with minor modifications. For each slide, bright field images of 1000x oil-immersion fields were acquired with a Sony DXC-390P 3CCD colour video camera (Scion Corporation, USA) connected to a

Leica Laborlux S microscope (Leica Microsystems UK Ltd., UK). The RGB video signal from the camera was digitised using Scion Image (Scion Corporation, USA) installed in a G4 Macintosh computer (Apple Computer, USA) fitted with a CG-7 frame grabber (Scion Corporation, USA).

For image acquisition, fields equivalent to 14.4 mm² were selected randomly by starting in the bottom left corner of each slide and moving through the slide in a defined way using the scale on the microscope stage. A field was discarded if the corneocyte layer was not confluent, the bacteria were poorly stained against the background or the field could not be focused properly.

The software used for quantification of bacterial adherence was set to calculate the percentage area that was covered by bacteria per confluent layer of corneocytes in a defined region of interest (ROI) of 500 μm^2 within each image field acquired. Previous studies by Forsythe *et al.* (2002) using the same technique and software have demonstrated that 15 replicates of each duplicate slide resulted in acceptable coefficients of variation of approximately 10%. In this study, 25 replicates of each slide were acquired and the overall mean percentage area of adherence was determined by calculating the mean of all replicates.

6.3.3 Canine keratinocyte cell culture adherence assay

6.3.3.1 Canine epidermal cell line

Canine epidermal keratinocyte progenitors (CPEK, CELLn-TEC Advanced Cell Systems, Switzerland), were grown in 25 cm² cell culture flasks (BD Falcon™ Cell Culture Flasks, Scientific Laboratory Supplies, UK) at 37°C with 5% CO_2. For each flask, 5 ml optimised medium was used (CnT-09 basal medium plus 'Supplement A' (fetal calf serum) and 'Supplement B' (200 mM L-Glutamine), CELLn-TEC Advanced Cell Systems, Switzerland). Every two days, cells were provided with fresh medium and examined for contamination and growth. When confluent, cells were passaged to a new flask. Briefly, cells were washed twice with pre-warmed PBS and detached from the surface of the flask by 10 min incubation at 37°C with trypsin-like enzyme (TrypLE Select, Invitrogen, UK). Cells were centrifuged for 5

min at 1200 rpm, pellets resuspended in 5 ml of pre-warmed culture medium and seeded at 1 x 10^5 per ml using a Neubauer chamber.

6.3.3.2 Canine epidermal cell line adherence assay

Canine keratinocytes were subcultured from a confluent flask into 16-well glass slides (Nunc™, Thermo Fisher Scientific, Denmark) at a density of 5 x 10^5 per ml using a Neubauer chamber. The total volume per well was 100 µl of cell culture medium (CnT-09 and supplements). Chamber slides were incubated for 36 h at 37°C with 5% CO_2 and examined for keratinocyte confluence and signs of contamination. Chamber slides were washed twice in pre-warmed bacterial growth media (BHI or GM17, Oxoid, UK). 100 µl of bacterial growth media was added to each well and the cells were incubated at 37°C and 5% CO_2 for 1 h. Stationary phase bacterial cultures (1 ml) were centrifuged at 4000 rpm for 5 min, washed once with PBS and resuspended in the appropriate growth media (BHI or GM17, Oxoid, UK) to a final OD_{600} of 0.5. Exponential phase cultures of *S. pseudintermedius* ED99 and *S. hominis* ATCC 27844 were prepared by transferring 125 µl of overnight culture into 25 ml of fresh BHI medium (Oxoid, UK), and allowing the bacteria to grow to an OD_{600} of 0.5 before adding the bacterial suspension directly to the chamber slides.

For incubation with bacterial cells, 20 µl medium was removed from each well of the chamber slides, followed by the addition of 20 µl of bacterial suspension. Canine keratinocytes were incubated with bacteria for 15 min at 37°C and 5% CO_2. After four washes with PBS, slides were air-dried and stained with modified Wright Giemsa stain (Diff-Quik® Stain, Dade Behring AG, Switzerland). Chambers were allowed to air-dry and the preparation was then mounted with DPX (Sigma-Aldrich, UK) and a cover slip (Scientific Laboratory Supplies, UK).

6.3.3.3 Quantification of adherent bacteria

Chamber slides were analysed by direct visualisation under a light microscope (Leica Laborlux S microscope, Leica Microsystems Ltd., UK). Adherent bacteria per microscopic field (14.4 mm^2) were counted visually and fields were selected randomly by starting in the bottom left corner of each chamber and moving through

the chamber in a defined way using the scale on the microscope stage. If the keratinocyte layer was not confluent or the field could not be properly focused, the field was not counted. The adherence per chamber was determined by acquiring 50 replicates and calculating the median and mean of all replicates.

6.3.4 Statistical analysis

Data were tested for normality by applying the Anderson-Darling Normality Test. Data were normally distributed and adherence to canine corneocytes was therefore analysed by the One-sample T-Test. A P-value of <0.05 was regarded as statistically significant. All data analyses were carried out using Minitab (Minitab Ltd., UK). Box plots, individual value plots, and histograms were created with Minitab (Minitab Ltd., UK), and bar graphs with Microsoft Office Excel (Microsoft Corporation, USA).

6.4 Results

6.4.1 *S. pseudintermedius* ED99 adheres to *ex vivo* canine corneocytes in exponential and stationary phases of growth

Previous adherence studies which employed *S. pseudintermedius* strain ED99 (formerly M732/99) utilised an approximate bacterial concentration of 3 x 10^8 colony-forming units (CFU) per ml, corresponding to an OD_{600} of 0.289 (Forsythe *et al.*, 2002; Simou *et al.*, 2005b; Simou *et al.*, 2005c). To optimise the assay for the current study, adherence of *S. pseudintermedius* strain ED99 at ODs_{600} of 0.289, 0.5, and 1.0 was tested. Based on visual microscopic analysis, an OD_{600} of 0.5 corresponding to an approximate bacterial concentration of 1 x 10^9 cells per ml resulted in optimal adherence levels (data not shown) and was used for all subsequent experiments. Additionally, viable colony counts confirmed that approximate bacterial concentrations of staphylococcal and lactococcal liquid cultures were comparable and correlated to the same spectrophotometric measurements (OD_{600}).

To confirm previous findings that *S. pseudintermedius* ED99 binds to canine corneocytes and to verify that *L. lactis* does not bind to canine corneocytes, *S. pseudintermedius* ED99 and *L. lactis* strain MG1363 with and without the empty pOri23 expression vector were grown to exponential and stationary phases of growth and tested for adherence to canine corneocytes. For microscopic quantification, 10 replicates of each duplicate slide were acquired and visually counted within the set ROI of 500 μm^2, and the mean adherence of a total of 20 replicates was included to create box plots (data not shown). *S. pseudintermedius* ED99 demonstrated adherence to *ex vivo* canine corneocytes in exponential and stationary phases of growth (mean adherence of 12.80 and 38.15 bacterial cells per ROI respectively), and *L. lactis* MG1363 with and without pOri23 did not adhere to the cells (mean adherence ranging from 0.00 to 0.30 bacterial cells per ROI).

6.4.2 The putative MSCRAMMs SpsD and SpsO, but not SpsL, mediate adherence of *L. lactis* to *ex vivo* canine corneocytes

L. lactis expressing SpsD, SpsL, and SpsO were tested for their ability to adhere to *ex vivo* canine corneocytes in comparison to *L. lactis* with the empty vector pOri23 and *S. pseudintermedius* ED99. *L. lactis* carrying the empty vector pOri23 adhered poorly to canine corneocytes (Figure 6.1). For *S. pseudintermedius* ED99, the mean percentage adherence to canine corneocytes was 4.24% which was significantly different to *L. lactis* carrying pOri23 alone (P=0.001) (Figure 6.1). *L. lactis* expressing SpsD and *L. lactis* expressing SpsO adhered to *ex vivo* canine corneocytes (Figure 6.1). The increase in adherence was approaching significance for SpsD (P=0.050), and was significant for SpsO when expressed in *L. lactis* compared to *L. lactis* carrying pOri23 alone (P=0.004). SpsD demonstrated considerable variability in adherence to corneocytes from different dogs (Figure 6.2) and for one animal (dog 3), SpsD-mediated adherence varied considerably between duplicates (Figure 6.2). Binding of *L. lactis* expressing SpsL was not significantly different to *L. lactis* carrying pOri23 alone (P=0.108), indicating that SpsL does not promote adherence to canine corneocytes (Figure 6.1). Representative photomicrographs are shown in Figure 6.3.

6.4.3 Development of a novel assay to examine the adherence of *S. pseudintermedius* ED99 to CPEK cells, a canine epidermal cell line

In order to investigate the interaction of *S. pseudintermedius* ED99 and *L. lactis* expressing SpsD, SpsL, and SpsO with canine keratinocytes *in vitro*, a novel adherence assay was developed using the CPEK epidermal cell line derived from the epidermis of an adult Beagle dog (CELLn-TEC Advanced Cell Systems, Switzerland).

The assay was optimised by testing *S. pseudintermedius* ED99 and *L. lactis* carrying the empty vector pOri23 at varying concentrations (5, 10, 20, and 50 µl of bacterial

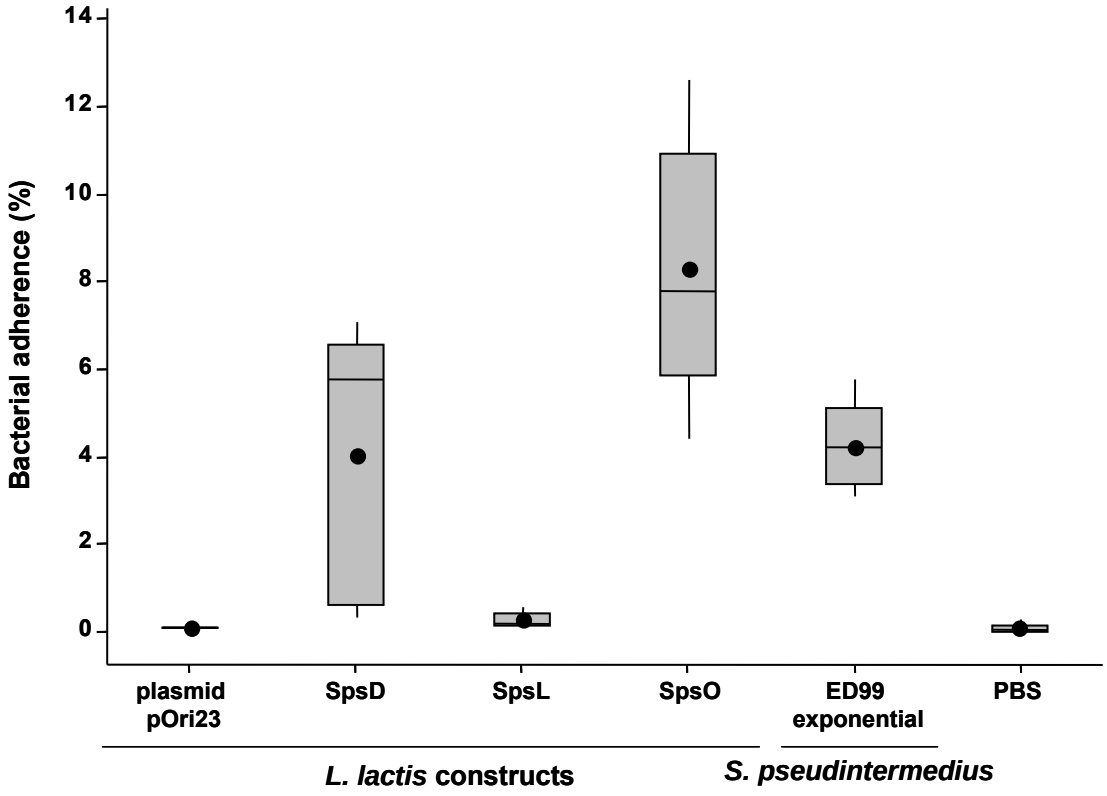


Figure 6.1. Adherence of *S. pseudintermedius* ED99 and *L. lactis* expressing different MSCRAMMs to canine corneocytes of five dogs. Bacterial adherence is calculated as percentage area covered with bacterial cells per field of corneocytes (ROI=500 μm^2). Results are based on the arithmetic mean of duplicate experiments. The bottom of each box represents the first quartile (Q1), the top of the box the third quartile (Q3), the bold lines the median, and the black circles the mean values. The whiskers define the range of the data.

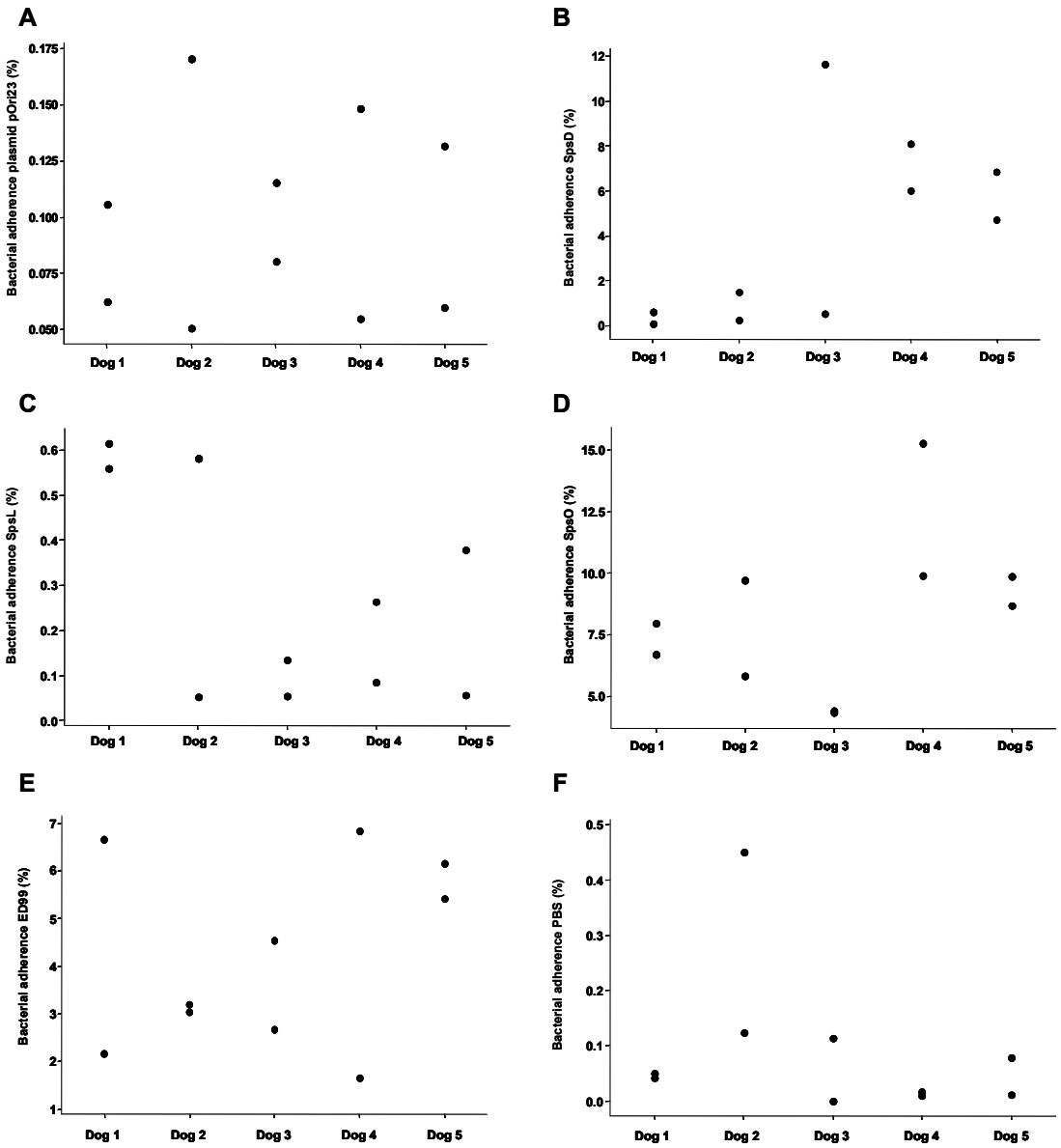


Figure 6.2. Variation in adherence of *S. pseudintermedius* ED99 and *L. lactis* expressing different MSCRAMMs to canine corneocytes of five dogs. Corneocyte adherence assays were carried out in duplicate with 25 replicates for each slide. Results are expressed as individual value plots, the black dots indicate the mean percentage adherence for each duplicate slide. The Y scales of the plots are adjusted to the different value ranges of the results. (A) *L. lactis* carrying pOri23 alone; (B) *L. lactis* expressing SpsD; (C) *L. lactis* expressing SpsL; (D) *L. lactis* expressing SpsO; (E) *S. pseudintermedius* ED99; (F) PBS.

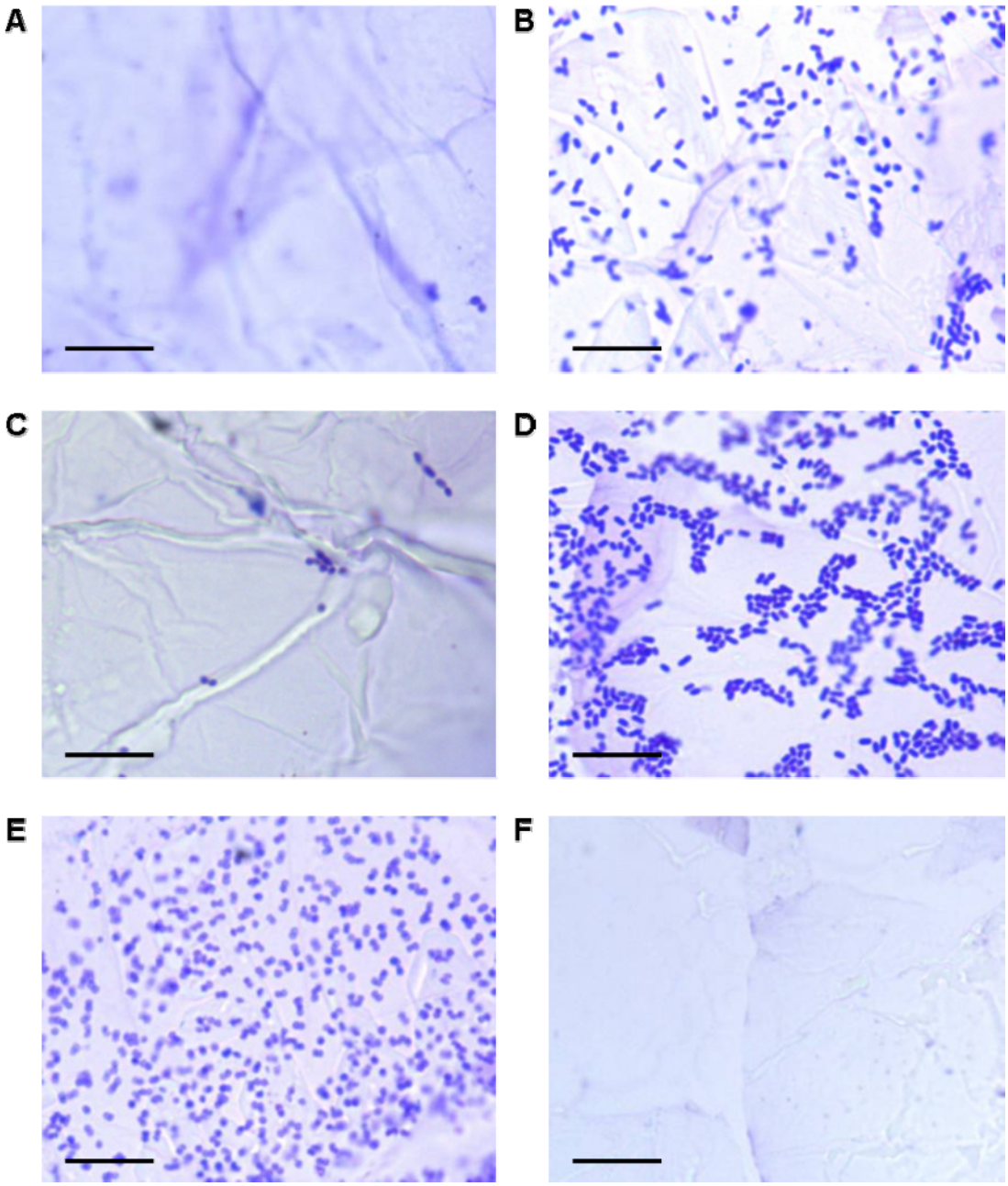


Figure 6.3. Representative photomicrographs of *S. pseudintermedius* ED99 and *L. lactis* constructs adhering to canine corneocytes. (A) *L. lactis* carrying pOri23 alone; (B) *L. lactis* expressing SpsD; (C) *L. lactis* expressing SpsL; (D) *L. lactis* expressing SpsO; (E) *S. pseudintermedius* ED99; (F) PBS. Stained crystal violet. x1000 original magnification. Bar=10 µm.

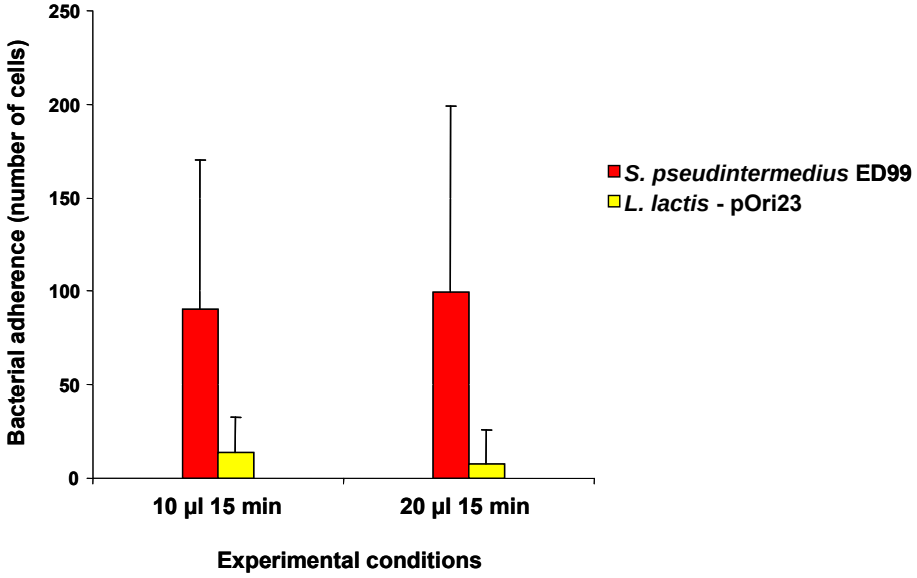
suspension, corresponding to an approximate bacterial concentration of 5 x 10^{10}, 1 x 10^{11}, 2 x 10^{11}, and 5 x 10^{11} CFU per ml), and at different incubation times (15, 30, 60 and 90 min at 37°C) in duplicate, for their adherence to CPEK cells grown in chamber slides. Initially, 10 random microscopic fields were counted for each chamber. To estimate background adherence, i.e. bacteria attaching non-specifically to the plastic surface of the chamber slides, the number of bacteria in 10 random microscopic fields which were at least 75% free of keratinocytes was counted. Incubation with 10 µl and 20 µl of bacterial suspension for 15 min resulted in low background (<20 bacteria per field). The mean adherence of *S. pseudintermedius* ED99 was 19.6 bacterial cells (median=16) for incubation with 10 µl bacterial suspension and 31.4 bacterial cells (median=26) for incubation with 20 µl bacterial suspension compared to mean adherence of 0.8 bacterial cells for incubation with 10 µl bacterial suspension and 2.5 bacterial cells for incubation with 20 µl bacterial suspension (median=0 for 10 µl and 20 µl, respectively) for *L. lactis* carrying pOri23 alone.

To determine the number of replicates required per chamber for statistical analysis and to decide on final experimental conditions, 60 replicates were counted for *S. pseudintermedius* ED99 and *L. lactis* carrying pOri23 alone for chambers with low background (Figure 6.4A). Based on the results, incubation with 20 µl of bacterial suspension for 15 min was selected for further experiments (Figure 6.4A). Stable results were obtained for 50 replicates or more, but the assay remained variable, independent of the number of replicates (high standard deviation, Figure 6.4B). For subsequent experiments, 50 replicates were counted for each duplicate chamber.

6.4.4 *S. pseudintermedius* ED99 adheres to canine keratinocyte cell culture in exponential and stationary phases of growth

The capacity of *S. pseudintermedius* ED99 and *S. hominis* ATCC 27844 to adhere to cultured canine keratinocytes was investigated for bacteria grown to exponential (OD600 of 0.5) and stationary phases of growth. *S. hominis* was employed as a negative control as a previous study indicated it binds poorly to canine corneocytes (Woolley *et al.*, 2008).

A



B

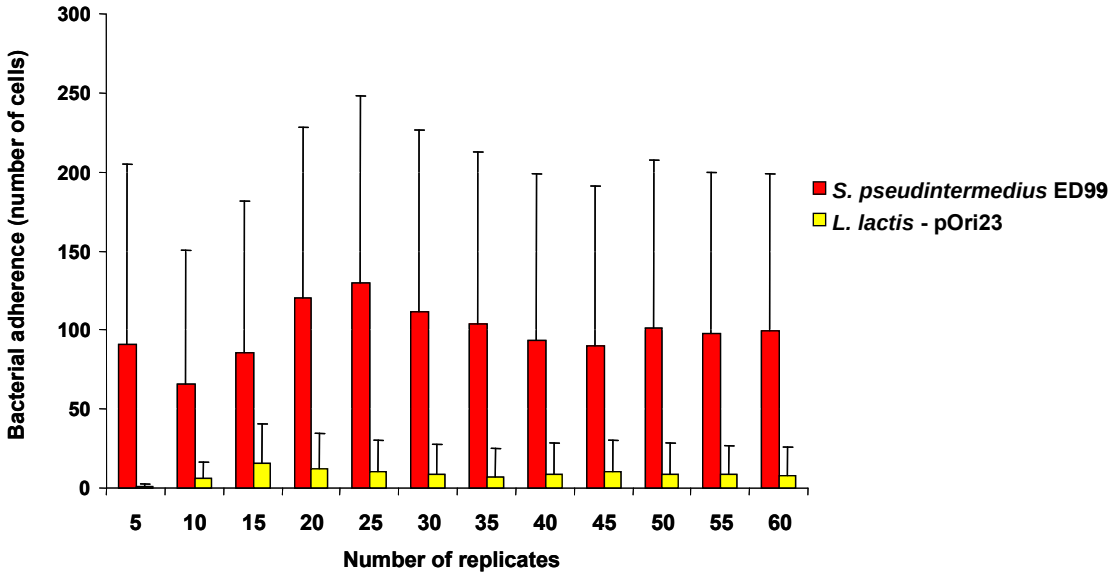


Figure 6.4. Adherence of *S. pseudintermedius* ED99 and *L. lactis* carrying pOri23 alone to CPEK cells. Columns represent the arithmetic mean (ROI=14.4 mm^2 per replicate); error bars indicate standard deviation. (A) comparison of different experimental conditions based on 60 replicates; (B) variation of results depending on the number of replicates for assay with 20 µl of bacterial suspension and 15 min incubation time.

S. pseudintermedius ED99 adhered to CPEK cells in exponential and stationary phases of growth to a similar degree, whereas *S. hominis* adhered poorly (Figure 6.5). Statistical analysis to generate *P*-values was not applied for this part of the study, as the sample size was one (cell culture) with 50 random replicates acquired from the same chamber, and the data distribution was found to be best represented by histograms (Figure 6.5). Representative photomicrographs are shown in Figure 6.6.

6.4.5 *L. lactis* constructs expressing selected MSCRAMMs lack the ability to adhere to canine keratinocyte cell culture

L. lactis expressing SpsD, SpsL, and SpsO were tested for their ability to adhere to cultured canine keratinocytes in comparison to *L. lactis* with the vector pOri23 alone. *S. pseudintermedius* ED99 grown to exponential phase of growth was included as a positive control.

L. lactis carrying the empty vector pOri23 adhered poorly to CPEK cells with a median value of 2.00 bacterial cells for 50 replicates in comparison to a median value of 69.50 for *S. pseudintermedius* ED99 (Figure 6.7).

The degree of adherence to CPEK cells for *L. lactis* expressing SpsD, SpsL, and SpsO was not considerably increased compared to *L. lactis* carrying pOri23 alone (Figure 6.7). The median values of 50 replicates were 8.00 bacterial cells for SpsD, 13.00 for SpsL, and 10.00 for SpsO, respectively, indicating that host cell factors required for adherence of *L. lactis* expressing SpsD and *L. lactis* expressing SpsO are not present in undifferentiated cultured canine keratinocytes. For the same reasons as mentioned in section 6.4.4, statistical analysis to generate *P*-values was not applied for this part of the study and histograms of the data are shown in Figure 6.7. Representative photomicrographs are shown in Figure 6.8.

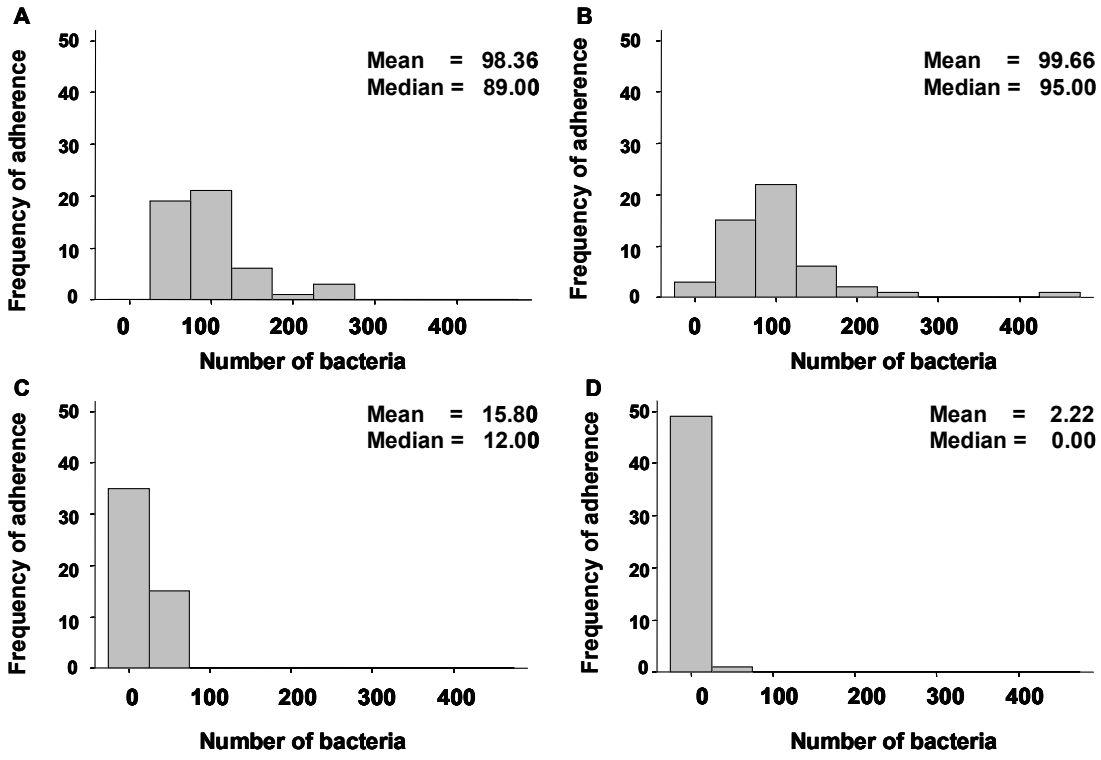


Figure 6.5. Adherence of *S. pseudintermedius* ED99 and *S. hominis* ATCC 27844 to cultured canine keratinocytes. Bacteria were grown to exponential and stationary phases of growth and 20 µl of bacterial suspension was added to CPEK cells in chamber slides for 15 min at 37°C. Bacteria were counted visually with 50 replicates per slide (ROI=14.4 mm²). Mean and median values are shown with each histogram. (A) *S. pseudintermedius* ED99, exponential phase of growth; (B) *S. pseudintermedius* ED99, stationary phase of growth; (C) *S. hominis* ATCC 27844, exponential phase of growth; (D) *S. hominis* ATCC 27844, stationary phase of growth. The experiment was done in duplicate with similar results (data not shown).

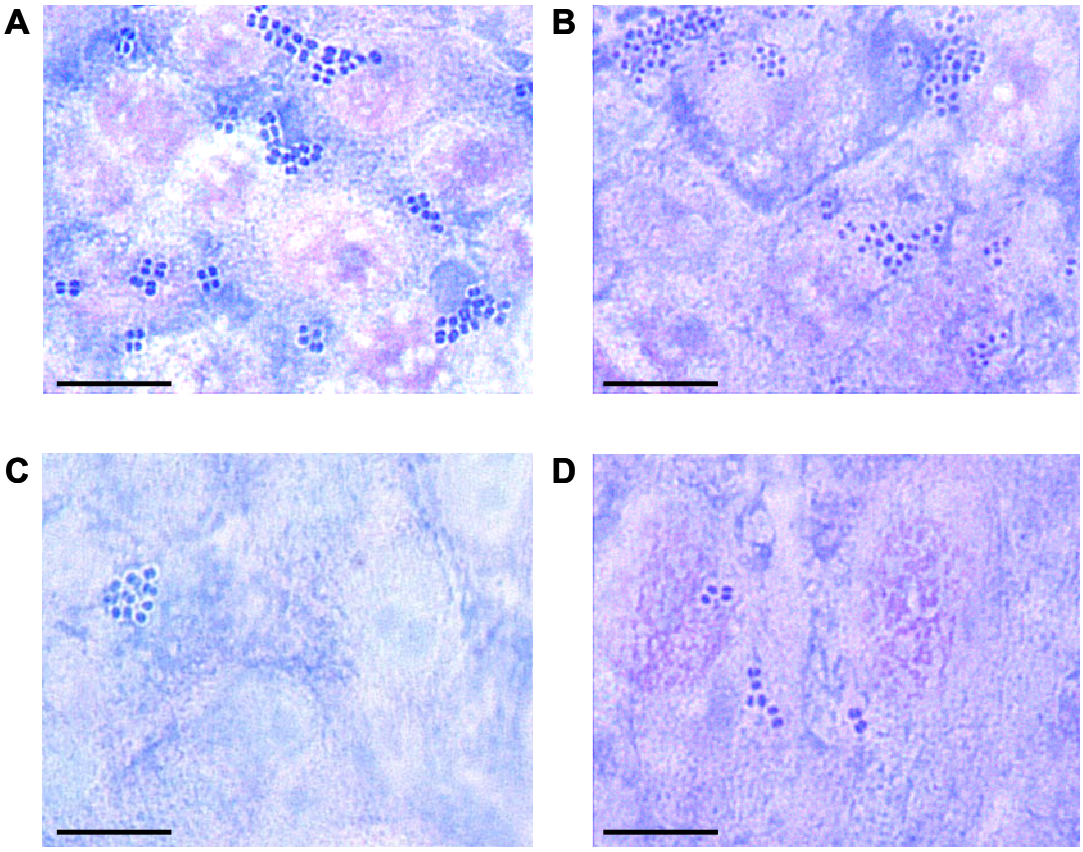


Figure 6.6. Representative photomicrographs showing adherence of *S. pseudintermedius* ED99 and *S. hominis* ATCC 27844 to cultured canine keratinocytes. (A) *S. pseudintermedius* ED99, exponential phase of growth; (B) *S. pseudintermedius* ED99, stationary phase of growth; (C) *S. hominis* ATCC 27844, exponential phase of growth; (D) *S. hominis* ATCC 27844, stationary phase of growth. Diff-Quik stain. x1000 original magnification. Bar=10 µm.

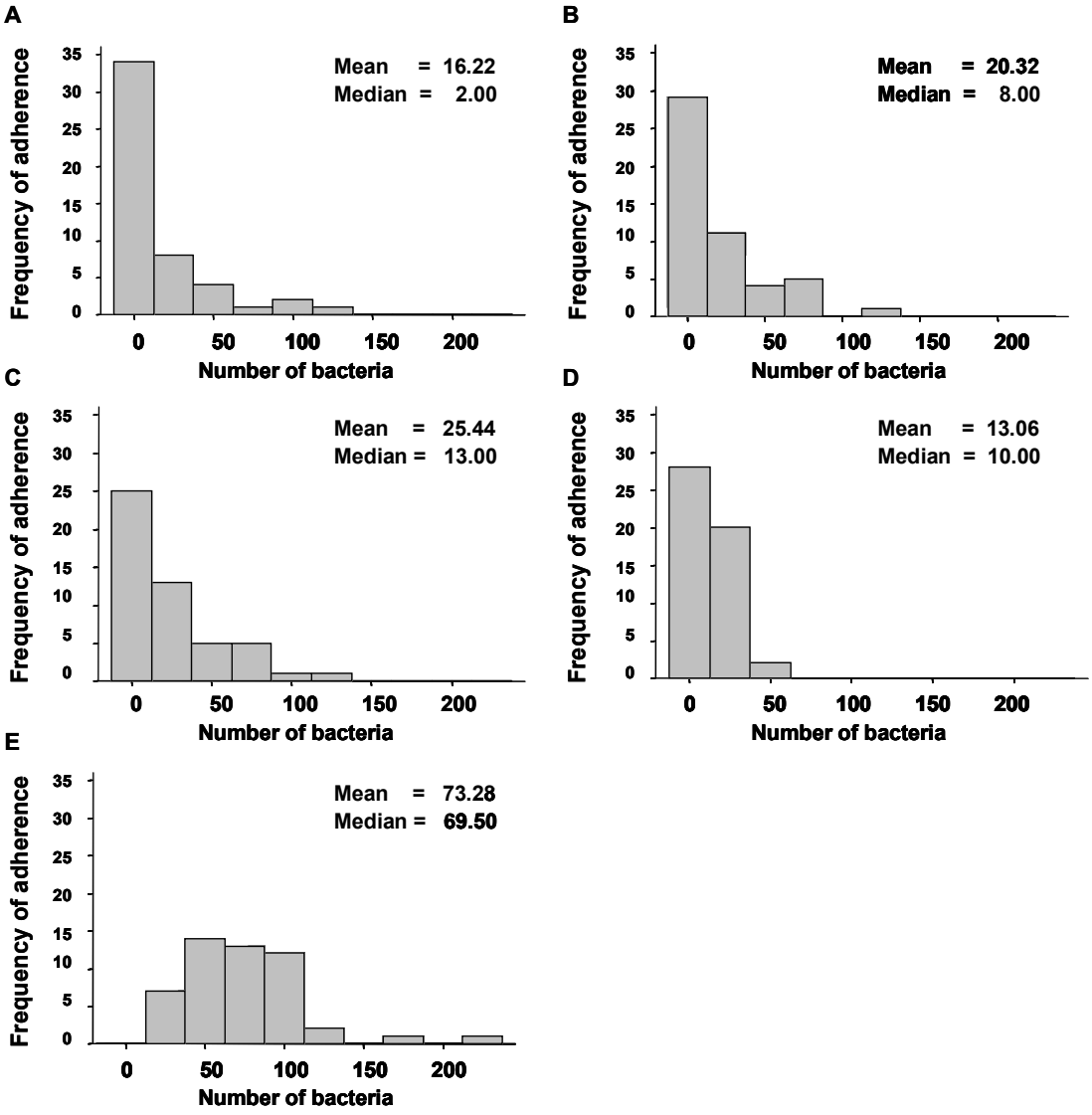


Figure 6.7. Adherence of *L. lactis* expressing individual MSCRAMMs to cultured canine keratinocytes. 20 µl of bacterial suspension was added to CPEK cells in chamber slides for 15 min at 37°C. Bacteria were counted visually with 50 replicates per slide (ROI=14.4 mm²). Mean and median values are shown with each histogram. (A) *L. lactis* carrying pOri23 alone; (B) *L. lactis* expressing SpsD; (C) *L. lactis* expressing SpsL; (D) *L. lactis* expressing SpsO; (E) *S. pseudintermedius* ED99. The experiment was done in duplicate with similar results (data not shown).

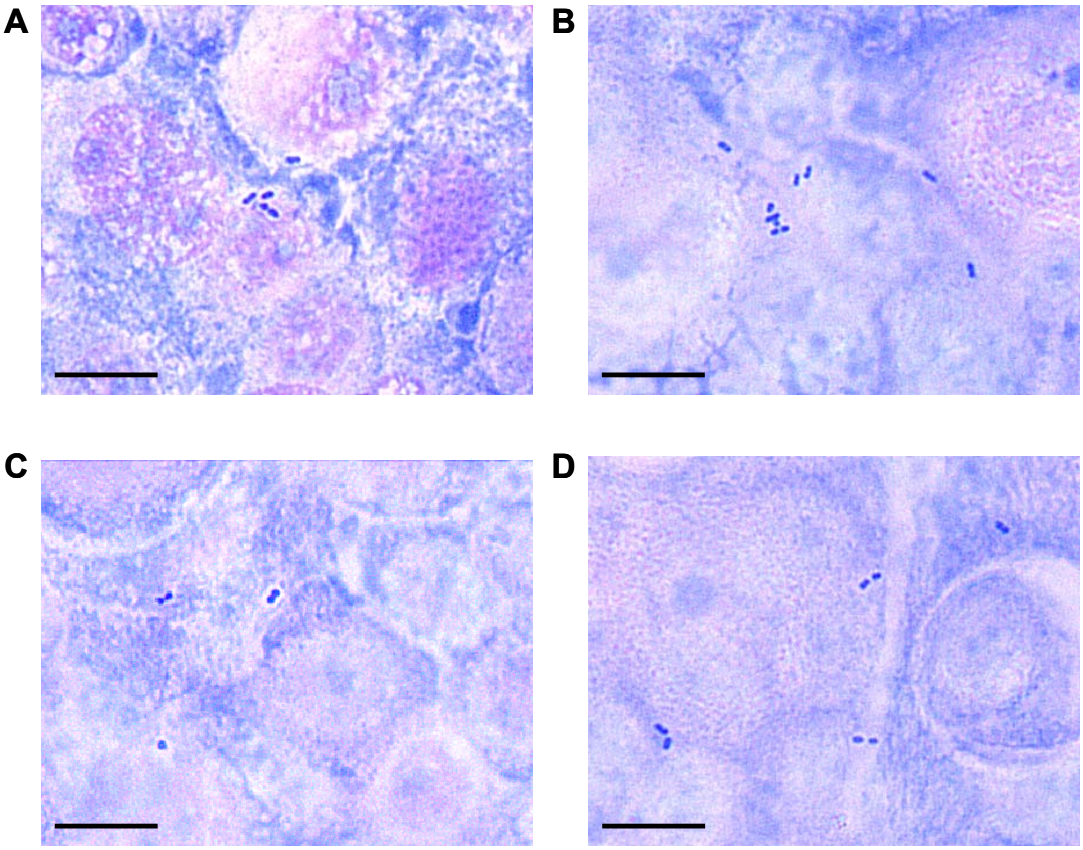


Figure 6.8. Representative photomicrographs showing adherence of *L. lactis* expressing individual MSCRAMMs to cultured canine keratinocytes. (A) *L. lactis* carrying pOri23 alone; (B) *L. lactis* expressing SpsD; (C) *L. lactis* expressing SpsL; (D) *L. lactis* expressing SpsO. Diff-Quik stain. x1000 original magnification. Bar=10 μm.

6.5 Discussion

S. pseudintermedius adherence to canine corneocytes has been demonstrated in numerous studies (McEwan, 2000; Forsythe *et al.*, 2002; Saijonmaa-Koulumies and Lloyd, 2002a; McEwan *et al.*, 2005; Simou *et al.*, 2005b; Simou *et al.*, 2005c; McEwan *et al.*, 2006a; Lu and McEwan, 2007; Woolley *et al.*, 2008), but the mechanism of adherence has not yet been determined. In a previous study, digestion of *S. pseudintermedius* with different concentrations of trypsin, a broad-spectrum proteinase, resulted in a dose-dependent loss of staphylococcal adherence to corneocytes, suggesting a protein-protein interaction between the bacteria and the host cell (Simou *et al.*, 2005a).

In the current study, two putative MSCRAMMs of *S. pseudintermedius*, SpsD and SpsO, mediated adherence to canine corneocytes when expressed on the surface of *L. lactis*. The SpsO-mediated percentage adherence of *L. lactis* was consistently high for all corneocyte slides, indicating that SpsO-mediated interaction occured with host cell receptors which were present on the corneocytes from all dogs included in the study. However, the specific host cell receptor for the SpsO-mediated interaction has not yet been identified. ClfB of *S. aureus* promotes adhesion to nasal epithelial cells via binding to CK10 (O'Brien *et al.*, 2002b; Walsh *et al.*, 2004; Wertheim *et al.*, 2008; Corrigan *et al.*, 2009). SpsO in the current study did not bind to CK10 (Chapter 5) indicating that a different receptor is involved in the interaction with canine corneocytes such as loricrin or involucrin. Recently, IsdA of *S. aureus* has been demonstrated to interact with these two components of the cornified envelope (Clarke *et al.*, 2009). It is also possible that SpsO interacts with a novel receptor not implicated previously in staphylococcal adherence to skin cells. Future studies are required to investigate the SpsO-corneocyte interaction further in order to identify the host receptor and SpsO binding site involved.

The second putative MSCRAMM mediating adherence of *L. lactis* to *ex vivo* canine corneocytes, SpsD, demonstrated considerable variation in adherence compared to SpsO. These results suggest that SpsD may enhance adherence to some dogs, and not to others, perhaps due to variation in availability of host cell receptors in different

animals. This possibility has been discussed in relation to *S. aureus* adherence to human epithelial cells, and it has been suggested that the expression level of CK10 may differ between human corneocyte donors, resulting in adherence variation (Corrigan *et al.*, 2009). Further studies are required to elucidate the role of SpsD in binding to canine corneocytes. In particular, the number of dogs and range of breeds sampled would have to be increased to draw conclusions about potential host factors such as breed which may influence the adherence of *L. lactis* expressing SpsD.

The corneocyte adherence assay in combination with computerised image analysis chosen for the current study was optimised and validated previously in our laboratories (Forsythe *et al.*, 2002). The approach is objective, fast, and less laborious compared with visual counting techniques. The images acquired can be stored for later analysis or reference and the software is user-friendly, but the results depend on a clear contrast between the bacterial cell and the background host cell. With the staining technique used here, the contrast between the rich-stained bacterial cells and the poorly stained corneocytes was well-defined. In contrast, the computerised image analysis software was not suitable for calculation of bacterial adherence percentage to the canine epidermal cell line (CPEK), because the keratinocyte cells were stained to a much greater extent than the corneocytes, precluding computer-based differentiation of bacteria. In keratinocytes, staining of the nuclei was an additional problem for which the software was not designed. Therefore, the more laborious approach of visual counting was chosen for the analysis of bacterial adherence to CPEK cells.

A considerable degree of variation was observed in both adherence assays employed, resulting in high standard deviation which could not be reduced by increasing the number of replicates. This phenomenon was observed in previous staphylococcal adherence studies, and it was reported that the number of bacterial cells adhering to individual corneocytes can differ markedly (Woolley *et al.*, 2008). Additionally, the adherence of *S. aureus* to human nasal epithelial cells of healthy volunteers varies considerably, depending on the donor (Weidenmaier *et al.*, 2008).

There are a number of limitations to the *ex vivo* corneocyte adherence assay employed in the current study. For example, the corneocyte sampling technique using double-sided adhesive tape exposes the lower surface of the corneocyte for interaction with the bacteria. Potential differences in bacterial adhesion between the upper and lower surfaces of canine corneocytes have not yet been investigated. Further, the approach depends on the availability of canine cell donors and the sampling of live animals may contribute to the variability of the assay. In the novel cell culture adherence assay developed and tested in the current study, the upper side of the keratinocyte is exposed to the environment, consistent with the situation *in vivo*. Further, using a cell line for the study of bacterial adherence eliminates host-dependent variability and provides an unlimited supply of host cells. In addition, keratinocyte culture allows the investigation of host response, e.g. by examining the stimulation of the immune system by *S. pseudintermedius* binding. However, a limitation of the assay is the lack of differentiation of the keratinocyte cell line used. Studies have shown that CPEK cells resemble keratinocytes of the basal epidermal layers including the expression of CK14 rather than CK10 (Shibata *et al.*, 2008). In the present study, the *S. pseudintermedius* strain ED99 adhered to CPEK cells, indicating that receptors for *S. pseudintermedius* adherence are present on basal keratinocytes. However, the putative MSCRAMMs SpsD and SpsO, which mediated adherence to *ex vivo* canine corneocytes, did not promote adherence to CPEK cells, suggesting that the factors required for adherence were not present. Adherence of *S. aureus* to human nasal epithelial cells involves multiple bacterial components (Weidenmaier *et al.*, 2008; Corrigan *et al.*, 2009). Similarly, the present study indicates that *S. pseudintermedius* adherence to canine epithelial cells involves several bacterial components, including SpsD, SpsO, and one or more unknown factors. Weidenmaier *et al.* (2008) investigated binding of *S. aureus* strain SA113 to cultured primary human nasal epithelial cells (HNECs) and to *ex vivo* HNECs obtained from nasal swabs, and reported *S. aureus* binding to differentiated and non-differentiated HNECs. Further, it has been speculated that *S. aureus* interaction with differentiated nasal epithelial cells may be crucial for initial adherence whereas less differentiated cells may be involved in persistent colonisation (Weidenmaier *et al.*, 2008). Subsequent studies are required to investigate the potential of the CPEK cell

line for studying bacterial adherence. Of potential importance, CPEK cells have been reported to differentiate after 18 days of continuous growth with frequent media changes, monitored by the increase of mRNA expression of envoplakin, a precursor protein of the cornified envelope (Yagihara *et al.*, 2009).

In summary, the current study resulted in the identification of two CWA proteins, SpsD and SpsO, which mediate binding to canine corneocytes, and could be described as putative *S. pseudintermedius* colonisation factors.

Chapter 7

General Discussion

Canine bacterial skin infection is frequently seen in veterinary practice, and for over three decades, *S. intermedius* was considered to be the most common causative pathogen (Scott *et al.*, 2001a). However, the species type strain was a pigeon isolate (Hajek, 1976), and considerable phenotypic and genotypic variation was noted within the species (Hajek, 1976; Hesselbarth and Schwarz, 1995; Chesneau *et al.*, 2000; Aarestrup, 2001; Bes *et al.*, 2002). The current study proposes a taxonomical re-classification of isolates previously identified as *S. intermedius* into three distinct species, including *S. intermedius*, *S. pseudintermedius*, and *S. delphini*, which are together referred to as the SIG. Further, the present study identifies *S. pseudintermedius* as the most common causative agent of canine pyoderma. The results presented here are consistent with the population structure identified concurrently among Japanese isolates by Sasaki *et al.* (2007b). With hindsight, the existence of two different species, specific for the pigeon and canine host, respectively, was consistent with the findings of several previous studies. For example, Futagawa-Saito *et al.* (2006) screened canine and pigeon isolates phenotypically identified as *S. intermedius* for selected virulence factors and did not detect clumping factor or Protein A in pigeon isolates. In contrast, over 24 (54.5%) of the canine isolates were positive for both, indicating a difference in the repertoire of virulence factors depending on host species. Further, Lautz *et al.* (2006) applied a previously developed multiplex PCR assay based on the thermonuclease (*nuc)* and 16s rRNA genes (Baron *et al.*, 2004) to investigate the diversity of clinical isolates of canine origin. Of the 49 canine isolates, 45 were identified as *S. intermedius* and the remaining 4 as *S. aureus* (Lautz *et al.*, 2006). However, the 4 *S. pseudintermedius* strains described originally by Devriese *et al.* (2005) were included, and resulted in PCR amplification of the *nuc* gene, indicating the close relatedness of *S. pseudintermedius* strains to the isolates identified as *S. intermedius*. Since the re-classification of the SIG, it has been proposed to describe all canine isolates as *S. pseudintermedius*, unless proven otherwise by genetic typing methods (Devriese *et al.*, 2009).

Epidemiological surveillance of the *S. pseudintermedius* population has become increasingly important with regard to antimicrobial resistance, including methicillin

resistance which has become a major problem in veterinary medicine worldwide (Werckenthin *et al.*, 2001; Guardabassi *et al.*, 2004a; Kania *et al.*, 2004; Ganiere *et al.*, 2005; Morris *et al.*, 2006b; Futagawa-Saito *et al.*, 2007; Jones *et al.*, 2007; Lloyd, 2007; Loeffler *et al.*, 2007; Sasaki *et al.*, 2007a; Weese and van Duijkeren, 2009). For example, a recent study among clinical isolates in Germany identified 72 MRSP isolates among 870 SIG isolates from dogs, cats, horses, and a donkey (Ruscher *et al.*, 2009), and three cases of feline urinary tract infection with MRSP were reported in Switzerland (Wettstein *et al.*, 2008). Further, a recent study screened 193 dogs which were presented to a Canadian veterinary hospital for reasons unrelated to staphylococcal infection by nasal, axillary, and rectal swabs, and identified 4 dogs as MRSP carriers (Hanselman *et al.*, 2008). Similar, *S. pseudintermedius* was isolated from 57 dogs in a Japanese veterinary hospital and 17 isolates (30%) were identified as MRSP (Sasaki *et al.*, 2007a). These studies highlight the increasing prevalence of antimicrobial resistance among *S. pseudintermedius* strains. However, the MRSP surveillance and screening methods in place to date are not satisfactory. For example, the identification guidelines for MRSP according to the Clinical and Laboratory Standards Institute (CLSI), published in 2008, were reported to lack sensitivity for the detection of MRSP (Schissler *et al.*, 2009), and a nationwide monitoring programme of bacterial resistance in companion animals in Germany, introduced by the German Federation for Animal Health in 2003, currently does not include *S. pseudintermedius* (Schwarz *et al.*, 2008). The identification of the common methicillin-resistant clones in the current study provides a framework for monitoring MRSP clonal dissemination, and to develop strategies for the control of MRSP infections. The data from the current study indicates two predominant MRSP lineages, including ST68 in the USA, and ST71 from Northern Europe. Moodley *et al.* (2009) discovered that all four ST68 isolates from the USA employed in their study belonged to the same *spa* type, whereas the eight ST71 Northern European isolates were distinguished into four closely related, and one singleton *spa* type (Moodley *et al.*, 2009).

Moodley *et al.* (2009) developed a *spa* typing method, based on *S. pseudintermedius* ED99 *spsQ* sequence, for monitoring MRSP dissemination. The authors tested their

spa typing approach in comparison to PFGE and SCC*mec* typing with 31 MRSP isolates, including 15 isolates belonging to STs 29, 68, 69, 70, and 71 as identified in the current study (Moodley *et al.*, 2009). Four of the 15 isolates (3279, Can6, Can10, HH1) represented major genotypes and were investigated further for the presence of the putative MSCRAMM-encoding genes identified in the present study, including the *spa* orthologues *spsP* and *spsQ*. All four strains were positive for *spsQ* by PCR screening and the data are consistent with the findings of Moodley *et al.* (2009). Overall, 55% (11/20) and 35% (7/20) of the strains tested in the present study were positive for *spsP* and *spsQ,* respectively. Moodley *et al.* (2009) investigated the presence and allelic variation of tandem repeats in the variable region X of *spsQ* and described 100% typeability. The current study uses upstream- and N-terminal-encoding sequences rather than the region X for gene detection. The differences in typeability by Moodley *et al.* (2009) and in the current study may be explained by different primer locations and sequence variation in the primer annealing site used in this study. Of note, Moodley *et al.* (2009) described a much lower typeability rate (58%) when methicillin-susceptible isolates were tested and hypothesised that *spsQ* might not be present in all *S. pseudintermedius* isolates and allelic variation might prevent detection by PCR when a single primer set is used. The distribution of *spsP* and *spsQ* needs to be investigated further to reveal sequence variation and to establish if some isolates are truly lacking these genes.

The multilocus sequence approach developed in the current study is proposed as a useful tool for species differentiation and identification of clonal lineages whereas other methods such as *spa* typing or an extended MLST typing scheme may be more appropriate for fine-structure analysis of the *S. pseudintermedius* population. Black *et al.* (2009) employed the multilocus sequence approach developed in the current study to 60 clinical isolates of canine origin in the USA and all isolates were identified as *S. pseudintermedius*. Further, 38 of the isolates were classified as MRSP with 37 (97.4%) belonging to ST68 (Black *et al.*, 2009), supporting the predominant role of this clonal lineage in the USA. Another 15 novel ST types, which were not identified in the present study, were described among methicillin-susceptible *S. pseudintermedius* isolates (MSSP) (Black *et al.*, 2009), indicating the clonal diversity

of MSSP. It will be of critical importance to monitor the spread of the common MRSP clones in the near future, but it will be equally important to carry out surveillance to ensure that newly emerging MRSP clones are identified rapidly and included in strategies for disease control.

The identification and analysis of clonal lineages is central to bacterial evolutionary and epidemiological research, but the definition of a clonal lineage is not uniform. Some authors refer to CCs determined by eBURST analysis as lineages ('genetic lineages') (Serrano *et al.*, 2005; Lindsay *et al.*, 2006; Moodley *et al.*, 2006; Miragaia *et al.*, 2007), whereas others define STs based on MLST analysis as lineages, sometimes in combination with other typing methods such as SCC*mec* typing (e.g. 'MRSP lineage') (Strommenger *et al.*, 2006; Faria *et al.*, 2008; Ruscher *et al.*, 2009). The current study identifies several *S. pseudintermedius* lineages using multilocus sequence analysis and assignment of STs. Further, eBURST analysis is employed to establish the relatedness between different STs. The eBURST algorithm divides ST data sets into non-overlapping clusters of related genotypes (Feil *et al.*, 2004). If eBURST is applied with default settings (most exclusive settings), the resulting groups are equal to CCs which are 'biologically meaningful cluster of STs that have diversified very recently from a common founder' (Feil *et al.*, 2004). If the settings are user-defined, eBURST places STs together according to manually selected group definition which is less powerful than the standard settings (Feil *et al.*, 2004). The current study uses a manually selected group definition at the most stringent setting possible (four of the five gene loci need to be identical with at least one other ST in the same group). The 61 STs are divided by eBURST into one major group comprising 47 STs, three minor groups including 2 STs each, and 8 singletons (see Figure 3.4, page 103). A single group based on ST20 contains the majority of STs (77%), indicating that one large genetic lineage is predominant among the *S. pseudintermedius* population. However, the resolution is limited using only five loci. A similar distribution pattern has been recognised among *S. epidermidis* isolates analysed using the eBURST algorithm (Miragaia *et al.*, 2007). The authors explain the existence of one major group by a relatively high recombination rate in the *S. epidermidis* population (Miragaia *et al.*, 2007). Interestingly, the eBURST algorithm

seems to become less reliable with an increased recombination to mutation ratio in a population (Turner *et al.*, 2007). Based on the current study, the recombination rate in the *S. pseudintermedius* population is low and therefore should not interfere with eBURST reliability. However, ST assignment indicates remarkable clonal diversity among *S. pseudintermedius* isolates resulting in identification of 61 STs. Further, the *agr* locus, which is most likely acquired by assortive recombination, is included in the ST allocation. The existence of 61 STs may suggest that very diverse STs exist among the *S. pseudintermedius* population sharing common alleles which are clustered together by eBURST. In summary, the vast majority of *S. pseudintermedius* isolates investigated in the current study cluster as one single genetic lineage when eBURST analysis is applied, but divide into several clonal lineages based on multilocus sequence analysis.

The *agr* locus is a global regulator of virulence found in all *Staphylococcus* species examined to date including *S. pseudintermedius* (Lyon and Novick, 2004; Sung *et al.*, 2006). The current study identified four predicted AIP variants among the SIG including *agr* type IV which had not been described previously. The AIP encoded among the SIG consists of a cyclic lactone rather than the thiolactone ring structure identified among other staphylococci. Novick and Geisinger (2008) hypothesise that the single nucleotide polymorphism leading to a serine-containing AIP unique to the SIG occurred after the divergence of carnivores from other mammals 55 million years ago, suggesting an evolutionary host adaptation event which happened prior to the divergence of different AIP subtypes among the SIG. In the present study, the *agr* types did not correlate with host, disease, or geographic origin. The *agr* system of *S. pseudintermedius* requires further investigation in relation to the regulation of virulence, interference between different *agr* types, and the possible disease association of different *agr* types. In *S. aureus*, specific *agr* types have demonstrated an association with certain human diseases, such as SSSS and TSS (Novick, 2003). In addition, *S. aureus agr* type IV is prevalent among highly virulent strains spreading in rabbitries, and *agr* type I can be associated with bovine intramammary infections, and possibly with penicillin resistance (Boyen *et al.*, 2009).

The discrimination of *S. pseudintermedius* from *S. intermedius* and *S. delphini* with biochemical tests is difficult (Sasaki *et al.*, 2007b; Devriese *et al.*, 2009). Therefore, a simple PCR-RFLP approach was developed in the current study. The technique was further tested by independent investigators in a diagnostic laboratory in Italy on a panel of 112 coagulase-positive staphylococci isolated from dogs, cats, cows, horses, wild foxes, and a captive bear (Baron *et al.*, 2004). In addition to dogs, *S. pseudintermedius* was also identified among cats, wild foxes, and a captive bear, indicating that *S. pseudintermedius* may be the most common coagulase-positive species associated with these animals (Bannoehr *et al.*, 2009). Further, two out of seven bovine isolates were identified as *S. pseudintermedius*, expanding its known host repertoire to cows (Bannoehr *et al.*, 2009). This simple diagnostic test should be useful for routine identification of *S. pseudintermedius* in veterinary and human clinical diagnostic laboratories.

In addition to canine pyoderma, *S. pseudintermedius* is also frequently isolated from canine ear infections (Oliveira *et al.*, 2008), is a frequent pathogen of wound infections in dogs (Ruscher *et al.*, 2009), and has been identified as the causative agent of a fatal case of canine necrotising fasciitis (Weese *et al.*, 2009). Furthermore, in 2006, the first case of human infection with *S. pseudintermedius* was reported which was isolated from a cardiac device related infection (Van Hoovels *et al.*, 2006). In spite of its wide clinical importance, the pathogenesis of *S. pseudintermedius* is poorly understood. The population genetic structure determined in the current study provides an excellent framework for investigations into the molecular basis of *S. pseudintermedius* pathogenesis. Importantly, strain ED99 was selected for genome sequencing and phenotypic characterisation, because it represents one of the most common widespread clones identified in the present study. Up to now, the cell wall-associated factors involved in adherence to corneocytes or ECM proteins had not been identified. Of the 17 putative CWA proteins encoded in the genome of *S. pseudintermedius* strain ED99, the current project focused on the characterisation of 3 which included SpsD, SpsL, and SpsO. The full-length *spsD*, *spsL*, and *spsO* genes were cloned into *L. lactis*. The constructs were verified by DNA sequencing and expressed on the surface of *L. lactis*. Ideally, the presence of

the Sps-proteins on the lactococcal surface would be demonstrated using specific laboratory methods. For example, Arrecubieta *et al.* (2007) employed whole cell FITC-labelling and flow cytometry with specific antibodies to detect staphylococcal protein expression on the lactococcal surface. The actual presence of SpsD, SpsL, and SpsO could not be demonstrated in the current study due to time restrictions, but Western blot analysis resulted in additional bands for SpsD and SpsL compared to the negative control. Furthermore, specific phenotypes were demonstrated for each of the *L. lactis* constructs, with the only difference between them being the presence or absence of the *sps* genes.

L. lactis expressing SpsD adhered strongly to all ECM proteins tested in this study (Fg, Fn, CK10), and SpsD mediated binding of *L. lactis* to Fg of human, canine, feline, and bovine origin and to Fn of human and canine source, in a non host-specific manner. The detection of *spsD* by Southern blot analysis in all members of the SIG suggests a conserved function, and is consistent with its binding to ligands from several different hosts. The binding of SpsD to multiple ligands indicates that it may belong to a growing list of 'multi ligand binding' MSCRAMMs, which includes proteins such as FnbpA of *S. aureus* (Greene *et al.*, 1995; Wann *et al.*, 2000; Roche *et al.*, 2004; Keane *et al.*, 2007a). SpsD demonstrated binding to *ex vivo* corneocytes from different dogs to varying degrees which may be explained by inconsistency in availability of host cell receptors in the canine host. Staphylococcal adherence has been shown to vary noticeably between healthy human donors (Weidenmaier *et al.*, 2008; Corrigan *et al.*, 2009), suggesting differences in host cell receptors. *L. lactis* expressing SpsD binds to CK10 which is expressed by differentiated keratinocytes in the upper layers of the epidermis (Suter *et al.*, 1997). It has been suggested that the expression level of CK10 may differ between human corneocyte donors, resulting in adherence variation (Corrigan *et al.*, 2009), and the same may apply to canine corneocyte donors. SpsD mediates binding to the plasma proteins Fg and Fn which may have been more readily available on some corneocyte slides, for example due to microlesions caused by the sampling procedure (particularly clipping). Further, staphylococcal adherence appears to be increased to corneocytes of certain dog breeds (Forsythe *et al.*, 2002) and there might be a breed-related element in SpsD-

mediated adherence to canine corneocytes. Biophysical canine skin parameters such as pH, hydration, and sebum production are breed- and gender-related (Young *et al.*, 2002) and affect the cutaneous protective barrier and may therefore influence SpsD-mediated bacterial adherence. Host factors such as innate / adaptive immunity and local microenvironment may play a role in SpsD-mediated adherence. One important defence mechanism of mammalian skin is the presence of antimicrobial peptides, e.g. cathelicidins and defensins which are synthesised by keratinocytes and can inhibit microbial growth (Braff *et al.*, 2005). Wingate *et al.* (2009) screened the canine genome for genes encoding putative antimicrobial peptides and detected 65 genes. The authors demonstrated the expression of two defensins and two protease inhibitors in the skin of normal dogs at various body sites including the ventral abdomen and inner thigh (Wingate *et al.*, 2009). It may be speculated that variation in the expression profile or quantity of antimicrobial peptides in the skin of different dogs or breeds influences SpsD-binding. However, sample size is one of the main limitations of the current study and further investigation of the influence of host factors in SpsD-mediated adherence is necessary, commencing with the investigation of the adherence pattern of *L. lactis* expressing SpsD in a corneocyte adherence assay with a larger sample size. For corneocytes of one animal, SpsD-mediated adherence varied considerably between duplicates. Remarkable variation of the number of bacteria binding to individual corneocytes of the same donor has been noted previously (Wooley *et al.*, 2008), indicating that host cell factors influencing bacterial adherence are not consistent even within the same animal. The variability within the same animal may be explained by a higher number of antimicrobial factors on some slides which could be caused by stress-induced increase of sweat production during the course of corneocyte sampling. In humans, sweat glands primarily serve thermoregulation by sweat production, but have an additional important role in local antimicrobial defence by expressing proteins such as dermcidin and cathelicidin LL-37 (Murakami *et al.*, 2002; Hata and Gallo, 2008). Dermcidin and LL-37 have been shown to reduce survival time of *S. aureus in vitro* (Murakami *et al.*, 2002; Hata and Gallo, 2008). In contrast to humans, thermoregulation in dogs is not based on sweat production, but on vasodilatation (heat loss) and vasoconstriction (heat preservation) of the cutaneous vascular system

(Guaguere *et al.*, 2008). Canine (epitrichial) sweat glands are distributed alongside sebaceous glands over the whole body surface; sweat gland secretions and sebum together form the surface hydrolipid film, a physical and chemical defence barrier (Guaguere *et al.*, 2008). The sweat component of the hydrolipid film contains several antimicrobial properties such as transferrin, fatty acids, and IgA (Garthwaite, 1983; Guaguere *et al.*, 2008). Sweat glands are triggered by circulating adrenalin and noradrenalin (Guaguere *et al.*, 2008), hence sweat production is increased in response to stress or fear. In the current study, exposure to the stressful situation of corneocyte sampling (restraint, clipping, lying still for a long time period) could have prompted sweat production in particular one dog, leading to increased antimicrobial properties and subsequent less bacterial adherence on some slides.

SpsO mediated adherence of *L. lactis* to *ex vivo* canine corneocytes, but *L. lactis* expressing SpsO did not interact with any of the ECM proteins tested (Fg, Fn, CK10). These data indicate that SpsO mediates binding via a distinct receptor not examined in the current study such as loricrin, involucrin, or laminin. In order to further investigate and to measure the role of SpsO in binding of *S. pseudintermedius* to canine corneocytes, additional studies are necessary such as inhibition assays with antibodies generated specifically against SpsO, inhibition of binding with purified recombinant protein, and binding studies using truncated versions of the protein to determine the binding site that interacts with the host cell. Further, generating knockout mutants of the wildtype strain would be useful to investigate the effect on corneocyte binding when *S. pseudintermedius* ED99 lacks SpsO. Similar experimental approaches have been used to elucidate the role of MSCRAMMs in other staphylococci species, for example of SdrF binding to collagen in *S. epidermidis* (Arrecubieta *et al.*, 2007) or ClfB binding to CK10 and nasal epithelial cells in *S. aureus* (O′Brien *et al.*, 2002b).

Of note, the gene encoding SpsO was only detected in 6 out of the 20 *S. pseudintermedius* isolates suggesting that it may provide a colonisation advantage to certain strains. The location of *spsO* in the *S. pseudintermedius* ED99 genome adjacent to transposases, and its presence in a sub-portion of strains suggests that it

was acquired by lateral gene transfer. Of note, SpsD and SpsO did not promote binding of *L. lactis* to cultured keratinocytes, indicating that the receptors involved are present on *ex vivo* corneocytes but not on *in vitro* undifferentiated keratinocytes. SpsL did not enhance adherence of *L. lactis* to canine keratinocytes. However, *L. lactis* expressing SpsL adhered to canine Fg in a host-specific manner, indicating that it is an MSCRAMM and that it contributes to host-pathogen interactions. The *spsL* gene was only found in *S. pseudintermedius* strains and not in the other members of the SIG, consistent with its canine host specific ligand-binding activity. The identification for the first time of *S. pseudintermedius* CWA proteins which mediate binding to ECM proteins is an important step towards an enhanced understanding of *S. pseudintermedius* pathogenesis. Although the data reported herein represent important initial discoveries regarding the function of the selected MSCRAMMs, further studies are required to identify the key sites within the MSCRAMMs and their host protein receptors which facilitate binding. In addition, it is required to screen additional host proteins such as collagen, loricrin, involucrin, and laminin for their capacity to interact with SpsD, SpsL, and SpsO.

The corneocyte binding data generated in the current study was statistically analysed by One-sample T-Test. For the binding data of *L. lactis* expressing SpsD, SpsL, and SpsO to ECM proteins and to keratinocyte cell culture descriptive statistical analysis was chosen as the sample size precluded statistical tests. Previous binding studies using *L. lactis* expression systems and *S. aureus* mutants have either applied statistical analysis (Student's T-test) to evaluate their data (Que *et al.*, 2001; O′Brien *et al.*, 2002b; Arrecubieta *et al.*, 2007) or have used descriptive statistics (Roche *et al.*, 2004; Walsh *et al.*, 2004). For the current study, a statistician's advice was given and it was recommended to evaluate the data by descriptive statistical methods based on the grounds that the sample size was too small (population size $n = 1$ for each *L. lactis* construct as repetition of the experiment on different days does not increase the sample size) (Dr. Darren Shaw, personal communication). In contrast, the corneocyte adherence study was eligible for statistical analysis as the *L. lactis* constructs were tested in a dog population ($n = 5$) (Dr. Darren Shaw, personal communication).

Considering that AD is an underlying condition in many dogs which develop pyoderma, it would be interesting to examine the role of the MSCRAMMs characterised in the current study in binding to corneocytes from atopic dogs. *S. pseudintermedius* adherence to corneocytes from atopic canine skin is elevated compared to corneocytes from healthy dogs (McEwan, 2000; McEwan *et al.*, 2005; Simou *et al.*, 2005c; McEwan *et al.*, 2006a), but the reasons for this are currently unknown. In skin sections prepared from biopsies of human atopic skin, Cho *et al.* (2001) detected Fn in the upper layers of the epidermis, which was not observed in biopsies from non-atopic human skin. A similar distribution pattern in canine atopic skin has been hypothesised (McEwan *et al.*, 2006a). Further, plasma proteins such as Fg and Fn exudate during inflammatory processes, and may therefore be found in the upper epidermal layers in atopic dogs (McEwan *et al.*, 2006a). Accordingly, it is feasible that SpsD and SpsL contribute to the enhanced adherence of *S. pseudintermedius* to atopic canine skin via Fg and Fn interactions.

Although the cell culture assay developed in the current study demonstrated differences in adherence phenotype in comparison to *ex vivo* corneocyte preparations, the assay provides a platform for further studies of canine host-pathogen interactions. Of particular interest, unlike the corneocyte assay, the host response to bacterial challenge could be tested by measuring the expression of host response markers such as cytokines. Furthermore, primary cells from canine skin biopsies could be cultured in a similar fashion, e.g. from atopic patients, and employed to study the connection of staphylococcal pyoderma and canine AD.

In the current study, immunoreactivity with canine sera was detected for SpsD and SpsL, but not for SpsO. The canine sera tested for specific antibody were obtained from staphylococcal pyoderma cases as the main purpose of this study was to establish whether SpsD, SpsL, and SpsO are likely to be expressed by *S. pseudintermedius* during infection rather than colonisation and to investigate their antigenic potential in disease to test their suitability as therapeutic targets. Further, the study was designed to compare the *L. lactis* constructs and to demonstrate expression of the *S. pseudintermedius* surface proteins on the lactococcal surface

rather than comparing host responses, i.e. dogs affected by staphylococcal pyoderma with dogs not suffering from skin disease. Therefore, sera of dogs not suffering from clinically manifested staphylococcal infection were not included in the study. Of note, Neuber *et al.* (2008) investigated IgG responses of dogs with and without pyoderma to *S. pseudintermedius* isolates and concluded that the IgG response was comparable. In the current study, the canine sera were obtained during clinically manifested infection, but the clonal origin and gene content of the *S. pseudintermedius* isolates that caused the infections are unknown. While genes for SpsD and SpsL were identified among all *S. pseudintermedius* strains examined, the gene for SpsO was detected in only six of the strains. It is therefore likely that the animals sampled in the current study had been exposed to *S. pseudintermedius* strains expressing SpsD and SpsL, but not SpsO.

The easy access and availability of genome sequence data has influenced and changed the development of novel vaccines and therapeutics, especially the concept of 'reverse vaccinology' (Capecchi *et al.*, 2004) has gained importance in recent years. In this approach, the genome of a bacterial or viral pathogen is screened for genes encoding proteins that may represent suitable vaccine targets (Seib *et al.*, 2009). The potential vaccine target is ideally a surface protein which is crucial for pathogenesis or pathogen survival and is expressed at a high level (Otto, 2008). With regard to *S. pseudintermedius* and potential vaccine development, it will be important to test whether the individual MSCRAMMs represent virulence factors using suitable animal models or the natural host in experimental infections with isogenic mutants. For example, experiments to test the role of SpsD, SpsL, and SpsO in *S. pseudintermedius* colonisation of dogs would be highly appropriate. Once the role of SpsD, SpsL, and SpsO as virulence factors of *S. pseudintermedius* is established *in vivo*, further studies would be necessary to investigate their potential as vaccine candidates and therapeutic targets. For example, SpsD, SpsL, and SpsO could be employed in passive and active immunisation studies to test their antigenic properties, either singular or in combination, as proposed for *S. aureus* ClfA (Josefsson *et al.*, 2001; Hall *et al.*, 2003; Patti, 2004; Nanra *et al.*, 2009). Of note, a combinatory vaccine of *S. aureus* surface proteins including IsdA, iron-regulated

surface determinant protein B (IsdB), SdrD, and SdrE has proven to be highly protective in a mouse infection model (Stranger-Jones *et al.*, 2006), demonstrating the potential of vaccine preparations containing multiple staphylococcal CWA proteins. In addition, MSCRAMMs with known ligands could be targets of anti-staphylococcal drug development by generating synthetic peptides based on the interacting ECM proteins which antagonise the MSCRAMM-host protein interaction but do not interfere profoundly with physiological processes in the host. An excellent example is provided by Ganesh *et al.* (2008) who demonstrated that synthetic peptides based on the Fg-binding site for ClfA hinder the ClfA interaction but do not block binding of the platelet integrin $\alpha_{IIb}\beta_3$ to Fg. However, vaccine development against staphylococci has been proven challenging and potential vaccinations against *S. aureus* in humans have so far failed the clinical trials even though animal studies had been promising (Otto, 2008). One possible explanation is that animal protection models are not very appropriate as animals are not colonised with *S. aureus* like humans and therefore do not reflect the actual situation (Otto, 2008). Further, since *S. aureus* colonises human skin, there is a risk of causing an iatrogenic imbalance of the cutaneous microflora by introducing a vaccine (Otto, 2008). Based on experiences with *S. aureus*, an appropriate animal model must be carefully chosen and the consequences on the canine cutaneous microflora must be considered for a potential *S. pseudintermedius* vaccine. Several murine models have been employed for investigations of *S. aureus* skin infections. Experimental procedures used include subcutaneous bacterial injection (Horsburgh *et al.*, 2002), subcutaneous implantation of bacterial-soaked suture material (Gisby and Bryant, 2000), and intradermal bacterial injection (Mölne and Tarkowski, 2000; Cho *et al.*, 2010). Immune responses were evaluated by serological analysis (Mölne and Tarkowski, 2000; Cho *et al.*, 2010). Similar infection models could be developed to test the *in vivo* immunogenicity of *S. pseudintermedius* and SpsD, SpsL, and SpsO in particular with regard to vaccine development. However, these models might not be as suitable for the investigation of *S. pseudintermedius* superficial skin infection as they require relatively invasive procedures and involve bacterial inoculation in and under the skin. Kugelberg *et al.* (2005) developed a murine 'tape strip' model for superficial *S. aureus* skin infection. In their model, the skin barrier was disrupted in anaesthetised

mice by applying elastic adhesive tape strips repeatedly in order to remove fur and epidermal layer (Kugelberg *et al.*, 2005). *S. aureus* infection was established by applying a droplet of bacterial suspension on the prepared site, resulting in local inflammation without sepsis and consequently, the effectiveness of topical treatment could be investigated (Kugelberg *et al.*, 2005). A similar approach in a *S. pseudintermedius* superficial infection model might be suitable to investigate the role of SpsD, SpsL, and SpsO under more natural conditions. Further, the development of novel (topical) therapeutics involving SpsD, SpsL, and SpsO might be tested in such a model. Previous studies have quantified the number of live bacteria at the infection site at different time points by counting CFUs post mortem (Gisby and Bryant, 2000; Mölne and Tarkowski, 2000; Kugelberg *et al.*, 2005) or by *in vivo* bioluminescence imaging (Cho *et al.*, 2010). The latter might be an elegant option to assess the effectiveness of novel therapeutics or vaccines based on SpsD, SpsL, and SpsO in *S. pseudintermedius* cutaneous infection models in the future.

Taken together, the current study has solved the population genetic structure of the SIG revealing important features of its ancient and recent evolution. Importantly, it was discovered that *S. pseudintermedius*, and not *S. intermedius*, is the major canine pyoderma pathogen. Further, new insights into the molecular basis of *S. pseudintermedius*-canine host interactions were revealed, including the identification and characterisation of three novel MSCRAMMs which represent putative new therapeutic targets and/or vaccine candidates for the control of this important animal pathogen.

References

Aarestrup, F. M. (2001) Comparative ribotyping of *Staphylococcus intermedius* isolated from members of the Canoidea gives possible evidence for host-specificity and co-evolution of bacteria and hosts. *Int J Syst Evol Microbiol,* 51**,** 1343-7.

Ahmed, S., Meghji, S., Williams, R. J., Henderson, B., Brock, J. H. & Nair, S. P. (2001) *Staphylococcus aureus* fibronectin binding proteins are essential for internalization by osteoblasts but do not account for differences in intracellular levels of bacteria. *Infect Immun,* 69**,** 2872-7.

Allaker, R. P., Lloyd, D. H. & Bailey, R. M. (1992a) Population sizes and frequency of staphylococci at mucocutaneous sites on healthy dogs. *Vet Rec,* 130**,** 303-4.

Allaker, R. P., Lloyd, D. H. & Simpson, A. I. (1992b) Occurrence of *Staphylococcus intermedius* on the hair and skin of normal dogs. *Res Vet Sci,* 52**,** 174-6.

Alves, P. D., McCulloch, J. A., Even, S., Le Marechal, C., Thierry, A., Grosset, N., Azevedo, V., Rosa, C. A., Vautor, E. & Le Loir, Y. (2009) Molecular characterisation of *Staphylococcus aureus* strains isolated from small and large ruminants reveals a host rather than tissue specificity. *Vet Microbiol,* 137**,** 190-5.

Arbeit, R. D., Karakawa, W. W., Vann, W. F. & Robbins, J. B. (1984) Predominance of two newly described capsular polysaccharide types among clinical isolates of *Staphylococcus aureus*. *Diagn Microbiol Infect Dis,* 2**,** 85-91.

Arciola, C. R., Campoccia, D., Gamberini, S., Baldassarri, L. & Montanaro, L. (2005) Prevalence of *cna*, *fnbA* and *fnbB* adhesin genes among *Staphylococcus aureus* isolates from orthopedic infections associated to different types of implant. *FEMS Microbiol Lett,* 246**,** 81-6.

Arrecubieta, C., Asai, T., Bayern, M., Loughman, A., Fitzgerald, J. R., Shelton, C. E., Baron, H. M., Dang, N. C., Deng, M. C., Naka, Y., Foster, T. J. & Lowy, F. D. (2006) The role of *Staphylococcus aureus* adhesins in the pathogenesis of ventricular assist device-related infections. *J Infect Dis,* 193**,** 1109-19.

Arrecubieta, C., Lee, M. H., Macey, A., Foster, T. J. & Lowy, F. D. (2007) SdrF, a *Staphylococcus epidermidis* surface protein, binds type I collagen. *J Biol Chem,* 282**,** 18767-76.

Arrecubieta, C., Toba, F. A., Von Bayern, M., Akashi, H., Deng, M. C., Naka, Y. & Lowy, F. D. (2009) SdrF, a *Staphylococcus epidermidis* surface protein, contributes to the initiation of ventricular assist device driveline-related infections. *PLoS Pathog,* 5**,** e1000411.

Baker, B. B., Maibach, H. I., Park, R. D., McFarland, L. Z. & O'Brien, T. R. (1973) Epidermal cell renewal in the dog. *Am J Vet Res,* 34**,** 93-4.

Baker, B. S. (2006) The role of microorganisms in atopic dermatitis. *Clin Exp Immunol,* 144**,** 1-9.

Balaban, N. & Rasooly, A. (2000) Staphylococcal enterotoxins. *Int J Food Microbiol,* 61**,** 1-10.

Bannoehr, J., Franco, A., Iurescia, M., Battisti, A. & Fitzgerald, J. R. (2009) Molecular diagnostic identification of *Staphylococcus pseudintermedius*. *J Clin Microbiol,* 47**,** 469-71.

Barkema, H. W., Schukken, Y. H. & Zadoks, R. N. (2006) Invited Review: The role of cow, pathogen, and treatment regimen in the therapeutic success of bovine *Staphylococcus aureus* mastitis. *J Dairy Sci,* 89**,** 1877-95.

Baron, F., Cochet, M. F., Pellerin, J. L., Ben Zakour, N., Lebon, A., Navarro, A., Proudy, I., Le Loir, Y. & Gautier, M. (2004) Development of a PCR test to differentiate between *Staphylococcus aureus* and *Staphylococcus intermedius*. *J Food Prot,* 67**,** 2302-5.

Bartual, S. G., Seifert, H., Hippler, C., Luzon, M. A., Wisplinghoff, H. & Rodriguez-Valera, F. (2005) Development of a multilocus sequence typing scheme for characterization of clinical isolates of *Acinetobacter baumannii*. *J Clin Microbiol,* 43**,** 4382-90.

Baumer, W. & Kietzmann, M. (2007) Effects of cyclosporin A and cilomilast on activated canine, murine and human keratinocytes. *Vet Dermatol,* 18**,** 107-14.

Beachey, E. H. (1981) Bacterial adherence: adhesin-receptor interactions mediating the attachment of bacteria to mucosal surface. *J Infect Dis,* 143**,** 325-45.

Becker, K., Bierbaum, G., Von Eiff, C., Engelmann, S., Gotz, F., Hacker, J., Hecker, M., Peters, G., Rosenstein, R. & Ziebuhr, W. (2007) Understanding the physiology and adaptation of staphylococci: a post-genomic approach. *Int J Med Microbiol,* 297**,** 483-501.

Becker, K., Keller, B., Von Eiff, C., Bruck, M., Lubritz, G., Etienne, J. & Peters, G. (2001) Enterotoxigenic potential of *Staphylococcus intermedius*. *Appl Environ Microbiol,* 67**,** 5551-7.

Ben Zakour, N. L., Guinane, C. M. & Fitzgerald, J. R. (2008) Pathogenomics of the staphylococci: insights into niche adaptation and the emergence of new virulent strains. *FEMS Microbiol Lett,* 289**,** 1-12.

Bes, M., Saidi Slim, L., Becharnia, F., Meugnier, H., Vandenesch, F., Etienne, J. & Freney, J. (2002) Population diversity of *Staphylococcus intermedius* isolates from various host species: typing by 16S-23S intergenic ribosomal DNA spacer polymorphism analysis. *J Clin Microbiol,* 40**,** 2275-7.

Bhakdi, S. & Tranum-Jensen, J. (1991) Alpha-toxin of *Staphylococcus aureus*. *Microbiol Rev,* 55**,** 733-51.

Bibel, D. J., Aly, R., Shinefield, H. R., Maibach, H. I. & Strauss, W. G. (1982) Importance of the keratinized epithelial cell in bacterial adherence. *J Invest Dermatol,* 79**,** 250-3.

Bingham, R. J., Rudino-Pinera, E., Meenan, N. A., Schwarz-Linek, U., Turkenburg, J. P., Hook, M., Garman, E. F. & Potts, J. R. (2008) Crystal structures of fibronectin-binding sites from *Staphylococcus aureus* FnBPA in complex with fibronectin domains. *Proc Natl Acad Sci U S A,* 105**,** 12254-8.

Birgersson, A., Jonsson, P. & Holmberg, O. (1992) Species identification and some characteristics of coagulase-negative staphylococci isolated from bovine udders. *Vet Microbiol,* 31**,** 181-9.

Bjorland, J., Steinum, T., Kvitle, B., Waage, S., Sunde, M. & Heir, E. (2005) Widespread distribution of disinfectant resistance genes among staphylococci of bovine and caprine origin in Norway. *J Clin Microbiol,* 43**,** 4363-8.

Black, C. C., Solyman, S. M., Eberlein, L. C., Bemis, D. A., Woron, A. M. & Kania, S. A. (2009) Identification of a predominant multilocus sequence type, pulsed-field gel electrophoresis cluster, and novel staphylococcal chromosomal cassette in clinical isolates of *mecA*-containing, methicillin-resistant *Staphylococcus pseudintermedius*. *Vet Microbiol*.

Boles, B. R. & Horswill, A. R. (2008) *agr*-mediated dispersal of *Staphylococcus aureus* biofilms. *PLoS Pathog,* 4**,** e1000052.

Borregaard, N., Theilgaard-Monch, K., Cowland, J. B., Stahle, M. & Sorensen, O. E. (2005) Neutrophils and keratinocytes in innate immunity-cooperative actions to provide antimicrobial defense at the right time and place. *J Leukoc Biol,* 77**,** 439-43.

Bowden, M. G., Chen, W., Singvall, J., Xu, Y., Peacock, S. J., Valtulina, V., Speziale, P. & Hook, M. (2005) Identification and preliminary characterization of cell-wall-anchored proteins of *Staphylococcus epidermidis*. *Microbiology,* 151**,** 1453-64.

Bowden, M. G., Heuck, A. P., Ponnuraj, K., Kolosova, E., Choe, D., Gurusiddappa, S., Narayana, S. V., Johnson, A. E. & Hook, M. (2008) Evidence for the "dock, lock, and latch" ligand binding mechanism of the staphylococcal microbial surface component recognizing adhesive matrix molecules (MSCRAMM) SdrG. *J Biol Chem,* 283**,** 638-47.

Boyen, F., Eeckhaut, V., Van Immerseel, F., Pasmans, F., Ducatelle, R. & Haesebrouck, F. (2009) Quorum sensing in veterinary pathogens: mechanisms, clinical importance and future perspectives. *Vet Microbiol,* 135**,** 187-95.

Braff, M. H., Bardan, A., Nizet, V. & Gallo, R. L. (2005) Cutaneous defense mechanisms by antimicrobial peptides. *J Invest Dermatol,* 125(1), 9-13.

Brenninkmeijer, E. E., Spuls, P. I., Legierse, C. M., Lindeboom, R., Smitt, J. H. & Bos, J. D. (2008) Clinical differences between atopic and atopiform dermatitis. *J Am Acad Dermatol,* 58**,** 407-14.

Bronner, S., Monteil, H. & Prevost, G. (2004) Regulation of virulence determinants in *Staphylococcus aureus*: complexity and applications. *FEMS Microbiol Rev,* 28**,** 183-200.

Brouillette, E. & Malouin, F. (2005) The pathogenesis and control of *Staphylococcus aureus*-induced mastitis: study models in the mouse. *Microbes Infect,* 7**,** 560-8.

Brouillette, E., Talbot, B. G. & Malouin, F. (2003) The fibronectin-binding proteins of *Staphylococcus aureus* may promote mammary gland colonization in a lactating mouse model of mastitis. *Infect Immun,* 71**,** 2292-5.

Bubeck Wardenburg, J., Palazzolo-Ballance, A. M., Otto, M., Schneewind, O. & Deleo, F. R. (2008) Panton-Valentine leukocidin is not a virulence determinant in murine models of community-associated methicillin-resistant *Staphylococcus aureus* disease. *J Infect Dis,* 198**,** 1166-70.

Camilli, A. & Bassler, B. L. (2006) Bacterial small-molecule signaling pathways. *Science,* 311**,** 1113-6.

Capecchi, B., Serruto, D., Adu-Bobie, J., Rappuoli, R. & Pizza, M. (2004) The genome revolution in vaccine research. *Curr Issues Mol Biol,* 6(1), 17-27.

Carlotti, D. N., Guaguere, E., Pin, D., Jasmin, P., Thomas, E. & Guiral, V. (1999) Therapy of difficult cases of canine pyoderma with marbofloxacin: a report of 39 dogs. *J Small Anim Pract,* 40**,** 265-70.

Castagliuolo, I., Piccinini, R., Beggiao, E., Palu, G., Mengoli, C., Ditadi, F., Vicenzoni, G. & Zecconi, A. (2006) Mucosal genetic immunization against four adhesins protects against *Staphylococcus aureus*-induced mastitis in mice. *Vaccine,* 24**,** 4393-402.

Cefai, C., Ashurst, S. & Owens, C. (1994) Human carriage of methicillin-resistant *Staphylococcus aureus* linked with pet dog. *Lancet,* 344**,** 539-40.

Chambers, H. F. (2001) The changing epidemiology of *Staphylococcus aureus*? *Emerg Infect Dis,* 7**,** 178-82.

Chavakis, T., Hussain, M., Kanse, S. M., Peters, G., Bretzel, R. G., Flock, J. I., Herrmann, M. & Preissner, K. T. (2002) *Staphylococcus aureus* extracellular adherence protein serves as anti-inflammatory factor by inhibiting the recruitment of host leukocytes. *Nat Med,* 8**,** 687-93.

Chavakis, T., Wiechmann, K., Preissner, K. T. & Herrmann, M. (2005) *Staphylococcus aureus* interactions with the endothelium: the role of bacterial "secretable expanded repertoire adhesive molecules" (SERAM) in disturbing host defense systems. *Thromb Haemost,* 94**,** 278-85.

Chesbro, W. R., Heydrick, F. P., Martineau, R. & Perkins, G. N. (1965) Purification of Staphylococcal Beta-Hemolysin and Its Action on Staphylococcal and Streptococcal Cell Walls. *J Bacteriol,* 89**,** 378-89.

Chesneau, O., Morvan, A., Aubert, S. & El Solh, N. (2000) The value of rRNA gene restriction site polymorphism analysis for delineating taxa in the genus *Staphylococcus*. *Int J Syst Evol Microbiol,* 50 Pt 2**,** 689-97.

Cheung, A. L., Bayer, A. S., Zhang, G., Gresham, H. & Xiong, Y. Q. (2004) Regulation of virulence determinants *in vitro* and *in vivo* in *Staphylococcus aureus*. *FEMS Immunol Med Microbiol,* 40**,** 1-9.

Cheung, A. L., Eberhardt, K. & Heinrichs, J. H. (1997) Regulation of protein A synthesis by the *sar* and *agr* loci of *Staphylococcus aureus*. *Infect Immun,* 65**,** 2243-9.

Cheung, A. L. & Fischetti, V. A. (1990) The role of fibrinogen in staphylococcal adherence to catheters *in vitro*. *J Infect Dis,* 161**,** 1177-86.

Cho, J. S., Pietras, E. M., Garcia, N. C., Ramos, R. I., Farzam, D. M., Monroe, H. R., Magorien, J. E., Blauvelt, A., Kolls, J. K., Cheung, A. L., Cheng, G., Modlin, R. L. & Miller, L. S. (2010) IL-17 is essential for host defense against cutaneous *Staphylococcus aureus* infection in mice. *J Clin Invest,* Apr 1. pii: 40891. doi: 10.1172/JCI40891.

Cho, S. H., Strickland, I., Boguniewicz, M. & Leung, D. Y. (2001) Fibronectin and fibrinogen contribute to the enhanced binding of *Staphylococcus aureus* to atopic skin. *J Allergy Clin Immunol,* 108**,** 269-74.

Clarke, S. R., Andre, G., Walsh, E. J., Dufrene, Y. F., Foster, T. J. & Foster, S. J. (2009) Iron-regulated surface determinant protein A mediates adhesion of *Staphylococcus aureus* to human corneocyte envelope proteins. *Infect Immun,* 77**,** 2408-16.

Clarke, S. R. & Foster, S. J. (2006) Surface adhesins of *Staphylococcus aureus*. *Adv Microb Physiol,* 51**,** 187-224.

Coenye, T. & Vandamme, P. (2003) Intragenomic heterogeneity between multiple 16S ribosomal RNA operons in sequenced bacterial genomes. *FEMS Microbiol Lett,* 228**,** 45-9.

Colombo, S., Hill, P. B., Shaw, D. J. & Thoday, K. L. (2007) Requirement for additional treatment for dogs with atopic dermatitis undergoing allergen-specific immunotherapy. *Vet Rec,* 160**,** 861-4.

Cone, L. A., Sontz, E. M., Wilson, J. W. & Mitruka, S. N. (2005) *Staphylococcus capitis* endocarditis due to a transvenous endocardial pacemaker infection: case report and review of *Staphylococcus capitis* endocarditis. *Int J Infect Dis,* 9**,** 335-9.

Corrigan, R. M., Miajlovic, H. & Foster, T. J. (2009) Surface proteins that promote adherence of *Staphylococcus aureus* to human desquamated nasal epithelial cells. *BMC Microbiol,* 9**,** 22.

Cox, H. U., Hoskins, J. D., Newman, S. S., Foil, C. S., Turnwald, G. H. & Roy, A. F. (1988) Temporal study of staphylococcal species on healthy dogs. *Am J Vet Res,* 49**,** 747-51.

Cree, R. G. & Noble, W. C. (1995) *In vitro* indices of tissue adherence in *Staphylococcus intermedius*. *Lett Appl Microbiol,* 20**,** 168-70.

Curtis, C. F., Lamport, A. I. & Lloyd, D. H. (2006) Masked, controlled study to investigate the efficacy of a *Staphylococcus intermedius* autogenous bacterin for the control of canine idiopathic recurrent superficial pyoderma. *Vet Dermatol,* 17**,** 163-8.

Davis, S. L., Gurusiddappa, S., McCrea, K. W., Perkins, S. & Hook, M. (2001) SdrG, a fibrinogen-binding bacterial adhesin of the microbial surface components recognizing adhesive matrix molecules subfamily from *Staphylococcus epidermidis*, targets the thrombin cleavage site in the Bbeta chain. *J Biol Chem,* 276**,** 27799-805.

De Haas, C. J., Veldkamp, K. E., Peschel, A., Weerkamp, F., Van Wamel, W. J., Heezius, E. C., Poppelier, M. J., Van Kessel, K. P. & Van Strijp, J. A. (2004) Chemotaxis inhibitory protein of *Staphylococcus aureus*, a bacterial antiinflammatory agent. *J Exp Med,* 199**,** 687-95.

De Jaham, C. (2003) Effects of an ethyl lactate shampoo in conjunction with a systemic antibiotic in the treatment of canine superficial bacterial pyoderma in an open-label, nonplacebo-controlled study. *Vet Ther,* 4**,** 94-100.

De Kimpe, S. J., Kengatharan, M., Thiemermann, C. & Vane, J. R. (1995) The cell wall components peptidoglycan and lipoteichoic acid from *Staphylococcus aureus* act in synergy to cause shock and multiple organ failure. *Proc Natl Acad Sci U S A,* 92**,** 10359-63.

DeBoer, D. J., Moriello, K. A., Thomas, C. B. & Schultz, K. T. (1990) Evaluation of a commercial staphylococcal bacterin for management of idiopathic recurrent superficial pyoderma in dogs. *Am J Vet Res,* 51**,** 636-9.

Deivanayagam, C. C., Rich, R. L., Carson, M., Owens, R. T., Danthuluri, S., Bice, T., Hook, M. & Narayana, S. V. (2000) Novel fold and assembly of the repetitive B region of the *Staphylococcus aureus* collagen-binding surface protein. *Structure,* 8**,** 67-78.

Deivanayagam, C. C., Wann, E. R., Chen, W., Carson, M., Rajashankar, K. R., Hook, M. & Narayana, S. V. (2002) A novel variant of the immunoglobulin fold in surface adhesins of *Staphylococcus aureus*: crystal structure of the fibrinogen-binding MSCRAMM, clumping factor A. *Embo J,* 21**,** 6660-72.

Deurenberg, R. H. & Stobberingh, E. E. (2008) The evolution of *Staphylococcus aureus*. *Infect Genet Evol,* 8**,** 747-63.

Devriese, L. A. & De Pelsmaecker, K. (1987) The anal region as a main carrier site of *Staphylococcus intermedius* and *Streptococcus canis* in dogs. *Vet Rec,* 121**,** 302-3.

Devriese, L. A., Hajek, V., Oeding, P., Meyer, S. A. & Schleifer, K. H. (1978) *Staphylococcus hyicus* (Sompolinsky 1953) comb. nov. and *Staphylococcus hyicus* subsp. *chromogenes* subsp. nov. *Int J Syst Bacteriol,* 28**,** 482-490.

Devriese, L. A., Hermans, K., Baele, M. & Haesebrouck, F. (2009) *Staphylococcus pseudintermedius* versus *Staphylococcus intermedius*. *Vet Microbiol,* 133**,** 206-7.

Devriese, L. A., Vancanneyt, M., Baele, M., Vaneechoutte, M., De Graef, E., Snauwaert, C., Cleenwerck, I., Dawyndt, P., Swings, J., Decostere, A. & Haesebrouck, F. (2005) *Staphylococcus pseudintermedius* sp. nov., a coagulase-positive species from animals. *Int J Syst Evol Microbiol,* 55**,** 1569-73.

Dillon, H. C., Jr. (1983) Treatment of staphylococcal skin infections: a comparison of cephalexin and dicloxacillin. *J Am Acad Dermatol,* 8**,** 177-81.

Dinges, M. M., Orwin, P. M. & Schlievert, P. M. (2000) Exotoxins of *Staphylococcus aureus*. *Clin Microbiol Rev,* 13**,** 16-34.

Donvito, B., Etienne, J., Greenland, T., Mouren, C., Delorme, V. & Vandenesch, F. (1997) Distribution of the synergistic haemolysin genes *hld* and *slush* with respect to *agr* in human staphylococci. *FEMS Microbiol Lett,* 151**,** 139-44.

Downer, R., Roche, F., Park, P. W., Mecham, R. P. & Foster, T. J. (2002) The elastin-binding protein of *Staphylococcus aureus* (EbpS) is expressed at the cell surface as an integral membrane protein and not as a cell wall-associated protein. *J Biol Chem,* 277**,** 243-50.

Dufour, P., Jarraud, S., Vandenesch, F., Greenland, T., Novick, R. P., Bes, M., Etienne, J. & Lina, G. (2002) High genetic variability of the *agr* locus in *Staphylococcus* species. *J Bacteriol,* 184**,** 1180-6.

Dunman, P. M., Murphy, E., Haney, S., Palacios, D., Tucker-Kellogg, G., Wu, S., Brown, E. L., Zagursky, R. J., Shlaes, D. & Projan, S. J. (2001) Transcription profiling-based identification of *Staphylococcus aureus* genes regulated by the *agr* and/or *sarA* loci. *J Bacteriol,* 183**,** 7341-53.

Dziewanowska, K., Edwards, V. M., Deringer, J. R., Bohach, G. A. & Guerra, D. J. (1996) Comparison of the beta-toxins from *Staphylococcus aureus* and *Staphylococcus intermedius*. *Arch Biochem Biophys,* 335**,** 102-8.

Dziewanowska, K., Patti, J. M., Deobald, C. F., Bayles, K. W., Trumble, W. R. & Bohach, G. A. (1999) Fibronectin binding protein and host cell tyrosine kinase are required for internalization of *Staphylococcus aureus* by epithelial cells. *Infect Immun,* 67**,** 4673-8.

Edwards, V. M., Deringer, J. R., Callantine, S. D., Deobald, C. F., Berger, P. H., Kapur, V., Stauffacher, C. V. & Bohach, G. A. (1997) Characterization of the canine type C enterotoxin produced by *Staphylococcus intermedius* pyoderma isolates. *Infect Immun,* 65**,** 2346-52.

Elasri, M. O., Thomas, J. R., Skinner, R. A., Blevins, J. S., Beenken, K. E., Nelson, C. L. & Smeltzer, M. S. (2002) *Staphylococcus aureus* collagen adhesin contributes to the pathogenesis of osteomyelitis. *Bone,* 30**,** 275-80.

Ellington, J. K., Harris, M., Webb, L., Smith, B., Smith, T., Tan, K. & Hudson, M. (2003) Intracellular *Staphylococcus aureus*. A mechanism for the indolence of osteomyelitis. *J Bone Joint Surg Br,* 85**,** 918-21.

Enright, M. C., Day, N. P., Davies, C. E., Peacock, S. J. & Spratt, B. G. (2000) Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. *J Clin Microbiol,* 38**,** 1008-15.

Enright, M. C., Robinson, D. A., Randle, G., Feil, E. J., Grundmann, H. & Spratt, B. G. (2002) The evolutionary history of methicillin-resistant *Staphylococcus aureus* (MRSA). *Proc Natl Acad Sci U S A,* 99**,** 7687-92.

Entenza, J. M., Foster, T. J., Ni Eidhin, D., Vaudaux, P., Francioli, P. & Moreillon, P. (2000) Contribution of clumping factor B to pathogenesis of experimental endocarditis due to *Staphylococcus aureus*. *Infect Immun,* 68**,** 5443-6.

Faires, M. C., Tater, K. C. & Weese, J. S. (2009) An investigation of methicillin-resistant *Staphylococcus aureus* colonization in people and pets in the same household with an infected person or infected pet. *J Am Vet Med Assoc,* 235**,** 540-3.

Faria, N. A., Carrico, J. A., Oliveira, D. C., Ramirez, M. & de Lencastre H. (2008) Analysis of typing methods for epidemiological surveillance of both methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* strains. *J Clin Microbiol,* 46(1), 136-44.

Farrell, D. H., Thiagarajan, P., Chung, D. W. & Davie, E. W. (1992) Role of fibrinogen alpha and gamma chain sites in platelet aggregation. *Proc Natl Acad Sci U S A,* 89**,** 10729-32.

Fazakerley, J., Nuttall, T., Sales, D., Schmidt, V., Carter, S. D., Hart, C. A. & McEwan, N. A. (2009) Staphylococcal colonization of mucosal and lesional skin sites in atopic and healthy dogs. *Vet Dermatol,* 20**,** 179-84.

Feil, E. J., Cooper, J. E., Grundmann, H., Robinson, D. A., Enright, M. C., Berendt, T., Peacock, S. J., Smith, J. M., Murphy, M., Spratt, B. G., Moore, C. E. & Day, N. P. (2003) How clonal is *Staphylococcus aureus*? *J Bacteriol,* 185**,** 3307-16.

Feil, E. J. & Enright, M. C. (2004) Analyses of clonality and the evolution of bacterial pathogens. *Curr Opin Microbiol,* 7**,** 308-13.

Feil, E. J., Li, B. C., Aanensen, D. M., Hanage, W. P. & Spratt, B. G. (2004) eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. *J Bacteriol,* 186**,** 1518-30.

Finlay, B. B. & Cossart, P. (1997) Exploitation of mammalian host cell functions by bacterial pathogens. *Science,* 276**,** 718-25.

Fitzgerald, J. R., Loughman, A., Keane, F., Brennan, M., Knobel, M., Higgins, J., Visai, L., Speziale, P., Cox, D. & Foster, T. J. (2006) Fibronectin-binding proteins of *Staphylococcus aureus* mediate activation of human platelets via fibrinogen and fibronectin bridges to integrin GPIIb/IIIa and IgG binding to the FcgammaRIIa receptor. *Mol Microbiol,* 59**,** 212-30.

Fitzgerald, J. R., Meaney, W. J., Hartigan, P. J., Smyth, C. J. & Kapur, V. (1997) Fine-structure molecular epidemiological analysis of *Staphylococcus aureus* recovered from cows. *Epidemiol Infect,* 119**,** 261-9.

Fitzgerald, J. R., Sturdevant, D. E., Mackie, S. M., Gill, S. R. & Musser, J. M. (2001) Evolutionary genomics of *Staphylococcus aureus*: insights into the origin of methicillin-resistant strains and the toxic shock syndrome epidemic. *Proc Natl Acad Sci U S A,* 98**,** 8821-6.

Flock, J. I., Froman, G., Jonsson, K., Guss, B., Signas, C., Nilsson, B., Raucci, G., Hook, M., Wadstrom, T. & Lindberg, M. (1987) Cloning and expression of the gene for a fibronectin-binding protein from *Staphylococcus aureus*. *Embo J,* 6**,** 2351-7.

Flock, J. I., Hienz, S. A., Heimdahl, A. & Schennings, T. (1996) Reconsideration of the role of fibronectin binding in endocarditis caused by *Staphylococcus aureus*. *Infect Immun,* 64**,** 1876-8.

Forsgren, A. & Sjoquist, J. (1966) "Protein A" from S. *aureus*. I. Pseudo-immune reaction with human gamma-globulin. *J Immunol,* 97**,** 822-7.

Forsythe, P. J., Hill, P. B., Thoday, K. L. & Brown, J. (2002) Use of computerized image analysis to quantify staphylococcal adhesion to canine corneocytes: does breed and body site have any relevance to the pathogenesis of pyoderma? *Vet Dermatol,* 13**,** 29-36.

Foster, A. P. (2004) Immunomodulation and immunodeficiency. *Vet Dermatol,* 15**,** 115-26.

Foster, G., Ross, H. M., Hutson, R. A. & Collins, M. D. (1997) *Staphylococcus lutrae* sp. nov., a new coagulase-positive species isolated from otters. *Int J Syst Bacteriol,* 47**,** 724-6.

Foster, T. J. (2005) Immune evasion by staphylococci. *Nat Rev Microbiol,* 3**,** 948-58.

Foster, T. J. & Hook, M. (1998) Surface protein adhesins of *Staphylococcus aureus*. *Trends Microbiol,* 6**,** 484-8.

Fowler, T., Wann, E. R., Joh, D., Johansson, S., Foster, T. J. & Hook, M. (2000) Cellular invasion by *Staphylococcus aureus* involves a fibronectin bridge between the bacterial fibronectin-binding MSCRAMMs and host cell beta1 integrins. *Eur J Cell Biol,* 79**,** 672-9.

Frank, L. A. & Kunkle, G. A. (1993) Comparison of the efficacy of cefadroxil and generic and proprietary cephalexin in the treatment of pyoderma in dogs. *J Am Vet Med Assoc,* 203**,** 530-3.

Fraser, J. D. & Proft, T. (2008) The bacterial superantigen and superantigen-like proteins. *Immunol Rev,* 225**,** 226-43.

Frenay, H. M., Bunschoten, A. E., Schouls, L. M., Van Leeuwen, W. J., Vandenbroucke-Grauls, C. M., Verhoef, J. & Mooi, F. R. (1996) Molecular typing of methicillin-resistant *Staphylococcus aureus* on the basis of protein A gene polymorphism. *Eur J Clin Microbiol Infect Dis,* 15**,** 60-4.

Freney, J., Bruin, Y., Bes, M., Meugnier, H., Grimont, F. & Grimont, P. A. D. (1988) *Staphylococcus lugdunensis* sp. nov and *Staphylococcus schleiferi* sp. nov., two species from human clinical specimens. . *Int J Syst Bacteriol,* 38**,** 168-172.

Fuchs, E. (1995) Keratins and the skin. *Annu Rev Cell Dev Biol,* 11**,** 123-53.

Futagawa-Saito, K., Ba-Thein, W. & Fukuyasu, T. (2007) High occurrence of multi-antimicrobial resistance in *Staphylococcus intermedius* isolates from healthy and diseased dogs and domesticated pigeons. *Res Vet Sci,* 83**,** 336-9.

Futagawa-Saito, K., Ba-Thein, W., Sakurai, N. & Fukuyasu, T. (2006) Prevalence of virulence factors in *Staphylococcus intermedius* isolates from dogs and pigeons. *BMC Vet Res,* 2**,** 4.

Futagawa-Saito, K., Sugiyama, T., Karube, S., Sakurai, N., Ba-Thein, W. & Fukuyasu, T. (2004a) Prevalence and characterization of leukotoxin-producing *Staphylococcus intermedius* in Isolates from dogs and pigeons. *J Clin Microbiol,* 42**,** 5324-6.

Futagawa-Saito, K., Suzuki, M., Ohsawa, M., Ohshima, S., Sakurai, N., Ba-Thein, W. & Fukuyasu, T. (2004b) Identification and prevalence of an enterotoxin-

related gene, se-int, in *Staphylococcus intermedius* isolates from dogs and pigeons. *J Appl Microbiol,* 96**,** 1361-6.
Ganesh, V. K., Rivera, J. J., Smeds, E., Ko, Y. P., Bowden, M. G., Wann, E. R., Gurusiddappa, S., Fitzgerald, J. R. & Hook, M. (2008) A structural model of the *Staphylococcus aureus* ClfA-fibrinogen interaction opens new avenues for the design of anti-staphylococcal therapeutics. *PLoS Pathog,* 4**,** e1000226.
Ganiere, J. P., Medaille, C., Limet, A., Ruvoen, N. & Andre-Fontaine, G. (2001) Antimicrobial activity of enrofloxacin against *Staphylococcus intermedius* strains isolated from canine pyodermas. *Vet Dermatol,* 12**,** 171-5.
Ganiere, J. P., Medaille, C. & Mangion, C. (2005) Antimicrobial drug susceptibility of *Staphylococcus intermedius* clinical isolates from canine pyoderma. *J Vet Med B Infect Dis Vet Public Health,* 52**,** 25-31.
Gao, J. & Stewart, G. C. (2004) Regulatory elements of the *Staphylococcus aureus* protein A (Spa) promoter. *J Bacteriol,* 186**,** 3738-48.
Garthwaite, G., Lloyd, D. H. & Thomsett, L. R. (1983) Location of immunoglobulins and complement (C3) at the surface and within the skin of dogs. *J Comp Pathol,* 93(2), 185-93.
Gaskin, D. K., Bohach, G. A., Schlievert, P. M. & Hovde, C. J. (1997) Purification of *Staphylococcus aureus* beta-toxin: comparison of three isoelectric focusing methods. *Protein Expr Purif,* 9**,** 76-82.
Gasson, M. J. (1983) Plasmid complements of *Streptococcus lactis* NCDO 712 and other lactic streptococci after protoplast-induced curing. *J Bacteriol,* 154**,** 1-9.
Gelse, K., Poschl, E. & Aigner, T. (2003) Collagens- structure, function, and biosynthesis. *Adv Drug Deliv Rev,* 55**,** 1531-46.
Geoghegan, J. A., Smith, E. J., Speziale, P. & Foster, T. J. (2009) *Staphylococcus pseudintermedius* expresses surface proteins that closely resemble those from *Staphylococcus aureus*. *Vet Microbiol,* 138**,** 345-52.
George, E. A. & Muir, T. W. (2007) Molecular mechanisms of *agr* quorum sensing in virulent staphylococci. *Chembiochem,* 8**,** 847-55.
Gillaspy, A. F., Lee, C. Y., Sau, S., Cheung, A. L. & Smeltzer, M. S. (1998) Factors affecting the collagen binding capacity of *Staphylococcus aureus*. *Infect Immun,* 66**,** 3170-8.
Gisby, J. & Bryant, J. (2000) Efficacy of a new cream formulation of mupirocin: comparison with oral and topical agents in experimental skin infections. *Antimicrob Agents Chemother,* 44(2), 255-60.
Goh, S. H., Santucci, Z., Kloos, W. E., Faltyn, M., George, C. G., Driedger, D. & Hemmingsen, S. M. (1997) Identification of *Staphylococcus* species and subspecies by the *chaperonin 60* gene identification method and reverse checkerboard hybridization. *J Clin Microbiol,* 35**,** 3116-21.
Gong, J. Q., Lin, L., Lin, T., Hao, F., Zeng, F. Q., Bi, Z. G., Yi, D. & Zhao, B. (2006) Skin colonization by *Staphylococcus aureus* in patients with eczema and atopic dermatitis and relevant combined topical therapy: a double-blind multicentre randomized controlled trial. *Br J Dermatol,* 155**,** 680-7.
Gotz, F. (2002) *Staphylococcus* and biofilms. *Mol Microbiol,* 43**,** 1367-78.
Gow, J. A. & Robinson, J. (1969) Properties of purified staphylococcal beta-hemolysin. *J Bacteriol,* 97**,** 1026-32.
Greene, C., McDevitt, D., Francois, P., Vaudaux, P. E., Lew, D. P. & Foster, T. J. (1995) Adhesion properties of mutants of *Staphylococcus aureus* defective in

fibronectin-binding proteins and studies on the expression of *fnb* genes. *Mol Microbiol,* 17**,** 1143-52.

Gross, T. L., Ihrke, P. J., Walder, E. J. & Affolter, V. K. (2005) Pustular and nodular diseases without adnexal destruction. *Skin diseases of the dog and cat: clinical and histopathologic diagnosis*, pp. 406-419. 2nd ed. Iowa, Blackwell Publishing.

Grundmann, H., Hori, S., Enright, M. C., Webster, C., Tami, A., Feil, E. J. & Pitt, T. (2002) Determining the genetic structure of the natural population of *Staphylococcus aureus*: a comparison of multilocus sequence typing with pulsed-field gel electrophoresis, randomly amplified polymorphic DNA analysis, and phage typing. *J Clin Microbiol,* 40**,** 4544-6.

Grundmeier, M., Hussain, M., Becker, P., Heilmann, C., Peters, G. & Sinha, B. (2004) Truncation of fibronectin-binding proteins in *Staphylococcus aureus* strain Newman leads to deficient adherence and host cell invasion due to loss of the cell wall anchor function. *Infect Immun,* 72**,** 7155-63.

Guaguere, E., Prelaud, P. & Craig, M. (2008) Structure and physiology of skin and hair. *A Practical Guide to Canine Dermatology*, pp. 17-30. 1st ed. Paris, Merial.

Guardabassi, L., Loeber, M. E. & Jacobson, A. (2004a) Transmission of multiple antimicrobial-resistant *Staphylococcus intermedius* between dogs affected by deep pyoderma and their owners. *Vet Microbiol,* 98**,** 23-7.

Guardabassi, L., Schwarz, S. & Lloyd, D. H. (2004b) Pet animals as reservoirs of antimicrobial-resistant bacteria. *J Antimicrob Chemother,* 54**,** 321-32.

Guss, B., Uhlen, M., Nilsson, B., Lindberg, M., Sjoquist, J. & Sjodahl, J. (1984) Region X, the cell-wall-attachment part of staphylococcal protein A. *Eur J Biochem,* 138**,** 413-20.

Hajek, V. (1976) *Staphylococcus intermedius*, a new species isolated from animals. *Int. J. Syst. Bacteriol.,* 26**,** 401-408.

Hall, A. E., Domanski, P. J., Patel, P. R., Vernachio, J. H., Syribeys, P. J., Gorovits, E. L., Johnson, M. A., Ross, J. M., Hutchins, J. T. & Patti, J. M. (2003) Characterization of a protective monoclonal antibody recognizing *Staphylococcus aureus* MSCRAMM protein clumping factor A. *Infect Immun,* 71**,** 6864-70.

Halliwell, R. (2006) Revised nomenclature for veterinary allergy. *Vet Immunol Immunopathol,* 114**,** 207-8.

Hanselman, B. A., Kruth, S. & Weese, J. S. (2008) Methicillin-resistant staphylococcal colonization in dogs entering a veterinary teaching hospital. *Vet Microbiol,* 126**,** 277-81.

Hanssen, A. M. & Ericson Sollid, J. U. (2006) SCC*mec* in staphylococci: genes on the move. *FEMS Immunol Med Microbiol,* 46**,** 8-20.

Haque, R. U. & Baldwin, J. N. (1969) Purification and properties of staphylococcal beta hemolysin. II. Purification of beta hemolysin. *J Bacteriol,* 100**,** 751-9.

Harraghy, N., Hussain, M., Haggar, A., Chavakis, T., Sinha, B., Herrmann, M. & Flock, J. I. (2003) The adhesive and immunomodulating properties of the multifunctional *Staphylococcus aureus* protein Eap. *Microbiology,* 149**,** 2701-7.

Hartford, O., Francois, P., Vaudaux, P. & Foster, T. J. (1997) The dipeptide repeat region of the fibrinogen-binding protein (clumping factor) is required for

functional expression of the fibrinogen-binding domain on the *Staphylococcus aureus* cell surface. *Mol Microbiol,* 25**,** 1065-76.

Hartford, O., McDevitt, D. & Foster, T. J. (1999) Matrix-binding proteins of *Staphylococcus aureus*: functional analysis of mutant and hybrid molecules. *Microbiology,* 145 (Pt 9)**,** 2497-505.

Hartford, O. M., Wann, E. R., Hook, M. & Foster, T. J. (2001) Identification of residues in the *Staphylococcus aureus* fibrinogen-binding MSCRAMM clumping factor A (ClfA) that are important for ligand binding. *J Biol Chem,* 276**,** 2466-73.

Hartleib, J., Kohler, N., Dickinson, R. B., Chhatwal, G. S., Sixma, J. J., Hartford, O. M., Foster, T. J., Peters, G., Kehrel, B. E. & Herrmann, M. (2000) Protein A is the von Willebrand factor binding protein on *Staphylococcus aureus*. *Blood,* 96**,** 2149-56.

Hata, T. R. & Gallo, R. L. (2008) Antimicrobial peptides, skin infections, and atopic dermatitis. *Semin Cutan Med Surg,* 27(2), 144-50.

Hauck, C. R. & Ohlsen, K. (2006) Sticky connections: extracellular matrix protein recognition and integrin-mediated cellular invasion by *Staphylococcus aureus*. *Curr Opin Microbiol,* 9**,** 5-11.

Hawiger, J., Timmons, S., Kloczewiak, M., Strong, D. D. & Doolittle, R. F. (1982) gamma and alpha chains of human fibrinogen possess sites reactive with human platelet receptors. *Proc Natl Acad Sci U S A,* 79**,** 2068-71.

Hendricks, A., Schuberth, H. J., Schueler, K. & Lloyd, D. H. (2002) Frequency of superantigen-producing *Staphylococcus intermedius* isolates from canine pyoderma and proliferation-inducing potential of superantigens in dogs. *Res Vet Sci,* 73**,** 273-7.

Hermans, K., Devriese, L. A. & Haesebrouck, F. (2003) Rabbit staphylococcosis: difficult solutions for serious problems. *Vet Microbiol,* 91**,** 57-64.

Herrmann, M., Hartleib, J., Kehrel, B., Montgomery, R. R., Sixma, J. J. & Peters, G. (1997) Interaction of von Willebrand factor with *Staphylococcus aureus*. *J Infect Dis,* 176**,** 984-91.

Hesselbarth, J. & Schwarz, S. (1995) Comparative ribotyping of *Staphylococcus intermedius* from dogs, pigeons, horses and mink. *Vet Microbiol,* 45**,** 11-7.

Hesselbarth, J., Witte, W., Cuny, C., Rohde, R. & Amtsberg, G. (1994) Characterization of *Staphylococcus intermedius* from healthy dogs and cases of superficial pyoderma by DNA restriction endonuclease patterns. *Vet Microbiol,* 41**,** 259-66.

Heying, R., Van De Gevel, J., Que, Y. A., Moreillon, P. & Beekhuizen, H. (2007) Fibronectin-binding proteins and clumping factor A in *Staphylococcus aureus* experimental endocarditis: FnBPA is sufficient to activate human endothelial cells. *Thromb Haemost,* 97**,** 617-26.

Heying, R., Van De Gevel, J., Que, Y. A., Piroth, L., Moreillon, P. & Beekhuizen, H. (2009) Contribution of (sub)domains of *Staphylococcus aureus* fibronectin-binding protein to the proinflammatory and procoagulant response of human vascular endothelial cells. *Thromb Haemost,* 101**,** 495-504.

Higgins, J., Loughman, A., Van Kessel, K. P., Van Strijp, J. A. & Foster, T. J. (2006) Clumping factor A of *Staphylococcus aureus* inhibits phagocytosis by human polymorphonuclear leucocytes. *FEMS Microbiol Lett,* 258**,** 290-6.

Hochkeppel, H. K., Braun, D. G., Vischer, W., Imm, A., Sutter, S., Staeubli, U., Guggenheim, R., Kaplan, E. L., Boutonnier, A. & Fournier, J. M. (1987) Serotyping and electron microscopy studies of *Staphylococcus aureus* clinical isolates with monoclonal antibodies to capsular polysaccharide types 5 and 8. *J Clin Microbiol,* 25**,** 526-30.

Horsburgh, M. J., Aish, J. L., White, I. J., Shaw, L., Lithgow, J. K. & Foster, S. J. (2002) sigmaB modulates virulence determinant expression and stress resistance: characterization of a functional *rsbU* strain derived from *Staphylococcus aureus* 8325-4. *J Bacteriol,* 184**,** 5457-67.

Horspool, L. J., Van Laar, P., Van Den Bos, R. & Mawhinney, I. (2004) Treatment of canine pyoderma with ibafloxacin and marbofloxacin- fluoroquinolones with different pharmacokinetic profiles. *J Vet Pharmacol Ther,* 27**,** 147-53.

Horvath, C. & Neuber, A. (2007) Management of canine pyoderma. *UK Vet,* 12**,** 1-7.

House-Pompeo, K., Xu, Y., Joh, D., Speziale, P. & Hook, M. (1996) Conformational changes in the fibronectin binding MSCRAMMs are induced by ligand binding. *J Biol Chem,* 271**,** 1379-84.

Hussain, M., Haggar, A., Heilmann, C., Peters, G., Flock, J. I. & Herrmann, M. (2002) Insertional inactivation of Eap in *Staphylococcus aureus* strain Newman confers reduced staphylococcal binding to fibroblasts. *Infect Immun,* 70**,** 2933-40.

Igimi, S., Kawamura, S., Takahashi, E. & Mitsuoka, T. (1989) *Staphylococcus felis*, a new species from clinical specimens from cats. *Int J Syst Bacteriol,* 39**,** 373-377.

Igimi, S., Takahashi, E. & Mitsuoka, T. (1990) *Staphylococcus* schleiferi subsp. *coagulans* subsp. nov., isolated from the external auditory meatus of dogs with external ear otitis. *Int J Syst Bacteriol,* 40**,** 409-11.

Jarraud, S., Lyon, G. J., Figueiredo, A. M., Gerard, L., Vandenesch, F., Etienne, J., Muir, T. W. & Novick, R. P. (2000) Exfoliatin-producing strains define a fourth *agr* specificity group in *Staphylococcus aureus*. *J Bacteriol,* 182**,** 6517-22.

Jensen, P. J. & Wheelock, M. J. (1996) The relationships among adhesion, stratification and differentiation in keratinocytes. *Cell Death Differ,* 3**,** 357-71.

Ji, G., Beavis, R. & Novick, R. P. (1997) Bacterial interference caused by autoinducing peptide variants. *Science,* 276**,** 2027-30.

Ji, G., Pei, W., Zhang, L., Qiu, R., Lin, J., Benito, Y., Lina, G. & Novick, R. P. (2005) *Staphylococcus intermedius* produces a functional *agr* autoinducing peptide containing a cyclic lactone. *J Bacteriol,* 187**,** 3139-50.

Joh, D., Speziale, P., Gurusiddappa, S., Manor, J. & Hook, M. (1998) Multiple specificities of the staphylococcal and streptococcal fibronectin-binding microbial surface components recognizing adhesive matrix molecules. *Eur J Biochem,* 258**,** 897-905.

Jonas, D., Speck, M., Daschner, F. D. & Grundmann, H. (2002) Rapid PCR-based identification of methicillin-resistant *Staphylococcus aureus* from screening swabs. *J Clin Microbiol,* 40**,** 1821-3.

Jones, R. D., Kania, S. A., Rohrbach, B. W., Frank, L. A. & Bemis, D. A. (2007) Prevalence of oxacillin- and multidrug-resistant staphylococci in clinical

samples from dogs: 1,772 samples (2001-2005). *J Am Vet Med Assoc,* 230**,** 221-7.

Jonsson, K., McDevitt, D., McGavin, M. H., Patti, J. M. & Hook, M. (1995) *Staphylococcus aureus* expresses a major histocompatibility complex class II analog. *J Biol Chem,* 270**,** 21457-60.

Jonsson, K., Signas, C., Muller, H. P. & Lindberg, M. (1991) Two different genes encode fibronectin binding proteins in *Staphylococcus aureus*. The complete nucleotide sequence and characterization of the second gene. *Eur J Biochem,* 202**,** 1041-8.

Josefsson, E., Hartford, O., O'Brien, L., Patti, J. M. & Foster, T. (2001) Protection against experimental *Staphylococcus aureus* arthritis by vaccination with clumping factor A, a novel virulence determinant. *J Infect Dis,* 184**,** 1572-80.

Josefsson, E., Higgins, J., Foster, T. J. & Tarkowski, A. (2008) Fibrinogen binding sites P336 and Y338 of clumping factor A are crucial for *Staphylococcus aureus* virulence. *PLoS One,* 3**,** e2206.

Josefsson, E., McCrea, K. W., Ni Eidhin, D., O'Connell, D., Cox, J., Hook, M. & Foster, T. J. (1998a) Three new members of the serine-aspartate repeat protein multigene family of *Staphylococcus aureus*. *Microbiology,* 144 (Pt 12)**,** 3387-95.

Josefsson, E., O'Connell, D., Foster, T. J., Durussel, I. & Cox, J. A. (1998b) The binding of calcium to the B-repeat segment of SdrD, a cell surface protein of *Staphylococcus aureus*. *J Biol Chem,* 273**,** 31145-52.

Kaneko, J. & Kamio, Y. (2004) Bacterial two-component and hetero-heptameric pore-forming cytolytic toxins: structures, pore-forming mechanism, and organization of the genes. *Biosci Biotechnol Biochem,* 68**,** 981-1003.

Kania, S. A., Williamson, N. L., Frank, L. A., Wilkes, R. P., Jones, R. D. & Bemis, D. A. (2004) Methicillin resistance of staphylococci isolated from the skin of dogs with pyoderma. *Am J Vet Res,* 65**,** 1265-8.

Keane, F. M., Clarke, A. W., Foster, T. J. & Weiss, A. S. (2007a) The N-terminal A domain of *Staphylococcus aureus* fibronectin-binding protein A binds to tropoelastin. *Biochemistry,* 46**,** 7226-32.

Keane, F. M., Loughman, A., Valtulina, V., Brennan, M., Speziale, P. & Foster, T. J. (2007b) Fibrinogen and elastin bind to the same region within the A domain of fibronectin binding protein A, an MSCRAMM of *Staphylococcus aureus*. *Mol Microbiol,* 63**,** 711-23.

Kerr, I. D., Dufourcq, J., Rice, J. A., Fredkin, D. R. & Sansom, M. S. (1995) Ion channel formation by synthetic analogues of staphylococcal delta-toxin. *Biochim Biophys Acta,* 1236**,** 219-27.

Khambaty, F. M., Bennett, R. W. & Shah, D. B. (1994) Application of pulsed-field gel electrophoresis to the epidemiological characterization of *Staphylococcus intermedius* implicated in a food-related outbreak. *Epidemiol Infect,* 113**,** 75-81.

Kloczewiak, M., Timmons, S., Lukas, T. J. & Hawiger, J. (1984) Platelet receptor recognition site on human fibrinogen. Synthesis and structure-function relationship of peptides corresponding to the carboxy-terminal segment of the gamma chain. *Biochemistry,* 23**,** 1767-74.

Kluytmans, J. A. & Wertheim, H. F. (2005) Nasal carriage of *Staphylococcus aureus* and prevention of nosocomial infections. *Infection,* 33**,** 3-8.

Kohler, H. B., Huchzermeyer, B., Martin, M., De Bruin, A., Meier, B. & Nolte, I. (2001) TNF-alpha dependent NF-kappa B activation in cultured canine keratinocytes is partly mediated by reactive oxygen species. *Vet Dermatol,* 12**,** 129-37.

Koreen, L., Ramaswamy, S. V., Graviss, E. A., Naidich, S., Musser, J. M. & Kreiswirth, B. N. (2004) *spa* typing method for discriminating among *Staphylococcus aureus* isolates: implications for use of a single marker to detect genetic micro- and macrovariation. *J Clin Microbiol,* 42**,** 792-9.

Kreikemeyer, B., McDevitt, D. & Podbielski, A. (2002) The role of the map protein in *Staphylococcus aureus* matrix protein and eukaryotic cell adherence. *Int J Med Microbiol,* 292**,** 283-95.

Kugelberg, E., Norström, T., Petersen, T. K., Duvold, T., Andersson, D. I. & Hughes, D. (2005) Establishment of a superficial skin infection model in mice by using *Staphylococcus aureus* and *Streptococcus pyogenes*. *Antimicrob Agents Chemother,* 49(8), 3435-41.

Kumar, S., Tamura, K. & Nei, M. (2004) MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Brief Bioinform,* 5**,** 150-63.

Kuroda, M., Ohta, T., Uchiyama, I., Baba, T., Yuzawa, H., Kobayashi, I., Cui, L., Oguchi, A., Aoki, K., Nagai, Y., Lian, J., Ito, T., Kanamori, M., Matsumaru, H., Maruyama, A., Murakami, H., Hosoyama, A., Mizutani-Ui, Y., Takahashi, N. K., Sawano, T., Inoue, R., Kaito, C., Sekimizu, K., Hirakawa, H., Kuhara, S., Goto, S., Yabuzaki, J., Kanehisa, M., Yamashita, A., Oshima, K., Furuya, K., Yoshino, C., Shiba, T., Hattori, M., Ogasawara, N., Hayashi, H. & Hiramatsu, K. (2001) Whole genome sequencing of meticillin-resistant *Staphylococcus aureus*. *Lancet,* 357**,** 1225-40.

Kuypers, J. M. & Proctor, R. A. (1989) Reduced adherence to traumatized rat heart valves by a low-fibronectin-binding mutant of *Staphylococcus aureus*. *Infect Immun,* 57**,** 2306-12.

Kwochka, K. W. & Kowalski, J. J. (1991) Prophylactic efficacy of four antibacterial shampoos against *Staphylococcus intermedius* in dogs. *Am J Vet Res,* 52**,** 115-8.

Kwochka, K. W. & Rademakers, A. M. (1989) Cell proliferation of epidermis, hair follicles, and sebaceous glands of beagles and cocker spaniels with healthy skin. *Am J Vet Res,* 50**,** 587-91.

Kwok, A. Y. & Chow, A. W. (2003) Phylogenetic study of *Staphylococcus* and Macrococcus species based on partial hsp60 gene sequences. *Int J Syst Evol Microbiol,* 53**,** 87-92.

Kwok, A. Y., Su, S. C., Reynolds, R. P., Bay, S. J., Av-Gay, Y., Dovichi, N. J. & Chow, A. W. (1999) Species identification and phylogenetic relationships based on partial *HSP60* gene sequences within the genus *Staphylococcus*. *Int J Syst Bacteriol,* 49 Pt 3**,** 1181-92.

Labandeira-Rey, M., Couzon, F., Boisset, S., Brown, E. L., Bes, M., Benito, Y., Barbu, E. M., Vazquez, V., Hook, M., Etienne, J., Vandenesch, F. & Bowden, M. G. (2007) *Staphylococcus aureus* Panton-Valentine leukocidin causes necrotizing pneumonia. *Science,* 315**,** 1130-3.

Ladhani, S. (2003) Understanding the mechanism of action of the exfoliative toxins of *Staphylococcus aureus*. *FEMS Immunol Med Microbiol,* 39**,** 181-9.

Lammers, A., Nuijten, P. J. & Smith, H. E. (1999) The fibronectin binding proteins of *Staphylococcus aureus* are required for adhesion to and invasion of bovine mammary gland cells. *FEMS Microbiol Lett,* 180**,** 103-9.

Lautz, S., Kanbar, T., Alber, J., Lammler, C., Weiss, R., Prenger-Berninghoff, E. & Zschock, M. (2006) Dissemination of the gene encoding exfoliative toxin of *Staphylococcus intermedius* among strains isolated from dogs during routine microbiological diagnostics. *J Vet Med B Infect Dis Vet Public Health,* 53**,** 434-8.

Le Loir, Y., Baron, F. & Gautier, M. (2003) *Staphylococcus aureus* and food poisoning. *Genet Mol Res,* 2**,** 63-76.

Lee, J. H. (2003) Methicillin (Oxacillin)-resistant *Staphylococcus aureus* strains isolated from major food animals and their potential transmission to humans. *Appl Environ Microbiol,* 69**,** 6489-94.

Lee, L. Y., Miyamoto, Y. J., McIntyre, B. W., Hook, M., McCrea, K. W., McDevitt, D. & Brown, E. L. (2002) The *Staphylococcus aureus* Map protein is an immunomodulator that interferes with T cell-mediated responses. *J Clin Invest,* 110**,** 1461-71.

Lindsay, J. A., Moore, C. E., Day, N. P., Peacock, S. J., Witney, A. A., Stabler, R. A., Husain, S. E., Butcher, P. D. & Hinds, J. (2006) Microarrays reveal that each of the ten dominant lineages of *Staphylococcus aureus* has a unique combination of surface-associated and regulatory genes. *J Bacteriol,* 188**,** 669-76.

Littlewood, J. D., Lakhani, K. H., Paterson, S., Wood, J. L. & Chanter, N. (1999) Clindamycin hydrochloride and clavulanate-amoxycillin in the treatment of canine superficial pyoderma. *Vet Rec,* 144**,** 662-5.

Liu, Y., Ames, B., Gorovits, E., Prater, B. D., Syribeys, P., Vernachio, J. H. & Patti, J. M. (2004) SdrX, a serine-aspartate repeat protein expressed by *Staphylococcus capitis* with collagen VI binding activity. *Infect Immun,* 72**,** 6237-44.

Lloyd, D. H. (2007) Reservoirs of antimicrobial resistance in pet animals. *Clin Infect Dis,* 45 Suppl 2**,** S148-52.

Lloyd, D. H., Allaker, R. P. & Pattinson, A. (1991) Carriage of *Staphylococcus intermedius* on the Ventral Abdomen of Clinically Normal Dogs and Those With Pyoderma. *Vet Dermatol,* 2**,** 161-164.

Lloyd, D. H. & Lamport, A. I. (1999) Activity of chlorhexidine shampoos *in vitro* against *Staphylococcus intermedius*, *Pseudomonas aeruginosa* and *Malassezia pachydermatis*. *Vet Rec,* 144**,** 536-7.

Lloyd, D. H., Lamport, A. I., Noble, W. C. & Howell, S. A. (1999) Fluoroquinolone resistance in *Staphylococcus intermedius*. *Vet Dermatol* 10**,** 249-51.

Lloyd, D. H., Viac, J., Werling, D., Reme, C. A. & Gatto, H. (2007) Role of sugars in surface microbe-host interactions and immune reaction modulation. *Vet Dermatol,* 18**,** 197-204.

Lodish, H., Berk, A., Zipursky, L. S., Matsudaira, P., Baltimore, D., & Darnell, J. (2000) Integrating Cells into Tissues. *Molecular cell biology*, pp. 197-244. 4th ed. New York, W. H. Freeman Company.

Loeffler, A., Boag, A. K., Sung, J., Lindsay, J. A., Guardabassi, L., Dalsgaard, A., Smith, H., Stevens, K. B. & Lloyd, D. H. (2005) Prevalence of methicillin-

resistant *Staphylococcus aureus* among staff and pets in a small animal referral hospital in the UK. *J Antimicrob Chemother,* 56**,** 692-7.

Loeffler, A., Kearns, A. M., Ellington, M. J., Smith, L. J., Unt, V. E., Lindsay, J. A., Pfeiffer, D. U. & Lloyd, D. H. (2009) First isolation of MRSA ST398 from UK animals: a new challenge for infection control teams? *J Hosp Infect,* 72**,** 269-71.

Loeffler, A., Linek, M., Moodley, A., Guardabassi, L., Sung, J. M., Winkler, M., Weiss, R. & Lloyd, D. H. (2007) First report of multiresistant, *mecA*-positive *Staphylococcus intermedius* in Europe: 12 cases from a veterinary dermatology referral clinic in Germany. *Vet Dermatol,* 18**,** 412-21.

Loughman, A., Fitzgerald, J. R., Brennan, M. P., Higgins, J., Downer, R., Cox, D. & Foster, T. J. (2005) Roles for fibrinogen, immunoglobulin and complement in platelet activation promoted by *Staphylococcus aureus* clumping factor A. *Mol Microbiol,* 57**,** 804-18.

Loughman, A., Sweeney, T., Keane, F. M., Pietrocola, G., Speziale, P. & Foster, T. J. (2008) Sequence diversity in the A domain of *Staphylococcus aureus* fibronectin-binding protein A. *BMC Microbiol,* 8**,** 74.

Lowy, F. D. (2000) Is *Staphylococcus aureus* an intracellular pathogen? *Trends Microbiol,* 8**,** 341-3.

Lu, Y. F. & McEwan, N. A. (2007) Staphylococcal and micrococcal adherence to canine and feline corneocytes: quantification using a simple adhesion assay. *Vet Dermatol,* 18**,** 29-35.

Lyon, G. J. & Novick, R. P. (2004) Peptide signaling in *Staphylococcus aureus* and other Gram-positive bacteria. *Peptides,* 25**,** 1389-403.

Maeda, S., Shibata, S., Chimura, N. & Fukata, T. (2009) House dust mite major allergen Der f 1 enhances proinflammatory cytokine and chemokine gene expression in a cell line of canine epidermal keratinocytes. *Vet Immunol Immunopathol*.

Manian, F. A. (2003) Asymptomatic nasal carriage of mupirocin-resistant, methicillin-resistant *Staphylococcus aureus* (MRSA) in a pet dog associated with MRSA infection in household contacts. *Clin Infect Dis,* 36**,** e26-8.

Martin, D. P., Williamson, C. & Posada, D. (2005) RDP2: recombination detection and analysis from sequence alignments. *Bioinformatics,* 21**,** 260-2.

Mascari, L. M. & Ross, J. M. (2003) Quantification of staphylococcal-collagen binding interactions in whole blood by use of a confocal microscopy shear-adhesion assay. *J Infect Dis,* 188**,** 98-107.

Massey, R. C., Kantzanou, M. N., Fowler, T., Day, N. P., Schofield, K., Wann, E. R., Berendt, A. R., Hook, M. & Peacock, S. J. (2001) Fibronectin-binding protein A of *Staphylococcus aureus* has multiple, substituting, binding regions that mediate adherence to fibronectin and invasion of endothelial cells. *Cell Microbiol,* 3**,** 839-51.

May, E. R., Hnilica, K. A., Frank, L. A., Jones, R. D. & Bemis, D. A. (2005) Isolation of *Staphylococcus schleiferi* from healthy dogs and dogs with otitis, pyoderma, or both. *J Am Vet Med Assoc,* 227**,** 928-31.

McAleese, F. M., Walsh, E. J., Sieprawska, M., Potempa, J. & Foster, T. J. (2001) Loss of clumping factor B fibrinogen binding activity by *Staphylococcus aureus* involves cessation of transcription, shedding and cleavage by metalloprotease. *J Biol Chem,* 276**,** 29969-78.

McCrea, K. W., Hartford, O., Davis, S., Eidhin, D. N., Lina, G., Speziale, P., Foster, T. J. & Hook, M. (2000) The serine-aspartate repeat (Sdr) protein family in *Staphylococcus epidermidis*. *Microbiology,* 146 (Pt 7)**,** 1535-46.

McDevitt, D. & Foster, T. J. (1995) Variation in the size of the repeat region of the fibrinogen receptor (clumping factor) of *Staphylococcus aureus* strains. *Microbiology,* 141 (Pt 4)**,** 937-43.

McDevitt, D., Francois, P., Vaudaux, P. & Foster, T. J. (1994) Molecular characterization of the clumping factor (fibrinogen receptor) of *Staphylococcus aureus*. *Mol Microbiol,* 11**,** 237-48.

McDevitt, D., Francois, P., Vaudaux, P. & Foster, T. J. (1995) Identification of the ligand-binding domain of the surface-located fibrinogen receptor (clumping factor) of *Staphylococcus aureus*. *Mol Microbiol,* 16**,** 895-907.

McDevitt, D., Nanavaty, T., House-Pompeo, K., Bell, E., Turner, N., McIntire, L., Foster, T. & Hook, M. (1997) Characterization of the interaction between the *Staphylococcus aureus* clumping factor (ClfA) and fibrinogen. *Eur J Biochem,* 247**,** 416-24.

McEwan, N. A. (2000) Adherence by *Staphylococcus intermedius* to canine keratinocytes in atopic dermatitis. *Res Vet Sci,* 68**,** 279-83.

McEwan, N. A., Kalna, G. & Mellor, D. (2005) A comparison of adherence by four strains of *Staphylococcus intermedius* and *Staphylococcus hominis* to canine corneocytes collected from normal dogs and dogs suffering from atopic dermatitis. *Res Vet Sci,* 78**,** 193-8.

McEwan, N. A., Mellor, D. & Kalna, G. (2006a) Adherence by *Staphylococcus intermedius* to canine corneocytes: a preliminary study comparing noninflamed and inflamed atopic canine skin. *Vet Dermatol,* 17**,** 151-4.

McEwan, N. A., Reme, C. A. & Gatto, H. (2006b) Sugar inhibition of adherence by *Staphylococcus intermedius* to canine corneocytes. *Vet Dermatol,* 17**,** 358.

Medleau, L., Long, R. E., Brown, J. & Miller, W. H. (1986) Frequency and antimicrobial susceptibility of *Staphylococcus* species isolated from canine pyodermas. *Am J Vet Res,* 47**,** 229-31.

Melchior, M. B., Vaarkamp, H. & Fink-Gremmels, J. (2006) Biofilms: a role in recurrent mastitis infections? *Vet J,* 171**,** 398-407.

Mellmann, A., Weniger, T., Berssenbrugge, C., Rothganger, J., Sammeth, M., Stoye, J. & Harmsen, D. (2007) Based Upon Repeat Pattern (BURP): an algorithm to characterize the long-term evolution of *Staphylococcus aureus* populations based on *spa* polymorphisms. *BMC Microbiol,* 7**,** 98.

Mendoza, M., Meugnier, H., Bes, M., Etienne, J. & Freney, J. (1998) Identification of *Staphylococcus* species by 16S-23S rDNA intergenic spacer PCR analysis. *Int J Syst Bacteriol,* 48 Pt 3**,** 1049-55.

Menestrina, G., Dalla Serra, M., Comai, M., Coraiola, M., Viero, G., Werner, S., Colin, D. A., Monteil, H. & Prevost, G. (2003) Ion channels and bacterial infection: the case of beta-barrel pore-forming protein toxins of *Staphylococcus aureus*. *FEBS Lett,* 552**,** 54-60.

Meyer, S. A. & Schleifer, K. H. (1978) Deoxyribonucleic acid reassociation in the classification of coagulase-positive staphylococci. *Arch Microbiol,* 117**,** 183-8.

Miragaia, M., Thomas, J. C., Couto, I., Enright M. C. & de Lencastre. H. (2007) Inferring a population structure for *Staphylococcus epidermidis* from multilocus sequence typing data. *J Bacteriol,* 189(6), 2540-52.

Miyake, Y., Sugai, M., Kohada, A., Minagi, S. & Suginaka, H. (1990) Adherence of *Staphylococcus aureus* to cultured epidermal cells during differentiation. *J Med Microbiol,* 32**,** 9-14.

Molne, L. & Tarkowski, A. (2000) An experimental model of cutaneous infection induced by superantigen-producing Staphylococcus aureus. *J Invest Dermatol,* 114(6), 1120-5.

Moodley, A., Stegger, M., Bagcigil, A. F., Baptiste, K. E., Loeffler, A., Lloyd, D. H., Williams, N. J., Leonard, N., Abbott, Y., Skov, R. & Guardabassi, L. (2006) *spa* typing of methicillin-resistant *Staphylococcus aureus* isolated from domestic animals and veterinary staff in the UK and Ireland. *J Antimicrob Chemother,* 58**,** 1118-23.

Moodley, A., Stegger, M., Ben Zakour, N. L., Fitzgerald, J. R. & Guardabassi, L. (2009) Tandem repeat sequence analysis of staphylococcal protein A (*spa*) gene in methicillin-resistant *Staphylococcus pseudintermedius*. *Vet Microbiol,* 135**,** 320-6.

Moreillon, P., Entenza, J. M., Francioli, P., McDevitt, D., Foster, T. J., Francois, P. & Vaudaux, P. (1995) Role of *Staphylococcus aureus* coagulase and clumping factor in pathogenesis of experimental endocarditis. *Infect Immun,* 63**,** 4738-43.

Morris, D. O., Mauldin, E. A., O'Shea, K., Shofer, F. S. & Rankin, S. C. (2006a) Clinical, microbiological, and molecular characterization of methicillin-resistant *Staphylococcus aureus* infections of cats. *Am J Vet Res,* 67**,** 1421-5.

Morris, D. O., Rook, K. A., Shofer, F. S. & Rankin, S. C. (2006b) Screening of *Staphylococcus aureus*, *Staphylococcus intermedius*, and *Staphylococcus schleiferi* isolates obtained from small companion animals for antimicrobial resistance: a retrospective review of 749 isolates (2003-04). *Vet Dermatol,* 17**,** 332-7.

Mosesson, M. W. (2005) Fibrinogen and fibrin structure and functions. *J Thromb Haemost,* 3**,** 1894-904.

Mosesson, M. W. & Amrani, D. L. (1980) The structure and biologic activities of plasma fibronectin. *Blood,* 56**,** 145-58.

Muller, A., Talon, D., Potier, A., Belle, E., Cappelier, G. & Bertrand, X. (2005) Use of intranasal mupirocin to prevent methicillin-resistant *Staphylococcus aureus* infection in intensive care units. *Crit Care,* 9**,** R246-50.

Murakami, M., Ohtake, T., Dorschner, R. A., Schittek, B., Garbe, C. & Gallo, R. L. (2002) Cathelicidin anti-microbial peptide expression in sweat, an innate defense system for the skin. *J Invest Dermatol,* 119(5), 1090-5.

Murray, P. R., Rosenthal, K. S., Kobayashi, G. S. & Pfaller, M. A. (2002) *Staphylococcus* and Related Organisms. *Medical Microbiology*, pp. 202-216. 4th ed. St. Louis, Missouri., Mosby, Inc.

Nair, S. P., Williams, R. J. & Henderson, B. (2000) Advances in our understanding of the bone and joint pathology caused by *Staphylococcus aureus* infection. *Rheumatology (Oxford),* 39**,** 821-34.

Nanra, J. S., Timofeyeva, Y., Buitrago, S. M., Sellman, B. R., Dilts, D. A., Fink, P., Nunez, L., Hagen, M., Matsuka, Y. V., Mininni, T., Zhu, D., Pavliak, V.,

Green, B. A., Jansen, K. U. & Anderson, A. S. (2009) Heterogeneous *in vivo* expression of clumping factor A and capsular polysaccharide by *Staphylococcus aureus*: implications for vaccine design. *Vaccine,* 27**,** 3276-80.

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

Neuber, A. E., Thoday, K. L. & Hill, P. B. (2008) Immunoglobulin G responses in 21 dogs with skin diseases to antigens from different isolates of *Staphylococcus intermedius*. *Vet Rec,* 162**,** 75-9.

Ni Eidhin, D., Perkins, S., Francois, P., Vaudaux, P., Hook, M. & Foster, T. J. (1998) Clumping factor B (ClfB), a new surface-located fibrinogen-binding adhesin of *Staphylococcus aureus*. *Mol Microbiol,* 30**,** 245-57.

Nishifuji, K., Sugai, M. & Amagai, M. (2008) Staphylococcal exfoliative toxins: "molecular scissors" of bacteria that attack the cutaneous defense barrier in mammals. *J Dermatol Sci,* 49**,** 21-31.

Novick, R. P. (2003) Autoinduction and signal transduction in the regulation of staphylococcal virulence. *Mol Microbiol,* 48**,** 1429-49.

Novick, R. P. & Geisinger, E. (2008) Quorum sensing in staphylococci. *Annu Rev Genet,* 42**,** 541-64.

O'Brien, L., Kerrigan, S. W., Kaw, G., Hogan, M., Penades, J., Litt, D., Fitzgerald, D. J., Foster, T. J. & Cox, D. (2002a) Multiple mechanisms for the activation of human platelet aggregation by *Staphylococcus aureus*: roles for the clumping factors ClfA and ClfB, the serine-aspartate repeat protein SdrE and protein A. *Mol Microbiol,* 44**,** 1033-44.

O'Brien, L. M., Walsh, E. J., Massey, R. C., Peacock, S. J. & Foster, T. J. (2002b) *Staphylococcus aureus* clumping factor B (ClfB) promotes adherence to human type I cytokeratin 10: implications for nasal colonization. *Cell Microbiol,* 4**,** 759-70.

O'Connell, D. P., Nanavaty, T., McDevitt, D., Gurusiddappa, S., Hook, M. & Foster, T. J. (1998) The fibrinogen-binding MSCRAMM (clumping factor) of *Staphylococcus aureus* has a Ca2+-dependent inhibitory site. *J Biol Chem,* 273**,** 6821-9.

O'Riordan, K. & Lee, J. C. (2004) *Staphylococcus aureus* capsular polysaccharides. *Clin Microbiol Rev,* 17**,** 218-34.

Oliveira, L. C., Leite, C. A., Brilhante, R. S. & Carvalho, C. B. (2008) Comparative study of the microbial profile from bilateral canine otitis externa. *Can Vet J,* 49**,** 785-8.

Olivry, T. (2008) Current issues of controversy in canine allergy. *Proceedings of the continuing education programme, World Congress of Veterinary Dermatology 6,* pp. 154-159. *19-22 November 2008.* Hong Kong.

Olivry, T., DeBoer, D. J., Griffin, C. E., Halliwell, R. E., Hill, P. B., Hillier, A., Marsella, R. & Sousa, C. A. (2001) The ACVD task force on canine atopic dermatitis: forewords and lexicon. *Vet Immunol Immunopathol,* 81**,** 143-6.

Otto, M. (2001) *Staphylococcus aureus* and *Staphylococcus epidermidis* peptide pheromones produced by the accessory gene regulator *agr* system. *Peptides,* 22**,** 1603-8.

Otto, M. (2008) Staphylococcal biofilms. *Curr Top Microbiol Immunol,* 322**,** 207-28.

Otto, M. (2008) Targeted immunotherapy for staphylococcal infections: focus on anti-MSCRAMM antibodies. *BioDrugs,* 22(1), 27-36.

Otto, M., Echner, H., Voelter, W. & Gotz, F. (2001) Pheromone cross-inhibition between *Staphylococcus aureus* and *Staphylococcus epidermidis*. *Infect Immun,* 69**,** 1957-60.

Palma, M., Haggar, A. & Flock, J. I. (1999) Adherence of *Staphylococcus aureus* is enhanced by an endogenous secreted protein with broad binding activity. *J Bacteriol,* 181**,** 2840-5.

Palmqvist, N., Foster, T., Tarkowski, A. & Josefsson, E. (2002) Protein A is a virulence factor in *Staphylococcus aureus* arthritis and septic death. *Microb Pathog,* 33**,** 239-49.

Pankov, R. & Yamada, K. M. (2002) Fibronectin at a glance. *J Cell Sci,* 115**,** 3861-3.

Park, P. W., Broekelmann, T. J., Mecham, B. R. & Mecham, R. P. (1999) Characterization of the elastin binding domain in the cell-surface 25-kDa elastin-binding protein of *Staphylococcus aureus* (EbpS). *J Biol Chem,* 274**,** 2845-50.

Park, P. W., Roberts, D. D., Grosso, L. E., Parks, W. C., Rosenbloom, J., Abrams, W. R. & Mecham, R. P. (1991) Binding of elastin to *Staphylococcus aureus*. *J Biol Chem,* 266**,** 23399-406.

Park, P. W., Rosenbloom, J., Abrams, W. R., Rosenbloom, J. & Mecham, R. P. (1996) Molecular cloning and expression of the gene for elastin-binding protein (*ebpS*) in *Staphylococcus aureus*. *J Biol Chem,* 271**,** 15803-9.

Patel, A. & Forsythe, P. J. (2008) Staphylococcal pyoderma. *Small Animal Dermatology*, pp. 161-168. 1st ed. Oxford, Saunders Elsevier.

Patti, J. M. (2004) A humanized monoclonal antibody targeting *Staphylococcus aureus*. *Vaccine,* 22 Suppl 1**,** S39-43.

Patti, J. M., Allen, B. L., McGavin, M. J. & Hook, M. (1994a) MSCRAMM-mediated adherence of microorganisms to host tissues. *Annu Rev Microbiol,* 48**,** 585-617.

Patti, J. M., Bremell, T., Krajewska-Pietrasik, D., Abdelnour, A., Tarkowski, A., Ryden, C. & Hook, M. (1994b) The *Staphylococcus aureus* collagen adhesin is a virulence determinant in experimental septic arthritis. *Infect Immun,* 62**,** 152-61.

Patti, J. M., Jonsson, H., Guss, B., Switalski, L. M., Wiberg, K., Lindberg, M. & Hook, M. (1992) Molecular characterization and expression of a gene encoding a *Staphylococcus aureus* collagen adhesin. *J Biol Chem,* 267**,** 4766-72.

Peacock, S. J., Foster, T. J., Cameron, B. J. & Berendt, A. R. (1999) Bacterial fibronectin-binding proteins and endothelial cell surface fibronectin mediate adherence of *Staphylococcus aureus* to resting human endothelial cells. *Microbiology,* 145 (Pt 12)**,** 3477-86.

Peacock, S. J., Moore, C. E., Justice, A., Kantzanou, M., Story, L., Mackie, K., O'Neill, G. & Day, N. P. (2002) Virulent combinations of adhesin and toxin genes in natural populations of *Staphylococcus aureus*. *Infect Immun,* 70**,** 4987-96.

Peikes, H., Morris, D. O. & Hess, R. S. (2001) Dermatologic disorders in dogs with diabetes mellitus: 45 cases (1986-2000). *J Am Vet Med Assoc,* 219**,** 203-8.

Perkins, S., Walsh, E. J., Deivanayagam, C. C., Narayana, S. V., Foster, T. J. & Hook, M. (2001) Structural organization of the fibrinogen-binding region of the clumping factor B MSCRAMM of *Staphylococcus aureus*. *J Biol Chem,* 276**,** 44721-8.

Pin, D. (2008) Bacterial overgrowth syndrome and mucocutaneous pyoderma. *Proceedings of the continuing education programme, World Congress of Veterinary Dermatology*, pp. 145-149. *19-22 November 2008.* Hong Kong.

Pin, D., Carlotti, D. N., Jasmin, P., DeBoer, D. J. & Prelaud, P. (2006) Prospective study of bacterial overgrowth syndrome in eight dogs. *Vet Rec,* 158**,** 437-41.

Pinchbeck, L. R., Cole, L. K., Hillier, A., Kowalski, J. J., Rajala-Schultz, P. J., Bannerman, T. L. & York, S. (2006) Genotypic relatedness of staphylococcal strains isolated from pustules and carriage sites in dogs with superficial bacterial folliculitis. *Am J Vet Res,* 67**,** 1337-46.

Plano, L. R. (2004) *Staphylococcus aureus* exfoliative toxins: how they cause disease. *J Invest Dermatol,* 122**,** 1070-7.

Ponnuraj, K., Bowden, M. G., Davis, S., Gurusiddappa, S., Moore, D., Choe, D., Xu, Y., Hook, M. & Narayana, S. V. (2003) A "dock, lock, and latch" structural model for a staphylococcal adhesin binding to fibrinogen. *Cell,* 115**,** 217-28.

Pottumarthy, S., Schapiro, J. M., Prentice, J. L., Houze, Y. B., Swanzy, S. R., Fang, F. C. & Cookson, B. T. (2004) Clinical isolates of *Staphylococcus intermedius* masquerading as methicillin-resistant *Staphylococcus aureus*. *J Clin Microbiol,* 42**,** 5881-4.

Prevost, G., Bouakham, T., Piemont, Y. & Monteil, H. (1995) Characterisation of a synergohymenotropic toxin produced by *Staphylococcus intermedius*. *FEBS Lett,* 376**,** 135-40.

Que, Y. A., Francois, P., Haefliger, J. A., Entenza, J. M., Vaudaux, P. & Moreillon, P. (2001) Reassessing the role of *Staphylococcus aureus* clumping factor and fibronectin-binding protein by expression in *Lactococcus lactis*. *Infect Immun,* 69**,** 6296-302.

Que, Y. A., Haefliger, J. A., Francioli, P. & Moreillon, P. (2000) Expression of *Staphylococcus aureus* clumping factor A in *Lactococcus lactis* subsp. *cremoris* using a new shuttle vector. *Infect Immun,* 68**,** 3516-22.

Que, Y. A., Haefliger, J. A., Piroth, L., Francois, P., Widmer, E., Entenza, J. M., Sinha, B., Herrmann, M., Francioli, P., Vaudaux, P. & Moreillon, P. (2005) Fibrinogen and fibronectin binding cooperate for valve infection and invasion in *Staphylococcus aureus* experimental endocarditis. *J Exp Med,* 201**,** 1627-35.

Raus, J. & Love, D. N. (1983) Characterization of coagulase-positive *Staphylococcus intermedius* and *Staphylococcus aureus* isolated from veterinary clinical specimens. *J Clin Microbiol,* 18**,** 789-92.

Raz, R., Colodner, R. & Kunin, C. M. (2005) Who are you- *Staphylococcus saprophyticus*? *Clin Infect Dis,* 40**,** 896-8.

Reilly, S. S., Hudson, M. C., Kellam, J. F. & Ramp, W. K. (2000) *In vivo* internalization of *Staphylococcus aureus* by embryonic chick osteoblasts. *Bone,* 26**,** 63-70.

Rhem, M. N., Lech, E. M., Patti, J. M., McDevitt, D., Hook, M., Jones, D. B. & Wilhelmus, K. R. (2000) The collagen-binding adhesin is a virulence factor in *Staphylococcus aureus* keratitis. *Infect Immun,* 68**,** 3776-9.

Rich, R. L., Deivanayagam, C. C., Owens, R. T., Carson, M., Hook, A., Moore, D., Symersky, J., Yang, V. W., Narayana, S. V. & Hook, M. (1999) Trench-shaped binding sites promote multiple classes of interactions between collagen and the adherence receptors, alpha(1)beta(1) integrin and *Staphylococcus aureus* cna MSCRAMM. *J Biol Chem,* 274**,** 24906-13.

Rivera, J., Vannakambadi, G., Hook, M. & Speziale, P. (2007) Fibrinogen-binding proteins of Gram-positive bacteria. *Thromb Haemost,* 98**,** 503-11.

Robinson, D. A., Monk, A. B., Cooper, J. E., Feil, E. J. & Enright, M. C. (2005) Evolutionary genetics of the accessory gene regulator (*agr*) locus in *Staphylococcus aureus*. *J Bacteriol,* 187**,** 8312-21.

Roche, F. M., Downer, R., Keane, F., Speziale, P., Park, P. W. & Foster, T. J. (2004) The N-terminal A domain of fibronectin-binding proteins A and B promotes adhesion of *Staphylococcus aureus* to elastin. *J Biol Chem,* 279**,** 38433-40.

Roche, F. M., Massey, R., Peacock, S. J., Day, N. P., Visai, L., Speziale, P., Lam, A., Pallen, M. & Foster, T. J. (2003) Characterization of novel LPXTG-containing proteins of *Staphylococcus aureus* identified from genome sequences. *Microbiology,* 149**,** 643-54.

Rodgers, U. R. & Weiss, A. S. (2005) Cellular interactions with elastin. *Pathol Biol (Paris),* 53**,** 390-8.

Rooney, M. M., Parise, L. V. & Lord, S. T. (1996) Dissecting clot retraction and platelet aggregation. Clot retraction does not require an intact fibrinogen gamma chain C terminus. *J Biol Chem,* 271**,** 8553-5.

Ruggeri, Z. M. & Ware, J. (1993) von Willebrand factor. *Faseb J,* 7**,** 308-16.

Ruscher, C., Lubke-Becker, A., Wleklinski, C. G., Soba, A., Wieler, L. H. & Walther, B. (2009) Prevalence of Methicillin-resistant *Staphylococcus pseudintermedius* isolated from clinical samples of companion animals and equidaes. *Vet Microbiol,* 136**,** 197-201.

Ryden, C., Tung, H. S., Nikolaev, V., Engstrom, A. & Oldberg, A. (1997) *Staphylococcus aureus* causing osteomyelitis binds to a nonapeptide sequence in bone sialoprotein. *Biochem J,* 327 (Pt 3)**,** 825-9.

Sabat, A., Melles, D. C., Martirosian, G., Grundmann, H., Van Belkum, A. & Hryniewicz, W. (2006) Distribution of the serine-aspartate repeat protein-encoding *sdr* genes among nasal-carriage and invasive *Staphylococcus aureus* strains. *J Clin Microbiol,* 44**,** 1135-8.

Saijonmaa-Koulumies, L. E. & Lloyd, D. H. (1996) Colonization of the canine skin with bacteria. *Vet Dermatol,* 7**,** 153-162.

Saijonmaa-Koulumies, L. E. & Lloyd, D. H. (2002a) Adherence of *Staphylococcus intermedius* to canine corneocytes *in vitro*. *Vet Dermatol,* 13**,** 169-76.

Saijonmaa-Koulumies, L. E. & Lloyd, D. H. (2002b) Colonization of neonatal puppies by *Staphylococcus intermedius*. *Vet Dermatol,* 13**,** 123-30.

Sakai, H., Procop, G. W., Kobayashi, N., Togawa, D., Wilson, D. A., Borden, L., Krebs, V. & Bauer, T. W. (2004) Simultaneous detection of *Staphylococcus aureus* and coagulase-negative staphylococci in positive blood cultures by real-time PCR with two fluorescence resonance energy transfer probe sets. *J Clin Microbiol,* 42**,** 5739-44.

Sakinc, T., Kleine, B. & Gatermann, S. G. (2006) SdrI, a serine-aspartate repeat protein identified in *Staphylococcus saprophyticus* strain 7108, is a collagen-binding protein. *Infect Immun,* 74**,** 4615-23.

Sandoe, J. A., Kerr, K. G., Reynolds, G. W. & Jain, S. (1999) *Staphylococcus capitis* endocarditis: two cases and review of the literature. *Heart,* 82**,** e1.

Saravia-Otten, P., Muller, H. P. & Arvidson, S. (1997) Transcription of *Staphylococcus aureus* fibronectin binding protein genes is negatively regulated by *agr* and an *agr*-independent mechanism. *J Bacteriol,* 179**,** 5259-63.

Sasaki, T., Kikuchi, K., Tanaka, Y., Takahashi, N., Kamata, S. & Hiramatsu, K. (2007a) Methicillin-resistant *Staphylococcus pseudintermedius* in a veterinary teaching hospital. *J Clin Microbiol,* 45**,** 1118-25.

Sasaki, T., Kikuchi, K., Tanaka, Y., Takahashi, N., Kamata, S. & Hiramatsu, K. (2007b) Reclassification of phenotypically identified *Staphylococcus intermedius* strains. *J Clin Microbiol,* 45**,** 2770-8.

Sato, H., Watanabe, T., Murata, Y., Ohtake, A., Nakamura, M., Aizawa, C., Saito, H. & Maehara, N. (1999) New exfoliative toxin produced by a plasmid-carrying strain of *Staphylococcus hyicus*. *Infect Immun,* 67**,** 4014-8.

Schaffer, A. C., Solinga, R. M., Cocchiaro, J., Portoles, M., Kiser, K. B., Risley, A., Randall, S. M., Valtulina, V., Speziale, P., Walsh, E., Foster, T. & Lee, J. C. (2006) Immunization with *Staphylococcus aureus* clumping factor B, a major determinant in nasal carriage, reduces nasal colonization in a murine model. *Infect Immun,* 74**,** 2145-53.

Schissler, J. R., Hillier, A., Daniels, J. B., Cole, L. K. & Gebreyes, W. A. (2009) Evaluation of Clinical Laboratory Standards Institute interpretive criteria for methicillin-resistant *Staphylococcus pseudintermedius* isolated from dogs. *J Vet Diagn Invest,* 21**,** 684-8.

Schlievert, P. M. (1982) Enhancement of host susceptibility to lethal endotoxin shock by staphylococcal pyrogenic exotoxin type C. *Infect Immun,* 36**,** 123-8.

Schmid-Grendelmeier, P., Simon, D., Simon, H. U., Akdis, C. A. & Wuthrich, B. (2001) Epidemiology, clinical features, and immunology of the "intrinsic" (non-IgE-mediated) type of atopic dermatitis (constitutional dermatitis). *Allergy,* 56**,** 841-9.

Schmitt, C. K., Meysick, K. C. & O'Brien, A. D. (1999) Bacterial toxins: friends or foes? *Emerg Infect Dis,* 5**,** 224-34.

Schwarz-Linek, U., Hook, M. & Potts, J. R. (2004) The molecular basis of fibronectin-mediated bacterial adherence to host cells. *Mol Microbiol,* 52**,** 631-41.

Schwarz-Linek, U., Hook, M. & Potts, J. R. (2006) Fibronectin-binding proteins of Gram-positive cocci. *Microbes Infect,* 8**,** 2291-8.

Schwarz-Linek, U., Plevin, M. J., Pickford, A. R., Hook, M., Campbell, I. D. & Potts, J. R. (2001) Binding of a peptide from a *Streptococcus dysgalactiae* MSCRAMM to the N-terminal F1 module pair of human fibronectin involves both modules. *FEBS Lett,* 497**,** 137-40.

Schwarz-Linek, U., Werner, J. M., Pickford, A. R., Gurusiddappa, S., Kim, J. H., Pilka, E. S., Briggs, J. A., Gough, T. S., Hook, M., Campbell, I. D. & Potts, J. R. (2003) Pathogenic bacteria attach to human fibronectin through a tandem beta-zipper. *Nature,* 423**,** 177-81.

Schwarz, S., Kadlec, K. & Strommenger, B. (2008) Methicillin-resistant *Staphylococcus aureus* and *Staphylococcus pseudintermedius* detected in the

BfT-GermVet monitoring programme 2004-2006 in Germany. *J Antimicrob Chemother,* 61**,** 282-5.

Scott, D. W., Beningo, K. E., Miller, W. H., Jr. & Rothstein, E. (1998) Efficacy of clindamycin hydrochloride capsules for the treatment of deep pyoderma due to *Staphylococcus intermedius* infection in dogs. *Can Vet J,* 39**,** 753-6.

Scott, D. W., Miller, W. H. & Griffin, C. E. (2001a) Bacterial Skin Diseases. *Muller & Kirk's small animal dermatology*, pp. 274-335. 6th ed. Philadelphia, W.B. Saunders.

Scott, D. W., Miller, W. H. & Griffin, C. E. (2001b) Structure and Function of the Skin. *Muller & Kirk's small animal dermatology*, pp. 1-70. 6th ed. Philadelphia, W.B. Saunders.

Seib, K. L., Dougan, G. & Rappuoli, R. (2009) The key role of genomics in modern vaccine and drug design for emerging infectious diseases. *PLoS Genet,* 5(10), e1000612.

Serra, M., Brazis, P., Puigdemont, A., Fondevila, D., Romano, V., Torre, C. & Ferrer, L. (2007) Development and characterization of a canine skin equivalent. *Exp Dermatol,* 16**,** 135-42.

Serrano, I., Melo-Cristino, J., Carriço, J. A. & Ramirez, M. (2005) Characterization of the genetic lineages responsible for pneumococcal invasive disease in Portugal. *J Clin Microbiol,* 43(4), 1706-15.

Sewell, C. M., Clarridge, J. E., Young, E. J. & Guthrie, R. K. (1982) Clinical significance of coagulase-negative staphylococci. *J Clin Microbiol,* 16**,** 236-9.

Sharpe, G. R., Gillespie, J. I. & Greenwell, J. R. (1989) An increase in intracellular free calcium is an early event during differentiation of cultured human keratinocytes. *FEBS Lett,* 254**,** 25-8.

Shearer, D. H. & Day, M. J. (1997) Aspects of the humoral immune response to *Staphylococcus intermedius* in dogs with superficial pyoderma, deep pyoderma and anal furunculosis. *Vet Immunol Immunopathol,* 58**,** 107-20.

Shibata, S., Maeda, S., Tsuchida, H. & Fukata, T. (2008) Phenotypic analysis for a cell line of canine epidermal keratinocytes. *J Vet Med Sci,* 70**,** 853-5.

Shopsin, B., Gomez, M., Montgomery, S. O., Smith, D. H., Waddington, M., Dodge, D. E., Bost, D. A., Riehman, M., Naidich, S. & Kreiswirth, B. N. (1999) Evaluation of protein A gene polymorphic region DNA sequencing for typing of *Staphylococcus aureus* strains. *J Clin Microbiol,* 37**,** 3556-63.

Signas, C., Raucci, G., Jonsson, K., Lindgren, P. E., Anantharamaiah, G. M., Hook, M. & Lindberg, M. (1989) Nucleotide sequence of the gene for a fibronectin-binding protein from *Staphylococcus aureus*: use of this peptide sequence in the synthesis of biologically active peptides. *Proc Natl Acad Sci U S A,* 86**,** 699-703.

Simoons-Smit, A. M., Savelkoul, P. H., Stoof, J., Starink, T. M. & Vandenbroucke-Grauls, C. M. (2000) Transmission of *Staphylococcus aureus* between humans and domestic animals in a household. *Eur J Clin Microbiol Infect Dis,* 19**,** 150-2.

Simou, C., Forsythe, P. J., Hill, P. B. & Thoday, K. L. (2005a) Adherence of *Staphylococcus intermedius* to canine corneocytes involves a protein-protein interaction that is sensitive to trypsin but resistant to cold. *Advances in*

Veterinary Dermatology Volume 5, pp. 270 – 276. Oxford, Blackwell Scientific Publications.

Simou, C., Hill, P. B., Forsythe, P. J. & Thoday, K. L. (2005b) Species specificity in the adherence of staphylococci to canine and human corneocytes: a preliminary study. *Vet Dermatol,* 16**,** 156-61.

Simou, C., Thoday, K. L., Forsythe, P. J. & Hill, P. B. (2005c) Adherence of *Staphylococcus intermedius* to corneocytes of healthy and atopic dogs: effect of pyoderma, pruritus score, treatment and gender. *Vet Dermatol,* 16**,** 385-91.

Sinha, B., Francois, P. P., Nusse, O., Foti, M., Hartford, O. M., Vaudaux, P., Foster, T. J., Lew, D. P., Herrmann, M. & Krause, K. H. (1999) Fibronectin-binding protein acts as *Staphylococcus aureus* invasin via fibronectin bridging to integrin alpha5beta1. *Cell Microbiol,* 1**,** 101-17.

Sinha, B., Francois, P., Que, Y. A., Hussain, M., Heilmann, C., Moreillon, P., Lew, D., Krause, K. H., Peters, G. & Herrmann, M. (2000) Heterologously expressed *Staphylococcus aureus* fibronectin-binding proteins are sufficient for invasion of host cells. *Infect Immun,* 68**,** 6871-8.

Sinha, B. & Herrmann, M. (2005) Mechanism and consequences of invasion of endothelial cells by *Staphylococcus aureus*. *Thromb Haemost,* 94**,** 266-77.

Smeltzer, M. S. & Gillaspy, A. F. (2000) Molecular pathogenesis of staphylcoccal osteomyelitis. *Poult Sci,* 79**,** 1042-9.

Smeltzer, M. S., Gillaspy, A. F., Pratt, F. L., Jr., Thames, M. D. & Iandolo, J. J. (1997) Prevalence and chromosomal map location of *Staphylococcus aureus* adhesin genes. *Gene,* 196**,** 249-59.

Smith, J. A. & Mac Namee, P. T. (2001) Staphylococci, Streptococci and Enterococci. *Poultry Diseases*, pp. 163-169. 5th ed. London, W. B. Saunders.

Sonnenberg, A., Calafat, J., Janssen, H., Daams, H., Van Der Raaij-Helmer, L. M., Falcioni, R., Kennel, S. J., Aplin, J. D., Baker, J., Loizidou, M. & *et al.* (1991) Integrin alpha 6/beta 4 complex is located in hemidesmosomes, suggesting a major role in epidermal cell-basement membrane adhesion. *J Cell Biol,* 113**,** 907-17.

Southern, E. M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol,* 98**,** 503-17.

Srinivasan, A., Dick, J. D. & Perl, T. M. (2002) Vancomycin resistance in staphylococci. *Clin Microbiol Rev,* 15**,** 430-8.

Staden, R. (1996) The Staden sequence analysis package. *Mol Biotechnol,* 5**,** 233-41.

Stranger-Jones, Y. K., Bae, T. & Schneewind, O. (2006) Vaccine assembly from surface proteins of *Staphylococcus aureus*. *Proc Natl Acad Sci U S A,* 103**,** 16942-7.

Strommenger, B., Kettlitz, C., Weniger, T., Harmsen, D., Friedrich, A. W. & Witte, W. (2006) Assignment of *Staphylococcus* isolates to groups by spa typing, SmaI macrorestriction analysis, and multilocus sequence typing. *J Clin Microbiol,* 44(7), 2533-40.

Stutzmann Meier, P., Entenza, J. M., Vaudaux, P., Francioli, P., Glauser, M. P. & Moreillon, P. (2001) Study of *Staphylococcus aureus* pathogenic genes by transfer and expression in the less virulent organism *Streptococcus gordonii*. *Infect Immun,* 69**,** 657-64.

Sung, J. M., Chantler, P. D. & Lloyd, D. H. (2006) Accessory gene regulator locus of *Staphylococcus intermedius*. *Infect Immun,* 74**,** 2947-56.

Suter, M. M., Crameri, F. M., Olivry, T., Mueller, E., Von Tscharner, C. & Jensen, P. J. (1997) Keratinocyte biology and pathology. *Vet Dermatol,* 8**,** 67-100.

Suter, M. M., Greenberger, L. J., Wilkinson, J. E. & Lewis, R. M. (1990) Differential expression of cell surface antigens of canine keratinocytes defined by monoclonal antibodies. *J Histochem Cytochem,* 38**,** 541-9.

Switalski, L. M., Speziale, P. & Hook, M. (1989) Isolation and characterization of a putative collagen receptor from *Staphylococcus aureus* strain Cowan 1. *J Biol Chem,* 264**,** 21080-6.

Symersky, J., Patti, J. M., Carson, M., House-Pompeo, K., Teale, M., Moore, D., Jin, L., Schneider, A., Delucas, L. J., Hook, M. & Narayana, S. V. (1997) Structure of the collagen-binding domain from a *Staphylococcus aureus* adhesin. *Nat Struct Biol,* 4**,** 833-8.

Takeuchi, F., Watanabe, S., Baba, T., Yuzawa, H., Ito, T., Morimoto, Y., Kuroda, M., Cui, L., Takahashi, M., Ankai, A., Baba, S., Fukui, S., Lee, J. C. & Hiramatsu, K. (2005) Whole-genome sequencing of *Staphylococcus haemolyticus* uncovers the extreme plasticity of its genome and the evolution of human-colonizing staphylococcal species. *J Bacteriol,* 187**,** 7292-308.

Talan, D. A., Goldstein, E. J., Staatz, D. & Overturf, G. D. (1989) *Staphylococcus intermedius*: clinical presentation of a new human dog bite pathogen. *Ann Emerg Med,* 18**,** 410-3.

Tanner, M. A., Everett, C. L. & Youvan, D. C. (2000) Molecular phylogenetic evidence for noninvasive zoonotic transmission of *Staphylococcus intermedius* from a canine pet to a human. *J Clin Microbiol,* 38**,** 1628-31.

Terauchi, R., Sato, H., Endo, Y., Aizawa, C. & Maehara, N. (2003a) Cloning of the gene coding for *Staphylococcus intermedius* exfoliative toxin and its expression in *Escherichia coli*. *Vet Microbiol,* 94**,** 31-8.

Terauchi, R., Sato, H., Hasegawa, T., Yamaguchi, T., Aizawa, C. & Maehara, N. (2003b) Isolation of exfoliative toxin from *Staphylococcus intermedius* and its local toxicity in dogs. *Vet Microbiol,* 94**,** 19-29.

Therrien, R., Lacasse, P., Grondin, G. & Talbot, B. G. (2007) Lack of protection of mice against *Staphylococcus aureus* despite a significant immune response to immunization with a DNA vaccine encoding collagen-binding protein. *Vaccine,* 25**,** 5053-61.

Thiemermann, C. (2002) Interactions between lipoteichoic acid and peptidoglycan from *Staphylococcus aureus*: a structural and functional analysis. *Microbes Infect,* 4**,** 927-35.

Ton-That, H., Faull, K. F. & Schneewind, O. (1997) Anchor structure of staphylococcal surface proteins. A branched peptide that links the carboxyl terminus of proteins to the cell wall. *J Biol Chem,* 272**,** 22285-92.

Traber, K. E., Lee, E., Benson, S., Corrigan, R., Cantera, M., Shopsin, B. & Novick, R. P. (2008) *agr* function in clinical *Staphylococcus aureus* isolates. *Microbiology,* 154**,** 2265-74.

Tsuchida, S., Bonkobara, M., McMillan, J. R., Akiyama, M., Yudate, T., Aragane, Y., Tezuka, T., Shimizu, H., Cruz, P. D., Jr. & Ariizumi, K. (2004) Characterization of Kdap, a protein secreted by keratinocytes. *J Invest Dermatol,* 122**,** 1225-34.

Tung, H., Guss, B., Hellman, U., Persson, L., Rubin, K. & Ryden, C. (2000) A bone

sialoprotein-binding protein from *Staphylococcus aureus*: a member of the staphylococcal Sdr family. *Biochem J,* 345 Pt 3**,** 611-9.

Turner, K. M., Hanage, W. P., Fraser, C., Connor, T. R. & Spratt, B. G. (2007) Assessing the reliability of eBURST using simulated populations with known ancestry. *BMC Microbiol,* 12;7, 30.

Uhlen, M., Guss, B., Nilsson, B., Gatenbeck, S., Philipson, L. & Lindberg, M. (1984) Complete sequence of the staphylococcal gene encoding protein A. A gene evolved through multiple duplications. *J Biol Chem,* 259**,** 1695-702.

Upton, A., Lang, S. & Heffernan, H. (2003) Mupirocin and *Staphylococcus aureus*: a recent paradigm of emerging antibiotic resistance. *J Antimicrob Chemother,* 51**,** 613-7.

Van Belkum, A., Melles, D. C., Nouwen, J., Van Leeuwen, W. B., Van Wamel, W., Vos, M. C., Wertheim, H. F. & Verbrugh, H. A. (2009) Co-evolutionary aspects of human colonisation and infection by *Staphylococcus aureus*. *Infect Genet Evol,* 9**,** 32-47.

Van Hoovels, L., Vankeerberghen, A., Boel, A., Van Vaerenbergh, K. & De Beenhouwer, H. (2006) First case of *Staphylococcus pseudintermedius* infection in a human. *J Clin Microbiol,* 44**,** 4609-12.

Van Leeuwen, W. B., Melles, D. C., Alaidan, A., Al-Ahdal, M., Boelens, H. A., Snijders, S. V., Wertheim, H., Van Duijkeren, E., Peeters, J. K., Van Der Spek, P. J., Gorkink, R., Simons, G., Verbrugh, H. A. & Van Belkum, A. (2005) Host- and tissue-specific pathogenic traits of *Staphylococcus aureus*. *J Bacteriol,* 187**,** 4584-91.

Van Leeuwen, W., Verbrugh, H., Van Der Velden, J., Van Leeuwen, N., Heck, M. & Van Belkum, A. (1999) Validation of binary typing for *Staphylococcus aureus* strains. *J Clin Microbiol,* 37**,** 664-74.

Varaldo, P. E., Kilpper-Balz, R., Biavasco, F., Satta, G. & Schleifer, K. H. (1988) *Staphylococcus delphini* sp. nov., a coagulase-positive species isolated from dolphins. *Int. J. Syst. Bacteriol.,* 38**,** 436-439.

Vaudaux, P. E., Francois, P., Proctor, R. A., McDevitt, D., Foster, T. J., Albrecht, R. M., Lew, D. P., Wabers, H. & Cooper, S. L. (1995) Use of adhesion-defective mutants of *Staphylococcus aureus* to define the role of specific plasma proteins in promoting bacterial adhesion to canine arteriovenous shunts. *Infect Immun,* 63**,** 585-90.

Vautor, E., Abadie, G., Guibert, J. M., Huard, C. & Pepin, M. (2003) Genotyping of *Staphylococcus aureus* isolated from various sites on farms with dairy sheep using pulsed-field gel electrophoresis. *Vet Microbiol,* 96**,** 69-79.

Villaruz, A. E., Wardenburg, J. B., Khan, B. A., Whitney, A. R., Sturdevant, D. E., Gardner, D. J., Deleo, F. R. & Otto, M. (2009) A Point Mutation in the *agr* Locus rather than Expression of the Panton-Valentine Leukocidin Caused Previously Reported Phenotypes in *Staphylococcus aureus* Pneumonia and Gene Regulation. *J Infect Dis,* 200**,** 724-734.

Villavicencio, R. T. & Wall, M. J., Jr. (1996) The pathogenesis of *Staphylococcus aureus* in the trauma patient and potential future therapies. *Am J Surg,* 172**,** 291-6.

Von Eiff, C., Becker, K., Machka, K., Stammer, H. & Peters, G. (2001) Nasal carriage as a source of *Staphylococcus aureus* bacteremia. Study Group. *N Engl J Med,* 344**,** 11-6.

Von Eiff, C., Peters, G. & Heilmann, C. (2002) Pathogenesis of infections due to coagulase-negative staphylococci. *Lancet Infect Dis,* 2**,** 677-85.

Wakita, Y., Shimizu, A., Hajek, V., Kawano, J. & Yamashita, K. (2002) Characterization of *Staphylococcus intermedius* from pigeons, dogs, foxes, mink, and horses by pulsed-field gel electrophoresis. *J Vet Med Sci,* 64**,** 237-43.

Waller, A. (2005) The creation of a new monster: MRSA and MRSI- important emerging veterinary and zoonotic diseases. *Vet J,* 169**,** 315-6.

Walsh, E. J., Miajlovic, H., Gorkun, O. V. & Foster, T. J. (2008) Identification of the *Staphylococcus aureus* MSCRAMM clumping factor B (ClfB) binding site in the alphaC-domain of human fibrinogen. *Microbiology,* 154**,** 550-8.

Walsh, E. J., O'Brien, L. M., Liang, X., Hook, M. & Foster, T. J. (2004) Clumping factor B, a fibrinogen-binding MSCRAMM (microbial surface components recognizing adhesive matrix molecules) adhesin of *Staphylococcus aureus*, also binds to the tail region of type I cytokeratin 10. *J Biol Chem,* 279**,** 50691-9.

Walter, J. H. (2001) Cytokeratins in the canine epidermis. *Vet Dermatol,* 12**,** 81-7.

Wang, J. E., Jorgensen, P. F., Almlof, M., Thiemermann, C., Foster, S. J., Aasen, A. O. & Solberg, R. (2000) Peptidoglycan and lipoteichoic acid from *Staphylococcus aureus* induce tumor necrosis factor alpha, interleukin 6 (IL-6), and IL-10 production in both T cells and monocytes in a human whole blood model. *Infect Immun,* 68**,** 3965-70.

Wann, E. R., Gurusiddappa, S. & Hook, M. (2000) The fibronectin-binding MSCRAMM FnbpA of *Staphylococcus aureus* is a bifunctional protein that also binds to fibrinogen. *J Biol Chem,* 275**,** 13863-71.

Wayne, R. K. (1993) Molecular evolution of the dog family. *Trends Genet,* 9**,** 218-24.

Weems, J. J., Jr., Steinberg, J. P., Filler, S., Baddley, J. W., Corey, G. R., Sampathkumar, P., Winston, L., John, J. F., Kubin, C. J., Talwani, R., Moore, T., Patti, J. M., Hetherington, S., Texter, M., Wenzel, E., Kelley, V. A. & Fowler, V. G., Jr. (2006) Phase II, randomized, double-blind, multicenter study comparing the safety and pharmacokinetics of tefibazumab to placebo for treatment of *Staphylococcus aureus* Bacteremia. *Antimicrob Agents Chemother,* 50**,** 2751-5.

Weese, J. S., Poma, R., James, F., Buenviaje, G., Foster, R. & Slavic, D. (2009) *Staphylococcus pseudintermedius* necrotizing fasciitis in a dog. *Can Vet J,* 50**,** 655-6.

Weese, J. S. & Van Duijkeren, E. (2009) Methicillin-resistant *Staphylococcus aureus* and *Staphylococcus pseudintermedius* in veterinary medicine. *Vet Microbiol*.

Weidenmaier, C., Kokai-Kun, J. F., Kulauzovic, E., Kohler, T., Thumm, G., Stoll, H., Gotz, F. & Peschel, A. (2008) Differential roles of sortase-anchored surface proteins and wall teichoic acid in *Staphylococcus aureus* nasal colonization. *Int J Med Microbiol,* 298**,** 505-13.

Werckenthin, C., Cardoso, M., Martel, J. L. & Schwarz, S. (2001) Antimicrobial resistance in staphylococci from animals with particular reference to bovine *Staphylococcus aureus*, porcine *Staphylococcus hyicus*, and canine *Staphylococcus intermedius*. *Vet Res,* 32**,** 341-62.

Wertheim, H. F., Verveer, J., Boelens, H. A., Van Belkum, A., Verbrugh, H. A. & Vos, M. C. (2005) Effect of mupirocin treatment on nasal, pharyngeal, and perineal carriage of *Staphylococcus aureus* in healthy adults. *Antimicrob Agents Chemother,* 49**,** 1465-7.

Wertheim, H. F., Walsh, E., Choudhurry, R., Melles, D. C., Boelens, H. A., Miajlovic, H., Verbrugh, H. A., Foster, T. & Van Belkum, A. (2008) Key role for clumping factor B in *Staphylococcus aureus* nasal colonization of humans. *PLoS Med,* 5**,** e17.

Wettstein, K., Descloux, S., Rossano, A. & Perreten, V. (2008) Emergence of methicillin-resistant *Staphylococcus pseudintermedius* in Switzerland: three cases of urinary tract infections in cats. *Schweiz Arch Tierheilkd,* 150**,** 339-43.

Wilkinson, J. E., Smith, C., Suter, M. & Lewis, R. M. (1987) Long-term cultivation of canine keratinocytes. *J Invest Dermatol,* 88**,** 202-6.

Williams, R. J., Henderson, B. & Nair, S. P. (2002) *Staphylococcus aureus* fibronectin binding proteins A and B possess a second fibronectin binding region that may have biological relevance to bone tissues. *Calcif Tissue Int,* 70**,** 416-21.

Wingate, K. V., Torres, S. M., Silverstein, K. A., Hendrickson, J. A. & Rutherford, M. S. (2009) Expression of endogenous antimicrobial peptides in normal canine skin. *Vet Dermatol,* 20(1), 19-26.

Wisplinghoff, H., Bischoff, T., Tallent, S. M., Seifert, H., Wenzel, R. P. & Edmond, M. B. (2004) Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. *Clin Infect Dis,* 39**,** 309-17.

Woodford, N. (2005) Biological counterstrike: antibiotic resistance mechanisms of Gram-positive cocci. *Clin Microbiol Infect,* 11 Suppl 3**,** 2-21.

Woolley, K. L., Kelly, R. F., Fazakerley, J., Williams, N. J., Nuttall, T. J. & McEwan, N. A. (2008) Reduced *in vitro* adherence of *Staphylococcus* species to feline corneocytes compared to canine and human corneocytes. *Vet Dermatol,* 19**,** 1-6.

Wright, J. S., 3rd, Traber, K. E., Corrigan, R., Benson, S. A., Musser, J. M. & Novick, R. P. (2005) The *agr* radiation: an early event in the evolution of staphylococci. *J Bacteriol,* 187**,** 5585-94.

Xu, Y., Rivas, J. M., Brown, E. L., Liang, X. & Hook, M. (2004) Virulence potential of the staphylococcal adhesin CNA in experimental arthritis is determined by its affinity for collagen. *J Infect Dis,* 189**,** 2323-33.

Yacoub, A., Lindahl, P., Rubin, K., Wendel, M., Heinegard, D. & Ryden, C. (1994) Purification of a bone sialoprotein-binding protein from *Staphylococcus aureus*. *Eur J Biochem,* 222**,** 919-25.

Yagihara, H., Kinjo, E., Kobayashi, Y., Tsuji, A., Nishimura, Y., Shinozaki, N., Tamura, K., Isotani, M., Nakagaki, K., Takahashi, K., Ono, K., Washizu, T. & Bonkobara, M. (2009) Expression of canine Kdap in normal, hyperplastic and neoplastic epidermis. *Vet J,* 180**,** 348-55.

Yagihara, H., Terada, Y., Sugimoto, S., Hidaka, F., Yamada, O., Ono, K., Washizu, T., Ariizumi, K. & Bonkobara, M. (2006) Identification and cornification-related gene expression of canine keratinocyte differentiation-associated protein, Kdap. *Vet J,* 172**,** 141-6.

Yamaguchi, T., Nishifuji, K., Sasaki, M., Fudaba, Y., Aepfelbacher, M., Takata, T., Ohara, M., Komatsuzawa, H., Amagai, M. & Sugai, M. (2002) Identification of the *Staphylococcus aureus etd* pathogenicity island which encodes a novel exfoliative toxin, ETD, and EDIN-B. *Infect Immun,* 70**,** 5835-45.

Yamazoe, K., Miyamoto, S., Hikosaka, Y., Kitagawa, K., Watanabe, K., Sakai, H. & Kudo, T. (2007) Three-dimensional culture of keratinocytes and the formation of basement membrane for canine footpad substitute. *J Vet Med Sci,* 69**,** 611-7.

Young, L. A., Dodge, J. C., Guest, K. J., Cline J. L. & Kerr, W. W. (2002) Age, breed, sex and period effects on skin biophysical parameters for dogs fed canned dog food. *J Nutr*, 132 (6 Suppl 2), 1695S-7S.

Zadoks, R. N., Schukken, Y. H. & Wiedmann, M. (2005) Multilocus sequence typing of *Streptococcus uberis* provides sensitive and epidemiologically relevant subtype information and reveals positive selection in the virulence gene *pauA*. *J Clin Microbiol,* 43**,** 2407-17.

Zadoks, R., Van Leeuwen, W., Barkema, H., Sampimon, O., Verbrugh, H., Schukken, Y. H. & Van Belkum, A. (2000) Application of pulsed-field gel electrophoresis and binary typing as tools in veterinary clinical microbiology and molecular epidemiologic analysis of bovine and human *Staphylococcus aureus* isolates. *J Clin Microbiol,* 38**,** 1931-9.

Zadoks, R. N., Van Leeuwen, W. B., Kreft, D., Fox, L. K., Barkema, H. W., Schukken, Y. H. & Van Belkum, A. (2002) Comparison of *Staphylococcus aureus* isolates from bovine and human skin, milking equipment, and bovine milk by phage typing, pulsed-field gel electrophoresis, and binary typing. *J Clin Microbiol,* 40**,** 3894-902.

Zong, Y., Xu, Y., Liang, X., Keene, D. R., Hook, A., Gurusiddappa, S., Hook, M. & Narayana, S. V. (2005) A 'Collagen Hug' model for *Staphylococcus aureus* CNA binding to collagen. *Embo J,* 24**,** 4224-36.

Appendix I

Published Papers

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Population Genetic Structure of the *Staphylococcus intermedius* Group: Insights into *agr* Diversification and the Emergence of Methicillin-Resistant Strains▽†

Jeanette Bannoehr,[1] Nouri L. Ben Zakour,[1] Andrew S. Waller,[2] Luca Guardabassi,[3] Keith L. Thoday,[4] Adri H. M. van den Broek,[4] and J. Ross Fitzgerald[1]*

Centre for Infectious Diseases, Royal (Dick) School of Veterinary Studies, The Chancellor's Building, New Royal Infirmary, University of Edinburgh, Edinburgh EH16 4SB, Scotland, United Kingdom[1]; Centre for Preventive Medicine, Animal Health Trust, Newmarket, Suffolk CB8 7UU,United Kingdom[2]; Department of Veterinary Pathobiology, Faculty of Life Science, University of Copenhagen, Frederiksberg C, Denmark[3]; and Dermatology Unit, Division of Veterinary Clinical Sciences, Royal (Dick) School of Veterinary Studies, Easter Bush Veterinary Centre, University of Edinburgh, Roslin, Midlothian, EH25 9RG, Scotland, United Kingdom[4]

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The population genetic structure of the animal pathogen *Staphylococcus intermedius* is poorly understood. We carried out a multilocus sequence phylogenetic analysis of isolates from broad host and geographic origins to investigate inter- and intraspecies diversity. We found that isolates phenotypically identified as *S. intermedius* are differentiated into three closely related species, *S. intermedius*, *Staphylococcus pseudintermedius*, and *Staphylococcus delphini*. *S. pseudintermedius*, not *S. intermedius*, is the common cause of canine pyoderma and occasionally causes zoonotic infections of humans. Over 60 extant STs were identified among the *S. pseudintermedius* isolates examined, including several that were distributed on different continents. As the *agr* quorum-sensing system of staphylococci is thought to have evolved along lines of speciation within the genus, we examined the allelic variation of *agrD*, which encodes the autoinducing peptide (AIP). Four AIP variants were encoded by *S. pseudintermedius* isolates, and identical AIP variants were shared among the three species, suggesting that a common quorum-sensing capacity has been conserved in spite of species differentiation in largely distinct ecological niches. A lack of clonal association of *agr* alleles suggests that assortive recombination may have contributed to the distribution of *agr* diversity. Finally, we discovered that the recent emergence of methicillin-resistant strains was due to multiple acquisitions of the *mecA* gene by different *S. pseudintermedius* clones found on different continents. Taken together, these data have resolved the population genetic structure of the *S. intermedius* group, resulting in new insights into its ancient and recent evolution.

Staphylococcus intermedius is a member of the normal flora of dogs and is also a major opportunistic pathogen responsible for the common canine skin condition pyoderma (19). *S. intermedius* has also been found in association with other animal species (1, 3, 12, 16) and can occasionally cause severe infections of humans (26, 29, 37).

Despite its prevalence among many members of the animal kingdom, an understanding of the population genetics of *S. intermedius* is lacking. Until very recently, studies of the diversity of *S. intermedius* populations have been limited to phenotypic and molecular typing approaches (1, 3, 5, 12, 16–18, 40). A relatively high degree of phenotypic diversity exists within the species, leading to the identification of different biotypes associated with specific animal hosts (16, 28), and ribotyping studies have shown that strains are typically associated with only a single host species (1, 5). Canine strains were represented by a small number of distinct ribotypes, in contrast to strains from other animals, which generally had greater diversity (17). The level of phenotypic and genotypic diversity observed among *S. intermedius* isolates has led some investigators to speculate that an *S. intermedius* group (SIG) consisting of several species or subspecies may exist (5, 28). A closely related species, *Staphylococcus pseudintermedius*, was recently described (7) and has been isolated from several animal species, including healthy dogs in a veterinary clinic in Japan (32), and from a human infection (38). Very recently, a study of Japanese isolates based on DNA sequences of *sodA* and *hsp60* genes identified three different species, *S. intermedius*, *S. pseudintermedius*, and *Staphylococcus delphini*, among isolates phenotypically identified as *S. intermedius* and suggested a reclassification of the species (33). However, the distribution of these different species among isolates from outside Japan was not examined.

Worryingly, methicillin-resistant strains of *S. intermedius* (MRSI) and *S. pseudintermedius* (MRSP) have recently emerged in veterinary clinics around the world (15, 22, 32, 44), but their evolutionary histories have not been examined. An understanding of the population genetics of *S. intermedius* is required to provide a much-needed framework for studies of the evolution, pathogenesis, and emerging methicillin resistance of *S. intermedius*. In order to investigate the population genetic structure of *S. intermedius*, we developed a multilocus

* Corresponding author. Mailing address: Laboratory for Bacterial Evolution and Pathogenesis, Centre for Infectious Diseases, The Chancellor's Building, New Royal Infirmary, University of Edinburgh, Edinburgh EH16 4SB, Scotland. Phone: 44 131 242 9376. Fax: 44 131 242 9385. E-mail: Ross.Fitzgerald@ed.ac.uk.

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sequencing approach that included five gene loci with a range of predicted nucleotide diversities to facilitate inter- and intraspecies differentiation. Broad new insights into the diversity of *S. intermedius* populations were obtained.

MATERIALS AND METHODS

Bacterial strains. In total, 105 isolates were examined, including 99 from an array of diseased and healthy animal species, such as dogs, humans, horses, camels, and pigeons, which were previously identified as *S. intermedius* in different centers in the United States, Canada, Japan, United Kingdom (including Scotland and England), France, Belgium, Czech Republic, Germany, and Sweden (see Table S1 in the supplemental material). Identification was carried out with standard phenotypic tests that varied slightly depending on the laboratory but included production of coagulase and anaerobic acid from mannitol, sucrose, and D-trehalose and lack of hyaluronidase production, lack of growth on P agar containing acriflavin, and the production of β-galactosidase when grown in *o*-nitrophenyl-β-D-galactopyranoside broth according to the original description of the species by Hajek (16). In addition, the type strain of *S. delphini*, ATCC 49171 (39); four strains of the recently described *S. pseudintermedius* species, including the type strain, LMG 22219^T (7); and the type strain of *Staphylococcus schleiferi* subsp. *schleiferi*, ATCC 43808 (11), were included in the study.

Bacterial growth conditions and genomic-DNA isolation. Staphylococcal strains were grown on tryptic soy agar (Oxoid) at 37°C overnight or in tryptic soy broth (Oxoid) at 37°C overnight with shaking at 200 rpm. Genomic-DNA extraction was carried out with a bacterial genomic-DNA purification kit (Edge Biosystems) according to the manufacturer's instructions. Prior to incubation at 37°C for 10 min, 125 μg/ml lysostaphin (Ambi products) was included.

Gene loci selected for DNA sequence analysis. Five loci, the 16S rRNA, *cpn60* (*hsp60*), *tuf*, *pta*, and *agrD* genes, were selected for DNA sequence analysis. 16S rRNA, *cpn60*, and *tuf* have been used previously in single-locus approaches to differentiating staphylococcal species (7, 13, 24, 25), and *cpn60*, *tuf*, and *pta* have been incorporated into multilocus sequence-typing schemes for differentiating strains of several different bacterial species (2, 10, 43). The accessory gene regulator (*agr*) quorum-sensing system found in the majority of staphylococcal species examined (8) is thought to have diversified along lines of speciation, giving rise to a number of subspecies groups that have the capacity for intra- and interspecies inhibition of virulence (30, 42). We examined the allelic variation of *agrD*, which encodes the autoinducing peptide (AIP), to further investigate both inter- and intraspecies differentiation.

PCR amplification of gene fragments. Oligonucleotide primers (Sigma-Genosys or Invitrogen) were designed for the 16S rRNA, *cpn60*, and *agrD* genes based on gene sequences available for *S. intermedius* in GenBank (accession numbers Z26897, AF019773, AY965912, and AF346723), and *pta* primers were designed based on the *S. intermedius pta* nucleotide sequence (unpublished data). Oligonucleotide sequences specific for the *tuf* and *mecA* genes were designed in previous studies (21, 31). The oligonucleotide sequences and predicted PCR product sizes for specified gene fragments were as follows: 16S rRNA (370 bp), 5′-CCT CTT CGG AGG ACA AAG TGA-3′ (forward) and 5′-GAC CCG GGA ACG TAT TCA CC-3′ (reverse); *tuf* (500 bp), 5′-CAA TGC CAC AAA CTC G-3′ (forward) and 5′-GCT TCA GCG TAG TCT A-3′ (reverse); *cpn60* (550 bp), 5′-GCG ACT GTA CTT GCA CAA GCA-3′ (forward) and 5′-AAC TGC AAC CGC TGT AAA T G-3′ (reverse); *pta* (570 bp), 5′-GTG CGT ATC GTA TTA CCA GAA GG-3′ (forward) and 5′-GCA GAA CCT TTT GTT GAG AAG C-3′ (reverse); *agrD* (300 bp), 5′-GGG GTA TTA TTA CAA TCA TTC-3′ (forward) and 5′-CTG ATG CGA AAA TAA AGG ATT G-3′ (reverse), and 5′-CTC ATG ACT ATT GCA TGC ATC G-3′ (reverse; pigeon isolates only); and *mecA* (310 bp), 5′-GTA GAA ATG ACT GAA CGT CCG ATAA-3′ (forward) and 5′-CCA ATT CCA CAT TGT TTC GGT CTA A-3′ (reverse). PCR mixtures for the 16S RNA, *tuf*, *cpn60*, *pta*, and *mecA* genes included 0.2 μM each primer, 0.025 U/μl *Taq* polymerase (Promega), 1.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates (Promega), and 1 μl genomic DNA template in 96-well PCR microplates (Axygen/Thistle Scientific). The thermocycler program for the 16S RNA, *tuf*, *cpn60*, and *pta* genes consisted of an initial denaturation for 2 min at 95°C, followed by 30 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 55°C, and an extension for 1 min at 72°C, and a final extension for 7 min at 72°C. The *mecA* thermocycler program consisted of an initial denaturation for 5 min at 95°C, followed by 30 cycles of denaturation for 45 s at 95°C, annealing for 45 s at 52°C, and an extension for 1 min at 72°C, and a final extension for 5 min at 72°C. For *agrD* amplification, the reaction mixtures included 0.5 μM each primer, 0.02 U/μl Vent DNA polymerase (New England Biolabs [NEB]), 1× ThermoPol Reaction Buffer (NEB), 0.2 mM deoxynucleoside triphosphates (Promega), and 1 μl genomic-DNA template. The thermocycler conditions were an initial denaturation for 2 min at 94°C, followed by 35 cycles of denaturation for 15 s at 94°C, annealing for 30 s at 45°C, and extension for 1 min at 72°C, with a final extension for 7 min at 72°C (36). PCR products were purified by incubation with exonuclease I and Antarctic phosphatase (NEB) at 37°C for 15 min, followed by inactivation at 80°C for 15 min.

DNA sequencing. Sequencing reactions were carried out using 5 μl purified PCR-amplified DNA (approximately 50 to 90 ng) plus 1 μl sequencing primer (3.2 pmol/μl) with the BigDye Terminator v3.0 Ready Reaction cycle sequencing kit (Applied Biosystems) according to the manufacturer's instructions. Each sequencing reaction included 25 cycles with denaturation for 30 s at 95°C, annealing for 20 s at 50°C, and extension for 4 min at 60°C. The nucleotide sequence was determined with a 3730 DNA analyzer (Applied Biosystems). For the 16S rRNA, *cpn60*, *tuf*, and *pta* genes, forward and reverse sequencing reactions were carried out with independently amplified PCR products to rule out the possibility of *Taq* polymerase-generated errors. For *agrD*, forward and reverse sequencing reactions were carried out from a single PCR product generated with Vent DNA polymerase (NEB), which contains proofreading activity and has a much lower predicted error rate than *Taq* polymerase.

DNA sequence and molecular evolutionary analyses. DNA sequences were assembled using BioEdit Sequence Alignment Editor software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and the Staden package (35). Nucleotide and amino acid sequences were aligned using AlignX in Vector NTI Advance10 (Invitrogen). Phylogenetic analyses were carried out with MEGA v.3.1 software (23). The neighbor-joining method was applied to construct phylogenetic trees using the Kimura two-parameter model, while the degree of statistical support for the nodes on the minimum evolution tree was evaluated by examining their percent recovery in 1,000 resample trees by the bootstrap test. For estimating events of recombination, RDP software, which includes the programs GENECONV, BOOTSCAN, MAXIMUM χ^2, CHIMAERA, and SISTER SCANNING, was used (27). The index of association standardized (I_A^S) between the different gene loci was calculated using the LIAN program (version 3.1; Department of Biotechnology and Bioinformatics, University of Applied Sciences, Weihenstephan, Germany) (http://adenine.biz.fh-weihenstephan.de/lian_3.1/). The value of the I_A^S would be expected to be zero for linkage equilibrium when recombination events occur frequently. If the I_A^S value differs significantly from zero ($P < 0.05$), recombination should be rare.

eBURST analysis. Predicted lines of evolutionary descent and clonal complexes in our collection of isolates were identified using the eBURST algorithm (http://eburst.mlst.net). Sequence types (STs) were included in the same group if they shared four of the five gene loci with at least one other ST within the group. Subgroups were defined by the existence of at least three single-locus variants.

Nucleotide sequence accession numbers. The DNA sequences generated in this study were deposited in GenBank, accession no. EU157195 to EU157715.

RESULTS

Multilocus sequence analysis of *S. intermedius*. Overall, 192,482 bp representing 521 sequences from 105 isolates, including 99 isolates phenotypically identified as *S. intermedius*, 4 isolates of *S. pseudintermedius*, and the type strains of *S. delphini* and *S. schleiferi* subsp. *schleiferi*, were generated. 16S rRNA gene sequences contained 1.37% variable sites and 7 alleles, *tuf* sequences contained 2.4% and 9 alleles, *cpn60* contained 12.76% and 28 alleles, *pta* contained 10.98% and 18 alleles, and *agrD* contained 13.04% polymorphic nucleotide sites and 9 alleles (Table 1). 16S rRNA gene sequence analysis revealed the existence of five ambiguous nucleotide sites characterized by a double peak for a single site in the sequencing trace for eight isolates. A mixed or contaminated template was ruled out by repurification of the strain to a single colony before genomic-DNA isolation, repeat PCR, and sequencing. These data are consistent with the existence of intragenomic 16S rRNA gene polymorphisms, which have been previously observed (6). Phylogenetic reconstructions were carried out using concatenated sequences, which included both possible 16S rRNA alleles to determine if they influenced tree topology, but no significant difference in topology was observed

TABLE 1. Summary of nucleotide sequence variation for the SIG and for the *S. pseudintermedius*, *S. delphini*, and *S. intermedius* phylotypes

Gene locus	Group	No. of strains	Total length of sequence (bp)	No. of variable sites (%)	No. of singleton variable sites (%)	No. of alleles[a]
16S rRNA	SIG	104	366	5 (1.37)	2 (0.55)	7
	S. pseudintermedius.	89		2 (0.55)	1 (0.27)	3
	S. delphini	11		3 (0.82)	1 (0.27)	5
	S. intermedius	4		1 (0.27)	0 (0.00)	1
tuf	SIG	104	417	10 (2.40)	4 (0.96)	9
	S. pseudintermedius	89		3 (0.72)	2 (0.48)	4
	S. delphini	11		3 (0.72)	2 (0.48)	4
	S. intermedius	4		1 (0.24)	1 (0.24)	2
cpn60	SIG	104	431	55 (12.76)	9 (2.10)	28
	S. pseudintermedius	89		14 (3.25)	3 (0.70)	20
	S. delphini	11		19 (4.41)	8 (1.86)	7
	S. intermedius	4		0 (0.00)	0 (0.00)	1
pta	SIG	104	492	54 (10.98)	11 (2.24)	18
	S. pseudintermedius	89		12 (2.44)	6 (1.22)	10
	S. delphini	11		8 (1.63)	7 (1.42)	6
	S. intermedius	4		4 (0.81)	4 (0.81)	2
agrD	SIG	104	138	18 (13.04)	4 (2.90)	9
	S. pseudintermedius	89		6 (4.35)	0 (0.00)	4
	S. delphini	11		11 (7.97)	5 (3.62)	5
	S. intermedius	4		0 (0.00)	0 (0.00)	1

[a] One 16S rRNA allele is shared by *S. pseudintermedius* and *S. delphini* and one by *S. delphini* and *S. intermedius*. *S. pseudintermedius* and *S. delphini* share one allele for the *tuf* gene and one for *agrD*.

(data not shown). The least common allelic alternative was selected for each strain and used in phylogenetic analysis and assignment of STs. The *cpn60* sequences for *S. delphini* ATCC 49171 and *S. schleiferi* ATCC 43808 (GenBank accession numbers AF053571 and AF033622) and the *agrD* sequence for *S. intermedius* NCTC 11048 (GenBank accession number AF346723) were obtained from the NCBI GenBank nucleotide database (http://www.ncbi.nlm.nih.gov).

Phylogenetic analysis reveals that *S. pseudintermedius* and not *S. intermedius* is the common canine pyoderma pathogen. Neighbor-joining trees were constructed with MEGA3.1 using the Kimura two-parameter model combined with 1,000 resample trees by the bootstrap test using sequences for each individual locus (see Fig. S1 in the supplemental material). Trees constructed with sequences from each housekeeping locus appeared to be generally in good agreement, indicating a robust phylogenetic signal (see Fig. S1 in the supplemental material). In contrast, the topology of the tree generated with the *agrD* nucleotide sequence was very different from those of trees generated with the other gene sequences, suggesting that recombination may have interfered with the phylogenetic signal (see Fig. S1 in the supplemental material). Accordingly, *agrD* sequences were not included in the concatenated sequence analysis (Fig. 1). Phylogenetic analysis based on the concatenated sequence data indicated the existence of three major phylotypes with strong confidence (bootstrap values, 100% for each node) (Fig. 1). For comparison, phylogenetic-tree reconstruction was also carried out using the Minimum Evolution and unweighted-pair group method using average linkages approaches, and each resulted in a topology that was cognate with the neighbor-joining trees (data not shown). The type strain of *S. intermedius* (NCTC 11048) belongs to a small phylogenetically distinct group of pigeon isolates that is not representative of the majority of isolates phenotypically identified as *S. intermedius*. All canine isolates ($n = 75$) belong to a single phylotype, which includes the four isolates previously identified as the novel species *S. pseudintermedius* (7) (Fig. 1). The *S. pseudintermedius* isolates were distributed among the canine isolates in the phylogenetic tree, including two strains, *S. pseudintermedius* LMG 22221 and *S. pseudintermedius* LMG 22222, that were genetically indistinguishable from canine isolates belonging to one of the most populous nodes (Fig. 1). These data indicate that the recently described *S. pseudintermedius* and not *S. intermedius* is the common canine pyoderma pathogen.

The third major phylotype was represented by isolates from horse, camel, and pigeon hosts and included the *S. delphini* type strain, ATCC 49171, isolated from a dolphin (39), suggesting that *S. delphini* may be commonly misidentified as *S. intermedius*. Taken together, these data indicate the existence of at least three closely related but distinct species among isolates that are identified as *S. intermedius* by phenotypic methods and that together could be described as the SIG.

***S. pseudintermedius* has a largely clonal population structure.** As mentioned above, trees constructed with sequences from each housekeeping locus appeared to be generally in good agreement, suggesting that recombination has not played a major role in the evolution of these genes and consistent with a clonal population structure (see Fig. S1 in the supplemental material). To further investigate the population structure, we calculated the degrees of linkage disequilibrium within the whole SIG and within the *S. pseudintermedius* and *S. delphini* species (Table 2). The value of the I_A^S for the SIG as a whole was 0.1510 ($P < 0.001$), and for the *S. pseudintermedius* and *S. delphini* species it was 0.0877 ($P < 0.001$) and 0.3699 ($P < 0.001$), respectively, indicating that there is linkage disequilibrium within each group tested (Table 2). In addition, the RDP2 program was used to search for evidence of recombination among the selected gene loci (27). For each locus, none of the recombination detection methods, GENECONV, BOOTSCAN,

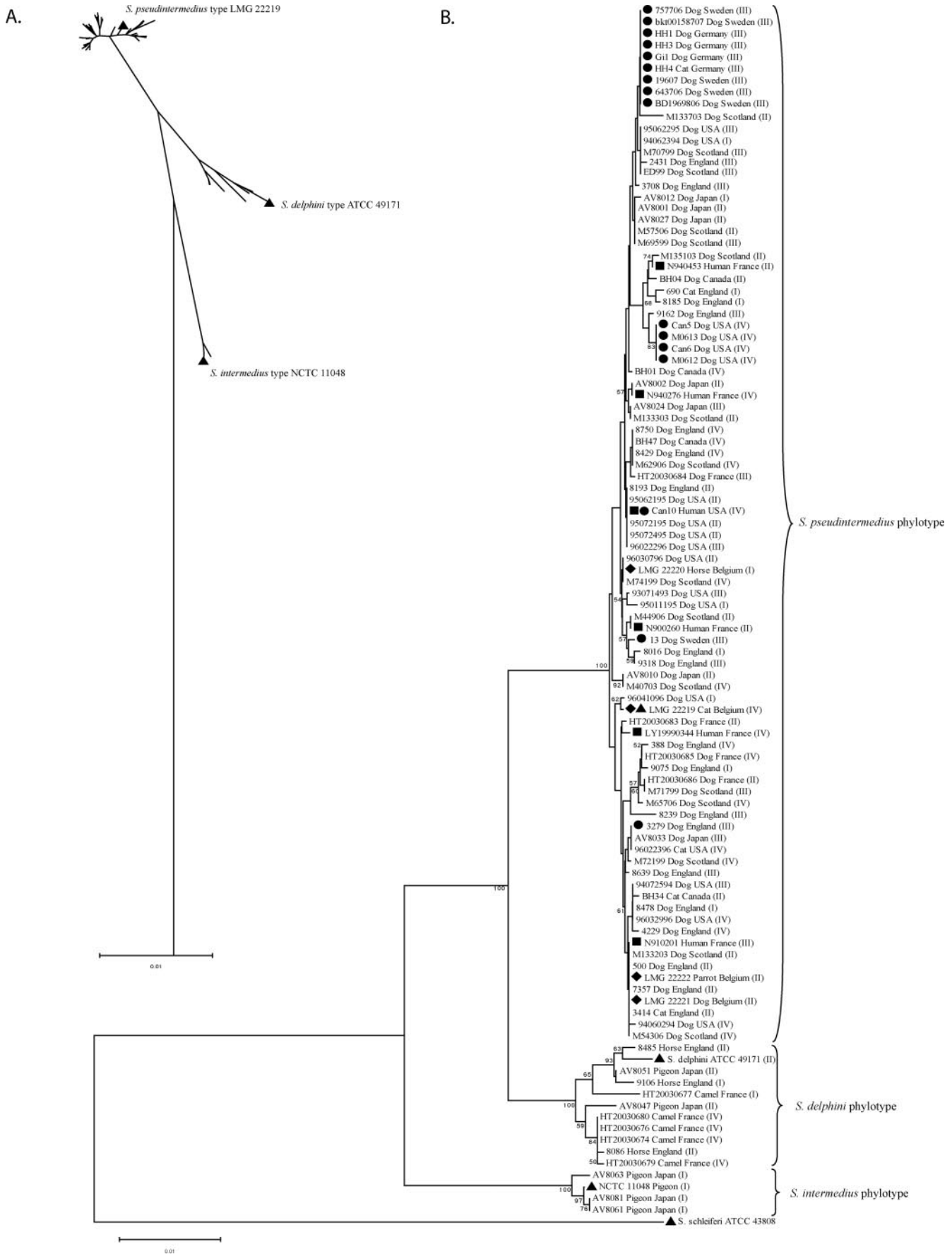
A.
S. pseudintermedius type LMG 22219
S. delphini type ATCC 49171
S. intermedius type NCTC 11048
0.01
B.
757706 Dog Sweden (III)
bkt00158707 Dog Sweden (III)
HH1 Dog Germany (III)
HH3 Dog Germany (III)
Gi1 Dog Germany (III)
HH4 Cat Germany (III)
19607 Dog Sweden (III)
643706 Dog Sweden (III)
BD1969806 Dog Sweden (III)
M133703 Dog Scotland (II)
95062295 Dog USA (III)
94062394 Dog USA (I)
M70799 Dog Scotland (III)
2431 Dog England (III)
ED99 Dog Scotland (III)
3708 Dog England (III)
AV8012 Dog Japan (I)
AV8001 Dog Japan (II)
AV8027 Dog Japan (II)
M57506 Dog Scotland (II)
M69599 Dog Scotland (III)
M135103 Dog Scotland (II)
N940453 Human France (II)
BH04 Dog Canada (II)
690 Cat England (I)
8185 Dog England (I)
9162 Dog England (III)
Can5 Dog USA (IV)
M0613 Dog USA (IV)
Can6 Dog USA (IV)
M0612 Dog USA (IV)
BH01 Dog Canada (IV)
AV8002 Dog Japan (II)
N940276 Human France (IV)
AV8024 Dog Japan (III)
M133303 Dog Scotland (II)
8750 Dog England (IV)
BH47 Dog Canada (IV)
8429 Dog England (IV)
M62906 Dog Scotland (IV)
HT20030684 Dog France (III)
8193 Dog England (II)
95062195 Dog USA (II)
Can10 Human USA (IV)
95072195 Dog USA (II)
95072495 Dog USA (II)
96022296 Dog USA (III)
96030796 Dog USA (II)
LMG 22220 Horse Belgium (I)
M74199 Dog Scotland (IV)
93071493 Dog USA (III)
95011195 Dog USA (I)
M44906 Dog Scotland (II)
N900260 Human France (II)
13 Dog Sweden (III)
8016 Dog England (I)
9318 Dog England (III)
AV8010 Dog Japan (II)
M40703 Dog Scotland (IV)
96041096 Dog USA (I)
LMG 22219 Cat Belgium (IV)
HT20030683 Dog France (II)
LY19990344 Human France (IV)
388 Dog England (IV)
HT20030685 Dog France (IV)
9075 Dog England (I)
HT20030686 Dog France (II)
M71799 Dog Scotland (III)
M65706 Dog Scotland (IV)
8239 Dog England (III)
3279 Dog England (III)
AV8033 Dog Japan (III)
96022396 Cat USA (IV)
M72199 Dog Scotland (IV)
8639 Dog England (III)
94072594 Dog USA (III)
BH34 Cat Canada (II)
8478 Dog England (I)
96032996 Dog USA (IV)
4229 Dog England (IV)
N910201 Human France (III)
M133203 Dog Scotland (II)
500 Dog England (II)
LMG 22222 Parrot Belgium (II)
7357 Dog England (II)
LMG 22221 Dog Belgium (II)
3414 Cat England (II)
94060294 Dog USA (IV)
M54306 Dog Scotland (IV)
8485 Horse England (II)
S. delphini ATCC 49171 (II)
AV8051 Pigeon Japan (II)
9106 Horse England (I)
HT20030677 Camel France (I)
AV8047 Pigeon Japan (II)
HT20030680 Camel France (IV)
HT20030676 Camel France (IV)
HT20030674 Camel France (IV)
8086 Horse England (II)
HT20030679 Camel France (IV)
AV8063 Pigeon Japan (I)
NCTC 11048 Pigeon (I)
AV8081 Pigeon Japan (I)
AV8061 Pigeon Japan (I)
S. schleiferi ATCC 43808
S. pseudintermedius phylotype
S. delphini phylotype
S. intermedius phylotype
0.01

TABLE 2. Analysis of linkage disequilibrium by calculation of I_A^S for the SIG and for the *S. pseudintermedius* and *S. delphini* phylotypes

Group selection	I_A^S	*P* value[a]	
		Parametric	Monte Carlo (100 iterations)
SIG	0.151	0.0000	0.001
S. pseudintermedius	0.0877	0.0000	0.001
S. delphini	0.3699	0.0000	0.001
S. intermedius[b]	ND	ND	ND

[a] The value of the I_A^S would be expected to be zero for linkage equilibrium when recombination events occur frequently. If the I_A^S ($P < 0.05$) value differs significantly from zero, recombination should be rare.
[b] The small number of isolates of the *S. intermedius* phylotype precluded carrying out this analysis. ND, not done.

TABLE 3. Amino acid sequences of the predicted *agrD*-encoded AIPs identified among the strains examined

agr type (AIP)	Amino acid sequence	No. of strains (%)
I	RIPTSTGFF	16 (15)
II	RIPISTGFF	31 (30)
III	KIPTSTGFF	29 (28)
IV[a]	KYPTSTGFF	28 (27)

[a] Novel AIP variant identified in the current study.

MAXIMUM χ^2, CHIMAERA, and SISTER SCANNING, detected any evidence for recombinant sequences among the isolates examined (data not shown). Taken together, these data indicate a largely clonal population structure for the SIG as a whole and for the *S. pseudintermedius* and *S. delphini* species.

***S. pseudintermedius* clonal diversity and intercontinental distribution.** We identified the number of alleles at each of the five gene loci and defined unique complements of alleles as STs (see Table S1 in the supplemental material). *S. pseudintermedius* is characterized by the existence of 61 different STs among 89 isolates examined, indicating considerable clonal diversity within the species (see Table S1 in the supplemental material). The limited number of *S. pseudintermedius* isolates that were from human and noncanine animal sources commonly shared identical or closely related genotypes with *S. pseudintermedius* canine commensal strains, consistent with zoonotic transfer from dogs (Fig. 1). In addition, identical or closely related *S. pseudintermedius* STs were found on different continents, indicating broad geographic dissemination of successful clones (Fig. 1).

Identification and distribution of *agr* types among the SIG of closely related species. DNA sequencing of the *agrD* locus revealed the presence of four predicted AIP variants among the strains examined, including a novel fourth variant that has not been described previously (designated type IV) which is specific to 27% of all strains (Table 3). The AIP variants may correspond to distinct *agr* interference groups (8, 30, 36, 42). All staphylococcal species examined to date have been shown to encode AIP peptides that are unique to each species. In contrast, the three closely related species examined in the current study had AIP peptide variants in common, suggesting the existence of a conserved *agr* quorum-sensing system. All four AIP variants (I to IV) were encoded among the *S. pseudintermedius* isolates; AIP variants I, II, and IV were identified among the *S. delphini* group of isolates; and the *S. intermedius* type strain and related pigeon isolates all encoded AIP type I (see Table S1 in the supplemental material).

A lack of clonal association of *agrD* alleles was observed, and several isolates shared identical genotypes by 16S rRNA gene, *tuf*, *cpn60*, and *pta* but encoded different AIP variants (Fig. 1). Further, the *agr* gene tree topology was markedly different from those of the other gene trees. These data and the lack of recombination breakpoints identified within *agrD* sequences using the RDP2 suite of programs suggest that assortive (whole-gene) recombination may have contributed to the distribution of *agr* alleles. There was no identifiable association between *agr* type and host, clinical, or geographic origin.

MRSP strains have evolved by multiple *mecA* gene acquisitions by different clones. The presence of the *mecA* gene in 16 of 105 isolates was detected by PCR (see Table S1 in the supplemental material). Sequencing of the *mecA* gene in representative isolates revealed a high degree of homology with *mecA* of *Staphylococcus aureus* origin (95 to 100%; data not shown). These strains had previously been identified as MRSI in the centers where they were isolated and had oxacillin MIC levels of >256 μg/ml for all strains except one (strain 13), which had an MIC of 0.75 μg/ml. All strains previously identified as MRSI belonged to the *S. pseudintermedius* phylotype and are therefore reclassified as MRSP. MRSP genotypes are distributed widely across the diversity identified within the *S. pseudintermedius* phylogenetic tree, including one isolate, 3279, that is genetically indistinguishable from methicillin-sensitive isolates (ST29), suggesting horizontal transfer of the *mecA* gene (Fig. 1). The existence of multiple MRSP clones is supported by eBURST analysis, which shows that MRSP isolates belong to five distinct STs (ST29, ST68, ST69, ST70, and ST71) that differ at two to four of the five loci examined, strongly suggesting that they do not share a very recent ancestor (Fig. 2). Taken together, these data suggest that the *mecA* gene has been acquired multiple times by different *S. pseudintermedius* strains. Of note, 9 out of 10 MRSP isolates from five different centers in Sweden and Germany belong to ST71, indicating that a common clone may predominate in North and Central Europe (see Table S1 in the supplemental material). There

FIG. 1. Phylogenetic tree constructed by the neighbor-joining method with 1,000 bootstrap replicates of concatenated 16S rRNA gene, *tuf*, *cpn60*, and *pta* sequences. (A) Radiating tree indicating the three major phylotypes, with species type strains indicated by triangles. (B) Branch style tree indicating the host and geographic origins of all isolates. Methicillin-resistant, *mecA*-positive isolates (dots); human isolates (squares); previously identified *S. pseudintermedius* isolates (diamonds); and species-type isolates (triangles) are indicated, along with the *agr* type in roman numerals in parentheses. Bootstrap values over 50% are indicated.

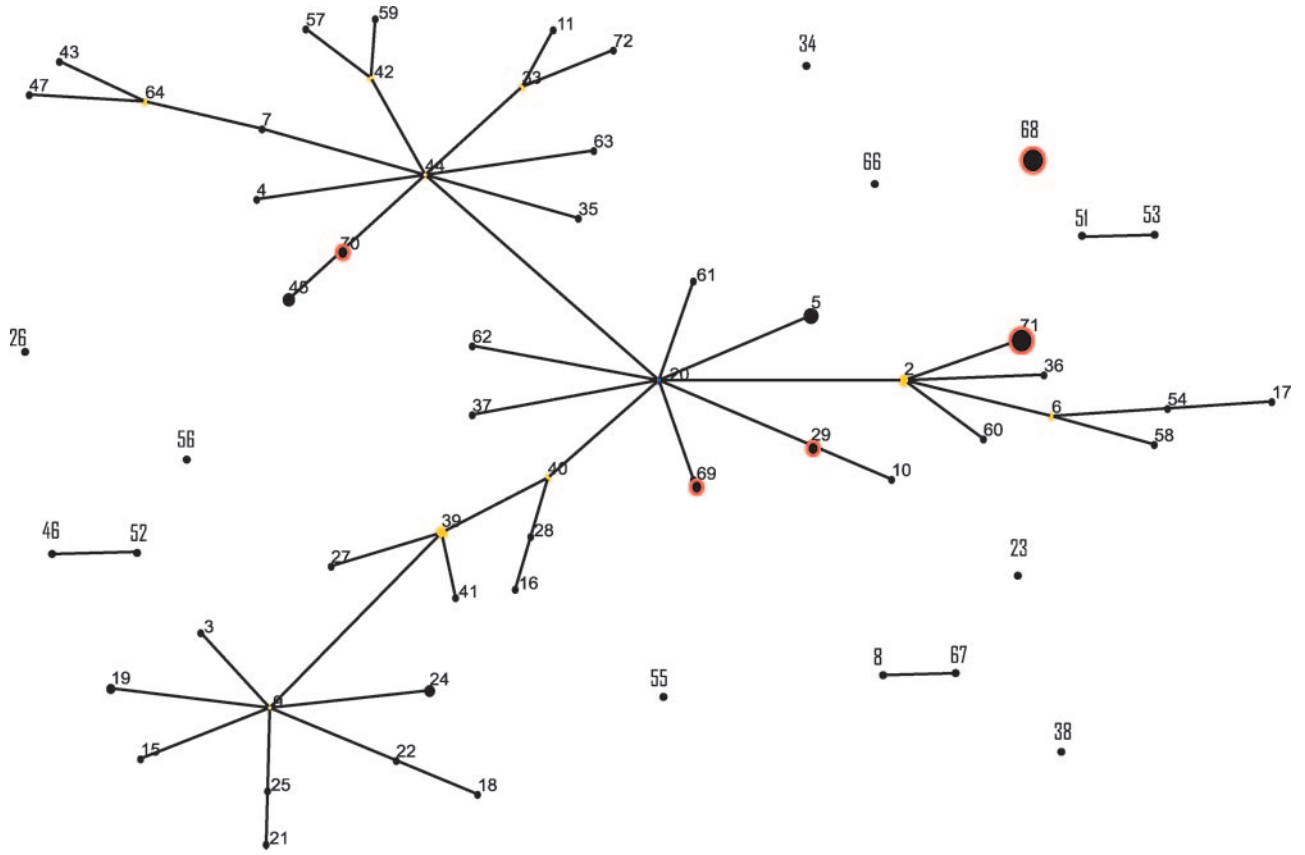

FIG. 2. Schematic diagram of the clonal relatedness of *S. pseudintermedius* STs predicted by eBURST analysis. Each ST is represented by a black dot, the size of which is proportional to the number of isolates of that ST. Blue and yellow dots denote the predicted group founder and subgroup founders, respectively. Single-locus variants are linked by lines. STs that include MRSP strains are indicated by red circles. STs that are not linked by branches to other STs do not share at least four identical alleles with any other ST.

was no sharing of STs among European and U.S. MRSP isolates (see Table S1 in the supplemental material).

DISCUSSION

Hajek and colleagues first described the novel coagulase-positive species *S. intermedius*, which was isolated from pigeons, dogs, minks, and horses in 1976 (16). The type strain, NCTC 11048 (also known as ATCC 29663, CCM 5739, or LMG 13351), isolated from a pigeon, has typically been used to represent *S. intermedius* in species differentiation studies (7, 11, 13, 24, 25). Very recently, Sasaki and colleagues carried out nucleotide sequence analysis of the *sodA* and *hsp60* (*cpn60*) genes and identified *S. intermedius*, *S. pseudintermedius*, and *S. delphini* among isolates from Japan phenotypically identified as *S. intermedius* (33). Here, we show that isolates obtained from 10 different countries on three continents have a population structure that is consistent with that identified among Japanese isolates by Sasaki et al. (33). Our data indicate that the pigeon isolate represents a distinct taxon that is not representative of the majority of isolates commonly identified as *S. intermedius*. All canine isolates examined ($n = 75$) belong to the phylotype that includes four isolates of the recently described *S. pseudintermedius* species, including the type strain (7). Importantly, two *S. pseudintermedius* strains (ST5) were genetically indistinguishable from canine isolates phenotypically identified as *S. intermedius*. These data indicate that the newly described *S. pseudintermedius* species and not *S. intermedius* is the common cause of canine pyoderma. *S. pseudintermedius* is characterized by over 60 different STs identified among the 89 isolates examined, revealing considerable clonal diversity within the species. Identical and closely related STs were identified in several countries on different continents, indicating global dissemination of the most successful clones. The clonal diversity and broad geographic distribution of *S. pseudintermedius* suggests that it has coevolved with its canine host for a long time in evolutionary terms and possibly since the evolution of dogs between 40 and 50 million years ago (20, 41). STs of *S. pseudintermedius* isolates infecting humans are identical or closely related to commensal isolates of dogs, suggesting that human infections are due to zoonotic transmission from a canine host. Indeed, transmission of *S. pseudintermedius* has been shown to occur frequently between dogs affected by deep pyoderma and their owners (14).

The third major phylotype found among the isolates previously identified as *S. intermedius* was associated with several different host species, including horse, camel, and pigeon, and was phylogenetically aligned with the *S. delphini* type strain isolated from a dolphin (39). A previous DNA-DNA hybridization analysis confirmed that the *S. delphini* type strain rep-

resented a distinct species compared to the recently described *S. pseudintermedius* and the *S. intermedius* type strain (7). Taken together, these data suggest that *S. delphini* is commonly misidentified as *S. intermedius* and may be more clinically important than was previously thought. In fact, very few studies have reported the identification of *S. delphini* strains since the original description of the species, but one study in Norway isolated *S. delphini* from a case of bovine mastitis, extending the broad host range observed in the current study (4). Of note, Sasaki et al. indicated that more than one species may exist among isolates genetically allied with *S. delphini* (33).

The accessory gene regulator (*agr*) is conserved throughout the staphylococci but has diverged along lines that appear to parallel speciation within the genus (8, 42). This divergence has given rise to a novel type of interstrain and interspecies cross-inhibition that likely represents an essential aspect of staphylococcal biology and may be a predominant feature of the evolutionary forces that have driven it. In order to further investigate the diversity of SIG populations, we examined the diversity of the *agrD* locus, which encodes the AIP (8, 20, 36, 42). Sung et al. identified three *agrD* alleles corresponding to *agr* specificity groups encoding different AIP variants among 20 strains of *S. intermedius* isolated from dogs in a single veterinary hospital in the United Kingdom (36) and demonstrated biological activity for two of them. Here, we identified the same three predicted AIPs, in addition to a novel fourth AIP, which was encoded by approximately 27% of strains (Table 3). Of note, all of the predicted AIP variants of the SIG contain a serine in place of the conserved cysteine found in the AIPs of the other staphylococcal species analyzed to date, resulting in a lactone rather than a thiolactone ring (20). *S. aureus* populations are also divided into four distinct groups based on *agr* allelic variation. Interference in virulence gene expression caused by different *S. aureus agr* groups has been suggested to be a mechanism for isolating bacterial populations and a fundamental basis for subdividing the species (42). All previously examined staphylococcal species encode AIPs that are unique to each species (8). However, we found that *agr* alleles were shared among the three closely related species of the SIG, indicating that a common quorum-sensing capacity has been maintained despite species differentiation in largely distinct ecological niches.

A previous study of *agr* evolution in *S. aureus* reported that genotypes were not associated with more than one *agr* type, indicating that *agr* radiation preceded clonal diversification and that recombination has played a very limited role in the distribution of *agr* diversity (42). The study concluded that the species was phylogenetically structured according to *agr* group (42). More recently, Robinson et al. provided evidence that the *S. aureus* species could be divided into two subgroups that both contain multiple clonal complexes and *agr* groups (30). The authors proposed that recombination events had resulted in the sharing of *agr* groups between the two subspecies but concluded that recombination of *agr* has not occurred very frequently within *S. aureus* populations, as *agr* and clone tree topologies within the two subspecies were in agreement (30). In contrast, the association of different *agr* alleles with strains of *S. pseudintermedius* of identical genotype identified in the current study suggests that assortive recombination has frequently contributed to the distribution of *agr* alleles among *S. pseudintermedius* populations. The markedly different topology of the phylogenetic tree constructed with *agrD* sequences compared to trees based on the other four gene loci is consistent with this theory (Fig. 1). Overall, we have found that *S. pseudintermedius* has a largely clonal population structure, but recombination appears to have played an important role in the distribution of *agr* alleles within the *S. pseudintermedius* species and the SIG as a whole. The sharing of *agr* alleles between different species of staphylococci has not been previously observed. This discovery indicates that *agr* differentiation has not occurred strictly along lines that parallel speciation. The lack of an association between *agr* type and SIG species, host, disease, and clinical or geographic origin identified in the current study leads to the question of what selective pressure is driving *agr* diversification. The basis for *agr* diversity and the importance of its biological activity in the SIG remain to be elucidated.

Recently, an increasing number of episodes of *S. intermedius* infections that were refractory to treatment with methicillin have been reported (15, 22, 32, 44). Here, we found that the methicillin-resistant SIG strains examined are all classified as *S. pseudintermedius* and have evolved by multiple acquisitions of the *mecA* gene by different *S. pseudintermedius* clones. Of note, a common clone was identified among isolates from five different centers in Sweden and Germany, indicating the existence of a widespread successful clone in Northern and Central Europe. MRSP clones were not shared between Europe and North America, indicating geographic restriction and probably reflecting the very recent emergence of methicillin-resistant strains. The identification of the common methicillin-resistant clones in the current study means that surveillance can be carried out to monitor MRSP clonal dissemination and strategies for the control of MRSP infections can be targeted to the most widespread clones.

Taken together, these data have resolved the population genetic structure of the SIG, resulting in broad new insights into the ancient and recent evolution of this important group of animal pathogens.

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REFERENCES

1. **Aarestrup, F. M.** 2001. Comparative ribotyping of *Staphylococcus intermedius* isolated from members of the Canoidea gives possible evidence for host-specificity and co-evolution of bacteria and hosts. Int. J. Syst. Evol. Microbiol. **51:**1343–1347.
2. **Bartual, S. G., H. Seifert, C. Hippler, M. A. Luzon, H. Wisplinghoff, and F. Rodriguez-Valera.** 2005. Development of a multilocus sequence typing scheme for characterization of clinical isolates of *Acinetobacter baumannii*. J. Clin. Microbiol. **43:**4382–4390.
3. **Bes, M., L. Saidi Slim, F. Becharnia, H. Meugnier, F. Vandenesch, J. Etienne, and J. Freney.** 2002. Population diversity of *Staphylococcus intermedius* isolates from various host species: typing by 16S-23S intergenic ribosomal DNA spacer polymorphism analysis. J. Clin. Microbiol. **40:**2275–2277.
4. **Bjorland, J., T. Steinum, B. Kvitle, S. Waage, M. Sunde, and E. Heir.** 2005. Widespread distribution of disinfectant resistance genes among staphylococci of bovine and caprine origin in Norway. J. Clin. Microbiol. **43:**4363–4368.
5. **Chesneau, O., A. Morvan, S. Aubert, and N. el Solh.** 2000. The value of rRNA gene restriction site polymorphism analysis for delineating taxa in the genus *Staphylococcus*. Int. J. Syst. Evol. Microbiol. **50:**689–697.

6. **Coenye, T., and P. Vandamme.** 2003. Intragenomic heterogeneity between multiple 16S ribosomal RNA operons in sequenced bacterial genomes. FEMS Microbiol. Lett. **228:**45–49.
7. **Devriese, L. A., M. Vancanneyt, M. Baele, M. Vaneechoutte, E. De Graef, C. Snauwaert, I. Cleenwerck, P. Dawyndt, J. Swings, A. Decostere, and F. Haesebrouck.** 2005. *Staphylococcus pseudintermedius* sp. nov., a coagulase-positive species from animals. Int. J. Syst. Evol. Microbiol. **55:**1569–1573.
8. **Dufour, P., S. Jarraud, F. Vandenesch, T. Greenland, R. P. Novick, M. Bes, J. Etienne, and G. Lina.** 2002. High genetic variability of the *agr* locus in *Staphylococcus* species. J. Bacteriol. **184:**1180–1186.
9. Reference deleted.
10. **Feil, E. J., J. E. Cooper, H. Grundmann, D. A. Robinson, M. C. Enright, T. Berendt, S. J. Peacock, J. M. Smith, M. Murphy, B. G. Spratt, C. E. Moore, and N. P. Day.** 2003. How clonal is *Staphylococcus aureus*? J. Bacteriol. **185:**3307–3316.
11. **Freney, J., Y. Brun, M. Bes, H. Meugnier, F. Grimont, P. A. D. Grimont, C. Nervi, and J. Fleurette.** 1988. *Staphylococcus lugdunensis* sp. nov. and *Staphylococcus schleiferi* sp. nov., two species from human clinical specimens. Int. J. Syst. Bacteriol. **38:**168–172.
12. **Futagawa-Saito, K., T. Sugiyama, S. Karube, N. Sakurai, W. Ba-Thein, and T. Fukuyasu.** 2004. Prevalence and characterization of leukotoxin-producing *Staphylococcus intermedius* in isolates from dogs and pigeons. J. Clin. Microbiol. **42:**5324–5326.
13. **Goh, S. H., Z. Santucci, W. E. Kloos, M. Faltyn, C. G. George, D. Driedger, and S. M. Hemmingsen.** 1997. Identification of *Staphylococcus* species and subspecies by the chaperonin 60 gene identification method and reverse checkerboard hybridization. J. Clin. Microbiol. **35:**3116–3121.
14. **Guardabassi, L., M. E. Loeber, and A. Jacobson.** 2004. Transmission of multiple antimicrobial-resistant *Staphylococcus intermedius* between dogs affected by deep pyoderma and their owners. Vet. Microbiol. **98:**23–27.
15. **Guardabassi, L., S. Schwarz, and D. H. Lloyd.** 2004. Pet animals as reservoirs of antimicrobial-resistant bacteria. J. Antimicrob. Chemother. **54:**321–332.
16. **Hajek, V.** 1976. *Staphylococcus intermedius*, a new species isolated from animals. Int. J. Syst. Bacteriol. **26:**401–408.
17. **Hesselbarth, J., and S. Schwarz.** 1995. Comparative ribotyping of *Staphylococcus intermedius* from dogs, pigeons, horses and mink. Vet. Microbiol. **45:**11–17.
18. **Hesselbarth, J., W. Witte, C. Cuny, R. Rohde, and G. Amtsberg.** 1994. Characterization of *Staphylococcus intermedius* from healthy dogs and cases of superficial pyoderma by DNA restriction endonuclease patterns. Vet. Microbiol. **41:**259–266.
19. **Hill, P. B., A. Lo, C. A. Eden, S. Huntley, V. Morey, S. Ramsey, C. Richardson, D. J. Smith, C. Sutton, M. D. Taylor, E. Thorpe, R. Tidmarsh, and V. Williams.** 2006. Survey of the prevalence, diagnosis and treatment of dermatological conditions in small animals in general practice. Vet. Rec. **158:**533–539.
20. **Ji, G., W. Pei, L. Zhang, R. Qiu, J. Lin, Y. Benito, G. Lina, and R. P. Novick.** 2005. *Staphylococcus intermedius* produces a functional *agr* autoinducing peptide containing a cyclic lactone. J. Bacteriol. **187:**3139–3150.
21. **Jonas, D., M. Speck, F. D. Daschner, and H. Grundmann.** 2002. Rapid PCR-based identification of methicillin-resistant *Staphylococcus aureus* from screening swabs. J. Clin. Microbiol. **40:**1821–1823.
22. **Kania, S. A., N. L. Williamson, L. A. Frank, R. P. Wilkes, R. D. Jones, and D. A. Bemis.** 2004. Methicillin resistance of staphylococci isolated from the skin of dogs with pyoderma. Am. J. Vet. Res. **65:**1265–1268.
23. **Kumar, S., K. Tamura, and M. Nei.** 2004. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform. **5:**150–163.
24. **Kwok, A. Y., and A. W. Chow.** 2003. Phylogenetic study of *Staphylococcus* and *Macrococcus* species based on partial *hsp60* gene sequences. Int. J. Syst. Evol. Microbiol. **53:**87–92.
25. **Kwok, A. Y., S. C. Su, R. P. Reynolds, S. J. Bay, Y. Av-Gay, N. J. Dovichi, and A. W. Chow.** 1999. Species identification and phylogenetic relationships based on partial HSP60 gene sequences within the genus *Staphylococcus*. Int. J. Syst. Bacteriol. **49:**1181–1192.
26. **Mahoudeau, I., X. Delabranche, G. Prevost, H. Monteil, and Y. Piemont.** 1997. Frequency of isolation of *Staphylococcus intermedius* from humans. J. Clin. Microbiol. **35:**2153–2154.
27. **Martin, D. P., C. Williamson, and D. Posada.** 2005. RDP2: recombination detection and analysis from sequence alignments. Bioinformatics **21:**260–262.
28. **Meyer, S. A., and K. H. Schleifer.** 1978. Deoxyribonucleic acid reassociation in the classification of coagulase-positive staphylococci. Arch. Microbiol. **117:**183–188.
29. **Pottumarthy, S., J. M. Schapiro, J. L. Prentice, Y. B. Houze, S. R. Swanzy, F. C. Fang, and B. T. Cookson.** 2004. Clinical isolates of *Staphylococcus intermedius* masquerading as methicillin-resistant *Staphylococcus aureus*. J. Clin. Microbiol. **42:**5881–5884.
30. **Robinson, D. A., A. B. Monk, J. E. Cooper, E. J. Feil, and M. C. Enright.** 2005. Evolutionary genetics of the accessory gene regulator (*agr*) locus in *Staphylococcus aureus*. J. Bacteriol. **187:**8312–8321.
31. **Sakai, H., G. W. Procop, N. Kobayashi, D. Togawa, D. A. Wilson, L. Borden, V. Krebs, and T. W. Bauer.** 2004. Simultaneous detection of *Staphylococcus aureus* and coagulase-negative staphylococci in positive blood cultures by real-time PCR with two fluorescence resonance energy transfer probe sets. J. Clin. Microbiol. **42:**5739–5744.
32. **Sasaki, T., K. Kikuchi, Y. Tanaka, N. Takahashi, S. Kamata, and K. Hiramatsu.** 2007. Methicillin-resistant *Staphylococcus pseudintermedius* in a veterinary teaching hospital. J. Clin. Microbiol. **45:**1118–1125.
33. **Sasaki, T., K. Kikuchi, Y. Tanaka, N. Takahashi, S. Kamata, and K. Hiramatsu.** 2007. Reclassification of phenotypically identified *Staphylococcus intermedius* strains. J. Clin. Microbiol. **45:**2770–2778.
34. Reference deleted.
35. **Staden, R.** 1996. The Staden sequence analysis package. Mol. Biotechnol. **5:**233–241.
36. **Sung, J. M., P. D. Chantler, and D. H. Lloyd.** 2006. Accessory gene regulator locus of *Staphylococcus intermedius*. Infect. Immun. **74:**2947–2956.
37. **Tanner, M. A., C. L. Everett, and D. C. Youvan.** 2000. Molecular phylogenetic evidence for noninvasive zoonotic transmission of *Staphylococcus intermedius* from a canine pet to a human. J. Clin. Microbiol. **38:**1628–1631.
38. **Van Hoovels, L., A. Vankeerberghen, A. Boel, K. Van Vaerenbergh, and H. De Beenhouwer.** 2006. First case of *Staphylococcus pseudintermedius* infection in a human. J. Clin. Microbiol. **44:**4609–4612.
39. **Varaldo, P. E., R. Kilpper-Balz, F. Biavasco, G. Satta, and K. H. Schleifer.** 1988. *Staphylococcus delphini* sp. nov., a coagulase-positive species isolated from dolphins. Int. J. Syst. Bacteriol. **38:**436–439.
40. **Wakita, Y., A. Shimizu, V. Hajek, J. Kawano, and K. Yamashita.** 2002. Characterization of *Staphylococcus intermedius* from pigeons, dogs, foxes, mink, and horses by pulsed-field gel electrophoresis. J. Vet. Med. Sci. **64:**237–243.
41. **Wayne, R. K.** 1993. Molecular evolution of the dog family. Trends Genet. **9:**218–224.
42. **Wright, J. S., III, K. E. Traber, R. Corrigan, S. A. Benson, J. M. Musser, and R. P. Novick.** 2005. The *agr* radiation: an early event in the evolution of staphylococci. J. Bacteriol. **187:**5585–5594.
43. **Zadoks, R. N., Y. H. Schukken, and M. Wiedmann.** 2005. Multilocus sequence typing of *Streptococcus uberis* provides sensitive and epidemiologically relevant subtype information and reveals positive selection in the virulence gene *pauA*. J. Clin. Microbiol. **43:**2407–2417.
44. **Zubeir, I. E., T. Kanbar, J. Alber, C. Lammler, O. Akineden, R. Weiss, and M. Zschock.** 2007. Phenotypic and genotypic characteristics of methicillin/oxacillin-resistant *Staphylococcus intermedius* isolated from clinical specimens during routine veterinary microbiological examinations. Vet. Microbiol. **121:**170–176.

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AUTHOR'S CORRECTION

Population Genetic Structure of the *Staphylococcus intermedius* Group: Insights into *agr* Diversification and the Emergence of Methicillin-Resistant Strains

Jeanette Bannoehr, Nouri L. Ben Zakour, Andrew S. Waller, Luca Guardabassi, Keith L. Thoday, Adri H. M. van den Broek, and J. Ross Fitzgerald

Centre for Infectious Diseases, Royal (Dick) School of Veterinary Studies, The Chancellor's Building, New Royal Infirmary, University of Edinburgh, Edinburgh EH16 4SB, Scotland, United Kingdom; Centre for Preventive Medicine, Animal Health Trust, Newmarket, Suffolk CB8 7UU, United Kingdom; Department of Veterinary Pathobiology, Faculty of Life Science, University of Copenhagen, Frederiksberg C, Denmark; and Dermatology Unit, Division of Veterinary Clinical Sciences, Royal (Dick) School of Veterinary Studies, Easter Bush Veterinary Centre, University of Edinburgh, Roslin, Midlothian, EH25 9RG, Scotland, United Kingdom

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Molecular Diagnostic Identification of *Staphylococcus pseudintermedius*▽

Jeanette Bannoehr,[1] Alessia Franco,[2] Manuela Iurescia,[2] Antonio Battisti,[2] and J. Ross Fitzgerald[1]*

The Roslin Institute and Centre for Infectious Diseases, Royal (Dick) School of Veterinary Studies, University of Edinburgh, Edinburgh, Scotland, United Kingdom,[1] and Istituto Zooprofilattico Sperimentale delle Regioni Lazio e Toscana, Via Appia Nuova 1411, 00178 Rome, Italy[2]

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We report the first diagnostic test for the identification of *Staphylococcus pseudintermedius* involving a simple PCR-restriction fragment length polymorphism approach. The method allows discrimination of *S. pseudintermedius* from the closely related members of the *Staphylococcus intermedius* group and other important staphylococcal pathogens of humans and dogs.

Until recently, *Staphylococcus intermedius* was considered responsible for most cases of canine pyoderma, a major skin disease of dogs (8). However, using a multilocus sequencing approach, independent research groups have demonstrated that isolates phenotypically identified as *Staphylococcus intermedius* consist of three distinct species, including *S. intermedius*, *Staphylococcus pseudintermedius*, and *Staphylococcus delphini*, which together represent the *S. intermedius* group (SIG) (1a, 4). Importantly, it was discovered that *S. pseudintermedius*, not *S. intermedius*, is the common canine pyoderma pathogen and that *S. delphini*, isolated from a variety of different animals, may be more clinically important than was previously thought (1a, 4). The recently identified *S. pseudintermedius* (5) is occasionally isolated from serious human infections, and the emergence and spread of methicillin-resistant *S. pseudintermedius* strains are major veterinary and public health issues (1a, 3, 4, 7, 11–13). Sasaki et al. could biochemically differentiate *S. intermedius* from the other SIG species but was unable to identify phenotypic markers to discriminate *S. pseudintermedius*, the most clinically relevant species, from *S. delphini* (12), and DNA sequencing is currently required to identify *S. pseudintermedius* (1a, 12). In order to address the need for a method of discriminating clinical isolates of *S. pseudintermedius*, we have developed a rapid, simple, and robust PCR-restriction fragment length polymorphism (RFLP) approach which has been validated independently in laboratories in separate countries.

Our previous population genetic study of SIG isolates examined sequence diversity at five gene loci among 104 isolates (1a). In the current study, sequence analysis of one of the loci, *pta*, which encodes the enzyme phosphoacetyltransferase, revealed the presence of an MboI restriction site in all *S. pseudintermedius* isolates, which was absent in all *S. intermedius* and *S. delphini* isolates examined. Based on this discovery we have designed a simple, robust, and inexpensive PCR-RFLP diagnostic test for the identification of *S. pseudintermedius*. Staphylococcal genomic DNA isolation was carried out as previously described (1a). PCR amplification of a 320-bp fragment of the *pta* gene was carried out in a 50-μl volume with a 0.2 μM concentration of each oligonucleotide primer (pta_f1, AAA GAC AAA CTT TCA GGT AA, and pta_r1, GCA TAA ACA AGC ATT GTA CCG), a 0.2 mM concentration of the de-

TABLE 1. Staphylococcal isolates previously identified to the species level by a DNA sequencing approach[a]

Strain	Species	Host origin	MboI restriction site[b]
ED99	*S. pseudintermedius*	Dog	+
AV8001	*S. pseudintermedius*	Dog	+
95072195	*S. pseudintermedius*	Dog	+
94062394	*S. pseudintermedius*	Dog	+
N900260	*S. pseudintermedius*	Human	+
HT20030686	*S. pseudintermedius*	Dog	+
M72199	*S. pseudintermedius*	Dog	+
3279 (MRSP)	*S. pseudintermedius*	Dog	+
3414	*S. pseudintermedius*	Cat	+
9075	*S. pseudintermedius*	Dog	+
690	*S. pseudintermedius*	Cat	+
8478	*S. pseudintermedius*	Dog	+
HH1	*S. pseudintermedius*	Dog	+
Can6	*S. pseudintermedius*	Dog	+
Can10	*S. pseudintermedius*	Human	+
BH47	*S. pseudintermedius*	Dog	+
AV8024	*S. pseudintermedius*	Dog	+
8016	*S. pseudintermedius*	Dog	+
LMG22219	*S. pseudintermedius*	Cat	+
LMG22220	*S. pseudintermedius*	Horse	+
ATCC 49171	*S. delphini*	Dolphin	−
HT20030680	*S. delphini*	Camel	−
9106	*S. delphini*	Horse	−
8485	*S. delphini*	Horse	−
AV8051	*S. delphini*	Pigeon	−
HT20030677	*S. delphini*	Camel	−
AV8047	*S. delphini*	Pigeon	−
HT20030676	*S. delphini*	Camel	−
HT20030674	*S. delphini*	Camel	−
8086	*S. delphini*	Horse	−
HT20030679	*S. delphini*	Camel	−
AV8061	*S. intermedius*	Pigeon	−
NCTC11048	*S. intermedius*	Pigeon	−
AV8063	*S. intermedius*	Pigeon	−
AV8063	*S. intermedius*	Pigeon	−
ATCC 43808	*S. schleiferi* subsp. *schleiferi*	Human	−
CCUG37248	*S. schleiferi* subsp. *coagulans*	Dog	−
Newman	*S. aureus*	Human	+
N315	*S. aureus*	Human	+

[a] The isolates were previously identified by Bannoehr et al. (1). +, present; −, absent.
[b] *S. pseudintermedius pta* MboI restriction fragments were 107 bp and 213 bp, and *S. aureus pta* MboI restriction fragments were 156 bp and 164 bp.

* Corresponding author. Mailing address: The Roslin Institute and Centre for Infectious Diseases, Royal (Dick) School of Veterinary Studies, University of Edinburgh, Chancellor's Building, Little France, Edinburgh E164SB, Scotland, United Kingdom. Phone: 44 131 2429376. Fax: 44 131 242 9385. E-mail: Ross.Fitzgerald@ed.ac.uk.
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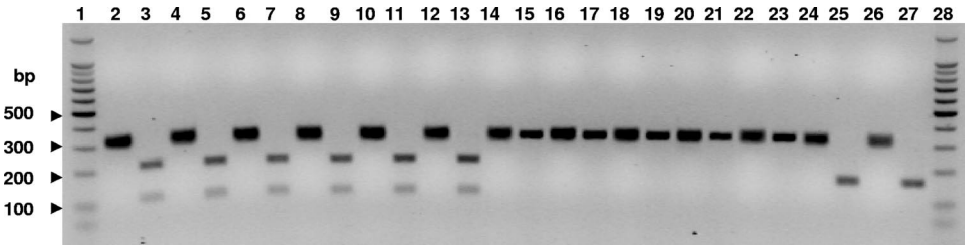

FIG. 1. Agarose gel electrophoresis of MboI restriction digest *pta* PCR products. Lane 1, 100-bp ladder; lane 2, *S. pseudintermedius* ED99; lane 3, *S. pseudintermedius* ED99, MboI digested; lane 4, *S. pseudintermedius* HH1; lane 5, *S. pseudintermedius* HH1, MboI digested; lane 6, *S. pseudintermedius* Can6; lane 7, *S. pseudintermedius* Can6, MboI digested; lane 8, *S. pseudintermedius* Can10; lane 9, *S. pseudintermedius* Can10, MboI digested; lane 10, *S. pseudintermedius* LMG22219; lane 11, *S. pseudintermedius* LMG22219, MboI digested; lane 12, *S. pseudintermedius* 3414; lane 13, *S. pseudintermedius* 3414, MboI digested; lane 14, *S. delphini* ATCC 49171; lane 15, *S. delphini* ATCC 49171, MboI digested; lane 16, *S. delphini* 9106; lane 17, *S. delphini* 9106, MboI digested; lane 18, *S. delphini* HT20030680; lane 19, *S. delphini* HT20030680, MboI digested; lane 20, *S. intermedius* NCTC11048; lane 21, *S. intermedius* NCTC11048, MboI digested; lane 22, *S. intermedius* AV8061; lane 23, *S. intermedius* AV8061, MboI digested; lane 24, *S. aureus* Newman; lane 25, *S. aureus* Newman, MboI digested; lane 26, *S. aureus* N315; lane 27, *S. aureus* N315, MboI digested; lane 28, 100-bp ladder.

oxynucleoside triphosphates, 1.5 mM $MgCl_2$, 0.5 U *Taq* DNA polymerase, and 50 ng DNA template, in a 1× reaction buffer. Thermocycling conditions included a 95°C incubation for 2 min followed by 30 cycles of 95°C for 1 min, 53°C for 1 min, and 72°C for 1 min, with a final incubation of 72°C for 7 min. Twenty-five-microliter samples of the PCR mixtures were incubated with 5 U of MboI and 5 μl of 5× digestion buffer for 2 h, and the digestion products were resolved in 2% (wt/vol) agarose by electrophoresis. We investigated the efficacy of the technique by the analysis of well-characterized isolates of *S. pseudintermedius*, *S. intermedius*, and *S. delphini* that represented the breadth of diversity identified within the SIG and that included the type strains (1). In addition, representative isolates of *Staphylococcus aureus*, *Staphylococcus schleiferi* subsp. *coagulans*, and *Staphylococcus schleiferi* subsp. *schleiferi*, which are associated with human and canine infections, were included (Table 1). A *pta* PCR product of 320 bp was successfully amplified from all strains examined. *S. pseudintermedius* PCR products all contained a single MboI site, resulting in two restriction fragments of 213 bp and 107 bp, respectively (Fig. 1). In contrast, SIG species *S. delphini* and *S. intermedius* and the *S. schleiferi* strains did not contain an MboI restriction site (Fig. 1). *S. aureus* isolates contained a unique MboI site, resulting in restriction fragments of 156 bp and 164 bp that appeared as a single band after agarose electrophoresis; this band was readily distinguishable from the *S. pseudintermedius* restriction fragment profile (Fig. 1). Our data indicate that the PCR-RFLP assay is an effective approach to *S. pseudintermedius* identification, allowing discrimination from the other SIG species and important staphylococcal pathogens of dogs (6, 9). In order to test the reproducibility of the method, independent investigators applied the approach in a diagnostic laboratory in Italy to a panel of 112 coagulase-positive staphylococcal field isolates which had been isolated from dogs, cats, cows, horses, wild foxes, and a captive bear and identified as either SIG or *S. aureus* (2). Of the 86 isolates from dogs, 85 were identified as *S. pseudintermedius* by the PCR-RFLP approach, consistent with our previous findings that *S. pseudintermedius* is a common commensal of dogs and the major canine pyoderma pathogen. The remaining isolate resulted in a *pta* restriction profile which indicated an *S. aureus* identity. All but 1 of the 14 isolates from cats, all 3 isolates from wild foxes, and the single isolate from a captive bear were identified as *S. pseudintermedius*. The remaining isolate of feline origin had an *S. aureus*-specific restriction profile. A previous study reported the isolation of *S. intermedius* from different species of the canoidea, including bears and foxes (1). Our data suggest that *S. pseudintermedius* may be the most common SIG species associated with these animals. All five isolates from equid genital swabs did not contain a *pta* MboI site, consistent with our previous findings that *S. delphini* is the SIG species commonly identified among horses, and three isolates from equine respiratory tracts and a skin lesion were identified as *S. aureus*. Finally, of the seven isolates from cows, two were *S. pseudintermedius* and five were identified as *S. aureus*. To our knowledge, this represents the first reported identification of *S. pseudintermedius* in association with cows.

Until now, the lack of unique phenotypic markers for *S. pseudintermedius* in comparison to the other SIG members has precluded its identification without DNA sequencing. Importantly, due to the presence of common phenotypic markers, *S. pseudintermedius* is occasionally misidentified as *S. aureus* in human clinical diagnostic laboratories (10). The simple PCR-RFLP approach presented here represents the first reported diagnostic method which is effective for the identification of *S. pseudintermedius* and for its discrimination from other SIG species and several other important staphylococcal pathogens. As such, the method should prove extremely useful in routine veterinary diagnostic and clinical microbiology laboratories.

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REFERENCES

1. **Aarestrup, F. M.** 2001. Comparative ribotyping of *Staphylococcus intermedius* isolated from members of the Canoidea gives possible evidence for host-specificity and co-evolution of bacteria and hosts. Int. J. Syst. Evol. Microbiol. **51:**1343–1347.
1a. **Bannoehr, J., N. L. Ben Zakour, A. S. Waller, L. Guardabassi, K. L. Thoday, A. H. van den Broek, and J. R. Fitzgerald.** 2007. Population genetic structure of the *Staphylococcus intermedius* group: insights into *agr* diversification and the emergence of methicillin-resistant strains. J. Bacteriol. **189:**8685–8692.
2. **Baron, F., M. F. Cochet, J. L. Pellerin, N. Ben Zakour, A. Lebon, A. Navarro, I. Proudy, Y. Le Loir, and M. Gautier.** 2004. Development of a PCR test to differentiate between Staphylococcus aureus and Staphylococcus intermedius. J. Food Prot. **67:**2302–2305.
3. **Descloux, S., A. Rossano, and V. Perreten.** 2008. Characterization of new staphylococcal cassette chromosome mec (SCCmec) and topoisomerase

genes in fluoroquinolone- and methicillin-resistant *Staphylococcus pseudintermedius*. J. Clin. Microbiol. **46:**1818–1823.
4. **Devriese, L. A., K. Hermans, M. Baele, and F. Haesebrouck.** 2008. Staphylococcus pseudintermedius versus Staphylococcus intermedius. Vet. Microbiol. **133:**206–207.
5. **Devriese, L. A., M. Vancanneyt, M. Baele, M. Vaneechoutte, E. De Graef, C. Snauwaert, I. Cleenwerck, P. Dawyndt, J. Swings, A. Decostere, and F. Haesebrouck.** 2005. *Staphylococcus pseudintermedius* sp. nov., a coagulase-positive species from animals. Int. J. Syst. Evol. Microbiol. **55:**1569–1573.
6. **Frank, L. A., S. A. Kania, K. A. Hnilica, R. P. Wilkes, and D. A. Bemis.** 2003. Isolation of Staphylococcus schleiferi from dogs with pyoderma. J. Am. Vet. Med. Assoc. **222:**451–454.
7. **Guardabassi, L., S. Schwarz, and D. H. Lloyd.** 2004. Pet animals as reservoirs of antimicrobial-resistant bacteria. J. Antimicrob. Chemother. **54:**321–332.
8. **Hajek, V.** 1976. *Staphylococcus intermedius*, a new species isolated from animals. Int. J. Syst. Bacteriol. **26:**401–408.
9. **Morgan, M.** 26 September 2008, posting date. Methicillin-resistant Staphylococcus aureus and animals: zoonosis or humanosis? J. Antimicrob. Chemother. [Epub ahead of print.]
10. **Pottumarthy, S., J. M. Schapiro, J. L. Prentice, Y. B. Houze, S. R. Swanzy, F. C. Fang, and B. T. Cookson.** 2004. Clinical isolates of *Staphylococcus intermedius* masquerading as methicillin-resistant *Staphylococcus aureus*. J. Clin. Microbiol. **42:**5881–5884.
11. **Sasaki, T., K. Kikuchi, Y. Tanaka, N. Takahashi, S. Kamata, and K. Hiramatsu.** 2007. Methicillin-resistant *Staphylococcus pseudintermedius* in a veterinary teaching hospital. J. Clin. Microbiol. **45:**1118–1125.
12. **Sasaki, T., K. Kikuchi, Y. Tanaka, N. Takahashi, S. Kamata, and K. Hiramatsu.** 2007. Reclassification of phenotypically identified *Staphylococcus intermedius* strains. J. Clin. Microbiol. **45:**2770–2778.
13. **Van Hoovels, L., A. Vankeerberghen, A. Boel, K. Van Vaerenbergh, and H. De Beenhouwer.** 2006. First case of *Staphylococcus pseudintermedius* infection in a human. J. Clin. Microbiol. **44:**4609–4612.