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Molecular Genetic Typing of *Staphylococcus aureus* from Cows, Goats, Sheep, Rabbits and Chickens

A thesis submitted for the degree of Doctor of Philosophy

by

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March 2006

DECLARATION

I hereby declare that this thesis has not been previously submitted for a Degree at this or any other University and that it represents my own unaided work except where duly acknowledged in the text.

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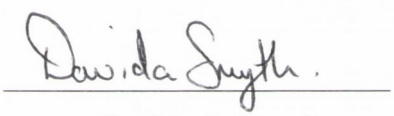

Davida S. Smyth

Contents

Summary

Staphylococcus aureus is an important pathogen of man causing a range of infections from boils, carbuncles and abscesses to more serious and life-threatening infections like endocarditis and osteomyelitis. *S. aureus* is also an important pathogen of animals. Bovine mastitis is one of the most important infectious diseases among dairy cattle worldwide resulting in several billion dollars of lost revenue to the dairy industry every year, with *S. aureus* being a common cause. Apart from mastitis in cows, sheep and goats, *S. aureus* can cause septicaemia, lameness and skeletal infections such as osteomyelitis in commercial broiler flocks. In rabbits *S. aureus* can infect small dermal lesions and invade subcutaneous tissue causing staphylococcosis.

In this work, *S. aureus* isolates from cows, goats, sheep, rabbits, chickens and a cat were analysed by a number of different genotyping methods in an attempt to characterise the clonal relationships of animal-associated *S. aureus.* Firstly, the prevalence of the genes encoding *S. aureus* superantigen (SAg) toxins and superantigen-like proteins (SAg-like), that can modulate the host's immune system, was determined. Secondly, the prevalence of the genes encoding the novel family of *S. aureus* proteins, the SET (SSL) proteins, that are also proposed to interact with the host's immune system was examined. This was achieved by the design of PCR primers and multiplex PCR assays. The genes encoded by the *egc* locus were demonstrated to be highly prevalent in *S. aureus* strains of animal origin, being found in 42.7 % of SAg and SAg-like gene-positive isolates. Every animal strain was found to contain a locus of genes encoding the SET (SSL) proteins. Mitogenicity assays showed that strains encoding SAg and SAg-like genes could stimulate the proliferation of human T lymphocytes, a hallmark of superantigenicity. These assays also showed that, despite encoding *set* (*ssl*) genes, strains that were negative for SAg and SAg-like protein encoding genes, were negative in terms of mitogenicity. These results add to the body of evidence to suggest that the SET (SSL) proteins are not superantigens.

To further assess the role of SAg, SAg-like and SET (SSL) proteins in animal infection, *E. coli* strains expressing recombinant *S. aureus* SAg, SAg-like and SET (SSL) proteins were used in Western immunoblotting experiments. Serum samples from cows with or without mastitis were screened for antibodies to the SAg, SAg-like and SET (SSL) proteins. The SAg and SAg-like proteins did not react with any of the serum samples implying that these proteins were either not expressed *in vivo* or expressed at very low levels, even with serum

from a cow that was infected with a strain encoding the SAg and SAg-like encoding genes. The SET (SSL) proteins, however, did react with the serum samples from cows that had mastitis. These data correlate well with studies of SAg, SAg-like and SET (SSL) proteins from human-associated *S. aureus* that suggest that the SAg and SAg-like proteins are expressed at low levels and that the SET (SSL) proteins are expressed during human infection.

Few epidemiological data were available on the relatedness of strains of *S. aureus* from different animal hosts. The techniques of Multilocus Sequence Typing (MLST), RAPD typing and *agr* typing were used to investigate the phylogeny of 123 isolates of *S. aureus* from different animal hosts from a number of different geographical locations. MLST revealed that a limited number of sequence types were responsible for the majority of cases of animal-associated infection with certain predominant clones being widespread amongst ungulate hosts. Although the commonest MLST type, ST133 was identified in 23 % of the animal strains tested, it was only associated with strains from sheep, goats and cows.

Forty-eight animal-associated isolates that represented the major clonal MLST/RAPD types were further typed using the nucleotide sequences of seven predicted surface proteins (*sas* typing) and using the polymorphic short sequence repeat region (SSr) of the protein A-encoding gene, *spa*. The *sas* typing revealed that animal-associated clones showed significant variation in terms of the nucleotide sequences of genes encoding putative surface proteins. The animal-associated strains generated many novel *sas* sequence types that had not been previously identified in human-associated *S. aureus*. The *spa* typing method was found to have the highest resolution of all the three sequence typing methods used. The combination of MLST and *spa* typing may prove to be a valuable alternative typing method to PFGE for the examination of animal-associated *S. aureus*.

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I dedicate this thesis to Mam and Dad

Publications

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Smyth, D., Hartigan, P.J., Meaney, W.J. and Smyth, C.J. (2003). Application of multiplex PCR to detect novel enterotoxin genes in strains of *Staphylococcus aureus* from goats, sheep, pigs and rabbits, *Proceedings of 1st FEMS Congress of European Microbiologists,* Ljubljana, Slovenia, June-July, 2003, 35 (Abstract A3–5).

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Smyth, D., Kennedy, J., Twohig, J, Bolton, D.J. and Smyth, C.J. (2004). *Staphylococcus aureus* isolates from Irish domestic fridges possess novel superantigen genes and are clonal in nature, *Proceedings of Society for Applied Microbiology Summer Conference "Dairy and Food Microbiology: Challenges and Opportunities"*, Cork, Ireland, July 2004, 35 (Abstract P33).

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Distinctions

2002 – Winner of Best Poster Prize at the *32nd Annual Food Science and Technology Conference*, Cork, Ireland, September 2002.

2003 – Winner of President's Prize for Best Oral Communication by a Postgraduate Student at the *57th Conference of the Association of Veterinary Teachers and Research Workers,* Scarborough, England, April 2003.

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Abbreviations

bp	base pair
DTT	dithiothreitol
g	gram
h	hour
kb	kilobases
kDa	kilodalton
M	molar
µ	micro
µl	microlitre
µg	microgram
µmol	micromole
mg	milligram
min	minute
ml	millilitre
NCTC	National Collection of Type Cultures
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PVDF	polyvinylidene diflouride
RAPD	random amplified polymorphic DNA
rpm	revolutions per minute
RT	room temperature
s	second
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TAE	Tris acetate buffer
TE	Tris EDTA buffer
vol	volume
w/v	weight per volume

Chapter 1

Introduction

1.1. Biology of the Staphylococci.

1.1.1. Classification and identification.

In 1880 Sir Alexander Ogston, a Scottish surgeon, discovered a cluster-forming, coccus-shaped organism that was capable of causing a number of pyogenic diseases in man (Linder, 1981). He subsequently named the organism 'staphylococcus' which is derived from the Greek word *staphyle*, meaning bunch of grapes and *coccus* meaning a grain or berry (Smith *et al.*, 1981). In a series of experiments he showed that when either pus containing staphylococci or a culture of the organism derived from pus were injected into mice, the mice developed the same symptoms as those of the original patients from which the pus was taken (Smith *et al.*, 1981; Baird-Parker *et al.*, 1990).

Staphylococci are defined as non-motile, non-sporing, spherical, Gram-positive cocci (0.5-1.5 μm in diameter) that characteristically divide in more than one plane to form irregular grape-like clusters. Their DNA has a low % G+C content of 30-39%. The closest relatives of the staphylococci are bacteria in the genera *Listeria*, *Enterococcus* and *Bacillus* as revealed by DNA sequence data and hybridisation. To date 37 species of staphylococci have been described; nine of these include two subspecies and one includes three subspecies. Most strains of staphylococci are catalase positive and oxidase negative, and are distinctively halotolerant, being able to withstand NaCl concentrations of up to 3.5 M (Wilkinson, 1997). The ability of *S. aureus* to grow on mannitol salt agar which contains 7.5 % NaCl is often used to distinguish staphylococci from other organisms. *S. aureus* forms glistening, smooth, raised, translucent colonies that often have a golden pigment. Staphylococci are ubiquitous commensals of the body including the mouth and throat and are commonly associated with the skin, skin glands and mucous membranes of warm-blooded animals. Based upon their ability to produce free coagulase, staphylococci can be divided into two main groups, coagulase-positive and coagulase-negative species.

At present coagulase-positive *S. aureus* and coagulase-negative *S. epidermidis* are the best studied of the staphylococci. To date the genomes of seven human–associated *S. aureus* strains have been published or are in the process of being published and that of strain RF122 from bovine mastitis infection is currently underway. Of two *S. epidermidis* strains, one is sequenced and one is underway (Table 1.1).

Table 1.1. Finished and incomplete genome sequences of staphylococci [Table adapted from Štěpán *et al.* (2004)].

Genome	Strain	Accession no.	Size, bp	Institution	Reference
S. aureus	N315	NC_002745	2,813,641	Juntendo University, Tokyo	Kuroda *et al.* (2001)
	Mu50	NC_002758	2,878,040	Juntendo University, Tokyo	Kuroda *et al.* (2001)
	MW2	NC_003923	2,820,462	Juntendo University, Tokyo	Baba *et al.* (2002)
	COL	NC_002951	2,813861	Institute for Genomic Research, Rockville, USA	Gill *et al.* (2005)
	8325	NC_002954	2,821,351	University of Oklahoma, Tulsa, USA	Not published
	MRSA252	NC_002952	2,902,619	Sanger Centre, Trinity College, Hinxton, UK	Holden *et al.* (2004)
	MSSA476	NC_002953	2,799,802	Sanger Centre, Trinity College, Hinxton, UK	Holden *et al.* (2004)
	RF122	NC_007622	2,742,531	University of Minnesota, Minneapolis,USA	Not published
S. epidermidis	RP62A	NC_002976	2,655,392	Institute for Genomic Research, Rockville, USA	Gill *et al.* (2005)
	ATCC 12228	NC_004461	2,499,279	Shanghai Medical University, Republic of China	Zhang *et al.* (2003)

All of the above genome sequences are available at http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Genome

1.1.2. Staphylococcal diseases

S. aureus is a major human pathogen that causes a wide variety of diseases ranging in severity from food-poisoning (McCormick *et al.*, 2001; Le Loir *et al.*, 2003) and life-threatening toxic shock syndrome (Llewelyn & Cohen, 2002; Proft & Fraser, 2003) to less serious infections, e.g., boils (Stulberg *et al.*, 2002).

Bovine mastitis is the most important infectious disease among dairy cattle worldwide and probably causes the single greatest economic loss in dairy farming (Beck *et al.*, 1992; Miles *et al.*, 1992; Wilson *et al.*, 1996). The infection results in reduced milk yield, lower quality milk, permanent damage to the udder, severe management problems, and on occasion the culling or death of affected animals. This has led to the introduction and widespread application of various control programmes aimed at preventing and reducing its prevalence. While these have reduced the overall prevalence of mastitis, a hidden or subclinical form is widespread and continues to constitute a major problem worldwide. Studies have shown that 30–40% of cows are affected by mastitis at any one time, mainly the subclinical form of the disease. *S. aureus*, one of the major causes of mastitis, continues to be largely refractory to therapy by antibiotics. After antibiotic therapy latent carriage of *S. aureus* in the udder and relapses of infection are common. A characteristic of mastitis infection is that the organism can persist in the udder for long periods of time in the form of a chronic infection which is subclinical for the most part but can flare up into a clinical condition.

Along with mastitis in goats, sheep and dairy cows, *S. aureus* can cause infections such as oedematous and necrotic dermatitis, septicaemia, abscesses and chondronecrosis in commercial broiler flocks (McCullagh *et al.*, 1998; McNamee *et al.*, 1998; Takeuchi *et al.*, 2002a). In rabbits *S. aureus* can infect small dermal lesions and invade subcutaneous tissue causing staphylococcosis (Hermans *et al.*, 1999). All *S. aureus* infections of rabbits have a similar clinical appearance with lesions of pododermatitis, subcutaneous abscesses, and mastitis (Okerman *et al.*, 1984). *S. aureus* can also cause tick-associated pyaemia in lambs (Webster & Mitchell, 1989) and pneumonia and osteomyelitis complex in turkeys (Huff *et al.*, 2000; Linares & Wigle, 2001).

1.1.3. Small colony variants.

Staphylococcal small colony variants (SCVs) are slow-growing subpopulations of *S. aureus* that are often associated with patients suffering from persistent or recurring infections (Baddour & Christensen, 1987, Proctor *et al.*, 1994; Proctor *et al.*, 1995; Kahl *et al.*, 1998; Looney, 2000). SCVs are characterised by a slow rate of growth and the formation of minute colonies on solid media, and can often be misidentified in the clinical laboratory (Proctor *et al.*, 1995; Kipp *et al.*, 2004). The phenotype of SCVs is caused by defects in menadione or haemin biosynthesis that disrupt the electron transport chain and cause a decrease in intracellular ATP (Proctor *et al.*, 1994). This defect can be reversed when haemin, thymidine and/or menadione are supplied (Balwit *et al.*, 1994; Kahl *et al.*, 1998). This alteration in metabolism causes many effects such as changes in pigmentation, carbohydrate utilisation profiles, production of exotoxins, and expression of adhesins (Vaudaux *et al.*, 2002). Especially of note is the increased resistance of SCVs to aminoglycoside antibiotics (Proctor *et al.*, 1998; Schaaff *et al.*, 2003). SCVs can survive within endothelial cells, possibly due to lower expression of the cytolytic α-toxin, and this ability may play a role in recurrent infection (Balwit *et al.*, 1994). It has been shown that the host intracellular environment can induce the formation of SCVs (Vesga *et al.*, 1996). SCVs induced by knockout of the *hemB* gene were more virulent per organism in a mouse model of septic arthritis than the isogenic parental strain (Jonsson *et al.*, 2003). Moreover, another study has shown that a *menD* mutation conferring an SCV phenotype allowed a survival advantage during antimicrobial therapy *in vivo* (Bates *et al.*, 2003).

1.2. Virulence factors of *S. aureus*.

The ability of bacteria to cause infection is multifactorial in the majority of cases and is rarely the property of a single determinant (Smith, 1977). The ability of *S. aureus* to cause infection is due to the large number of extracellular virulence factors and surface-associated proteins that it produces to colonise and survive within the host.

1.2.1. Extracellular toxins.

Staphylococci secrete a large number of toxins that serve various cytotoxic and other functions and have been implicated in different staphylococcal disease phenotypes.

1.2.1.1. α-, β-, δ-Toxins.

Staphylococcal α-toxin is considered to be the prototype of oligomerising pore-forming cytotoxins. Encoded on the chromosome, the *hla* gene is regulated at the level of transcription by the accessory gene regulator (*agr*). α-Toxin is synthesised as a 319-amino acid long precursor molecule with an N-terminal signal sequence of 26 amino acid residues (Foster *et al.*, 1990; Bhakdi & Tranum-Jensen, 1991; Hildebrand *et al.*, 1991). The affinity of α-toxin for cells ranges from high affinity, as is seen for rabbit erythrocytes, human platelets and human monocytes, to low affinity, as is seen for human erythrocytes (Bhakdi & Tranum-Jensen, 1991; Hildebrand *et al.*, 1991). The water-soluble monomer is secreted and upon contacting the membrane oligomerises to form a ring-shaped heptameric transmembrane pore, the crystal structure of which has been resolved (Song *et al.*, 1996). Several animal models have shown a role for α-toxin in *S. aureus* virulence (Jonsson *et al.*, 1985; Patel *et al.*, 1987; Bramley *et al.*, 1989; Foster *et al.*, 1990; Bhakdi & Tranum-Jensen, 1991; Callegan *et al.*, 1994; O'Callaghan *et al.*, 1997). It is considered to be an important virulence factor in bovine mastitis, being produced by between 20% – 50% of bovine *S. aureus* isolates (Matsunaga *et al.*, 1993; Sutra & Poutrel, 1994; Fitzgerald *et al.*, 1997). In a study of *S. aureus* from a variety of birds all strains possessed the gene for α-toxin (El-Sayed *et al.*, 2005).

β-toxin, encoded by the *hlb* gene, is an Mg^{2+}-dependent sphingomyelinase that causes invaginations of selected regions of the host cell membrane. Its cytolytic activity depends on the sphingomyelin content of mammalian cells (Arbuthnott, 1973). It acts as a type C phosphatase, hydrolysing sphingomeylin to phosphorylcholine and ceramide (Low & Freer, 1977). It is produced by most *S. aureus* from mastitic infections but the role of this toxin in mastitis has yet to be determined (Fitzgerald *et al.*, 1997). Cow, sheep and goat erythrocytes are most sensitive to β-toxin, whilst human erythrocytes display intermediate sensitivity. β-toxin causes "hot-cold" haemolysis (Smyth *et al.*, 1975). The *hlb* gene carries the attachment site for the serological group F *S. aureus* converting bacteriophages (Coleman *et al.*, 1989) which can mediate the simultaneous triple lysogenic conversion with respect to enterotoxin A, staphylokinase and β-toxin production.

δ-Toxin, encoded by the *hld* gene, is a small heat-stable 26 amino acid residue peptide that is thought to insert into a variety of membranes including animal erythrocytes, human cells and bacterial membranes (Mellor *et al.*, 1988; Clyne *et al.*, 1992). The *hld* gene is located

near the 5′-end of the *agr* (accessory gene regulator) RNAIII transcript. It is produced by nearly all *S. aureus* isolates (Donvito *et al.*, 1997). A role for δ-toxin in pathogenicity has not been demonstrated.

1.2.1.2. Synergohymenotrophic toxins.

The synergohymenotrophic toxins are a family of bi-component toxins that include Panton-Valentine Leucocidin (PVL), encoded by the *luk-PV* locus, and γ-toxin encoded by the *hlg* locus (Supersac *et al.*, 1993; Prévost *et al.*, 1995b). Members of this family of toxins contain two synergistically acting proteins, namely, an S component (LukS-PV, HlgA and HlgC) and an F component (LukF-PV and HlgB). The genes encoding the two components of PVL are co-transcribed and both components act together to damage the membranes of neutrophils and macrophages but not of erythrocytes. The γ-toxin locus consists of three open-reading frames; the *hlgC* and *hlgA* genes are co-transcribed (similarly to the *lukS-PV* and *lukF-PV* genes), whilst the *hlgB* gene is separately transcribed. The PVL protein is associated with severe skin infections such as furunculosis and is active against both human and rabbit leucocytes (Foster *et al.*, 1990; Gillet *et al.*, 2002). γ-Toxin is active against erythrocytes. Studies have shown that about 90% of *S. aureus* isolates carry the *hlg* locus, while only 2% carry the *luk-PV* locus (Prévost *et al.*, 1995a). Recently methicillin-resistant *S. aureus* (MRSA) isolates from companion animals have been shown to possess the *luk-PV* genes (Rankin *et al.*, 2005). Also there has been an emergence of community-acquired MRSA encoding the *luk-PV* genes (Vandenesch *et al.*, 2003).

1.2.1.3. Epidermolytic (exfoliative) toxin.

The exfoliative toxins of *S. aureus* are responsible for staphylococcal scalded skin syndrome (SSSS), a blistering skin disorder that particularly affects infants and young children, as well as adults with underlying disease (Bailey *et al.*, 1995; Gemmell, 1995). *S. aureus* produces two antigenically distinct forms of exfoliative toxin (ETA and ETB). The gene encoding ETA is located on the chromosome, while the gene encoding ETB is plasmid encoded. Their three-dimensional structure is similar to other glutamate-specific, trypsin-like, serine proteases with two substrate-binding domains and a serine-histidine-aspartate catalytic triad (H-72, D-120 and S-195) that forms the active site. However, unlike other serine proteases, the exfoliative toxins possess a highly charged N-terminal α-

helix and a unique orientation of a critical peptide bond, which blocks the active site of the toxins so that, in their native state, they do not possess any significant enzymatic activity. The target for the toxins has been identified as desmoglein-1, a desmosomal glycoprotein that plays an important role in maintaining cell-to-cell adhesion in the superficial layers of the epidermis. It is speculated that binding of the N-terminal α-helix to desmoglein-1 results in a conformational change that opens the active site of the toxin to cleave the extracellular part of desmoglein-1 between its third and fourth domains, resulting in disruption of intercellular adhesion and formation of superficial blisters (Amagai *et al.*, 2000; Amagai *et al.*, 2002; Hanakawa *et al.*, 2002; Yamaguchi *et al.*, 2002; Hanakawa *et al.*, 2003).

1.2.1.4. Enterotoxins and toxic shock syndrome toxin-1.

The staphylococcal enterotoxins (SEs) belong to a family of proteins termed superantigens (SAgs) (Llewelyn & Cohen, 2002; Proft & Fraser, 2003). SAgs are produced by different organisms including *S. aureus* and *Streptococcus pyogenes*. SAgs cause a massive T-cell proliferation by simultaneously binding to major histocompatibility complex class II (MHCII) molecules on antigen-presenting cells (APCs) outside of the peptide-binding groove and to the variable region of the β-chain of the T-cell receptor (TCRs) on T lymphocytes (Petersson *et al.*, 2004). SAgs interact with a subset of TCR Vβ-domains. There are about 50 different Vβ-regions in the human T-cell repertoire and consequently a large number of T-cells, as high as 20 % of the T-cell population, can be stimulated by SAgs. A conventional antigen will activate only about 1 in 10^5–10^6 T-cells. The interaction of the SAg with the TCR and MHCII complex leads to a systemic release of pro-inflammatory cytokines such as tumour necrosis factor-alpha (TNF-α) and interleukin-beta (IL-1β) (Fig. 1.1).

Among superantigens only SEs possess emetic activity. A highly flexible disulphide loop in the N-terminal domain of SEs has been linked with emetic properties. Neither the exact mechanism that leads to emesis nor a specific receptor molecule has yet been identified (Harris *et al.*, 1995; Hu *et al.*, 2001). The release of serotonin, a neurotransmitter, by both rat RBL-2H3 mast cells and murine peritoneal cells after SEB stimulation in culture was demonstrated (Komisar *et al.*, 1992). Serotonin has been shown to be released by the enterochromaffin cells that are present in the gut, and to bind to the receptor 5-HT_3 located on the extrinsic nerves in the gut, rapidly leading to nausea and vomiting

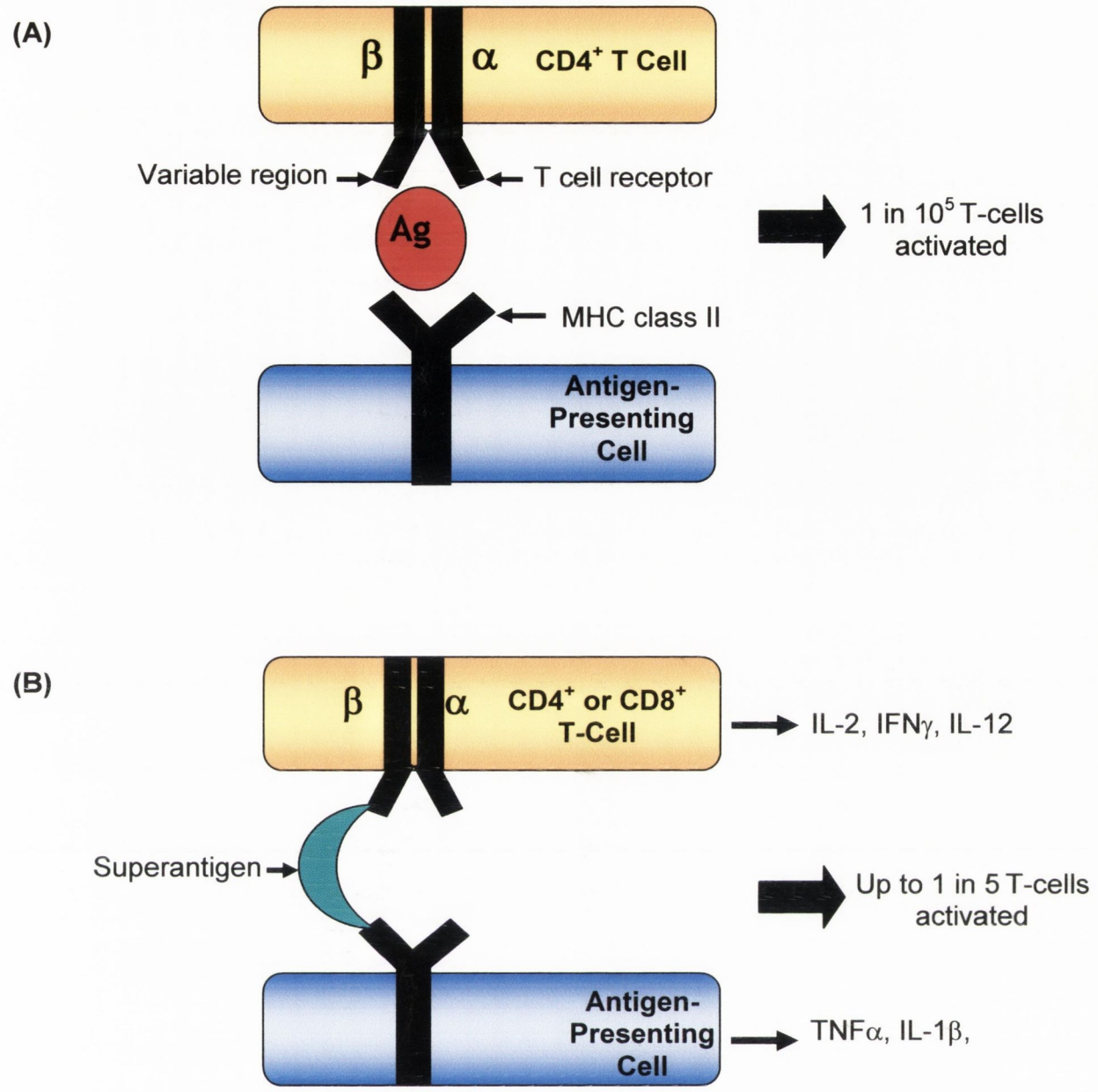


Fig 1.1. Interaction of a conventional antigen and of a superantigen with MHCII complex of APCs and the TCR of T lymphocytes.

Panel (A) shows the interaction of a conventional antigen. Panel (B) shows the interaction of a superantigen. Figure adapted from Llewelyn & Cohen (2002).

(http://www.hospprac.com/issues/1999/07/gershon.htm). A more recent study by Hu *et al.* (2005), demonstrated that SEA caused an increase in intracellular Ca^{2+} in human intestinal epithelial cells that was released from intracellular stores. The release of Ca^{2+} from intracellular stores was also induced by TNF-α or IFN-γ, both of which are produced by T-cells and APCs following superantigenic activity such as is caused by SEA. The release of Ca^{2+} was inhibited by treating cells with nitric oxide synthase (NOS) inhibitors, implying that the release of Ca^{2+} caused by SEA was dependent on NOS. Ca^{2+} is involved in neurotransmitter release (Perney *et al.*, 1986). Nitric oxide is an intracellular and intercellular messenger that functions in a number of physiological processes within gastroenterology, including mucosal function, inflammatory responses, blood flow regulation and gastrointestinal tract motility. It is possible that SEB stimulates neurons in the gut by causing the release of serotonin, whilst SEA may cause direct or indirect changes (mediated by TNF-α or IFN-γ) in Ca^{2+} concentrations in gut neurons leading to an emetic response. These studies infer that there may be a relationship between NOS expression, Ca^{2+} release, serotonin and emesis in the mechanism of SE induced food-poisoning.

The SAgs are often associated with mobile genetic elements. The gene for staphylococcal enterotoxin A (SEA) is encoded by a family of serotype F bacteriophages which insert into the β-toxin locus (Coleman *et al.*, 1989), the genes for SED and SElJ are plasmid encoded (Zhang *et al.*, 1998), and a number of the SE-encoding genes are associated with pathogenicity islands such as SaPIbov which encodes SE and SE-like genes, namely, *sec*-bov and *sell,* and the toxic shock syndrome toxin-1 gene *tst* (Fitzgerald *et al.*, 2001a).

Toxic shock syndrome toxin-1 (TSST-1) is a member of the SAg family of bacterial proteins. The TSST-1 protein shares sequence and structural similarities to the SEs. However, TSST-1 is not associated with emesis. It is best known for its involvement in toxic shock syndrome (TSS), which manifests as fever, rash, desquamation, and hypotension (Musser *et al.*, 1990; Booth *et al.*, 2001; Jarraud *et al.*, 2002). This toxin is responsible for 90% of menstrual and 50% of non-menstrual TSS cases (Hu *et al.*, 2003). The number of studies have suggested that at least a portion of atopic dermatitis, Kawasaki syndrome, some forms of psoriasis, neonatal TSS-like exanthematous disease, and other illnesses might be associated with TSST-1 (Leung *et al.*, 1993; Kotzin *et al.*, 1993; Takahashi *et al.*, 1998).

A decade ago only seven SEs were recognised, namely, SEA, SEB, SEC1, SEC2, SEC3, SED and SEE (Table 1.2). Since 1993, through gene sequencing and partial and complete genome sequencing, thirteen enterotoxins (SEG – SEQ, SER and SEU), three further variants of SEC (SEC-bovine, SEC-ovine and SEC-caprine), and variant enterotoxins encoded by the *egc* locus (enterotoxin gene cluster – SEGv, SEIv, SENv and SEUv) have been added (Table 1.2). The SAgs are further discussed in Chapter 3.

1.2.2. Surface components.

1.2.2.1. Surface-associated proteins.

Initial attachment of *S. aureus* to host tissues is considered to be a crucial step in the pathogenesis of infection. It allows the bacteria to resist the mechanical defences of the host's body, enables colonisation and growth, and may play a role in the invasion of host cells. Surface-associated adhesins of *S. aureus* with their ligands are shown in Table 1.3. Many surface proteins of *S. aureus* consist of a ~40 amino acid N-terminal secretory signal sequence (S), repeat domains, a proline-rich wall-spanning region (W), a wall-anchoring LPXTG motif, a hydrophobic transmembrane region (M), and a positively charged cytoplasmic tail (C) (Fischetti *et al.*, 1990) (Fig. 1.2). The members of the Sdr (serine-aspartic acid repeat) family of proteins, including the fibrinogen-binding proteins ClfA and ClfB of *S. aureus*, and SdrG of *S. epidermidis* contain a Ser-Asp (SD) repeat region (R region) just outside the cell wall-anchoring region (Josefsson *et al.*, 1998). This region acts as a stalk projecting the ligand-binding domain through the cell wall, thereby allowing it to be functionally expressed (Hartford *et al.*, 1997). The ligand-binding activities of most *S. aureus* surface-associated proteins have been localised to the N-terminal A-regions that are approximately 500 amino acids long (McDevitt *et al.*, 1995; Davis *et al.*, 2001).

1.2.2.2. Anchoring of surface proteins to the cell wall.

The anchoring of surface proteins to the cell wall in *S. aureus* requires both an N-terminal signal (leader) peptide and a C-terminal cell-wall sorting signal (LPXTG motif) (Schneewind *et al.*, 1992). Proteins are synthesised in the cytoplasm and then exported via a cleavable N-terminal signal peptide through the *sec* pathway (Schneewind *et al.*, 1992). Positively charged residues at the C-terminus of the protein allow it to be retained by the cell wall with the hydrophobic residues spanning the membrane (Fig. 1.3). The 35-residue

Table 1.2. Staphylococcal enterotoxins.

Enterotoxin	Gene	Location	Reference
SEA	*sea*	Serotype F Bacteriophages	Coleman *et al.* (1989)
SEB	*seb*	Pathogenicity island	Ranelli *et al.* (1985)
SEC1	*sec1*	Pathogenicity island	Bohach & Schlievert (1987)
SEC2	*sec2*	Pathogenicity island	Direct Submission to NCBI
SEC3	*sec3*	Pathogenicity island	Couch & Betley (1989)
SED	*sed*	Plasmid	Bayles & Iandolo (1989)
SEE	*see*	Chromosome	Couch *et al.* (1988)
SEC-bovine	*sec-bovine*	SaPIbov	Marr *et al.* (1993)
SEC-ovine	*sec-ovine*	Pathogenicity island ?	Marr *et al.* (1993)
SEC-caprine	*sec-caprine*	Pathogenicity island ?	Marr *et al.* (1993)
SEG	*seg*	*egc* pathogenicity island	Munson *et al.* (1998)
SEGv	seg_v	*egc* pathogenicity island	Blaiotta *et al.* (2004)
SEH	*seh*	Chromosome	Su & Wong (1995)
SEI	*sei*	*egc* locus	Munson *et al.* (1998)
SEIv	sei_v	*egc* locus	Blaiotta *et al.* (2004)
SEJ	*sej*	Plasmid pIB485	Zhang *et al.* (1998)
SEL	*sel*	SaPIbov	Fitzgerald *et al.* (2001)
SEK	*sek*	SaPI3	Orwin *et al.* (2001)
SEM	*sem*	*egc* locus	Jarraud *et al.* (2001)
SEN	*sen*	*egc* locus	Jarraud *et al.* (2001)
SENv	sen_v	*egc* locus	Blaiotta *et al.* (2004)
SEO	*seo*	*egc* locus	Jarraud *et al.* (2001)
SEP	*sep*	Chromosome	Kuroda *et al.* (2001)
SEQ	*seq*	SaPI3	Yarwood *et al.* (2002)
SER	*ser*	Plasmid pIB485-like	Omoe *et al.* (2003)
SEU	*seu*	*egc* locus	Letertre *et al.* (2003a)
SEUv	seu_v	*egc* locus	Blaiotta *et al.* (2004)

Table 1.3. *S. aureus* surface-associated, LPXTG-motif adhesins [Table adapted from Jutti (2005)].

Protein*	Ligand	References	*In vivo* model used	Virulence	References
SpA	Fc part of IgG	Forsgren & Sjöquist (1966); Löfdahl *et al.* (1983); Sjödahl (1977);	Mouse septic arthritis & mouse septic death	+	Palmqvist *et al.* (2002)
			Mouse skin lesion	+	Patel *et al.* (1987)
	Fab of IgG	Uhlén *et al.* (1984); Inganäs *et al.* (1980); Roben *et al.* (1995)	Mouse intraperitoneal infection	+	Patel *et al.* (1987)
	Complement receptor gC1qR/p33, e.g. on activated platelets	Nguyen *et al.* (2000)			
	von Willebrand factor	Hartleib *et al.* (2000)			
ClfB*	Fibrinogen	Ní Eidhin *et al.* (1998)	Rat endocarditis	+/-	Entenza *et al.* (2000)
	Type 1 cytokeratin 10	O'Brien *et al.* (2002)			

Protein	Ligand	References	*In vivo* model used	Virulence	References
Cna	Collagen	Patti *et al.* (1992); Patti *et al.* (1993); Switalski *et al.* (1989)	Mouse septic arthritis	+	Patti *et al.* (1994)
			Mouse septic death	Immunisation protective	Nilsson *et al.* (1998)
			Rabbit keratitis	+	Rhem *et al.* (2000)
			Rat endocarditis	+	Hienz *et al.* (1996)
			Mouse osteomyelitis	+	Elasri *et al.* (2002)
			Mouse septic arthritis	-	Elasri *et al.* (2002)
ClfA*	Fibrinogen Platelet membrane 118-kDa protein	McDevitt *et al.* (1994) Siboo *et al.* (2001)	Mouse septic arthritis	+	Josefsson *et al.* (2001)
			Rat endocarditis	+	Moreillon *et al.* (1995); Que *et al.* (2001) ; Stutzmann-Meier *et al.* (2001)
Bap	Attachment to inert surfaces, cell-cell adhesion	Cucarella *et al.* (2001)	Ovine mastitis	Early adherence reduced, persistence increased	Cucarella *et al.* (2002); Cucarella *et al.*(2004)

Protein	Ligand	References	*In vivo* model used	Virulence	References
FnBPA/ FnBPB	Fibronectin	Flock *et al.* (1987); Greene *et al.* (1995); Jönsson *et al.* (1991); Kuusela (1978); Massey *et al.* (2001); Signäs *et al.* (1989)	Rat endocarditis	+	Kuypers & Proctor (1989); Que *et al.* (2001)
			Rat endocarditis	-	Flock *et al.* (1996)
			Rat endocarditis	Immunisation protective	Schennings *et al.* (1993)
	Elastin	Roche *et al.* (2004)	Rat pneumonia	Reduced virulence	McElroy *et al.* (2002)
	Fibrinogen	Wann *et al.* (2000)	Mouse mastitis	+	Brouillette *et al.* (2003)
	Hsp60	Dziewanowska *et al.* (2000)			
Bbp*	Bone sialoprotein	Tung *et al.* (2000); Yacoub *et al.* (1994)			

* These proteins are members of the Sdr family.

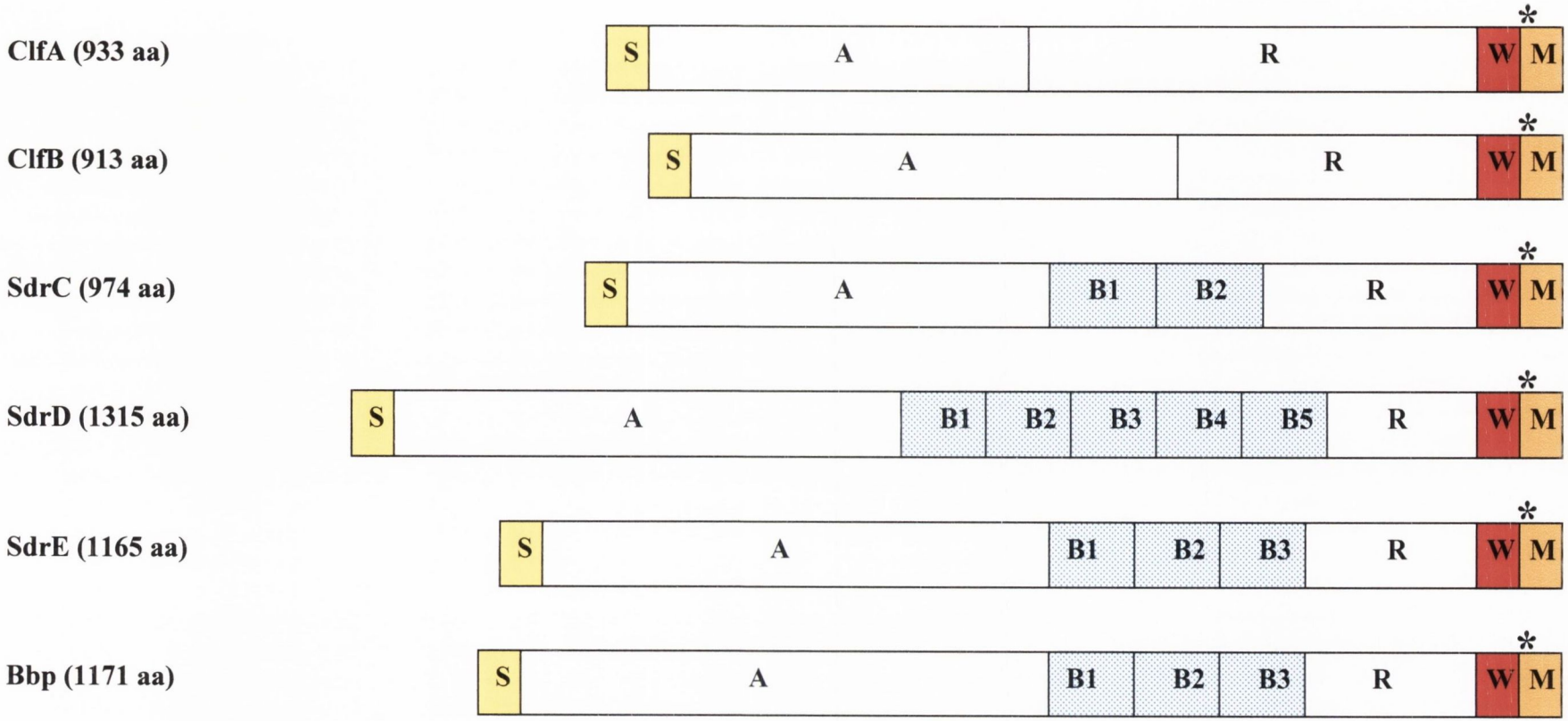
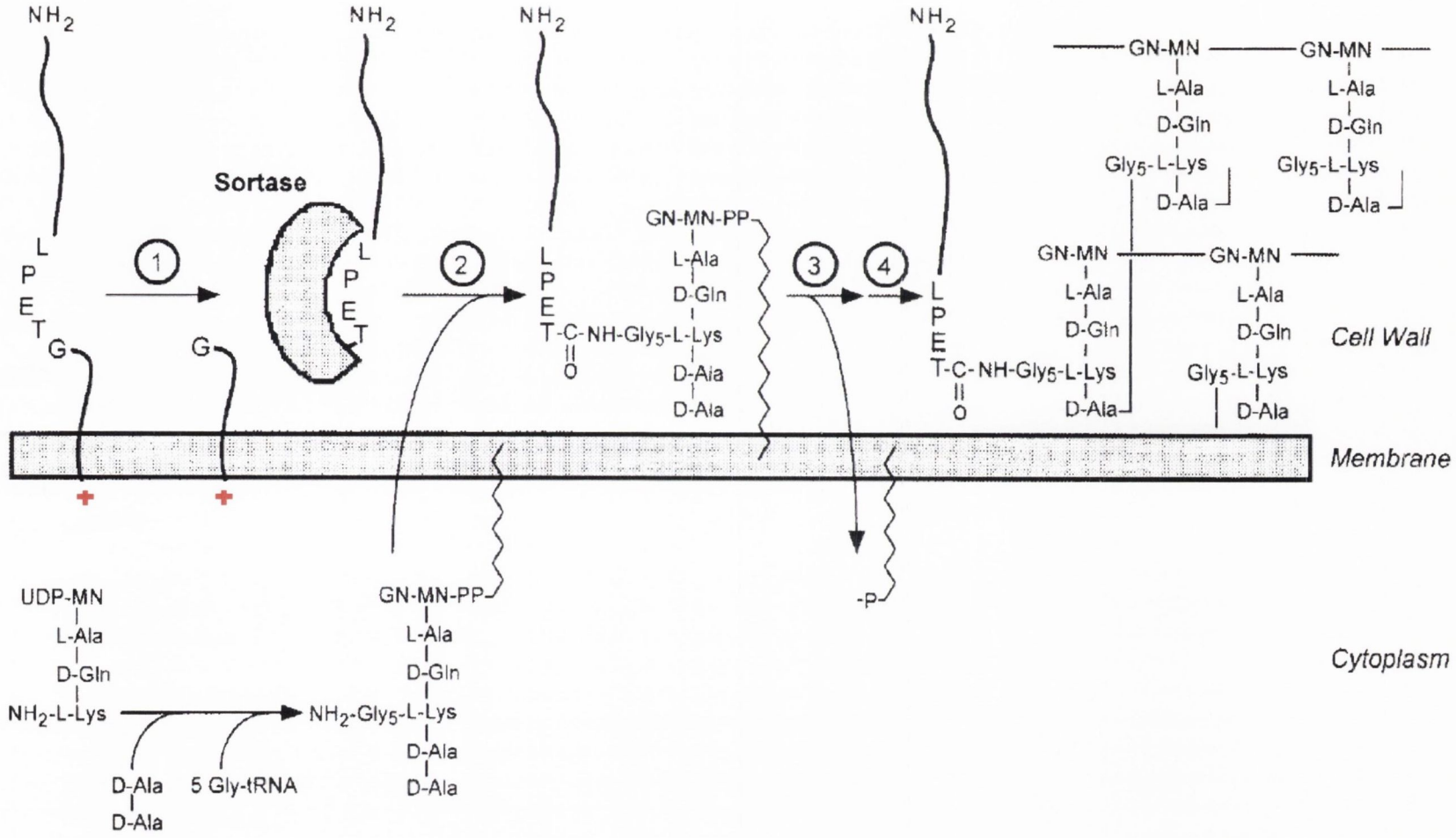


Fig. 1.2. Schematic of *S. aureus* surface proteins.

Many surface proteins of *S. aureus* consist of a ~40 amino acid N-terminal secretory signal sequence (S), repeat domains (R), a proline-rich wall-spanning region (W), a wall-anchoring LPXTG motif, a hydrophobic transmembrane region (M), and a positively charged cytoplasmic tail (Fischetti *et al.*, 1990). The ligand-binding activity of most surface proteins is found in the A domain and a number of surface proteins have additional repeats (B). The LPXTG motif is indicated by *.

Fig. 1.3. Model for sorting of cell-wall-associated proteins [Figure from Mazmanian *et al.* (1999)].

Proteins are synthesised in the cytoplasm and then exported via a cleavable N-terminal signal peptide through the *sec* pathway (Schneewind *et al.*, 1992). Positively charged residues at the C-terminus of the protein (indicated in red) allow it to be retained by the cell wall with the hydrophobic residues spanning the membrane. The 35-residue sorting signal contains an LPXTG sequence motif that is conserved within the sorting signals of more than 100 surface proteins of Gram-positive bacteria (Schneewind *et al.*, 1992; Mazmanian *et al*, 1999; Fischetti *et al.*, 1990). The motif serves as the recognition sequence for proteolytic cleavage between the threonine (T) and glycine (G) residues by the enzyme, Sortase. Sortase links the carboxyl group of the threonine in an amide linkage to the free amino group of the pentaglycine cross-bridge of peptidoglycan precursor, thus anchoring the protein to the cell wall peptidoglycan.


NH2
LPETG
Sortase
1
2
3
4
LPET-C-NH-Gly5-L-Lys
D-Ala
D-Ala
GN-MN-PP
L-Ala
D-Gln
GN-MN
Gly5-L-Lys
Cell Wall
Membrane
Cytoplasm
UDP-MN
NH2-L-Lys
NH2-Gly5-L-Lys
5 Gly-tRNA
-P

sorting signal harbours an LPXTG sequence motif that is conserved within the sorting signals of more than 100 surface proteins of Gram-positive bacteria (Fischetti *et al.*, 1990; Schneewind *et al.*, 1992; Mazmanian *et al.*, 1999). It serves as the recognition sequence for proteolytic cleavage between the threonine (T) and glycine (G) residues by the enzyme sortase (Navarre & Schneewind, 1994). Sortase links the carboxyl group of the threonine in an amide linkage to the free amino group of the pentaglycine cross-bridge of peptidoglycan precursor, thus anchoring the protein to the cell wall peptidoglycan (Fig. 1.3). Sortase mutant strains of *S. aureus* that fail to anchor Protein A and the ClfA protein to the cell wall are less virulent in animal models of infection (Mazmanian *et al.*, 2000). Sortase and surface proteins with LPXTG motifs are found in many Gram-positive bacteria (Navarre & Schneewind, 1999; Pallen *et al.*, 2001). More recently a novel sortase system, sortase B, has been described which facilitates the sorting of iron-responsive proteins via a NPQTN motif (Mazmanian *et al.*, 2002) (Fig. 1.4).

1.2.2.3. Protein A.

Protein A is a member of the family of adhesins termed MSCRAMMs (Microbial Surface Component Recognising Adhesive Matrix Molecules). These proteins mediate adherence to extracellular matrix (ECM) proteins (Patti *et al.*, 1994; Foster & Höök, 1998). Protein A is considered to be the archetypal cell-wall-associated protein; it was the first cell-wall-associated protein of *S. aureus* to be characterised (Söjdahl *et al.*, 1977; Moks *et al.*, 1986). Protein A has a domain structure, the S domain encodes the signal peptide, the domains A-E encode the immunoglobulin-binding domains, X_r encodes the SSRs (short sequence repeats), and X_c encodes the cell-wall attachment sequence. While the biological function of the X region is not known, it has been proposed that this domain may serve to extend the N-terminal immunoglobulin G-binding portion of protein A through the cell wall (von Heijne *et al.*, 1987). Protein A binds IgG in the orientation opposite to that required for IgG function, thereby inhibiting phagocytosis by disguising the organism from the innate immune system (Forsgren & Sjöquist, 1966; Löfdahl *et al.*, 1983). Protein A can also bind to the Fab region of IgG, mediating the proliferation of B cells in a manner similar to that of SAgs (Graille *et al.*, 2000). It has been recently shown to bind von Willebrand factor (Hartleib *et al.*, 2000) (Table 1.3). Protein A is a virulence factor (Patel *et al.*, 1987; Palmqvist *et al.*, 2002). It binds to platelets (Herrmann *et al.*, 1993; Nguyen *et al.*, 2000) and may play a role in their activation, a phenomenon important in the development of infective endocarditis. Protein A has been detected on 50–60% of bovine *S. aureus* strains

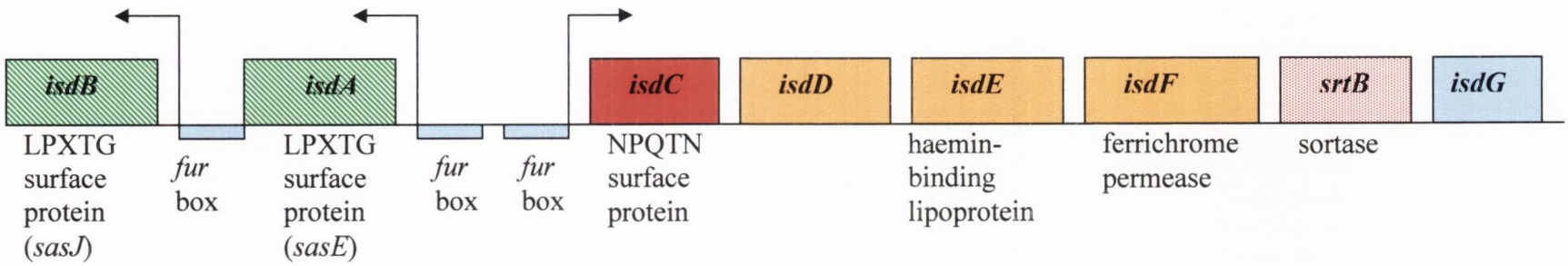


Fig. 1.4. Genomic organisation of the *S. aureus* iron regulated *isd* locus [Figure adapted from Mazmanian *et al.* (2002)].

The locus includes a number of iron-regulated genes that include two of the *sas* genes, *sasJ* and *sasE*. The proteins encoded by the *isdB* and *isdA* genes (in green) are processed by sortase A. The IsdC protein encoded by the *isdC* gene (in red) is processed by sortase B, the gene for which (*srtB*) is encoded within the locus. It is hypothesised that the products of the *isdC*, *isdD* and *isdE* genes (in orange) are involved in haeme-transport across the plasma membrane and that the *isdG* gene (in blue) encodes a haeme-degrading enzyme (Skaar & Schneewind, 2004). The *fur* box is a target for the ferric uptake regulator (Fur) of *S. aureus*, indicating that this locus is regulated by Fur (Xiong *et al.*, 2000).

(Sutra & Poutrel, 1994) though the prevalence of the gene was not investigated. The production of Protein A by only 50-60% of bovine strains may indicate altered expression profiles of bovine isolates.

1.2.2.4. Collagen-binding protein.

S. aureus can express a collagen-binding MSCRAMM called Cna. The Cna protein has been shown to be necessary and sufficient for *S. aureus* to attach to cartilage *in vitro*, showing that Cna can act as an adhesin (Switalski *et al.,* 1993) (Table 1.3). The Cna adhesin has also been shown to be a virulence factor in experimental septic arthritis where strains expressing Cna showed substantially increased virulence compared with knockout mutants, as demonstrated by macroscopic clinical evaluation and histopathological analysis of the joints (Patti *et al.*, 1994). Vaccination with a recombinant fragment of the Cna adhesin protected mice from septic death induced by intravenously administered *S. aureus* (Nilsson *et al.*, 1998).

1.2.2.5. Fibronectin-binding proteins.

The cell-wall-attached, fibronectin-binding proteins A and B (FnbpA and FnbpB) mediate attachment of *S. aureus* to host fibronectin (Fn) and are encoded by two closely related genes (Flock *et al.*, 1987; Jönsson *et al.*, 1991; Foster & Höök, 1998) (Table 1.3). The ability of *S. aureus* to bind to fibronectin contributes to its adherence to plasma clots and to *ex vivo* biomaterial, thus playing a role in the initiation of foreign body infection (Greene *et al.*, 1995; Vaudaux *et al.*, 1995). The binding of FnBPs to fibronectin is mediated by interactions between multiple fibronectin-binding repeat domains of the bacterial proteins connecting as extended antiparallel β-strands to the fibronectin type-1 modules in the N-terminal domain of fibronectin (Schwarz-Linek *et al.*, 2003). Peptides mirroring the repeat domains can block *S. aureus* binding (Joh *et al.*, 1999). The Fnbp proteins have been shown to form a complex between themselves, fibronectin and the α5β1 integrin that facilitates binding to and invasion of epithelial and endothelial cells (Sinha *et al.*, 1999). The Fnbp proteins are also important in the internalisation of *S. aureus* by osteoblasts (Ahmed *et al.*, 2001). It is not entirely clear whether or not these proteins are important virulence factors in rat endocarditis due to conflicting results from animal studies (Table 1.3).

1.2.2.6. Fibrinogen-binding proteins.

S. aureus expresses several different proteins that bind to fibrinogen. The cell-wall-anchored proteins clumping factor A (ClfA) and B (ClfB), along with the fibronectin binding proteins A and B (FnbpA, and FnbpB), recognise fibrinogen. The SD repeat protein of *S. epidermidis*, the SdrG protein, also recognises fibrinogen.

The ClfA, FnbpA, and FnbpB adhesins bind to the extreme C-terminal residues of the γ-chain of fibrinogen (McDevitt *et al.*, 1994; McDevitt *et al.*, 1997; Wann *et al.*, 2000) whereas the ClfB adhesin binds in the centre of the α-chain and SdrG binds in close proximity to the thrombin cleavage site of the β-chain of fibrinogen (Ní Eidhin *et al.*, 1998; Davis *et al.*, 2001). The ClfA and ClfB adhesins also promote binding to fibrinogen-coated biomaterials (McDevitt *et al.*, 1994; McDevitt *et al.*, 1995; Ní Eidhin *et al.*, 1998). The binding of the ClfA protein to fibrinogen is inhibited by Ca^{2+} (O'Connell *et al.*, 1998). The ClfB protein has been shown to bind to cytokeratin 10, an interaction that is probably responsible for *S. aureus* adherence to desquamated human nasal epithelial cells *in vivo* (O'Brien *et al.*, 2002; Walsh *et al.*, 2004).

S. aureus secretes several other fibrinogen-binding proteins, including Map/Eap (Jönsson *et al.*, 1995; Palma *et al.*, 1999), Efb (Palma *et al.*, 2001), and Emp (Hussain *et al.*, 2001a).

1.2.2.7. Other surface-located, ligand-binding proteins

S. aureus produces a range of other surface-located proteins that facilitate adherence to a multitude of host molecules, including elastin, vitronectin, fibrinogen and collagen. These are shown in Table 1.4.

1.2.2.8. Capsular polysaccharide.

Capsular polysaccharides are produced by more than 90% of *S. aureus* strains *in vitro* and eleven capsular serotypes have been identified (Sordelli *et al.*, 2000). However, the majority of the human-associated strains are either of serotype 5 or 8 (Arbeit *et al.*, 1984; Boutonnier *et al.*, 1989). The type of capsule produced by the majority of strains is known as a microcapsule and is very thin (<0.05 μm). The role of microcapsule in virulence remains controversial. The production of capsular polysaccharide is encoded by the genes

Table 1.4. *S. aureus* surface-associated adhesive proteins [Table adapted from Jutti (2005)].

Protein	Ligand	References	*In vivo* model used	Role in virulence	References
EbpS	Elastin	Downer *et al.* (2002); Park *et al.* (1991); Park *et al.* (1996)			
Emp	Fibronectin, fibrinogen, collagen, vitronectin	Hussain *et al.* (2001)			
Ebh	Fibronectin	Clarke *et al.* (2002)	Mouse skin abscess	-	Clarke *et al.* (2002)
VnBP	Vitronectin, heparin sulphate	Liang *et al.* (1995); Liang *et al.* (1992)			
PIA	Cell-cell adhesion	Cramton *et al.* (1999)	Mouse kidney abscess	Immunisation protective	McKenney *et al.* (1999)

of the *cap* locus which is located on the chromosome (Fig. 1.5). There are 16-open reading frames within the *cap* locus. Gene products encoded by 12 of the 16 genes of the *cap5* and *cap8* loci share significant amino acid identity (CapA–CapG and CapL–CapP), the four central genes (*CapH–CapK*) being type-specific (Sau *et al.*, 1997). *S. aureus* strains expressing a capsule are more resistant to phagocytosis, while anti-capsular antibodies opsonize encapsulated strains for phagocytic killing (Karakawa *et al.*, 1988; Xu *et al.*, 1992; Nilsson *et al.*, 1997; Thakker *et al.*, 1998). In animal models, anti-capsular antibodies protected animals against death, bacteraemia, endocarditis, and metastasis to the spleen, liver and kidneys.

Evaluation of capsule production in *S. aureus* strains from cows has shown varying results. In a French study 69% of 212 isolates from cows' milk were either of serotype 5 or 8 (Poutrel *et al.*, 1988), while only 3 of 17 (17.6%) isolates from bovine mastitis in Israel (Sompolinsky *et al.*, 1985) and only 27 of 195 (13.8%) bovine isolates in an Argentinian study were of either serotype 5 or 8 (Sordelli *et al.*, 2000). A comprehensive study of 636 bovine isolates (274 isolates from Europe and 362 from the USA) showed that almost half of the European isolates were of serotype 8, while 42 % of the USA isolates were either of serotype 5 or 8. It is clear that not all isolates from bovine mammary infection are of capsular serotype 5 or 8 and that there is considerable variability in bovine *S. aureus* from different geographical regions (Tollersrud *et al.*, 2000).

1.2.2.9. Biofilm formation.

The ability of bacteria to form biofilms is dependent on two properties, namely, the adherence of cells to a surface via an adhesin and an intercellular aggregation substance that allows the bacteria to accumulate and to form multilayered cell clusters (Mack *et al.*, 1999; Götz, 2002). Because of the size of the bacterial aggregate, biofilms have been shown to be resistant to some antibiotics and resistant to phagocytosis by macrophages (Cucarella *et al.*, 2004). The *ica* locus, a cluster of five genes, that is present in *S. epidermidis* and *S. aureus*, encodes biofilm matrix polysaccharide (Heilmann *et al.*, 1996). The polysaccharide is composed of linear β-1→6-linked *N*-acetylglucosamine residues (Mack *et al.*, 1996). Mutations in the *ica* locus lead to cells that are biofilm negative, less virulent, and less adhesive on hydrophilic surfaces (Götz, 2002). The expression of *ica* genes is regulated by the σ^B sigma factor. Other proteins that are involved in biofilm formation include the ClfA adhesin, the accumulation-associated protein (Aap), and the

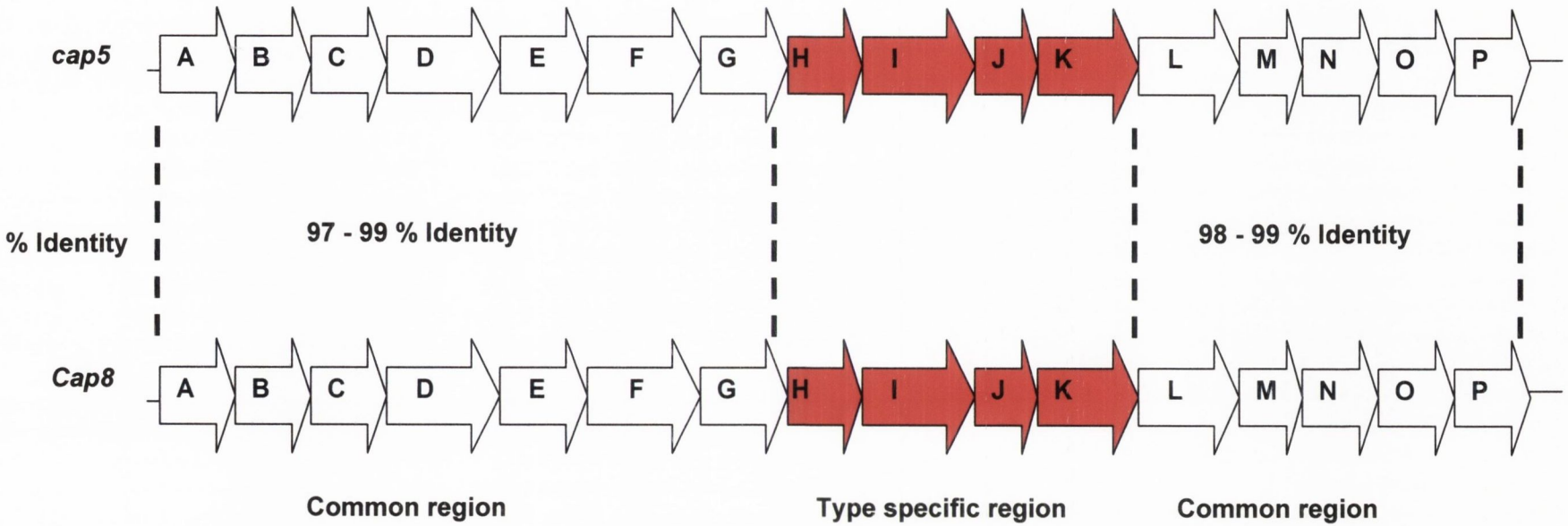


Fig. 1.5. Structural organisation of the *cap5* and *cap8* loci of *S. aureus* [Figure adapted from Sau *et al.* (1997)].

The *cap* locus is chromosomally encoded and consists of 16 orfs, with the gene products of 12 of the 16 genes (white arrows), *capA – capG* and *capL – capP* encoding significant amino-acid identity; the remaining four genes (red arrows) *capH – capK* are type-specific genes.

biofilm-associated protein (Bap). The Bap protein is involved in biofilm formation in bovine isolates. Bap-positive isolates have an elevated capacity to infect and to persist in the mammary gland (Cucarella *et al.*, 2004). The Bap protein promotes attachment to inert surfaces whereas PIA (polysaccharide intercellular adhesin) seems to be involved in intercellular adhesion. Bap is encoded by pathogenicity island SaPIbov2 (Úbeda *et al.*, 2003) and *bap* orthologue genes have been found in several staphylococcal species, including *S. epidermidis, S. chromogenes*, *S. xylosus* and *S. simulans* (Tormo *et al.*, 2005).

1.2.3. Extracellular proteins and enzymes.

1.2.3.1. Secreted ligand-binding proteins.

Coagulase is an extracellular protein produced by most *S. aureus* strains and its activity is used to distinguish *S. aureus* from coagulase-negative *S. epidermidis* (Phonimdaeng *et al.*, 1990). Coagulase binds specifically to prothrombin in a 1:1 molar ratio to form a complex called staphylothrombin that stimulates clotting of plasma by converting fibrinogen to fibrin (Phonimdaeng *et al.*, 1990) (Table 1.5). The function of coagulase in infection has not been clearly defined. Most studies have disagreed as to whether or not coagulase is involved in the infective process (Phonimdaeng *et al.*, 1990; Baddour *et al.*, 1994; Sawai *et al.*, 1997) (Table 1.5). Coagulase is the prototype of a new family of proteins designated the zymogram activator and adhesion protein family (ZAAP) (Panizzi *et al.*, 2004).

Staphylokinase is a 136-amino acid-long protein produced by lysogenic strains of *S. aureus*. The gene for staphylokinase (*sak*) is carried by a group of serotype F bacteriophages that insert into the *hlb* gene (Coleman *et al.*, 1989). Double-converting serotype F phages encode *sak* but not *sea*. Triple and quadruple-converting serotype F phages encode *sak* and *sea*. The synthesis of staphylokinase is under the positive control of the accessory gene regulator (*agr*) and the negative control of the staphylococcal accessory regulator (*sar*). Staphylokinase is not an enzyme. Staphylokinase forms a 1:1 stoichiometric complex with plasminogen that converts other plasminogen molecules to plasmin, an enzyme with proteolytic activity which can cause the dissolution of fibrin clots (Jin *et al.*, 2004). Mutants lacking the *sak* gene are not demonstrably less virulent than the wild-type parent strain in animal models (Vaudaux *et al.*, 1998).

The Map (MHC class II analogue protein) adhesin, also referred to as EAP and p70, is a secreted protein that binds to extracellular matrix components, including fibronectin,

Table 1.5. *S. aureus* secreted adhesive proteins.

Protein	Ligand	References	*In vivo* model used	Role in virulence	References
Efb (Fib)	Fibrinogen	Bodén & Flock (1994)	Rat wound infection	+	Palma *et al.* (1996)
	Complement C3	Lee *et al.* (2004)	Rat endocarditis	-	Palma *et al.* (1996)
Coagulase	Prothrombin, fibrinogen	Bodén & Flock (1989); McDevitt *et al.* (1992); Phonimdaeng *et al.* (1988)	Rat endocarditis	-	Moreillon *et al.* (1995a); Stuzmann Meier *et al.* (2001)
			Mouse haematogenous pneumonia	+	Sawai *et al.* (1997)
Map (Eap)	Fibronectin, fibrinogen, vitronectin, thrombospondin, bone sialoprotein,	Chavakis *et al.* (2002); Flock & Flock, (2001); McGavin *et al.* (1993); Jönsson *et al.* (1995); Palma *et al.* (1999);	Mouse wound infection	-	Chavakis *et al.* (2002)
			Mouse kidney abscess	-	
			Mouse peritonitis	+	
			Mouse acute septic	-	Lee *et al.* (2002)

	collagen, osteopontin, prothrombin, neutral phosphatase, ICAM-1, Eap	Hussain *et al.* (2002a); Kreikemeyer *et al.* (2002); reviewed by Harraghy *et al.* (2003)	arthritis, osteomyelitis and abscess Mouse chronic septic arthritis, osteomyelitis and abscess	+	Lee *et al.* (2002)
vWbp	von Willebrand factor	Bjerketorp *et al.* (2002)			

fibrinogen, vitronectin, bone sialoprotein, and thrombospondin (Chhatwal *et al.*, 1987; Herrmann *et al.*, 1991; Hudson *et al.*, 1999). However, isogenic Map-deficient *S. aureus* mutants were not impaired in their adhesion to these extracellular matrix proteins, thus acknowledging that other adhesins mediate this interaction and that Map may have an alternative function (Chavakis *et al.*, 2002; Kreikemeyer *et al.*, 2002; Haggar *et al.*, 2003; Harraghy *et al.*, 2003). The Map adhesin has been shown to induce Ig synthesis and to cause proliferation of mononuclear cells, in addition to shifting T-cell responses in a Th2 direction (Lee *et al.*, 2002). The Map protein may play a role in persistent *S. aureus* infection. Recently an analogue of Map, termed MapW, was described in the genome of strain N315 (Buckling *et al.*, 2005).

Other secreted ligand-binding proteins are shown in Table 1.5.

Chavakis *et al.* (2005) have proposed the grouping of secreted ligand-binding proteins of *S. aureus*, such as Coa, Efb (extracellular fibrinogen-binding protein) and Map, into a family of " secretable expanded repertoire adhesive molecules" (SERAM). It is not clear whether or not this new family of proteins, with no significant homology to each other, will be accepted as a family such as the MSCRAMMs.

1.2.3.2. Chips.

The chemotaxis inhibitory protein (Chips) of *S. aureus* is a secreted protein that is produced *in vivo* and by greater than 60% of clinical isolates (de Haas *et al.*, 2004a). It is a potent and specific inhibitor of neutrophil and monocyte chemotaxis toward the chemoattractants anaphylatoxin C5a and formylated peptides, bacterial excretion products like fMLP (*N*-formyl-peptide) (Postma *et al.*, 2004). These chemoattractants play a key role in the recruitment of neutrophils from the circulation to the site of infection. It is hypothesised that Chips production by *S. aureus* strains may stall the immune response at the early stages of infection (Postma *et al.*, 2004).

1.2.3.3. Proteases.

S. aureus secretes several proteases that include serine protease, thiol-protease, and metalloprotease (aureolysin) (Drapeau *et al.*, 1972; Arvidson, 1973a, 1973b, 1973c). The serine protease (V8 protease) is an endopeptidase that cleaves peptide bonds on the C-

terminal side of glutamic acid. It has been found that expression of V8 protease is essential for full virulence in animal models of infection (Coulter *et al.*, 1998). Aureolysin is expressed throughout the growth cycle of *S. aureus*, unlike the other proteases which are expressed during stationary phase. There is evidence to suggest that proteases can cleave both bacterial and host proteins (McAleese *et al.*, 2001). *S. aureus* has been shown to express two HtrA-like (high temperature requirement) surface proteases ($HtrA_1$ and $HtrA_2$) that have been shown play a role in the stress response, virulence, and production of secreted virulence factors (Rigoulay *et al.*, 2005).

1.3. Regulation of gene expression.

The expression of surface-associated and secreted *S. aureus* exoproteins is controlled by a complex regulatory network (Fig. 1.6) (Novick, 2003a; Yarwood *et al.*, 2003; Bronner *et al.*, 2004; Cheung *et al.*, 2004). The network is composed of two component regulatory, systems such as *agr*, *saeRS*, *srrAB*, *arlRS*, *lytRS*, and DNA-binding proteins, such as SarA and its homologues (SarR, Rot, SarS, SarT and SarU).

1.3.1. Two component regulatory systems.

The best characterised two component regulatory system is *agr* (accessory gene regulator) (See Chapter 5, Fig. 5.2). Two transcripts are divergently produced from the *agr* locus promoters P2 and P3. It is the products of the P2 transcript which act as a two component system. The AgrD protein is processed and transported out of the cell by the AgrB protein. The processed octapeptide binds to the transmembrane protein AgrC causing its autophosphorylation and leading to phosphorylation of the intracellular protein AgrA. The AgrA protein further activates the promoters P2 and P3 (Novick, 2003a). The transcript from promoter P3, RNAIII, is activated by two component systems AgrC–AgrA and TRAP-RAP [target of RNAIII-activating protein (TRAP)-RNAIII-activating protein (RAP)] (Gilot *et al.*, 2002). The RNAIII transcript encodes δ-toxin and is the effector molecule that during the exponential growth phase, up-regulates genes encoding many extracellular proteins and down-regulates genes encoding many surface proteins (Novick *et al.*, 1993) (Fig. 5.2). The *agr* system is discussed in more detail in Chapter 5.

Another two component system, SaeRS (*S. aureus* exoprotein expression), upregulates the production of several secreted proteins primarily at the transcriptional level (Giraudo *et al.*,

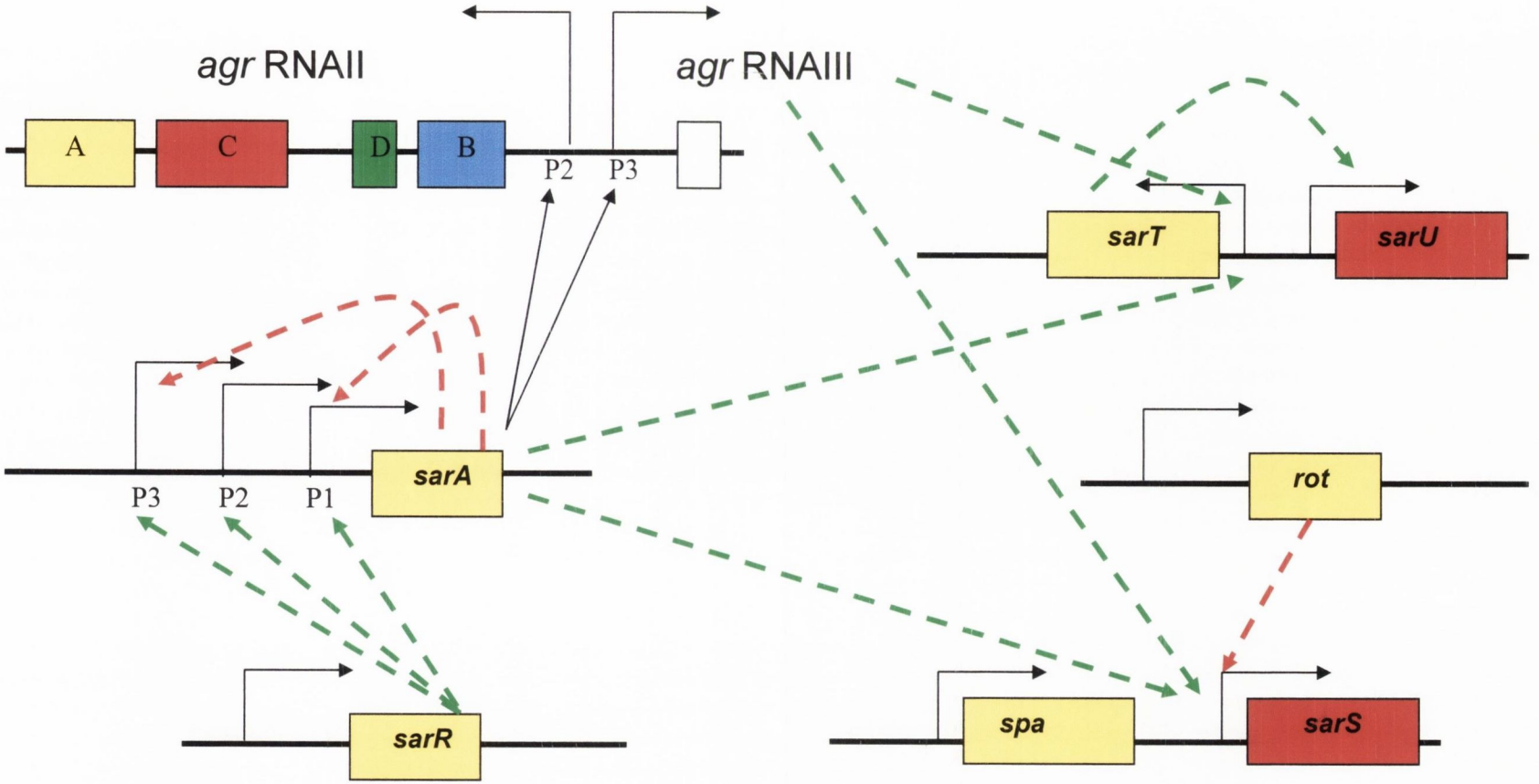


Fig. 1.6. Regulatory role of the *agr* and *sar* family.

Members of the *agr* and *sar* family of gene regulators are shown. Some of the interactions between these systems are indicated by dashed arrows. Red arrows indicate upregulation of transcription while green arrows indicate downregulation of transcription.

1994; Giraudo *et al.*, 1999). The *sae* locus comprises two co-transcribed genes; *saeR* encodes the response regulator and *saeS* encodes the histidine protein kinase. SaeRS does not affect *agr* or *sarA* expression but is essential for the transcription of the genes encoding α-toxin, β-toxin and coagulase. The SrrAB (staphylococcal respiratory response) two component system is also involved in regulation and influenced by environmental oxygen conditions. It may repress virulence factors such as TSST-1 under low oxygen conditions (Yarwood *et al.*, 2001). The ArlSR regulatory system (Autolysis-related locus) is a two component system that may be involved in cell growth and division, and in attachment to polymer surfaces (Fournier & Hooper, 2000). It is indirectly involved in the down-regulation of the transcription of some virulence genes such as the α- and β-toxin genes. Like ArlRS, the LytRS system is also involved in autolysis.

1.3.2. Transcription factors.

A number of transcription factors act in global regulation of virulence factors. The SarA protein (staphylococcal accessory regulator A) is a transcription factor up-regulating production of several surface proteins. It is also required for the expression of the *agr* locus (Cheung *et al.*, 1992; Cheung & Projan, 1994; Novick, 2003a). The SarA protein activates its own expression and is down-regulated by SarR. It is a DNA-binding protein that binds to AT-rich recognition motifs in the promoters of target genes. The SarA protein binds to the P2 and P3 promoter regions of the *agr* locus, activating the locus and altering the synthesis of virulence factors (Morfeldt *et al.*, 1996; Cheung *et al.*, 1997). SarA has a number of homologues. The SarR protein down-regulates SarA expression (Manna & Cheung, 2001). The SarS protein (also known as SarH1) is chromosomally encoded between the iron-regulated genes and the *spa* gene and is transcribed by two promoters, namely, a σ^A-dependent promoter and a σ^B-dependent stress response promoter (Bronner *et al.*, 2004). Expression of the SarS protein is repressed by SarA and by *agr* RNAIII. SarS activates expression of the *spa* gene. Other SarA homologues are SarH (Schmidt *et al.*, 2001) and SarU (Manna & Cheung, 2003). The Rot protein (repressor of toxins), another transcription factor, up-regulates surface adhesin production and down-regulates several secreted proteins (McNamara *et al.*, 2000; Saïd-Salim *et al.*, 2003).

1.3.3. The sigma factor, σ^B.

Additionally, an alternative sigma factor, Sigma B, is directly and indirectly through SarA, involved in regulation of virulence genes (Deora *et al.*, 1997; Kullik *et al.*, 1998).

The interaction of regulatory systems in *S. aureus* is shown in Fig. 1.6.

1.3.4. Superantigens.

Two of the staphylococcal SAg toxins, TSST-1 and SEB, act as global repressors of most exoprotein genes at the level of transcription and are also repressors of their own expression (Vojtov *et al.*, 2002). The cytoplasmic forms of these proteins, rather than their mRNA transcripts, act as the inhibitor (Vojtov *et al.*, 2002).

1.4. Identification of genes expressed or required *in vivo.*

The identification and characterisation of genes allowing a bacterial pathogen to survive and replicate in the host are essential for understanding the pathogenesis of an organism. In recent years two methods have been developed that determine the role of genes in virulence in living animals

1.4.1. Signature tagged mutagenesis.

Signature tagged mutagenesis has been applied to identify important virulence-associated genes in *S. aureus* in mouse models of bacteraemia, abscesses and wound infection (Mei *et al.*, 1997; Schwan *et al.*, 1998). Signature tagged mutagenesis allows the screening of a large number of insertional mutant strains for loss of virulence simultaneously in the same animal (Hensel *et al.*, 1995). *S. aureus* insertional mutants are generated using transposon Tn*917*. Each insertional mutation is flagged by a different DNA tag (40 bp), allowing the mutants to be distinguished from each other (Mei *et al.*, 1997). The DNA tags are flanked by conserved 20-bp regions that are amplifiable by PCR, allowing the central region to be amplified. Mutant strains with DNA tags are placed into microtitre plates and pooled into an inoculum pool that is used in infection studies. After a period of time the mutants are recovered (recovered pool). Both the tags in the inoculum and in the recovered pool are amplified by PCR, labelled, and used to probe filters arrayed with tags representing the mutants in the inoculum. Mutants that have attenuated virulence generate hybridisation signals when probed with tags from the inoculum pool but not from the recovered pool.

This technique has shown that several genes are important for *in vivo* growth of *S. aureus*. These include the *femA*, *femB* and *femX* (involved in peptidoglycan biosynthesis), *recA*, *lsp* (encodes the *S. aureus* prolipoprotein signal peptidase), and *putP* (proline permease) genes (Mei *et al.*, 1997; Schwan *et al.*, 1998; Coulter *et al.*, 1998). One of the disadvantages of signature tagged mutagenesis is that it does not identify secreted proteins as virulence factors due to cross complementation by neighbouring bacterial cells in the infection pool.

1.4.2. *In vivo* expression technology.

Unlike signature tagged mutagenesis, *in vivo* expression technology does not rely on mutagenesis. This method identifies genes specifically expressed during infection that are relatively inactive at other times (Mahan *et al.*, 1993; Lowe *et al.*, 1998). *In vivo* expression technology allows the detection of any gene, including those encoding secreted factors, that is strongly expressed during infection. *In vivo* expression technology uses genetic recombination as a reporter of gene activation *in vivo* (Camilli *et al.*, 1994). A library of bacterial genomic DNA is constructed in which the fragments are ligated to a promoterless copy of the *tnpR* gene (*tnpR* encodes resolvase of transposons γδ) (Newman & Grindley, 1984). A plasmid containing a kanamycin resistance cassette flanked by restriction sites which act as a substrate for resolvase is integrated into the chromosome. Thus cells that contain the plasmid are kanamycin resistant. DNA fragments which are trancriptionally active in the animal will cause the excision of the kanamycin cassette from the chromosome mediated by resolvase. Thus kanamycin-resistant cells are selected following recovery from the animal. *In vivo* expressed genes that have been identified using this approach include the *cap8C* (type 8 capsule production), *pbp2'* (penicillin-binding protein 2), and *agrA* (accessory gene regulator) genes (Lowe *et al.*, 1998).

1.5. Epidemiological typing of *S. aureus*.

Microbial typing is a useful tool for defining the source and route of transmission of bacterial pathogens and for studying the persistence, re-infection rate, and clonal selection of bacterial pathogens in the host. The evolution of bacterial pathogens can also be examined using typing techniques. Any typing method is rated on the basis of seven criteria (Maslow *et al.*, 1993): (*i*) typeability, (*ii*) reproducibility, (*iii*) stability, (*iv*) discriminatory power, (*v*) epidemiological concordance, (*vi*) ease of interpretation, and

(*vii*) ease of use. The various methods used in genotyping and phenotyping of *S. aureus* have been reviewed (Štěpán *et al.*, 2004) and are also described in Chapters 5 and 6.

1.5.1. Phenotypic methods for typing for staphylococci.

The phenotyping methods that have been applied to *S. aureus* include testing growth and biochemical characteristics (Kloos *et al.*, 1991; Kloos & Bannerman, 1999), conventional or commercial identification kits, biotyping (Devriese, 1984), phage typing (Blair & Williams, 1961, Tenover *et al.*, 1994), antibiotic susceptibility testing (NCCLS, 2000; Tenover *et al.*, 1994), whole cell protein electrophoresis (Clink & Pennington, 1987), serotyping and capsule typing (Karakawa *et al.*, 1985; Fattom *et al.*, 1992; Schlichting *et al.*, 1993; Guidry *et al.*, 1998). MLEE (Multilocus enzyme electrophoresis) has been successfully applied to both studies of bovine-associated *S. aureus* (Kapur *et al.*, 1995; Fitzgerald *et al.*, 1997) and human-associated *S. aureus* (Tenover *et al.*, 1994).

1.5.2. Genotypic methods used for staphylococci.

1.5.2.1. Methods without DNA amplification.

A large number of methods that do not require prior amplification of bacterial DNA by PCR have been used to type *S. aureus* from humans and animals, including macrorestriction analysis, selective restriction fragment hybridisation, and binary typing.

Macrorestriction analysis or pulsed field gel electrophoresis (PFGE) has been used to type *S. aureus* from a range of hosts and continues to be a standard method for bacterial typing in the USA and Europe (Richardson & Reith, 1993; Cox *et al.*, 1995, van Belkum *et al.*, 1998; Chang *et al.*, 2000, von Eiff *et al.*, 2001). In PFGE, bacteria contained within agarose plugs are lysed and the released high molecular weight DNA is cut with a rare-cutting restriction enzyme such as *Sma*I or *Csp*I. The agarose plugs with the restricted DNA are embedded in an agarose gel that is then subjected to electrophoresis. PFGE allows the resolution of very large DNA fragments (up to 10 Megabase pairs) unlike conventional agarose gel electrophoresis (up to 50 kb). Isolates with the same restriction profile are considered to be of the same PFGE type (pulsotype), isolates differing by one or two bands are considered to be subtypes (these changes are consistent with a single genetic event), and isolates differing by more than two bands are considered to be different PFGE

types (Tenover *et al.*, 1995). PFGE has a high discriminatory power and harmonised PFGE protocols for the epidemiological typing of MRSA in Europe have been described (Murchan *et al.*, 2003). PFGE has been used to type bovine *S. aureus* (Annemuller *et al.*, 1999; Zadoks *et al.*, 2000; Nagase *et al.*, 2002; Cabral *et al.*, 2004; Sabour *et al.*, 2004), chicken-associated *S. aureus* (McCullagh *et al.*, 1998; Rodgers *et al.*, 1999), sheep-associated *S. aureus* (Vautor *et al.*, 2003; Mørk *et al.*, 2005a), MRSA in a dog (Manian, 2003), MRSA in horses (Weese *et al.*, 2005b), goat-associated *S. aureus* (Mørk *et al.*, 2005), and human-associated *S. aureus* (Tenover *et al.*, 1994; Grundmann *et al.*, 2002a; Peacock *et al.*, 2002; Ip *et al.*, 2003). A number of studies have compared *S. aureus* from different animal hosts and humans using PFGE (Rodgers *et al.*, 1999; Zadoks *et al.*, 2002; Manian, 2003; Mørk *et al.*, 2005b; O'Mahoney *et al.*, 2005; Weese *et al.*, 2005c). A study by Hennekinne *et al.* (2003) used PFGE to resolve *S. aureus* biotypes (as described by Devriese, 1984).

Selective restriction fragment hybridisation involves the detection of restriction fragment length polymorphism (RFLP) by Southern hybridisation. Ribotyping is a modified version of this technique. Ribotyping involves the restriction digestion of total genomic DNA, agarose gel electrophoresis of the restriction fragments, transfer of the DNA fragments to a membrane, and the subsequent probing of the membrane with probes amplified from 16S rRNA and/or the 23S rRNA genes from *E. coli* (Thompson-Carter *et al.*, 1989). This method exhibits good reproducibility, stability and high discriminatory power. Ribotyping has been applied to bovine *S. aureus* (Fitzgerald *et al.*, 1997; Rivas *et al.*, 1997; Larsen *et al.*, 2000b; Pereira *et al.*, 2002) and human-associated *S. aureus* (De Buyser *et al.*, 1989; Blumberg *et al.*, 1992; de Lencastre *et al.*, 1994 ; Tenover *et al.*, 1994 and Pereira *et al.*, 2002). Oliveira & Ramos (2002a) developed a PCR-based ribotyping method that involves the amplification of the polymorphic genomic 16S-23S rRNA region.

Binary typing (BT) involves the hybridisation of restricted genomic DNA with a series of strain differentiating probes generated by arbitrarily primed PCR (random amplified polymorphic DNA) (van Leeuwen *et al.*, 1996, 1998, 1999). This method is highly reproducible, stable, and highly discriminatory (van Leeuwen *et al.*, 2002). Binary typing has been applied to bovine *S. aureus* (Zadoks *et al.*, 2000).

Plasmid profiling involves isolating plasmids from the bacteria, and analysing the size and number of plasmids by agarose gel electrophoresis. In restriction endonuclease analysis of

plasmids (REAP), the plasmids are digested with restriction enzymes prior to electrophoresis (Tenover *et al.*, 1994). Owing to the instability of plasmids this method shows only moderate reproducibility and is not widely used.

1.5.2.2. Methods involving DNA amplification.

A large number of methods that require prior amplification of bacterial genomic DNA by PCR have been used to type *S. aureus* from humans and animals, including PCR-RFLP, multiplex PCR, arbitrarily primed PCR (AP-PCR), and amplified fragment length polymorphism (AFLP).

PCR-RFLP typing involves the amplification of a gene and subsequent restriction digestion of the amplicon to generate a specific RFLP pattern following agarose gel electrophoresis. This method is rapid, simple, and reproducible, but has low discriminatory ability. Target genes amplified and digested in this way include the gene encoding coagulase (*coa*) (Goh *et al.*, 1992; Kobayashi *et al.*, 1995; Hookey *et al.*, 1998; Annemuller *et al.*, 1999), protein A (*spa*) (Frénay *et al.*, 1994; Sabat *et al.*, 2003), 5-enolpyruvylshikimate-3-phosphate synthase (*aroA*) (Marcos *et al.*, 1999), and clumping factors A and B (*clfA*, *clfB*) (Sabat *et al.*, 2003). Diep *et al.* (2003) developed multilocus restriction fragment typing (MLRFT), which involves the amplification of the genes used in multilocus sequence typing (MLST) (Enright *et al.*, 2000) and their subsequent digestion with restriction enzymes. This approach is significantly less expensive than sequencing. Takeuchi *et al.* (2002b) developed a PCR-RFLP typing assay for the aureolysin gene (*aur*) and examined strains from cows, pigs and chickens.

Multiplex PCR assays allow the simultaneous amplification of several genes in the one PCR reaction. This technique has been used to detect MSCRAMM-encoding genes (Mason *et al.*, 2001) and SAg and SAg-like genes (herein, Chapter 3) (Smyth *et al.*, 2005). Multiple locus variable number of tandem repeats (VNTR) analysis or MLVA is a multiplex PCR-based method that simultaneously amplifies the variable repeat regions of several surface-protein-encoding genes (Sabat *et al.*, 2003; Smyth *et al.*, 2004; Malachowa *et al.*, 2005). MLVA has all the advantages of PCR-based methods and also a discriminatory power similar to PFGE. This method has been automated (François *et al.*, 2005) and may prove to be a valuable tool in future outbreak investigations.

Arbitrarily primed PCR (AP-PCR), also known as random amplified polymorphic DNA analysis (RAPD), involves the use of short random oligonucleotide primers in PCR reactions that include both low and high stringency cycles. AP-PCR is rapid and discriminatory and has been used to type *S. aureus* from a range of hosts, including bovine *S. aureus* (Lam *et al.*, 1996; Lipman *et al.*, 1996; Fitzgerald *et al.*, 1997; Pereira *et al.*, 2002; Reinoso *et al.*, 2004), rabbit *S. aureus* (Hermans *et al.*, 2000, 2001), and human *S. aureus* (van Leeuwen *et al.*, 1996; Tambic *et al.*, 1997).

Amplified fragment length polymorphism (AFLP) is an innovative PCR-based technique with high resolution and reproducibility. The technique involves restriction digestion of total genomic DNA and attaching of oligonucleotide adapters, selective amplification of sets of restriction fragments, and electrophoresis of amplified fragments. This method has high discriminatory power and enables species or strain differentiation of the staphylococci (Sloos *et al.*, 1998, 2000). AFLP has been used to investigate human-associated *S. aureus* (Melles *et al.*, 2004) and animal-associated *S. aureus* (Cuteri *et al.*, 2004). More recently an oligoarray-based, high throughput AFLP method has been used to look at animal-associated *S. aureus* in combination with MLST (van Leeuwen *et al.*, 2005). Fluorescent-amplified fragment length polymorphism (FAFLP) has been developed for *S. aureus* and used to type isolates of MRSA (Ip *et al.*, 2003).

1.5.2.3. Methods based on direct sequence determination.

These methods involve the direct sequencing of target genes to detect polymorphisms at the genetic level. Methods that involve direct sequencing include *spa* typing, *coa* typing, *clfB* typing, *sas* typing, and multilocus sequence typing. These techniques are discussed in more detail in Chapters 5 and 6.

1.5.3. Innovative high-throughput molecular methods.

A number of novel and innovative techniques have been applied to *S. aureus* and have been recently reviewed (Štěpán *et al.*, 2004). These include denaturing gradient gel electrophoresis (DGGE), DNA microarrays, fluorescence *in situ* hybridisation with peptide nucleic acid probes (FISH PHA), and matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS).

1.6. Genetic structure of *S. aureus* populations.

Three types of population structure have been described by Smith (1995), namely, clonal, panmictic and epidemic. Most species of bacteria are clonal in structure; recombination and horizontal gene transfer is rare. In panmictic populations, recombination is frequent. A frequently recombining population is said to be epidemic when particular clones expand and spread rapidly allowing the population to be transiently clonal.

Many studies on the population structure of *S. aureus* have used techniques such as MLEE, MLST, and whole genome microarrays. These studies have shown that *S. aureus* conforms to a clonal population structure with mobile genetic elements such as bacteriophages, plasmids and pathogenicity islands contributing to diversity within clones. Feil *et al.* (2003) showed that point mutations give rise to new types at least 15-fold more frequently than recombination. In the long term, however, homologous recombination does contribute to the evolution of *S. aureus*. Robinson & Enright (2004a) described two occurrences of single chromosomal rearrangements (one of ~244 kb and one of ~557 kb), one of which occurred in a pandemic MRSA clonal lineage. Such replacements would have a dramatic impact on the evolution of *S. aureus* despite being rare occurrences.

1.6.1 *S. aureus* carriage.

Carriage of *S. aureus* appears to play a key role in the epidemiology and pathogenesis of infection. The ecological niche of *S. aureus* is moist squamous epithelium of the anterior nares of the nose. This part of the nose is lined by a fully keratinized epidermis with hairs, sebaceous glands, and sweat glands. *S. aureus* also colonizes the axillae and the perineum with nasal carriage seemingly a prerequisite for colonization of these sites (Walsh *et al.*, 2004). In healthy subjects, over time, three patterns of carriage can be distinguished: about 20% of people are persistent carriers, 60% are intermittent carriers, and approximately 20% almost never carry *S. aureus*. The molecular basis of the carrier state remains to be elucidated. However recent studies have shown that ClfB binds to cytokeratin 10 in the moist squamous epithelium allowing *S. aureus* to adhere (Walsh *et al.*, 2004).

The role of nasal carriage in infection has been reviewed by Wertheim *et al.* (2005). It has been shown in many different studies that nasal carriage of *S. aureus* is associated with increased risk of acquiring an infection. Eradication of *S aureus* from nasal carriers has

been shown to prevent infection in specific patient categories—eg, haemodialysis and general surgery patients.

1.6.2. Animal-associated *S. aureus*.

S. aureus strains from bovine mastitis have been shown to have a clonal population structure. Studies of strains from diverse geographical regions have revealed that only a few *S. aureus* clones are responsible for most cases of mastitis, and that these clones have a wide geographical distribution (Fitzgerald *et al.*, 1997; Annemüller *et al.*, 1999; Lange *et al.*, 1999). The main reservoirs for the disease appear to be the infected quarters and the skin of the udders and teats, with milking machines thought to be the main vectors of the infection (Myllss *et al.*, 1997).

The theory that human and bovine *S. aureus* have evolved separately is supported by a number of studies involving phage typing and ribotyping of Danish strains (Larsen *et al.*, 2000b), MLEE of isolates from a broad geographical distribution (Kapur *et al.*, 1995), and PFGE and binary typing of isolates from the Netherlands (Zadoks *et al.*, 2000). The study of Fitzgerald *et al.* (2001b) using whole genome microarrays showed that at least one bovine strain was more closely related to a human strain than other bovine strains; the authors implied that this bovine strain evolved from a human strain. The study of the epidemiology of bovine *S. aureus* requires the use of more discriminatory methods, such as sequence typing methods to verify these theories.

PFGE analysis of *S. aureus* strains from chickens and hatcheries in Northern Ireland showed that the majority of chicken-associated *S. aureus* strains from clinical infections and hatcheries had PFGE types related to a particular strain, strain 24 (McCullagh *et al.*, 1998), indicating a clonal population.

In Belgium rabbit-associated *S. aureus* strains, a cause of pododermatitis, subcutaneous abscesses and mastitis (Okerman *et al.*, 1984; Devriese *et al.*, 1996), have been analysed by RAPD typing by Hermans *et al.* (1999). The study revealed that 23 of 53 strains from 13 different rabbitries generated the same RAPD profile indicating a clonal population. This RAPD type belonged to a particular biotype termed 'mixed CV-C' that is known to be highly virulent and epidemic among the rabbit population.

1.6.3. Antibiotic-resistant *S. aureus*.

Methicillin-resistant *S. aureus* (MRSA) now represents one of the major challenges to microbiologists. Since the the 1980's MRSA isolates have been increasingly isolated in hospitals in Europe, the USA and many other countries.

The first MRSA strain was isolated in 1961, only 2 years after the introduction of methicillin (Jevons, 1961). The strain spread rapidly and became a problem in the USA in the 1970's. It was subsequently shown that MRSA strains had acquired a gene, *mecA,* which encoded an altered penicillin-binding protein, PBP2′ that rendered the strain resistant to methicillin and virtually all other β-lactams (Matsuhashi *et al.,* 1986). The *mecA* gene is encoded by a genetic element that has been named the staphylococcal cassette chromosome *mec* (SCC*mec*). It ranges in size from 21–67 kb and integrates into the chromosome near the origin of replication into an orf of unknown function (Ito *et al.*, 1999). Five distinct SCC*mec* types have been described (Ito *et al.*, 1999; Hiramatsu *et al.*, 2001; Ito *et al.*, 2001; Oliveira *et al.*, 2001a, 2002b; Ito *et al.*, 2003). The SCC*mec* type IV has two subtypes that have been described in community-acquired MRSA (Hiramatsu *et al.*, 2001; Ma *et al.*, 2002). More recently several different variants of SCC*mec* have been described in MRSA isolates from Ireland (Shore *et al.*, 2005). It is thought that the *mecA* gene has been acquired from another species, most probably *S. sciuri* (Wu *et al.*, 2001). The *mecA* gene has been identified in several staphylococcal species (Couto *et al.*, 1996).

Several different groups have investigated the population structure of MRSA by a number of different typing methods, including MLEE, MLST, ribotyping, PFGE, DNA microarray analysis, and *spa* typing (Musser & Kapur, 1992; Hiramatsu *et al.*, 1995; Roberts *et al.*, 1998; Enright *et al.*, 2000; Fitzgerald *et al.*, 2001b; Shopsin & Kreisworth, 2001). At present, it is believed that there are five major clonal lineages of MRSA which have evolved through multiple horizontal acquisitions of the *mec* element by diverse MSSA strains.

Community-acquired MRSA is a worrying problem and infections with community acquired MRSA have risen dramatically (Chambers, 2001; Hussain *et al.*, 2001b). These strains appear to differ from hospital-acquired MRSA in terms of phenotype and genotype. Community-acquired MRSA is noted for carrying the phage-borne Panton-Valentine leucocidin (PVL) toxin and a particular allele of the staphylococcal chromosomal cassette

mec (SCC*mec* type IV) (Robinson *et al.*, 2005). Outbreaks of MRSA soft tissue infections have been reported in certain segments of the population (eg, football players, wrestlers and prison inmates) and even in the animal population that often do not have the known risk factors for MRSA. In general *S. aureus* tends to infect immunocompromised individuals. Whether or not these strains have originated in a hospital setting, it is clear that these strains are successfully spreading within the community and affecting otherwise healthy individuals. The notion of *S. aureus* as an opportunistic pathogen must be re-evaluated. These strains may represent worryingly aggressive pathogens which anybody can acquire.

Antibiotic-resistant *S. aureus* have been isolated from animals. In a study by Lee *et al.* (2003), 28 *mecA*-positive strains were identified among 1913 isolates of *S. aureus* from cattle, pigs and chickens. The RAPD profiles of the *mecA*-positive isolates were identical and the antibiotypes of these animal-associated MRSA strains were found to be similar to those of human MRSA strains, indicating that animals could be a possible source of MRSA infection in humans.

Vancomycin-resistant *S. aureus* (VRSA) is an even more worrying threat. In the case of VRSA, the organism resists the drug not by inactivating the drug or altering the drug's target but by reducing access of vancomycin or other glycopeptide antibiotics by increasing the thickness of the cell wall or changing the components of the cell wall (Hanaki *et al.*, 1998; Oliveira *et al.*, 2001b; Cui *et al.*, 2003). Reports of VRSA have been rare, the first reported case occuring in July, 1996 (Hiramatsu *et al.*, 1997). Since then eighteen VRSA infections have been reported worldwide (Cui & Hiramatsu, 2003).

1.7. Approaches to combating staphylococcal infections.

1.7.1. Antibiotic therapy.

The emergence of antimicrobial-resistant strains of *S. aureus* in the early 1970's has greatly reduced the number of antibiotics capable of treating *S. aureus* infections. As a result several other avenues of anti-staphylococcal therapies have been investigated in order to complement existing effective antimicrobial chemotherapies, including vaccination and passive immunotherapy.

1.7.2. Vaccines.

1.7.2.1. Whole cell lysates.

Due to the low immunogenicity of capsular polysaccharides and the lack of in-depth knowledge as to which staphylococcal antigens elicit immunity against staphylococcal disease in cattle, vaccines for bovine isolates have been composed of whole bacterial lysates and bacterial extracts (Watson, 1984, 1992a, 1992b; Watson *et al.*, 1996). To date, one bovine vaccine, MASTIVAC I (VIREO, Israel), has been brought to veterinary challenge and field trials. This vaccine has proved to be successful in protecting cattle against *S. aureus* challenge (Leitner *et al.*, 2003b, c). The MASTIVAC preparation also elicited a broad spectrum of antibodies that protected mice against *S. aureus* challenge in mouse models of infection (Leitner *et al.*, 2003a). The regulations governing human vaccines are far more stringent and require exact specifications of the composition of the vaccine administered. These factors cannot be exactly controlled with bacterial lysates, which are subject to variability depending on day-to-day growth conditions.

1.7.2.2. Capsular polysaccharide.

S. aureus of capsular polysaccharide types 5 and type 8 comprise about 85% of human-associated strains. Thus they represent a good vaccine target. However, while antibodies to capsular polysaccharides can protect against *S. aureus* challenge (Fattom *et al.*, 1996, 1998), the polysaccharides are poorly immunogenic and vaccination with the polysaccharides alone has not been observed to yield long-lasting immunity. The capsular type 5 and type 8 polysaccharides were subsequently conjugated to *Pseudomonas* exotoxin A which improved their antigenicity. This conjugate vaccine has been developed as an anti-staphylococcal vaccine, STAPHVAX (Nabi Biopharmaceuticals). Clinical trials of STAPHVAX have been successful in generating specific antibodies to capsular polysaccharides in ~75% of patients tested (Shinefield *et al.*, 2002). However, no significant difference in the incidence of infection between the vaccinated and non-vaccinated groups was observed. In a recent phase III clinical study in haemodialysis patients, STAPHVAX was found to prevent *S. aureus* bacteraemia for up to 10 months following a single immunisation (Fattom *et al.*, 2004a; 2004b).

1.7.2.3. Staphylococcal proteins.

Proteins that play a role in adherence to host tissues provide an interesting target for a potential vaccine. Antibodies against MSCRAMM proteins have been shown to possess two important biological properties. Firstly antibodies to MSCRAMMS have been shown to prevent microbial adherence (McDevitt *et al.*, 1995; Mohamed *et al.*, 1999; Rennermalm *et al.*, 2001), as well as re-colonisation of host tissues or biomaterials. Secondly, anti-MSCRAMM antibodies bound to the bacterial cell wall facilitate rapid clearance of the organism through opsonophagocytosis (Rozalska *et al.*, 1993; Nilsson *et al.*, 1998).

Several MSCRAMMs have been investigated as potential vaccine candidate antigens, among them the collagen-binding protein which provided protection against *S. aureus* strains expressing the *cna* gene (Nilsson *et al.*, 1998). Unfortunately, as the *cna* gene is carried on a mobile genetic element that is present in only 40–60% of *S. aureus* strains, it does not represent a viable vaccine target. The fibronectin-binding protein (FnbpA) was also successfully investigated as a potential vaccine candidate (Schennings *et al.*, 1993; Mamo *et al.*, 1994). The *clfA* gene is present in 100% of human-associated invasive isolates and 98% of human-associated carriage isolates of *S. aureus* (Peacock *et al.*, 2002). The ClfA protein is expressed during infection and its expression is independent of *agr* regulation (Etz *et al.*, 2002; Weichhart *et al.*, 2003). Knock-out *clfA* mutants have been shown to be attenuated in virulence (Que *et al.*, 2000; Stutzman *et al.*, 2001). Studies have shown that immunisation with the ClfA protein can protect against lethal challenge with *S. aureus* (Brouillette *et al.*, 2002) and that the severity of disease is markedly reduced in a mouse model of septic arthritis (Josefsson *et al.*, 2001).

Staphylococcal SAgs also represent good vaccine candidate antigens as they are expressed by a wide variety of strains and contribute to the severity of many staphylococcal disease phenotypes. Also the increasing number of newly described SAgs and SAg-like proteins could potentially increase the range of vaccine candidates. However, toxins cannot be injected into the host in their native form as they would mediate significant damage to the host either by their superantigenic nature as in the case of the SEs and TSST-1, or cytotoxicity as in the case of α-toxin. To avoid the deleterious effects of these toxins on the host, recombinant mutants have been developed that lack superantigenicity/toxicity but are still immunogenic (Ulrich *et al.*, 1995; Bavart *et al.*, 1996). This approach has been

successful in protecting against lethal challenge with *S. aureus* (Ulrich *et al.*, 1995; Nilsson *et al.*, 1999).

1.7.2.4. DNA vaccines.

DNA vaccines consist of plasmid vectors derived from bacteria that contain genes inserted under the control of a eukaryotic promoter, thereby allowing expression of the protein in mammalian cells (Davis, 1997). Vaccination involves the intra-muscular injection of this non-replicating bacterial plasmid encoding the antigen.

To date two anti-staphylococcal DNA vaccination strategies have been used. The first approach involved immunisation with a plasmid that contained the *clfA* gene under the control of the cytomegalovirus promoter (Brouillette *et al.*, 2002). Mice immunised in this way generated a strong and specific antibody response to the ClfA protein and preincubation of *S. aureus* with these antibodies inhibited adherence to fibrinogen up to 92%. However, this form of vaccination did not protect mice against intraperitoneal challenge with *S. aureus*.

The second approach involved vaccination with a plasmid encoding the *mecA* gene which encodes the PBP2′ protein responsible for methicillin resistance in MRSA (Senna *et al.*, 2003). This approach appeared to provide protection against a sub-lethal dose of MRSA injected intraperitoneally. DNA vaccination may prove to be a successful approach in future investigations.

1.7.2.5. Passive immunization.

Passive immunisation is the administration of exogenously produced polyclonal or monoclonal antibodies in order to prevent infections in exposed individuals, to inactivate bacterial toxins, or to remedy hypogammaglobulinaemia in immunocompromised patients. During bacterial infections administered antibodies act to neutralise bacteria, toxins and superantigens, to facilitate opsonisation and complement activation, and to modulate cytokine production. A number of different approaches have been taken to combat staphylococcal infections by passive immunotherapy, each targeting different cellular components of staphylococci. Higuchi *et al.* (1994) used monthly injections of IVIG (intra venous immunoglobulin) to successfully treat patients susceptible to recurrent episodes of

toxic shock. Clinical trials have been done to evaluate the safety and efficacy of this donor-selected IVIG in the prevention of staphylococcal infections in very low birth-weight infants (Bloom *et al.*, 2005).

The *clfA* gene is present in 100% of invasive strains tested and is routinely detected as being expressed during infection (Etz *et al.*, 2002; Peacock *et al.*, 2002; Weichhart *et al.*, 2003). Therefore the ClfA protein represents a viable target for passive immunotherapy. Joseffson *et al.* (2001) showed that both human and rat antibody preparations containing high titres of anti-ClfA-specific IgG could be used prophylactically to decrease mortality in a murine model of *S. aureus* sepsis. Inhibitex Inc. has also developed a hyperimmunoglobulin, SA-IGIV, derived from plasma donors with high titres of anti-ClfA IgG, for the treatment of staphylococcal infections in very low birth-weight infants (Vernachio *et al.*, 2003). This IgG preparation recognised the *S. aureus* cell surface, could inhibit *S. aureus* adherence to immobilised fibrinogen, acted as an efficient opsonin in an *in vitro* phagocytosis assay, and finally, when used in conjunction with vancomycin, was therapeutically effective in a rabbit model of catheter-induced infective endocarditis caused by MRSA. A clinical trial of SA-IVIG is being undertaken to evaluate its safety and efficacy.

Inhibitex, Inc. has also developed a monoclonal antibody product, Aurexis. This monoclonal antibody was raised against residues 200–559 of the ligand-binding A domain of the ClfA protein and inhibits ClfA-mediated adherence to fibrinogen. Consequently, when this antibody was tested in *S. aureus* animal models of infection by passive transfer, it efficiently protected against intravenous challenge (Hall *et al.*, 2003) and significantly enhanced the therapeutic efficacy of vancomycin in a model of already established infectious endocarditis (Domanski *et al.*, 2005).

The neutralisation of staphylococcal toxins plays an important role in the treatment of *S. aureus* infections and several studies have used anti-toxin antibodies to passively treat animal models of infection (Gauduchon *et al.*, 2004). Passive transfer of immunoglobulin Y antibody generated in chickens against whole staphylococcal enterotoxin B suppressed cytokine responses and was protective in mice. Furthermore rhesus monkeys treated with the IgY specific for SEB for up to 4 h after challenge survived lethal SEB aerosol exposure (LeClaire *et al.*, 2002). Antibodies against non-toxic recombinant mutant toxins that retain their antigenicity have also been shown to confer protection. Hu *et al.* (2003) showed that

passive transfer of antibodies specific for a non-toxic mutant TSST-1 toxin provided protection against *S. aureus*-induced septic death in a mouse model of infection and protection has also been conferred in mouse models of infection by antibodies generated against a non-toxic mutant α-toxin and SEA devoid of superantigenicity (Menzies *et al.*, 1996; Nilsson *et al.*, 1999).

1.7.3. Novel therapy.

Broad-spectrum bacteriocins produced by lactic acid bacteria have been examined for potential therapy for *S. aureus* mastitis. These proteins are natural inhibitors that are already being used as preservatives in foods. Nicin is a bacteriocin that is effective against a range of Gram-positive pathogens (Broadbent *et al.*, 1989). Nicin in combination with the enzyme lysostaphin has been shown to be an effective treatment for staphylococcal mastitis giving a cure rate of 100% (Oldham *et al.*, 1991). A novel bacteriocin, lacticin 3147, has been impregnated into a commercial teat seal, the oil-based formulation that provides a physical barrier against infection of the teat canal and sinus (Meaney, & Nash, 1977). It also provided protection against another mastitis-causing organism *Streptococcus dysgalactiae* in an artificial infection model (Ryan *et al.*, 1998).

Phage therapy is another developing area of *S. aureus* research. In a study by Wills (2005) bacteriophage was found to prevent abscess formation in rabbits, when injected simultaneously with *S. aureus*. Also O'Flaherty *et al.* (2005a, 2005b) have described a lytic bacteriophage K that is active against drug-resistant *S. aureus.* However it should be noted that *S. aureus* can become resistant to bacteriophage. The failure of phage typing is due to the resistance of *S. aureus* to the lytic bacteriophages used in the typing procedure. As a result it remains to be seen how successful this approach to therapy will be.

1.8. Aims, objectives and rationale of this study.

The aims of this study were:

1. Development and application of a multiplex PCR for rapid screening for the genes encoding production of the recently described staphylococcal superantigens (SEG–SEO and SEQ for which no commercial serological kit is available.

2. Development of a PCR-based method for screening of isolates for the presence or absence of the newly described staphylococcal exotoxin-like genes [*set1–set25*].

3. Screening of *S. aureus* isolates, primarily from cows but also from rabbits, sheep and goats using the PCR methods developed, to determine the frequencies of the newer staphylococcal superantigen genes and *set* genes in *S. aureus* of animal origin. No such data existed at the outset of this project that would allow assessment of their potential role(s) in staphylococcal persistence and mastitis or of food safety risks in relation to food products from these animals and their milk.

4. Mitogenicity testing of bovine-associated *S. aureus* and screening of serum samples from cows with and without mastitis for antibodies to both the SET/SSL proteins and SAg-like proteins. This would reveal if novel enterotoxins were expressed by *S. aureus* strains and immunogenic in the host.

5. Previously several molecular fingerprinting techniques had been applied to the characterisation and differentiation of bovine isolates of *S. aureus*, namely, RAPD, RFLP analysis, ribotyping and PFGE. MLST (Multilocus Sequence Typing), *sas* typing and *spa* typing had been applied to population analysis of antibiotic-resistant *S. aureus* but not to *S. aureus* associated with animal infection. Evaluation of these molecular typing methods for *S. aureus* isolates of animal origin would provide important novel epidemiological data and give insight into the population structure and clonal nature of animal-associated *S. aureus*.

Chapter 2

Materials and Methods

2.1. *Staphylococcus aureus* strains.

A total of 191 *S. aureus* animal isolates from cows, goats, sheep, chickens, rabbits and a cat were used in these studies (See Chapter 3 for details). These isolates were obtained from animals with *S. aureus* infection.

Control *S. aureus* strains are listed where appropriate in the text.

2.2. Culture of *Staphylococcus aureus.*

S. aureus strains were routinely grown either on Trypticase Soy Agar (TSA, Oxoid, Fannin Healthcare, Sandyford Industrial Estate, Foxrock, Dublin 18, Ireland) or Trypticase Soy Broth (TSB, Oxoid) with shaking at 37 °C. The identification of *Staphylococcus aureus* strains was further verified by testing their ability to grow and produce acid on mannitol salt agar, their ability to produce acetoin, their ability to grow on TSA supplemented with 7 μg acriflavin/ml, and a negative PCR reaction for the *S. intermedius* 16S rRNA gene.

2.3. *Escherichia coli* strains.

Strains DH5α (Invitrogen, Bio Sciences Ltd., Dun Laoghaire, Ireland) and BL21 (Stratagene, Amsterdam, The Netherlands) were used as transformation recipient and expression strains. The genotypes of these strains are as follows: DH5α, F^- Φ80*lacZ*ΔM15Δ(*lacZYA-argF*)U169 *deoR recA1 endA1 hsdR17*(rk^-, mk^+) *phoA supE44 thi*-1 *gyrA96 relA1* λ^- and BL21, *E. coli* B F^- *dcm ompT hsdS*(rB^- mB^-) *gal*. *E. coli* strains were cultured in L-broth and on L-agar containing appropriate selective antibiotics. Antibiotics were incorporated into the medium where appropriate: ampicillin (Amp), 100 μg/ml and tetracycline (Tet), 2 μg/ml. Antibiotics were purchased from Sigma-Aldrich, Ireland Limited (Tallaght, Dublin 24, Ireland). Stocks of bacterial strains were maintained as frozen cultures at –70 °C in L-broth or TSB containing 20% (v/v) glycerol.

2.4. Routine molecular techniques.

Routine molecular techniques, e.g., restriction endonuclease digestion, DNA ligation, phenol-chloroform extraction of DNA and DNA precipitation, were all carried out using manufacturers' instructions and according to standard protocols (Ausubel *et al.,* 1987;

Sambrook & Russell, 2001). Commercially purchased kits were also utilised for certain applications and were used according to the accompanying specifications. Enzymes for DNA manipulations were purchased from Sigma-Aldrich, Roche Diagnostics Ltd. (Bell Lane, Lewes, East Sussex , UK), New England Biolabs (ISIS Ltd., Bray, Co. Wicklow, Ireland), Stratagene (Techno-Path, National Technology Park, Plassey, Limerick, Ireland) and Promega (Medical Supply Co. Ltd., Damastown, Mulhuddart, Dublin 15, Ireland) and were used according to the manufacturers' instructions.

2.5. DNA isolation and analysis.

2.5.1. *S. aureus* DNA extraction.

Extraction of genomic DNA from *Staphylococcus aureus* was performed as previously described with some modifications (Fitzgerald *et al.*, 1997). A single colony was used to inoculate 2 ml TSB and incubated at 37 °C with shaking. The cells were pelleted at maximum speed (12,300 × *g*) for 10 min in a bench-top microfuge (Sorvall Microspin 24, DuPont, Wilmington, USA). The pellets were resuspended in 200 µl Tris EDTA buffer (TE) to which 10 µl of lysostaphin (Sigma-Aldrich) was added (1 mg/ml in 10 mM Tris-HCl buffer, pH 8.0) and the tubes incubated at 37 °C for 30 min. Following this incubation 20 µl of proteinase K (Promega) was added (20 mg/ml in 10 mM Tris-HCl buffer, pH 8.0) and the tubes incubated at 37 °C for 30 min. Then 20 µl of 0.5 M EDTA and 20 µl of 10% (w/v) Sarkosyl™ were added, and the suspensions mixed gently and kept in ice for 2 h. The tubes were then incubated at 55 °C for 1 h. To each tube 200 µl of TE buffer was added, followed by 90 µl 5 M NaCl. The tube contents were mixed thoroughly and then 80 µl of prewarmed 10% (w/v) CTAB (cetyltrimethylammonium bromide) in 0.7 M NaCl was added. The tubes were incubated at 65 °C for 10 min and then cooled to room temperature. The tube contents were extracted once using 1 vol. chloroform:isoamyl alcohol (24:1) and three times using 1 vol. phenol:chloroform:isoamyl alcohol (25:24:1). The DNA was precipitated using 2 vol. absolute ethanol and the DNA pellets washed with 70% (v/v) ethanol. The pellets were dried and resuspended in 50 µl TE. The quantity and quality of the extracted DNA were estimated by running 1 µl of the DNA preparation on a 1% (w/v) agarose gel alongside DNA standards of known concentration namely the 2-Log DNA Ladder (Promega).

2.5.2. Small scale preparation of plasmid DNA by alkaline lysis.

Typically, 2 ml L-broth with the appropriate antibiotic(s) was inoculated with the strain containing the plasmid of interest and incubated at 37 °C and 250 r.p.m. for 16 h. After overnight incubation, 1.5 ml of the culture was poured into a microfuge tube and centrifuged at 12,300 × *g* for 30 s. The medium was decanted. The plasmid DNA was then extracted using a modification of the method of Birnboim & Doly (1979) as described by Sambrook & Russell (2001). The bacterial pellet was resuspended in 100 µl GET buffer [50 mM glucose, 25 mM Tris-Cl (pH 8.0), 10 mM EDTA (pH 8.0)] by vigorous vortexing. After resupending the bacterial pellet, 200 µl of a freshly prepared solution containing 0.2 M NaOH and 1% (w/v) SDS was added. The contents of the tube were mixed by rapid inversion and then placed on ice. A 150-µl aliquot of 'Solution III' [5 M potassium acetate (60 ml), glacial acetic acid (11.5 ml) and H_2O (28.5 ml)] was added and the contents of the tube mixed by vortexing briefly. The tubes were placed on ice for 5 min followed by centrifugation at 12,300 × *g* for 5 min. The supernatant fraction was then transferred to a fresh tube. Plasmid DNA was precipitated by the addition of 2 vol. absolute ethanol followed by vortexing. The mixture was left at room temperature for 2 min followed by centrifugation at 12,300 × *g* for 5 min. After removal of the supernatant fraction the pellet was rinsed with 1 ml 70% ethanol and again centrifuged briefly. The supernatant fraction was removed and the pellet allowed to air-dry. The extracted plasmid DNA was dissolved in 50 µl TE buffer (pH 8.0) containing DNase-free RNase (20 µl/ml). For sequencing plasmid DNA was prepared using the High Pure Plasmid Isolation Kit (Roche Diagnostics Ltd.).

2.5.3. Polymerase chain reaction.

PCR was performed on chromosomal or plasmid DNA of *S. aureus* or *E. coli* strains. Appropriate positive and negative control strains were included in each reaction. The sequences of the oligonucleotides used as primers and their corresponding PCR product sizes are shown in tables in the 'Methods' section of the relevant chapters. In general PCR reactions were carried out in a final volume of 25 µl containing 1 µl DNA template (100 ng/µl), 3 mM $MgCl_2$, 10 pmol of each primer, 2.5 U *Taq* DNA polymerase, 80 µM each of dATP, dGTP, dCTP and dTTP in 10 mM Tris-HCl buffer (pH 8.3) containing 50 mM KCl and 0.001% (w/v) gelatin. All PCR reagents were purchased from Promega. PCR was performed in a PTC-100 thermocycler (MJ Research, Nevada, USA) with a heated lid

under the following conditions, unless otherwise specified: 10 min at 95 °C, then cycles of [1 min at 94 °C, 1 min at 50–52 °C (depending on primers used), and 1 min at 72 °C]. This was followed by 10 min at 72 °C. The reaction products were stored at 4 °C.

2.5.4. Agarose gel electrophoresis.

Genomic DNA, PCR products, plasmids preparations and digested genomic DNA were electrophoresed through agarose gels according to standard protocols (Sambrook & Russell, 2001) using 0.5 × Tris-Borate-EDTA buffer (TBE) [1 litre 10 × stock, 890-mM Tris base, 890 mM boric acid, 40 ml 0.5 M EDTA, pH 8.0] or 1 × Tris-Acetate-EDTA (TAE) buffer [1 litre 50 × stock, 2 M Tris base, 57.1 ml glacial acetic acid, 0.1 M $Na_2EDTA.2H_2O$, pH ~ 8.5] where appropriate. Agarose gels were routinely cast with agarose at a 1–2% (w/v) concentration in buffer and contained ethidium bromide at a final concentration of 0.5 μg/ml. Electrophoresed DNA was visualised by exposure of the stained gel to ultra-violet light using an ultraviolet transilluminator. Horizontal gel tanks (Gibco BRL, Life Technologies) of various sizes were routinely used.

2.5.5. Southern hybridisation analysis.

2.5.5.1. Capillary blotting.

Total genomic DNA (~10 μg) was digested with restriction endonuclease *Hin*dIII for 16 h and resolved by electrophoresis in 1.8% (w/v) agarose gels (Roche) in 1 × TAE buffer. Following electrophoresis, DNA fragments were depurinated (0.2 M HCl, 10 min), denatured (1.5 M NaCl, 0.5 M NaOH, 45 min), rinsed briefly in distilled H_2O, and neutralised (1.5 M NaCl, 1-M Tris-HCl, pH 7.4, 45 min), with gentle shaking. DNA was then transferred from the gel for 16 h to a positively charged nylon membrane (Gelman Sciences) by capillary transfer using 20 × SSC (3 M NaCl, 0.3 M trisodium citrate) as previously described (Southern, 1975). DNA fragments were fixed to the membrane by incubation at 80 °C for 2 h.

2.5.5.2. Probe hybridisation.

DNA probes labelled with digoxigenin (DIG, Roche Diagnostics) were used throughout these studies. Probes were constructed using the PCR DIG-labelling mix (Roche

Diagnostics) in PCR reactions that incorporated DIG-labelled dUTP into the synthesised DNA. The membrane was prehybridised in DIG Easy Hyb Solution (Roche Diagnostics) for 2 h at 42 °C and then hybridised for 16 h with the probe (0.5 μg DIG-labelled PCR product/ml prehybridisation solution) at 42 °C. Following hybridisation, the membrane was washed twice at room temperature with 2 × SSC containing 0.1% (w/v) SDS and twice at 50 °C with 0.5 × SSC containing 0.1% (w/v) SDS. The membrane was then washed for 5 min with wash buffer [0.1 M maleic acid, 0.15 M NaCl, pH 7.5, Tween 20 (0.3% v/v)]. Blocking was performed for 30 min with a 1% (w/v) solution of blocking reagent (Roche) diluted in maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5) and then the membrane was incubated for 30 min with a 1:10,000 solution of anti-DIG Fab fragments (Roche) diluted in maleic acid buffer as recommended by the manufacturer. Unbound antibody was removed by washing twice in wash buffer [0.1 M maleic acid, 0.15 M NaCl, pH 7.5, Tween 20 (0.3% v/v)]. The membrane was equilibrated in 100 mM Tris-HCl buffer, pH 9.5, containing 100 mM NaCl and then incubated with the chemiluminescent substrate CDP-Star (Roche), as recommended by the manufacturer. Finally, hybridisation of the DNA probe was detected by exposure of the membrane to X-ray film (X-Omat, Kodak).

2.5.6. DNA sequencing and oligonucleotide synthesis.

Oligonucleotides (primers) were synthesised by MWG-Biotech, Germany. Automated nucleotide sequencing of PCR products was carried out by MWG-Biotech, unless otherwise indicated in the text.

2.6. Phylogenetic analysis.

2.6.1. Generation of multiple alignments and phylogenetic trees.

Multiple alignments were generated using the ClustalW web-based program (http://www.ebi.ac.uk/clustalW) (Thompson *et al.*, 1994) or the downloadable version ClustalX (1.81) (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/). Protein or nucleotide sequences were obtained in fasta format using their accession numbers from PubMed (http://www.ncbi.nlm.nih.gov). Phylogenetic trees were constructed using ClustalX.

Chapter 3

Novel and Putative Superantigen Genes in Strains of *Staphylococcus aureus* from Cows, Goats, Sheep, Rabbits and Chickens

3.1. Introduction.

Staphylococcus aureus is a major human pathogen that causes a wide variety of diseases ranging in severity from food-poisoning (McCormick *et al.*, 2001; Le Loir *et al.*, 2003) and life-threatening toxic shock syndrome (Llewelyn & Cohen, 2002; Proft & Fraser, 2003) to less serious infections, e.g., boils (Stulberg *et al.*, 2002). *S. aureus* can also cause a number of infections in animals such as tick-associated pyaemia in lambs (Webster & Mitchell, 1989), staphylococcosis in rabbits (Hermans *et al.*, 2003), oedematous and necrotic dermatitis, septicaemia, abscesses and chondronecrosis in chickens (McCullagh *et al.*, 1998; McNamee *et al.*, 1998; Takeuchi *et al.*, 2002), and pneumonia and osteomyelitis complex in turkeys (Huff *et al.*, 2000; Linares & Wigle, 2001). *S. aureus* is the most frequent cause of bovine mastitis, a disease that is of economic importance worldwide (Beck *et al.*, 1992; Miles *et al.*, 1992). Typically staphylococcal mastitis is chronic in nature, with subclinical mastitis being the most common form (Gruet *et al.*, 2001). The organism may survive for long periods of time in the host without causing overt symptoms of disease (Fitzgerald *et al.*, 1997).

The ability of *S. aureus* to cause disease is thought to be due to a combination of virulence factors, namely, toxins, cell-surface-associated adhesins and secreted exoproteins (Peacock *et al.*, 2002). The classical staphylococcal enterotoxins (SEA–SEE) cause staphylococcal food-poisoning (Balaban & Rasooly, 2000; Jablonski & Bohach, 2001), a food-borne intoxication characterised by a short incubation period, nausea, vomiting, abdominal pain and diarrhoea. SEA, SEB and SED are the most common enterotoxins detected in outbreaks. In a study of *S. aureus* from 359 outbreaks of food-poisoning between 1969 and 1990 in the U.K., 79 % of the strains were found to produce SEA alone or together with another toxin (Wieneke *et al.*, 1993). *S. aureus* is estimated by the Centers for Disease Control and Prevention (CDC) to cause 185,000 cases of food-poisoning in the USA annually (Mead *et al.*, 1999).

Although humans are the main reservoir of *S. aureus* associated with food-poisoning, animals are often heavily colonised, leading to contamination of foodstuffs during processing of domestic animals and their products. Due to its osmotolerance *S. aureus* can be isolated from many types of food products, e.g., bakery products, beef, pork sausages, poultry, cooked meats, dairy products, egg products and seafood (Jablonski & Bohach, 2001). SEC or SED are produced by 10–30% of bovine isolates (Kenny *et al.*, 1993;

Matsunga *et al.*, 1993, Fitzgerald *et al.*, 2000; Cenci-Goga *et al.*, 2003) and SED by 35 % and SEC by 22 % of isolates from sheep milk (Bautista *et al.*, 1988). In the study of Foschino *et al.* (2002) the incidence of enterotoxin C producers among caprine *S. aureus* strains was 23%.

The SEs belong to a large family of staphylococcal and streptococcal pyrogenic exotoxins. They are involved in other types of toxigenic illnesses with symptoms of shock in humans and animals (Michie & Cohen 1998; McCormick *et al.*, 2001; Llewelyn & Cohen, 2002). These proteins share common structural features, sequence similarity and phylogenetic relationships (Figs. 3.1 and 3.2). The SE genes are often associated with mobile genetic elements such as bacteriophages, transposons and plasmids (Lindsay *et al.*, 1998; Hentschel & Hacker, 2001; Novick *et al.*, 2001). One such element is the bovine pathogenicity island, SaPIbov, that encodes the three superantigens SEC-bovine, TSST-1 and the staphylococcal enterotoxin-like protein SElL (Fitzgerald *et al.*, 2001a, Lina *et al.*, 2004). The enterotoxin gene cluster (*egc*) that was identified by Jarraud *et al.* (2001) comprises two staphylococcal enterotoxin (*se*) and three staphylococcal enterotoxin-like (*sel*) genes *seg, sei, selm, seln* and *selo*.

These enterotoxins, enterotoxin-like proteins, and TSST-1 can act as superantigens (SAgs) that stimulate T-cell proliferation (Alouf & Müller-Alouf, 2003; Proft & Fraser, 2003; Petersson *et al.*, 2004). SAgs bypass conventional antigen presentation. They interact simultaneously with the major histocompatibility complex class II (MHCII) molecule of the antigen-presenting cells and the Vβ-domain of the lymphocyte T-cell receptor (TCR) forming trimolecular complexes (Petersson *et al.*, 2004) (See Chaper 1, Fig. 1.1). This interaction activates as many as one in five T-cells, whereas conventional antigen presentation activates 1 in 10,000 T-cells. This explains the release of massive amounts of cytokine and the subsequent immunomodulative and other deleterious effects brought about by SAgs (Alouf & Müller-Alouf, 2003; Proft & Fraser, 2003).

Eighteen SE and SEl genes have been identified to date, namely, *sea–see*, *seg–sei, selj–selr* and *selu*, (Fitzgerald *et al.*, 2001a; Jarraud *et al.*, 2001; Kuroda *et al.*, 2001; Orwin *et al.*, 2001, 2003; Yarwood *et al.*, 2002; Letertre *et al.*, 2003c; Omoe *et al.*, 2003; Lina *et al.*, 2004, Omoe *et al.*, 2005). The enterotoxicity (emetic effect) and superantigenicity of SEs appear to be distinct properties of these molecules (Schlievert *et al.*, 2000; Proft & Fraser, 2003). Not all of the currently identified SEs and SEls are emetic, although all exhibit

lymphocyte mitogenic activity towards human and/or other mammalian lymphocytes. SEI, SElK and SElQ have been shown to lack the cysteine loop structure important for emetic activity, resulting in reduced activity in SEI and a lack of activity in SElK and SElQ (Orwin *et al.*, 2002) (Figs 3.1 and 3.2).

At present little is known about the occurrence and significance of the novel SEs and SEls (SEG-SEI, SElJ-SElR, SElU) in strains of *S. aureus* from animal infection (Akineden *et al.*, 2001; Larsen *et al.*, 2002). Some of these novel SEs and SELs may contribute to the persistence of *S. aureus* in sub-clinical mastitis. Because of the putative significance of these enterotoxins and enterotoxin-like proteins for public health and food safety, greater knowledge of their prevalence and an efficient means of screening for their genes is needed (Wieneke *et al.*, 1993; Mead *et al.*, 1999; Shimizu *et al.*, 2000; Chen *et al.*, 2004). Changes in food processing and retailing over recent decades have the potential to introduce toxigenic strains *S. aureus* of animal origin into the food chain (Rosec & Gigaud, 2002; Woteki & Kineman, 2003; Chen *et al.*, 2004). Since several of these SEs and SEls have been discovered in the last five years, there is reason to believe that, as research on pathogenic *S. aureus* isolates continues, additional SEs and SEls will be described. The present study was designed to investigate the frequency of genes encoding the SEs and SEls by means of multiplex PCR in strains of *S. aureus* from cows, goats, sheep, rabbits and chickens.

3.2. Methods.

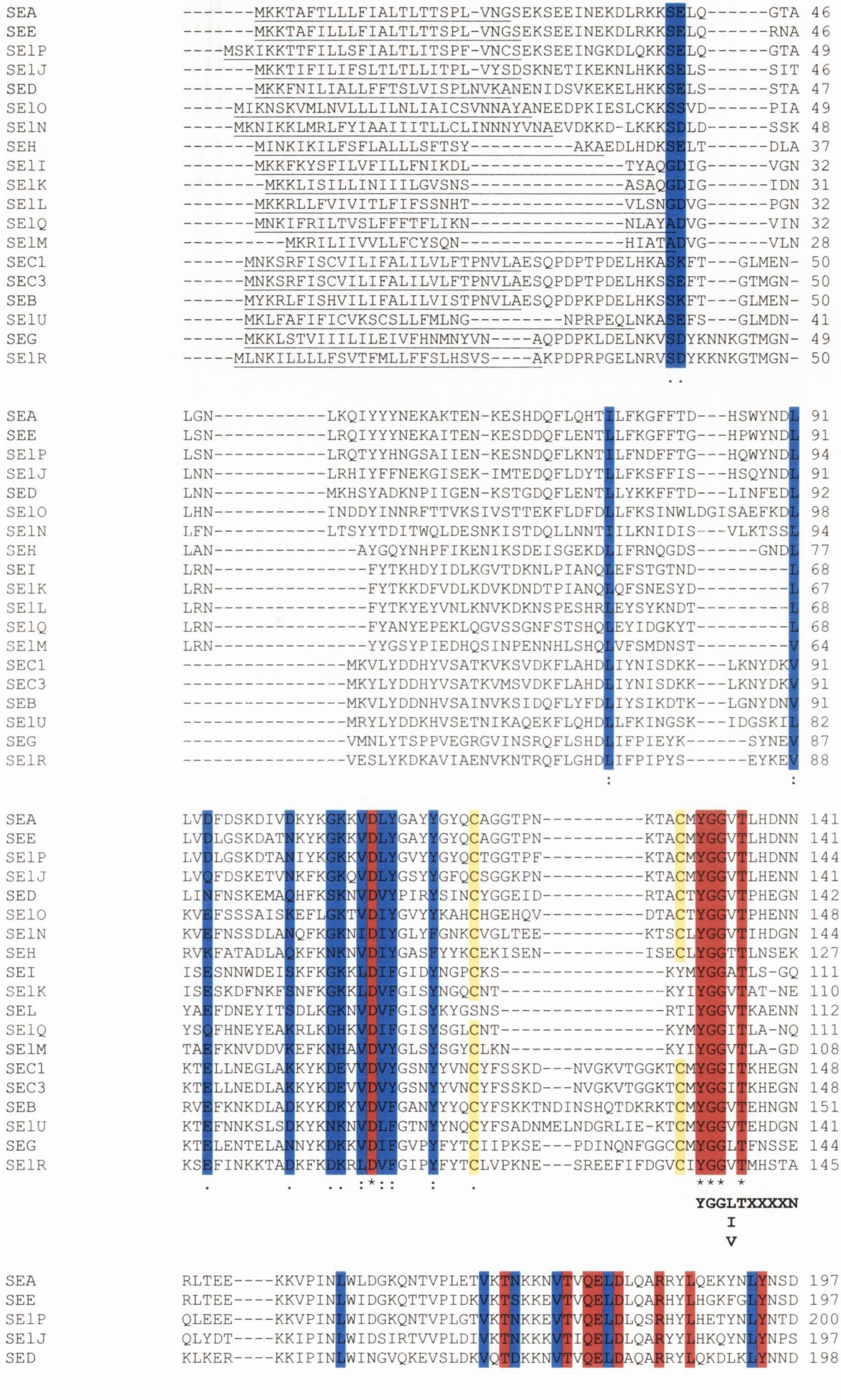
3.2.1. Bacterial strains and DNA isolation.

A total of 191 *S. aureus* isolates were analysed – 99 strains from cows, 39 strains from goats, 23 strains from sheep, 15 strains from chickens and 15 strains from rabbits (Table 3.1). One Swedish feline isolate was also included. *S. aureus* strains were routinely grown in TSB or on TSA at 37 °C. Staphylococcal genomic DNA was isolated as described in Chapter 2.

Strain COL was used as a control for the *selk* and *selq* genes (http://www.tigr.org/tdb/mdb/mdbcomplete.html), strain RF122 as a control for the *sec*-bovine, *sell* and *tst* genes encoded by the bovine pathogenicity island SaPIbov (Fitzgerald *et al.*, 2001a), strain NY19 as a *seb*-positive control, strain FRI913 as a *sea*-, *sec3*-, *see*-

Fig. 3.1. Multiple protein sequence alignment of known staphylococcal enterotoxins and enterotoxin-like proteins generated by ClustalX (1.81).

Functionally conserved or semi-conserved residues are indicated below the alignment by : or . and are highlighted in blue. Identical residues are indicated below the alignment by * and are highlighted in red. The predicted signal peptides are underlined (predicted by SignalP). Cysteine residues considered to be important for emetic activity are highlighted in yellow. The staphylococcal and streptococcal exotoxin consensus signatures 1 (residues 132-141) and 2 (residues 171-194) are shown underneath the alignment in bold. Residues 152-161 of SEB have been shown to be important for transcystosis across epithelial monolayers and are highlighted in green (Shupp *et al.*, 2002). Genbank accession numbers: SEA (AAA26681), SEE (AAA26617), SElP, (NP_375057), SElJ (AAC78590), SED (AAB06195), SElO (SEO-AF285760), SElN (SEN-AF285760), SEH (AAA19777), SEI (AAC26661), SElK (SEK-AF410775), SElL (SEL-AF217235), SElQ (SEQ-AF410775), SElM (SEM-AF285760), SEC1 (CAA29260), SEC3 (AAA26624), SEB (AAA88550), *seu* (AY158703), *seg* (AAC26660), *ser* (BAC97795).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SEE -------MKKTAFILLLFIALTLTTSPL-VNGSEKSEEINEKDLRKKSELQ------RNA 46
SElP ----MSKIKKTTFILLSFIALTLITSPF-VNCSEKSEEINGKDLQKKSELQ------GTA 49
SElJ -------MKKTIFILIFSLTLTLLITPL-VYSDSKNETIKEKNLHKKSELS------SIT 46
SED -------MKKFNILIALLFFTSLVISPLNVKANENIDSVKEKELHKKSELS------STA 47
SElO -----MIKNSKVMLNVLLLILNLIAICSVNNAYANEEDPKIESLCKKSSVD------PIA 49
SElN -----MKNIKKLMRLFYIAAIIITLLCLINNNYVNAEVDKKD-LKKKSDLD------SSK 48
SEH -------MINKIKILFSFLALLLSFTSY----------AKAEDLHDKSELT------DLA 37
SElI -------MKKFKYSFILVFILLFNIKDL---------------TYAQGDIG------VGN 32
SElK --------MKKLISILLINIIILGVSNS---------------ASAQGDIG------IDN 31
SElL -------MKKRLLFVIVITLFIFSSNHT---------------VLSNGDVG------PGN 32
SElQ -------MNKIFRILTVSLFFFTFLIKN---------------NLAYADVG------VIN 32
SElM ----------MKRILIIVVLLFCYSQN----------------HIATADVG------VLN 28
SEC1 ------MNKSRFISCVILIFALILVLFTPNVLAESQPDPTPDELHKASKFT---GLMEN- 50
SEC3 ------MNKSRFISCVILIFALILVLFTPNVLAESQPDPTPDELHKSSEFT---GTMGN- 50
SEB ------MYKRLFISHVILIFALILVISTPNVLAESQPDPKPDELHKSSKFT---GLMEN- 50
SElU ------MKLFAFIFICVKSCSLLFMLNG---------NPRPEQLNKASEFS---GLMDN- 41
SEG ------MKKLSTVIIILILEIVFHNMNYVN----AQPDPKLDELNKVSDYKNNKGTMGN- 49
SElR -----MLNKILLLLFSVTFMLLFFSLHSVS----AKPDPRPGELNRVSDYKKNKGTMGN- 50
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SEE LSN----------LRQIYYYNEKAITEN-KESDDQFLENTLLFKGFFTG---HPWYNDL 91
SElP LSN----------LRQTYYHNGSAIIEN-KESNDQFLKNTILFNDFFTG---HQWYNDL 94
SElJ LNN----------LRHIYFFNEKGISEK-IMTEDQFLDYTLLFKSFFIS---HSQYNDL 91
SED LNN----------MKHSYADKNPIIGEN-KSTGDQFLENTLLYKKFFTD---LINFEDL 92
SElO LHN----------INDDYINNRFTTVKSIVSTTEKFLDFDLLFKSINWLDGISAEFKDL 98
SElN LFN----------LTSYYTDITWQLDESNKISTDQLLNNTIILKNIDIS---VLKTSSL 94
SEH LAN--------------AYGQYNHPFIKENIKSDEISGEKDLIFRNQGDS------GNDL 77
SEI LRN---------------FYTKHDYIDLKGVTDKNLPIANQLEFSTGTND---------L 68
SElK LRN---------------FYTKKDFVDLKDVKDNDTPIANQLQFSNESYD---------L 67
SElL LRN---------------FYTKYEYVNLKNVKDKNSPESHRLEYSYKNDT---------L 68
SElQ LRN---------------FYANYEPEKLQGVSSGNFSTSHQLEYIDGKYT---------L 68
SElM LRN---------------YYGSYPIEDHQSINPENNHLSHQLVFSMDNST---------V 64
SEC1 ----------------MKVLYDDHYVSATKVKSVDKFLAHDLIYNISDKK---LKNYDKV 91
SEC3 ----------------MKYLYDDHYVSATKVMSVDKFLAHDLIYNISDKK---LKNYDKV 91
SEB ----------------MKVLYDDNHVSAINVKSIDQFLYFDLIYSIKDTK---LGNYDNV 91
SElU ----------------MRYLYDDKHVSETNIKAQEKFLQHDLLFKINGSK---IDGSKIL 82
SEG ----------------VMNLYTSPPVEGRGVINSRQFLSHDLIFPIEYK------SYNEV 87
SElR ----------------VESLYKDKAVIAENVKNTRQFLGHDLIFPIPYS------EYKEV 88
SEA LVDFDSKDIVDKYKGKKVDLYGAYYGYQCAGGTPN----------KTACMYGGVTLHDNN 141
SEE LVDLGSKDATNKYKGKKVDLYGAYYGYQCAGGTPN----------KTACMYGGVTLHDNN 141
SElP LVDLGSKDTANIYKGKKVDLYGVYYGYQCTGGTPF----------KTACMYGGVTLHDNN 144
SElJ LVQFDSKETVNKFKGKQVDLYGSYYGFQCSGGKPN----------KTACMYGGVTLHENN 141
SED LINFNSKEMAQHFKSKNVDVYPIRYSINCYGGEID----------RTACTYGGVTPHEGN 142
SElO KVEFSSSAISKEFLGKTVDIYGVYYKAHCHGEHQV----------DTACTYGGVTPHENN 148
SElN KVEFNSSDLANQFKGKNIDIYGLYFGNKCVGLTEE----------KTSCLYGGVTIHDGN 144
SEH RVKFATADLAQKFKNKNVDIYGASFYYKCEKISEN----------ISECLYGGTTLNSEK 127
SEI ISESNNWDEISKFKGKKLDIFGIDYNGPCKS----------------KYMYGGATLS-GQ 111
SElK ISESKDFNKFSNFKGKKLDVFGISYNGQCNT----------------KYIYGGVTAT-NE 110
SEL YAEFDNEYITSDLKGKNVDVFGISYKYGSNS----------------RTIYGGVTKAENN 112
SElQ YSQFHNEYEAKRLKDHKVDIFGISYSGLCNT----------------KYMYGGITLA-NQ 111
SElM TAEFKNVDDVKEFKNHAVDVYGLSYSGYCLKN----------------KYIYGGVTLA-GD 108
SEC1 KTELLNEGLAKKYKDEVVDVYGSNYYVNCYFSSKD---NVGKVTGGKTCMYGGITKHEGN 148
SEC3 KTELLNEDLAKKYKDEVVDVYGSNYYVNCYFSSKD---NVGKVTGGKTCMYGGITKHEGN 148
SEB RVEFKNKDLADKYKDKYVDVFGANYYYQCYFSKKTNDINSHQTDKRKTCMYGGVTEHNGN 151
SElU KTEFNNKSLSDKYKNKNVDLFGTNYYNQCYFSADNMELNDGRLIE-KTCMYGGVTEHDGN 141
SEG KTELENTELANNYKDKKVDIFGVPYFYTCIIPKSE---PDINQNFGGCCMYGGLTFNSSE 144
SElR KSEFINKKTADKFKDKRLDVFGIPYFYTCLVPKNE---SREEFIFDGVCIYGGVTMHSTA 145
YGGLTXXXXN
I
V
SEA RLTEE----KKVPINLWLDGKQNTVPLETVKTNKKNVTVQELDLQARRYLQEKYNLYNSD 197
SEE RLTEE----KKVPINLWIDGKQTTVPIDKVKTSKKEVTVQELDLQARHYLHGKFGLYNSD 197
SElP QLEEE----KKVPINLWIDGKQNTVPLGTVKTNKKEVTVQELDLQSRHYLHETYNLYNTD 200
SElJ QLYDT----KKIPINLWIDSIRTVVPLDIVKTNKKKVTIQELDLQARYYLHKQYNLYNPS 197
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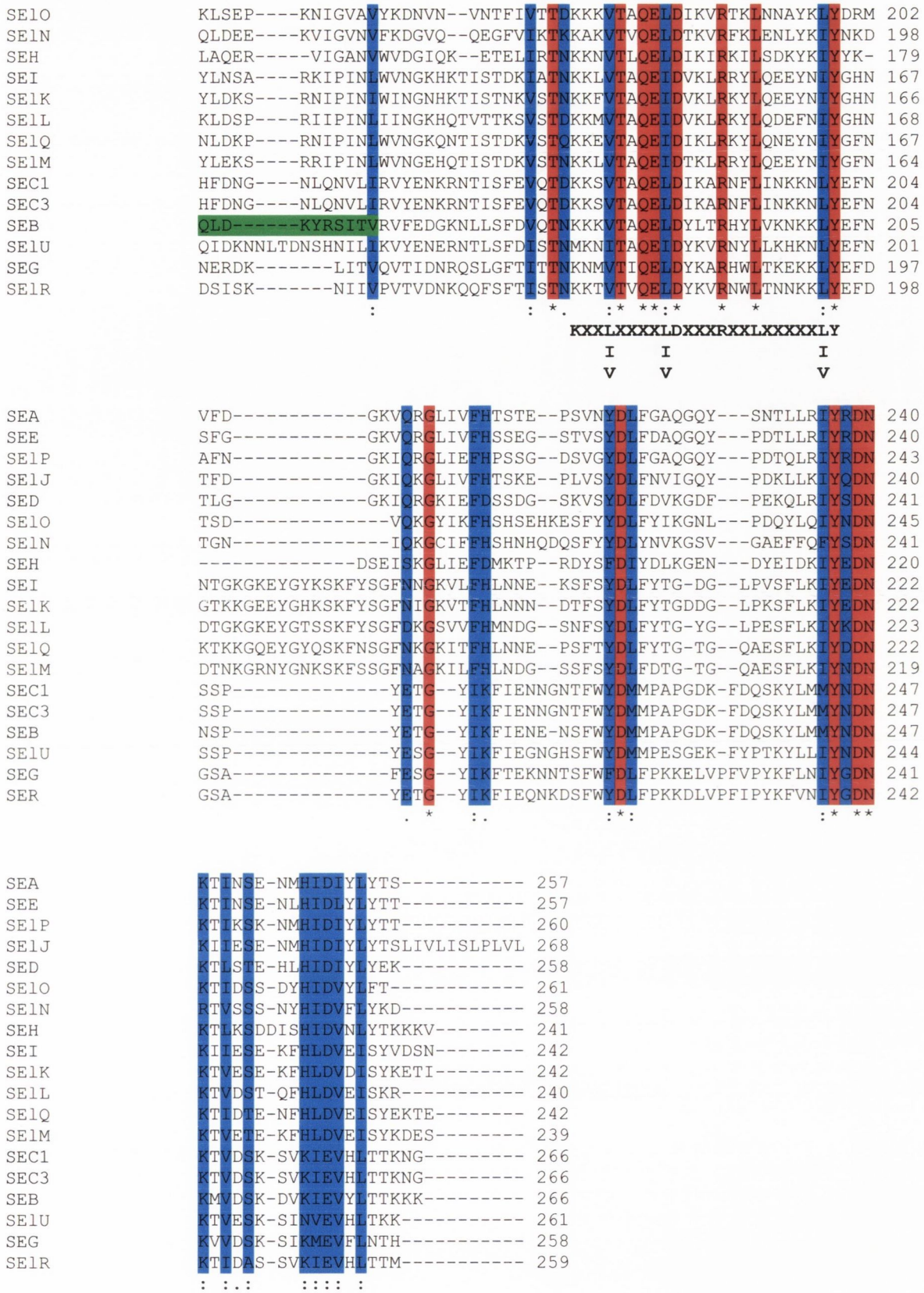
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SEH LAQER-----VIGANVWVDGIQK--ETELIRTNKKNVTLQELDIKIRKILSDKYKIYYK- 179
SEI YLNSA----RKIPINLWVNGKHKTISTDKIATNKKLVTAQEIDVKLRRYLQEEYNIYGHN 167
SElK YLDKS----RNIPINIWINGNHKTISTNKVSTNKKFVTAQEIDVKLRKYLQEEYNIYGHN 166
SElL KLDSP----RIIPINLIINGKHQTVTTKSVSTDKKMVTAQEIDVKLRKYLQDEFNIYGHN 168
SElQ NLDKP----RNIPINLWVNGKQNTISTDKVSTQKKEVTAQEIDIKLRKYLQNEYNIYGFN 167
SElM YLEKS----RRIPINLWVNGEHQTISTDKVSTNKKLVTAQEIDTKLRRYLQEEYNIYGFN 164
SEC1 HFDNG----NLQNVLIRVYENKRNTISFEVQTDKKSVTAQELDIKARNFLINKKNLYEFN 204
SEC3 HFDNG----NLQNVLIRVYENKRNTISFEVQTDKKSVTAQELDIKARNFLINKKNLYEFN 204
SEB QLD------KYRSITVRVFEDGKNLLSFDVQTNKKKVTAQELDYLTRHYLVKNKKLYEFN 205
SElU QIDKNNLTDNSHNILIKVYENERNTLSFDISTNMKNITAQEIDYKVRNYLLKHKNLYEFN 201
SEG NERDK-------LITVQVTIDNRQSLGFTITTNKNMVTIQELDYKARHWLTKEKKLYEFD 197
SElR DSISK-------NIIVPVTVDNKQQFSFTISTNKKTVTVQELDYKVRNWLTNNKKLYEFD 198
KXXLXXXXLDXXXRXXLXXXXXLY
SEA VFD----------GKVQRGLIVFHTSTE--PSVNYDLFGAQGQY---SNTLLRIYRDN 240
SEE SFG----------GKVQRGLIVFHSSEG--STVSYDLFDAQGQY---PDTLLRIYRDN 240
SElP AFN----------GKIQRGLIEFHPSSG--DSVGYDLFGAQGQY---PDTQLRIYRDN 243
SElJ TFD----------GKIQKGLIVFHTSKE--PLVSYDLFNVIGQY---PDKLLKIYQDN 240
SED TLG----------GKIQRGKIEFDSSDG--SKVSYDLFDVKGDF---PEKQLRIYSDN 241
SElO TSD----------VQKGYIKFHSHSEHKESFYYDLFYIKGNL---PDQYLQIYNDN 245
SElN TGN----------IQKGCIFFHSHNHQDQSFYYDLYNVKGSV---GAEFFQFYSDN 241
SEH ----------DSEISKGLIEFDMKTP--RDYSFDIYDLKGEN---DYEIDKIYEDN 220
SEI NTGKGKEYGYKSKFYSGFNNGKVLFHLNNE--KSFSYDLFYTG-DG--LPVSFLKIYEDN 222
SElK GTKKGEEYGHKSKFYSGFNIGKVTFHLNNN--DTFSYDLFYTGDDG--LPKSFLKIYEDN 222
SElL DTGKGKEYGTSSKFYSGFDKGSVVFHMNDG--SNFSYDLFYTG-YG--LPESFLKIYKDN 223
SElQ KTKKGQEYGYQSKFNSGFNKGKITFHLNNE--PSFTYDLFYTG-TG--QAESFLKIYDDN 222
SElM DTNKGRNYGNKSKFSSGFNAGKILFHLNDG--SSFSYDLFDTG-TG--QAESFLKIYNDN 219
SEC1 SSP----------YETG--YIKFIENNGNTFWYDMMPAPGDK-FDQSKYLMMYNDN 247
SEC3 SSP----------YETG--YIKFIENNGNTFWYDMMPAPGDK-FDQSKYLMMYNDN 247
SEB NSP----------YETG--YIKFIENE-NSFWYDMMPAPGDK-FDQSKYLMMYNDN 247
SElU SSP----------YESG--YIKFIEGNGHSFWYDMMPESGEK-FYPTKYLLIYNDN 244
SEG GSA----------FESG--YIKFTEKNNTSFWFDLFPKKELVPFVPYKFLNIYGDN 241
SER GSA----------YETG--YIKFIEQNKDSFWYDLFPKKDLVPFIPYKFVNIYGDN 242
SEA KTINSE-NMHIDIYLYTS---------- 257
SEE KTINSE-NLHIDLYLYTT---------- 257
SElP KTIKSK-NMHIDIYLYTT---------- 260
SElJ KIIESE-NMHIDIYLYTSLIVLISLPLVL 268
SED KTLSTE-HLHIDIYLYEK---------- 258
SElO KTIDSS-DYHIDVYLFT---------- 261
SElN RTVSSS-NYHIDVFLYKD---------- 258
SEH KTLKSDDISHIDVNLYTKKKV-------- 241
SEI KIIESE-KFHLDVEISYVDSN-------- 242
SElK KTVESE-KFHLDVDISYKETI-------- 242
SElL KTVDST-QFHLDVEISKR---------- 240
SElQ KTIDTE-NFHLDVEISYEKTE-------- 242
SElM KTVETE-KFHLDVEISYKDES-------- 239
SEC1 KTVDSK-SVKIEVHLTTKNG--------- 266
SEC3 KTVDSK-SVKIEVHLTTKNG--------- 266
SEB KMVDSK-DVKIEVYLTTKKK--------- 266
SElU KTVESK-SINVEVHLTKK---------- 261
SEG KVVDSK-SIKMEVFLNTH---------- 258
SElR KTIDAS-SVKIEVHLTTM---------- 259

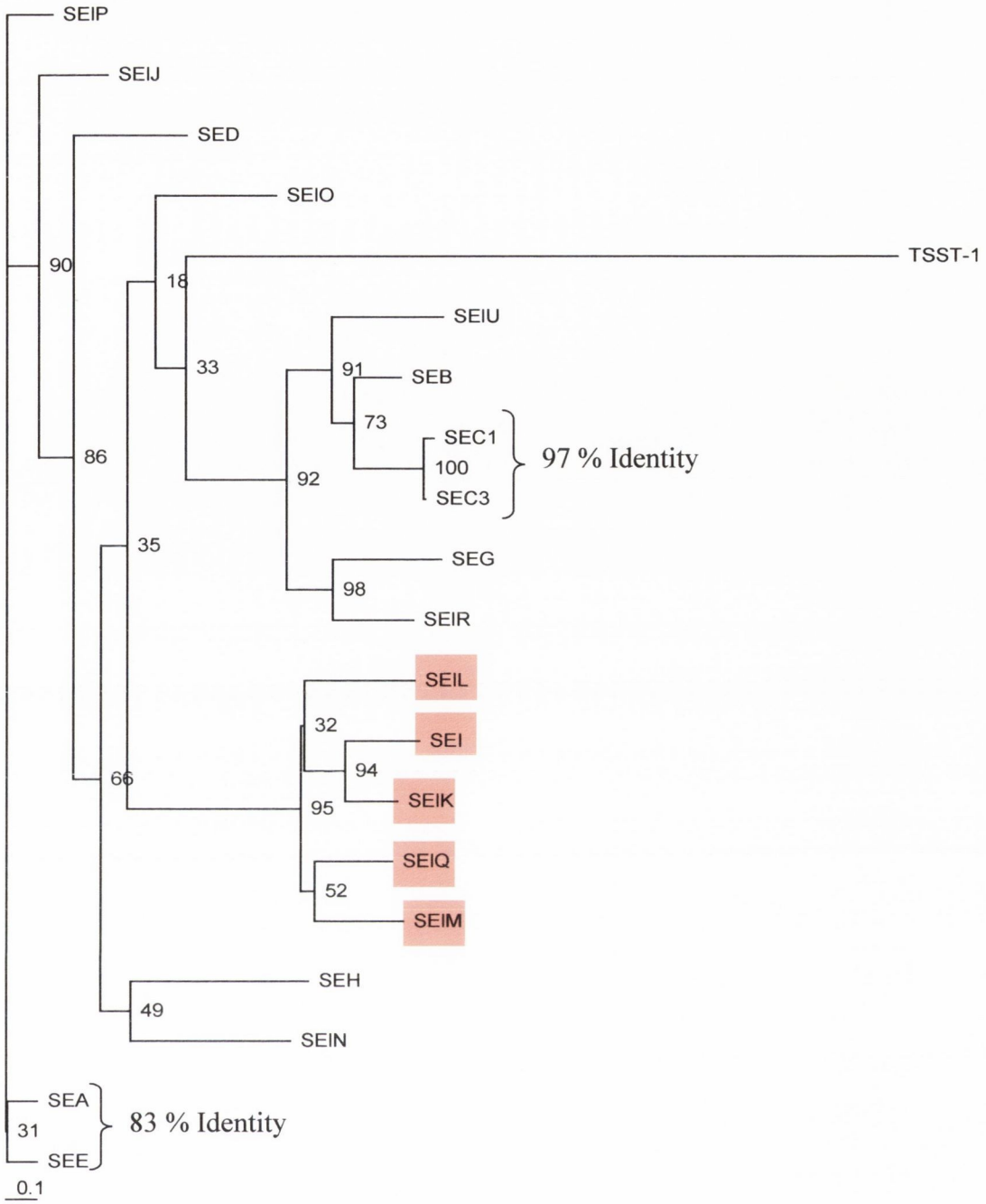


Fig. 3.2. Phylogenetic tree of the staphylococcal enterotoxins, enterotoxin-like proteins and toxic shock syndrome toxin 1 generated using ClustalX (1.81).

The SEs and SEls highlighted in pink lack cysteine residues which are considered to be important for emetic activity; these proteins have yet to be demonstrated as being emetic. The SEA and SEE proteins have an amino acid sequence similarity of 83 %. The allelic variants SEC1 and SEC3 have 97 % amino acid sequence similarity. TSST-1 has the least amino acid sequence similarity with the SEs and SEls and it is most similar to the SEC proteins showing similarity of 17 % at the amino acid level. Bootstrap values are indicated on the nodes.

Table 3.1. Bacterial strains toxinotyped in this work.

Code	Strain	Host	Country	Source	Disease	Enterotoxins	Reference	Supplier
DS71	DS71	Cat	Sweden	Uterus	Septicaemia	*sed, selj*	None	P. Winter
DS72	496	Chicken	N. Ireland	Bone	Osteomyelitis	*seh, selk, selq*	Rodgers *et al.*, 1999	J. Rodgers
DS67	72	Chicken	N. Ireland	Bone	Osteomyelitis	*seg, sei, selm, seln, selo*	Rodgers *et al.*, 1999	J. Rodgers
DS63	47	Chicken	N. Ireland	Liver	Osteomyelitis	*seg, sei, selm, seln, selo*	Rodgers *et al.*, 1999	J. Rodgers
DS55	11	Chicken	N. Ireland	Joint	Osteomyelitis	*seg, sei, selm, seln, selo*	Rodgers *et al.*, 1999	J. Rodgers
DS56	18	Chicken	N. Ireland	Bone	Osteomyelitis	*seg, sei, selm, seln, selo*	Rodgers *et al.*, 1999	J. Rodgers
DS58	31	Chicken	N. Ireland	Bone	Osteomyelitis	*seg, sei, selm, seln, selo*	Rodgers *et al.*, 1999	J. Rodgers
DS60	39	Chicken	N. Ireland	Bone	Osteomyelitis	*seg, sei, selm, seln, selo*	Rodgers *et al.*, 1999	J. Rodgers
DS61	70	Chicken	N. Ireland	Bone	Osteomyelitis	*seg, sei, selm, seln, selo*	Rodgers *et al.*, 1999	J. Rodgers
DS66	589	Chicken	N. Ireland	Joint	Osteomyelitis	*seg, sei, selm, seln, selo*	Rodgers *et al.*, 1999	J. Rodgers
DS62	34	Chicken	N. Ireland	Liver	Osteomyelitis	*seg, sei, selm, seln, selo*	Rodgers *et al.*, 1999	J. Rodgers
DS65	564	Chicken	N. Ireland	Liver	Osteomyelitis	*seg, sei, selm, seln, selo*	Rodgers *et al.*, 1999	J. Rodgers
DS57	24	Chicken	N. Ireland	Bone	Osteomyelitis	*seg, sei, selm, seln, selo*	Rodgers *et al.*, 1999	J. Rodgers
DS59	33	Chicken	N. Ireland	Bone	Osteomyelitis	*seg, sei, selm, seln, selo*	Rodgers *et al.*, 1999	J. Rodgers
DS64	563	Chicken	N. Ireland	Bone	Osteomyelitis	*seg, sei, selm, seln, selo*	Rodgers *et al.*, 1999	J. Rodgers
DS68	82	Chicken	N. Ireland	Bone	Osteomyelitis	Negative	Rodgers *et al.*, 1999	J. Rodgers
DS33	PS19	Rabbit	Spain	Nk	Staphylococcosis	Negative	Nk	J. Penadéz
DS47	KH275	Rabbit	Belgium	Nk	Staphylococcosis	Negative	Vancraeynest *et al.*, 2004	D. Vancraeynest
DS48	KH21	Rabbit	Belgium	Nk	Staphylococcosis	Negative	Vancraeynest *et al.*, 2004	D. Vancraeynest
DS52	KH15	Rabbit	Belgium	Nk	Staphylococcosis	Negative	Nk	D. Vancraeynest
DS34	PS21	Rabbit	Spain	Nk	Staphylococcosis	Negative	Nk	J. Penadéz

Code	Strain	Host	Country	Source	Disease	Enterotoxins	Reference	Supplier
DS54	KH16	Rabbit	Belgium	Nk	Staphylococcosis	Negative	Vancraeynest *et al.*, 2004	D. Vancraeynest
DS50	KH17	Rabbit	Belgium	Nk	Staphylococcosis	Negative	Vancraeynest *et al.*, 2004	D. Vancraeynest
DS49	KH473	Rabbit	Belgium	Nk	Staphylococcosis	*sed*, *selj*	Vancraeynest *et al.*, 2004	D. Vancraeynest
DS51	KH365	Rabbit	Belgium	Nk	Staphylococcosis	*seh*	Vancraeynest *et al.*, 2004	D. Vancraeynest
DS32	PS17	Rabbit	Spain	Nk	Staphylococcosis	*seh*	Nk	J. Penadéz
DS53	KH513	Rabbit	Belgium	Nk	Staphylococcosis	*seg*, *sei*, *selm*, *seln*, *selo*	Nk	D. Vancraeynest
DS46	KH454	Rabbit	Belgium	Nk	Staphylococcosis	*seg*, *sei*, *selm*, *seln*, *selo*	Nk	D. Vancraeynest
DS31	PS15	Rabbit	Spain	Nk	Staphylococcosis	*seg*, *sei*, *selm*, *seln*, *selo*	Nk	J. Penadéz
DS44	KH103	Rabbit	Belguim	Nk	Staphylococcosis	*seg*, *sei*, *selm*, *seln*, *selo*	Vancraeynest *et al.*, 2004	D. Vancraeynest
DS45	KH276	Rabbit	Belgium	Nk	Staphylococcosis	*seg*, *sei*, *selm*, *seln*, *selo*	Vancraeynest *et al.*, 2004	D. Vancraeynest
DS80	232-11	Goat	Norway	Milk	Mastitis	Negative	Nk	T. Tollersrud
DS1	DS1	Goat	Austria	Milk	Mastitis	Negative	Nk	P. Winter
DS7	St57	Goat	Italy	Milk	Mastitis	Negative	Foschino *et al.*, 2002	R. Foschino
DS9	St60	Goat	Italy	Milk	Mastitis	Negative	Foschino *et al.*, 2002	R. Foschino
DS11	St108	Goat	Italy	Milk	Mastitis	Negative	Foschino *et al.*, 2002	R. Foschino
DS18	St36	Goat	Italy	Milk	Mastitis	Negative	Foschino *et al.*, 2002	R. Foschino
DS17	St11	Goat	Italy	Milk	Mastitis	Negative	Foschino *et al.*, 2002	R. Foschino
DS24	St16	Goat	Italy	Milk	Mastitis	Negative	Foschino *et al.*, 2002	R. Foschino
DS12	St134	Goat	Italy	Milk	Mastitis	Negative	Foschino *et al.*, 2002	R. Foschino
DS20	St66	Goat	Italy	Milk	Mastitis	Negative	Foschino *et al.*, 2002	R. Foschino
DS19	St65	Goat	Italy	Milk	Mastitis	Negative	Foschino *et al.*, 2002	R. Foschino
DS23	St140	Goat	Italy	Milk	Mastitis	Negative	Foschino *et al.*, 2002	R. Foschino

Code	Strain	Host	Country	Source	Disease	Enterotoxins	Reference	Supplier
DS21	St67	Goat	Italy	Milk	Mastitis	Negative	Foschino *et al.*, 2002	R. Foschino
DS10	St107	Goat	Italy	Milk	Mastitis	Negative	Foschino *et al.*, 2002	R. Foschino
DS22	St137	Goat	Italy	Milk	Mastitis	Negative	Foschino *et al.*, 2002	R. Foschino
DS5	St5	Goat	Italy	Milk	Mastitis	Negative	Foschino *et al.*, 2002	R. Foschino
DS8	St59	Goat	Italy	Milk	Mastitis	Negative	Foschino *et al.*, 2002	R. Foschino
DS25	St24	Goat	Italy	Milk	Mastitis	*sec, tst*	Foschino *et al.*, 2002	R. Foschino
DS28	St152	Goat	Italy	Milk	Mastitis	*sec, tst*	Foschino *et al.*, 2002	R. Foschino
DS73	1618-2	Goat	Norway	Milk	Mastitis	*sec, tst, sell*	Nk	T. Tollersrud
DS75	1589-1	Goat	Norway	Milk	Mastitis	*sec, tst, sell*	Nk	T. Tollersrud
DS76	1589-2	Goat	Norway	Milk	Mastitis	*sec, tst, sell*	Nk	T. Tollersrud
DS77	213-10	Goat	Norway	Milk	Mastitis	*sec, tst, sell*	Nk	T. Tollersrud
DS78	213-8	Goat	Norway	Milk	Mastitis	*sec, tst, sell*	Nk	T. Tollersrud
DS79	232-21	Goat	Norway	Milk	Mastitis	*sec, tst, sell*	Nk	T. Tollersrud
DS81	243-7	Goat	Norway	Milk	Mastitis	*sec, tst, sell*	Nk	T. Tollersrud
DS82	243-2	Goat	Norway	Milk	Mastitis	*sec, tst, sell*	Nk	T. Tollersrud
DS6	St31	Goat	Italy	Milk	Mastitis	*sec, tst, sell*	Foschino *et al.*, 2002	R. Foschino
DS14	St34	Goat	Italy	Milk	Mastitis	*sec, tst, sell*	Foschino *et al.*, 2002	R. Foschino
DS16	St144	Goat	Italy	Milk	Mastitis	*sec, tst, sell*	Foschino *et al.*, 2002	R. Foschino
DS13	St7	Goat	Italy	Milk	Mastitis	*sec, tst, sell*	Foschino *et al.*, 2002	R. Foschino
DS15	St62	Goat	Italy	Milk	Mastitis	*sec, tst,sell*	Foschino *et al.*, 2002	R. Foschino
DS2	DS2	Goat	Austria	Milk	Mastitis	*sec, tst, sell*	Nk	P. Winter
DS4	DS4	Goat	Austria	Milk	Mastitis	*sec, tst, sell*	Nk	P. Winter

Code	Strain	Host	Country	Source	Disease	Enterotoxins	Reference	Supplier
DS3	DS3	Goat	Italy	Milk	Mastitis	*sec, tst, sell*	Foschino *et al.*, 2002	P. Winter
DS30	St153	Goat	Italy	Milk	Mastitis	*seg, sei, selm, seln, selo*	Foschino *et al.*, 2002	R. Foschino
DS27	St125	Goat	Italy	Milk	Mastitis	*seg, sei, selm, seln, selo*	Foschino *et al.*, 2002	R. Foschino
DS26	St49	Goat	Italy	Milk	Mastitis	*seg, sei, selm, seln, selo*	Foschino *et al.*, 2002	R. Foschino
DS29	St51	Goat	Italy	Milk	Mastitis	*seg, sei, selm, seln, selo*	Foschino *et al.*, 2002	R. Foschino
DS99	57-33	Sheep	Finland	Milk	Mastitis	Negative	Nk	T. Tollersrud
DS101	2038-3	Sheep	Sweden	Milk	Mastitis	Negative	Nk	T. Tollersrud
DS96	2	Sheep	Denmark	Milk	Mastitis	Negative	Nk	T. Tollersrud
DS100	99-49	Sheep	Finland	Milk	Mastitis	*seb*	Nk	T. Tollersrud
DS105	2463	Sheep	Iceland	Milk	Mastitis	*seh*	Nk	T. Tollersrud
DS98	12(02)	Sheep	Finland	Milk	Mastitis	*sed, selj*	Nk	T. Tollersrud
DS104	2114	Sheep	Iceland	Milk	Mastitis	*sec, tst*	Nk	T. Tollersrud
DS106	2539	Sheep	Iceland	Milk	Mastitis	*sec, tst*	Nk	T. Tollersrud
DS84	890-1	Sheep	Norway	Milk	Mastitis	*sec, tst*	Nk	T. Tollersrud
DS94	680-2	Sheep	Norway	Milk	Mastitis	*sec, tst*	Nk	T. Tollersrud
DS87	681-2	Sheep	Norway	Milk	Mastitis	*sec, tst, sell*	Nk	T. Tollersrud
DS83	891-1	Sheep	Norway	Milk	Mastitis	*sec, tst, sell*	Nk	T. Tollersrud
DS89	586-2	Sheep	Norway	Milk	Mastitis	*sec, tst, sell*	Nk	T. Tollersrud
DS85	585-1	Sheep	Norway	Milk	Mastitis	*sec, tst, sell*	Nk	T. Tollersrud
DS86	887-1	Sheep	Norway	Milk	Mastitis	*sec, tst, sell*	Nk	T. Tollersrud
DS88	571-2	Sheep	Norway	Milk	Mastitis	*sec, tst, sell*	Nk	T. Tollersrud
DS91	896-1	Sheep	Norway	Milk	Mastitis	*sec, tst, sell*	Nk	T. Tollersrud

Code	Strain	Host	Country	Source	Disease	Enterotoxins	Reference	Supplier
DS92	897-2	Sheep	Norway	Milk	Mastitis	*sec, tst, sell*	Nk	T. Tollersrud
DS93	894-2	Sheep	Norway	Milk	Mastitis	*sec, tst, sell*	Nk	T. Tollersrud
DS97	23	Sheep	Denmark	Milk	Mastitis	*sei, selm, selo, seln*	Nk	T. Tollersrud
DS95	1	Sheep	Denmark	Milk	Mastitis	*seg, sei, selm, seln, selo*	Nk	T. Tollersrud
DS103	7062-4	Sheep	Sweden	Milk	Mastitis	*seg, sei, selm, seln, selo*	Nk	T. Tollersrud
DS90	895-1	Sheep	Norway	Milk	Mastitis	*sec, tst, sel, seg, sei, selm, seln, selo*	Nk	T. Tollersrud
DS39	CH497	Cow	Argentina	Milk	Mastitis	Negative	Nk	J. Penadéz
DS40	CH498	Cow	Argentina	Milk	Mastitis	Negative	Nk	J. Penadéz
DS41	CH501	Cow	Argentina	Milk	Mastitis	Negative	Nk	J. Penadéz
RF101	RF101	Cow	Ireland	Milk	Mastitis	Negative	Fitzgerald *et al.*, 1997	J.R. Fitzgerald
RF103	RF103	Cow	Ireland	Milk	Mastitis	Negative	Fitzgerald *et al.*, 1997	J.R. Fitzgerald
RF104	RF103	Cow	Ireland	Milk	Mastitis	Negative	Fitzgerald *et al.*, 1997	J.R. Fitzgerald
RF105	RF105	Cow	Ireland	Milk	Mastitis	Negative	Fitzgerald *et al.*, 1997	J.R. Fitzgerald
RF106	RF123	Cow	Ireland	Milk	Mastitis	Negative	Fitzgerald *et al.*, 1997	J.R. Fitzgerald
RF107	RF123	Cow	Ireland	Milk	Mastitis	Negative	Fitzgerald *et al.*, 1997	J.R. Fitzgerald
RF123	RF123	Cow	Ireland	Milk	Mastitis	Negative	Fitzgerald *et al.*, 1997	J.R. Fitzgerald
RF195	RF195	Cow	Ireland	Milk	Mastitis	Negative	None	J.R. Fitzgerald
RF196	RF196	Cow	Ireland	Milk	Mastitis	Negative	None	J.R. Fitzgerald
RF25	RF25	Cow	Ireland	Milk	Mastitis	Negative	Fitzgerald *et al.*, 2000	J.R. Fitzgerald
RF26	RF26	Cow	Ireland	Milk	Mastitis	Negative	Fitzgerald *et al.*, 2000	J.R. Fitzgerald
RF30a	RF30a	Cow	Ireland	Milk	Mastitis	Negative	Fitzgerald *et al.*, 2000	J.R. Fitzgerald
RF31a	RF31a	Cow	Ireland	Milk	Mastitis	Negative	Fitzgerald *et al.*, 2000	J.R. Fitzgerald

Code	Strain	Host	Country	Source	Disease	Enterotoxins	Reference	Supplier
RF27	RF27	Cow	Ireland	Milk	Mastitis	Negative	Fitzgerald *et al.*, 2000	J.R. Fitzgerald
RF28	RF28	Cow	Ireland	Milk	Mastitis	Negative	Fitzgerald *et al.*, 2000	J.R. Fitzgerald
RF29	RF29	Cow	Ireland	Milk	Mastitis	Negative	Fitzgerald *et al.*, 2000	J.R. Fitzgerald
RF33a	RF33a	Cow	Ireland	Milk	Mastitis	Negative	Fitzgerald *et al.*, 2000	J.R. Fitzgerald
RF34a	RF34a	Cow	Ireland	Milk	Mastitis	Negative	Fitzgerald *et al.*, 2000	J.R. Fitzgerald
RF35a	RF35a	Cow	Ireland	Milk	Mastitis	Negative	Fitzgerald *et al.*, 2000	J.R. Fitzgerald
RF36a	RF36a	Cow	Ireland	Milk	Mastitis	Negative	Fitzgerald *et al.*, 2000	J.R. Fitzgerald
RF41a	RF41a	Cow	Ireland	Milk	Mastitis	Negative	Fitzgerald *et al.*, 2000	J.R. Fitzgerald
RF45	RF45	Cow	Ireland	Milk	Mastitis	Negative	Fitzgerald *et al.*, 2000	J.R. Fitzgerald
RF47	RF47	Cow	Ireland	Milk	Mastitis	Negative	Fitzgerald *et al.*, 2000	J.R. Fitzgerald
RF50	RF50	Cow	Ireland	Milk	Mastitis	Negative	Fitzgerald *et al.*, 2000	J.R. Fitzgerald
RF79	RF79	Cow	Ireland	Milk	Mastitis	Negative	Fitzgerald *et al.*, 2000	J.R. Fitzgerald
RF116	RF116	Cow	Ireland	Milk	Mastitis	Negative	Fitzgerald *et al.*, 1997	J.R. Fitzgerald
RF117	RF117	Cow	Ireland	Milk	Mastitis	Negative	Fitzgerald *et al.*, 1997	J.R. Fitzgerald
RF115	RF115	Cow	Ireland	Milk	Mastitis	Negative	Fitzgerald *et al.*, 1997	J.R. Fitzgerald
RF111	RF111	Cow	Ireland	Milk	Mastitis	Negative	Fitzgerald *et al.*, 1997	J.R. Fitzgerald
RF288	RF288	Cow	Ireland	Milk	Mastitis	Negative	Nk	J.R. Fitzgerald
RF290	RF290	Cow	Ireland	Milk	Mastitis	Negative	Nk	J.R. Fitzgerald
DS36	V329	Cow	Spain	Milk	Mastitis	Negative	Cucarella *et al.*, 2001	J. Penadéz
DS38	V315	Cow	Spain	Milk	Mastitis	Negative	Nk	J. Penadéz
MSA948	MSA948	Cow	USA	Milk	Mastitis	Negative	Fitzgerald *et al.*, 2003	J.R. Fitzgerald
MSA951	MSA951	Cow	USA	Milk	Mastitis	Negative	Fitzgerald *et al.*, 2003	J.R. Fitzgerald

Code	Strain	Host	Country	Source	Disease	Enterotoxins	Reference	Supplier
MSA1401	MSA1401	Cow	USA	Milk	Mastitis	Negative	Fitzgerald *et al.*, 2003	J.R. Fitzgerald
MSA1521	MSA1521	Cow	USA	Milk	Mastitis	Negative	Fitzgerald *et al.*, 1997	J.R. Fitzgerald
MSA916	MSA916	Cow	USA	Milk	Mastitis	Negative	Fitzgerald *et al.*, 1997	J.R. Fitzgerald
MSA1011	RF1011	Cow	USA	Milk	Mastitis	Negative	Fitzgerald *et al.*, 1997	J.R. Fitzgerald
MSA961	MSA961	Cow	USA	Milk	Mastitis	Negative	Fitzgerald *et al.*, 2003	J.R. Fitzgerald
MSA1003	MSA1003	Cow	USA	Milk	Mastitis	Negative	Fitzgerald *et al.*, 1997	J.R. Fitzgerald
MSA1468	MSA1468	Cow	USA	Milk	Mastitis	Negative	Fitzgerald *et al.*, 1997	J.R. Fitzgerald
MSA1547	MSA1547	Cow	USA	Milk	Mastitis	Negative	Fitzgerald *et al.*, 1997	J.R. Fitzgerald
MSA927	MSA927	Cow	USA	Milk	Mastitis	Negative	Fitzgerald *et al.*, 1997	J.R. Fitzgerald
MSA932	MSA932	Cow	USA	Milk	Mastitis	Negative	Fitzgerald *et al.*, 1997	J.R. Fitzgerald
MSA1534	MSA1534	Cow	USA	Milk	Mastitis	Negative	Fitzgerald *et al.*, 1997	J.R. Fitzgerald
MSA77	MSA77	Cow	USA	Milk	Mastitis	Negative	Fitzgerald *et al.*, 1997	J.R. Fitzgerald
MSA1455	MSA1455	Cow	USA	Milk	Mastitis	Negative	Fitzgerald *et al.*, 1997	J.R. Fitzgerald
MSA1058	MSA1058	Cow	USA	Milk	Mastitis	Negative	Fitzgerald *et al.*, 1997	J.R. Fitzgerald
MSA20.1	MSA20.1	Cow	USA	Milk	Mastitis	Negative	Fitzgerald *et al.*, 1997	J.R. Fitzgerald
MSA1006	MSA1006	Cow	USA	Milk	Mastitis	*sed, selj*	Fitzgerald *et al.*, 2003	J.R. Fitzgerald
DS69	DS69	Cow	Sweden	Milk	Mastitis	*sed, selj*	Nk	T. Tollersrud
DS70	DS70	Cow	Sweden	Milk	Mastitis	*sed, selj*	Nk	T. Tollersrud
RF291	RF291	Cow	Ireland	Milk	Mastitis	*sec, tst, sell*	Nk	J.R. Fitzgerald
RF282	RF282	Cow	Ireland	Milk	Mastitis	*sec, tst, sell*	Nk	J.R. Fitzgerald
RF283	RF283	Cow	Ireland	Milk	Mastitis	*sec, tst, sell*	Nk	J.R. Fitzgerald
RF284	RF284	Cow	Ireland	Milk	Mastitis	*sec, tst, sell*	Nk	J.R. Fitzgerald

Code	Strain	Host	Country	Source	Disease	Enterotoxins	Reference	Supplier
RF286	RF286	Cow	Ireland	Milk	Mastitis	*sec*, *tst*, *sell*	Nk	J.R. Fitzgerald
RF287	RF287	Cow	Ireland	Milk	Mastitis	*sec*, *tst*, *sell*	Nk	J.R. Fitzgerald
RF285	RF285	Cow	Ireland	Milk	Mastitis	*sec*, *tst*, *sell*	Nk	J.R. Fitzgerald
RF289	RF289	Cow	Ireland	Milk	Mastitis	*sec*, *tst*, *sell*	Nk	J.R. Fitzgerald
DS35	1A	Cow	Spain	Milk	Mastitis	*sec, tst, sell*	Nk	J. Penadéz
PSA5	PSA5	Cow	USA	Milk	Mastitis	*sei, selm, seln, selo*	Fitzgerald *et al*., 1997	J.R. Fitzgerald
PSA6.1	PSA6.1	Cow	USA	Milk	Mastitis	*sei, selm, seln, selo*	Fitzgerald *et al*., 1997	J.R. Fitzgerald
MSA148	MSA148	Cow	USA	Milk	Mastitis	*sei, selm, seln, selo*	Fitzgerald *et al*., 1997	J.R. Fitzgerald
MSA1047	MSA1047	Cow	USA	Milk	Mastitis	*sei, selm, seln, selo*	Fitzgerald *et al*., 1997	J.R. Fitzgerald
MSA1014	MSA1014	Cow	USA	Milk	Mastitis	*sec*, *sed*, *selj*, *tst*, *sell*	Fitzgerald *et al*., 1997	J.R. Fitzgerald
RF114	RF114	Cow	Ireland	Milk	Mastitis	*seg, sei, selm, seln, selo*	Fitzgerald *et al*., 1997	J.R. Fitzgerald
RF120	RF120	Cow	Ireland	Milk	Mastitis	*seg, sei, selm, seln, selo*	Fitzgerald *et al*., 1997	J.R. Fitzgerald
RF121	RF121	Cow	Ireland	Milk	Mastitis	*seg, sei, selm, seln, selo*	Fitzgerald *et al*., 1997	J.R. Fitzgerald
DS37	V299	Cow	Spain	Milk	Mastitis	*seg, sei, selm, seln, selo*	Cucarella *et al*., 2004	J. Penadéz
MSA1363	MSA1363	Cow	USA	Milk	Mastitis	*seg, sei, selm, seln, selo*	Fitzgerald *et al*., 1997	J.R. Fitzgerald
MSA915	MSA915	Cow	USA	Milk	Mastitis	*seg, sei, selm, seln, selo*	Fitzgerald *et al*., 2003	J.R. Fitzgerald
MSA17.1	MSA17.1	Cow	USA	Milk	Mastitis	*seg, sei, selm, seln, selo*	Fitzgerald *et al*., 1997	J.R. Fitzgerald
MSA103.14	MSA103.14	Cow	USA	Milk	Mastitis	*seg, sei, selm, seln, selo*	Fitzgerald *et al*., 1997	J.R. Fitzgerald
MSA72.3	MSA72.3	Cow	USA	Milk	Mastitis	*seg, sei, selm, seln, selo*	Fitzgerald *et al*., 1997	J.R. Fitzgerald
MSA1460	MSA1460	Cow	USA	Milk	Mastitis	*seg, sei, selm, seln, selo*	Fitzgerald *et al*., 1997	J.R. Fitzgerald
MSA10.6	MSA10.6	Cow	USA	Milk	Mastitis	*seg, sei, selm, seln, selo*	Fitzgerald *et al*., 1997	J.R. Fitzgerald
MSA73.2	MSA73.2	Cow	USA	Milk	Mastitis	*seg, sei, selm, seln, selo*	Fitzgerald *et al*., 1997	J.R. Fitzgerald

Code	Strain	Host	Country	Source	Disease	Enterotoxins	Reference	Supplier
MSA31	MSA31	Cow	USA	Milk	Mastitis	*seg, sei, selm, seln, selo*	Fitzgerald *et al.*, 1997	J.R. Fitzgerald
MSA26.1	MSA26.1	Cow	USA	Milk	Mastitis	*seg, sei, selm, seln, selo*	Fitzgerald *et al.*, 1997	J.R. Fitzgerald
MSA12.1	MSA12.1	Cow	USA	Milk	Mastitis	*seg, sei, selm, seln, selo*	Fitzgerald *et al.*, 1997	J.R. Fitzgerald
MSA930	MSA930	Cow	USA	Milk	Mastitis	*seg, sei, selm, seln, selo*	Fitzgerald *et al.*, 1997	J.R. Fitzgerald
MSA1005	MSA1005	Cow	USA	Milk	Mastitis	*seg, sei, selm, seln, selo*	Fitzgerald *et al.*, 1997	J.R. Fitzgerald
DS42	CH505	Cow	Argentina	Milk	Mastitis	*sed, seg, sei, selj, selm, seln, selo*	Nk	J. Penadéz
MSA1007	MSA1007	Cow	USA	Milk	Mastitis	*sed, seg, sei, selj, selm, seln, selo*	Fitzgerald *et al.*, 2003	J.R. Fitzgerald
KB92D	KB92D	Cow	Denmark	Milk	Mastitis	*sec, seg, sei, selm, seln, selo, tst*	Larsen et al., 2000	H.D. Larsen
DS43	CH509	Cow	Argentina	Milk	Mastitis	*sec, seg, sei, sell, selm, seln, selo, tst*	Nk	J. Penadéz
RF122	RF122	Cow	Ireland	Milk	Mastitis	*sec, seg, sei, sell, selm, seln, selo, tst*	Fitzgerald *et al.*, 1997	J.R. Fitzgerald
RF124	RF124	Cow	Ireland	Milk	Mastitis	*sec, seg, sei, sell, selm, seln, selo, tst*	Fitzgerald *et al.*, 2000	J.R. Fitzgerald
RF102	RF102	Cow	Ireland	Milk	Mastitis	*sec, seg, sei, sell, selm, seln, selo, tst*	Fitzgerald *et al.*, 1997	J.R. Fitzgerald
RF108	RF108	Cow	Ireland	Milk	Mastitis	*sec, seg, sei, sell, selm, seln, selo, tst*	Fitzgerald *et al.*, 1997	J.R. Fitzgerald
RF110	RF110	Cow	Ireland	Milk	Mastitis	*sec, seg, sei, sell, selm, seln, selo, tst*	Fitzgerald *et al.*, 1997	J.R. Fitzgerald
RF80	RF80	Cow	Ireland	Milk	Mastitis	*sec, seg, sei, sell, selm, seln, selo, tst*	Fitzgerald *et al.*, 2000	J.R. Fitzgerald
RF113	RF113	Cow	Ireland	Milk	Mastitis	*sec, seg, sei, sell, selm, seln, selo, tst*	Fitzgerald *et al.*, 1997	J.R. Fitzgerald

Nk – Not known

It is important to note that although the above strains were isolated from animal infection, it is not clear as to whether or not these strains were animal in origin or that the animals were infected by *S. aureus* strains of human origin. However, 92.4 % of the Irish and American bovine isolates from Fitzgerald *et al.*, (2000) produced βtoxin that is rarely produced by human *S. aureus* isolates.

and *tst*-positive control, strain FRI569 as a *seh*-positive control, strain FRI472 as a *sed*-, *seg*-, *sei*- and *selj*-positive control (Monday & Bohach, 1999), and strain A900322 as a control for the *seg*, *sei*, *selm*, *seln* and *selo* genes, i.e., the *egc* cluster (Jarraud *et al.*, 2001).

3.2.2. Superantigen primer design and amplification of bacterial DNA.

Primers for SE and SEl genes *sea*, *sec–see*, *seg–sei* and *selj* and for the TSST-1 gene *tst* were designed in a previous study (Monday & Bohach, 1999). The *sec* primers used in sequencing analysis amplify variants of the *sec* gene including the bovine variant (Marr *et al.*, 1993; Fitzgerald *et al.*, 2001a). The *seg* primers used amplify known variants of the *seg* gene (Blaiotta *et al.*, 2004). Nucleotide sequences for each of the novel enterotoxin genes *selk, sell, selm, seln, selo* and *selq* were obtained from GenBank by using their specific accession numbers (Table 3.2). The sequences were aligned and compared using ClustalW (http://www.ebi.ac.uk/clustalW) (Thompson *et al.*, 1994). All primer sets were designed to anneal to unique internal regions of these genes and generated amplification products that allowed identification of each enterotoxin gene based on the size of its PCR product (Table 3.2). To ensure that gene-negative samples were interpreted correctly and that a sufficient quantity of template DNA was present, each PCR included a primer set that anneals to the *S. aureus* 16S rRNA gene generating a 228-bp amplicon.

The primers were combined in three multiplex PCR reactions to ensure reliability and reproducibility. Multiplex PCR 1 contained primers for the *sea, sec, sed, see* and 16S rRNA genes, multiplex PCR 2 contained primers for the *seg, seh, sei, selj, tst* and 16S rRNA genes and multiplex PCR 3 contained primers for the *sell, selm, seln, selo*, *selq* and 16S rRNA genes. The *seb* primer set from a previous study (Mehrotra *et al.*, 2000) and the *selk* primer set designed in this study were used individually. Each set of PCR reactions also included appropriate SE gene-positive and -negative controls. The multiplex PCR conditions were as previously described (Monday & Bohach, 1999) with the following cycling parameters: 95 °C for 10 min, 15 cycles of [95 °C for 1 min, 58 °C for 45 s, and 72 °C for 1 min] and 16 cycles of [95 °C for 1 min, 54 °C for 45 s, and 72 °C for 1 min]. The reaction was terminated with a 10-min incubation at 72 °C. PCR products were resolved by electrophoresis in 1.5% (w/v) agarose gels (0.5 × Tris-boric acid-EDTA) (Sambrook & Russell, 2001) at 90 V (constant voltage), stained with ethidium bromide and visualized using UV light. Product sizes were determined by comparison with a 100-bp ladder (Promega).

Table 3.2. Primers designed for genes encoding novel SEs and SEIs used in single locus PCR or multiplex PCR and those used for *sec* gene sequencing.

Primer	Primer sequence (5′–3′)*	Product size (bp)
selk forward	TTA GGT GTC TCT AAT AGT GCC AGC	278
selk reverse	AGC TGT GAC TCC GCC ATA TAT GTA	
sell forward	ATC ACA CCG CTT AGA ATA CTC	198
sell reverse	GGT ATT ATT CTT GGC GAA TCT	
selm forward	TGC TAT TCG CAA AAT CAT ATC GCA ACC	683
selm reverse	TCA ACT TTC GTC CTT ATA AGA TAT TTC TA	
seln forward	GCT TAT GAG ATT GTT CTA CAT AGC TGC	448
seln reverse	CAT TAA CGC CTA TAA CTT TCT CTT CAT C	
selo forward	GAG AGT TTG TGT AAG AAG TCA AGT G	556
selo reverse	GAT TCT TTA TGC TCC GAA TGA GAA	
selq forward	ATT TAG AAT ACT CAC TGT TAG CTT G	124
selq forward	GAA GCT TTT CAG GTT CAT AAT TAG C	
sec forward†	GGG AAG CTT GTA ATT TTG ATA TTC GCA CT	800
sec reverse	CCC GGA TCC TAT CAA AAT CGG ATT AAC A	

* Genbank accession numbers: *selk* (*sek*-AF410775), *sell* (*sel*-AF217235), *selm* (*sem*-AF285760), *seln* (*sen*-AF285760), *selo* (*seo*-AF285760) and *selq* (*seq*-AF410775).

† The *sec* primers used were those of Marr *et al.* (1993).

3.2.3. Sequencing of *sec* gene PCR products.

Single locus PCR was performed for the *sec* gene using seven strains, namely, three goat isolates [1 Italian (strain DS25), 1 Norwegian (strain DS77) and 1 Austrian (strain DS3)], two cow isolates [both Irish (RF124 and RF283)] and two sheep isolates [1 Norwegian (strain DS88) and 1 Icelandic (strain DS104)]. The same conditions as for the multiplex PCR reactions were used with the *sec* gene primers (Marr *et al.*, 1993). The PCR products were ethanol precipitated and sequenced by MWG-Biotech. The resulting sequences were aligned with those of known *sec* gene variants using ClustalW.

3.2.4. Southern hybridisation analysis.

PCR results using novel SE primers designed in this study were confirmed using genomic DNA from a number of representative Irish bovine isolates and control strains FRI913, FRI472, FRI569, COL and RF122. Genomic DNA was digested with restriction endonuclease *Hin*dIII and resolved by electrophoresis in 0.8% (w/v) agarose. Southern hybridisation analysis was performed using standard methods (Sambrook & Russell, 2001) and the DIG-labelling system as described in Chapter 2 (Roche). Genomic DNA from strains that were PCR-negative for the *seg* gene were subjected to dot blot analysis using DIG-labelled probes to the *seg* gene to confirm the result.

3.2.5. Mitogenicity testing of culture supernatant fluids.

3.2.5.1. Crude toxin isolation.

Seventy-nine of the bovine isolates were tested (29 from U.S.A. and 50 from Ireland). One millilitre of overnight culture was precipitated with 5 vol. ethanol and placed at −20 °C for 1 h. Samples were centrifuged and supernatant fractions were discarded. The pellets were air-dried, resuspended in 50 µl sterile distilled water and clarified by centrifugation at maximum speed in a bench-top centrifuge (1–2 min). Supernatant fractions were removed and tested for their ability to induce lymphocyte proliferation. The preparation of samples for mitogenicity assays was performed by Claudia Deobald at the University of Idaho, Moscow, U.S.A.

3.2.5.2. Mitogenicity assay.

Crude toxin preparations from the bovine isolates were diluted 10-, 100- and 1,000-fold in sterile distilled water and were assayed for their ability to induce proliferation of human T-lymphocytes by a standard 4-day mitogenicity assay (Poindexter & Schlievert, 1985). Background levels of proliferation were determined by testing identically prepared samples from the non-toxin-producing strain *S. aureus* RN4220 (Monday & Bohach, 1999). Lymphocytes were isolated from heparinised human blood that was fractionated by centrifugation through a Ficoll-Paque™ PLUS gradient (Amersham Biosciences). Lymphocytes were washed in RPMI 1640 medium (Gibco/Invitrogen Corporation) and suspended to a concentration of 1.0×10^6 cells/ml in RPMI 1640 medium supplemented with 2% (v/v) foetal bovine serum, 2 mM glutamine, 200 U sodium penicillin G/ml, and 200 μg streptomycin sulphate/ml. The T-lymphocytes were distributed into 96-well plates (200 μl per well) and exposed to diluted crude toxin preparations (25 μl per well). Plates were incubated at 37 °C, 6% (v/v) CO_2, for 72 h and then 3.7×10^4 Bq [^{3}H]thymidine (Moravek, Brea, CA) was added to each well. After incubation for a further 18–24 h, DNA was harvested with a Packard BioScience Filtermate Harvester (Meriden, CT). The amount of ^{3}H-radiolabel incorporation into cellular DNA was measured using a Packard BioScience TopCount NXT scintillation and luminescence counter. The mitogenicity assays were performed by Claudia Deobald at the University of Idaho, Moscow, U.S.A.

3.3. Results.

3.3.1. PCR detection of SE and SEl genes and the *tst* gene.

PCR analysis using control strains showed that the primers produced amplicons consistent with their predicted sizes (Figs. 3.3 and 3.4). PCR products of SE genes were used as DNA probes in Southern blot experiments with control strain genomic DNA and representative Irish bovine strain genomic DNA. Southern blotting confirmed the multiplex PCR results (Fig. 3.5).

Of the 191 animal isolates tested in this study, 110 were positive for at least one SAg gene (57.6 %) (Table 3.3). Only seven of the 15 SE and SEl genes and the TSST-1 gene screened for were present in more than 23 % of the strains, namely, *sec*, *seg*, *sei*, *sell*, *selm*, *seln*, *selo* and *tst* (Table 3.3). The *sea* and *see* genes were not detected in any of the

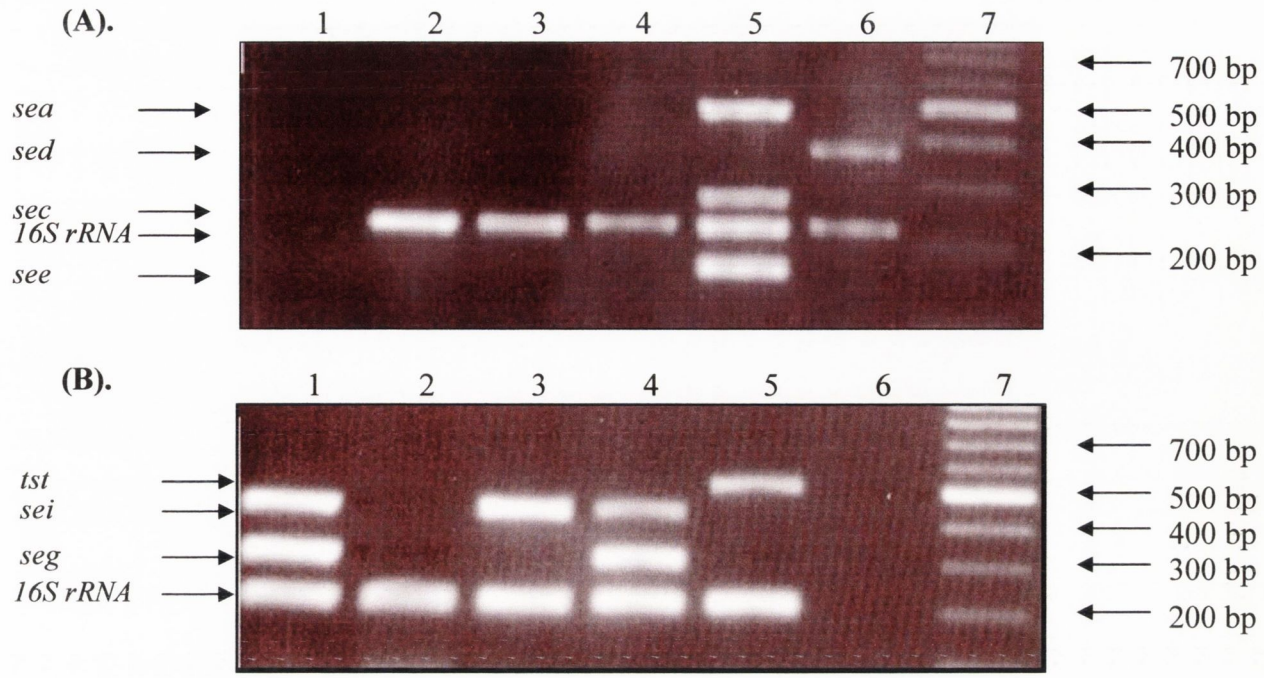


Fig. 3.3. Multiplex PCR for SE and SEl genes.

Panel (A) shows results for multiplex primer mix 1 (*sea*, *sec*, *sed*, *see* and 16S rRNA genes). Lane 1 contains a negative control and lane 8 contains a 100-bp ladder (Promega). Lanes 2–4 contain PCR products from bovine strains: 2, strain PSA6.1 (negative); 3, strain MSA1003 (negative); 4, strain MSA1455 (negative); lanes 5 and 6 contain PCR products from control strains FRI913 (*sea*, *sec*, *see*) and strain FRI472 (*sed*), respectively.

Panel (B) shows results for multiplex primer mix 2 (*seg*, *seh*, *sei*, *selj*, *tst* and 16S rRNA genes). Lanes 1–5 contain PCR products from bovine strains: 1, strain A900322 (*seg*, *sei*); 2, strain RF1003 (negative); 3, strain PSA6.1 (*sei*); 4, strain RF120 (*seg, sei*) and 5, strain MSA1369 (*tst*). Lane 6 contains a negative control and lane 7 contains 100-bp ladder (Promega).

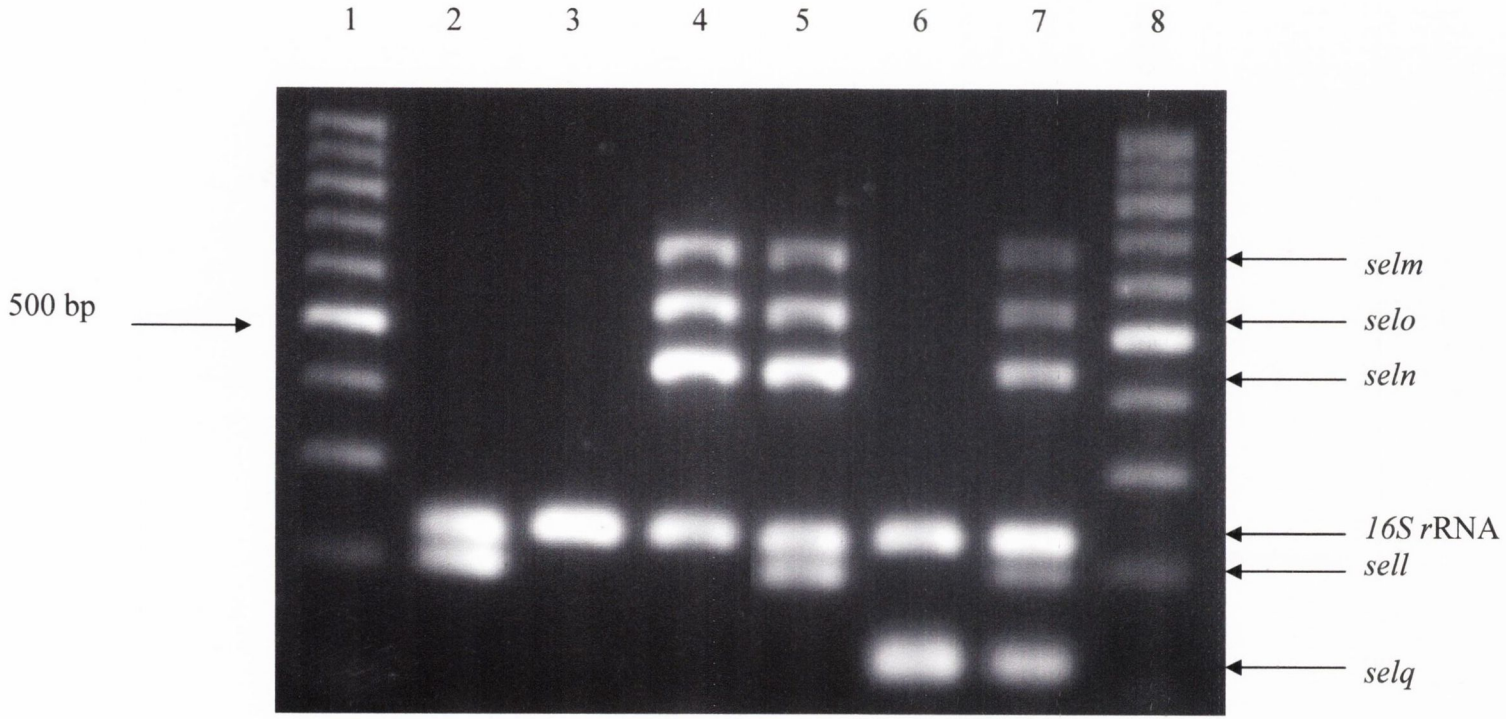


Fig. 3.4. Multiplex PCR products for animal-associated strains and controls using multiplex primer mix 3 (*sell*, *selm*, *seln*, *selo*, *selq* and 16S rRNA genes)

Lanes 1 and 8 contain 100-bp ladder (Promega). Lanes 2–5 contain PCR products from bovine strains: 2, strain RF283 (*sell*); 3, strain RF103 (negative); 4, strain RF120 (*selm*, *seln*, *selo*); and 5, strain RF122 (*sell*, *selm*, *seln*, *selo*). Lane 6 contains amplicons from strain COL (*selq*) and lane 7 contains PCR products from control strain DNA mixture of strains COL and RF122 (*sell*, *selm*, *seln*, *selo, selq*).

Fig. 3.5. Southern blots for SE and SEl genes using bovine RAPD type 7 strain DNA and control strain DNA.

Panel (A) shows *Hin*dIII-digested DNA from bovine strain RF102 and control strains probed with a DIG-labelled *selk* PCR product from strain COL. Strains COL, MSSA and FRI913 are positive for the *selk* gene. The probe hybridized to 8-kb, 4.5-kb and 3.5-kb *Hin*dIII fragments in these three strains, respectively. These strains were also positive for the *selq* gene which is present along with the *selk* gene on pathogenicity island SaPI4.

Panel (B) shows *Hin*dIII-digested DNA from seven bovine strains and control strains probed with a DIG-labelled *selm* PCR product from strain A900322. Strains EMRSA16 and NCTC6571 were positive for the *selm* gene along with all the bovine strains. The probe hybridized to an approximate 6-kb band in strains EMRSA16 and NCTC6571 and to a 5-kb band in the bovine strains. All these *selm* gene-positive strains were also positive for the *seg*, *sei*, *seln* and *selo* genes which are encoded on the enterotoxin gene cluster (*egc*).

Panel (C) shows *Hin*dIII-digested DNA from seven bovine strains and control strains probed with a DIG-labelled *sell* PCR product from bovine strain RF122. Strain FRI913, along with 5 of the 7 bovine strains were positive for the *sell* gene with the probe hybridising to a 10-kb band in strain FRI913 and to 3.5-kb bands in each of the bovine strains. The *sell* gene is present on a bovine-associated pathogenicity island, SaPIbov. The presence of the *sell* gene in some but not all RAPD type 7 bovine strains gives evidence of horizontal gene transfer between strains. The approximate sizes of the *Hin*dIII-digested DNA fragments were estimated by running a 10 kb-DNA ladder (NEB) alongside the fragments on the agarose gel, cutting the gel at the position of each of the bands in the DNA ladder and post-transfer, marking the position of the bands of the DNA ladder on the nylon membrane.

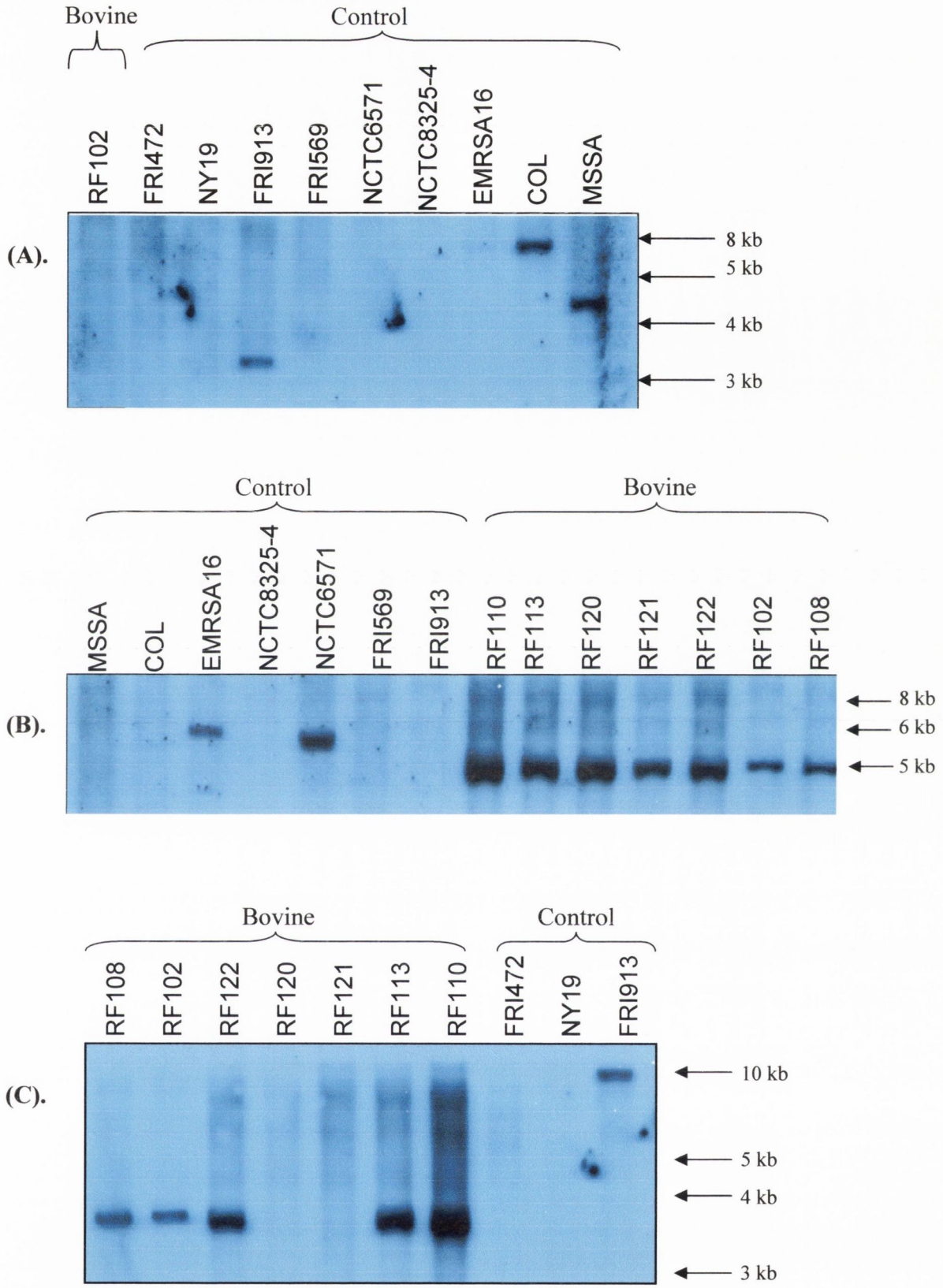

Bovine
Control
RF102
FRI472
NY19
FRI913
FRI569
NCTC6571
NCTC8325-4
EMRSA16
COL
MSSA
(A).
8 kb
5 kb
4 kb
3 kb
Control
Bovine
MSSA
COL
EMRSA16
NCTC8325-4
NCTC6571
FRI569
FRI913
RF110
RF113
RF120
RF121
RF122
RF102
RF108
(B).
8 kb
6 kb
5 kb
Bovine
Control
RF108
RF102
RF122
RF120
RF121
RF113
RF110
FRI472
NY19
FRI913
(C).
10 kb
5 kb
4 kb
3 kb

Table 3.3. PCR analysis for SAg genes in animal-associated strains of *S. aureus*.

SAg gene	Cow (n=99)		Goat (n=39)		Sheep (n=23)		Rabbit (n=15)		Chicken (n=15)		Total (n=191)		
	n*	%	n	%	n	%	n	%	n	%	n	% Total	% SAg + Strains
SE & SEl genes	45	45.5%	22	56.4%	20	87%	8	53.3%	14	93.3%	110	57.6%	100%
sea	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0%
seb	0	0%	0	0%	1	4.3%	0	0%	0	0%	1	0.5%	0.9%
sec	19	19.2%	18	46.2%	14	60.9%	0	0%	0	0%	51	26.7%	46.4%
sed	6	6.10%	0	0%	1	4.3%	1	6.7%	0	0%	8	4.2%	7.3%
see	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0%
seg	22	22.2%	4	10.3%	3	13%	5	33.3%	13	86.7%	47	24.6%	42.7%
seh	0	0%	0	0%	1	4.3%	2	13.3%	1	6.7%	4	2.1%	3.6%
sei	32	32.3%	4	10.3%	4	17.4%	5	33.3%	13	86.7%	58	30.4%	52.7%
selj	6	6.1%	0	0%	1	4.3%	1	6.7%	0	0%	8	4.2%	7.3%
selk	0	0%	0	0%	0	0%	0	0%	1	6.7%	1	0.5%	0.9%
sell	18	18.2%	16	41%	10	43.5%	0	0%	0	0%	44	23.0%	40.0%
selm	32	32.3%	4	10.3%	4	17.4%	5	33.3%	13	86.7%	58	30.4%	52.7%
seln†	32	32.3%	4	10.3%	4	17.4%	5	33.3%	13	86.7%	58	30.4%	52.7%
selo	32	32.3%	4	10.3%	4	17.4%	5	33.3%	13	86.7%	58	30.4%	52.7%

SAg gene	Cow (n=99)		Goat (n=39)		Sheep (n=23)		Rabbit (n=15)		Chicken (n=15)		Total (n=191)		
	n	%	n	%	n	%	n	%	n	%	n	% Total	% SAg + Strains
selq	0	0%	0	0%	0	0%	0	0%	1	6.7%	1	0.5%	0.9%
TSST-1 gene	19	19.2%	18	46.2%	14	60.9%	0	0%	0	0%	51	26.7	46.4%

* Total number of animal-associated strains positive for any SE and SEl genes.

† 7 of the bovine strains and 1 of the sheep strains did not generate an amplicon for *seln* in multiplex PCR but were found to produce amplicons in single locus PCR

strains. The most common combination of enterotoxin genes observed was the *egc* gene cluster that was present in 42.7 % of the SAg gene-positive strains (Table 3.4). All 51 animal–associated strains that possessed the *sec* gene also had the *tst* gene; in those lacking the *sec* gene, the *tst* gene was also absent. The *sec, sell* and *tst* genes (SaPIbov pathogenicity island combination of genes) were found in 40 % of the SAg gene-positive strains. The combination of *sec* and *tst* genes without the *sell* gene was found in a further 7 strains (6.4 % of SAg gene-positive isolates). The combination of *sei, selm, seln* and *selo* genes (*egc* cluster lacking the *seg* gene) was found in 11 strains (10 % of the SAg gene-positive strains). The combination of *sed* and *selj* genes was found in 7.3 % of SAg gene-positive strains.

The ninety-nine bovine isolates included the main RAPD types (Random Amplified Polymorphic DNA types) associated with mastitis in the USA (RAPD types 1–4 and 9–12) and the Republic of Ireland (RAPD types 4, 5 and 7) that had previously been analysed for the presence of the classical enterotoxins and TSST-1 by RPLA (Reversed Passive Latex Agglutination) (Fitzgerald *et al.*, 2000). In the latter study 87.5 % (21 of 24) of Irish RAPD type 7 isolates were found to produce SEC and TSST-1. In the current study 45 bovine isolates (45.5 %) were positive for at least one SE and SEl gene (Table 3.3). Of these 45 strains, 24 (3 American, 2 Argentinian, 1 Danish, 15 Irish, 1 Spanish and 2 Swedish, 53.3 %) were positive for any of the classical SE genes (*sec*, 18; *sed*, 5; *sec* and *sed*, 1; Table 3.3). The genes encoded by the SaPIbov pathogenicity island (Fitzgerald *et al.,* 2001a), namely, *sec*-bovine, *sell* and *tst*, were present in 18 of the SAg gene-positive strains [15 Irish (including 7 known RAPD type 7 strains), 1 American, 1 Spanish, 1 Argentinian, 40 %] and one Danish strain had the *sec* and *tst* genes only. Six of the SAg gene-positive strains (3 American, 1 Argentinian, 2 Swedish, 13.3 %) harboured the *sed* and *selj* genes. The genes encoded by the *egc* gene cluster were found in 22 of the SAg gene-positive strains (8 American, 2 Argentinian, 1 Danish, 10 Irish, 1 Spanish, 48.9 %), with a further 10 American strains containing the *sei, selm, seln* and *selo* genes only (22.2 %, Table 3.3). RAPD types 4 and 5 isolates (31 strains) that had previously been analysed for the production of classical enterotoxins and TSST-1 by RPLA (Fitzgerald *et al.*, 2000) were negative for the presence of all 15 SE and SEl genes and the TSST-1 gene tested for.

Of the 39 caprine strains analysed, 22 (3 Austrian, 11 Italian, 8 Norwegian, 56.4 %) were positive for at least one SAg gene, with 18 strains of these 22 being positive for one of the classical SE genes (*sec*, 18; 81.8 %; Table 3.3). Sixteen of the 22 SAg gene-positive

Table 3.4. Combinations of SAg genes in 110 SE and SEl gene-positive and *tst*-positive strains of *S. aureus* of animal origin.

Country of origin	No. of strains	Animal Source(n)*	Gene combination
Argentina	1	B	*(sec, sell, tst)* †, *(seg, sei, selm, seln, selo)* ‡
Argentina	1	B	*sed, selj*, (*seg, sei, selm, seln, selo*)
Austria	3	G	*(sec, sell, tst)*
Belgium	1	R	*seh*
Belgium	1	R	*sed, selj*
Belgium	4	R	(*seg, sei, selm, seln, selo*)
Denmark	1	S	(*seg, sei, selm, seln, selo*)
Denmark	1	S	*sei, selm, seln, selo*§
Denmark	1	B	*sec, tst*, (*seg, sei, selm, seln, selo*)
Finland	1	S	*seb*
Finland	1	S	*sed, selj*
Iceland	1	S	*seh*
Iceland	2	S	*sec, tst*
Italy	5	G	*(sec, sell, tst)*
Italy	2	G	*sec, tst*
Italy	4	G	(*seg, sei, selm, seln, selo*)
Northern Ireland	13	C	(*seg, sei, selm, seln, selo*)
Northern Ireland	1	C	*seh, selk, selq*
Norway	17	G (8), S (9)	*(sec, sell, tst)*
Norway	1	S	*(sec, sell, tst), (seg, sei, selm, seln, selo)*
Norway	2	S	*sec, tst*
Republic of Ireland	8	B	*(sec, sell, tst)*
Republic of Ireland	7	B	*(sec, sell, tst), (seg, sei, selm, seln, selo)*
Republic of Ireland	3	B	(*seg, sei, selm, seln, selo*)
Spain	1	R	*seh*
Spain	1	B	*(sec, sell, tst)*
Spain	2	B (1), R(1)	(*seg, sei, selm, seln, selo*)

Country of origin	Number	Animal Source(n)*	Gene combination
Sweden	3	B (2), Cat (1)	*sed*, *selj*
Sweden	1	S	(*seg*, *sei*, *selm*, *seln*, *selo*)
USA	1	B	*(sec*, *sell*, *tst)* , *sed*, *selj*
USA	1	B	*sed*, *selj*
USA	1	B	*sed*, *selj*, (*seg*, *sei*, *selm*, *seln*, *selo*)
USA	7	B	(*seg*, *sei*, *selm*, *seln*, *selo*)
USA	10	B	*sei*, *selm*, *seln*, *selo*

*Animal source: B, bovine; G, goat; S, sheep; R, rabbit; C, chicken.
†SaPIbov pathogenicity island combination of genes
‡Complete *egc* cluster
§ *egc* cluster gene combination lacking the *seg* gene

strains (3 Austrian, 5 Italian, 8 Norwegian, 72.7 %) contained the three SAg genes associated with the SaPIbov pathogenicity island, with a further two Italian strains possessing the *sec* and *tst* genes only (9.1 %; Table 3.3). Four Italian strains (18.2 % of SAg gene-positive isolates) were positive for the entire *egc* cluster.

Of the 23 isolates of ovine origin tested, 20 (87 %) were positive for at least one SAg gene (2 Danish, 2 Finnish, 3 Icelandic, 12 Norwegian, 1 Swedish); sixteen of these 20 isolates were positive for one of the classical SE genes (*seb*, 1; sec, 14; *sed*, 1; 80 % of SAg gene-positive isolates, Table 3.3). Ten Norwegian strains (50 % of SAg gene-positive isolates) harboured the SaPIbov pathogenicity island-associated genes, with an additional four strains (2 Italian, 2 Norwegian, 20 %) containing the *sec* and *tst* genes only (Table 3.3). One Finnish strain possessed the *seb* gene, one Icelandic strain contained the *seh* gene and one Finnish strain had the *sed* and *selj* genes in combination. Three Scandinavian strains (15 % of SAg gene-positive strains) contained the *egc* cluster in its entirety, with one Danish strain containing the *sei, selm, seln* and *selo* genes only.

Of the 15 strains from rabbits analysed, eight (4 Belgian, 2 Spanish, 53.3 %) were positive for at least one SAg gene (Table 3.3). Only one Belgian strain contained a classical SE gene, *sed,* in combination with *selj.* Two strains (1 Belgian, 1 Spanish) possessed the *seh* gene, while five strains (4 Belgian, 1 Spanish, 62.5 % of SAg gene-positive isolates) strains harboured the *egc* locus in its entirety (Table 3.3).

Of the 15 poultry strains tested, none contained a classical SE gene. Fourteen (93.3 %) were positive for one or more of the SE and SEl genes (Table 3.3). One strain from poultry was positive for the *seh* gene, together with the *selk* and *selq* genes, while thirteen of the SAg gene-positive strains (92.9 %) contained the *egc* locus (Table 3.3).

One Swedish cat isolate was tested. This strain was positive for the *sed* and *selj* genes.

An online Chi-Square Calculator was used to examine whether the differences in the frequencies of superantigen genes between host populations were statistically significant (http://www.georgetown.edu/faculty/ballc/webtools/web_chi.html) (Fig. 3.6). The differences in the frequencies of SAg genes between bovine isolates and those from sheep or chickens are statistically significant (χ^2-test, both $P<0.001$). The differences in the frequencies of SAg genes between goat isolates and those from sheep or chickens are also

Web Chi Square Calculator: Results

Frequencies of Classical Enterotoxin Genes in SAg + isolates

	Positive	Negative	Total
Bovine	24	21	45
Caprine	18	4	22
Total	42	25	67

Degrees of freedom: 1
Chi-square = 5.12564502164502
p is less than or equal to 0.025.
The distribution is significant.

Fig. 3.6. Example of output from web-based Chi-Square Calculator.

The online calculator was used to determine if the differences in the presence of the classical enterotoxin genes (*sea–see*) in this example between host SAg-positive populations, were significant. The calculator is available at the following link: http://www.georgetown.edu/faculty/ballc/webtools/web_chi.html.

both statistically significant (χ^2-test, $P<0.025$ and $P<0.01$, respectively) and between rabbit and sheep or chicken isolates (χ^2-test, both $P<0.025$).

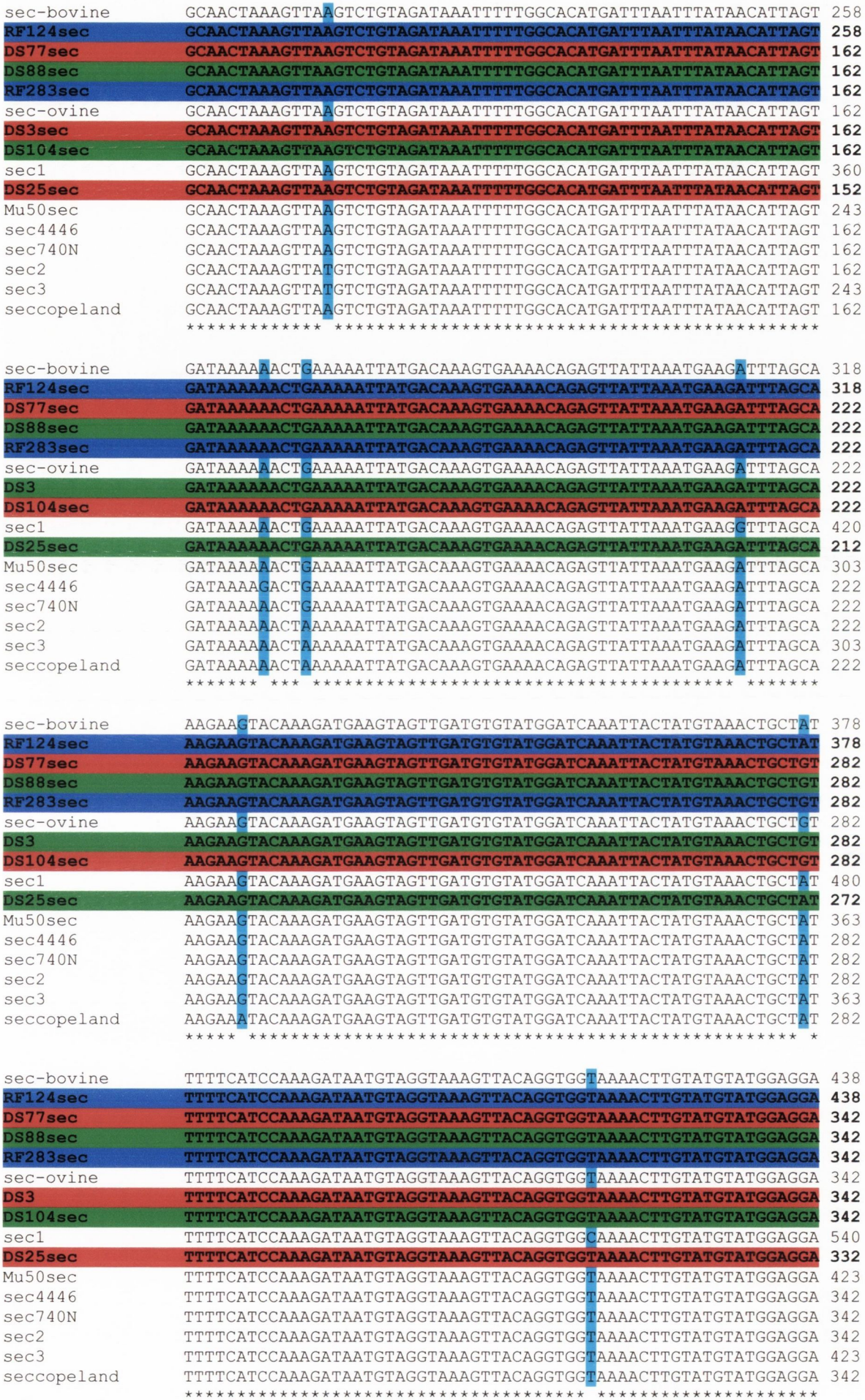
The percentages of SAg gene-positive isolates from sheep or goats harbouring at least one SAg gene or a classical SE gene (*sea–see*), or the *sec* and *tst* genes together or the complete *egc* cluster of genes are quite similar and not statistically different. The differences in the frequencies of genes encoding the classical SEs in SAg gene-positive strains between the bovine isolates and the sheep or goat isolates are statistically significant (χ^2-test, $P<0.05$ and $P<0.025$, respectively). The higher occurrences of the *sec* and *tst* genes among the SAg gene-positive sheep or goat isolates compared to bovine isolates are statistically significant (χ^2-test, $P<0.05$ and $P<0.01$). The lower occurrences of the *egc* gene cluster among the SAg gene-positive sheep or goat isolates compared to bovine isolates are statistically significant (χ^2-test, $P<0.01$ and $P<0.025$, respectively). When complete and incomplete *egc* gene clusters are considered together, the differences between SAg gene-positive sheep or goat isolates and bovine isolates attain higher statistical significance (χ^2-test, both $P<0.001$).

3.3.2. *sec* gene sequencing.

Several SEC variants have been described (Marr *et al.*, 1993) including SEC-bovine associated with the SaPIbov pathogenicity island (Fitzgerald *et al.*, 2001a). Sequencing of the *sec* gene was performed for seven representative animal-associated strains, three from goats, two from cows and two from sheep. The sequencing data were aligned with the sequences of known *sec* alleles, namely, *sec1*, *sec2*, *sec3*, *sec*-MNCopeland, *sec*-4446, *sec*740N, Mu50sec, *sec*-bovine and *sec*-ovine (Marr *et al.*, 1993; Fitzgerald *et al.*, 2001a) using ClustalW (Fig. 3.7). The two ovine (DS88 and DS104), one of the bovine (RF283) and two of the caprine sequences (DS3 and DS77) had 100 % identity to *sec*-ovine. The *sec* sequence of the remaining bovine strain (RF124) was identical to *sec*-bovine. The *sec* sequence of the remaining goat isolate DS25 (Italian) was identical to the *sec3* variant of the human-associated strain Mu50 genome sequence but exhibited a single nucleotide gap at position 596 in the alignment based on duplicate sequencing of both strands of PCR products (Accession no. NC_002758) (Fig. 3.7).

Fig. 3.7. Part of an alignment of the *sec* gene sequences of four animal-associated *S. aureus* strains with known *sec* variants.

Differences in nucleotide sequences between *sec* alleles are highlighted in light blue. Bovine strains are highlighted in dark blue, goat strains in red and sheep strains in green. Asterixes underneath the alignments indicate identical residues. Strains RF124 and RF283 are Irish bovine strains (highlighted in blue), strain DS3 is an Austrian goat strain (highlighted in red), strain DS25 is an Italian goat strain (highlighted in red), strain DS77 is a Norwegian goat strain (highlighted in red), strain DS88 is a Norwegian sheep strain (highlighted in green) and strain DS104 is an Icelandic sheep strain (highlighted in green). The *sec* sequences of goat strains DS3 and DS77 and of sheep strains DS88 and DS104 are identical to that of *sec*-ovine. The *sec* gene of bovine strain RF124 is identical to *sec*-bovine, unlike bovine strain RF283 whose *sec* gene is identical to *sec*-ovine. The *sec* gene of strain DS25 is identical to that of strain Mu50 but has a single nucleotide gap at position 596.


sec-bovine GCAACTAAAGTTAAGTCTGTAGATAAATTTTTGGCACATGATTTAATTTATAACATTAGT 258
RF124sec GCAACTAAAGTTAAGTCTGTAGATAAATTTTTGGCACATGATTTAATTTATAACATTAGT 258
DS77sec GCAACTAAAGTTAAGTCTGTAGATAAATTTTTGGCACATGATTTAATTTATAACATTAGT 162
DS88sec GCAACTAAAGTTAAGTCTGTAGATAAATTTTTGGCACATGATTTAATTTATAACATTAGT 162
RF283sec GCAACTAAAGTTAAGTCTGTAGATAAATTTTTGGCACATGATTTAATTTATAACATTAGT 162
sec-ovine GCAACTAAAGTTAAGTCTGTAGATAAATTTTTGGCACATGATTTAATTTATAACATTAGT 162
DS3sec GCAACTAAAGTTAAGTCTGTAGATAAATTTTTGGCACATGATTTAATTTATAACATTAGT 162
DS104sec GCAACTAAAGTTAAGTCTGTAGATAAATTTTTGGCACATGATTTAATTTATAACATTAGT 162
sec1 GCAACTAAAGTTAAGTCTGTAGATAAATTTTTGGCACATGATTTAATTTATAACATTAGT 360
DS25sec GCAACTAAAGTTAAGTCTGTAGATAAATTTTTGGCACATGATTTAATTTATAACATTAGT 152
Mu50sec GCAACTAAAGTTAAGTCTGTAGATAAATTTTTGGCACATGATTTAATTTATAACATTAGT 243
sec4446 GCAACTAAAGTTAAGTCTGTAGATAAATTTTTGGCACATGATTTAATTTATAACATTAGT 162
sec740N GCAACTAAAGTTAAGTCTGTAGATAAATTTTTGGCACATGATTTAATTTATAACATTAGT 162
sec2 GCAACTAAAGTTATGTCTGTAGATAAATTTTTGGCACATGATTTAATTTATAACATTAGT 162
sec3 GCAACTAAAGTTATGTCTGTAGATAAATTTTTGGCACATGATTTAATTTATAACATTAGT 243
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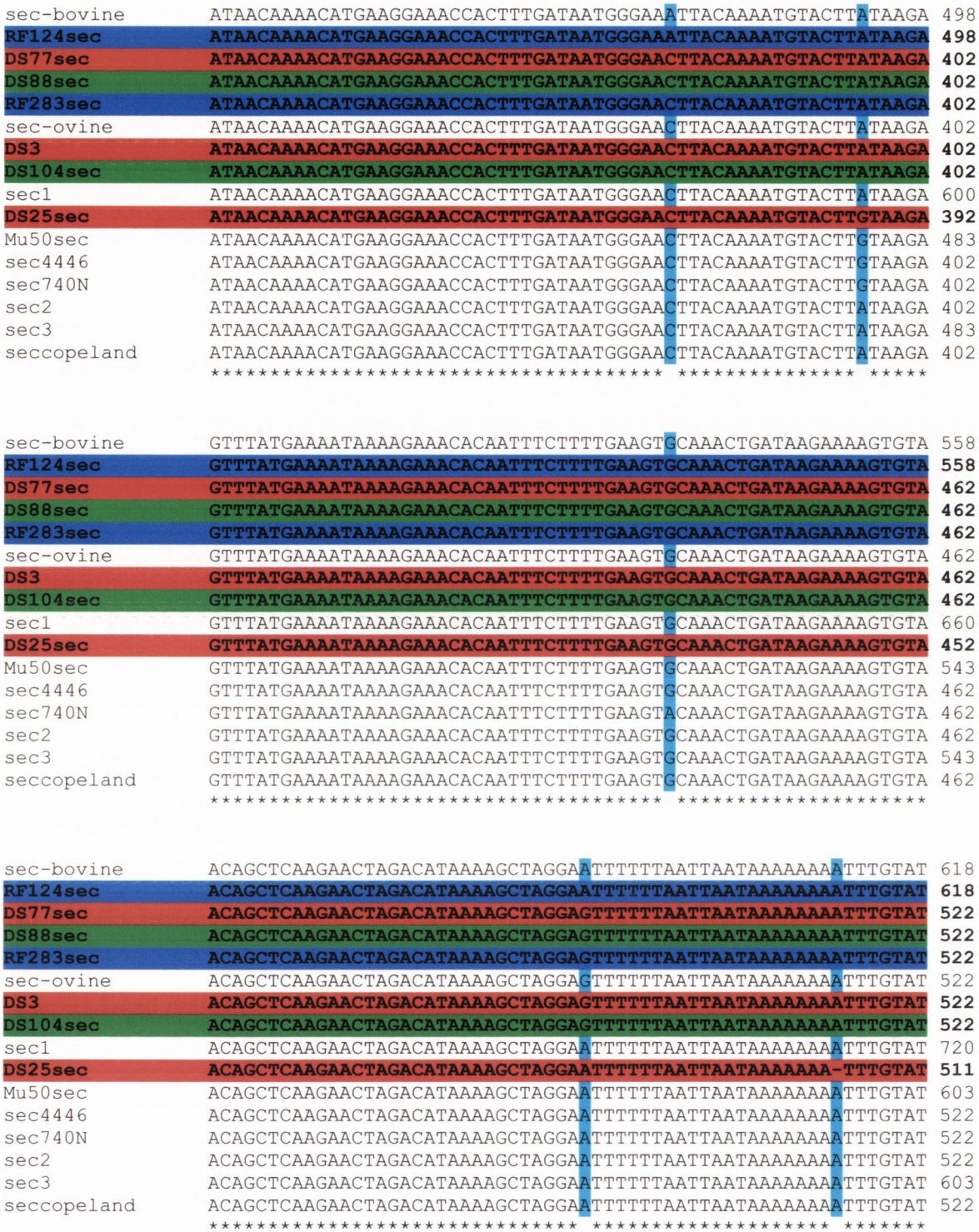
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RF124sec ATAACAAAACATGAAGGAAACCACTTTGATAATGGGAAATTACAAAATGTACTTATAAGA 498
DS77sec ATAACAAAACATGAAGGAAACCACTTTGATAATGGGAACTTACAAAATGTACTTATAAGA 402
DS88sec ATAACAAAACATGAAGGAAACCACTTTGATAATGGGAACTTACAAAATGTACTTATAAGA 402
RF283sec ATAACAAAACATGAAGGAAACCACTTTGATAATGGGAACTTACAAAATGTACTTATAAGA 402
sec-ovine ATAACAAAACATGAAGGAAACCACTTTGATAATGGGAACTTACAAAATGTACTTATAAGA 402
DS3 ATAACAAAACATGAAGGAAACCACTTTGATAATGGGAACTTACAAAATGTACTTATAAGA 402
DS104sec ATAACAAAACATGAAGGAAACCACTTTGATAATGGGAACTTACAAAATGTACTTATAAGA 402
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Mu50sec ATAACAAAACATGAAGGAAACCACTTTGATAATGGGAACTTACAAAATGTACTTGTAAGA 483
sec4446 ATAACAAAACATGAAGGAAACCACTTTGATAATGGGAACTTACAAAATGTACTTGTAAGA 402
sec740N ATAACAAAACATGAAGGAAACCACTTTGATAATGGGAACTTACAAAATGTACTTGTAAGA 402
sec2 ATAACAAAACATGAAGGAAACCACTTTGATAATGGGAACTTACAAAATGTACTTATAAGA 402
sec3 ATAACAAAACATGAAGGAAACCACTTTGATAATGGGAACTTACAAAATGTACTTATAAGA 483
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sec-bovine GTTTATGAAAATAAAAGAAACACAATTTCTTTTGAAGTGCAAACTGATAAGAAAAGTGTA 558
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DS88sec GTTTATGAAAATAAAAGAAACACAATTTCTTTTGAAGTGCAAACTGATAAGAAAAGTGTA 462
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sec-ovine GTTTATGAAAATAAAAGAAACACAATTTCTTTTGAAGTGCAAACTGATAAGAAAAGTGTA 462
DS3 GTTTATGAAAATAAAAGAAACACAATTTCTTTTGAAGTGCAAACTGATAAGAAAAGTGTA 462
DS104sec GTTTATGAAAATAAAAGAAACACAATTTCTTTTGAAGTGCAAACTGATAAGAAAAGTGTA 462
sec1 GTTTATGAAAATAAAAGAAACACAATTTCTTTTGAAGTGCAAACTGATAAGAAAAGTGTA 660
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sec4446 GTTTATGAAAATAAAAGAAACACAATTTCTTTTGAAGTGCAAACTGATAAGAAAAGTGTA 462
sec740N GTTTATGAAAATAAAAGAAACACAATTTCTTTTGAAGTACAAACTGATAAGAAAAGTGTA 462
sec2 GTTTATGAAAATAAAAGAAACACAATTTCTTTTGAAGTGCAAACTGATAAGAAAAGTGTA 462
sec3 GTTTATGAAAATAAAAGAAACACAATTTCTTTTGAAGTGCAAACTGATAAGAAAAGTGTA 543
seccopeland GTTTATGAAAATAAAAGAAACACAATTTCTTTTGAAGTGCAAACTGATAAGAAAAGTGTA 462
sec-bovine ACAGCTCAAGAACTAGACATAAAAGCTAGGAATTTTTTAATTAATAAAAAAAATTTGTAT 618
RF124sec ACAGCTCAAGAACTAGACATAAAAGCTAGGAATTTTTTAATTAATAAAAAAAATTTGTAT 618
DS77sec ACAGCTCAAGAACTAGACATAAAAGCTAGGAGTTTTTTAATTAATAAAAAAAATTTGTAT 522
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RF283sec ACAGCTCAAGAACTAGACATAAAAGCTAGGAGTTTTTTAATTAATAAAAAAAATTTGTAT 522
sec-ovine ACAGCTCAAGAACTAGACATAAAAGCTAGGAGTTTTTTAATTAATAAAAAAAATTTGTAT 522
DS3 ACAGCTCAAGAACTAGACATAAAAGCTAGGAGTTTTTTAATTAATAAAAAAAATTTGTAT 522
DS104sec ACAGCTCAAGAACTAGACATAAAAGCTAGGAGTTTTTTAATTAATAAAAAAAATTTGTAT 522
sec1 ACAGCTCAAGAACTAGACATAAAAGCTAGGAATTTTTTAATTAATAAAAAAAATTTGTAT 720
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sec2 ACAGCTCAAGAACTAGACATAAAAGCTAGGAATTTTTTAATTAATAAAAAAAATTTGTAT 522
sec3 ACAGCTCAAGAACTAGACATAAAAGCTAGGAATTTTTTAATTAATAAAAAAAATTTGTAT 603
seccopeland ACAGCTCAAGAACTAGACATAAAAGCTAGGAATTTTTTAATTAATAAAAAAAATTTGTAT 522

3.3.3. Geographical distribution of SE, SEl and TSST-1 genes in animal isolates.

Ninety-five percent of strains from Norway, 93 % of the Northern Ireland strains, 100 % of strains from Iceland, 80 % of strains from Sweden and 75 % of strains from Denmark were positive for SAg genes, while only 36 % of strains from the Republic of Ireland and 54.1 % of strains from the USA were positive for enterotoxin genes. However, the isolates from Norway were from sheep and goats that had a high frequency of SAg genes (Tables 3.3 and 3.4). Likewise the Northern Ireland strains that were from chickens also had a high frequency of SAg genes. The isolates with a low frequency of SAg genes were from the Republic of Ireland and the USA, in both cases from cows. However, it is important to note that 54 % of the Irish isolates tested herein were of RAPD types 4 and 5 that were negative for all sixteen SAg genes. Thus the differences in the frequencies of SAg genes on a geographical basis probably reflect the clonality of strains infecting cows, sheep and goats in these countries.

3.3.4. Mitogenicity assays.

Since mitogenicity assays on a *sec/tst* knockout mutant of a bovine strain aided the discovery of the SaPIbov pathogenicity island (Fitzgerald *et al.*, 2001a), seventy-nine of the ninety-nine bovine isolates surveyed for SAg genes were screened to determine whether the presence of SAg genes correlated with expression of mitogenic activity and whether or not SAg gene-negative isolates were mitogenic (Table 3.5). Thirty-six strains induced T-cell proliferation, of which 32 had at least one or more SAg genes while the four remaining strains did not possess the SAg genes tested for. Twenty-nine isolates, that did not possess the SAg genes tested for, did not induce T-cell proliferation. The remaining fourteen isolates exhibited borderline mitogenicity; 12 of these 14 isolates did not have one of the SAg genes tested for while two possessed at least one SAg gene.

3.4. Discussion.

3.4.1. PCR detection of SE/SEl genes and the *tst* gene.

Most previous studies have indicated that in animal-associated isolates of *S. aureus* the *sea*, *seb*, *see* and *seh* genes are absent or rarely occurring, namely in < 5 % of tested strains (Larsen *et al.*, 2000a, 2002; Akineden *et al.*, 2001; Hazariwala *et al.*, 2002), or that animal-

Table 3.5. Mitogenicity testing of bovine-associated *S. aureus*

Strain	Country	RAPD Type	Enterotoxins	Mitogenicity
RF111	Ireland	4	Negative	-
RF115	Ireland	4	Negative	-
RF116	Ireland	4	Negative	-
RF117	Ireland	4	Negative	-
RF101	Ireland	5	Negative	-
RF103	Ireland	5	Negative	+
RF104	Ireland	5	Negative	+
RF105	Ireland	5	Negative	±
RF106	Ireland	5	Negative	-
RF107	Ireland	5	Negative	-
RF123	Ireland	5	Negative	-
RF195	Ireland	ND	Negative	-
RF196	Ireland	ND	Negative	-
RF25	Ireland	4	Negative	-
RF26	Ireland	4	Negative	±
RF27	Ireland	4	Negative	-
RF28	Ireland	4	Negative	±
RF29	Ireland	4	Negative	-
RF30a	Ireland	4	Negative	±
RF31a	Ireland	4	Negative	±
RF33a	Ireland	4	Negative	-
RF34a	Ireland	5	Negative	-
RF35a	Ireland	5	Negative	-
RF36a	Ireland	5	Negative	-
RF39	Ireland	5	Negative	-
RF41a	Ireland	5	Negative	-
RF45	Ireland	5	Negative	-
RF47	Ireland	5	Negative	-
RF50	Ireland	4	Negative	-

Strain	Country	RAPD Type	Enterotoxins	Mitogenicity
RF79	Ireland	5	Negative	+
RF288	Ireland	ND	Negative	±
RF290	Ireland	ND	Negative	+
MSA916	USA	12	Negative	-
MSA927	USA	4	Negative	-
MSA1011	USA	4	Negative	-
MSA1547	USA	11	Negative	±
MSA932	USA	1	Negative	±
MSA1534	USA	1	Negative	±
MSA77	USA	1	Negative	-
MSA1455	USA	1	Negative	-
MSA1521	USA	1	Negative	±
MSA1003	USA	11	Negative	-
MSA1058	USA	4	Negative	-
MSA1468	USA	1	Negative	±
MSA20.1	USA	1	Negative	±
RF282	Ireland	ND	*sec*, *sell*, *tst*	+
RF283	Ireland	ND	*sec, sell, tst*	+
RF284	Ireland	ND	*sec, sell, tst*	+
RF285	Ireland	ND	*sec, sell, tst*	+
RF286	Ireland	ND	*sec, sell, tst*	+
RF287	Ireland	ND	*sec, sell, tst*	+
RF291	Ireland	ND	*sec, sell, tst*	±
RF289	Ireland	ND	*sec, sell, tst*	+
RF102	Ireland	7	*sec, sell, tst, seg, sei, selm, seln, selo*	+
RF108	Ireland	7	*sec, sell, tst, seg, sei, selm, seln, selo*	+
RF110	Ireland	7	*sec, sell, tst, seg, sei, selm, seln, selo*	+
RF113	Ireland	7	*sec, sell, tst, seg, sei, selm, seln, selo*	+
RF122	Ireland	7	*sec, sell, tst, seg, sei, selm, seln, selo*	+
RF124	Ireland	7	*sec, sell, tst, seg, sei, selm, seln, selo*	+

Strain	Country	RAPD Type	Enterotoxins	Mitogenicity
RF80	Ireland	7	*sec, sell, tst, seg, sei, selm, seln, selo*	+
RF114	Ireland	7	*seg, sei, selm, seln, selo*	+
RF120	Ireland	7	*seg, sei, selm, seln, selo*	+
RF121	Ireland	7	*seg, sei, selm, seln, selo*	+
MSA17.1	USA	3	*seg, sei, selm, seln, selo*	±
MSA72.3	USA	2	*seg, sei, selm, seln, selo*	+
MSA103.14	USA	2	*seg, sei, selm, seln, selo*	+
MSA1460	USA	2	*seg, sei, selm, seln, selo*	+
MSA10.6	USA	2	*seg, sei, selm, seln, selo*	+
MSA1363	USA	1	*seg, sei, selm, seln, selo*	+
PSA5	USA	9	*sei, selm, selo* (*seln*)	+
PSA6.1	USA	9	*sei, selm, selo* (*seln*)	+
MSA148	USA	9	*sei, selm, selo* (*seln*)	+
MSA73.2	USA	9	*sei, selm, selo* (*seln*)	+
MSA31	USA	9	*sei, selm, selo* (*seln*)	+
MSA26.1	USA	9	*sei, selm, selo* (*seln*)	+
MSA12.1	USA	9	*sei, selm, selo* (*seln*)	+
MSA930	USA	10	*sei, selm, seln, selo*	+
MSA1005	USA	10	*sei, selm, seln, selo*	+
MSA1047	USA	10	*sei, selm, seln, selo*	+

* RAPD types were defined by Fitzgerald *et al.* (1997). ND – Not done by Fitzgerald *et al.* (1997).

associated strains are rarely producers of the SEA, SEB, SEE and SEH toxins (Kenny *et al.*, 1993; Fitzgerald *et al.*, 2000; Foschino *et al.*, 2002; Cenci-Goga *et al.*, 2003). Likewise herein only 4.5 % of SAg-positive strains possessed these genes. These general findings contrast with the Swiss study of Scherrer *et al.* (2004) in which 10.9 % of isolates from bulk-tank milk samples from sheep and goats possessed either the *sea* or *seb* gene. Moreover, a Polish study (Kuźma *et al.*, 2003) demonstrated the presence of the *sea* and *seb* genes in 18.8 % and of the *seh* gene in 34.9 % of tested bovine strains.

Of the genes encoding the classical SEs, the *sec* gene has been reported with the highest frequency in strains from ungulates. In the case of bovine strains Larsen *et al.* (2002) reported a frequency of 26.6 % and Akineden *et al.* (2001) of 15.5 % versus 19.2 % in the present study. However, in a Danish study the *sec* gene was detected at a frequency of 0.25 % (Larsen *et al.*, 2000a). Scherrer *et al.* (2004) detected the *sec* gene in 42 % of sheep and goat isolates compared to 51.6 % herein. The frequency in poultry isolates is lower, 2.9 % in Hazariwala *et al.* (2002) and 0 % herein. The reported frequencies of the *sed* gene among isolates from sheep, goats and poultry of < 2 % agree with the present findings (Hazariwala *et al.*, 2002; Scherrer *et al.*, 2004). The frequencies of occurrence of the *sed* gene vary greatly among bovine isolates from different countries (Larsen *et al.*, 2002). Low frequencies (< 6 %) have been reported for Scandinavian countries, Germany and Ireland versus 17–35 % in Finland, Iceland, Switzerland and USA. These differences probably reflect the principal clonal types causing bovine mastitis.

Co-occurrence of the *sec* and *tst* genes in the present study was 100%. The co-occurrence of the *sec* and *tst* genes in bovine isolates from ten countries has been noted (Larsen *et al.*, 2002, 95.6 %) and also from Germany (Akinedén *et al.*, 2001, 100 %; Zschöck *et al.*, 2004, 100 %) or co-production of SEC and TSST-1 (Orden *et al.*, 1992, ~ 90%; Kenny *et al.*, 1993, 97.7 %; Fitzgerald *et al.*, 2000, 100% in Irish RAPD type 7 strains). This contrasts with findings of rare co-occurrence of the *sec* and *tst* genes in human *S. aureus* isolates of blood and nasal origin (Peacock *et al.*, 2002; Becker *et al.*, 2003; Nashev *et al.*, 2004). Fitzgerald *et al.* (2001a) discovered the SaPIbov pathogenicity island harbouring the *sec*-bovine and *tst* genes along with a novel enterotoxin-like gene *sell*. The present study extends the co-occurrence of these genes to isolates from goats and sheep and concurs with the report of Scherrer *et al.* (2004) who observed 95.9 % co-occurrence of the *sec* and *tst* genes in sheep and goat isolates. It is noteworthy that Orden *et al.* (1992) observed 95.3 %

co-production of SEC and TSST-1 in sheep and goat strains. Kúzma *et al.* (2003) noted that all isolates containing the *tst* gene were simultaneously positive for the *sec* gene.

3.4.2. *sec* gene sequencing.

While it is almost certain that the *sec*-bovine variant occurs in the majority of the bovine isolates containing the *sec* and *tst* genes, the primers used in the present study and by Scherrer *et al.* (2004) would detect all the known *sec* variants (Marr *et al.*, 1993; Fitzgerald *et al.*, 2001a). In the case of two sheep (DS88-*sell*$^+$ and DS104-*sell*$^-$) and two goat (DS3-*sell*$^+$ and DS77-*sell*$^+$) isolates investigated herein, the *sec*-ovine variant was confirmed (Marr *et al.*, 1993), irrespective of the presence of the *sell* gene. These limited data suggest that *sec*-ovine maybe present on a pathogenicity island with the *tst* gene in the presence or absence of the *sell* gene. In the remaining goat isolate examined, DS25, a *sec3* variant gene was found to have a single base-pair deletion mutation. In the case of the bovine isolates, strain RF124 (*sell*$^+$) was found to contain the *sec*-bovine variant, while strain RF283 (*sell*$^+$) was found to contain the *sec*-ovine variant. Apart from the *sec*-bovine gene, the *sec1* gene had been shown to be present on a pathogenicity island currently being sequenced termed SaPI4 along with genes encoding three other novel enterotoxins designated *sek*, *sel* and *sem* in Novick, (2003b). In the genome sequence of *S. aureus* strain Mu50, the genes *sell* and *sec3* are contiguous with the *sec3* gene separated from the *tst* gene by one open reading frame encoding a hypothetical protein (Kuroda *et al.*, 2001; Accession no. NC_002758). The close proximity of these genes suggests their co-occurrence on a chromosomal genetic element.

3.4.3. SE and SEl gene combinations.

The present study is the most comprehensive survey for SAg genes in *S. aureus* strains of animal origin to date. Examining animal-associated strains herein for the classical SE genes and the *tst* gene and for the novel SE and SElgenes increased the number of SAg gene-positive isolates from 60 strains (31.4 %) to 110 strains (57.6 %). The commonest SE-SEl gene combination was the *egc* cluster, namely, *seg*, *sei*, *selm*, *seln* and *selo* with or without the *seg* gene (52.7 % of SAg gene-positive isolates). Akineden *et al.* (2001) only screened bovine mastitis isolates for the *sea–selj* genes. Strains possessing the *seg* and *sei* genes, i.e., putatively the *egc* locus, accounted for 48 % of SAg gene-positive strains (48.9 % of bovine SAg gene-positive strains herein). Strains that were *sei* gene-positive but

lacked the *seg* gene, i.e., putatively possessed the *egc* locus minus the *seg* gene, accounted for 22.7 % of SAg gene-positive isolates (22.2 % of bovine isolates herein). The *seg* primers used in the current study would amplify the known variants of the *seg* gene (Blaiotta *et al.*, 2004) and should not account for the *seg* negative results. In two studies on human wound and nasal isolates, approximately 75 % of SAg gene-positive strains possessed both the *seg* and *sei* genes (Becker *et al.*, 2003; Nashev *et al.*, 2004). In a study by Becker *et al.* (2004), the *selm*, *seln* and *selo* genes were tested for in 219 *S. aureus* blood isolates and 210 nasal *S. aureus* isolates. They found that 294 of the 429 strains possessed at least one of the three genes tested for, with a number of different toxin genotypes being present: *selm*+, *seln*+, *and selo*+ (186 strains); *seln* only (62 strains); *seln* and *selo* only (32 strains); *selm* and *seln* only (7 strains); *selm* and *selo* only (5 strains); *selm* only (1 strain) and *selo* (1 strain). Omoe *et al.* (2005) demonstrated the *egc* gene cluster alone in 16.5% of human nasal isolates.

In a recent survey of 55 human isolates of *S. aureus* from food-poisoning outbreaks that were negative for classical SEs, only eight strains possessed the *seg*, *seh* or *sei* genes (Chen *et al.*, 2004). Of 139 isolates from food samples, 13 possessed the *seg*, *seh* or *sei* genes without classical SE genes, 15 strains possessed one or more of those genes in combination with the *sea* gene and one strain in combination with the *sed* gene (Chen *et al.*, 2004). Omoe *et al.* (2005) tested 69 isolates from food-poisoning outbreaks. Of these 69 isolates, 7 were found to contain the *egc* cluster of genes without any of the classical SEs. A further 11 of the 69 isolates were found to encode the *egc* locus of genes plus the *selp* gene (8 of the 11 strains) and the *egc* locus of genes plus the *selj* and the *selr* gene (3 of the 11 strains). A food-poisoning outbreak in Norway has been linked to *seh* gene-positive isolates of *S. aureus* present in mashed potato (Jørgensen *et al.*, 2004).

The *sed* and *selj* genes have been localised to a plasmid (Zhang *et al.*, 1998). The *sed* and *selj* genes in combination were present in nine animal isolates, in two instances with the *egc* locus and one with the SaPIbov pathogenicity island. Six of these nine strains were of bovine origin. Akineden *et al.* (2001) reported the *sed/selj* gene combination in 28 % of SAg gene-positive bovine strains (13.3 % herein). Scherrer *et al.* (2004) reported this combination in four sheep or goat isolates (2.1 % versus 1.6 % herein).

3.4.3. Mitogenicity testing.

Of the thirty-four bovine isolates harbouring one or more of SAg gene, 94.1 % were unequivocally mitogenic for human T-cells. The other two isolates possessing SAg genes may express SAgs at low levels leading to the borderline findings. Of the forty-five SAg gene-negative isolates four were mitogenic and twelve potentially mitogenic. The latter findings suggest that these strains may harbour genes for as yet unidentified SAgs or the *selp*, *selr* and *selu* genes, the latter two of which were identified during the present study (Kuroda *et al.*, 2001; Letertre *et al*, 2003c; Omoe *et al*, 2003). The *selr* gene localises to a plasmid that encodes the *sed* and *selj* genes. Accordingly *sed-selj*-positive isolates would be anticipated to also harbour the *selr* gene. The *selu* gene has only been reported in some strains that contain the *egc* cluster. Thus, it is likely that some of the animal strains analysed in the present study may contain the *selu*, *selr* and *selp* genes.

This work has extended a previously existing multiplex PCR to screen for novel SAg genes. Sergeev *et al.* (2004) have developed an assay which involves PCR amplification of SE genes using degenerate primers, followed by characterisation of the amplicons by microchip hybridisation with oligonucleotide probes specific for each SE gene. This assay has the advantage that it can detect previously unidentified SE genes. Letertre *et al.* (2003b) have developed a real-time PCR that can detect and toxin-type strains for the *sea* to *selj* genes. Letertre *et al.* (2003a) have also developed a 5′-nuclease assay that comprises three triplex PCRs for the amplification of the *sea* to *selj* genes. Martin *et al.* (2004) have used molecular typing methods along with multiplex PCR for SE genes to investigate the epidemiology of foodborne outbreaks of *S. aureus*. All of these newly developed or extended methods provide a range of strategies to allow different laboratories to rapidly screen *S. aureus* strains of animal and human origin for SE and SEl genes. The present multiplex assays could be readily adapted to screen for additional genes including the newly described *selp*, *selr* and *selu* genes as well as others that may be described in the future.

The demonstration herein that the *egc* cluster of SE and SEl genes and the SaPIbov SE and SEl genes are the commonest in clinical *S. aureus* isolates of animal origin raises questions about the potential roles of these SAgs in the variety of infections caused by *S. aureus* in their host animal species. The statistically significant higher frequency of the *egc* locus with or without the *seg* gene and the lower frequency of the *sec* and *tst* genes in bovine

isolates compared to those from sheep or goats suggest that *egc*-associated SAgs may be more important as putative virulence factors in bovine mastitis. One or more of these *egc*-associated SAgs may confer a survival advantage in the udder through modulation of the immune response. The ability of *S. aureus* to persist in the mammary gland in the chronic disease state is a conundrum that needs to be resolved for the development of an effective vaccine.

Chapter 4

An Investigation into the Presence and Composition of the Staphylococcal Exotoxin-Like Gene Locus [*set* (*ssl*)] in strains of *S. aureus* of Animal Origin.

4.1. Introduction.

Staphylococcus aureus causes a wide variety of clinical syndromes, ranging from uncomplicated infections of the skin, such as boils and carbuncles, to life-threatening infections, such as endocarditis and toxic shock syndrome (McCormick *et al.*, 2001; Proft & Fraser, 2003). Among the many known virulence factors of *S. aureus* are three groups of staphylococcal exoproteins: the enzymes such as hyaluronidase, proteases and nucleases, the non-enzymatic activators of plasma clotting (coagulase) and fibrinolysis (staphylokinase), and the exotoxins such as exfoliative toxins, leukocidins, enterotoxins and toxic shock syndrome toxin 1. The enterotoxins and TSST-1 belong to the SAg family.

In recent years due to major advances in staphylococcal genome sequencing, 18 staphylococcal enterotoxins (SEs) and enterotoxin-like proteins (SEls) as well as a number of variants of some of these have been described, as discussed in Chapter 3. It is likely that additional SEs and SEls will be discovered. In addition to these superantigens, *S. aureus* also produces a family of exoproteins that have been shown to have sequence similarity and three-dimensional structural similarities to the SE superantigens, namely, the staphylococcal exotoxin-like proteins (SETs). These proteins were first identified by Williams *et al.* (2001) who described the presence of a cluster of *set* genes in *S. aureus* strain NCTC6571 which encoded five SETs (SET1 to SET5). The *set1* gene was cloned and expressed. Recombinant SET1 protein was shown to be able to stimulate the secretion of proinflammatory cytokines, namely, interleukin 1β, interleukin-6 and tumour necrosis factor alpha by human peripheral blood mononuclear cells (PBMCs) (Williams *et al.*, 2000). Allelic variants of the SET1 protein were shown to have differing cytokine-inducing potencies (Williams *et al.*, 2000) (Fig. 4.1).

Upon completion of two *S. aureus* genome sequences by Kuroda *et al.* (2003), the *set* cluster was found to contain 10 *set* genes in one strain (N315) and nine in the other (Mu50). The *set* genes were found to show sequence similarity of 36–67 %. The *set* cluster was located on a novel pathogenicity island, SaPIn2, which contained nine lipoprotein (*lpl)* genes downstream of the *set* genes, and was flanked upstream by a putative transposase and downstream by an incomplete restriction and modification system (*hsdS* and *hsdM*), which are part of a three-component restriction/modification system and believed to maintain the locus in the genome (Kuroda *et al.*, 2003).

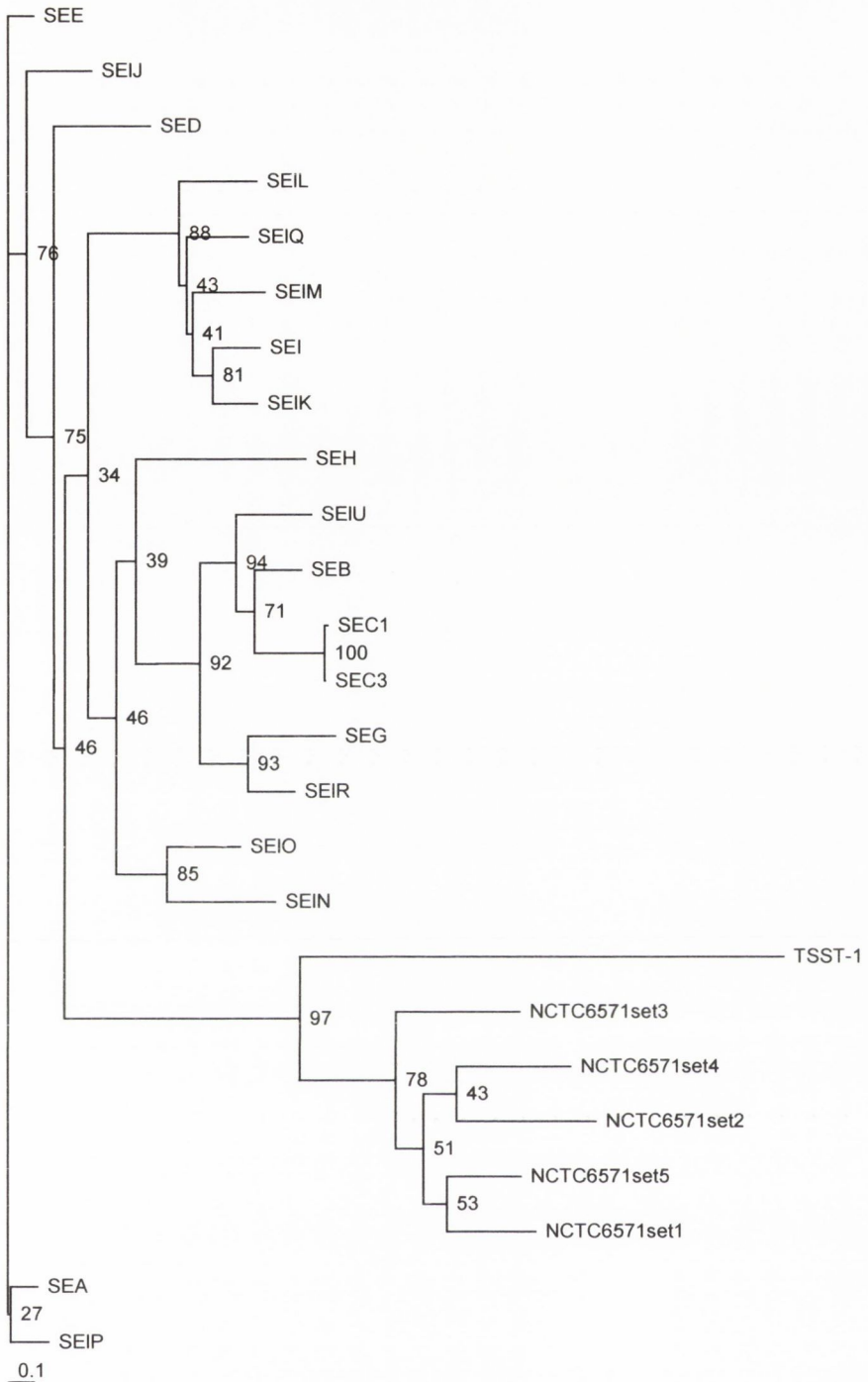


Fig. 4.1. ClustalX-generated phylogenetic tree of the staphylococcal enterotoxins with superantigen TSST-1 and the SET proteins from strain NCTC6571 (Williams *et al.*, 1999).

Although the SET proteins have sequence similarity to and the consensus signatures of the staphylococcal enterotoxins, the similarity ends here as they do not share a function and are not considered to be superantigens. The SET proteins have three-dimensional structural similarities to the enterotoxins and are most like superantigen TSST-1 in structure. The phylogenetic tree hints at this as the SETs cluster with superantigen TSST-1.

Subsequently Arcus *et al.* (2002) resolved the crystal structure of a member of the SET family. The SET3 protein was shown to have folding characteristics and the classic two-domain structure of the SAg family; most of the N-terminal region was folded into a 5-stranded β-barrel called an OB-fold and the C-terminal half formed a β-grasp domain. The structure, however, had significant differences. Specifically, the protein when forming a dimer could potentially create a large positively charged saddle-shaped surface which might bind to negatively charged molecules such as DNA. The protein also had widespread positive charges all over its surface. Arcus *et al.* (2002) also showed that recombinant SET1, SET3 and SET15 proteins could not induce T-cell proliferation which is a hallmark of superantigenicity. In the same study it was also shown that the SET3 protein was secreted by *S. aureus* and recognised by antibodies in human sera.

A DNA microarray study of *S. aureus* revealed the presence of 18 chromosomal regions of difference among 36 strains from humans, cows and goats (Fitzgerald *et al.*, 2001b). One region designated as RD13 varied in size from 12–17 kb and was predicted to contain considerable sequence variation. Region RD13 corresponded to the *set* cluster region of pathogenicity islands SaPIn2 and SaPIm2. Fitzgerald *et al.* (2001b) found that region RD13 was present in 63 representative strains of *S. aureus* but displayed high levels of sequence variation. Sequence analysis of the central variable domain of region RD13 led to the theory that horizontal gene transfer, recombination events, and loss of *set* genes had led to the diversity observed in region RD13. The *set* genes were found to be under selective constraint thus maintaining SET protein function. The expression of *set* genes was demonstrated with strain COL. Moreover Western immunoblot analysis using human sera confirmed the expression of several SETs during human infections.

Al-Shangiti *et al.* (2005) determined the structure of exoprotein SET1 in order to gain better insight into the relationships among the different members of the SET family, and also into the tissue tropism of exoprotein SET1 and its close homologue exoprotein SET5, the structure of which had been resolved by Arcus *et al.* (2002), for different cells of the immune system. The results demonstrated that both the SET1 and SET5 exoproteins interacted with human antigen-presenting cells (monocytes and dendritic cells), although they interacted with different sites on the cell surface, leading to uptake of the SET proteins by the cells. The ability of these secreted proteins to target antigen-presenting cells was theorized to enhance a misplaced antibody response against the proteins that

might facilitate bacterial colonisation rather than contribute to host protection. Thus, like SAgs, the SET exoproteins may distract the host's immune system, but do so via entirely different molecular mechanisms to SAgs.

Langley *et al.* (2005) demonstrated that immobilised SET1 protein routinely bound four polypeptides from human sera. The two predominant polypeptides possessed molecular masses of ~60 and ~27 kDa under reducing conditions and a joint molecular mass of ~170 kDa under non-reducing SDS-PAGE, consistent with their being immunoglobulin in nature. These polypeptides were identified as human IgA H-chain and IgA L-chain, respectively, by peptide mass spectroscopy fingerprinting. In addition to IgA H- and L-chains, two additional polypeptides with molecular masses of 110 kDa and 75 kDa were also bound by the SET1 protein. These were identified as human complement component C5α chain (115 kDa) and C5β chain (75 kDa) by mass spectrometry of tryptic peptides. The SET1 protein was shown to be able to bind to Ig at the site where Ig binds to the immunoglobulin A Fc alpha receptor (FcαRI) and to complement from different species. Allelic variants of the SET1 protein were not altered in Ig- or complement-binding activities. The SET1 protein was shown to effectively inhibit complement-mediated haemolysis and to enhance survival of *E. coli* at concentrations similar to the predicted serum concentration of complement component C5. These studies described for the first time the Ig- and complement-binding activity of one of the staphylococcal exotoxin-like proteins and suggested that superantigens are but a subgroup of a much larger family of molecules with varied activities designed to bind serum components involved in host immunity.

The International Nomenclature Committee for Staphylococcal Superantigens has recently recommended that the SET proteins should be renamed staphylococcal superantigen-like proteins (SSLs) and that the genes should be designated *ssl1* to *ssl11* in clockwise order from the replication origin of the chromosome based on homology to the full complement of genes found in strain MW2 (Lina *et al.*, 2004). This nomenclature is essentially that described by Fitzgerald *et al.* (2003), except that the numbering of the genes is in the opposite direction. To differentiate between allelic variants, the *ssl* gene designation is preceded by the strain designation. Both the SET and the SSL nomenclatures are used where possible henceforth throughout this chapter as the scientific literature does not as yet embody the recommendation of the INCSS.

At the start of this study little data were available on the frequency of the *ssl* genes in strains of *S. aureus* from human and animal sources. However, since commencing the study, the *set* (*ssl*) gene locus has been demonstrated to be present in all human and some animal-associated *S. aureus* examined. As of yet this has not been done specifically in strains from cows, goats, sheep, rabbits and chickens. The aims of this study were to develop a PCR-based screening method for *set* (*ssl*) genes and to investigate the frequency of these genes in strains of *S. aureus* of animal origin.

4.2. Methods.

4.2.1. Analysis of the *S. aureus* genome sequences using Artemis and BLAST.

The seven available *S. aureus* genome sequences for strains NCTC8325, N315, Mu50, COL, EMRSA16, MW2 and MSSA (the accession numbers for these genomes are in Table 1.2) along with the *set* (*ssl*) reference gene sequences from strain NCTC6571 (Accession numbers AF094826 and AF188835), strain FRI326 (Accession number AF188836), and strain NCTC8325 (Accession number AF188837) were downloaded and analysed by Artemis, a freeware sequence analysis tool available at the Sanger Centre website (www.sanger.ac.uk) and Blastn (http://www.ncbi.nlm.nih.gov/BLAST/) to determine the composition of the *set* (*ssl*) locus in each strain. An example of the output from Artemis using the genome sequences of strains N315 and COL is shown in Fig. 4.2. The sequence of the *set* (*ssl*) locus of strain RF122, a bovine strain (Fitzgerald *et al.*, 2000), was determined by PCR herein and by L. Herron (Department of Microbiology, College of Veterinary Medicine, University of Minnesota, Minneapolis, USA) (personal communication) was included for comparison.

Artemis allows the user to input large sequences of DNA and to graphically display putative open reading frames (orfs). These orfs can be retrieved and submitted using Blastn to the microbial genome database for comparison with known exotoxin gene sequences. Once a putative identity had been assigned to each orf of each genome sequence, the orfs from each genome were aligned to each other using ClustalW (www.ebi.ac.uk/clustalW) and ClustalX (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/).

4.2.2. Homology modelling of SET (SSL) proteins.

Fig. 4.2. Output from Artemis for genome sequences of *S. aureus* strains N315 and COL.

This figure shows Artemis output for genome sequence N315 on top and genome sequence COL on bottom. Strain N315 encodes 10 *set* (*ssl*) alleles, *set6–10* (*ssl1–ssl5*) and *set11–15* (*ssl7–ssl11*) and COL encodes 7 *set* (*ssl*) alleles, *set6–9* (*ssl1–4*) and *set13–15* (*ssl9–11*).

Strain N315

Strain COL

Pdb files of the TSST-1 (2QIL), SEC3 (1CK1), SET3/SSL7 (1M4V) and SET1/SSL5 (1V10) crystal structures were downloaded from the protein database at http://www.rcsb.org/pdb/. The designations in brackets refer to the pdb file entries in the database. PyMOL was used to produce cartoon structures to aid in the visualisation and comparison of the structures. Sequences of the SET11 (SSL5) and SET10 (SSL7) proteins of the strain N315, allelic variants of the SET1 (SSL5) and SET3 (SSL7) proteins, respectively, were submitted to PHYRE (http://www.sbg.bio.ic.ac.uk/~phyre/) which automatically generates a model of the crystal structure of submitted proteins based on 'template' proteins with >30% identity (template proteins are proteins, of which the crystal structures have been resolved and deposited in the pdb database). Once a putative model was obtained, the pdb file was input to PyMOL and compared to the other structures.

4.2.3. Design of primers and PCR analysis.

Using ClustalW-generated alignments it was possible to locate unique regions of each *set*/*ssl* gene that was present in all allelic variants of each *set* (*ssl*) gene. These unique regions were used to design primers for the *set* (*ssl*) alleles that were used in PCR and to construct probes for use in Southern hybridisation experiments (Table 4.1). Each set of primers used the same PCR reaction mix comprising 10 pmol forward and reverse primers, 200 μM each of dNTP, dTTP, dCTP and dATP, 1 × magnesium-free buffer (Promega), 1.5 U *Taq* DNA polymerase (Promega), 3 mM $MgCl_2$ and 50–100 ng DNA. Each PCR reaction also included appropriate *set* (*ssl*) gene-positive and -negative controls. The PCR reactions used the following cycling parameters: 95 °C for 10 min, 15 cycles of [95 °C for 1 min, 58 °C for 45 s, and 72 °C for 1 min] and 16 cycles of [95 °C for 1 min, 54 °C for 45 s, and 72 °C for 1 min]. The reaction was terminated with a 10-min incubation at 72 °C. PCR products were resolved by electrophoresis in 1.5% (w/v) agarose gels (0.5 × Tris-boric acid-EDTA buffer) (Sambrook & Russell, 2001) at 90 V (constant voltage), stained with ethidium bromide, and visualized using UV light. Product sizes were determined by comparison with a 100-bp ladder (Promega).

PCR analysis was performed on 52 animal–associated strains plus 6 human control strains that represented major clonal types associated with animal *S. aureus* infection. These strains included 5 control strains (EMRSA-16, NCTC8325-4, MSSA 476, COL and NCTC6571) and one clinical human isolate along with 42 strains from cows, 2 from chickens, 4 from goats, 3 from rabbits and 1 from a sheep.

Table 4.1. Newly designed primers used in *set* (*ssl*) gene PCR.

ssl gene	*set* gene	Primer Sequence (5′–3′)	Product size (bp)	T_m (°C)
10	*set4/14* for	AACAACAGGAACTTTAACAACAGA	397	57.6
	set4/14 rev	ACCTTTCACAAAAGCATCTTCACC		
9	*set5/13* for	GGTATAACAAAGAAAAATGTGAGATC	193	55
	set5/13 rev	CCTTTATACAATCTATATTTTTCGA		
8	*set12* for	GAA CCG TTT ATA TGA TAC AAA C	296	52.8
	set12 rev	GTT CTT AGA GAC TCA AAT AAT G		
7	*set1/11* for	CAA GAG AGA GTA CAA CAT TTA	242	52.4
	set1/11 rev	CCG TTT GGA TCA ATT AAT TCT T		
5	*set3/10* for	TGA AAA TGT GAC AAA AGA TAT CTT	103	53.9
	set3/10 rev	TGA AAA TGT GAC AAA AGA TAT CTT		
4	*set9* for	CGC GAC AAC ACC ATC TTC CAC T	550	58.4
	set9 rev	CTT TAG TAA TTC TTA CTC CTG C		
3	*set8* for	CAC AAG ACA AGA ACG CAC GCC T	820	62.1
	set8 rev	CTG CCA TAC GAT GCT CTT GCA G		
4	*set2* for	ATG CAG AAG TAG CAT CAG CAC T	783	57.15
	set2 rev	GAT ACT AGT GCC ATC TAT GAC		

Not all *set* (*ssl*) genes were tested for (*ssl1*, *ssl2*, *ssl6* and *ssl15* were omitted). It was not possible to design a primer to amplify both *set9* and *set2* (*ssl4*) so individual primers were used. Primers would amplify all allelic variants of *set/ssl* genes, e.g., the *set4/14* (*ssl*10) primers would amplify both the *set4/ssl10* allele of strain EMRSA16 and the *set14/ssl10* allele of NCTC8325.

PCR results using novel *set* (*ssl*) primers designed in this study were confirmed using Southern blots with genomic DNA from a number of representative Irish bovine isolates and human-associated control strains. Genomic DNA was digested with restriction endonuclease *Hin*dIII and resolved by electrophoresis in 0.8% (w/v) agarose. Southern hybridisation analysis was performed using standard methods (Sambrook & Russell, 2001) and the DIG-labelling system (Roche) as described in Chapter 2. Probes were constructed using the PCR DIG-labelling mix (Roche) in PCR reactions that incorporated DIG-labelled dUTP into the synthesised DNA. Probes for the *set1* (*ssl7*) and *set3* (*ssl5*) genes were amplified using DNA from strain NCTC6571 and a probe for the *set12* (*ssl8*) using DNA from strain RF122.

4.2.4. Characterisation of *S. aureus* strains from recent bovine mastitis infection.

Quarter milk and serum samples from four cows with mastitis were obtained from Teagasc Dairy Production Centre in Moorepark, Fermoy, Co. Cork. Also four samples of serum from cows with low somatic cell counts and considered to be free of mastitis infection were also obtained. The serum samples were used in Western immunoblot experiments in Chapter 8. The milk samples from the four cows were analysed for the presence of *S. aureus*. Of each quarter milk sample, 100 µl was dispensed onto the centre of a mannitol salt agar (MSA) plate and spread. Colonies were counted and putative *S. aureus* colonies were Gram-stained, replated onto MSA agar plates, regrown on sheep blood agar, tested by Staphaurex™ (Murex) and stored on TSA. DNA was extracted from *S. aureus* isolates and subjected to RAPD typing. The RAPD profiles obtained for the new isolates were compared to the RAPD profiles previously obtained for Irish bovine isolates (Fitzgeralad *et al.*, 1997). These isolates were also tested for the presence of the *set* (*ssl*) genes as described herein and for the SAg and SAg-like genes as described in Chapter 3.

4.3. Results.

4.3.1. *S. aureus* genomes.

From the analysis using Artemis and Blastn, it was observed that the *set* (*ssl*) genes of each sequenced *S. aureus* strain varied in number and composition and that there were a number of allelic variants of certain *set* (*ssl*) genes. The *set* (*ssl*) locus was found to have an

upstream putative transposase gene and an incomplete restriction and modification cassette in every sequenced strain (the *hsdS* gene is absent in the sequenced strains except for strain RF122; the significance of this remains unresolved). The *set* (*ssl*) locus was found to be located upstream of a cluster of lipoprotein genes that Kuroda *et al.* (2001) hypothesised might be involved in virulence. There were also a number of similar genes approximately 1.5 kb from the α-toxin locus. These genes have low sequence similarity to the *set* (*ssl*) genes of about 9–24% (Fig. 4.3 and 4.4). A schematic of the *set* (*ssl*) loci from the 8 human-associated *S. aureus* strains and the unfinished genome of bovine strain RF122 is shown in Fig. 4.5. The *set* (*ssl*) genes from strains N315 and NCTC6571 have sequence similarities ranging from 29–66 % between individual pairs of genes whereas the allelic variants within these strains have higher degrees of similarity (76–89%). These data are represented by Table 4.2. The *set* (*ssl*) genes from strain NCTC6571 were identical to those of strain EMRSA-16.

4.3.2. Homology modelling of SET (SSL) proteins.

The crystal structures of superantigen TSST-1, which clusters with the SET (SSL) proteins in phylogenetic trees (Fig. 4.1), and of enterotoxin SEC3 were compared to those of SET3 (SSL5) and SET1 (SSL7) and to the PHYRE-generated models of the allelic variants SET10 (SSL5) and SET11 (SSL7) from strain N315 using PyMOL (Fig. 4.6). The SET (SSL) proteins adopted the three-dimensional structure of the superantigen family forming a two-domain structure. It had been found from studies involving the crystallisation of the SET (SSL) proteins that the majority of conserved residues were involved in the structure of the proteins (Al-Shangiti *et al.*, 2002). The allelic variants were found to have the same two-domain structures.

4.3.3. PCR for *set* (*ssl*) genes.

To analyse the frequency of *set* (*ssl*) genes in strains of *S. aureus* from animals, PCR primers were developed. Initially PCR was done on strains NCTC8325-4, MSSA, MRSA, COL and NCTC6571 of known genome sequences and *set* (*ssl*) gene contents. The *set* (*ssl*) genes tested for were all amplifiable by PCR with the exception of *set9* (*ssl4*) gene which generated non-specific products of varying sizes. The PCR results for these strains were consistent with their genome sequences.

Fig. 4.3. Overview screen of the genome sequence of strain N315.

There is a user friendly interface available for certain *S. aureus* genome sequences. This is a display of the interface for strain N315. Open reading frame SA1007 is the α-toxin precursor of strain N315. Orf SA1006 is a putative transposase gene. The gene products of orfs SA1009, SA1010 and SA1011 have low sequence similarity to the SET (SSL) proteins of approximately 9–24 %. An alignment of these putative SET (SSL) proteins with the SET (SSL) proteins of strain NCTC6571 is shown in Fig. 4.4.

Summary sequence view - Microsoft Internet Explorer

File Edit View Favorites Tools Help

Back Address http://www.ncbi.nlm.nih.gov/genomes/altvik.cgi?gi=179&db=g&from=1115561&to=1165560 Go

Google Search Web Search Site PageRank 334 blocked Options

Views

Graphical View

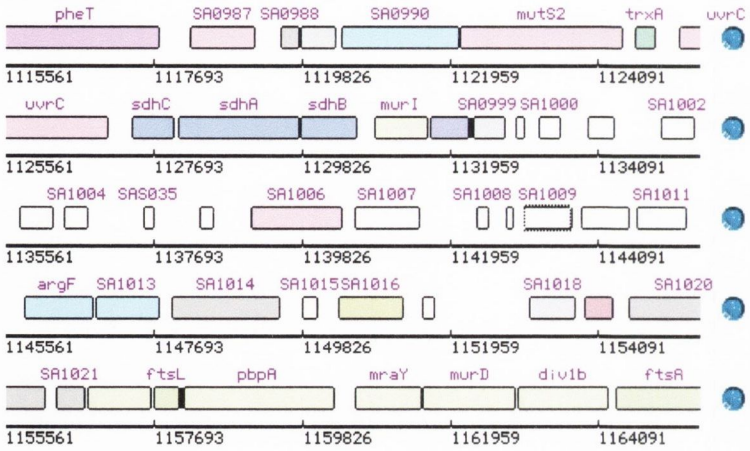
Coding regions

RNA Genes

Start from : Go Search for gene Find

46 protein coding genes Find Open Reading Frames

Click on the rectangle to get BLAST neighbors for the gene of interest
or click on the overview below to see a distant region



Translation, ribosomal structure and biogenesis
Transcription
DNA replication, recombination and repair
Cell division and chromosome partitioning
Posttranslational modification, protein turnover
Cell envelope biogenesis, outer membrane
Cell motility and secretion
Inorganic ion transport and metabolism
Signal transduction mechanisms

Internet

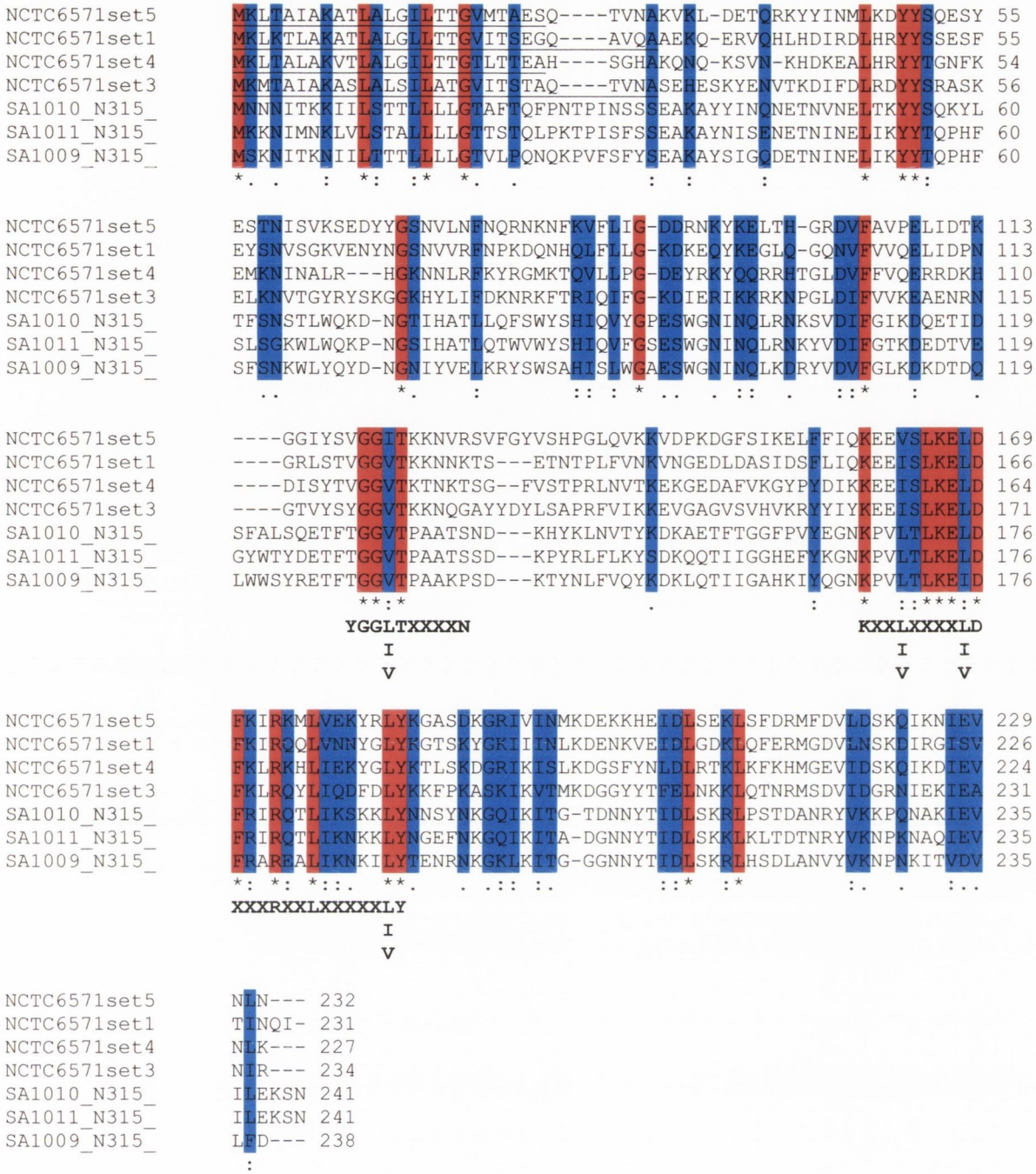


Fig. 4.4. Alignment of the SET (SSL) proteins encoded approximately 1.5 kb from the α-toxin locus in strain N315 with the SET (SSL) proteins of strain NCTC6571.

The signal peptides as predicted by SignalP of the SET (SSL) proteins are underlined. Identical residues are highlighted in red and highly similar residues are highlighted in blue. The SET (SSL) proteins in strain N315 have only 9–24 % identity to those of strain NCTC6571. The staphylococcal and streptococcal exotoxin consensus signatures 1 and 2 are shown underneath the alignment in bold.

Fig. 4.5. Schematic of the *set* (*ssl*) locus in available *S. aureus* genome sequences.

The old nomenclature is used in this figure to demonstrate the existence of allelic variants of *set* (*ssl*) genes. The *ssl* nomenclature is indicated in red at the top of the figure. The staphylococcal genomes show variation in the number and complement of *set* (*ssl*) alleles. There is a central variable region which contains allelic variants of *set* (*ssl*) genes, for example, *ssl5* (formerly *set3* and *set10*) and *ssl7* (formerly *set1* and *set11*) have variants. Deletions of *set* (*ssl*) genes occur also, for example, *ssl6* formerly indicated by an * is only present in strains NCTC8325, MSSA and MW2.

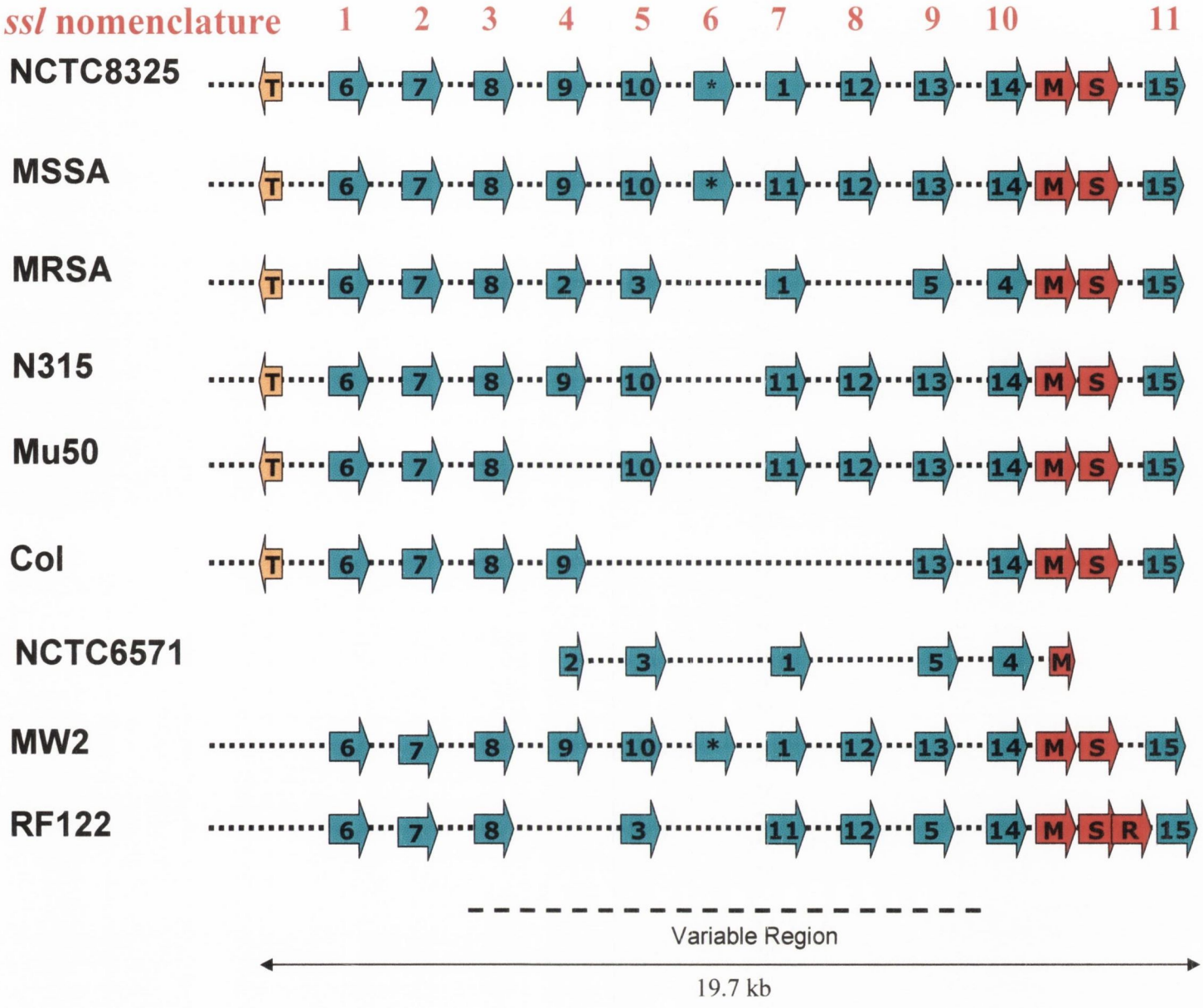

ssl nomenclature
1
2
3
4
5
6
7
8
9
10
11
NCTC8325
MSSA
MRSA
N315
Mu50
Col
NCTC6571
MW2
RF122
T
6
7
8
9
10
*
1
12
13
14
M
S
15
11
2
3
5
4
R
Variable Region
19.7 kb

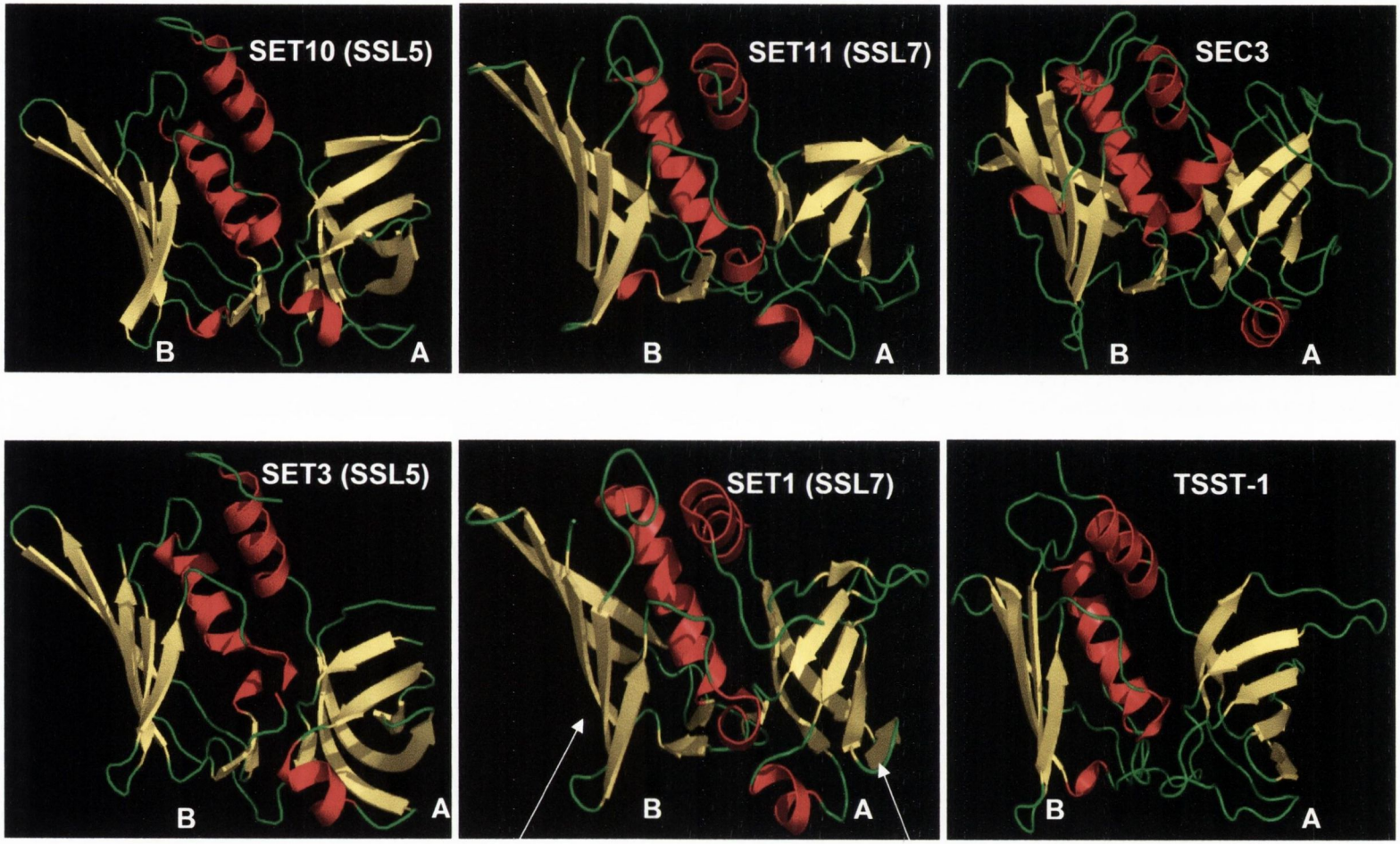
Table 4.2. ClustalW-generated alignment of the amino acid sequences of the strain NCTC6571 SET proteins (SET1–5) and the strain N315 SET proteins (SET6–15). The sequences of these proteins are available in Genbank (Accession numbers, AF094826 and NC_002745).

	% Sequence similarity														
Toxin	SET1	SET2	SET3	SET4	SET5	SET6	SET7	SET8	SET9	SET10	SET11	SET12	SET13	SET14	SET15
SET1	100	32	38	44	52	34	35	37	37	38	85	50	52	40	40
SET2		100	38	35	40	42	45	87	76	37	34	39	40	32	41
SET3			100	40	38	43	40	42	41	87	37	37	37	37	48
SET4				100	48	34	30	33	35	39	44	46	50	82	39
SET5					100	36	32	38	39	37	55	66	89	44	39
SET6						100	34	41	41	42	37	37	35	35	46
SET7							100	46	49	38	35	35	32	29	38
SET8								100	65	38	39	38	38	32	46
SET9									100	39	38	34	41	33	40
SET10										100	36	37	36	37	47
SET11											100	51	57	42	40
SET12												100	65	43	40
SET13													100	46	40
SET14														100	39
SET15															100

The SET proteins from strains NCTC6571 and N315 have sequence identities of 32–87% to each other. Blue indicates the presence of allelic variation in an SET protein, for example, SET3 and SET10 are 87% identical at the amino-acid level and occupy the same position in the *set/ssl* locus.

Fig. 4.6. Pymol-generated figures of the crystal structures of SET (SSL) proteins, enterotoxin SEC and superantigen TSST-1.

The SET (SSL) proteins have folding characteristics and the classic two-domain structure (domain A and B) of the SAg family. Most of the N-terminal region (domain B) is folded into a 5-stranded β-barrel called an OB-fold and the C-terminal half (domain A) forms a β-grasp domain. The two domains are bridged by an N-terminal helix.


SET10 (SSL5)
B
A
SET11 (SSL7)
B
A
SEC3
B
A
SET3 (SSL5)
B
A
SET1 (SSL7)
B
A
TSST-1
B
A
β-grasp domain
5-stranded β-barrel
– OB-fold

PCR analysis of the animal–associated *S. aureus* strains showed that all of them contained at least one *set* (*ssl*) gene and that there was variation in the number of *set* (*ssl*) genes encoded by the animal-associated strains (including those from recent mastitis infection). Clonally related strains had the same complement of *set* (*ssl*) genes (Fig. 4.7, Table 4.3). The *set4/14* (*ssl10*), *set3/10* (*ssl5*), *set12* (*ssl8*) and *set1/11* (*ssl7*) genes were present in 100% of the animal-associated strains. The *set5/13* (*ssl9*) gene was present in all strains but one bovine isolate, namely, MSA1455. The *set8* (*ssl3*) gene was present in 50 of the 52 animal-associated strains tested (except for one bovine and one caprine isolate). Although PCR for the *set9* gene generated non-specific products, the *set2* allelic variant was tested for and was found to be present in only 1 of the 52 animal-associated strains tested (DS25 from a goat). It is highly likely that some of the 52 animal isolates are positive for the *set9* allelic variant.

4.3.4. Southern blots for *set (ssl)* genes

Southern hybridisation experiments showed a similar restriction fragment length polymorphism (RFLP) in the clonally related strains analysed, whereas unrelated strains had a dissimilar RFLP (Fig. 4.8). The PCR assay has as yet to be extended to screen for the uncharacterized *set* (*ssl*) gene marked * (Fig. 4.5, strains NCTC8325, MSSA and MW2) as it may be this gene that contributes to differences in the RFLP patterns seen in Southern blotting experiments. The probes appeared to be specific for their target *set* (*ssl*) genes. Because the RFLP patterns are maintained in strains of the same clonal type, this may have useful applications in epidemiology and typing.

4.3.5. Strains from cows with recent mastitis infection.

A small number of *S. aureus* isolates obtained from the quarter-milk samples from cows with mastitis were analysed by RAPD typing, and for SAg and SAg-like genes and for *set* (*ssl*) genes. One *S. aureus* isolate from cow 2521 (3^{rd} quarter sample), four *S. aureus* isolates from cow 2242 (2 from the 3^{rd} quarter and 2 from the 4^{th} quarter), one *S. aureus* isolate from cow 2480 (4^{th} quarter) and two from cow 2487(3^{rd} quarter) were tested (Table 4.3).

The isolates obtained from cows with recent infection were found to be of RAPD types not previously seen in Irish cows, i.e., not of RAPD types 4, 5 or 7 as described by Fitzgerald

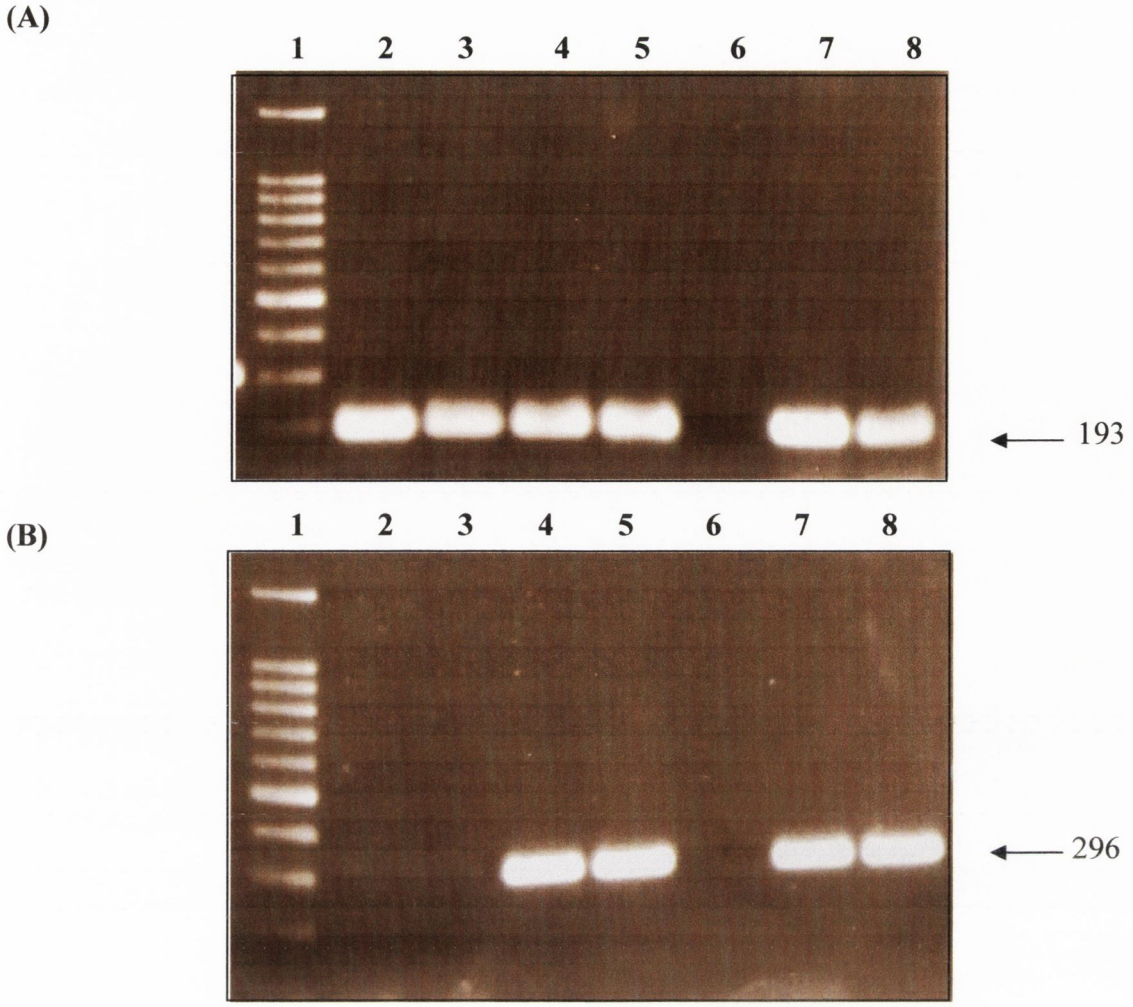


Fig 4.7. PCR of genome-sequenced *S. aureus* strains and bovine strains for *set* (*ssl*) genes.

Panel (A) shows results for *set5/13* (*ssl9*) PCR. Lane 1 contains 100-bp ladder (Promega). Lanes 2–5 contain PCR products from control strain DNA: 2, strain MSSA; 3, strain NCTC8325-4; 4, strain EMRSA-16; and 5, strain COL. Lane 6 contains a negative control. Lanes 7 and 8 contain PCR products from bovine strains: 7, strain MSA1468 and 8, strain MSA13.1.

Panel (B) shows results for *set12* (*ssl8*) PCR. Lane 1 contains 100-bp ladder (Promega). Lanes 2–5 contain PCR products from control strain DNA: 2, strain COL(negative); 3, strain EMRSA-16 (negative); 4, strain MSSA; and 5, strain NCTC8325-4. Lane 6 contains a negative control. Lanes 7 and 8 contain PCR products from bovine strains: 7, strain MSA1006; and 8, strain MSA1058.

Table 4.3. Combinations of *set* (*ssl*) genes in 57 strains of *S. aureus* of animal origin and control strains.

Strain ‡	Origin	Host	MLST Type	ET:RAPD type ¥	*set* genes §	*ssl* genes §	Mitogenicity †	SAg and SAg-like genes
NCTC8325	USA	Human	8	ND:ND	14/4,13/5,12,11/1,10/3,9,8	10,9,8,7,5,4,3		
MSSA	UK	Human	1	ND:ND	14/4,13/5,12,11/1,10/3,9,8	10,9,8,7,5,4,3		
EMRSA16	UK	Human	36	ND:ND	14/4,13/5, ,11/1,10/3,2,8	10,9, ,7,5,4,3		
COL	USA	Human	250	ND:ND	14/4,13/5, , , ,9,8	10,9, , , ,4,3		
NCTC6571	USA	Human	ND	ND:ND	14/4,13/5, ,11/1,10/3,2,8	10,9, ,7,5,4,3		
MSA2335	USA	Human	39	ND:ND	14/4,13/5,12,11/1,10/3,2,	10,9,8,7,5,4,		
RF111	IRL	Cow	97	3:4	14/4,13/5,12,11/1,10/3, ,8	10,9,8,7,5, ,3	-	Negative
RF115	IRL	Cow	97	3:4	14/4,13/5,12,11/1,10/3, ,8	10,9,8,7,5, ,3	-	Negative
RF116	IRL	Cow	97	3:4	14/4,13/5,12,11/1,10/3, ,8	10,9,8,7,5, ,3	-	Negative
RF117	IRL	Cow	97	41:4	14/4,13/5,12,11/1,10/3, ,8	10,9,8,7,5, ,3	-	Negative
RF101	IRL	Cow	71	3:5	14/4,13/5,12,11/1,10/3, ,8	10,9,8,7,5, ,3	-	Negative
RF103	IRL	Cow	71	3:5	14/4,13/5,12,11/1,10/3, ,8	10,9,8,7,5, ,3	+	Negative
RF107	IRL	Cow	71	3:5	14/4,13/5,12,11/1,10/3, ,8	10,9,8,7,5, ,3	-	Negative
RF123	IRL	Cow	71	3:5	14/4,13/5,12,11/1,10/3, ,8	10,9,8,7,5, ,3	-	Negative
RF102	IRL	Cow	151	3:7	14/4,13/5,12,11/1,10/3, ,8	10,9,8,7,5, ,3	+	SaPIbov, *egc*
RF108	IRL	Cow	151	3:7	14/4,13/5,12,11/1,10/3, ,8	10,9,8,7,5, ,3	+	SaPIbov, *egc*

Strain	Origin	Host	MLST Type	ET:RAPD type	*set* genes	*ssl* genes	Mitogenicity	Enterotoxins
RF110	IRL	Cow	151	3:7	14/4,13/5,12,11/1,10/3, ,8	10,9,8,7,5, ,3	+	SaPIbov, *egc*
RF120	IRL	Cow	151	3:7	14/4,13/5,12,11/1,10/3, ,8	10,9,8,7,5, ,3	+	*egc*
RF121	IRL	Cow	151	3:7	14/4,13/5,12,11/1,10/3, ,8	10,9,8,7,5, ,3	+	*egc*
RF122	IRL	Cow	151	3:7	14/4,13/5,12,11/1,10/3, ,8	10,9,8,7,5, ,3	+	SaPIbov, *egc*
2 (2521-3rd) *	IRL	Cow	ND	ND:ND	14/4,13/5,12,11/1,10/3, ,8	10,9,8,7,5, ,3		
6 (2242-3rd) *	IRL	Cow	ND	ND:ND	14/4,13/5,12,11/1,10/3, ,8	10,9,8,7,5, ,3		
7 (2242-3rd) *	IRL	Cow	ND	ND:ND	14/4,13/5,12,11/1,10/3, ,8	10,9,8,7,5, ,3		
10 (2242-4th) *	IRL	Cow	ND	ND:ND	14/4,13/5,12,11/1,10/3, ,8	10,9,8,7,5, ,3		
12 (2242-4th) *	IRL	Cow	ND	ND:ND	14/4,13/5,12,11/1,10/3, ,8	10,9,8,7,5, ,3		
14 (2480-4th)#	IRL	Cow	ND	ND:ND	14/4,13/5,12,11/1,10/3, ,8	10,9,8,7,5, ,3		
21 (2487-3rd)	IRL	Cow	ND	ND:ND	14/4,13/5,12,11/1,10/3, ,8	10,9,8,7,5, ,3		
22 (2487-3rd)	IRL	Cow	ND	ND:ND	14/4,13/5,12,11/1,10/3, ,8	10,9,8,7,5, ,3		
DS51	BEL	Rabbit	1	ND:ND	14/4,13/5,12,11/1,10/3, ,8	10,9,8,7,5, ,3		
DS67	N.IRL	Chicken	5	ND:ND	14/4,13/5,12,11/1,10/3, ,8	10,9,8,7,5, ,3		
DS60	N.IRL	Chicken	5	ND:ND	14/4,13/5,12,11/1,10/3, ,8	10,9,8,7,5, ,3		
MSA1006	USA	Cow	8	ND:ND	14/4,13/5,12,11/1,10/3, ,8	10,9,8,7,5, ,3		
DS47	BEL	Rabbit	8	ND:ND	14/4,13/5,12,11/1,10/3, ,8	10,9,8,7,5, ,3		
MSA1363	USA	Cow	25	7:1	14/4,13/5,12,11/1,10/3, ,8	10,9,8,7,5, ,3	+	*egc*
DS25	ITALY	Goat	39	ND:ND	14/4,13/5,12,11/1,10/3,2,	10,9,8,7,5,4,		

Strain	Origin	Host	MLST Type	ET:RAPD type	*set* genes	*ssl* genes	Mitogenicity	Enterotoxins
DS46	BEL	Rabbit	121	ND:ND	14/4,13/5,12,11/1,10/3, ,8	10,9,8,7,5, ,3		
MSA948	USA	Cow	126	ND:ND	14/4,13/5,12,11/1,10/3, ,8	10,9,8,7,5, ,3		
RF285	IRL	Cow	133	ND:13	14/4,13/5,12,11/1,10/3, ,8	10,9,8,7,5, ,3	+	SaPIbov
RF286	IRL	Cow	133	ND:13	14/4,13/5,12,11/1,10/3, ,8	10,9,8,7,5, ,3	+	SaPIbov
DS74	NOR	Goat	133	ND:ND	14/4,13/5,12,11/1,10/3, ,8	10,9,8,7,5, ,3		
DS90	NOR	Sheep	133	ND:ND	14/4,13/5,12,11/1,10/3, ,8	10,9,8,7,5, ,3		
DS20	ITALY	Goat	682	ND:ND	14/4,13/5,12,11/1,10/3, ,8	10,9,8,7,5, ,3		
DS19	ITALY	Goat	682	ND:ND	14/4,13/5,12,11/1,10/3, ,8	10,9,8,7,5, ,3		
MSA13.1	USA	Cow	672	1:6	14/4,13/5,12,11/1,10/3, ,8	10,9,8,7,5, ,3		
MSA930	USA	Cow	ND	1:10	14/4,13/5,12,11/1,10/3, ,8	10,9,8,7,5, ,3	+	*egc* without *seg*
MSA916	USA	Cow	133	1:12	14/4,13/5,12,11/1,10/3, ,8	10,9,8,7,5, ,3	-	Negative
MSA103.14	USA	Cow	ND	2:2	14/4,13/5,12,11/1,10/3, ,8	10,9,8,7,5, ,3	+	*egc*
MSA1460	USA	Cow	ND	2:2	14/4,13/5,12,11/1,10/3, ,8	10,9,8,7,5, ,3	+	*egc*
MSA1369	USA	Cow	71	3:5	14/4,13/5,12,11/1,10/3, ,8	10,9,8,7,5, ,3		
MSA17.1	USA	Cow	669	5:3	14/4,13/5,12,11/1,10/3, ,8	10,9,8,7,5, ,3	±	*egc*
MSA1058	USA	Cow	ND	3:4	14/4,13/5,12,11/1,10/3, ,8	10,9,8,7,5, ,3	-	Negative
MSA927	USA	Cow	ND	5:4	14/4,13/5,12,11/1,10/3, ,8	10,9,8,7,5, ,3	-	Negative

Strain	Origin	Host	MLST Type	ET:RAPD type	*set* genes	*ssl* genes	Mitogenicity	Enterotoxins
MSA1003	USA	Cow	352	2:11	14/4,13/5,12,11/1,10/3, ,	10,9,8,7,5, ,	-	Negative
MSA1468	USA	Cow	670	6:1	14/4,13/5,12,11/1,10/3, ,8	10,9,8,7,5, ,3	±	Negative
MSA148	USA	Cow	ND	36:9	14/4,13/5,12,11/1,10/3, ,8	10,9,8,7,5, ,3	+	*egc* without *seg*
MSA1455	USA	Cow	ND	7:1	14/4, ,12,11/1,10/3, ,8	10, ,8,7,5, ,3	-	Negative
MSA12.1	USA	Cow	ND	36:9	14/4,13/5,12,11/1,10/3, ,8	10,9,8,7,5, ,3	+	*egc* without *seg*
MSA915	USA	Cow	115	ND:ND	14/4,13/5,12,11/1,10/3, ,8	10,9,8,7,5, ,3		

§ Not all of the genes encoded by the *set/ssl* locus were investigated. Seven of the genes that lie in the variable region of the *set/ssl* locus were tested for.

† Mitogenicity results for the 29 strains tested in the previous chapter are indicated along with whether or not they encoded SAg and SAg-like genes.

‡ Strains 2, 6, 7, 10, 12, 14, 21 and 22 were isolated from milk samples from cows with mastitis. The numbers in brackets refer to the cow and quarter from which the milk sample was obtained.

was positive for the *egc* cluster of genes (*seg*, *sei*, *selm*, *seln*, *selo*)

* was positive for *seh*

¥ ET:RAPD type – as defined by Fitzgerald *et al*., (1997), ND – not done

Fig. 4.8. Southern blot analysis of the *set* (*ssl*) locus in bovine-associated and human control strains.

Panel (A) membrane was probed for the *set1* (*ssl7*) gene. Bovine strains of major RAPD types, along with control strains MSSA and EMRSA16, all had the *set1* (*ssl7*) gene. Also the size of the hybridising band appeared to be maintained in strains of the same RAPD type.

Panel (B) was probed for the *set3* (*ssl5*). Once again the probe hybridised to approximately same size fragments in each RAPD type (data not shown).

Panel (C) was probed for the *set12* (*ssl8*) gene. Unlike the *set1* (*ssl7*) and the *set3* (*ssl5*) genes, this gene was not present in every strain. The RAPD types of strains are shown in parenthesis after strain designations. However, strains of the same RAPD type (MSA1455 and MSA77) showed a hybridising band of the same size.

The RAPD types of these strains were defined by Fitzgerald *et al.* (1997)

(A)
RAPD 7
RAPD 5
RAPD 4
Control Strains
RF124
RF114
RF113
RF108
RF106
RF105
RF103
RF117
RF116
RF115
MSSA
EMRSA16
10 kb
6 kb
5 kb
(B)
RAPD 7
Control Strains
RF122
RF121
RF120
RF113
RF110
FRI472
NY19
FRI913
FRI569
NCTC657
EMRSA1
MSSA
10 kb
(C)
RF122(7)
EMRSA16
A900322
COL
PSA6.1(9)
MSA930(10)
MSA1058(4)
MSA1369(5)
MSA1455(1)
MSA77(1)
MSA72.3(2)
10 kb

et al. (1997). Isolates 6 and 7 (from the 3rd quarter of cow 2242), isolates 10 and 12 (from the 4th quarter of cow 2242), and isolate 2 (from the 3rd quarter of cow 2521) were of the same RAPD type. Isolates 21 and 22 (from the same quarter of cow 2487) were of a different but identical RAPD type. Isolate 14 was of a unique RAPD type. Isolates 2, 6, 7, 10 and 12 were found to be positive for the *seh* gene and isolate 14 was found to possess the *egc* locus, i.e. the *seg*, *sei*, *selm*, *seln* and *selo* genes. Isolates 21 and 22 were negative for SAg genes.

4.3.6. Mitogenicity.

In the previous chapter, 79 bovine-associated *S. aureus* strains were tested for the ability to stimulate the proliferation of human T-lymphocytes. These isolates represented major clonal types of *S. aureus* associated with bovine mastitis and encoded a range of SAg and SAg-like genes. The results of the mitogenicity assays showed that, for most of the strains, strains with SAg or SAg-like genes displayed mitogenicity, whereas strains without SAg or SAg-like genes were negative for mitogenicity. Twenty-nine of those strains were tested herein for the presence of *set* (*ssl*) genes and all 29 encoded a *set* (*ssl*) locus. Of the 29 strains, 12 were non-mitogenic and negative for SAg and SAg-like genes. Fourteen of the 29 strains were mitogenic and did encode SAg and SAg-like genes. One strain (RF103) was mitogenic and negative for SAg and SAg-like genes. The remaining two strains exhibited borderline mitogenicity, one of which (MSA17.1) possessed SAg and SAg-like genes and one (MSA1468) did not.

4.4. Discussion.

4.4.1. Genomic location of the *set* (*ssl*) locus.

Superantigen genes can be associated with mobile genetic elements such as bacteriophage (*sea*), plasmids (*sed*, *selj*), and pathogenicity islands (*sec*-bovine, *tst*) (Novick *et al.*, 2001). The *set* (*ssl*) locus of genes, however, appears to be stable in the chromosome, possibly facilitated by the presence of a restriction and modification system, the genes of which have been shown to be expressed *in vitro* (Fitzgerald *et al.*, 2003). As a result the *set* (*ssl*) locus may not be horizontally acquired. The putative transposase gene that flanks the *set* (*ssl*) locus may have once facilitated horizontal transfer. However, this putative gene has

been lost from the genome of the recently sequenced strain MW2, suggesting that the horizontal transfer of this locus has become a redundant and unnecessary function.

Fitzgerald *et al.* (2003) have proposed that the *set* (*ssl*) locus has evolved by multiple deletion and recombination events and has most likely evolved from a locus with a full complement of *set (ssl)* genes. The locus has been shown to be under selective constraint to maintain function. It is likely that deletion and recombination events, centred on the variable region of the locus (Fig. 4.5), have contributed to the variation seen today. However, it is interesting to note that one the bovine isolates tested herein (MSA1455, Table 4.3) lacks the *set5/13* (*ssl9*) allele that has been shown to be present in most strains.

In the current study, the *set* (*ssl*) locus was found to be present in every animal-associated strain, to show minor variation in gene content by PCR, and to show variation in RFLP patterns in Southern blots. In similar studies the *set (ssl)* locus has been found in all 63 strain tested (49 *S. aureus* strains from humans, 8 from cows, 2 from sheep, 2 form poultry and 2 of unknown origin) (Fitzgerald *et al.*, 2003). However, no group has looked for the individual *set* (*ssl*) genes with the exception of the *set1* (*ssl7*) gene, the archetypal *set* gene (Williams *et al.* 2001). The number and combination of *set* (*ssl*) genes in strains of animal origin seems to reflect clonal relationships. This would also support the theory that the locus is stable in the genome. The enterotoxins genes, some of which are capable of horizontal transfer such as *sec*, *sell* and *tst* that are on SaPIbov, have been shown to be variably present in strains of the same clonal type, as demonstrated in Chapter 3.

4.4.2. Expression of the *set (ssl)* genes.

It has been demonstrated that strain COL expresses its *set* (*ssl*) genes concurrently and that multiple *set* (*ssl*) genes are expressed during human infection by Western immunoblot using sera from acute and convalescent patients (Fitzgerald *et al.*, 2003). However, not all of the SET (SSL) proteins are immunogenic. Expression of the *set* (*ssl*) genes has yet to be shown for isolates from animal infection; neither has *in vivo* expression been demonstrated. If multiple *set* (*ssl*) genes are concurrently expressed during animal infection, the *set* (*ssl*) genes may play an important role in staphylococcal pathogenicity. This is given credence by the fact that every strain tested has a *set* (*ssl*) locus suggesting that the *set* (*ssl*) locus has possibly a role in virulence. It is of note that a number of the strains tested for herein were analysed for mitogenicity in the Chapter 3. SAg and SAg-

like gene-negative isolates that have been shown to encode a *set* (*ssl*) locus herein were found to be non-mitogenic. Considering that mitogenicity is a hallmark of superantigenic activity, these strains and their SET (SSL) proteins (if expressed by animal-associated strains) should have exhibited a mitogenic effect, if these proteins were indeed superantigens.

4.4.3. Function of SET (SSL) proteins and host specificity.

It has been previously shown that, despite the structural similarity of the SET (SSL) proteins to the SAg family and the presence of regions of sequence similarity such as the presence of consensus signature sequences, the SET (SSL) proteins are not superantigens (Arcus *et al.*, 2002; Fitzgerald *et al.*, 2003; Al-Shangiti *et al.*, 2005). It appears that the conserved residues seen in SET (SSL) proteins are necessary to maintain structure; in contrast residues important for superantigenic activity are not maintained.

In the seminal paper in which the *set* (*ssl*) genes were described, the production of proinflammatory cytokines was observed from human PBMCs exposed to recombinant SET1/SSL7 protein (Williams *et al.*, 1999). This experimental result has been retracted by the authors as having been due to the presence of contaminating LPS in the recombinant protein preparation (Al-Shangiti *et al.*, 2005). Several groups have attempted to demonstrate superantigenic activity in recombinant SET proteins and have found none (Arcus et al., 2002; Fitzgerald *et al.*, 2003; Al-Shangiti *et al.*, 2005).

Recent studies have shown a role for SET (SSL) proteins in interacting with varying components of the immune system. This is consistent with the theory that the SET (SSL) proteins have evolved to have related but distinct functions. The SET1 (SSL7) protein has been shown to interact with IgA and complement factor C5 from human serum as well as both serum and secreted IgA and complement factor C5 from different animal species (Langley *et al.*, 2005). The reactivity for complement factor C5 was independent of that of IgA suggesting a different interaction mechanism. Importantly the ability of SET1 (SSL7) to bind to IgA was found at similar levels for human, chimpanzee, baboon, and pig sera, at lower levels for horse and rat sera, and was undetectable for cow, sheep, mouse, rabbit or goat sera. This may reflect the host specificity of the SET1 (SSL7) protein as the *set1* (*ssl7*) gene was cloned from a human-associated clinical strain. It is possible that animal-specific alleles of the *set (ssl)* genes exist, as have been described for staphylococcal

enterotoxins such as SEC-ovine and SEC-bovine, the gene products of which have particular functions in the infection of difference animal hosts. Perhaps recombinant SET1/SSL7 from a bovine-associated strain would show higher affinity for bovine IgA.

Al-Shangiti *et al.* (2005) have shown that SET (SSL) proteins can bind to and be taken up by dendritic cells independently of receptors on the their surface and can stimulate T-cell responses. It remains to be seen if this function is of benefit to the bacteria or the host.

Due to the fact that little data are known about the immunogenicity of the SET (SSL) proteins in the animal host, *in vivo* expression experiments such as Western blots using serum from infected and uninfected cows, would reveal whether or not certain SET (SSL) proteins are expressed and hence potentially important in infection. This theory is explored in Chapter 7.

Chapter 5

Multi-locus Sequence Typing, Random Amplified Polymorphic DNA Typing and *agr* Typing of *Staphylococcus strains* Associated with Animals

5.1. Introduction

Microbial typing is a useful tool for defining the source and route of transmission of bacterial pathogens and for studying the persistence, re-infection rate and clonal selection of bacterial pathogens in the host. It also facilitates studies of bacterial evolution. The reproducibility, discriminatory power, ease of performance and interpretation, and cost associated with a particular typing method must be considered when evaluating a typing technique.

A wide variety of typing methods, including biochemical and molecular methods, have been used to investigate the population structure of human- and animal-associated *S. aureus*, e.g., bacteriophage typing, antibiotic susceptibility typing, biotyping, plasmid/chromosomal profiling with restriction enzymes, ribotyping, RAPD (Random Amplified Polymorphic DNA) typing, MLEE (Multi-locus Enzyme Electrophoresis), MLVA (Multi-locus Variable Number Tandem Repeat Analysis), PFGE (Pulsed Field Gel Electrophoresis), REP (Repetitive Element PCR), AFLP (Amplified Fluorescent Length Polymorphism) and Binary typing (reviewed by Štèpán *et al.*, 2004). These methods are discussed in Chapter 1.

The most widely used molecular typing methods, such as PFGE and RAPD typing, rely on the comparison of DNA fragments or amplicon patterns in agarose gels. These methods are discriminatory and can indicate the accumulation of genetic variation that is important in short-term epidemiological studies. However, these techniques are observer reliant and dependent on laboratory conditions. Accordingly it can be difficult to reproduce results from laboratory to laboratory. These techniques are not useful for long-term global studies as they mask broader, global genetic relationships.

Global epidemiology requires a method that can distinguish a vast number of genetic types but relies on genetic variation that accumulates very slowly. Genes that have this property are so-called 'housekeeping genes' for normal cellular function. Because these genes evolve slowly, using one gene would result in poor discriminatory ability. Accordingly analysis of multiple loci is used. MLEE was developed on this principle. However, MLEE is limited by its laborious nature and the difficulty of comparing results from different gels and between different laboratories. MLST (Multi-locus Sequence Typing), a relatively recently developed technique, is based on MLEE and has been widely applied to

the study of *S. aureus* populations, in particular of nosocomial and antibiotic-resistant *S. aureus* (Urwin & Maiden, 2003; Robinson & Enright, 2004b). It relates organisms on the basis of the nucleotide sequences of internal fragments of seven conserved housekeeping genes. A numerical profile is created from the sequences and is used to generate an unambiguous sequence type (ST) (Fig. 5.1). Seven loci are used in MLST with an average of 35 alleles per locus, allowing MLST to potentially resolve more than 20 billion sequence types. An internet database is available to check and annotate the sequences of alleles and another is available to indicate STs (http://www.mlst.net).

MLST was originally developed for *Neisseria meningitidis* (Maiden *et al.*, 1998). It has since been applied to *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Campylobacter jejuni* and *S. aureus* (Enright & Spratt, 1998; Dingle *et al.*, 2001; Enright *et al.*, 2000; Enright *et al.*, 2001). The major advantages of MLST are its high discrimination, reliability and electronic portability. The major disadvantage of MLST is the cost, as sequencing remains expensive. This may limit its application where cost is a factor.

RAPD typing is a PCR-based fingerprinting method that utilises non-specific random primers that amplify random segments of the genome generating a genotype-specific banding pattern upon agarose gel electrophoresis. RAPD typing is rapid and discriminatory and has been used to type *S. aureus* from a range of hosts including bovine *S. aureus* (Fitzgerald *et al.*, 1997; Pereira *et al.*, 2002; Lam *et al.*, 1996; Lipman *et al.*, 1996; Reinoso *et al.*, 2004), rabbit *S. aureus* (Hermans *et al.*, 2000, 2001), human *S. aureus* (van Leeuwen *et al.*, 1996; Tambic *et al.*, 1997).

Control of virulence gene expression by *S. aureus* is an area of intense research activity and a number of regulatory loci have been described (Novick, 2003; Yarwood *et al.*, 2003; Bronner *et al.*, 2004; Cheung *et al.*, 2004). Typing methods based on regulatory loci have also been evaluated. The most widely characterised of the staphylococcal regulatory loci is the accessory gene regulator (*agr*), which has been shown to regulate the synthesis of many virulence factors during bacterial growth (Fig. 5.2). The *agr* system co-ordinately down-regulates the production of cell-wall-associated proteins and up-regulates the production of secreted proteins at late stationary to early exponential growth phase *in vitro* (Bronner *et al.*, 2004). The locus encodes a two-component signal transduction system consisting of two divergently transcribed units driven by promoters P2 and P3 (Fig. 5.2). The P3 operon encodes RNAIII which is the effector of the *agr* response while the P2

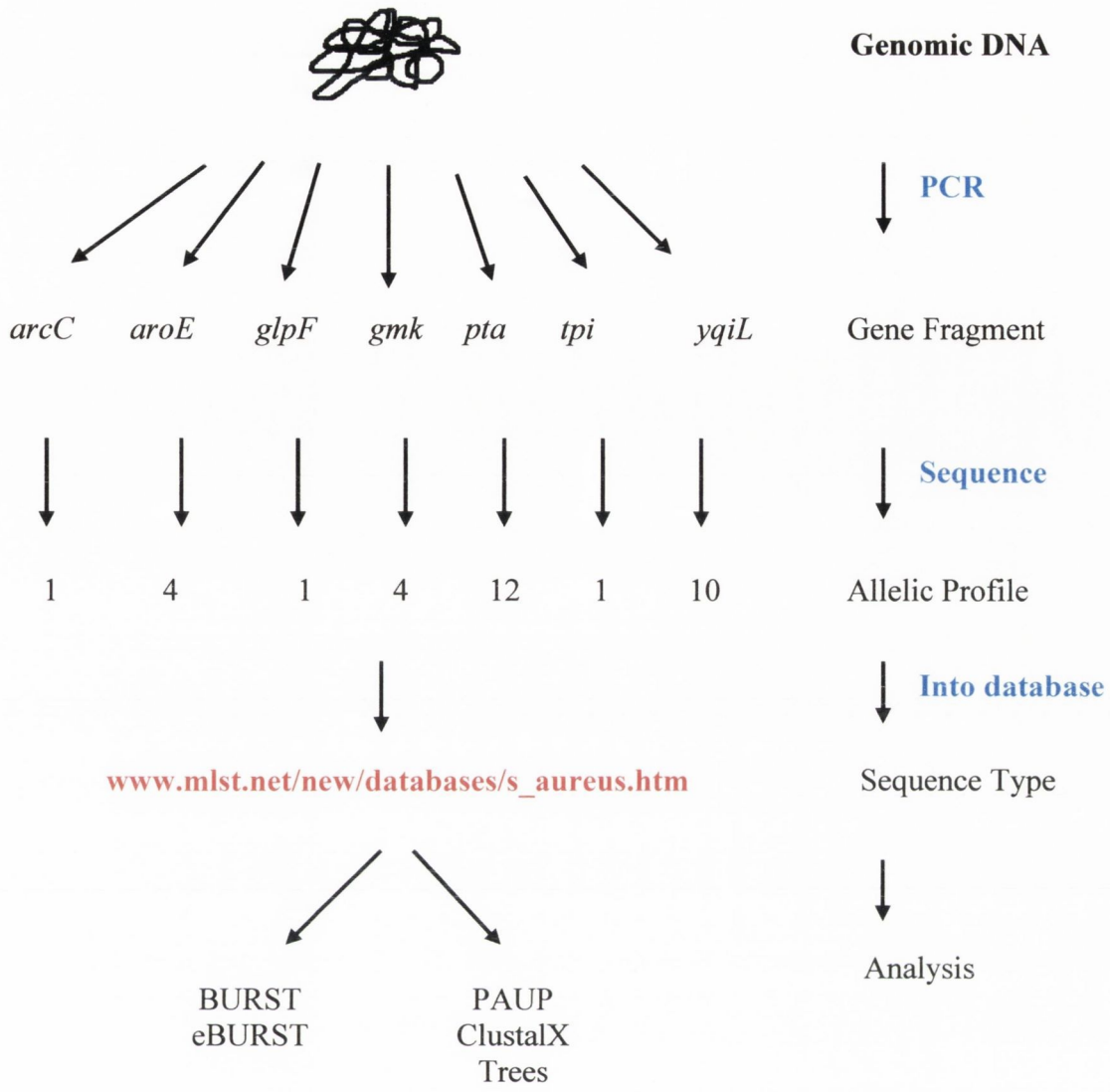
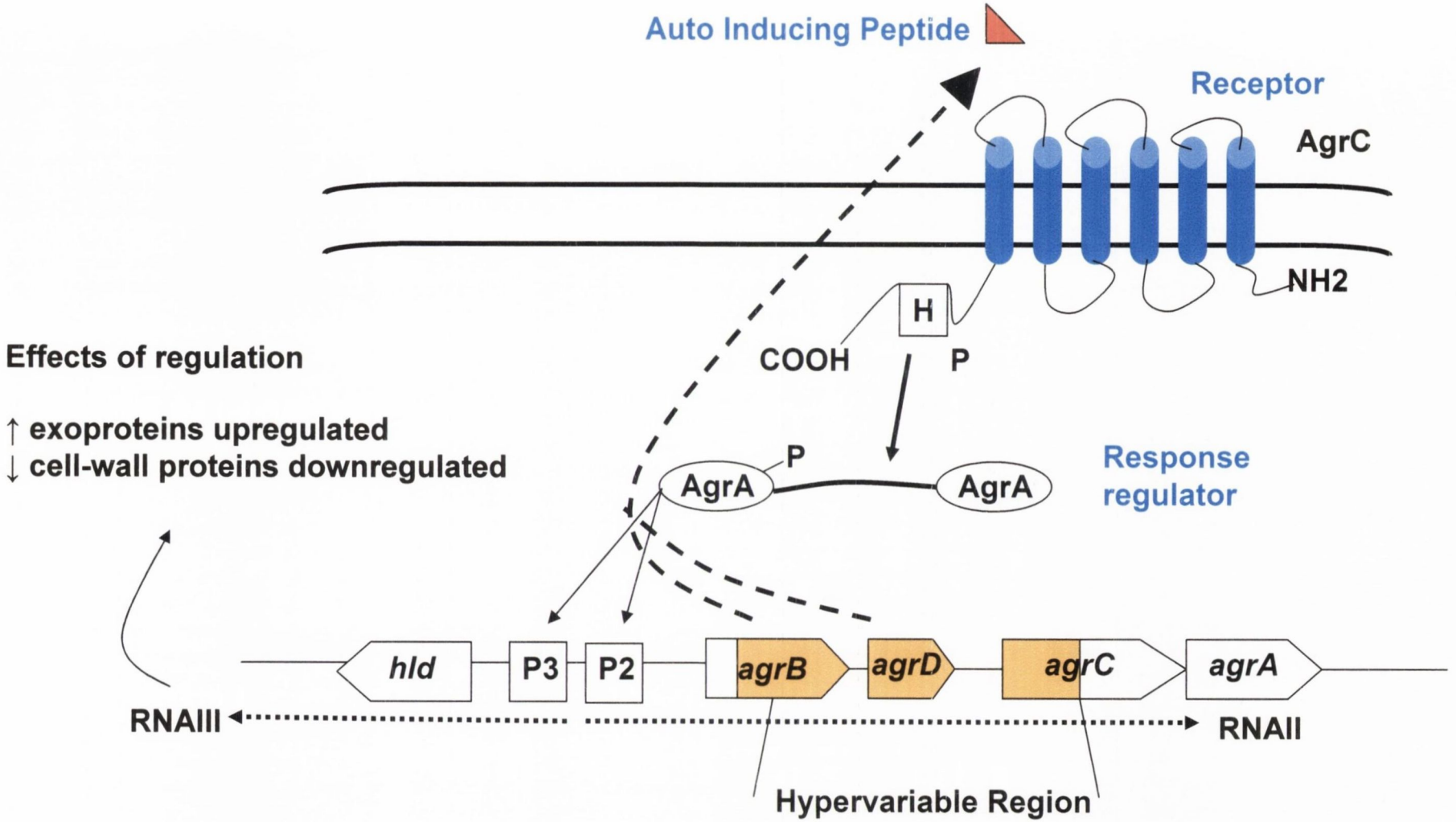


Fig. 5.1. Flow chart of the processes involved in MLST.

The MLST process from start to finish can take as little as two days if sequencing facilities are easily available. This method is reproducible, electronically portable and unambiguous. Briefly, seven 'housekeeping genes' are amplified by PCR from genomic DNA. The PCR products are sequenced and the resulting sequences are submitted to the MLST database. The sequences are compared to those in the database and are given a number. The seven numbers correspond to an allelic profile. The seven numbers of the allelic profile correspond to the sequence type. The sequence types can be analysed by eBURST and the nucleotide sequences themselves can be analysed by generating phylogenetic trees.

Fig. 5.2. Schematic of the *agr* locus.

One of several regulatory loci in *S. aureus*. The *agr* system co-ordinately down-regulates the production of cell wall-associated proteins and up-regulates the production of secreted proteins at late stationary to early exponential growth phase *in vitro*. The locus encodes a two-component signal transduction system consisting of two divergently transcribed units driven by promoters P2 and P3. The P3 operon encodes RNAIII which is the effector of the *agr* response while the P2 operon contains the genes *agrA*, *agrB*, *agrC* and *agrD*. The *agrB* and *agrD* genes generate an autoinducing peptide (AIP) that acts as an activating ligand for AgrC which is a transmembrane receptor histidine kinase. Histidine kinase AgrC phosphorylates the regulatory protein AgrA which activates transcription at promoters P2 and P3. The hypervariable region composing of almost two thirds of the *agrB* gene, half of the *agrC* gene and all of the *agrD* gene can be amplified with type-specific primers.


Auto Inducing Peptide
Receptor
AgrC
NH2
H
COOH
P
Effects of regulation
↑ exoproteins upregulated
↓ cell-wall proteins downregulated
P
AgrA
AgrA
Response
regulator
hld
P3
P2
agrB
agrD
agrC
agrA
RNAIII
RNAII
Hypervariable Region

operon contains the genes *agrA*, *agrB*, *agrC* and *agrD*. The *agrB* and *agrD* genes generate an autoinducing peptide (AIP) that acts as an activating ligand for the *agrC* gene which encodes a transmembrane receptor histidine kinase (Fig. 5.2). Histidine kinase AgrC phosphorylates the regulatory protein AgrA which activates transcription at promoters P2 and P3. It has been reported that the AIP produced by a given strain of *S. aureus* may activate its own *agr* locus but inhibit that of other strains (Gilot *et al.*, 2002; Goerke *et al.*, 2003). This is due to a region of the *agr* locus which is variable comprising of the *agrD* gene, two-thirds of the 5′-end of the *agrB* gene and a portion of the 3′-half of the *agrC* gene.

This phenomenon has been used to classify *S. aureus* into four main *agr* types (Fig. 5.3). Some research groups have completed more detailed analysis and have subtyped within the four *agr* types (Gilot *et al.*, 2002; 2004). This *agr* type variation may be involved in bacterial interference when the bacterium is attempting to colonise a host site. Links between a particular *agr* type and a specific staphylococcal syndrome have been shown for toxic shock syndrome (TSS) and staphylococcal scalded skin syndrome (SSSS) (Jarraud *et al.*, 2002). TSST-1 toxin-producing isolates belong to *agr* type 3 and are mostly of the same clonal type as shown by MLEE and PFGE. Most exfoliative toxin-producing strains that cause SSSS belong to *agr* type 4. However, the clonality of the latter strains has not been investigated. In one study of 38 representative MSSA (methicillin-sensitive *Staphylococcus aureus*) strains, 61% were of *agr* type 1, 26% were of *agr* type 2, and 13% were of *agr* type 3. None were of *agr* type 4. In another study, endocarditis-associated strains were shown to be of *agr* types 1 and 2 (Jarraud *et al.*, 2002). However, these data do not show a direct role for the *agr* genes in human disease.

Few data are available on the distribution of *agr* types in *S. aureus* isolates from animal infection. One study using bovine mastitis-associated strains has shown that certain *agr* types predominate in mastitis infection (Gilot *et al.*, 2002). In another study bovine strains with the predominant *agr* type were able to resist neutrophil bactericidal activity and the strains often possessed enterotoxin genes (Mullarky *et al.*, 2001).

This present work represented the first study to investigate the phylogenetic relationships of *S. aureus* from cows, goats, sheep, rabbits and chickens by MLST, RAPD typing and *agr* typing. Due to the availability of a large database of human-associated *S. aureus* it was possible to compare the results of MLST analysis of the animal-associated *S. aureus*

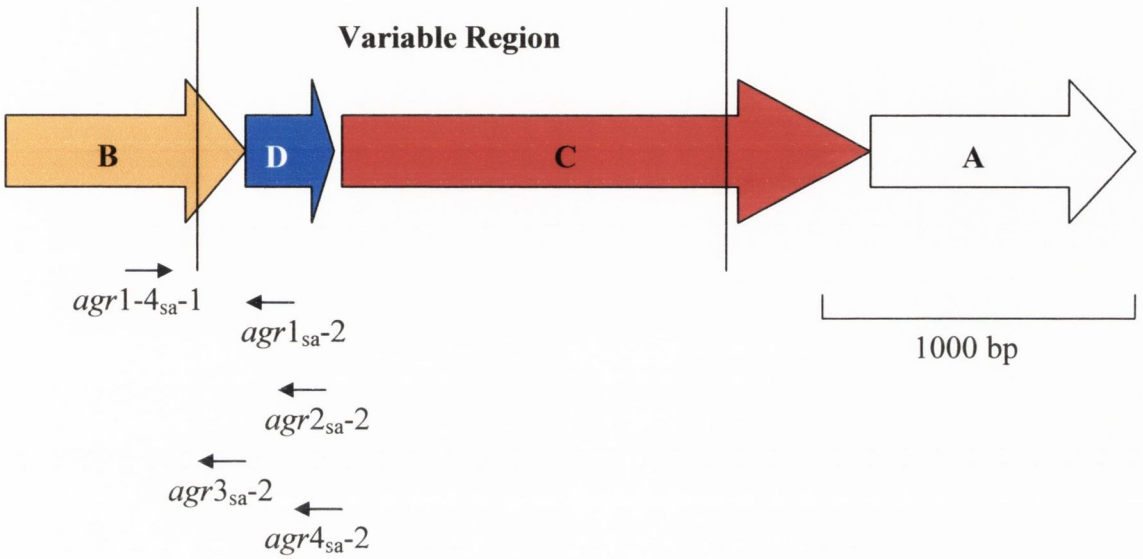


Fig. 5.3. Approximate binding location of the *agr* multiplex primers within the variable region of the *agr* locus [Figure adapted from Gilot *et al.* (2002)].

The universal forward primer *agr*1-4$_{sa}$-1 binds to the *agrB* gene and the four *agr* type-specific reverse primers (*agr*1$_{sa}$-2, *agr*2$_{sa}$-2, *agr*3$_{sa}$-2, *agr*4$_{sa}$-2) bind to regions within the *agrD* gene.

with human strains. BURST analysis was used to group the animal-associated STs along with human-associated STs into clonal complexes. The discriminatory power of MLST was compared to RAPD typing. Herein *agr* typing was used in an attempt to identify the distribution of *agr* types among strains from different animal hosts and also to see if the type of infection and distribution of toxin genes were associated with particular *agr* types.

5.2. Methods.

5.2.1. Multi-locus sequence typing (MLST).

5.2.1.1. DNA extraction and PCR.

One hundred and twenty animal infection-associated isolates and 3 human infection-associated isolates were analysed by MLST (See Table 5.3). All twelve chicken strains were from Northern Ireland. Fifty-two cow strains were analysed, 28 of which were characterised by Fitzgerald *et al.* (2000) [3 were from Argentina, 4 from Spain, 2 from Sweden, 19 from the USA and 24 were from the Republic of Ireland (6 RAPD type 4, 5 RAPD type 5, 6 RAPD type 7, 7 RAPD type not determined)]. Of the 33 goat- associated strains, 4 were from Austria, 21 were from Italy, and 8 were from Norway. Of the 12 rabbit-associated strains, 4 were from Spain and 8 were from Belgium. Of the 10 sheep-associated strains, 2 were from Iceland, 1 was from Sweden, and 7 were from Norway. The three human-associated strains were from the USA and the cat isolate was from Sweden.

Strains were plated onto sheep blood TSA plates and incubated overnight at 37 °C. Approximately one square inch of confluent growth was removed and resuspended in 400 µl of lysis solution [9.9 ml TE (Tris-EDTA) buffer, 10 µl lysostaphin (2 units/ml) (Sigma-Aldrich), 0.1 g lysozyme (Sigma-Aldrich)]. The bacterial suspensions were vortexed and incubated at 37 °C in a water-bath for 1 h. DNA was extracted using the DNeasy Genomic DNA Extraction Kit (Qiagen) following the manufacturer's instructions. The concentration of the DNA preparation was assessed by electrophoresis alongside DNA standards of known concentration as described in Chapter 2.

High-throughput PCR of the seven housekeeping genes listed in Table 5.1 was done using a 96-well plate format. PCRs were carried out in a final volume of 50 µl consisting of 1 µl

Table 5.1. Sequences of MLST primers used in this work.

Gene	Primer	Primer sequence (5′–3′)	PCR Product (bp)
Carbamate kinase (*arcC*)	*arcC* for	TTG ATT CAC CAG CGC GTA TTG TC	456
	arcC rev	AGG TAT CTG CTT CAA TCA GCG	
Shikimate dehydrogenase (*aroE*)	*aroE* for	ATC GGA AAT CCT ATT TCA CAT TC	456
	aroE rev	GGT GTT GTA TTA ATA ACG ATA TC	
Glycerol kinase (*glpF*)	*glpF* for	CTA GGA ACT GCA ATC TTA ATC C	465
	glpF rev	TGG TAA AAT CGC ATG TCC AAT TC	
Guanylate kinase (*gmk*)	*gmk* for	ATC GTT TTA TCG GGA CCA TC	429
	gmk rev	TCA TTA ACT ACA ACG TAA TCG TA	
Phosphate acetyltransferase (*pta*)	*pta* for	GTT AAA ATC GTA TTA CTT GAA GG	474
	pta rev	GAC CCT TTT GTT GAA AAG CTT AA	
Triosephosphate isomerase (*tpi*)	*tpi* for	TCG TTC ATT CTG AAC GTC GTG AA	402
	tpi rev	TTT GCA CCT TCT AAC AAT TGT AC	
Acetyl coenzyme A acetyltransferase (*yqiL*)	*yqil* for	CAG CAT ACA GGA CAC CTA TTG GC	516
	yqil rev	CGT TGA GGA ATC GAT ACT GGA AC	

chromosomal DNA (approximately 0.5 μg), 10 pmol of each forward and reverse primer, 1 U of *Taq* DNA polymerase (Promega), 5 μl of 10 × buffer (Promega), 5 μl of 3 mM $MgCl_2$ buffer (Promega), and 0.2 mM deoxynucleoside triphosphates. In each case the DNA was dispensed into the wells, the PCR mix prepared, and these PCR mixtures vortexed and added to each well. Plates were sealed using adhesive polypropylene covers (ABGene, Advanced Biotechnologies Ltd, New York, USA) and centrifuged at 1,000 r.p.m. (201 × *g*) for 10 s . PCR was performed in a PTC-200 DNA engine (MJ Research, Boston, MA) with an initial 5 min denaturation of 95 °C, followed by 30 cycles of [95 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min], and a final extension of 72 °C for 5 min.

The PCR products were checked by electrophoresis on a 1% (w/v) agarose gel and subsequently purified (Fig. 5.4). To each well 60 μl of 20% (w/v) PEG (polyethylene glycol 8000) in 2.5 M NaCl was added. The plate was vortexed, centrifuged at 1,000 r.p.m. (201 × *g*) for 10 s and incubated at room temperature for 30 min. The DNA was pelleted by centrifuging the plate at 3,500 r.p.m. (2465 × *g*) for 30 min. The pellets were washed with 150 μl 70% (v/v) ethanol and the plate centrifuged at 2465 × *g* for 30 min. The plates were then inverted onto tissue and allowed to drain. The previous two steps were repeated twice. The pellets were dried on the DNA engine (PTC-200 DNA engine) at 37 °C for 2 min. To each well 12 μl of sterile distilled water was added. The plates were sealed, vortexed and placed at 4 °C for 10 min. The plates were then vortexed and placed at 4 °C for 10 min; this was repeated three times. The cleaned PCR products were checked by electrophoresis as before.

5.2.1.2. Cycle sequencing of PCR products.

Of each cleaned-up PCR product 1 μl was dispensed into the bottom of each well (2 wells per isolate: forward and reverse sequencing) of 96-well plates. To each well 1 pmol of forward or reverse MLST gene primer was added along with 1 μl *Taq* FS-Big Dye polymerase (Applied Biosystems). The plates were sealed and briefly centrifuged at 1,000 r.p.m. before loading onto the DNA engine. The cycle sequencing parameters were: an initial 10 s denaturation at 96 °C, followed by cooling to 50 °C for 5 s, and extension at 60 °C for 2 min. This was repeated 24 times after which the plates were stored at 4 °C. PCR products for sequencing were cleaned up by ethanol precipitation. Plates were loaded onto an ABI 3700 Prism Sequencer by Paul Wilkinson (Department of Biology and Biochemistry, University of Bath).

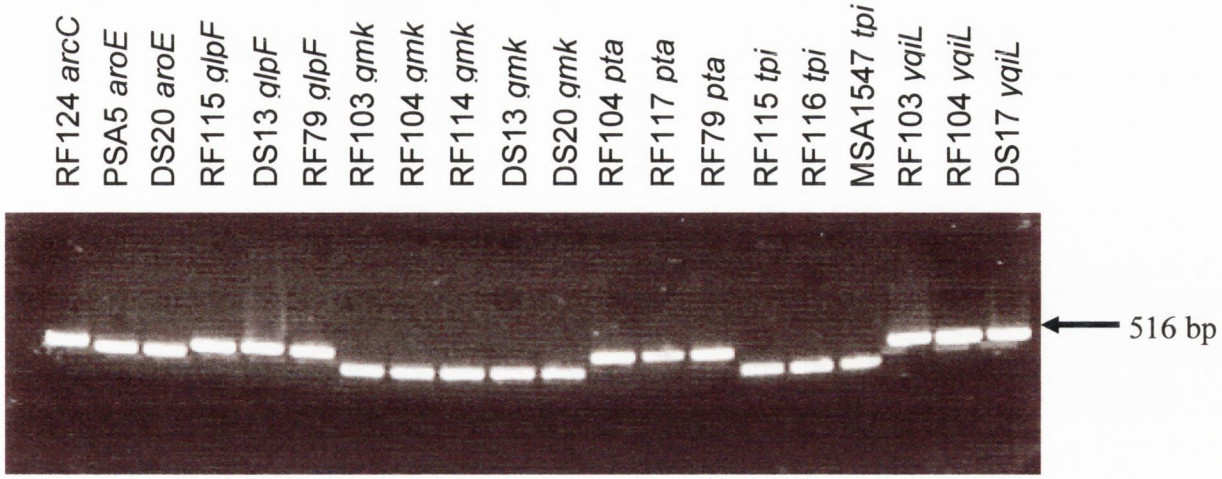


Fig. 5.4. PCR of MLST genes from animal-associated *S. aureus*.

The PCR reactions generate specific products that can be directly sequenced. The PCR products were resolved on 1% agarose gels prior to cleaning-up. All the housekeeping gene internal fragments amplified are in the 402–516 bp range. The housekeeping gene and the strain it was amplified from are indicated above the gel.

5.2.1.3. Sequence assembly.

Following sequencing, the data were assembled using the SeqMan program (DNASTAR Inc. WI, USA) (Fig. 5.5). Individual files containing the traces of both strands along with a reference sequence were compiled; the traces were trimmed to the desired length. Conflicts between the traces were resolved, and the resulting sequences were saved as text files and stored for comparison and analysis. The *S. aureus* MLST website (http://saureus.mlst.net/sql/singlelocus.asp) was used to analyse each gene sequence. The new sequences were either the same as a sequence in the database or different by one base pair or more. In the latter instance a new allele number was assigned to the sequence. The combined allele numbers for all seven housekeeping genes corresponded to the allelic profile of the isolate. Allelic profiles for each isolate were submitted to the database to obtain a sequence type number (ST). At the time of writing of this thesis there were 82 *arcC* alleles, 123 *aroE* alleles, 92 *glpF* alleles, 66 *gmk* alleles, 89 *pta* alleles, 92 *tpi* alleles, and 85 *yqil* alleles in the MLST database. A total of 668 sequence types (STs) had been submitted.

*5.2.1.4. e*BURST analysis.

The clustering of animal-associated sequence types was analysed using the eBURST (Based Upon Related Sequence Types) algorithm. BURST was originally designed by Dr. Edward Feil (Department of Biology and Biochemistry, University of Bath) specifically for use with MLST-generated data. It assembles related sequence types into clonal complexes. A clonal complex consists of a set of sequence types (STs) in which every ST shares at least any five loci of the seven in common with at least one other ST of the group. Ancestral STs are identified as consensus clones (placed in the central ring of the clonal complex), which differ from the highest number of other STs in respect of one allelic type out of seven (the Ancestral ST has the highest number of single locus variants). Once the ancestral type has been identified, the other STs are related to it and assigned to the clonal complex. Single locus variants (SLVs) differ at one locus from the ancestral ST and double locus variants (DLVs) differ at two loci. The relationships of STs in clonal complexes are displayed as rings, the ancestral ST being placed in the central ring, with SLVs in the next ring and DLVs in the next, implying radial spread from the ancestral ST (Fig. 5.6).

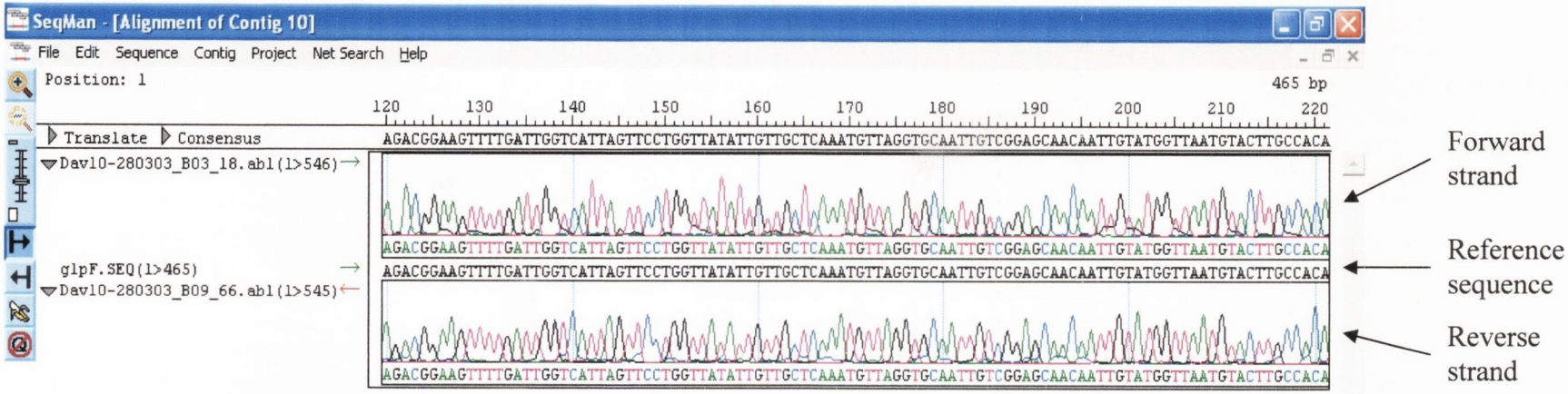


Fig. 5.5. Output of SeqMan (DNASTAR Technologies).

This program allows the user to input raw ABI files (forward and reverse sequencing reads of PCR products) and align the sequencing traces directly against a reference sequence. The sequencing reads are trimmed to the appropriate size. The program also displays conflicts for the user to resolve. The sequences once trimmed and checked are exported as text files and are submitted to the MLST database to assess which allele is present in the strain.

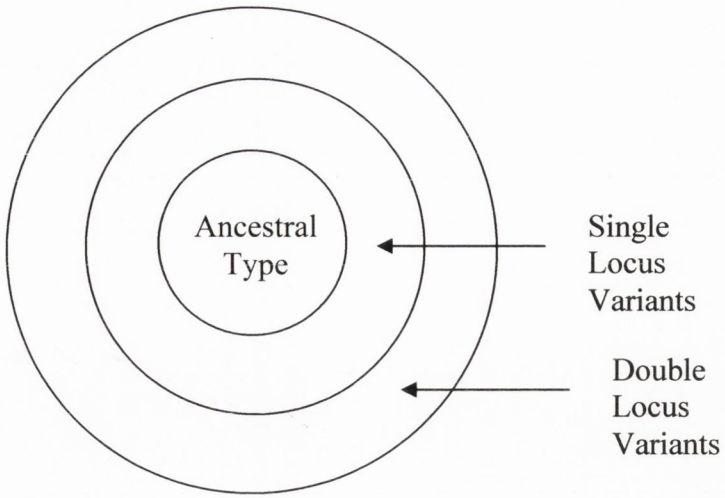
Fig. 5.6. Explanation of BURST and eBURST clonal complexes.

A clonal complex consists of a set of sequence types (STs) in which every ST shares at least five loci in common with at least one other ST of the group. Ancestral STs are identified as clones (placed in the centre of the clonal complex), which differ from the highest number of other STs in respect of one allelic type out of seven. Once the ancestral type has been identified, the other STs are related to it and assigned to the clonal complex.

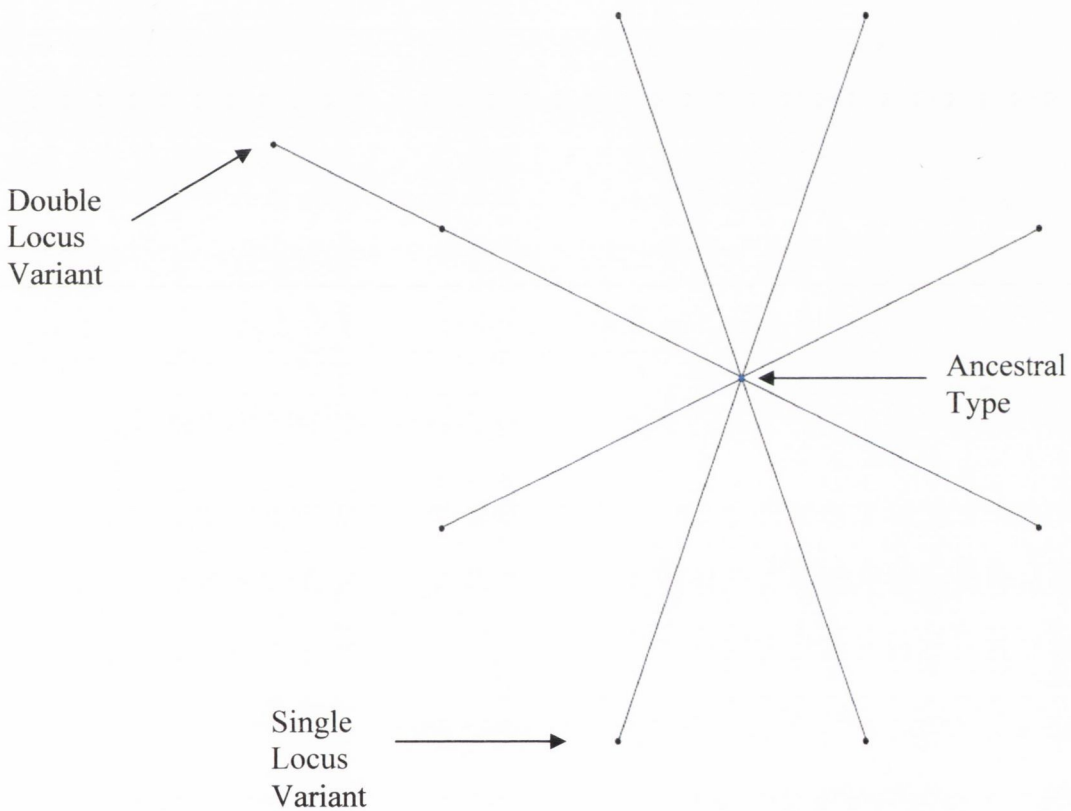
Panel (A) shows a BURST clonal complex. Single locus variants (SLVs) differ at one locus from the ancestral ST and are placed in the ring beyond the ancestral type ring. Double locus variants (DLVs) differ at two loci and are placed in the ring beyond that containing the SLVs.

Panel (B) shows an eBURST clonal complex. eBURST uses lines which radiate from a central point to show the relationships of SLVs and DLVs to the ancestral ST (primary founder). In eBURST DLVs of the ancestral type that are not related to any of the SLVs in the compex are displayed as separate dots.

(A)


Ancestral
Type
Single
Locus
Variants
Double
Locus
Variants

(B)


Double
Locus
Variant
Ancestral
Type
Single
Locus
Variant

eBURST v2 is an improved version of BURST developed by Feil *et al.* (2004) which displays and generates clonal complexes differently to BURST. Instead of arranging clonal complexes in concentric rings, eBURST arranges the clonal complexes with SLVs represented as dots on lines radiating from a central ancestral (primary founder) ST which is represented by a blue dot. There may be SLVs associated with an ancestral type that have themselves a large number of SLVs. These generate subgroups which are similar to the main group except that the subgroup founder type is represented by a yellow dot. eBURST also uses a bootstrapping process that gives an estimate of the level of confidence in assigning the primary founder ST.

All 668 STs in the MLST database along with the 19 novel STs identified in this study (temporarily annotated in Table 5.4 of Results section) were entered into eBURST.

5.2.1.5. Levels of discrimination.

To determine the level of discrimination achieved by MLST and RAPD typing, the index of diversity was calculated (Grundmann *et al.*, 2001, 2002). This method calculates the probability that two random isolates will be of different types by relating the number of clusters (groups) produced to the number of isolates within each cluster. To calculate the index of discrimination the following equation was used:

$$\mathbf{D=1-[(1/N(N-1)) \times \Sigma n_i(n_i-1)]}$$

Where D = index of discrimination,
N = number of isolates in the sample,
and n_i = number of isolates in group i.

5.2.2. Random Amplified Polymorphic DNA (RAPD) typing.

A number of the animal-associated strains used in this work had been previously successfully examined by RAPD typing (Fitzgerald *et al.*, 1997). To avoid confusion all the isolates were re-typed and given new RAPD type numbers. A 23-bp primer D11344 5′-AGTGAATTCGCGGTGAGATGCCA-3′ from Marshall *et al.* (1995) was used. RAPD typing was carried out in a final volume of 25 µl consisting of 1 µl chromosomal DNA

(approximately 100 ng), 2 µl of primer D11344 (20 pmol/µl), 2.5 U of *Taq* DNA polymerase (Promega), 2.5 µl of 10 × buffer (Promega), 3 µl of 25 mM $MgCl_2$ buffer (Promega), and 0.2 mM deoxynucleoside triphosphates. The PCR program used was 4 cycles of [94 °C for 5 min, 40 °C for 5 min, 72 °C for 5 min] and then 30 cycles of [94 °C for 1 min, 55 °C for 1 min and 72°C for 2 min]. Of the PCR product 5 µl was run on a 2% (w/v) agarose gel. Strains that generated the same banding pattern were considered to be of the same clonal (RAPD) type.

5.2.3. *agr* typing.

The multiplex PCR primers of Lina *et al.* (2004) were used (Table 5.2 and Fig. 5.3). This method utilises a universal forward primer (*agr*1-4_{sa}-1) and four *agr* type-specific primers (*agr*1_{sa}-2, *agr*2_{sa}-2, *agr*3_{sa}-2 and *agr*4_{sa}-2). Each type generates a different sized *agr* type-specific PCR product. PCRs were carried out in a final volume of 25 µl consisting of 1 µl chromosomal DNA (approximately 100 ng), 2 µl of primer mix [made up of 40 µl of stock *agr*1-4_{sa}-1 (100pmol/µl), 20 µl *agr*2_{sa}-2 (100 pmol/µl), 20 µl *agr*3_{sa}-2 (100 pmol/µl) and 10 µl *agr*1_{sa}-2 and *agr*4_{sa}-2 (both at 100 pmol/µl)], 2.5 U of *Taq* DNA polymerase (Promega), 2.5 µl of 10 × buffer (Promega), 3 µl of 25 mM $MgCl_2$ buffer (Promega), and 0.2 mM deoxynucleoside triphosphates. The PCR reaction comprised an initial 5-min denaturation of 95 °C, followed by 25 cycles of [94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min] and a final extension of 72 °C for 10 min. The PCR products were checked by electrophoresis through a 1% (w/v) agarose gel at 90 V.

5.3. Results.

5.3.1. Multi-locus sequence typing.

All 123 isolates (120 of animal and 3 of human origin) were typeable by MLST. The data are presented in Table 5.3 and Fig. 5.7. The 120 animal isolates generated 39 types of which 18 were newly described types. A number of STs had more than one representative isolate: ST1, 3 (2 %); ST5, 10 (8%); ST8, 2 (1.6%); ST25, 2 (1.6%); ST71, 10 (8%); ST96, 4 (3%); ST97, 8 (6.5%); ST121, 3 (2%); ST126, 10 (8%); ST133, 28 (23%); ST151, 6 (5%); ST352, 3 (2%); ST522, 2 (1.6%), and a SLV of ST522, 4 (3%). The remaining STs, including novel STs, had only one representative strain in the collection of strains

Table 5.2. Sequences of *agr* multiplex primers from Lina *et al.* (2003) used in this work.

Gene	Primer	Primer sequence (5′–3′)	PCR Product (bp)
agr_{sa}	$agr1\text{-}4_{sa}\text{-}1$	ATG CAC ATG GTG CAC ATG C	
$agr\text{-}1_{sa}$	$agr1_{sa}\text{-}2$	GTC ACA AGT ACT TAT AAG CTG CGA T	439
$agr\text{-}2_{sa}$	$agr2_{sa}\text{-}2$	TAT TAC TAA TTG AAA AGT GCC ATA GC	572
$agr\text{-}3_{sa}$	$agr3_{sa}\text{-}2$	GTA ATG TAA TAG CTT GTA TAA TAA TAC CCA G	321
$agr\text{-}4_{sa}$	$agr4_{sa}\text{-}2$	CGA TAA TGC CGT AAT ACC CG	657

The accession numbers for the sequences of *agr* alleles *agr*-1*sa* to *agr*-4*sa* are X52543, AF001782, AF001783 and AF288215, respectively.

Table 5.3. MLST, RAPD typing, and *agr*-typing results for 123 strains from animals.

Code	Strain	Host	Country	*arcC*	*aroE*	*glpF*	*gmk*	*pta*	*tpi*	*yqiL*	ST	RAPD	*agr*
DS71	DS71	Cat	Sweden	13(99.8)	13	1	1	12	11	13	15slv	9	2
DS72	496	Chicken	N. Ireland	1	1	1	1	1	1	1	1	1	3
DS67	72	Chicken	N. Ireland	1	4	1	4	12	1	10	5	2	2
DS63	47	Chicken	N. Ireland	1	4	1	4	12	1	10	5	2	2
DS55	11	Chicken	N. Ireland	1	4	1	4	12	1	10	5	2	2
DS56	18	Chicken	N. Ireland	1	4	1	4	12	1	10	5	2	2
DS58	31	Chicken	N. Ireland	1	4	1	4	12	1	10	5	2	2
DS60	39	Chicken	N. Ireland	1	4	1	4	12	1	10	5	2	2
DS61	70	Chicken	N. Ireland	1	4	1	4	12	1	10	5	2	2
DS66	589	Chicken	N. Ireland	1	4	1	4	12	1	10	5	2	2
DS62	34	Chicken	N. Ireland	1	4	1	4	12	1	10	5	2	2
DS65	564	Chicken	N. Ireland	1	4	1	4	12	1	10	5	2	2
DS68	82	Chicken	N. Ireland	12	89	1	1	4	5	7(99.4)	Unknown	39	1
DS40	CH498	Cow	Argentina	1	1	1	1	1	1	1	1	1	3
MSA1006	MSA1006	Cow	USA	3	3	1	1	4	4	3	8	3	1
DS37	V299	Cow	Spain	4	9	1	8	1	10	8	20	4	1
MSA1363	MSA1363	Cow	USA	4	1	4	1	5	5	4	25	6	1
RF79	RF79	Cow	Ireland	18	1	1	1	1	5	3	71	11	1

Code	Strain	Host	Country	*arcC*	*aroE*	*glpF*	*gmk*	*pta*	*tpi*	*yqiL*	ST	RAPD	*Agr*
RF103	RF103	Cow	Ireland	18	1	1	1	1	5	3	71	11	1
RF105	RF105	Cow	Ireland	18	1	1	1	1	5	3	71	11	1
RF123	RF123	Cow	Ireland	18	1	1	1	1	5	3	71	11	1
RF104	RF104	Cow	Ireland	18	1	1	1	1	5	3	71	11	1
RF291	RF291	Cow	Ireland	18	1	1	1	1	5	3	71	11	1
DS38	V315	Cow	Spain	18	1	1	1	1	5	3	71	11	1
MSA1369	MSA1369	Cow	USA	18	1	1	1	1	5	3	71	11	1
RF26	RF26	Cow	Ireland	3	1	1	1	1	5	3	97	16	1
RF31a	RF31a	Cow	Ireland	3	1	1	1	1	5	3	97	16	1
RF28	RF28	Cow	Ireland	3	1	1	1	1	5	3	97	16	1
RF116	RF116	Cow	Ireland	3	1	1	1	1	5	3	97	16	1
RF117	RF117	Cow	Ireland	3	1	1	1	1	5	3	97	16	1
RF115	RF115	Cow	Ireland	3	1	1	1	1	5	3	97	16	1
MSA915	MSA915	Cow	USA	3	1	1	1	1	5	53	115	16	1
DS36	V329	Cow	Spain	3	68	1	4	1	5	40	126	19	2
MSA948	MSA948	Cow	USA	3	68	1	4	1	5	40	126	19	2
MSA951	MSA951	Cow	USA	3	68	1	4	1	5	40	126	19	2
MSA1401	MSA1401	Cow	USA	3	68	1	4	1	5	40	126	19	2
MSA1521	MSA1521	Cow	USA	3	68	1	4	1	5	40	126	20	2

Code	Strain	Host	Country	*arcC*	*aroE*	*glpF*	*gmk*	*pta*	*tpi*	*yqiL*	ST	RAPD	*agr*
RF283	RF283	Cow	Ireland	6	66	46	2	7	50	18	133	23	1
RF286	RF286	Cow	Ireland	6	66	46	2	7	50	18	133	23	1
RF287	RF287	Cow	Ireland	6	66	46	2	7	50	18	133	23	2
RF285	RF285	Cow	Ireland	6	66	46	2	7	50	18	133	23	1
RF282	RF282	Cow	Ireland	6	66	46	2	7	50	18	133	23	1
MSA916	MSA916	Cow	USA	6	66	46	2	7	50	18	133	24	2
RF289	RF289	Cow	Ireland	6	66	46	2	7	50	18	133	25	1
DS69	DS69	Cow	Sweden	6	66	46	2	7	50	18	133	26	1
RF124	RF124	Cow	Ireland	6	72	12	43	49	67	59	151	33	1
RF80	RF80	Cow	Ireland	6	72	12	43	49	67	59	151	33	1
RF113	RF113	Cow	Ireland	6	72	12	43	49	67	59	151	33	1
RF114	RF114	Cow	Ireland	6	72	12	43	49	67	59	151	33	1
RF122	RF122	Cow	Ireland	6	72	12	43	49	67	59	151	33	1
RF121	RF121	Cow	Ireland	6	72	12	43	49	67	59	151	33	2
MSA1011	RF1011	Cow	USA	3	78	1	1	1	5	3	352	16	2
MSA961	MSA961	Cow	USA	3	78	1	1	1	5	3	352	16	2
MSA1003	MSA1003	Cow	USA	3	78	1	1	1	5	3	352	16	2
DS42	CH505	Cow	Argentina	52	79	54	18	56	32	65	479	40	1
MSA17.1	MSA17.1	Cow	USA	3	1(99.8)	1	37	1	5	3	124slv	35	1
MSA1468	MSA1468	Cow	USA	3	68	1	4	1	5	40(99.8)	126slv	21	1

Code	Strain	Host	Country	*arcC*	*aroE*	*glpF*	*gmk*	*pta*	*tpi*	*yqiL*	ST	RAPD	*agr*
MSA13.1	MSA13.1	Cow	USA	6	66	46	2(99.8)	7(99.8)	50	18	133sat	31	1
DS35	1A	Cow	Spain	6	66	46	19	7	50	18	133slv	31	1
MSA1047	MSA1047	Cow	USA	6	87	51	47	7	70	61	350slv	34	2
DS70	DS70	Cow	Sweden	3	78	1	1	1	5	3(99.8)	352dlv	16	1
PSA5	PSA5	Cow	USA	16	16(99.8)	12	2	13	13	15	50slv	15	4
MSA1007	MSA1007	Cow	USA	1	4	1(99.8)	4	12	1	10	5slv	2	2
MSA1547	MSA1547	Cow	USA	1	89	1	15	1	4	3	72sat	36	3
DS41	CH501	Cow	Argentina	3	1	1	1	1(99.8)	5	62(99.0)	97dlv	44	1
DS30	St153	Goat	Italy	7	6	1	5	8	8	6	22	5	1
DS27	St125	Goat	Italy	4	1	4	1	5	5	4	25	8	1
DS25	St24	Goat	Italy	2	2	2	2	2	2	2	39	7	3
DS1	DS1	Goat	Austria	18	1	1	1	1	5	3	71	11	1
DS29	St51	Goat	Italy	3	1	14	15	11	19	3	101	17	1
DS7	St57	Goat	Italy	3	68	1	4	1	5	40	126	19	2
DS9	St60	Goat	Italy	3	68	1	4	1	5	40	126	19	2
DS11	St108	Goat	Italy	3	68	1	4	1	5	40	126	19	2
DS6	St31	Goat	Italy	3	68	1	4	1	5	40	126	21	2
DS18	St36	Goat	Italy	3	68	1	4	1	5	40	126	22	2
DS14	St34	Goat	Italy	6	66	46	2	7	50	18	133	25	1

Code	Strain	Host	Country	*arcC*	*aroE*	*glpF*	*gmk*	*pta*	*tpi*	*yqiL*	ST	RAPD	*agr*
DS16	St144	Goat	Italy	6	66	46	2	7	50	18	133	25	1
DS13	St7	Goat	Italy	6	66	46	2	7	50	18	133	25	1
DS15	St62	Goat	Italy	6	66	46	2	7	50	18	133	25	1
DS74	1563-4	Goat	Norway	6	66	46	2	7	50	18	133	26	1
DS28	St152	Goat	Italy	6	66	46	2	7	50	18	133	26	1
DS75	1589-1	Goat	Norway	6	66	46	2	7	50	18	133	27	1
DS73	1518-2	Goat	Norway	6	66	46	2	7	50	18	133	27	1
DS76	1589-2	Goat	Norway	6	66	46	2	7	50	18	133	27	1
DS78	213-8	Goat	Norway	6	66	46	2	7	50	18	133	28	1
DS77	213-10	Goat	Norway	6	66	46	2	7	50	18	133	28	1
DS81	243-7	Goat	Norway	6	66	46	2	7	50	18	133	29	1
DS2	DS2	Goat	Austria	6	66	46	2	7	50	18	133	31	1
DS80	232-11	Goat	Norway	6	57	63	2	7	58	52	480	37	1
DS4	DS4	Goat	Austria	18	95	45	2	7	15	5	522	41	1
DS17	St11	Goat	Italy	18	95	45	2	7	15	5	522	42	1
DS24	St16	Goat	Italy	6	57	45	2	7	58(99.8)	52	130slv	37	3
DS3	DS3	Goat	Italy	6	66(99.8)	46	2	7	50	18	133slv	29	1
DS12	St134	Goat	Italy	3	95(99.8)	45	2	7	15	5	522dlv	43	1
DS20	St66	Goat	Italy	18	95(99.8)	45	2	7	15	5	522slv	42	1

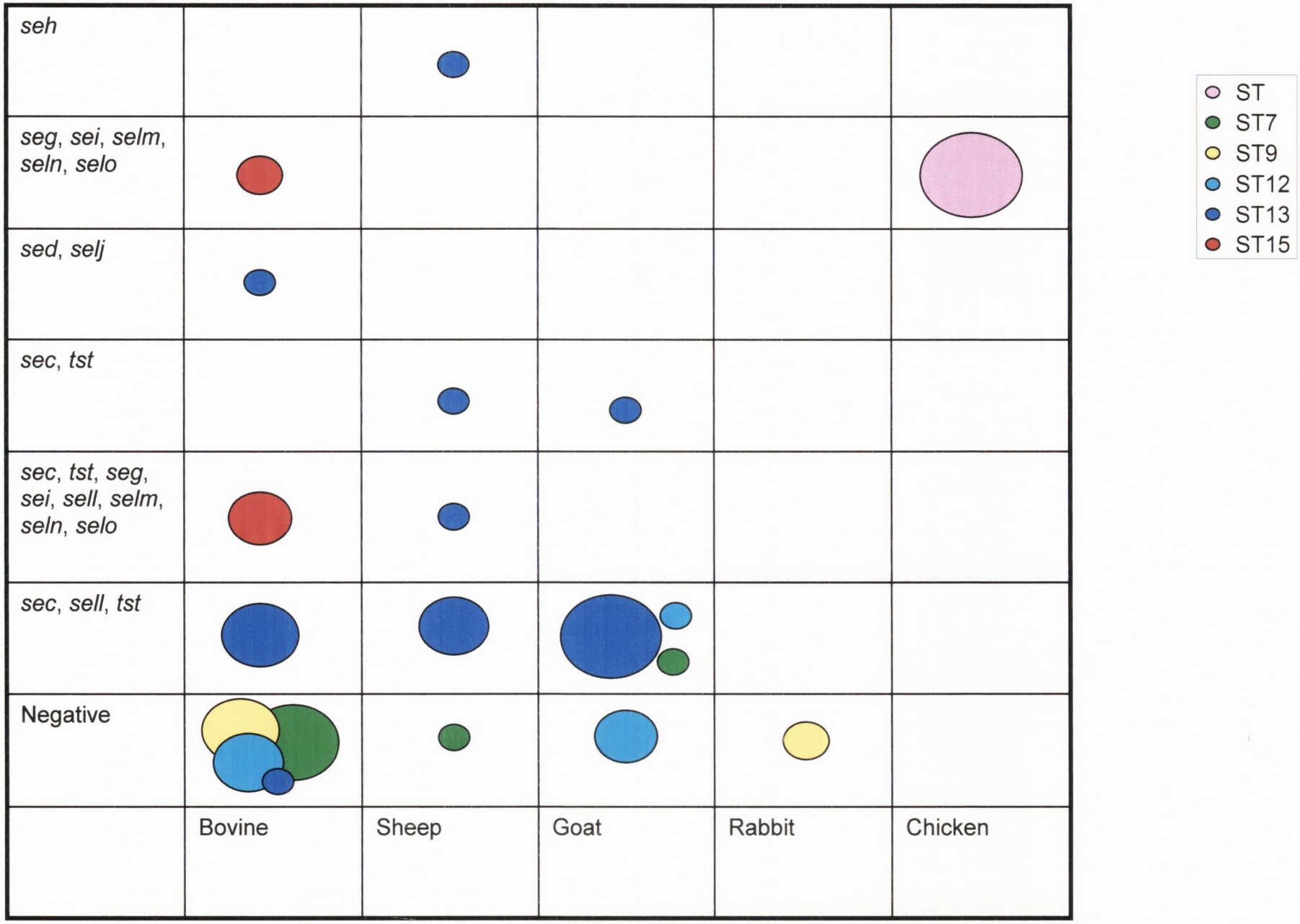
Code	Strain	Host	Country	*arcC*	*aroE*	*glpF*	*gmk*	*pta*	*tpi*	*yqiL*	ST	RAPD	*agr*
DS19	St65	Goat	Italy	18	95(99.8)	45	2	7	15	5	522slv	42	1
DS23	St140	Goat	Italy	18	95(99.8)	45	2	7	15	5	522slv	42	1
DS21	St67	Goat	Italy	18	95(99.8)	45	2	7	15	5	522slv	42	1
MSA2120	MSA2120	Human	USA	4	9	1	8	1	10	8	20	4	1
MSA2335	MSA2335	Human	USA	2	2	2	2	2	2	2	39	10	3
MSA2885	MSA2885	Human	USA	2	2	2	48	2	3	2	30dlv	14	3
DS51	KH365	Rabbit	Belgium	1	1	1	1	1	1	1	1	1	3
DS47	KH275	Rabbit	Belgium	3	3	1	1	4	4	3	8	3	1
DS48	KH21	Rabbit	Belgium	12	1	1	15	11	1	40	96	12	3
DS52	KH15	Rabbit	Belgium	12	1	1	15	11	1	40	96	12	3
DS34	PS21	Rabbit	Spain	12	1	1	15	11	1	40	96	12	3
DS32	PS17	Rabbit	Spain	12	1	1	15	11	1	40	96	13	3
DS54	KH16	Rabbit	Belgium	3	1	1	1	1	5	3	97	16	1
DS50	KH17	Rabbit	Belgium	3	1	1	1	1	5	3	97	16	1
DS53	KH513	Rabbit	Belgium	6	5	6	2	7	14	5	121	18	4
DS46	KH454	Rabbit	Belgium	6	5	6	2	7	14	5	121	18	4
DS31	PS15	Rabbit	Spain	6	5	6	2	7	14	5	121	18	4
DS33	PS19	Rabbit	Spain	3	3	57	1	4	4	67	407	3	1
DS96	2	Sheep	Denmark	18	1	1	1	1	5	3	71	32	1

Code	Strain	Host	Country	*arcC*	*aroE*	*glpF*	*gmk*	*pta*	*tpi*	*yqiL*	ST	RAPD	*Agr*
DS90	895-1	Sheep	Norway	6	66	46	2	7	50	18	133	29	1
DS83	891-1	Sheep	Norway	6	66	46	2	7	50	18	133	29	1
DS88	571-2	Sheep	Norway	6	66	46	2	7	50	18	133	29	1
DS89	586-2	Sheep	Norway	6	66	46	2	7	50	18	133	29	1
DS105	2463	Sheep	Iceland	6	66	46	2	7	50	18	133	30	1
DS104	2114	Sheep	Iceland	6	66	46	2	7	50	18	133	30	1
DS87	681-2	Sheep	Norway	6	66	46	2	7	50	18	133	31	1
DS102	6659-2	Sheep	Sweden	6	72	50	43	49	67	59	151slv	33	2
DS95	1	Sheep	Denmark	3(99.8)	3	1	1	1	1	10	9slv	38	2

Numbers in brackets indicate the percentage identity of the animal strain allele to the reference allele in the database.

Fig. 5.7. Graphical representation of the toxin and MLST types of the major animal-associated clonal types.

The size of the circles is representative of the number of strains of each toxin and MLST type. It is apparent that MLST ST133 was the most widely distributed ST type amongst the animal strains being present in cows, sheep and goats. This ST also showed the greatest number of varying toxin types.


seh
seg, sei, selm, seln, selo
sed, selj
sec, tst
sec, tst, seg, sei, sell, selm, seln, selo
sec, sell, tst
Negative
Bovine
Sheep
Goat
Rabbit
Chicken
ST
ST7
ST9
ST12
ST13
ST15

examined. MLST type ST133, the commonest type, was only found in strains from sheep [7 (5.7%)], goats [13 (10.5%)] and cows [8 (6.5%)].

The twelve chicken isolates generated three sequence types (STs), namely ST1, ST5 and a novel type (ST687) accounting for 1 (8.3%), 10 (83%) and 1 (8.3%) of the strains, respectively. The 52 bovine-associated strains generated 22 STs; ST71, ST97, ST126, ST133, ST151 and a ST352 accounted for 8 (15.4%), 6 (11.5%), 5 (9.6%), 8 (15.4%), 6 (12%) and 3 (5.8%) of the bovine strains, respectively. The remaining 16 bovine isolates belonged to individual STs, 10 of which were novel STs (ST669, ST670, ST672, ST673, ST678, ST679, ST680, ST683, ST684 and ST685; as designated in Table 5.4). The 33 goat-associated strains generated 13 STs of which ST126, ST133, ST522 and a SLV of ST522 (subsequently designated as ST682) accounted for 5 (15.2%), 13 (39.4%), 2 (6.1%) and 4 (12.1%) of the goat strains, respectively. The remaining 9 goat-associated strains generated individual STs, 3 of which were novel STs (ST671, ST674 and ST681). The 12 rabbit-associated strains generated 6 STs with ST96, ST97 and ST121 accounting for 4 (33.3%), 2 (16.7%) and 3 (25%) of the strains, respectively. The remaining 3 strains generated individual STs, none of which were novel. The 10 sheep-associated isolates generated 4 STs, with ST133 accounting for 7 (70%) of the isolates. The remaining 3 isolates generated individual STs, two of which were novel STs (ST675 and ST686). The cat isolate and 3 human isolates generated 4 STs, the ST of the cat isolate (ST676) and that of one of the human isolates (ST677) being novel .

Novel alleles and STs were given numbers that carried on the sequence of numbers given to MLST alleles and STs in the database. These new allele numbers and STs are displayed in Table 5.4. Five of the 19 new STs had new combinations of known alleles at the time these studies commenced. Fifteen new alleles were described from the gene sequencing data – 2 *arcC*, 4 *aroE*, 1 *glpF*, 1 *gmk*, 2 *pta*, 1 *tpi* and 4 *yqiL* alleles. Of the remaining 14 new STs, 12 possessed single new alleles and two possessed 2 new alleles (ST672 – *gmk67*, *pta90* and ST685 – *pta91* and *yqiL88*).

eBURST was performed (using 5 identical loci out of 7 to assign relationships) on the 687 STs, 668 STs of which were already in the MLST database and 19 novel STs identified herein. eBURST generated 34 groups from 654 STs, with groups 1, 2, 3, 4, 5 and 6 accounting for 314 STs (45.7%), 80 (11.6%), 43 (6.3%), 21 (3.1%), 16 (2.3%) and 14 (2.0%) of the total STs, respectively Thirty-three singletons (4.8%) were identified; these

Table 5.4. Novel MLST allele numbers and novel STs of animal-associated *S. aureus* identified in this work.

Code	Strain	Host	Country	*arcC*	*aroE*	*glpF*	*gmk*	*pta*	*tpi*	*yqiL*	Novel ST
DS71	DS71	Cat	Swe	83[a]	13	1	1	12	11	13	676
DS68	82	Chicken	N. Ire	12	89	1	1	4	5	89[a]	687
MSA17.1	MSA17.1	Cow	USA	3	124[a]	1	37	1	5	3	669
MSA1468	MSA1468	Cow	USA	3	68	1	4	1	5	86[a]	670
MSA13.1	MSA13.1	Cow	USA	6	66	46	67[a]	90[a]	50	18	672
DS35	1A	Cow	Sp	6	66	46	19	7	50	18	673
MSA1047	MSA1047	Cow	USA	6	87	51	47	7	70	61	678
DS70	DS70	Cow	Swe	3	78	1	1	1	5	87[a]	679
PSA5	PSA5	Cow	USA	16	126[a]	12	2	13	13	15	680
MSA1007	MSA1007	Cow	USA	1	4	93[a]	4	12	1	10	683
MSA1547	MSA1547	Cow	USA	1	89	1	15	1	4	3	684
DS41	CH501	Cow	Arg	3	1	1	1	91[a]	5	88[a]	685
DS24	St16	Goat	It	6	57	45	2	7	93[a]	52	671
DS3	DS3	Goat	It	6	125[a]	46	2	7	50	18	674
DS12	St134	Goat	It	3	127[a]	45	2	7	15	5	681
DS20	St66	Goat	It	18	127[a]	45	2	7	15	5	682
DS19	St65	Goat	It	18	127[a]	45	2	7	15	5	682

DS23	St140	Goat	It	18	127[a]	45	2	7	15	5	682
DS21	St67	Goat	It	18	127[a]	45	2	7	15	5	682
MSA2885	MSA2885	Human	USA	2	2	2	48	2	3	2	677
DS102	6659-2	Sheep	Swe	6	72	50	43	49	67	59	675
DS95	1	Sheep	Den	84[a]	3	1	1	1	1	10	686

[a] denotes novel alleles identified in this work

are STs with no SLVs or DLVs in the database. The output of eBURST analyses is shown in Figures 5.8, 5.9 and 5.10.

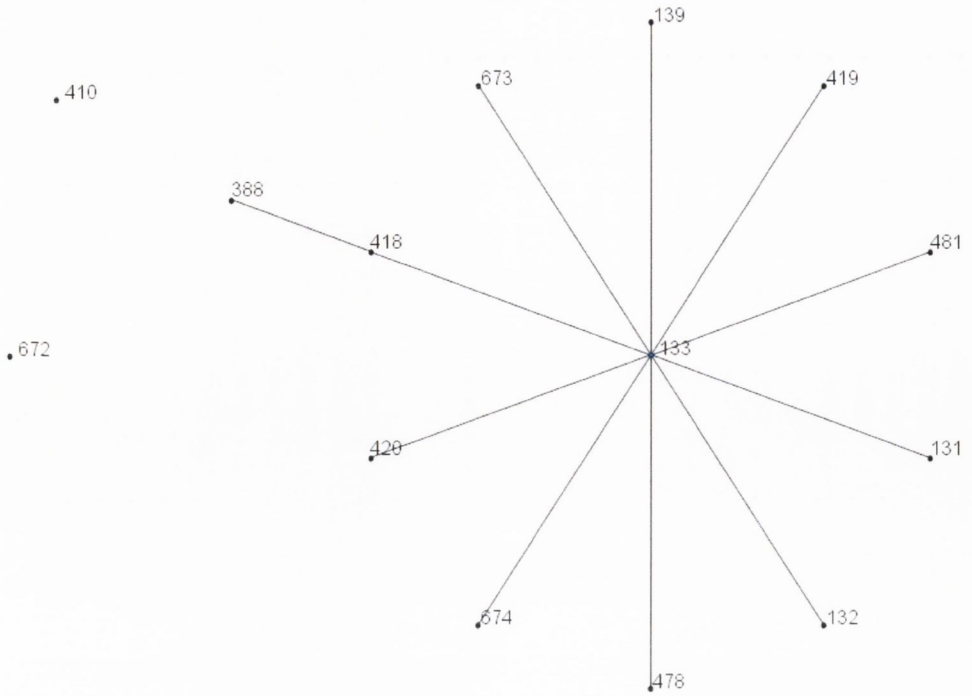
5.3.2. RAPD typing.

All strains were typeable by RAPD typing. This revealed 44 RAPD types amongst the 123 isolates examined (Fig. 5.11 and 5.12). In all, 23 RAPD types were represented by single strains. The most prevalent RAPD types were: RAPD types 2, 11 strains (8.9%); 11, 9 strains (6.3%); 16, 13 strains (10.6%); 19, 7 strains (7.3%); 29, 6 strains (4.9%), and 33, 7 strains (5.7%). These six types accounted for 53 (43%) of the total strains.

The twelve chicken isolates comprised 3 RAPD types; RAPD type 2 accounted for 10 (83.3%) of the chicken-associated isolates, the remaining two isolates generated individual RAPD types. The 52 bovine-associated isolates were broken down into 22 RAPD types with RAPD types 11, 16 and 4 accounting for 8 (15.4%), 11 (21.2%) and 19 (7.7%) of the bovine strains, respectively. The remaining bovine strains yielded individual RAPD types. The 33 goat strains fell into 18 types: RAPD type 19, 3 strains (9.1%); 25, 4 strains (12.1%); 26, 2 strains (6.1%); 27, 3 strains (9.1%); 28, 2 strains (6.1%); 29, 2 strains (6.1%); 37, 2 strains (6.1%), and 42, 5 strains (15.2%). The remaining goat strains generated individual RAPD types. The twelve rabbit-associated strains belonged to 6 RAPD types, of which RAPD types 3, 12, 16 and 18 accounted for 2 (16.7%), 3 (25%), 2 (16.7%) and 3 (25%) of the strains, respectively. The remaining two rabbit strains had individual RAPD types. The 10 sheep-associated isolates were of 6 RAPD types. RAPD types 29 and 30 accounted for 4 (40%) and 2 (20%) of the rabbit strains, respectively. The remaining rabbit strains possessed individual RAPD types. The one cat and 3 human-associated strains generated individual RAPD types.

5.3.3. *agr* typing.

All isolates were typeable by *agr* typing (Fig. 5.13 and 5.14). The isolates were predominately of *agr* type 1 [74 strains (60.2%)] and *agr* type 2 [33 strains (26.8%)], but several strains of *agr* type 3 [12 strains (9.8%)] and *agr* type 4 [4 strains (3.3%)] were identified.



	arcC	*aroE*	*glpF*	*gmk*	*pta*	*tpi*	*yqiL*
ST133	6	66	46	2	7	50	18
ST131	40	66	46	2	7	50	18
ST132	6	67	46	2	7	50	18
ST410	6	66	46	43	7	14	18
ST672	6	66	46	67	90	50	18

Fig. 5.8. eBURST-generated clonal complex containing the major animal-associated ST 133.

MLST type ST133 was the most prevalent ST identified in this work accounting for 23% of the strains and forms the primary founder of this group. It was associated with cows, goats and sheep. The table shows the allelic profiles of some of the STs in the complex. This group also contains ST131 and ST132, SLVs of ST133 which have previously been shown to be associated with cows. ST672 (from a cow), ST673 (from a cow) and ST674 (from a goat) are novel STs identified in this study. ST410 and ST672 are DLVs of ST133.

Fig. 5.9. eBURST-generated clonal groups containing the major animal-associated STs 1, 5, 8, 25, 71 and 97.

MLST type ST5 is the predicted primary founder of this group of clonal complexes. It associates with a large number of other significant clonal complexes and subgroups containing animal-associated STs. All the STs of interest have been pulled out so they can be easily viewed. The major animal-associated STs 1, 5, 8, 25, and 97 are founders of clonal complexes (blue dots) and subgroups in the case of ST71 (yellow dot).

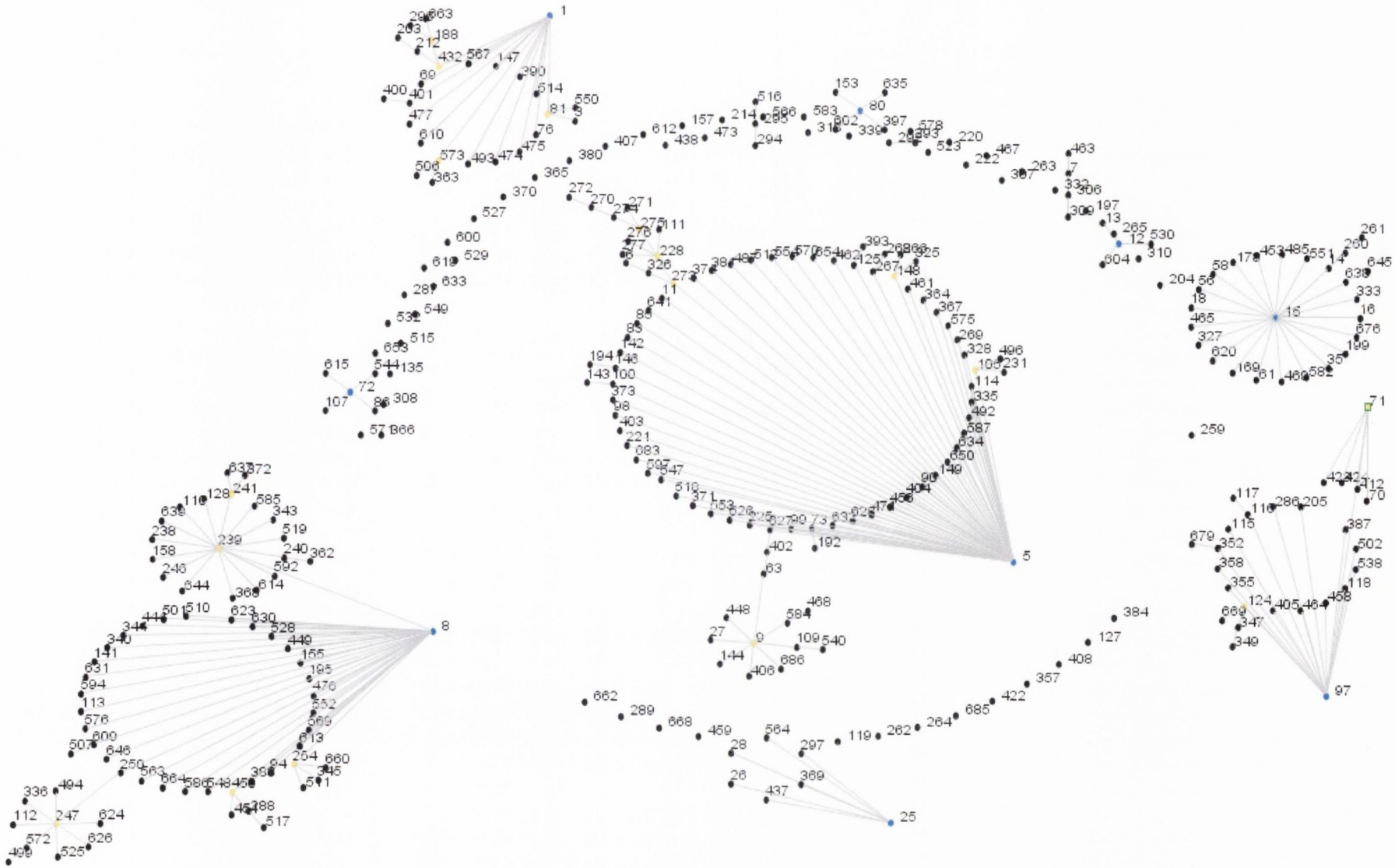
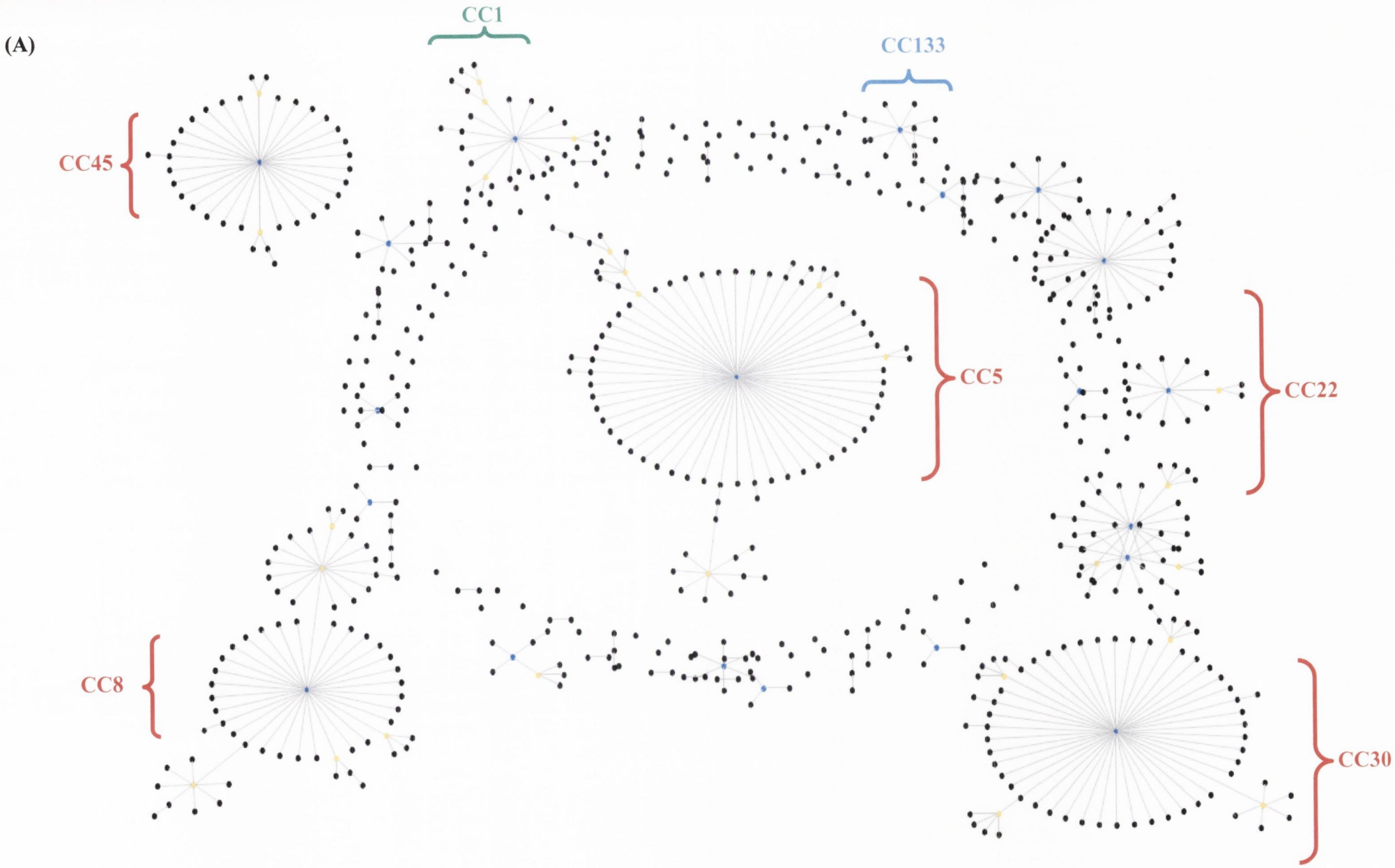


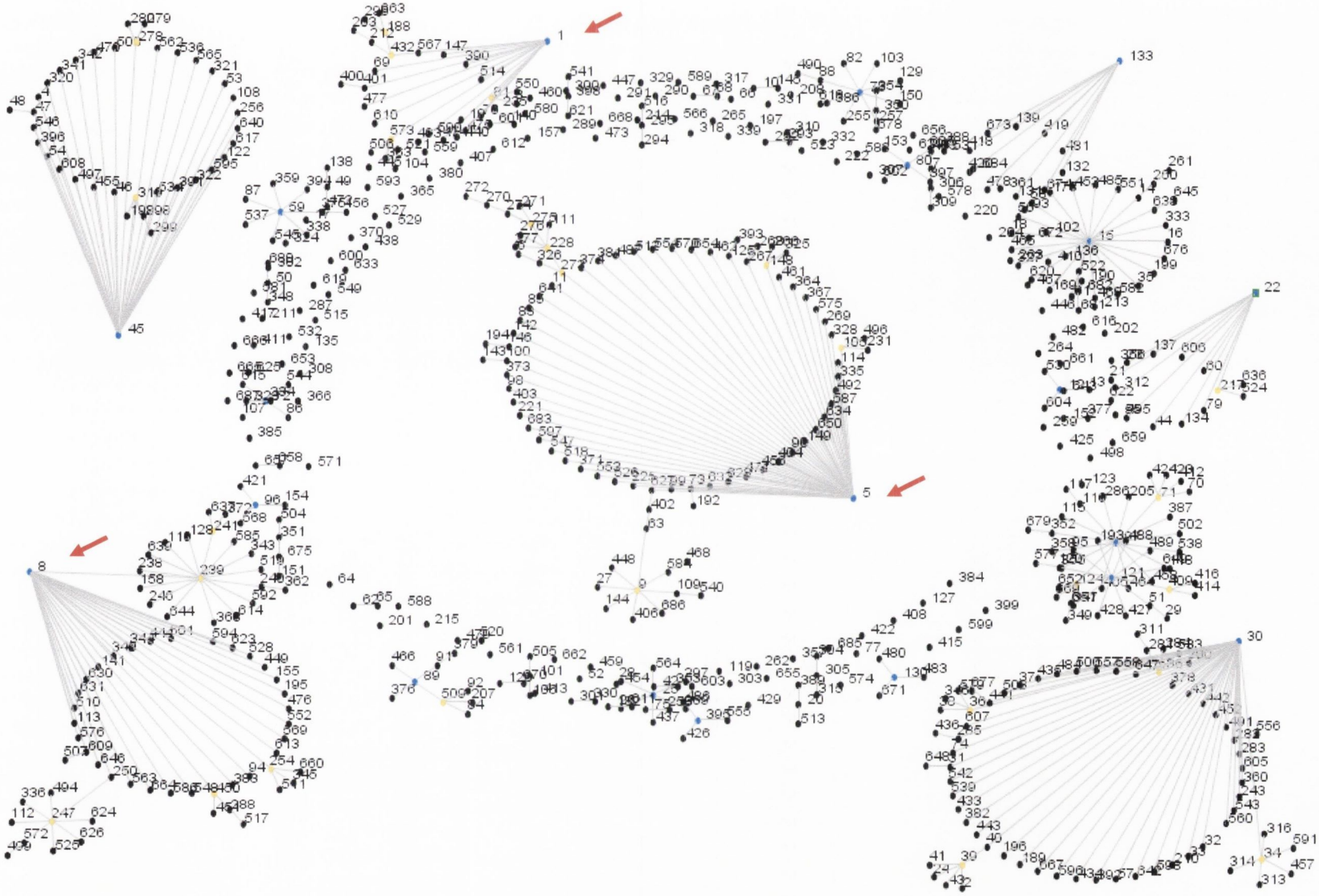
Fig. 5.10. Population snapshot of all 687 STs using eBURST.

These figures were generated in eBURST allowing 0 out of 7 loci for group definition.

Panel (A) shows all 687 STs represented as dots. Primary founders are represented by blue dots and subgroup founders by yellow dots. The major MRSA-containing clonal complexes (CC) are indicated by red brackets. The major animal-associated clonal complex containing ST133 is highlighted by blue brackets.

Panel (B) shows the same snapshot as panel (A) with the ST numbers intact and the primary founders pulled out to help in viewing. Animal-associated STs are found in clonal complexes CC5 (ST5, ST683), CC1 (ST1) and CC8 (ST8) [arrowed].


(A)
CC1
CC133
CC45
CC5
CC22
CC8
CC30



(B)

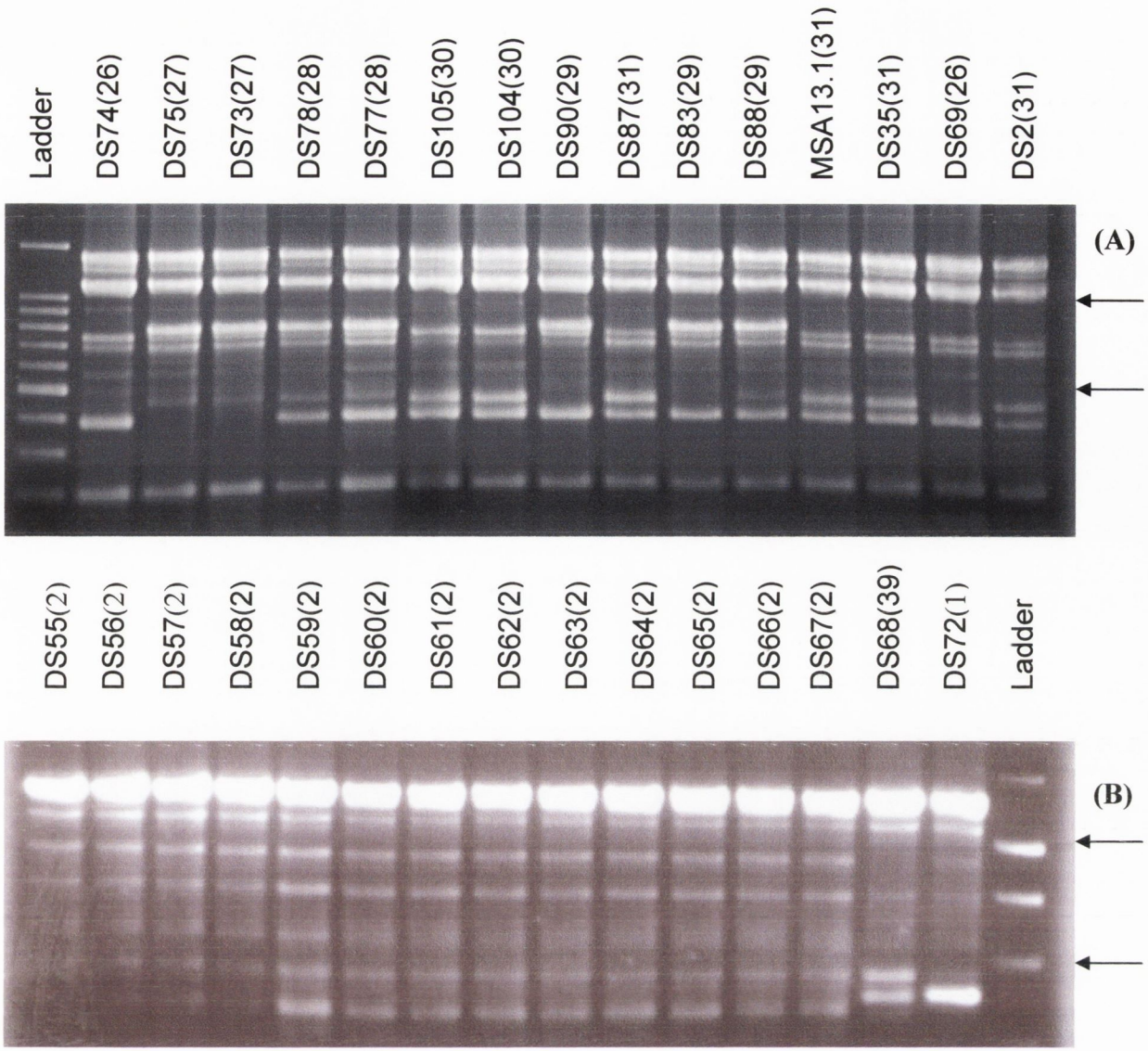
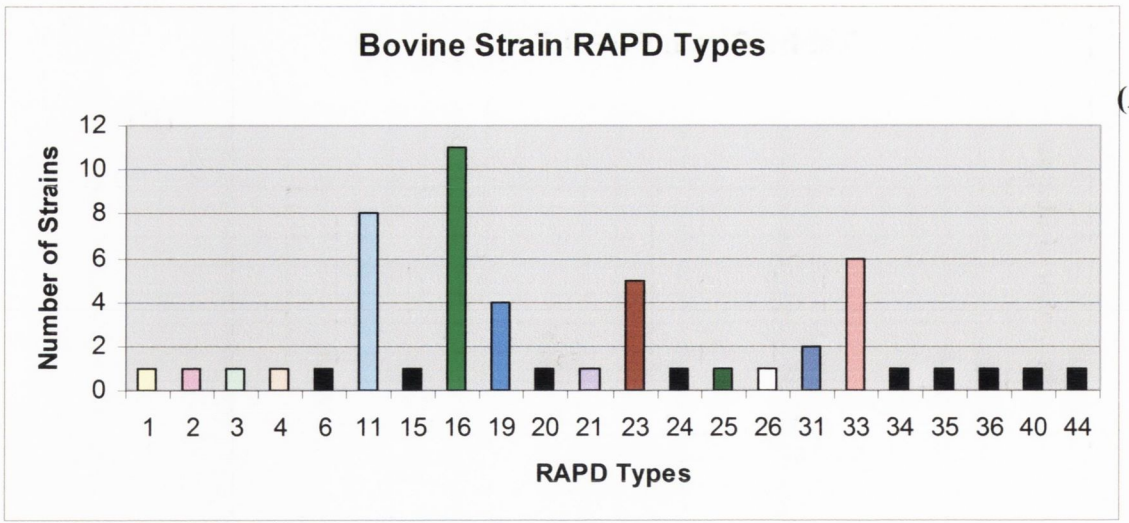


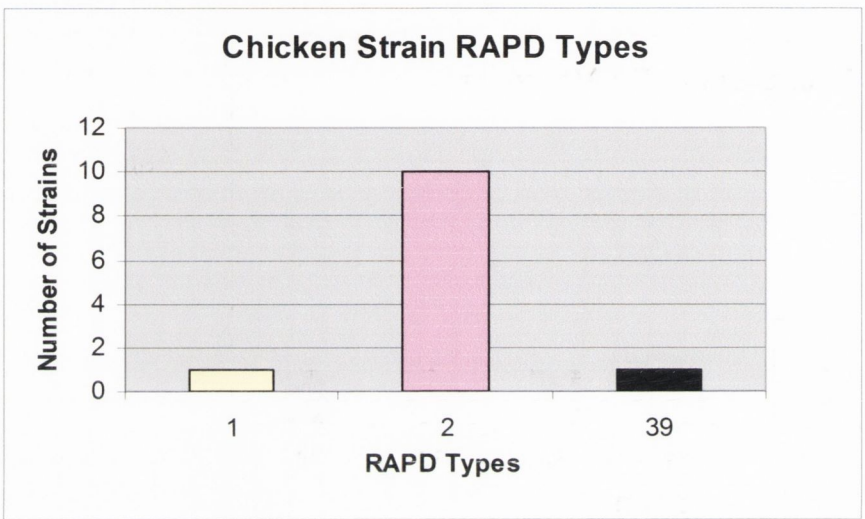
Fig. 5.11. RAPD typing of animal-associated *S. aureus*.

Panel (A) shows the RAPD profiles of strains from goats, sheep and cows. These strains were of the same MLST type, ST133, except strain MSA13.1 (cow) and strain DS35 (cow) which are variants of ST133. Many different RAPD types were observed. The RAPD types are indicated after the strain names in brackets.

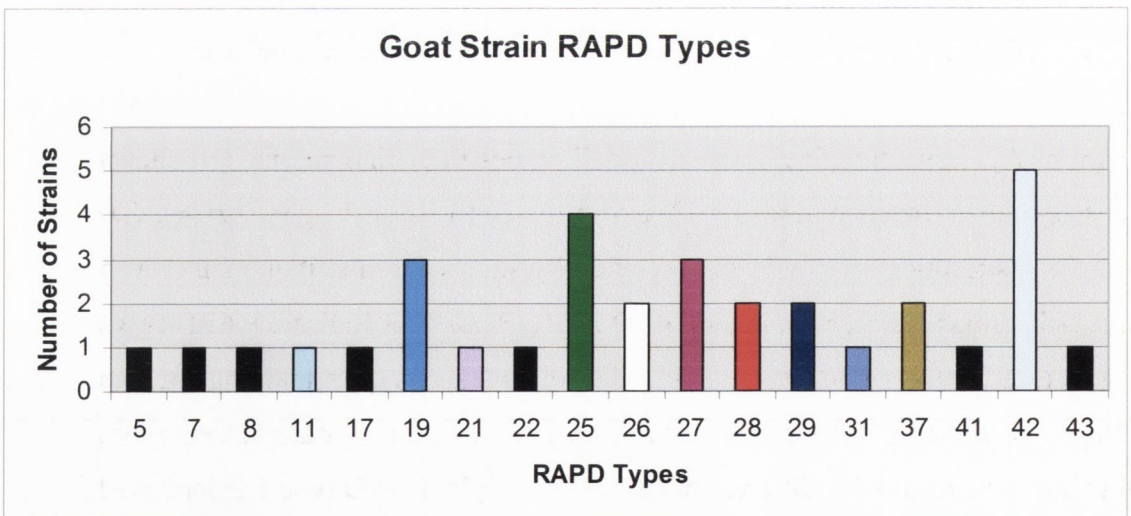
Panel (B) shows the RAPD profiles of chicken-associated *S. aureus*. These strains were of the same MLST type, ST5, except strains DS68 and DS72. The RAPD types are indicated in brackets after the strain name. The arrows indicate the position of the 1000-bp band (upper arrow) and the 500-bp band (lower arrow) in the DNA ladder. MLST had a lower discriminatory power than RAPD typing. This may be due to the fact that MLST measures the variability of seven housekeeping genes, whereas RAPD samples the variability of the whole genome.


Bovine Strain RAPD Types
Number of Strains
0
2
4
6
8
10
12
1 2 3 4 6 11 15 16 19 20 21 23 24 25 26 31 33 34 35 36 40 44
RAPD Types

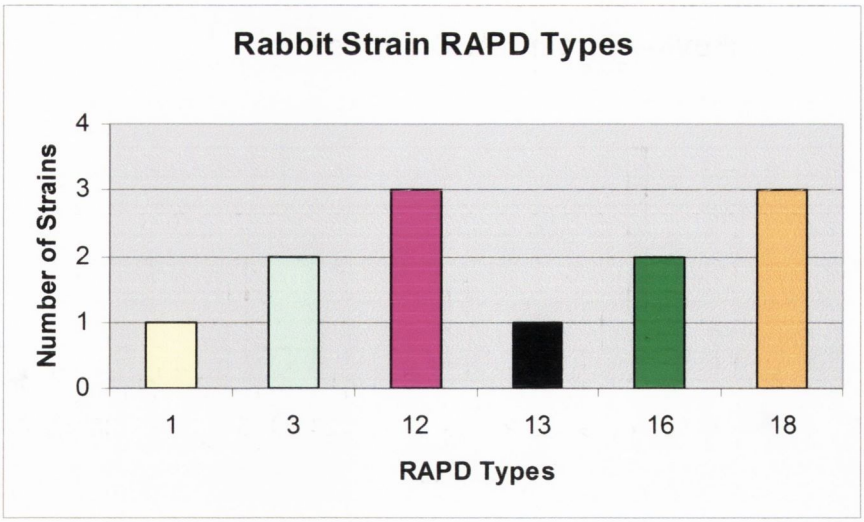
(A)


Chicken Strain RAPD Types
Number of Strains
0
2
4
6
8
10
12
1 2 39
RAPD Types

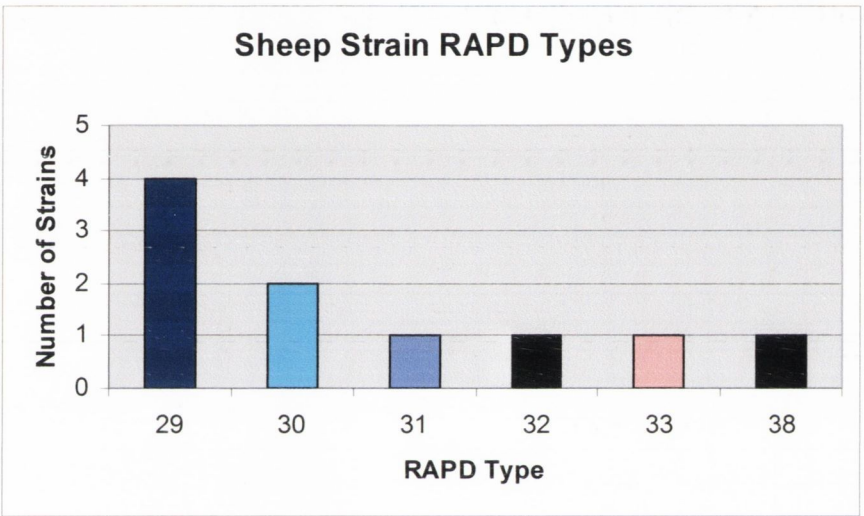
(B)


Goat Strain RAPD Types
Number of Strains
0
1
2
3
4
5
6
5 7 8 11 17 19 21 22 25 26 27 28 29 31 37 41 42 43
RAPD Types

(C)



(D)



(E)

Fig. 5.12. Distribution of RAPD types amongst strains of animal origin.

Histogram (A) shows the distribution of RAPD types in bovine strains, (B) shows the distribution in chicken strains, (C) shows the distribution in goat strains, (D) shows the distribution in rabbit strains, and (E) shows the distribution in sheep strains. All strains were typeable by RAPD PCR. Black bars indicate RAPD types that were represented by only one strain. The colors used on the remaining bars are specific to each RAPD type and help to display the fact that certain RAPD types were found associated with different hosts. For example, RAPD type 1 is indicated by light yellow bars and this type was represented by a rabbit-, chicken- and a bovine-associated strain.

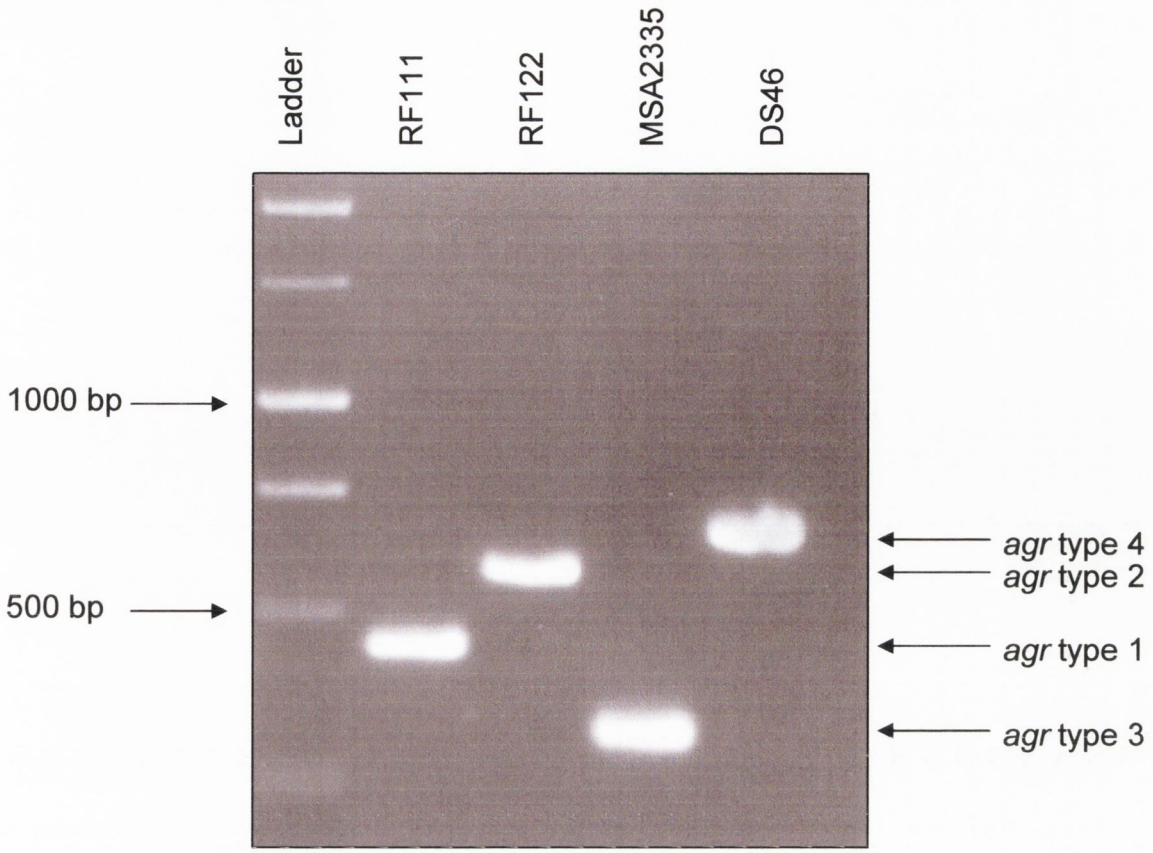


Fig. 5.13. *agr* typing of animal-associated *S. aureus*.

Examples of each *agr* type amplified using animal-associated strain DNA. Strain RF111 is of *agr* type 1, strain RF122 is of *agr* type 2, strain MSA2335 is of *agr* type 3 and strain DS46 is of *agr* type 4. Only bovine strains had examples of all *agr* types.

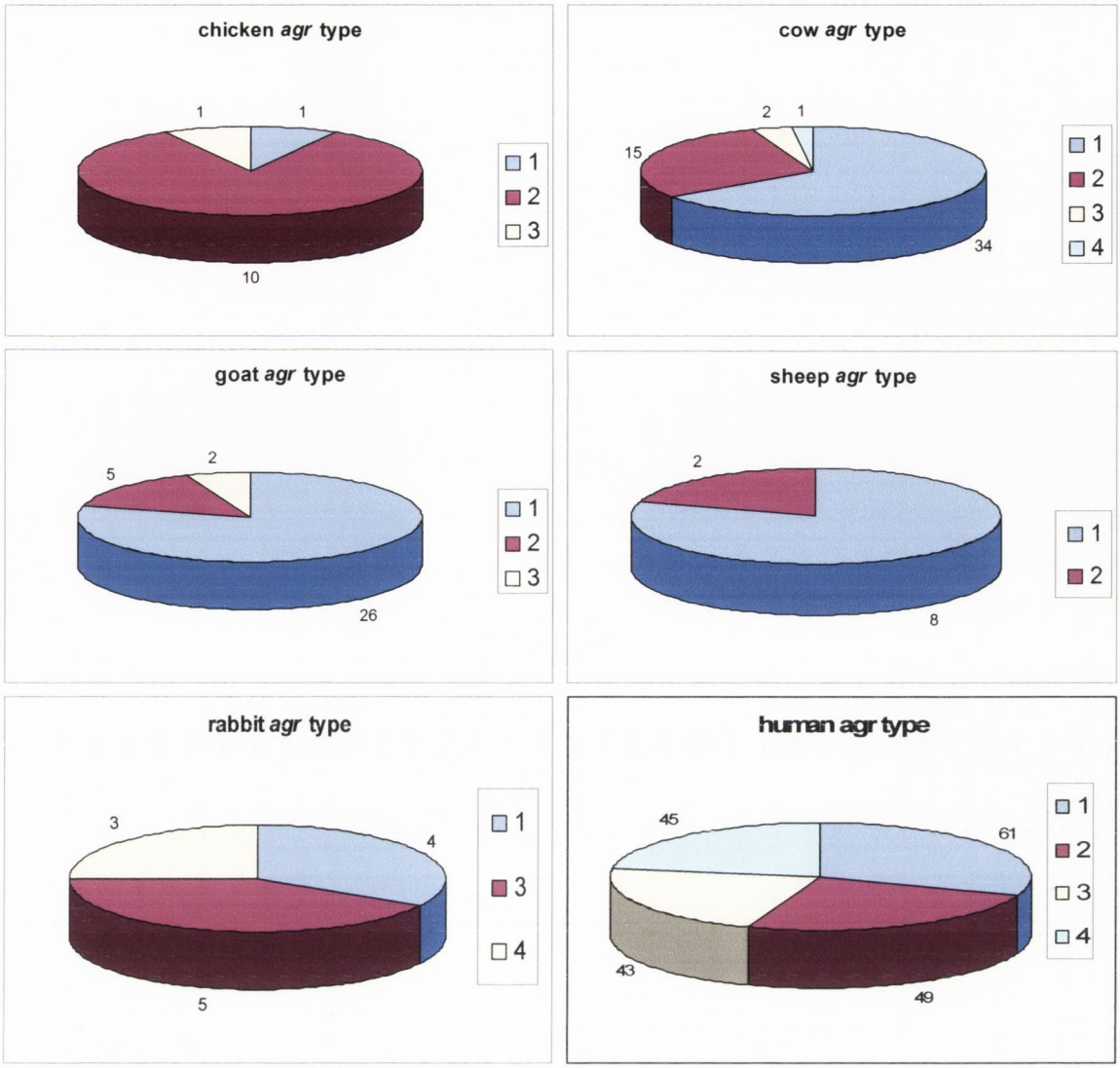


Fig. 5.14. Distribution of *agr* types amongst *S. aureus* of animal and of human origin.

Of the animal–associated strains only the bovine strains had representative isolates of all four *agr* types. The cow, sheep and goat strains were mostly *agr* type 1 (65.4%, 80% and 78.8% respectively). The chicken isolates were mostly *agr* type 2 (83.3%). The rabbit isolates had an almost equal distribution of *agr* types 1, 2 and 3 (33.3%, 41.7%, and 25%, respectively). The number of strains of each *agr* type is indicated beside each sector. The distribution of *agr* types amongst human *S. aureus* was obtained from the study of Jarraud *et al.* (2002) in which 198 isolates including 14 isolates from asymptomatic carriers, 66 isolates from patients with suppurative infection and 114 from patients with acute toxemia were examined.

Of the 52 bovine isolates, 34 were *agr* type 1 (65.4%), 15 were *agr* type 2 (28.8%), 2 were *agr* type 3 (3.8%) and 1 was *agr* type 4 (1.9%). Of the 10 sheep isolates, 8 were of *agr* type 1 (80%) and 2 were of *agr* type 2 (20%). Of the 33 goat isolates tested 26 were of *agr* type 1 (78.8%), 5 were of *agr* type 2 (15.2%) and 2 were of *agr* type 3 (6.1%). Of the 12 poultry isolates tested, 1 was *agr* type 1 (8.3%), 10 were of *agr* type 2 (83.3%) [all RAPD type 2], and 1 was *agr* type 3 (8.3%). Of the 12 rabbit isolates tested, 4 were of *agr* type 1 (33.3%), 5 were of *agr* type 3 (41.7%) and 3 were of *agr* type 4 (25%). The cat isolate tested was of *agr* type 2. Two of the 3 human control strains were of *agr* type 3 and 1 of *agr* type 1. Only the bovine-associated strains had representative strains of each *agr* type. The distributions of *agr* types amongst animal-associated strains are shown in Figure 5.13.

5.3.4. Correlation of major MLST types with *agr* types, RAPD types and SAg gene profiles.

The *agr* types, RAPD types and SAg gene profiles of the six major MLST types are shown in Table 5.5. For the major MLST STs ST5 (10 strains from chickens), ST97 (6 from cows and 2 from rabbits), strains of the same MLST ST were of the same *agr* type, RAPD type and possessed the same SAg gene profiles. However, MLST ST71, ST126, ST133 and ST151 exhibited more variability. All strains within ST71 (8 from cows, 1 from a sheep and 1 from a goat), ST126 (5 strains from cows and 5 from goats), ST133 (8 from cows, 8 from sheep and 12 from goats) and ST151 (6 strains from cows) were of the same *agr* type. The RAPD types showed higher diversity, in particular within MLST ST133, which could be broken down into 9 RAPD types. The presence of SAg and SAg-like genes was also variable in ST71, ST126 and ST133.

All 10 MLST ST5/RAPD type 2 isolates from chickens were *agr* type 2 and encoded the *egc* locus. All 8 bovine MLST ST71/RAPD type 11 isolates were agr type 1 and negative for SAg and SAg-like genes. The MLST ST71/RAPD type 32 sheep isolate and MLST ST71/RAPD type 11 goat isolate were negative for SAg and SAg-like genes and positive for the *sec*, *sell* and *tst* genes respectively. All MLST ST97/RAPD 16 isolates from cows and rabbits were *agr* type 1 and negative for SAg and SAg-like genes. Of the MLST ST133 isolates, RAPD types 23 (5 from cows), 25 (1 from a cow, 4 from goats), 27 (3 from goats), 28 (2 from goats), 29 (1 from goats, 3 from sheep) and 31 (1 from a sheep), were all *agr* type 1 and positive for the *sec*, *sell* and *tst* combination of genes. Of the *agr*

Table 5.5. Correlation of major MLST types with *agr* types, RAPD types and SAg and SAg-like genes.

MLST ST	Number	RAPD type	*agr* type	Host *	SAg and SAg-like genes #
ST5	10	2	2	Chicken	*seg*, *sei*, *selm*, *seln*, *selo*
ST71	8	11	1	Bovine	Negative
	1	32	1	Sheep	Negative
	1	11	1	Goat	*sec*, *sell*, *tst*
ST97	8	16	1	6 Bovine, 2 Rabbit	Negative
ST126	7	19	2	4 Bovine, 3 Goat	Negative
	1	20	2	Bovine	Negative
	1	21	2	Goat	*sec*, *sell*, *tst*
	1	22	2	Goat	Negative
ST133	5	23	1	5 Bovine	*sec*, *sell*, *tst*
	1	24	1	Bovine	Negative
	5	25	1	1 Bovine, 4 Goat	*sec*, *sell*, *tst*
	1	26	1	Bovine	*sed*, *selj*
	1	26	1	Goat	Nd
	1	26	1	Goat	*sec*, *tst*
	3	27	1	3 Goat	*sec*, *sell*, *tst*
	2	28	1	2 Goat	*sec*, *sell*, *tst*
	4	29	1	1 Goat, 3 Sheep	*sec*, *sell*, *tst*
	1	29	1	Sheep	*sec*, *sell*, *tst*, *seg*, *sei*, *selm*, *seln*, *selo*
	1	30	1	Sheep	*seh*
	1	30	1	Sheep	*sec*, *tst*
	2	31	1	Sheep	*sec*, *sell*, *tst*
ST151	4	33	2	Bovine	*sec, sell, tst, seg, sei, selm, seln, selo*
	2	33	2	Bovine	*seg, sei, selm, seln, selo*

* The numbers of strains of each genetic type from each host
# Nd: strain was not tested for SE and SE-like genes

type 2, MLST ST151/RAPD type 33 bovine isolates 4 encoded the *egc* locus and the *sec*, *sell* and *tst* genes, with a further 2 encoding the *egc* locus only.

5.3.5. Indexes of diversity.

Tables 5.6 and 5.7 show the calculations involved for RAPD typing and MLST. RAPD typing had a higher index of diversity (0.96) than MLST (0.92) suggesting that RAPD typing was more discriminatory than MLST.

5.4. Discussion.

5.4.1. Multi-locus sequence typing.

The MLST data herein show that animal-associated strains of *S. aureus* are clonal, with a small number of clones being responsible for the majority of infections in different animal hosts and in different geographical locations. These predominant clones represent successful lineages of both human-associated and animal-associated strains as evidenced by the eBURST analysis. This agrees with previously existing phylogenetic data for bovine *S. aureus* (Kapur *et al.*, 1995). Also a number of novel sequence types were discovered and have been submitted to the MLST database. These STs could be found in clonal complexes with other animal-associated and human-associated STs. Approximately 5 % of strains were MLST singletons. These novel sequence types have diverged as there are no closely related STs in the human isolate database (relatedness of strains was determined by eBURST, showing the relationships of STs in clonal complexes having at least 5 of the 7 loci allelic types in common) and may represent animal-specific clonal types. This gives credence to the theory that bovine *S. aureus,* and hence animal-associated *S. aureus* in general, represent subpopulations that have evolved specifically to infect the mammary gland and to cause other infections in different species.

eBURST analysis showed that the animal-associated STs identified in this study are members and founders of major *S. aureus* clonal complexes. MLST type ST133, which was the major ST identified in this work, was an ancestral ST from which the ST131, ST132 and ST139 (single locus variants of ST133) had evolved. Type ST133 has not been found to be associated with humans. The other bovine STs could be found in clonal complexes with other human isolates.

Table 5.6. Calculations of the Index of Diversity for RAPD typing.

RAPD Type	Number of Strains	n(n-1)
1	3	6
2	11	110
3	3	6
4	2	2
5	1	0
6	1	0
7	1	0
8	1	0
9	1	0
10	1	0
11	9	72
12	3	6
13	1	0
14	1	0
15	1	0
16	13	156
17	1	0
18	3	6
19	7	42
20	1	0
21	2	2
22	1	0
23	5	20
24	1	0
25	5	20
26	3	6
27	3	6
28	2	2
29	6	30
30	2	2

31	4	12
32	1	0
33	7	42
34	1	0
35	1	0
36	1	0
37	2	2
38	1	0
39	1	0
40	1	0
41	1	0
42	5	20
43	1	0
44	1	0
Σ	123	570

Using the formula the index of diversity for RAPD typing is:

$D= 1-[(1/123(123-1) \times 570)] = 0.96$

Table 5.7. Calculations of the Index of Diversity for MLST.

MLST ST	Number of Strains	n(n-1)
1	3	6
5	10	90
8	2	2
20	2	2
22	1	0
25	2	2
39	2	2
71	10	90
96	4	12
97	8	56
101	1	0
115	1	0
121	3	6
126	10	90
133	28	756
151	6	30
352	3	6
407	1	0
479	1	0
480	1	0
522	2	2
669	1	0
670	1	0
671	1	0
672	1	0
673	1	0
674	1	0
675	1	0
676	1	0

677	1	0
678	1	0
679	1	0
680	1	0
681	1	0
682	4	12
683	1	0
684	1	0
685	1	0
686	1	0
687	1	0
Σ	123	1164

Using the formula the index of diversity for MLST typing is:

$D = 1-[(1/123(123-1) \times 1164)] = 0.92$

Interestingly the poultry strains were predominantly of type ST5. This ST is a very successful human ST, and forms Clonal Complex 5, one of the five major MRSA clonal complexes. Of the chicken isolates used in this study 10 were biotyped and tested for their susceptibility to penicillin as part of another study (Smyth *et al.*, in press). All the chicken isolates were susceptible to penicillin and 6 of these were of the poultry biotype. The remaining four were designated to be of a non-host-specific biotype. It is very likely that strains of this ST were introduced to hatchery and broiler hens by workers colonised by type ST5 *S. aureus*. Although genetically identical to human ST5, phenotypically these chicken isolates have adapted to colonise chickens. Human-associated MLST type ST5 *S. aureus* were not available for comparison herein.

The current study represents the largest study to use sequence typing methods to analyse *S. aureus* from different animals. At the time of writing this thesis, only four other sequence typing studies of animal-associated *S. aureus* using MLST are recorded. In one study of pigs and pig-farm workers (Armand-Lefevre *et al.*, 2005), four STs were found to be associated with pigs, namely, ST9, ST97, ST398 and ST433. MLST type ST97 was found herein to be also associated with cows and rabbits. Type ST97 has also been shown to be associated with humans. In a recent study of 77 animal-associated strains of *S. aureus* using AFLP, van Leeuwen *et al.* (2005) showed that MLST types ST1, ST7, ST8, ST9, ST15, ST22, ST30 and ST45 were associated with animals. Of the 24 isolates from pet animals (cats, dogs, guinea-pigs and rabbits), 87.4% were represented in AFLP cluster 1 among the 42 strains in this cluster. Five different sequence types occurred in AFLP cluster 1 namely, ST1, ST7, ST8, ST9, and ST15. In AFLP cluster IVa, geographically unrelated isolates from goats, sheep and cows that caused mastitis were prominent (14 of 22 strains, 63.6%). MLST analysis on ten of the 22 AFLP cluster IVa strains revealed only clonal complex CC22 (ST22). Only one goat isolate in the current study was of type ST22. As with other STs in the present studies, MLST type ST22 is a clonal type causing mastitis in animal udders. In a recent study of 258 bovine *S. aureus* from the USA (227 isolates), Chile (20) and the United Kingdom (11), only 25 different MLST STs were identified (Smith *et al.*, 2005), the major ST was ST97, which was found in 6.5 % of the strains herein. In a parallel study of 191 bovine *S. aureus* collected at an organic farm, only seven MLST types were identified, emphasising the clonality of bovine *S. aureus* (Smith *et al.*, 2005).

5.4.2. RAPD typing.

Although the discriminatory power of this method was found to be higher than that of MLST, this method has technical failings, such as difficulties with interpreting banding patterns on gels. RAPD typing has not proved to be easily reproducible between laboratories and on different thermocyclers and may require extensive optimisation to work in every laboratory environment. MLST on the other hand, despite cost and time taken, is easier to interpret and analyse and easily transported to other laboratories. Also the database of MLST types that is freely available on the Internet facilitates easy comparisons with other studies and groups of strains.

5.4.3. *agr* typing.

The vast majority of isolates were of *agr* types 1 and 2 which agreed with previous studies using animal- and human-associated *S. aureus* (Gilot *et al.*, 2002; Goerke *et al.*, 2003; Lina *et al.*, 2003; Gilot *et al.*, 2004), although there have been rare occurrence isolations of strains of *agr* types 3 and 4. The most prevalent ST (ST133) was of *agr* type 1. Type ST151 isolates which are known to be TSST-1 producers were of *agr* type 2. TSST-1 producing strains from human infection have been shown to be *agr* type 3 in previous studies with exfoliative toxin producing strains that cause SSSS in humans being *agr* type 4 (Ji *et al.*, 1997; Jarraud *et al.*, 2000). The vast majority of ST133 strains were positive for the SaPIbov combination of genes. The other major MLST types ST97 and ST71 were negative for enterotoxins and of *agr* type 1. All clonally related strains appeared to be the same *agr* type.

In the study of Jarraud *et al.* (2002), strains from a wide range of infection and disease types, the four *agr* types were relatively evenly distributed. However a link between genetic background and *agr* type was observed. In the current study there was a strong correlation of MLST and *agr* typing with all strains of the same MLST ST being the same *agr* type.

To examine the correlation of the typing methods, the major MLST types were examined in detail in terms of their RAPD types, *agr* types and SAg gene profiles. Two of the six major MLST types showed 100 % correlation between all typing methods. The remaining three types showed variation in their RAPD types and also in terms of SAg genes being present. SAg genes are often horizontally acquired, so variation in SAg gene content in strains of the same MLST type is also not unprecedented.

Chapter 6

Typing of *Staphylococcus aureus* Strains of Animal Origin using the Nucleotide Sequences of Putative Surface Proteins (*sas* typing) and the Short Sequence Repeat Region (*ssr*) of the Protein A Gene (*spa* typing).

6.1. Introduction.

S. aureus genotyping has become an important tool in the study of the origin of strains, the relationships of clones, and the epidemiology of outbreaks. Genotyping is of the utmost importance in hospital investigations, as MRSA is now endemic or epidemic in many institutions, present in the community, and even in the animal population (Rankin *et al.*, 2005; O'Mahoney *et al.*, 2005; Loeffler *et al.*, 2005).

Although several different phenotypic and, more recently, molecular techniques are available for differentiating *S. aureus* (See Chapters 1 and 5), no method has been shown to be superior under all conditions. Currently PFGE is considered to be the gold standard *S. aureus* typing method at the Center for Disease Control and Prevention (CDC) and has been used successfully to study strain dissemination, especially in nosocomial outbreaks (Bannerman *et al.*, 1995; Tenover *et al.*, 1995; Roberts *et al.*, 1998). However, while PFGE has excellent discriminatory power, it is labour intensive, time consuming, and difficult to standardise among different laboratories (van Belkum *et al.*, 1998). As with other gel-based typing systems, the interpretation of the results of PFGE is often subjective (van Belkum *et al.*, 1998).

DNA sequencing is a powerful approach to strain typing with advantages in speed, unambiguous data interpretation, and simplicity of large-scale database creation. These advantages have been described in Chapter 5 where MLST was used to compare the DNA sequences of seven housekeeping genes of strains of animal origin.

DNA sequencing of the polymorphic X region, or of the short sequence repeat region (SSR) of the protein A gene (*spa*) has been used as an alternative to current techniques for the typing of *S. aureus* (Shopsin *et al.*, 1999; Koreen *et al.*, 2004). The polymorphic X region (Fig. 6.1A) consists of a variable number of 24-bp repeats and is located immediately upstream of the region encoding the C-terminal cell-wall attachment sequence. The diversity of the repeat region seems to have arisen from deletion and duplication of the repetitive units and by point mutation (Brígido *et al.*, 1991). While the biological function of the X region is not known, it has been proposed that this domain may serve to extend the N-terminal immunoglobulin G-binding portion of protein A through the cell wall (von Heijne *et al.*, 1987). The existence of well-conserved regions flanking the X region sequence in the *spa* gene allows the use of primers to the conserved

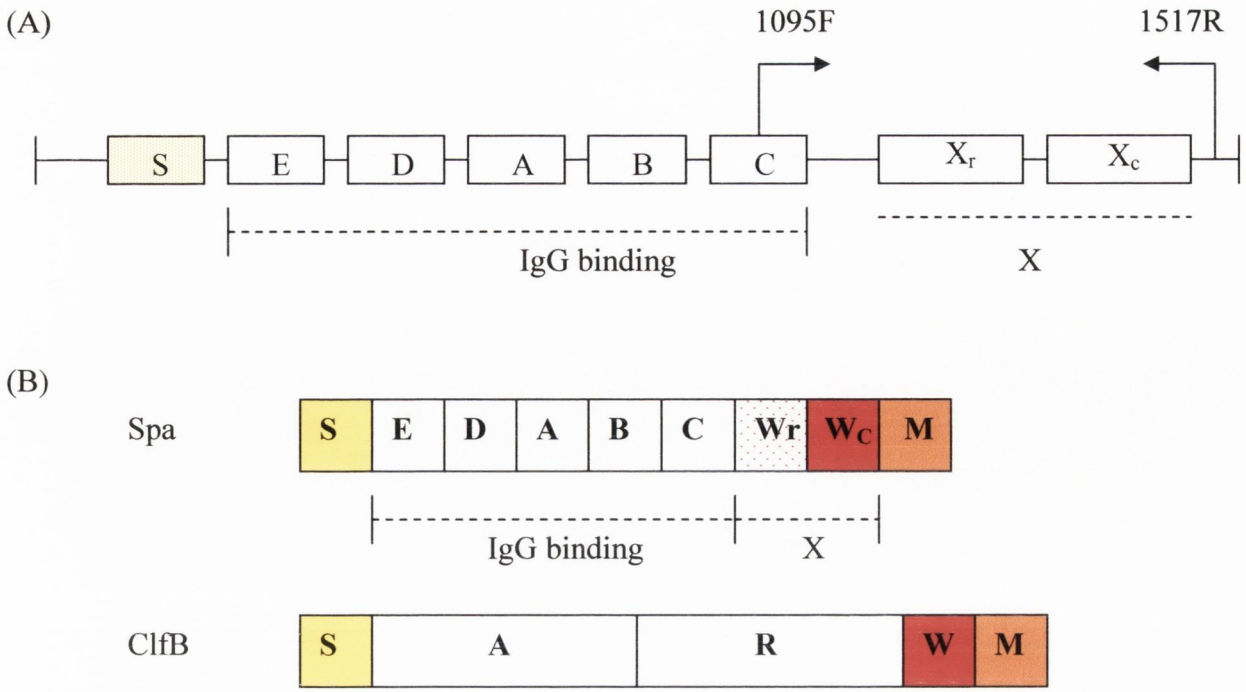


Fig. 6.1. Schematic of the surface-associated proteins Spa, Coa and ClfB.

Panel (A) shows a schematic of the regions of the *spa* gene, adapted from Shopsin *et al.* (1999). The X region of the *spa* gene is polymorphic and made up of variable repeats. The primers 1095F and 1517R amplify the entire X region. The S domain encodes the signal peptide, the domains A-E encode the immunoglobulin-binding domains, X_r encodes the SSRs (short sequence repeats), and X_c encodes the cell-wall attachment sequence.

Panel (B) shows the schematics of the Spa and ClfB proteins. Both proteins have the domain structure of *S. aureus* surface proteins, namely, a signal sequence (S) and wall- and membrane-spanning domains (W, M). The Spa protein binds IgG by means of multiple IgG-binding domains (A–E), whereas ClfB binds fibrinogen in region A. Both proteins have repeat regions. Wr of the Spa protein corresponds to Xr of the *spa* gene and the repeat region is designated R in the ClfB protein. Both repeat regions can be used as targets in typing *S. aureus*.

region for PCR amplification and direct sequence typing (Fig. 6.1A). The sequencing of the *spa* gene short sequence repeats combines many of the advantages of a sequencing-based system such as MLST but is more rapid and convenient since *spa* typing involves a single locus. Since the development of *spa* sequence typing, many studies have used *spa* typing for investigating *S. aureus* populations (Tang *et al.*, 2000; Montesinos *et al.*, 2002; Said-Salim *et al.*, 2003; Koreen *et al.*, 2004; Arakere *et al.*, 2005; El-Sayed *et al.*, 2005; Faria *et al.*, 2005; Malachowa *et al.*, 2005; Mellmann *et al.*, 2005; Witte *et al.*, 2005). When used in combination, *spa* typing has been shown to greatly improve the discriminatory power of other typing methods, e.g., MLST (Robinson & Enright, 2003).

The SD [serine- and aspartic acid-rich (SD)] repeat region of the gene encoding clumping factor B (*clfB*) has been used as a target for sequence typing of *S. aureus* (Koreen *et al.*, 2005; Harmsen *et al.*, 2005). The ClfB protein is a member of the SD-repeat family of *S. aureus* surface proteins (Josefsson *et al.*, 1998) (Fig. 6.1B). Like *spa* typing, *clfB* typing involves sequencing of the repeat region and annotating the types of repeats present. The combination of *clfB* typing and *spa* typing had a discriminatory power greater than *spa* typing alone and almost as high as that of whole genome microarrays (Koreen *et al.*, 2005). The major problem with this method is that it is technically more difficult than *spa* typing owing to the fact that the average *clfB* repeat size (677 bp) is much larger than that of the *spa* repeats observed in region X_r (24bp). However, the combination of *spa* and *clfB* typing may prove to be a suitable alternative to the more labour intensive PFGE for use in outbreak investigation (Koreen *et al.*, 2005).

The polymorphic region of the gene encoding coagulase, *coa*, has also been proposed as an addition to *spa* typing in evaluating outbreaks (Shopsin *et al.*, 2000). The variable region of the *coa* gene is comprises 81-bp tandem short sequence repeats (SSRs) that can be revealed by restriction fragment length polymorphism analysis of PCR products (Goh *et al.*, 1992; Hookey *et al.*, 1999; Hookey *et al.*, 1998). Like *spa* and *clfB* typing, *coa* typing involves sequencing of the repeat region and annotation of the types of repeats present.

Another novel sequence typing method that has been recently developed uses the nucleotide sequences of several *S. aureus* putative surface-associated protein (*sas*) genes (Robinson & Enright, 2003) (Fig. 6.2). The principle is similar to MLST in that the DNA sequences of seven genes are analysed (Fig. 6.3). The seven genes chosen for typing were the least characterized of the *sas* genes each of which encodes a LPXTG motif. These

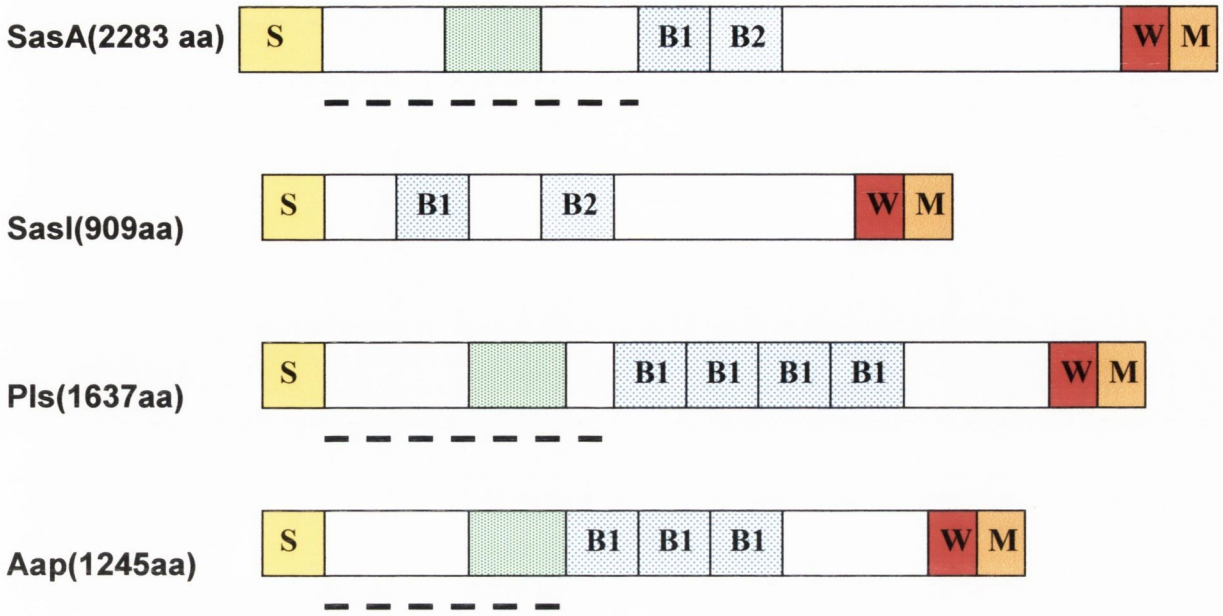


Fig. 6.2. Schematic of the novel SasA and SasI proteins (the genes of which are sequenced in *sas* typing) alongside two novel *S. aureus* surface proteins Pls and Aap [Figure adapted from Roche *et al.* (2003)].

Like *S. aureus* surface proteins, the SasA and SasI proteins have a domain organisation including putative ligand-binding domains (indicated by dashed lines under schematics), wall- and membrane-spanning domains (W and M) and signal sequences (S) that allow sorting to the cell wall. There is a conserved domain within Pls (Plasmin-sensitive protein), Aap (Accumulation associated protein from *S. epidermidis*) and SasA denoted by a green box. The SasA protein has an unusually long signal sequence (90 residues) suggesting that an accessory secretory system (SecA2/SecY2) is involved in export of SasA. The SasI protein lacks a typical A domain.

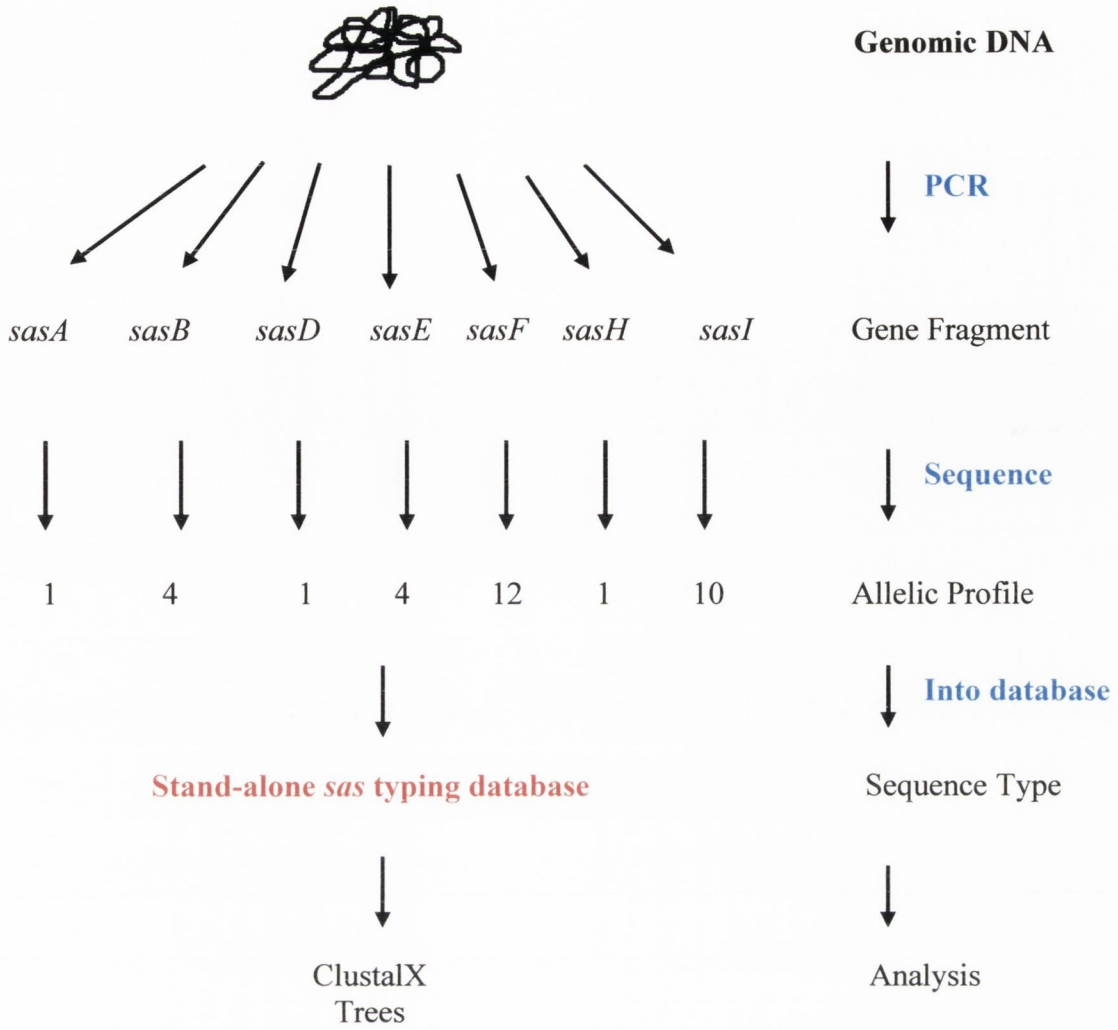


Fig. 6.3. Flow chart of the processes involved in *sas* typing.

The *sas* typing procedure can take as little as two days from start to finish if sequencing facilities are readily available. This method is reproducible, electronically portable and unambiguous. Used in combination with MLST this method greatly increases the discriminatory power of the methods. Briefly seven *sas* genes are amplified from genomic DNA, the amplified PCR products are sequenced on both strands, and the sequences are input into a database. The database assigns numerical nomenclature to the *sas* gene allele if it is in the database. The seven digit allelic profile number corresponds to a *sas* type.

genes were designated *sasA*, *sasB*, *sasD*, *sasE*, *sasF*, *sasH*, and *sasI* (Mazmanian *et al.* 2001). The *sasB* gene shares sequence similarity with the phosphoglucomutase-like gene, *mrp* (98% identity determined by BlastX) from strain COL (Accession number: CAB55329) and the *fmtB* gene (a homologue of the *mrp* gene) (98% identity determined by BlastX) from strain N315 (Accession number: NP_372684). Both genes are involved in methicillin resistance. The *sasE* gene may play a role in cell-envelope biogenesis and has been renamed the *isdA* gene (iron-responsive determinant). The *sasI* and *sasJ* genes have sequence similarity to other *isd* genes, namely, *isdH* and *isdB*. The location of the Isd proteins in the cell envelope is shown in Fig. 6.4. These iron-regulated proteins putatively play a role in haeme-iron acquisition (Skaar & Schneewind, 2004). The *sasH* gene is similar to a gene that encodes a 5′-nucleotidase (Robinson & Enright, 2003). The *sas* genes were further characterised by Roche *et al.* (2003). Certain *sas* genes, *sasG* and *sasH*, were found to be more often associated with invasive disease isolates than nasal carriage isolates (Roche *et al.*, 2003). The SasG protein has been shown to promote binding of *S. aureus* to nasal epithelial cells, although the receptor on nasal epithelial cells has yet to be identified (Roche *et al.*, 2003).

Little data are available on the possible host specificity and variability of the *sas* and *spa* genes in *S. aureus* strains from cows, goats, sheep, rabbits and chickens. The present study was undertaken to evaluate *spa* typing and *sas* typing based on discriminatory power, reproducibility, and ease of interpretation using a small number of representative isolates from different animal hosts. A comparison between the results herein and the MLST data in the previous chapter was also undertaken.

6.2. Methods.

6.2.1. *sas* and *spa* PCR.

The primers of Shopsin *et al.* (1999) were used to amplify the short sequence repeats of the Protein A (*spa*) gene (Table 6.1). Seven *S. aureus* surface protein (*sas*) genes that encode a LPXTG cell-wall attachment motif were chosen from a total of 19 *sas* genes (Accession numbers AY175407-AY175464) by Robinson & Enright (2003). Primers for amplification of the *sas* genes are listed in Table 6.1 and were designed to amplify ~ 450-bp internal fragments.

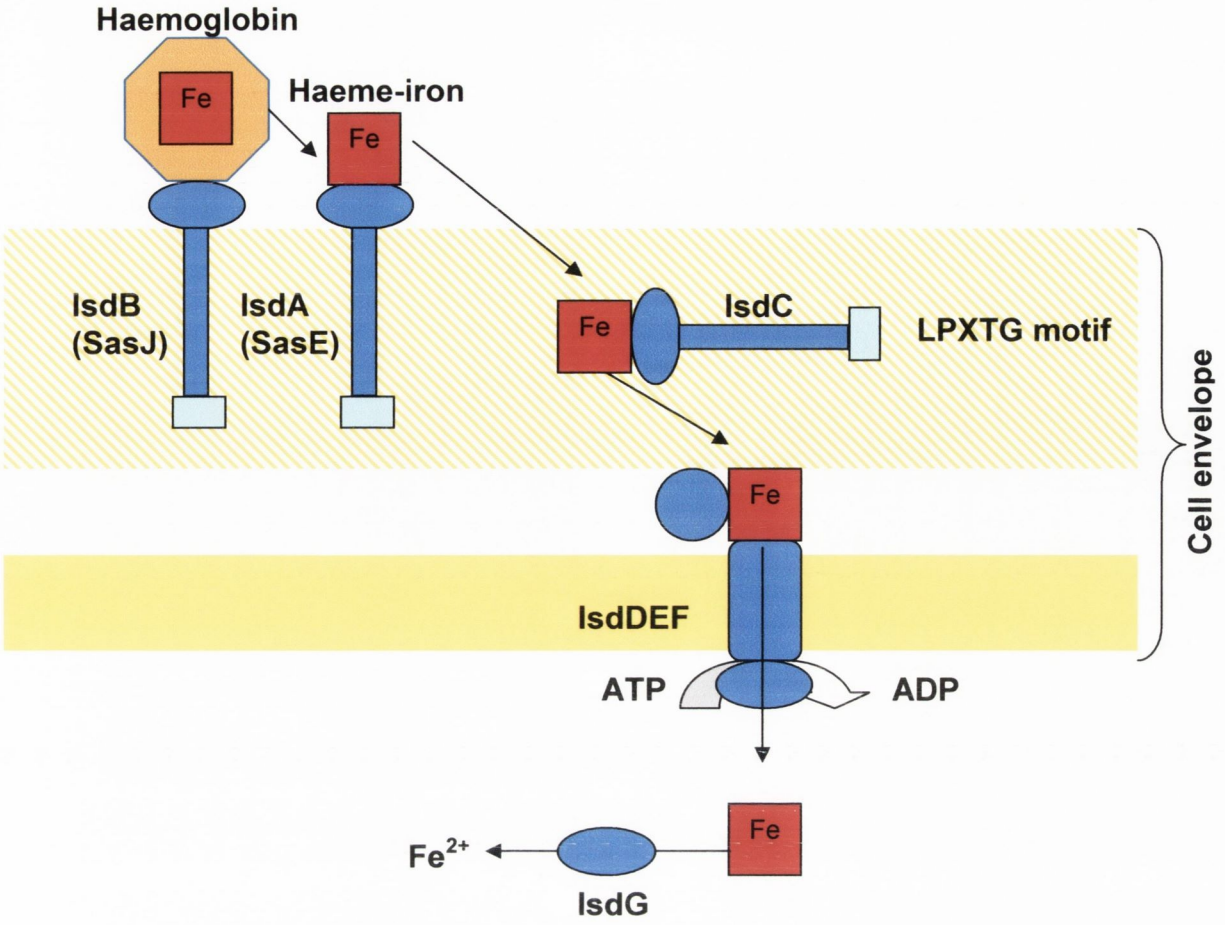


Fig. 6.4. Putative pathway of haeme-iron acquisition in *S. aureus* [Figure adapted from Skaar & Schneewind (2004)].

The Isd proteins are a group of iron-regulated, wall-attached or wall-associated secreted proteins that assemble in the cell envelope and may play a role in haeme-iron acquisition. Two of the Sas proteins, SasE and SasJ, that were described by Mazmanian *et al.* (2001) have since been renamed IsdA and IsdB, respectively. Homologues of the Isd proteins are found in *S. epidermidis*, *Listeria monocytogenes* and *Bacillus anthracis*.

Table 6.1. Primers used for *sas* and *spa* typing.

Primer	Primer sequence (5′–3′)
sasA for	TCA ACA TCC TCA AAG AAT ACT ACA
sasA rev	ATG CGT TAC TTA AGC CAC CAA TAC
sasB for	GTT GCA GCG CTT GTG ACT
sasB rev	ATT TTT GAG ATT TCT TCG TTT TTA
sasD for	GGC GGA GTA GTA CCA CAA GGA A
sasD rev	AAT GCT AAG AAT AAC CCA GAT ACT
sasE for	TTA CAA TGC AAA CAA TCA AGA
sasE rev	GTT TAG GCG TTT CGT TAT GTT TT
sasF for	GGA TAG CAA AGA CAA TAA AAG TTC
sasF rev	TGA TAT GTG TAA TGT TGC GTT GAG
sasH for	CGC ACC AAC TAA CAA ACC AAC TAC
sasH rev	TAC GCC AAT AAT TCC ATA ACG A
sasI for	ATA CTA TCA CTT TTT CAG CAT CAA
sasI rev	TCA TTC GTT TTA TCG TTA GTA TTA
*spa*1095F	AGA CGA TCC TTC GGT GA
*spa*1517R	CAG CAG TAG TGC CGT TTG

Forty-eight strains that were representative of the major clonal types defined by MLST (Chapter 5) were analysed in this work. PCR was carried out in a final volume of 50 µl consisting of 1 µl chromosomal DNA (100 ng), 10 pmol of each forward and reverse primer for the seven *sas* genes, 100 pmol of the forward and reverse primers for the *spa* gene, 1 U of *Taq* DNA polymerase (Promega), 5 µl of 10 × buffer (Promega), 5 µl of 3 mM $MgCl_2$ buffer (Promega), and 0.2 mM deoxynucleoside triphosphates. PCR was performed in a PTC100 thermocycler (MJ Research, Boston, MA) with an initial 5 min-denaturation at 95 °C, followed by 30 cycles of [95 °C for 1 min, 55 °C for 1 min for the *spa* gene or 45 °C for the *sas* genes, 72 °C for 1 min], and a final extension of 72 °C for 5 min. The PCR products were checked by electrophoresis on a 1% (w/v) agarose gel. The sizes of the PCR products were determined using a 100-bp ladder (Promega). The PCR products were subsequently purified using a High Pure PCR Purification Kit (Roche) as recommended by the manufacturer. The purified PCR products were sequenced on both strands by MWG-Biotech.

6.2.2. Analysis of the *spa* repeats.

A freely available database of *spa* types was not available at the time of writing this thesis. A Javascript algorithm was designed (with the help of Malachy McKay, BT) to determine the presence and number of repeats in the sequenced *spa* gene products. Essentially the algorithm was designed in such a way that the animal-associated *spa* gene sequence was input into the Javascript file within a html file. The *spa* repeats are output as html files that are viewed using a web browser. Each *spa* repeat is designated by a letter (and sometimes a number), with the pattern of letters corresponding to the *spa* type. The designed algorithm only assigned nomenclature to known *spa* repeats as defined by Shopsin *et al.* (1999) and Koreen *et al.* (2004). Novel repeats were temporarily assigned the nomenclature herein.

6.2.3. Analysis of the *sas* genes.

A standalone database of *sas* gene alleles for each of the seven *sas* genes (*sasA*, *sasB*, *sasD*, *sasE*, *sasF*, *sasH* and *sasI*) was provided by Dr. Ashley Robinson (Department of Biology and Biochemistry, University of Bath). At the time of writing of this thesis there were 23 *sasA* alleles, 25 *sasB* alleles, 17 *sasD* alleles, 24 *sasE* alleles, 31 *sasF* alleles, 35 *sasH* alleles, and 25 *sasI* alleles in the *sas* typing stand-alone database. A total of 82 *sas*

sequence types (STs) had been described in human-associated *S. aureus*. Sequences of *sas* genes obtained from animal-associated strains were checked against this database and novel *sas* alleles were submitted to Dr. Ashley Robinson to be verified and added to the database. Novel *sas* alleles were given the temporary numbers herein from 50 onwards. Novel *sas* STs (containing novel *sas* alleles or new combinations of known *sas* alleles) were given the temporary numbers herein from 100 onwards.

6.2.4. Phylogenetic analysis.

The sequences of the *sas* genes were aligned and checked using ClustalX with default parameters, followed by manual inspection. ClustalX was used to produce phylogenetic trees. MEGA3.1 was used to generate a UPGMA tree based on the concatenated nucleotide sequences of all seven *sas* genes (Kumar *et al.*, 2004).

6.3. Results.

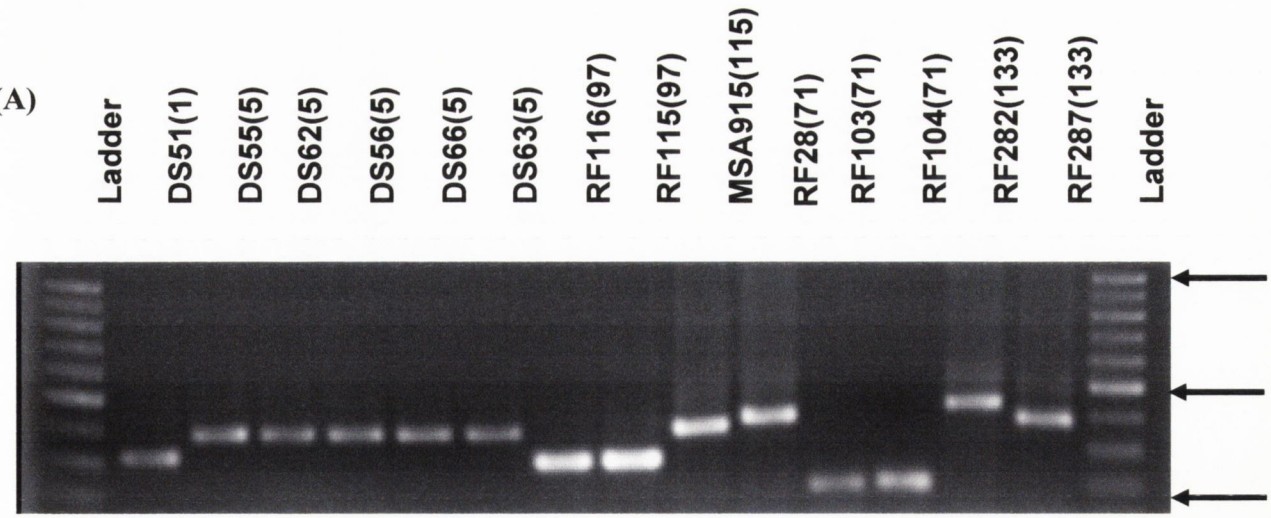
The *sas* typing and *spa* typing methods were applied to 48 animal isolates (22 from cows, 11 from goats, 5 from rabbits, 1 from a cat, 1 from a sheep and 8 from chickens) that had been analysed in the previous chapter and were representative of the MLST types observed.

6.3.1. *spa* typing.

The *spa* typing procedure involves the PCR amplification of the polymorphic SSR region of the *spa* gene and the subsequent sequencing of the PCR product. Due to the presence of differing numbers of repeats in this region of the gene, the PCR reaction generates products that vary in size (Figs. 6.5A and 6.5B). The *spa* repeats vary in length from 21 – 24 bp and are given designations in the form of letters (and sometimes numbers) (Table 6.2). The Javascript algorithm designed herein successfully typed 44 of the animal-associated strains using the known repeats of Koreen *et al.* (2004) (Table 6.3). Four of the animal-associated strains generated new *spa* repeats (A4, B4, C4 and D4) (Table 6.2).

The animal-associated strains generated 35 *spa* repeat profiles. A database of *spa* types was not available so the 35 *spa* repeat profiles were temporarily given *spa* type numbers from 1 to 35. Six *spa* repeat profiles accounted for 19 strains, namely:

(A)



(B)

Strain	*spa* repeats	Number of repeats
DS51	UJFKBPE	7
DS55	TJMBMDMGMK	10
DS62	TJMBMDMGMK	10
DS56	TJMBMDMGMK	10
DS66	TJMBMDMGMK	10
DS63	TJMBMDMGMK	10
RF116	UJBBPB	6
RF115	UJBBPB	6
RF915	UJGFMBBBPB	10
RF28	TJGFMBBBBPB	11
RF103	ZM	2
RF104	ZM	2
RF282	D2KGFMJEMMMJQ	12
RF287	D2KFMJEMMJQ	10

Fig. 6.5. *spa* tying of animal-associated *S. aureus* strains.

Panel (A) shows the results of *spa* gene PCR. The generated PCR products vary in size according to the numbers of repeats present in the *spa* gene. The MLST types of the strains are indicated in parentheses. The 1-kb, 500-bp and 200-bp bands in the ladder (top to bottom) are indicated with arrows.

Panel (B) tabulates the sequences and numbers of repeats present in each strain in the above gel.

Table 6.2. The *spa* repeat code table including novel repeats identified in this study.

spa repeat code	1	2	3	4	5	6	7	8
D1	AAA	GAA	GAC	AAC	AAC	AAA	CCT	GGC
A1	AAA	GAA	GAC	AAC	AAA	AAA	CCT	GGC
H1	AAA	GAA	GAC	AAT	AAC	AAG	CCT	GGC
F1	AAA	GAA	GAC	AAC	AAC	AAG	CCT	GGC
C1	AAA	GAA	GAC	AAC	AAA	AAG	CCT	GGC
F2	AAA	GAA	GAC	AAC	AAA	AAG	CCT	AGC
B1	AAA	GAA	GAC	AAC	AAA	AAA	CCT	GGT
E1	AAA	GAA	GAC	AAC	AAC	AAA	CCT	GGT
Z2	AAA	GAA	GAC	AAC		AAG	CCT	GGT
G1	AAA	GAA	GAC	AAC	AAC	AAG	CCT	GGT
C2	AAA	GAA	GAC	AAT	AAC	AAG	CCT	GGT
E2	AAA	GAA	GAC	AGC	AAC	AAG	CCT	GGC
J1	AAA	GAA	GAC	GGC	AAC	AAA	CCT	GGC
G2	AAA	GAA	GAC	GGC	AAA	AAA	CCT	GGC
L1	AAA	GAA	GAC	GGC	AAC	AAG	CCT	GGC
K1	AAA	GAA	GAC	GGC	AAC	AAA	CCT	GGT
M1	AAA	GAA	GAC	GGC	AAC	AAG	CCT	GGT
D4	**AAA**	**GAA**	**GAC**	**AGC**	**AAC**	**AAA**	**CCT**	**GGC**
N1	AAA	GAA	GAT	GGC	AAC	AAA	CCT	GGC
P1	AAA	GAA	GAT	GGC	AAC	AAG	CCT	GGC
R1	AAA	GAA	GAT	GGT	AAC	AAA	CCT	GGC
O1	AAA	GAA	GAT	GGC	AAC	AAA	CCT	GGT
Q1	AAA	GAA	GAT	GGC	AAC	AAG	CCT	GGT
H2	AAA	GAA	GAT	GGC	AAC	AAG	CCT	AGT
B2	AAA	GAA	GAT	AAC	AAC	AAG	CCT	GGT
I1	AAA	GAA	GGC	AAC	AAA	AAA	CCT	GGT
B4	**AAA**	**GAT**	**GAC**	**GGC**	**AAC**	**AAG**	**CCT**	**GGT**
C4	**AAA**	**GAT**	**GAC**	**GGC**	**AAC**	**AAA**	**CCT**	**GGT**
U2	CAA	GAA	GAC	GGC	AAC	AAG	CCT	GGT
V2	CAA	GAA	GAC	AAC	AAC	AAG	CCT	GGT
I2	GAG	GAA	GAC	AAC	AAC	AAA	CCT	GGC
Y2	GAG	GAA	GAC	AAC	AAA		CCT	GGC
Y1	GAG	GAA	GAC	AAT	AAC	AAG	CCT	GGC
W1	GAG	GAA	GAC	AAC	AAC	AAG	CCT	GGC
V1	GAG	GAA	GAC	AAC	AAC	AAG	CCT	AGC
T1	GAG	GAA	GAC	AAC	AAA	AAA	CCT	GGT
U1	GAG	GAA	GAC	AAC	AAC	AAA	CCT	GGT
D2	GAG	GAA	GAC	AAT	AAC	AAA	CCT	GGT
Z1	GAG	GAA	GAC	AAT	AAC	AAG	CCT	GGT
X1	GAG	GAA	GAC	AAC	AAC	AAG	CCT	GGT
A2	GAG	GAA	GAC	GGC	AAC	AAA	CCT	GGT
A4	**GAG**	**GAA**	**GAC**	**AAT**	**AGC**	**AAG**	**CCT**	**GGC**

This table shows the DNA sequences of 38 *spa* repeats identified to date in *S. aureus* and novel *spa* repeats identified in the current study (red text) (Koreen *et al.*, 2004). These repeat sequences were used to design a Javascript algorithm that determines the number and types of repeats in *spa* gene sequences of typed animal-associated *S. aureus* strains.

Table 6.3. Results of *sas* and *spa* typing using representative animal-associated strains.

Strain	Host §	Country	MLST-ST	RAPD type	*sas* profile †							*sas* type*	*Spa* repeat profile	*spa type*
RF103	C	Ireland	71	11	13	10	3	8	16	15	10	100	ZM	1
RF104	C	Ireland	71	11	13	10	3	8	16	15	10	100	ZM	1
RF28	C	Ireland	97	16	2	10	9	8	27	19	10	60	TJGFMBBBBPB	2
RF116	C	Ireland	97	16	2	10	9	8	27	19	10	60	UJBBPB	3
RF115	C	Ireland	97	16	2	10	9	8	27	19	10	60	UJBBPB	3
RF287	C	Ireland	133	23	23	51	3	55	56	15	50	101	D2KFMJEMMJQ	4
RF282	C	Ireland	133	23	23	51	3	55	56	15	50	101	D2KGFMJEMMMJQ	5
RF80	C	Ireland	151	33	59	NA	54	54	52	55	54	102	ZB	6
RF122	C	Ireland	151	33	59	NA	54	54	52	55	54	102	ZB	6
DS35	C	Spain	673	39	23	51	3	55	56	15	50	101	D2KGFMJEMMMJQ	5
DS69	C	Sweden	133	40	58	51	3	55	56	15	50	103	D2JQ	7
DS70	C	Sweden	679	49	2	10	50	8	27	19	10	104	JFKBBPE	8
MSA1006	C	USA	8	3	2	2	3	1	50	6	3	105	A4HGFMBQBLO	9
MSA1363	C	USA	25	6	7	15	9	8	28	22	12	38	ZFGU2DMGM	10
MSA915	C	USA	115	16	50	10	9	8	27	19	10	106	UJGFMBBBPB	11
MSA948	C	USA	126	19	7	10	14	57	51	50	59	107	UJ	12

Strain	Host §	Country	MLST-ST	RAPD type	*sas* profile †							*sas* type *	*Spa* repeat profile	*spa type*
MSA1047	C	USA	678	34	57	54	55	52	55	54	55	108	DB4NC4MKKK	13
MSA1468	C	USA	670	36	7	10	14	57	51	50	10	107	UJ	12
PSA5	C	USA	680	47	15	18	12	15	54	18	9	109	ZDMO	14
MSA1007	C	USA	683	2	2	3	2	1	6	5	57	110	JMK	15
MSA1547	C	USA	684	48	19	3	51	50	18	52	5	111	UGJAGJB	16
MSA961	C	USA	352	49	2	10	50	8	27	19	10	104	UKBBPB	17
DS71	Ca	Sweden	676	45	18	14	10	12	7	13	58	112	UJGBBGGJAGJ	18
DS63	Ch	N. Ireland	5	2	2	3	2	1	6	5	5	1	TJMBMDMGMK	19
DS55	Ch	N. Ireland	5	2	2	3	2	1	6	5	5	1	TJMBMDMGMK	19
DS56	Ch	N. Ireland	5	2	2	3	2	1	6	5	5	1	TJMBMDMGMK	19
DS58	Ch	N. Ireland	5	2	2	3	2	1	6	5	5	1	TJMBMDMGMK	19
DS66	Ch	N. Ireland	5	2	2	3	2	1	6	5	5	1	TJMBMDMGMK	19
DS62	Ch	N. Ireland	5	2	2	3	2	1	6	5	5	1	TJMBMDMGMK	19
DS65	Ch	N. Ireland	5	2	2	3	2	1	6	5	5	1	TJMBMDMGMK	19
DS68	Ch	N. Ireland	687	51	52	21	51	51	58	51	10	113	TJGBFMBOM	20
DS30	G	Italy	22	5	3	7	3	4	5	1	2	21	TJEJNCMOMOKR	21
DS27	G	Italy	25	7	7	15	9	8	28	22	12	38	ZFGU2DMGGM	22
DS25	G	Italy	39	9	5	5	4	7	2	4	8	14	XKKAMQ	23
DS29	G	Italy	101	17	19	3	9	12	7	23	6	74	ZDGMDMGMM	24

Strain	Host §	Country	MLST-ST	RAPD type	*sas* profile †							*sas* type *	*Spa* repeat profile	*spa type*
DS13	G	Italy	133	25	23	51	3	55	56	15	50	101	D2KKMMMJQ	25
DS24	G	Italy	671	37	54	53	52	56	53	53	50	114	ZD4MOM	26
DS20	G	Italy	682	54	53	53	3	53	NA	15	53	115	ZNMOMM	27
DS17	G	Italy	522	54	53	53	3	53	NA	15	53	115	ZNMOMMM	28
DS12	G	Italy	681	55	51	50	14	57	51	15	60	116	UJ	12
DS74	G	Norway	133	26	58	51	3	55	56	15	50	103	D2	29
DS80	G	Norway	480	37	56	52	53	56	53	53	52	117	ZD4MOMOOOKM	30
DS51	R	Belgium	1	1	11	3	8	1	18	12	17	63	UJFKBPE	31
DS48	R	Belgium	96	12	19	56	9	1	10	56	56	118	UJFMBBBBBPB	32
DS50	R	Belgium	97	16	2	10	9	8	27	19	10	60	U	33
DS46	R	Belgium	121	18	55	55	9	11	21	15	11	119	I2Z2EGMJH2M	34
DS33	R	Spain	407	3	2	2	3	1	57	6	3	120	YGGFMBQBLO	35
DS83	S	Norway	133	32	23	51	3	55	56	15	50	101	D2KGFMJEMMMJQ	5

† NA – Not amplifiable. It was not possible to generate PCR products for these strains.

Red text indicates new *sas* allelic type, *sas* ST or *spa* short sequence repeat. Novel *sas* alleles were numbered from 50 onwards and novel * *sas* types were numbered from 100 onwards temporarily.

§ C – Cow, Ca – Cat, Ch – Chicken, G – Goat, R – Rabbit, and S - Sheep

D2KGFMJEMMMJQ – *spa* type 5 (3 strains), TJMBMDMGMK – *spa* type 19 (7 strains), UJ – *spa* type 12 (3 strains), UJBBPB – *spa* type 3 (2 strains), ZB – *spa* type 1 (2 strains) and ZM – *spa* type 6 (2 strains). The smallest number of repeats was 1 (strain DS74, *spa* repeat profile D2, *spa* type 29) and the largest number of repeats was 12 (strain RF282, *spa* repeat profile, D2KGFMJEMMMJQ, *spa* type 5).

The 22 cow-associated strains generated 18 *spa* repeat profiles with five profiles accounting for 10 strains, namely: ZM (*spa* type 6), 2 strains, (MLST ST71); UJBBPB (*spa* type 3), 2 strains, (MLST ST97); ZB (*spa* type 1), 2 strains, (MLST ST151); UJ (*spa* type 12), 2 strains, (MLST ST126 and an SLV of ST126); and D2KGFMJEMMMJQ (*spa* type 5), 2 strains, (MLST ST133 and a SLV of ST133). The eight chicken-associated strains generated only 2 *spa* repeat profiles, 1 of which – TJMBMDMGMK (*spa* type 19) – accounted for 7 of the strains (all MLST ST5). The 11 goat-associated strains generated 11 different *spa* repeat profiles. The 5 rabbit-associated strains generated 5 different *spa* repeat profiles. The single sheep-associated strain generated the D2KGFMJEMMMJQ *spa* repeat profile (*spa* type 5). The cat isolate, DS71, generated a unique *spa* repeat profile.

6.3.2. *sas* typing.

Analysis of the nucleotide sequences of the *sas* genes of the 48 animal-associated *S. aureus* generated 28 *sas* ST types for the 48 strains, 21 of which were novel *sas* types (numbered 100 – 121). Four of the strains failed to generate a PCR product for a single *sas* allele, namely, strains RF122 and RF80 did not generate a *sasB* gene PCR product and strains DS17 and DS20 failed to generate a PCR product for the *sasF* gene. Ten *sas* ST types accounted for 30 of the strains, namely: *sas* ST1 (7 strains), *sas* ST38 (2 strains), *sas* ST60 (4 strains), *sas* ST100 (2 strains), *sas* ST101 (5 strains), *sas* ST102 (2 strains), *sas* ST103 (2 strains), *sas* ST104 (2 strains), *sas* ST107 (2 strains), and *sas* ST115 (2 strains). Many new *sas* gene alleles were identified, namely, 10 *sasA* alleles (*sasA50–sasA59*), 7 *sasB* alleles (*sasB50–sasB56*), 6 *sasD* alleles (*sasD50–sasD55*), 8 *sasE* alleles (*sasE50–sasE57*), 8 *sasF* alleles (*sasF50–sasF57*), 7 *sasH* alleles (*sasH50–sasH56*) and 10 *sasI* alleles (*sasI50–sasI60*).

The 22 cow-associated strains generated 14 *sas* STs, i.e., 2 known *sas* STs and 12 novel *sas* STs. Six STs accounted for 14 of the 22 strains: *sas* ST60 (3 strains); *sas* ST100 (2 strains); *sas* ST101 (3 strains); *sas* ST102 (2 strains); *sas* ST104 (2 strains); and *sas* ST107

(2 strains). The remaining 8 of the bovine strains were of different novel *sas* STs. The 8 chicken-associated strains generated two *sas* STs, namely, *sas* ST1 (7 strains) and a novel *sas* ST113 (1 strain). The 11 goat-associated strains generated 10 *sas* STs, four of which had been previously found in human strains. Two of the goat strains were of the same *sas* novel ST (*sas* ST115), the rest had individual *sas* STs. For the 5 rabbit strains analysed, 5 different *sas* STs were identified, three of which were novel *sas* STs (*sas* ST118, *sas* ST119 and *sas* ST120). The single sheep isolate generated the novel *sas* ST, ST101. The single cat isolate also had a novel *sas* type, *sas* ST112.

6.3.3. Discriminatory power.

In order to assess which novel sequence typing method had the highest resolution and discriminatory power, the index of diversity of each method was calculated as described in Chapter 5. The index of diversity of the methods combined was also determined. The indexes of diversity are given in Table 6.4. From the table it is clear that the single method with the highest resolution is *spa* typing (Index of diversity, 0.9725), followed by *sas* typing (Index of diversity, 0.9654), and then MLST (Index of diversity, 0.9627). The combination of *spa* typing and MLST and the combination of *spa* typing and *sas* typing had higher resolutions than either *sas* typing or MLST alone. The combination of MLST and *spa* typing had a higher resolution power than *spa* typing alone and the same resolution power as using all three methods.

6.3.4. Phylogenetic analysis.

Phylogenetic trees were generated using the nucleotide data for the *sas* genes using ClustalX (Fig. 6.6). The trees display the number of novel *sasA, sasB, sasD* and *sasE* alleles that were identified in this work (highlighted in blue in Fig. 6.6). The *sasA*, *sasB* and *sasE alleles* showed a similar degree of variation, whereas the variability of the *sasD* gene was much less. This would imply that mutations in the *sasD* gene occur at a slower rate than the other alleles. Robinson & Enright (2003) found that the variation at *sasD* appeared to be similar to that found in the MLST-associated housekeeping genes.

A UPGMA tree generated using MEGA3.1 and the concatenated sequences of the seven *sas* genes gave an impression of the correlation of *sas* typing with MLST typing and *spa* typing (Fig. 6.7). The tree shows that strains of the same *sas* ST were in general of the

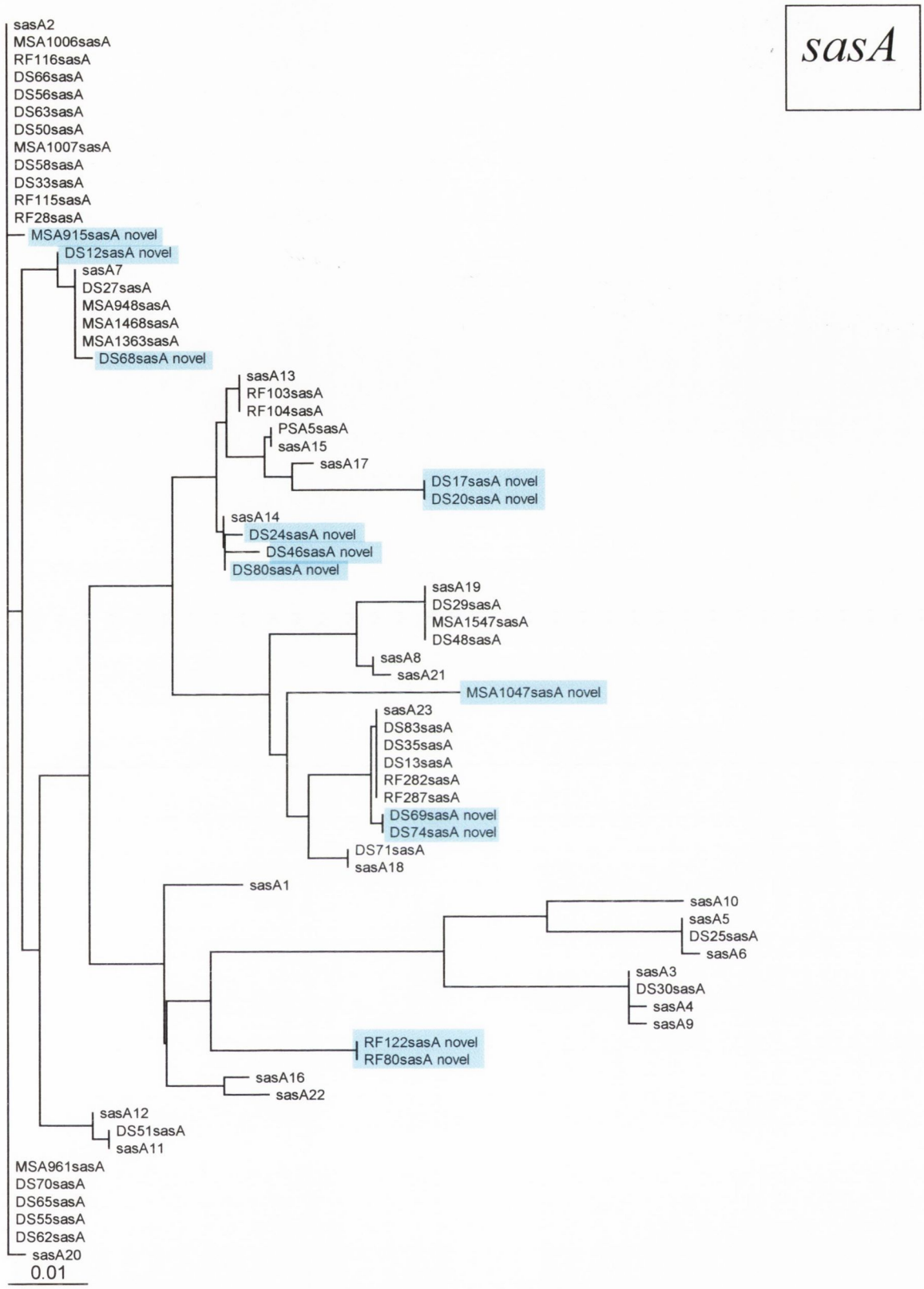
Table 6.4. Resolution of sequence based typing methods.

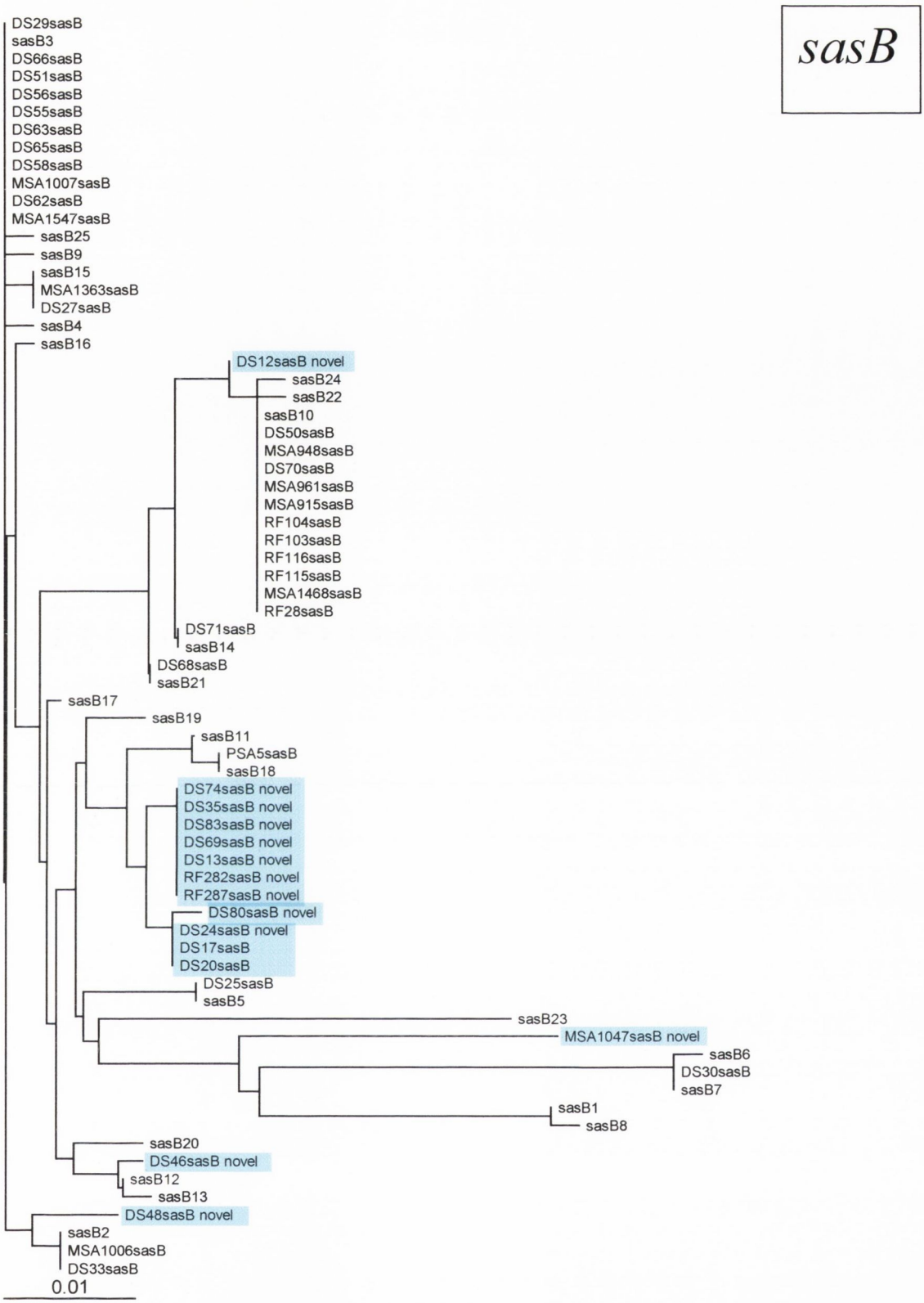
Typing method	No. of types	Index of diversity
spa typing	35	0.9725
sas typing	28	0.9654
MLST	31	0.9627
MLST + *sas*	32	0.9671
MLST + *spa*	38	0.9778
sas + *spa* typing	36	0.9751
sas + *spa* typing + MLST	38	0.9778

The index of diversity was calculated as described in Chapter 5. The closer the index of diversity is to 1, the higher the resolution power of the method.

Fig. 6.6. ClustalX-generated phylogenetic trees of the *sasA*, *sasB*, *sasD* and *sasE* of 48 animal-associated *S. aureus* strains.

The animal-associated strains generated many new *sas* allelic types and *sas* sequence types that are possibly unique to the animal-associated *S. aureus* population. The novel *sas* allele sequences are highlighted in blue. The *sasA*, *sasB* and *sasE* nucleotide sequences showed a lot more variation than *sasD* sequences. The *sasD* gene is acquiring mutations and variations at a slower rate than the *sasA*, *sasB* and *sasE* genes.


sasA
sasA2
MSA1006sasA
RF116sasA
DS66sasA
DS56sasA
DS63sasA
DS50sasA
MSA1007sasA
DS58sasA
DS33sasA
RF115sasA
RF28sasA
MSA915sasA novel
DS12sasA novel
sasA7
DS27sasA
MSA948sasA
MSA1468sasA
MSA1363sasA
DS68sasA novel
sasA13
RF103sasA
RF104sasA
PSA5sasA
sasA15
sasA17
DS17sasA novel
DS20sasA novel
sasA14
DS24sasA novel
DS46sasA novel
DS80sasA novel
sasA19
DS29sasA
MSA1547sasA
DS48sasA
sasA8
sasA21
MSA1047sasA novel
sasA23
DS83sasA
DS35sasA
DS13sasA
RF282sasA
RF287sasA
DS69sasA novel
DS74sasA novel
DS71sasA
sasA18
sasA1
sasA10
sasA5
DS25sasA
sasA6
sasA3
DS30sasA
sasA4
sasA9
RF122sasA novel
RF80sasA novel
sasA16
sasA22
sasA12
DS51sasA
sasA11
MSA961sasA
DS70sasA
DS65sasA
DS55sasA
DS62sasA
sasA20
0.01


sasB
DS29sasB
sasB3
DS66sasB
DS51sasB
DS56sasB
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DS63sasB
DS65sasB
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MSA1007sasB
DS62sasB
MSA1547sasB
sasB25
sasB9
sasB15
MSA1363sasB
DS27sasB
sasB4
sasB16
DS12sasB novel
sasB24
sasB22
sasB10
DS50sasB
MSA948sasB
DS70sasB
MSA961sasB
MSA915sasB
RF104sasB
RF103sasB
RF116sasB
RF115sasB
MSA1468sasB
RF28sasB
DS71sasB
sasB14
DS68sasB
sasB21
sasB17
sasB19
sasB11
PSA5sasB
sasB18
DS74sasB novel
DS35sasB novel
DS83sasB novel
DS69sasB novel
DS13sasB novel
RF282sasB novel
RF287sasB novel
DS80sasB novel
DS24sasB novel
DS17sasB
DS20sasB
DS25sasB
sasB5
sasB23
MSA1047sasB novel
sasB6
DS30sasB
sasB7
sasB1
sasB8
sasB20
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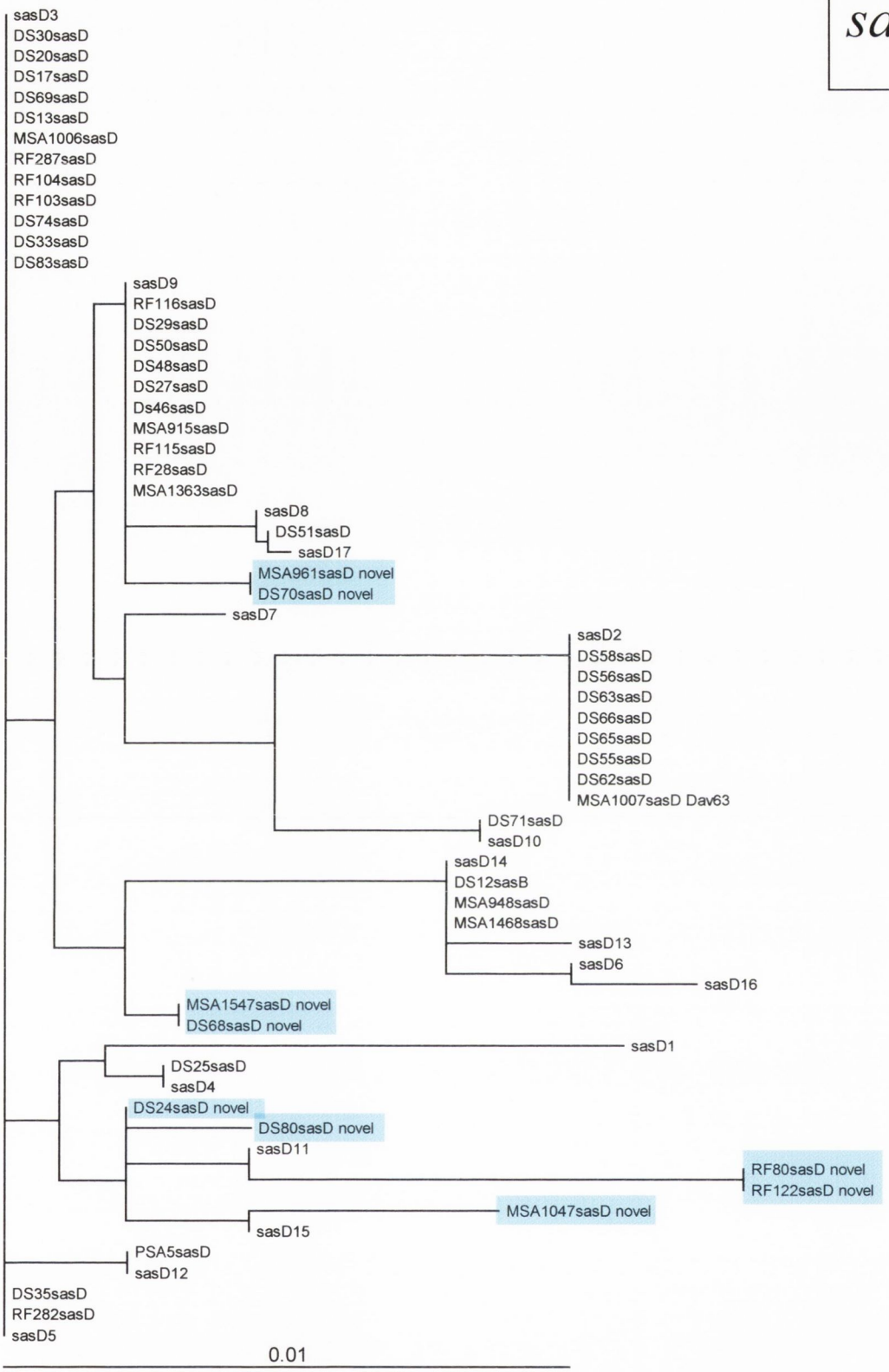
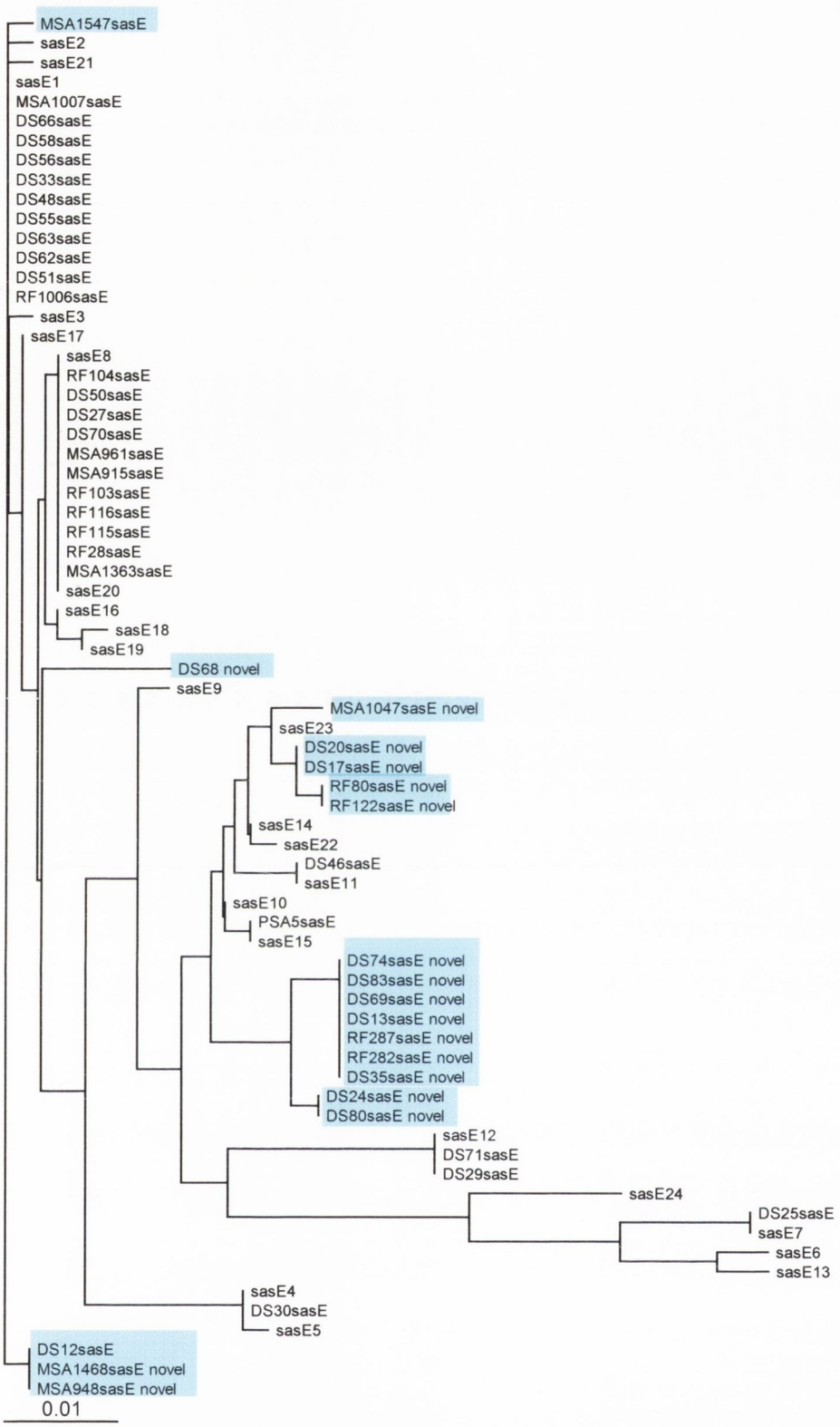
sasB13
DS48sasB novel
sasB2
MSA1006sasB
DS33sasB
0.01

sasD


sasD3
DS30sasD
DS20sasD
DS17sasD
DS69sasD
DS13sasD
MSA1006sasD
RF287sasD
RF104sasD
RF103sasD
DS74sasD
DS33sasD
DS83sasD
sasD9
RF116sasD
DS29sasD
DS50sasD
DS48sasD
DS27sasD
Ds46sasD
MSA915sasD
RF115sasD
RF28sasD
MSA1363sasD
sasD8
DS51sasD
sasD17
MSA961sasD novel
DS70sasD novel
sasD7
sasD2
DS58sasD
DS56sasD
DS63sasD
DS66sasD
DS65sasD
DS55sasD
DS62sasD
MSA1007sasD Dav63
DS71sasD
sasD10
sasD14
DS12sasB
MSA948sasD
MSA1468sasD
sasD13
sasD6
sasD16
MSA1547sasD novel
DS68sasD novel
sasD1
DS25sasD
sasD4
DS24sasD novel
DS80sasD novel
sasD11
RF80sasD novel
RF122sasD novel
MSA1047sasD novel
sasD15
PSA5sasD
sasD12
DS35sasD
RF282sasD
sasD5
0.01


sasE
MSA1547sasE
sasE2
sasE21
sasE1
MSA1007sasE
DS66sasE
DS58sasE
DS56sasE
DS33sasE
DS48sasE
DS55sasE
DS63sasE
DS62sasE
DS51sasE
RF1006sasE
sasE3
sasE17
sasE8
RF104sasE
DS50sasE
DS27sasE
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MSA915sasE
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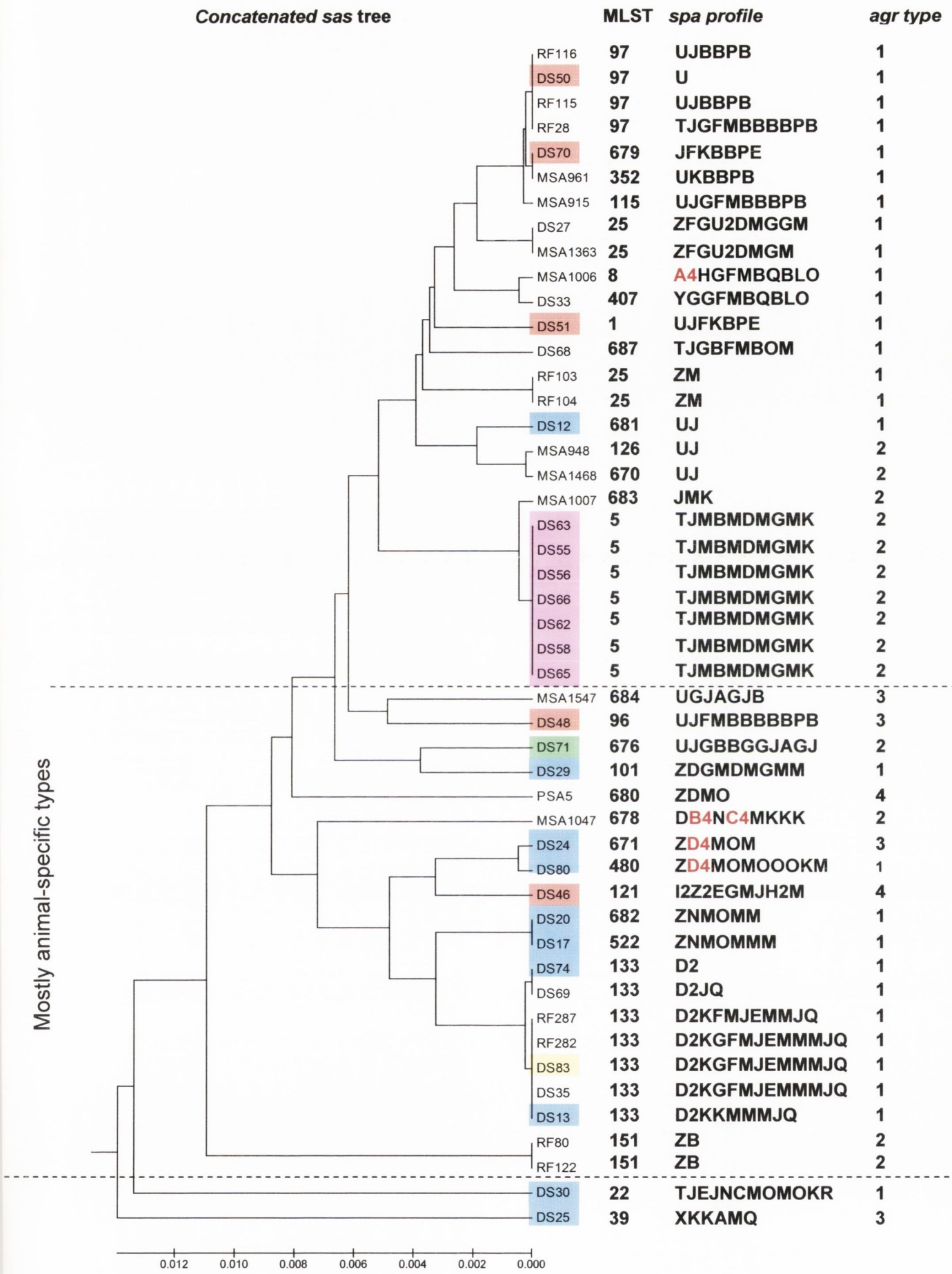
RF116sasE
RF115sasE
RF28sasE
MSA1363sasE
sasE20
sasE16
sasE18
sasE19
DS68 novel
sasE9
MSA1047sasE novel
sasE23
DS20sasE novel
DS17sasE novel
RF80sasE novel
RF122sasE novel
sasE14
sasE22
DS46sasE
sasE11
sasE10
PSA5sasE
sasE15
DS74sasE novel
DS83sasE novel
DS69sasE novel
DS13sasE novel
RF287sasE novel
RF282sasE novel
DS35sasE novel
DS24sasE novel
DS80sasE novel
sasE12
DS71sasE
DS29sasE
sasE24
DS25sasE
sasE7
sasE6
sasE13
sasE4
DS30sasE
sasE5
DS12sasE
MSA1468sasE novel
MSA948sasE novel
0.01

Fig. 6.7. MEGA 3.1-generated tree of the concatenated nucleotide sequences of the seven *sas* gene sequences from 48 animal-associated strains.

Strains from chickens are highlighted in pink, strains from goats are highlighted in blue, the strain from a cat is highlighted in green, and the strain from a sheep is highlighted in yellow. Strains from cows are not highlighted. The MLST sequence type, *spa* repeat profile and *agr* type of each strain are shown alongside the tree. In general strains of the same *sas* ST were of the same MLST ST, e.g., DS63, DS56, DS55 were all *sas* ST1 and MLST ST5. Also strains of the same *sas* ST and MLST ST showed the same or similar *spa* repeat profiles.


Concatenated sas tree
MLST
spa profile
agr type
RF116 97 UJBBPB 1
DS50 97 U 1
RF115 97 UJBBPB 1
RF28 97 TJGFMBBBBPB 1
DS70 679 JFKBBPE 1
MSA961 352 UKBBPB 1
MSA915 115 UJGFMBBBPB 1
DS27 25 ZFGU2DMGGM 1
MSA1363 25 ZFGU2DMGM 1
MSA1006 8 A4HGFMBQBLO 1
DS33 407 YGGFMBQBLO 1
DS51 1 UJFKBPE 1
DS68 687 TJGBFMBOM 1
RF103 25 ZM 1
RF104 25 ZM 1
DS12 681 UJ 1
MSA948 126 UJ 2
MSA1468 670 UJ 2
MSA1007 683 JMK 2
DS63 5 TJMBMDMGMK 2
DS55 5 TJMBMDMGMK 2
DS56 5 TJMBMDMGMK 2
DS66 5 TJMBMDMGMK 2
DS62 5 TJMBMDMGMK 2
DS58 5 TJMBMDMGMK 2
DS65 5 TJMBMDMGMK 2
MSA1547 684 UGJAGJB 3
DS48 96 UJFMBBBBBPB 3
DS71 676 UJGBBGGJAGJ 2
DS29 101 ZDGMDMGMM 1
PSA5 680 ZDMO 4
MSA1047 678 DB4NC4MKKK 2
DS24 671 ZD4MOM 3
DS80 480 ZD4MOMOOOKM 1
DS46 121 I2Z2EGMJH2M 4
DS20 682 ZNMOMM 1
DS17 522 ZNMOMMM 1
DS74 133 D2 1
DS69 133 D2JQ 1
RF287 133 D2KFMJEMMJQ 1
RF282 133 D2KGFMJEMMMJQ 1
DS83 133 D2KGFMJEMMMJQ 1
DS35 133 D2KGFMJEMMMJQ 1
DS13 133 D2KKMMMJQ 1
RF80 151 ZB 2
RF122 151 ZB 2
DS30 22 TJEJNCMOMOKR 1
DS25 39 XKKAMQ 3
Mostly animal-specific types
0.012
0.010
0.008
0.006
0.004
0.002
0.000

same or a related MLST ST. Strains of the same MLST and *sas* type were of the same *agr* type. The area of the tree inside the two dashed lines contained mostly strains that were of *sas* STs and MLST STs that had not been found in the human *S. aureus* population.

6.4. Discussion.

Due to advances in DNA sequencing, bioinformatics and bacterial genomics, a plethora of novel bacterial typing methods have been developed to simplify the work of bacterial epidemiologists, hospital outbreak investigators and evolutionary biologists. In the current study two sequence typing methods were used to investigate the clonality of isolates of *S. aureus* of animal origin.

6.4.1. *spa* typing.

spa typing has been shown to be effective in many different settings and to have a high discriminatory power similar to that of whole genome microarray. Whereas MLST has been shown to be discriminatory and unambiguous, it is expensive, relatively lengthy and quite labour intensive (not to the extent of PFGE, however). *spa* typing involves the examination of a single polymorphic locus and as a result has advantages over MLST. It also has a higher discriminatory ability than MLST (Table 6.4). However, *spa* typing used on its own, would not be sufficient to determine the broader global clonal relationships of strains without a method like MLST, PFGE or RAPD typing. It has been shown that *spa* typing can distinguish between strains of the same clonal MLST or PFGE type. It is thus an invaluable tool in an outbreak investigation. One of the problems with *spa* typing is the lack of a freely available database of repeats and types. Ridom StaphType, a software tool designed by Harmsen *et al.* (2003), has been used in the literature in assigning *spa* types, but is expensive and beyond the budget of the present research.

6.4.2. *sas* typing.

In the study of Robinson & Enright (2003) a high resolution multilocus sequence typing method was developed. The combination of seven putative *S. aureus* surface-(Sas)-protein-encoding genes with the seven MLST-associated genes was used to construct evolutionary models of the evolution of MRSA. It was thought that surface-protein-encoding genes would accumulate more genetic variations due to the pressure of the host

immune system. The *sas* genes had more polymorphic sites than the MLST housekeeping genes, the *sasD* and *sasF* genes having deletion/insertion polymorphisms. Despite this, no evidence of diversifying selection was observed. The d_s/d_n ratio was virtually the same for *sas* typing and MLST.

The current study is the first investigation to apply *sas* typing to *S. aureus* strains of animal origin and only the third study to apply this method to *S. aureus* (Robinson & Enright 2003; Robinson *et al.*, 2005). The *sas* genes were found to be present in almost all strains typed and it was possible to produce nucleotide sequences for the vast majority of the *sas* genes. Many novel *sas* types were observed in the animal-associated strains that had not been previously identified in the human-associated *S. aureus* population.

Roche *et al.* (2003) found that certain *sas* genes, *sasG* and *sasH*, were more often associated with invasive disease isolates than nasal carriage isolates. In the current study, the *sasH* gene was found to be present in all strains regardless of the animal host from which they were obtained. The *sasB* gene was not amplifiable from strains RF122 and RF80 and the *sasF* gene was not amplifiable from strains DS17 and DS20. This could mean that the genes were not present in the genome of these strains. Due to time constraints, Southern hybridisation analysis was not performed to confirm this. However, it is possible that the primers could not anneal to the template DNA due to low sequence similarity of the template to the primers, or due to the PCR conditions. The latter theory is more probable due to the fact that in the paper of Robinson & Enright (2003) all 136 strains, representing major MRSA and MSSA clonal MLST types that were subjected to *sas* typing, generated PCR products for all seven *sas* genes.

6.4.3. Comparison of sequencing methods.

The resolution power of *sas* typing and *spa* typing as well as MLST were compared using Simpson's Index of Diversity. The diversity resolved by *spa* typing was higher than for MLST, RAPD typing or *sas* typing. The diversities of MLST and *sas* typing were similar to each other. When used in combination, *sas* typing and MLST had a much higher resolution than *spa* typing or MLST alone and a resolution similar to *spa* typing. These data, along with the results of the study of Robinson & Enright (2003), suggest that MLST and/or *sas* typing along with *spa* typing could serve as an alternative to methods such as

PFGE for investigating genetically similar strains. These methods would be of use in analysing large collections for population-based studies.

However, in most laboratories the sequencing of seven MLST genes, seven *sas* genes and the repeats of *spa* gene would simply not be an option. The cost of this combination of methods is beyond the means of most budgets and not really suitable for routine diagnostics.

Sequence-based methods continue to be invaluable when examining the evolutionary relationships of populations of bacteria and global movements of clonal types. As DNA sequencing becomes less expensive these methods will no doubt become used more prevalently. The use of nucleotide sequences facilitates database creation. The use of a single polymorphic locus, such as *spa*, does not seem to be from the current study a suitable substitute for MLST or *sas* typing, but used in combination these methods may prove to be the gold standard methods of the next decade.

Chapter 7

Analysis of the *In Vivo* Expression of the SET (SSL) Proteins and of the Superantigen and Superantigen-Like Proteins Encoded by the *egc* Locus

7.1. Introduction

The screening of convalescent patients' sera for antibodies against staphylococcal proteins has proved an invaluable tool for assessing whether a particular gene is expressed *in vivo* and during the infectious process (Dryla *et al.*, 2005).

Several studies have used patients' sera to screen recombinant *S. aureus* surface proteins (Roche *et al.*, 2003, McCrea *at al.*, 2000), staphylococcal protein fragment expression libraries (Etz *et al.*, 2002, Weichhart *et al.*, 2003) or 2D-PAGE proteomic analysis of staphylococcal lysates (Vytvytska *et al.*, 2002) in order to identify which staphylococcal proteins are expressed during infections. A study by Lorenz *et al.* (2000) used human sera to detect *in vivo* expressed MRSA proteins, CspA (cold shock protein), and Hpr (phosphocarrier protein).

Convalescent and healthy donor sera can be compared to determine the preferential expression of proteins during infection. These *in vivo* expressed proteins are likely to be important virulence factors. Etz *et al.* (2002) used such an approach to identify proteins that are preferentially expressed during infections, such as SdrD, IsaA, Protein A, FnbpA and FnbpB (Etz *et al.*, 2002). The functions of many of the identified proteins were known. However, several proteins of unknown function were also identified. These proteins of known and unknown function could warrant further investigation to evaluate their potential as vaccine candidates.

The screening of patients' sera has also revealed the *in vivo* expression of secreted toxins. The *in vivo* expression of members of the novel *S. aureus* superantigen-like proteins [SET (SSLs)] has been demonstrated (Arcus *et al.*, 2002; Fitzgerald *et al.*, 2003). Although the functions of these proteins are not fully understood, they appear to interact with cells of the immune system such as monocytes and dendritic cells (Al-Shangiti *et al.*, 2005). In Chapter 4 screening for the *set* (*ssl*) genes in animal-associated *S. aureus* showed that every strain of *S. aureus* from several animal hosts possessed a cluster of *set* (*ssl*) genes. The fact that every strain contained at least one *set* (*ssl*) gene, and that the SET (SSL) proteins are expressed in the human host *in vivo* (Arcus *et al.*, 2002; Fitzgerald *et al.*, 2003), infers that these proteins could be potential vaccine candidates. However, the *in vivo* expression of these proteins has yet to be evaluated in animals.

In Chapter 3, animal-associated *S. aureus* were screened for the presence of SAg and SAg-like genes. It was found that the SAg and SAg-like genes associated with the *egc* locus (*seg*, *sei*, *selm*, *seln* and *selo*) were highly prevalent in strains of *S. aureus* of animal origin (Fig. 7.1). No data exist on the *in vivo* expression of these novel SAg and SAg-like genes in the animal host. In the absence of such data, mitogenicity assays were employed to investigate the ability of strains encoding these SAg and SAg-like genes to cause the proliferation of T cells, thus implying superantigenic activity.

In the study of Fitzgerald *et al.* (2003), the pET system was used to generate recombinant SET (SSL) proteins (Fig. 7.2). The *set* (*ssl*) genes from strains COL and NCTC8325 were cloned into the pET21b vector. *E. coli* lysates containing recombinant SET/SSL proteins were employed in Western immunoblotting experiments using acute and convalescent sera from human patients recovering from staphylococcal infections including abscesses, cellulitis, pneumonia, and purulent arthritis to investigate if the SET (SSL) proteins were expressed during infection.

The pET system, one of the most efficient systems developed for the cloning and expression of recombinant proteins in *E. coli*, was used herein as the inducible expression system. This system is based on a strategy developed by Studier *et al.* (1990). In the pET system target genes are cloned into plasmids under the control of a strong bacteriophage T7 promoter and expression is induced by providing a source of T7 RNA polymerase in the host cell that limits the number of host strains that can be used (Novagen, 2002). When fully induced, the T7 RNA polymerase directs almost all of the cell's resources into target gene expression and as a result the desired protein can comprise more than 50% of the total cell protein within a few hours after induction. The level of expression can be regulated by lowering or raising the level of the inducer (in this case the inducer is IPTG – isopropyl β-D-thiogalactopyranoside) or by altering the temperature during induction (Schein & Noteborn 1989; Novagen, 2002). In the initial stages target genes are cloned into vectors and host strains, such as pBluescript (*E coli* vector) and strain DH5α (host), that do not contain the T7 RNA polymerase gene. The plasmid is more stable as a result.

Once established in a non-expression host, target protein expression may be initiated either by infecting the host with bacteriophage λCE6, a phage that carries the T7 RNA polymerase gene under the control of the λ *p*L and *p*I promoters, or by transferring the

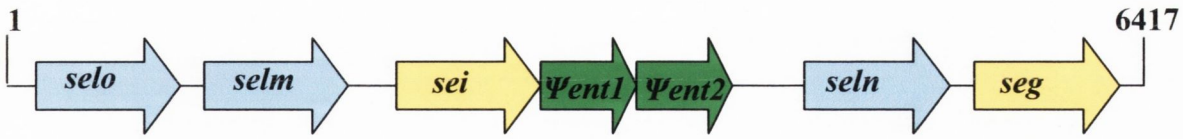


Fig 7.1. Schematic of the enterotoxin gene cluster (*egc*).

The *egc* cluster of genes encodes two SAgs (in yellow), three SAg-like genes (blue) as well as two pseuodgenes (green). This cluster of genes was found to be highly prevalent in strains of *S. aureus* from animals (Chapter 3). In the current chapter the *egc*-encoded genes from *S. aureus* bovine strain RF122 were cloned into pET22b vectors.

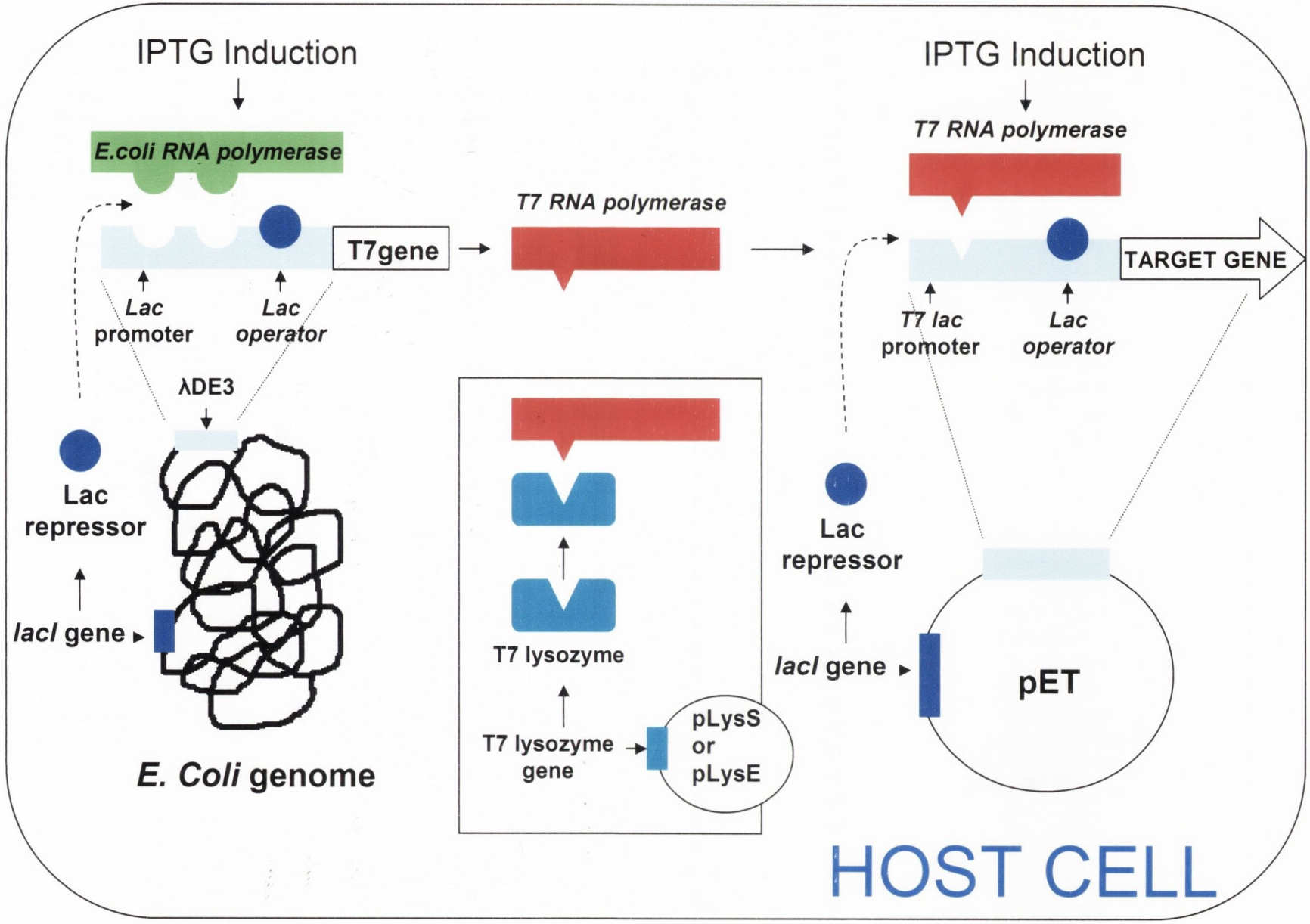
Fig. 7.2. Control elements of the pET system [Figure adapted from Novagen (2002)].

In the pET system, target genes are cloned in pET plasmids under the control of strong bacteriophage T7 promoters; expression is induced by providing a source of T7 RNA polymerase in the *E. coli* host cell. Host cells for expression bear the T7 RNA polymerase gene encoded on *E. coli* genome (λDE3 lysogen). In λDE3 lysogens the T7 polymerase gene is under the control of the *lacUV5* promoter, which is controlled by the binding of Lac repressor protein at the operator site suppressing basal expression in uninduced cells. The gene encoding the Lac repressor protein is present in the genome of λDE3 lysogen strains.

For more stringent control of expression, host strains bearing the pLysS or pLysE plasmids are available that encode T7 lysozyme, a natural inhibitor of T7 RNA polymerase. T7 lysozyme reduces the ability of T7 RNA polymerase to transcribe target genes in the uninduced state. The T7*lac* promoter in the pET vector contains a 25 bp *lac* operator sequence downstream from the *lac* promoter region. Binding of the Lac repressor protein at the operator site reduces the transcription by T7 RNA polymerase, again suppressing basal levels of expression. pET vectors also contain the gene for the Lac repressor protein.

IPTG induction removes the Lac repressor protein from the operator sites and allows expression of target genes to proceed.

T7 RNA polymerase is so selective and active that, when fully induced, almost all of the cell's resources are converted to target gene expression; the desired product can comprise more than 50% of the total cell protein within a few hours after induction. It is also possible to attenuate expression levels simply by lowering the concentration of inducer.


IPTG Induction
E.coli RNA polymerase
T7gene
Lac promoter
Lac operator
λDE3
Lac repressor
lacI gene
E. Coli genome
T7 RNA polymerase
T7 lysozyme
T7 lysozyme gene
pLysS or pLysE
IPTG Induction
T7 RNA polymerase
TARGET GENE
T7 lac promoter
Lac operator
Lac repressor
lacI gene
pET
HOST CELL

plasmid into an expression host containing a chromosomal copy of the T7 RNA polymerase gene under *lacUV5* control (Novagen, 2002).

Two types of T7 promoter and several hosts that differ in their stringency of suppressing basal expression levels are available, providing great flexibility and the ability to optimize the expression of a wide variety of target genes. Also a great variety of pET vectors are available that have different features such as antibiotic resistance cassettes or fusion tags, giving the user great choice and flexibility.

The aim of the this work was to investigate if the SAg and SAg-like genes associated with the *egc* cluster (*seg*, *sei*, *selm*, *seln* and *selo*) and the *set* (*ssl*) genes were expressed *in vivo* during bovine mastitis. This work also aimed to reveal if these proteins were potentially important virulence factors during infection and accordingly if they might have potential as vaccine candidates. To this end the SAg-like genes associated with the *egc* cluster (*seg*, *sei*, *selm*, *seln* and *selo*) were cloned into pET vectors. In Chapter 4, quarter milk and serum samples from four cows with mastitis were obtained from Teagasc Dairy Production Centre, Moorepark, Fermoy, Co. Cork. Four samples of serum from cows with low somatic cell counts and considered to be free of mastitis infection were also obtained. *S. aureus* strains were cultured from the milk samples from the cows with mastitis and were RAPD typed and tested for the presence of SAg, SAg-like and *set* (*ssl*) genes in Chapter 4. The serum samples were to be used in the present investigation in Western immunoblotting experiments using lysates of *E. coli* strains bearing pET vectors with cloned SAg, SAg-like encoding genes and *set* (*ssl*) genes.

7.2. Methods.

7.2.1. Cloning of genomic *S. aureus* fragments.

7.2.1.1. Construction of pET22b plasmids containing SAg and SAg-like genes.

The pET vectors [all pET21b except pET15 for SET10 (SSL5)] containing the *set* (*ssl*) genes from human-associated strains COL and NCTC8325 were provided by Dr J. Ross Fitzgerald, Zoonotic and Animal Pathogens Research Laboratory, Royal (Dick) School of Veterinary Studies, University of Edinburgh, Scotland (Fitzgerald *et al.*, 2003).

The signal sequences of the SAg and the SAg-like proteins encoded by the *egc* locus were detected by SignalP (http://www.cbs.dtu.dk/services/SignalP/). Primers were designed on the basis of the published sequence of the *egc* locus (Accession number: AF285760) to amplify the SAg and the SAg-like genes minus their signal sequences to ensure the direction of the recombinant protein to the periplasm and to include *Bam*HI and *Hin*dIII restriction sites to facilitate cloning into the *Bam*HI and *Hin*dIII cleavage sites of the multiple cloning site (MCS) of the pET vector (Table 7.1).

The SAg and SAg-like genes were cloned in frame with the vector without a signal sequence to retain the protein in the cytoplasm and without the terminator codon allowing the addition of the His tag. This tag would facilitate future purification of recombinant protein using a Ni^{2+} column (Xiong *et al.*, 2000). The purification of the SAg and SAg-like proteins was not attempted herein due to the fact that the constructs provided by Dr. J. Ross Fitzgerald were cloned in such a way as to remove the His tag from the recombinant protein. As a result only the constructs generated herein could have been purifed in this way.

The figure below indicates the structure of one of the forward primers.

CCCGGATCCAXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX...

Start of SAg gene sequence with signal sequence removed

CCC – clamp
GGATCC – *Bam*HI resctriction site
└─┘ - denotes the frame
A – this base was added to put the cloned gene 'in-frame' with the vector
X – primer sequence

The reverse primers included a *Hin*dIII site but not the terminator codon so that the His-tag would be included to facilitate future purification of the protein.

XXXXXXXXXXXXXXXXXXXXXXXXXXXACAAAGCTTCCC

CCC – clamp
AAGCTT – *Hin*dIII restriction site
└─┘ - denotes the frame
ACA – this codon was the codon before the terminator codon
X – primer sequence

Table 7.1. Primers used in cloning *egc*-encoded SAg and SAg-like genes.

Gene	Primer Sequence (5′–3′)	T_m (°C)
seg for	CCCGGATCCACAACCCGATCTTAAATTAGACGAACT	70.6
seg rev	GGGAAGCTTGTGAGTATTAAGAAATACTTCCAT	64.5
sei for	CCCGGATCCACAAGGTGATATTGGTGTAGGTAACTTAAGAAATTTC	72.1
sei rev	GGGAAGCTTGTTACTATCTACATATGATATTTCGACATCAAGATG	69.4
selm for	CCCGGATCCAGATGTCGGAGTTTTGAATCTTAGGAACTATTATGGTAG	73.7
selm rev	GGGAAGCTTACTTTCGTCCTTATAAGATATTTCTACATCTAAATGGAA	69.4
seln for	CCCGGATCCAGAAGTAGACAAAAAAGATTTAAAGAAAAAATCTGATC	69.4
seln rev	GGGAAGCTTATCTTTATATAAAAATACATCAATATGATAATTAGATGAGC	67.0
selo for	CCCGGATCCATGTAGTGTAAACAATGCATATGCAAATG	69.5
selo rev	GGGAAGCTTTGTAAATAAATAAACATCAATATGATAGTC	64.2
T7 promoter primer	TAATACGACTCACTATAGG	50.2
T7 terminator primer	CTAGTTATTGCTCAGCGG	53.7

The *Bam*HI and *Hin*dIII restriction sites are underlined. The T7 promoters amplify the multiple cloning site of the pET22b vector.

Initially attempts were made to clone the SAg and SAg-like genes from bovine strain RF122 into pET21b (Fig 7.3a). However, these attempts failed. Another pET vector, pET22b was used as a substitute (Fig 7.3b, Fig. 7.4). Plasmid pET22b has a *pelB* leader sequence which directs the recombinant protein to the periplasm unlike plasmid pET21b which directs the recombinant protein to the cytoplasm. The five SAg and SAg-like genes were amplified using *Pfu* DNA polymerase (Promega) and genomic DNA from bovine strain RF122. Each set of primers used the same PCR reaction mix comprising 10 pmol forward and reverse primers, 200 μM each of dNTP, dTTP, dCTP and dATP, 1 × magnesium-free buffer, 1.5 U *Pfu* DNA polymerase (Promega), 3 mM $MgCl_2$ and 50–100 ng DNA. The PCR reactions used the following cycling parameters: 94 °C for 10 min, 3 cycles of [94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min] and 27 cycles of [94 °C for 1 min, 65 °C for 1 min, and 72 °C for 1 min]. The reaction was terminated with a 10-min incubation at 72 °C. PCR products were resolved by electrophoresis in 1.5% (w/v) agarose gels (0.5 × Tris-boric acid-EDTA) (Sambrook & Russell, 2001) at 90 V (constant voltage), stained with ethidium bromide, and visualized using UV light. Product sizes were determined by comparison with a 100-bp ladder (Promega). The quantified PCR products were purified by gel extraction using the High Pure PCR Purification kit according to the manufacturer's instructions (Roche) and resolved by electrophoresis as before (Fig 7.5A).

Following electrophoresis, the PCR products and the pET22b vector were digested with restriction enzymes *Bam*HI and *Hin*dIII at 37 °C overnight, purified by gel extraction and resolved by electrophoresis (Fig 7.5B).

Ligation reactions comprised the following in a total volume of 20 μl: 3 μl of digested pET22b vector, 5 μl of each digested PCR product, 2 μl 10 × buffer (Roche) and 1 μl T4 DNA ligase (Roche). Ligation reactions were allowed to stand overnight at room temperature.

7.2.1.2. Preparation and transformation of competent bacteria

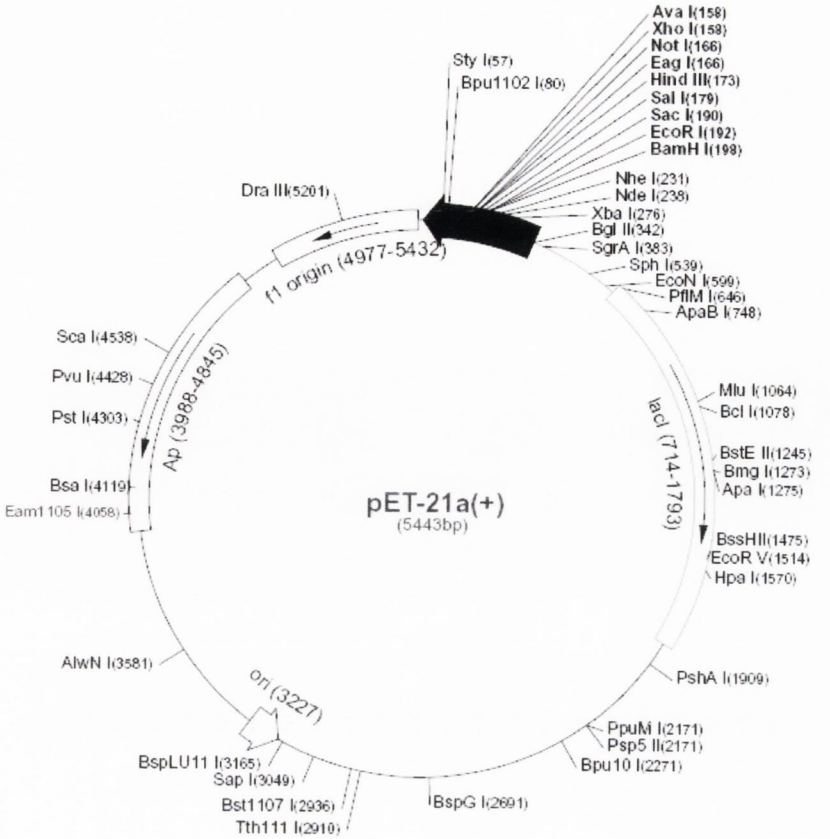
Competent strain DH5α or strain BL21 *E. coli* cells were prepared as follows. L-broth supplemented with appropriate antibiotics (400 ml) was inoculated with 2 ml of an overnight culture. Cells were incubated at 37 °C with shaking until mid-log growth phase (approx. 2–2.5 h, OD_{600}=0.4–0.5). The cells were chilled on ice for 1 h. The culture was

Fig. 7.3. Maps of the pET vectors used in this work.

Panel (A) shows vector pET21b. This vector carries an N-terminal T7-tag sequence, as well as an optional C-terminal His-tag sequence and encodes ampicillin resistance. The *set/ssl* genes used in this Chapter were cloned into vector pET21b by Dr. J. Ross Fitzgerald. The genes were cloned into the *Nde*I and *Bam*HI sites, thus removing the T7-tag, and included a stop codon, thus removing the His-tag. The b of the vector name refers to the frame of the vector.

Panel (B) shows plasmid pET22b. This vector is very similar to plasmid vector pET21b but includes a *pelB* leader sequence in the cloning/expression region. This directs the recombinant protein to the periplasm as opposed to the cytoplasm.

(A)


Ava I(158)
Xho I(158)
Not I(166)
Eag I(166)
Hind III(173)
Sal I(179)
Sac I(190)
EcoR I(192)
BamH I(198)
Sty I(57)
Bpu1102 I(80)
Nhe I(231)
Nde I(238)
Xba I(276)
Bgl II(342)
SgrA I(383)
Sph I(539)
EcoN I(599)
PflM I(646)
ApaB I(748)
Dra III(5201)
f1 origin (4977-5432)
Sca I(4538)
Pvu I(4428)
Pst I(4303)
Ap (3988-4845)
Bsa I(4119)
Eam1105 I(4058)
Mlu I(1064)
Bcl I(1078)
lacI (714-1793)
BstE II(1245)
Bmg I(1273)
Apa I(1275)
pET-21a(+)
(5443bp)
BssHII(1475)
EcoR V(1514)
Hpa I(1570)
AlwN I(3581)
ori (3227)
PshA I(1909)
PpuM I(2171)
Psp5 II(2171)
Bpu10 I(2271)
BspLU11 I(3165)
Sap I(3049)
Bst1107 I(2936)
Tth111 I(2910)
BspG I(2691)

(B)

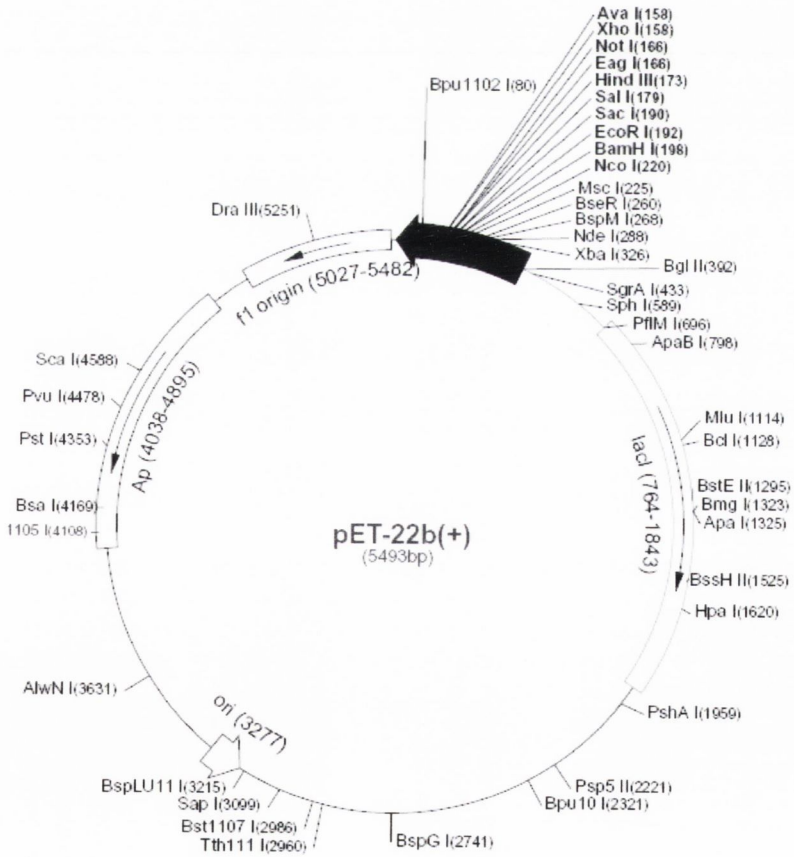
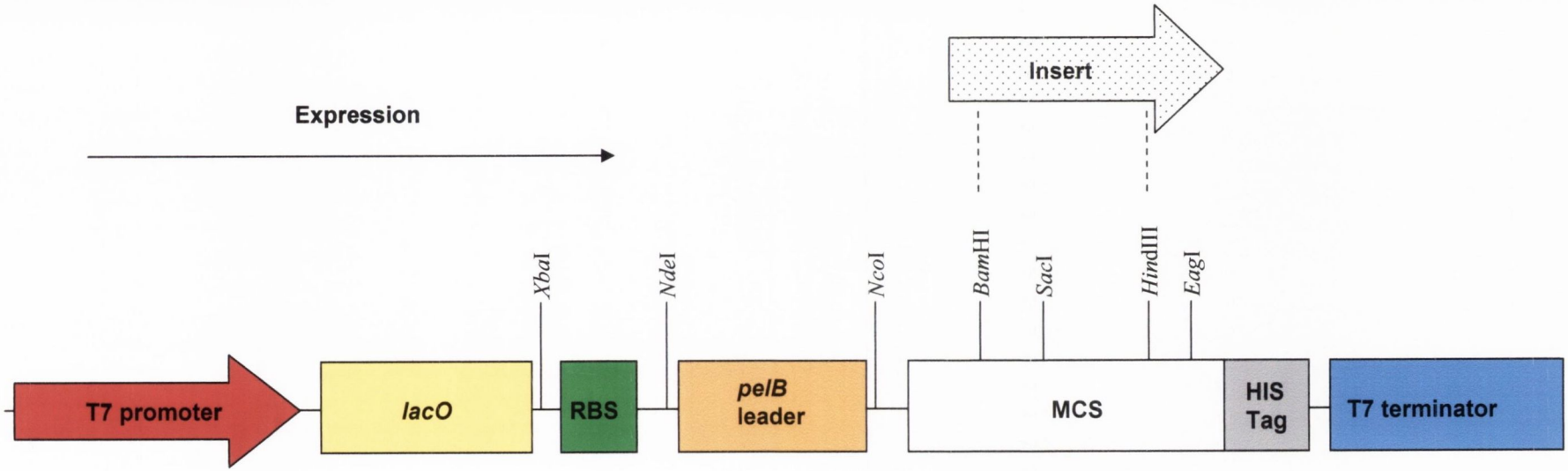
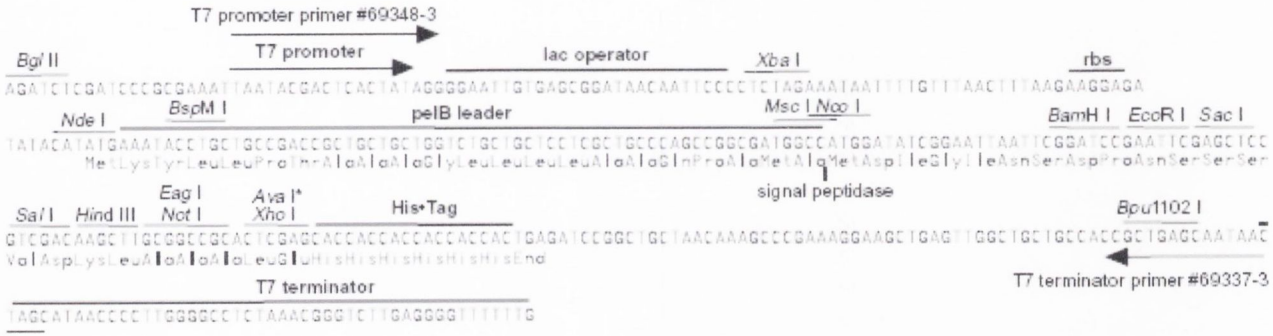

Ava I(158)
Xho I(158)
Not I(166)
Eag I(166)
Hind III(173)
Sal I(179)
Sac I(190)
EcoR I(192)
BamH I(198)
Nco I(220)
Bpu1102 I(80)
Msc I(225)
BseR I(260)
BspM I(268)
Nde I(288)
Xba I(326)
Bgl II(392)
Dra III(5251)
f1 origin (5027-5482)
SgrA I(433)
Sph I(589)
PflM I(696)
ApaB I(798)
Sca I(4588)
Pvu I(4478)
Pst I(4353)
Ap (4038-4895)
Bsa I(4169)
1105 I(4108)
Mlu I(1114)
Bcl I(1128)
lacI (764-1843)
BstE II(1295)
Bmg I(1323)
Apa I(1325)
pET-22b(+)
(5493bp)
BssH II(1525)
Hpa I(1620)
AlwN I(3631)
ori (3277)
PshA I(1959)
BspLU11 I(3215)
Sap I(3099)
Psp5 II(2221)
Bpu10 I(2321)
Bst1107 I(2986)
Tth111 I(2960)
BspG I(2741)

Fig. 7.4. General schematic of the pET22b vector expression system.

The five genes from the enterotoxin-gene cluster (*egc*) from *S. aureus* bovine strain RF122 were cloned 'in-frame' into the *Bam*HI and *Hin*dIII restriction sites of the multiple cloning site (MCS) of the pET22b vector minus the terminator codon, therefore facilitating the inclusion of a His-Tag onto the recombinant protein.





pET-22b(+) cloning/expression region

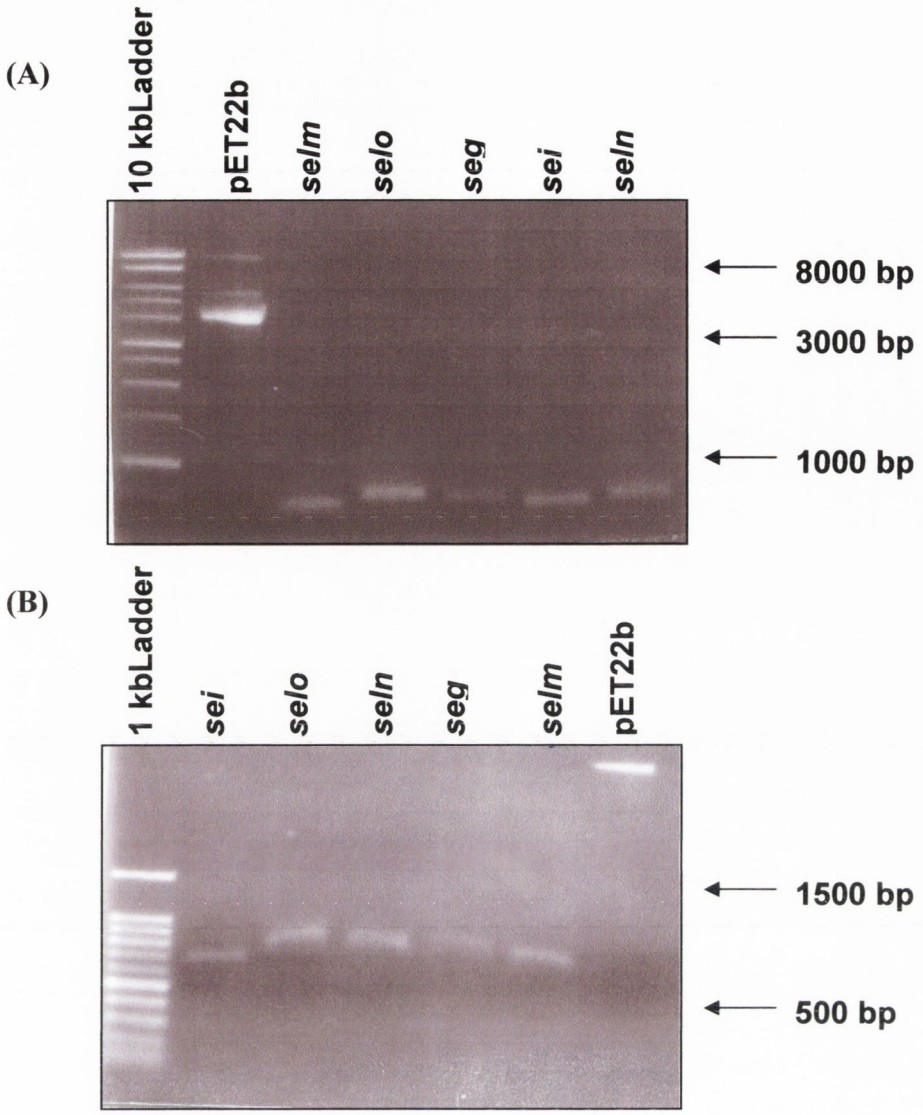


Fig.7.5. Agarose gel electrophoresis of amplified SAg and SAg-like PCR products.

Panel (A) shows gel-purified PCR products of SAg (*seg*, *sei*) and SAg-like (*selm*, *seln* and *selo*) genes from strain RF122.

Panel (B) shows gel-purified *Bam*HI- and *Hin*dIII-digested PCR products of SAg (*seg*, *sei*) and SAg-like (*selm*, *seln* and *selo*) genes from strain RF122.

decanted into 250-ml bottles and centrifuged at 2603 × *g* using a Sorvall GSA rotor for 10 min at 4 °C. The cells were gently resuspended in 2 × 30 ml ice-cold 100 mM $MgCl_2$ and recentrifuged in the GSA rotor for 10 min at 4 °C. The cells were gently resuspended in 2 × 30 ml ice cold 100 mM $CaCl_2$, placed on ice for 10 min and harvested as before. Cells were resuspended in 2 × 50 ml ice cold 60 mM $CaCl_2$, 10 mM PIPES, 15 % (v/v) glycerol and pelleted as before. The cells were resuspended with 2 × 20 ml ice cold 60 mM $CaCl_2$, 10 mM PIPES, 15 % (v/v) glycerol. Ice-cold 80 % (v/v) glycerol was gradually added with swirling to 10 % final solution. The suspension was aliquoted into 500 µl amounts in Eppendorf tubes and placed on ice. The tubes were snap-frozen in liquid nitrogen and immediately placed at –70 °C in a pre-chilled container.

Recombinant pET22b plasmids containing cloned SAg and SAg-like genes were transformed into competent cells as follows. Of the vector DNA, 100–200 ng was added to 200 µl competent *E. coli* (strain DH5α for storage of the plasmids or strain BL21 for expression) and kept on ice for 30 min. The cells were then placed in a water bath at 42 °C for 45 s and then placed on ice for 2 min. To this 1 ml of prewarmed L-broth was added and the cells were shaken at 37 °C for 1 h. Of the cell suspension, 100 µl was pipetted onto the centre of a prewarmed L-agar plate containing ampicillin (100 µg/ml) and the remainder harvested in a benchtop centrifuge (Sorvall Microspin, 12,300 × *g*) resuspended in 100 µl L-broth and pipetted onto the centre of a prewarmed L-agar plate as before. Plasmids without insert DNA were also subjected to the above procedure to check the competency of the cells.

Transformants were screened using a colony PCR screening procedure. Briefly the colony was toothpicked onto an agar plate with ampicillin (100 µg/ml) and the remainder of the colony was emulsified in 20 µl of sterile distilled water and boiled for 10 min. Of this boiled lysate 5 µl was used as template in a PCR reaction using primers specific to the pET22b vector backbone (Table 7.1) that would amplify across the multiple cloning site containing the insert of interest (Fig 7.6). Insert-positive pET22b vectors were purified using the High Pure Plasmid Isolation kit (Roche), precipitated with ethanol and sent to MWG-Biotech for sequencing. The sequences obtained were aligned with the sequences of the published *egc* cluster encoded genes using ClustalX to verify and check the sequences (Fig. 7.7) and analysed by BlastP to investigate the level of identity of the SAg and SAg-like genes from the bovine strain to other published SAg and SAg-like genes.

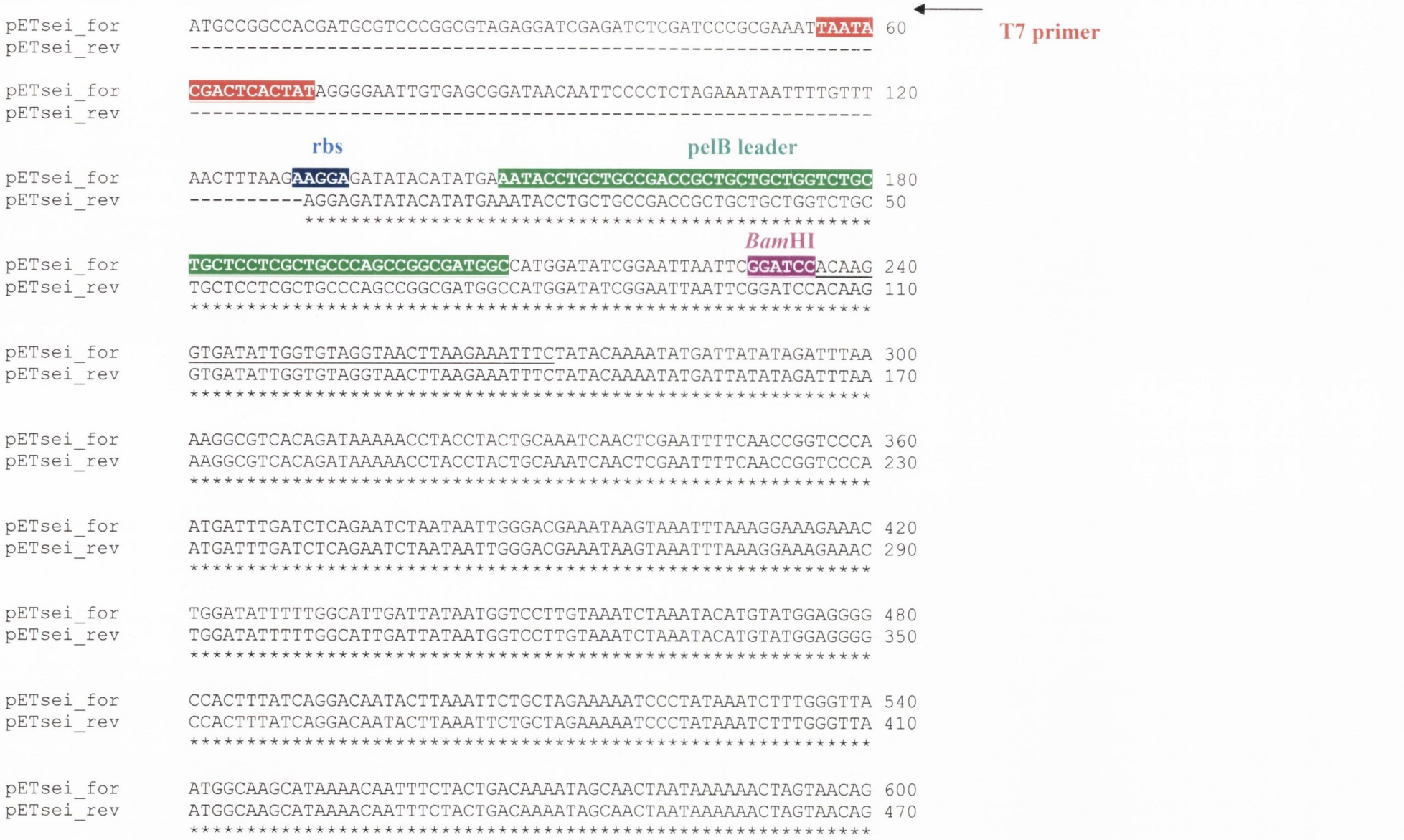
Fig.7.6. Agarose gel electrophoresis of PCR products from transformants bearing pET22b constructs with cloned *egc* locus genes.

Panel (A): Lanes 1 and 2 were transformants from ligations using restricted *seg*-bearing DNA; lanes 3 and 4 were from transformants from ligations using *selm* gene DNA. pET22b was used as a control in the PCR reactions and generated an approx. 300-bp-product.

Panel (B): Lanes 1 – 9 were transformants from ligations using *sei* gene DNA. pET22b was used as a control in the PCR reactions and generated an approx. 300-bp-product.

Panel (C): Lanes 1 – 5 were transformants from ligations using *selo* gene DNA, lanes 6 – 10 were transformants from ligations using *seln* gene DNA. A negative control using sterile distilled water was included.

(C)
10 kbp Ladder
1
2
3
4
5
6
7
8
9
10
Neg control
250 bp
1000 bp
(B)
10 kbp Ladder
1
2
3
4
5
6
7
8
9
pET22b
250 bp
1000 bp
(A)
1 kbp Ladder
1
2
3
4
pET22b
250 bp
1000 bp


T7 primer
rbs
pelB leader
BamHI
pETsei_for ATGCCGGCCACGATGCGTCCCGGCGTAGAGGATCGAGATCTCGATCCCGCGAAATTAATA 60
pETsei_rev --
pETsei_for CGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTT 120
pETsei_rev --
pETsei_for AACTTTAAGAAGGAGATATACATATGAATACCTGCTGCCGACCGCTGCTGCTGGTCTGC 180
pETsei_rev ----------AGGAGATATACATATGAAATACCTGCTGCCGACCGCTGCTGCTGGTCTGC 50
****
pETsei_for TGCTCCTCGCTGCCCAGCCGGCGATGGCCATGGATATCGGAATTAATTCGGATCCACAAG 240
pETsei_rev TGCTCCTCGCTGCCCAGCCGGCGATGGCCATGGATATCGGAATTAATTCGGATCCACAAG 110
****
pETsei_for GTGATATTGGTGTAGGTAACTTAAGAAATTTCTATACAAAATATGATTATATAGATTTAA 300
pETsei_rev GTGATATTGGTGTAGGTAACTTAAGAAATTTCTATACAAAATATGATTATATAGATTTAA 170
****
pETsei_for AAGGCGTCACAGATAAAAACCTACCTACTGCAAATCAACTCGAATTTTCAACCGGTCCCA 360
pETsei_rev AAGGCGTCACAGATAAAAACCTACCTACTGCAAATCAACTCGAATTTTCAACCGGTCCCA 230
****
pETsei_for ATGATTTGATCTCAGAATCTAATAATTGGGACGAAATAAGTAAATTTAAAGGAAAGAAAC 420
pETsei_rev ATGATTTGATCTCAGAATCTAATAATTGGGACGAAATAAGTAAATTTAAAGGAAAGAAAC 290
****
pETsei_for TGGATATTTTTGGCATTGATTATAATGGTCCTTGTAAATCTAAATACATGTATGGAGGGG 480
pETsei_rev TGGATATTTTTGGCATTGATTATAATGGTCCTTGTAAATCTAAATACATGTATGGAGGGG 350
****
pETsei_for CCACTTTATCAGGACAATACTTAAATTCTGCTAGAAAAATCCCTATAAATCTTTGGGTTA 540
pETsei_rev CCACTTTATCAGGACAATACTTAAATTCTGCTAGAAAAATCCCTATAAATCTTTGGGTTA 410
****
pETsei_for ATGGCAAGCATAAAACAATTTCTACTGACAAAATAGCAACTAATAAAAAACTAGTAACAG 600
pETsei_rev ATGGCAAGCATAAAACAATTTCTACTGACAAAATAGCAACTAATAAAAAACTAGTAACAG 470
****

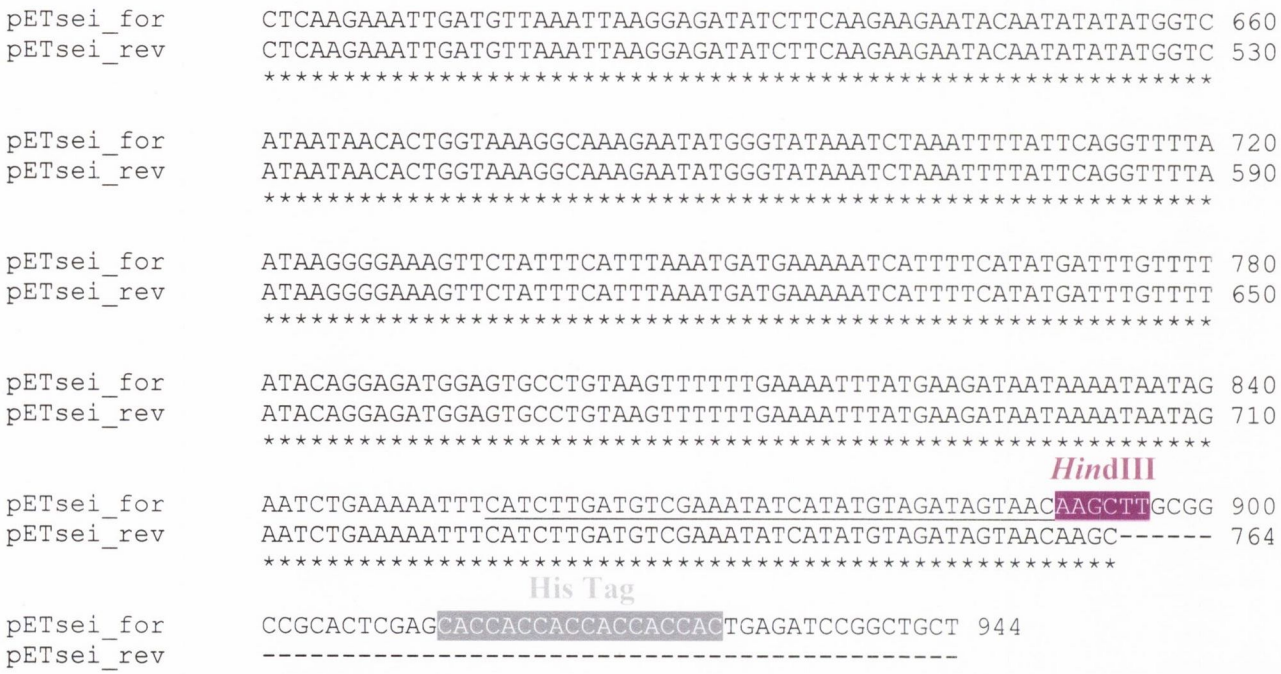


Fig. 7.7. ClustalW-generated alignments of both strands of the sequenced pET*sei* vector.

pET22b vectors with cloned SAg and SAg-like genes were amplified by PCR using primers specific to the vector backbone and sequenced on both strands. The sequences were aligned and checked to verify the inclusion of the cloned gene product and to ensure that the sequences were in the correct frame.

7.2.2. Protein analysis.

7.2.2.1. Induction of cultures and preparation of E. coli cells for SDS-PAGE.

Aliquots (3 ml) of L-broth containing ampicillin (100 μg/ml) were each inoculated with a colony of BL21 cells containing pET21b vectors with cloned *set* (*ssl*) genes, pET22b vectors containing SAg and SAg-like genes or pET21b/pET22b with no insert. The tubes were incubated at 37 °C with shaking until an OD_{600} of approximately 0.5 was reached. Then the entire 3 ml of culture was added to 100 ml LB containing ampicillin (100 μg/ml). The 100 ml culture was shaken for 2–3 h until the OD_{600} was approximately 0.5–1.0. At this point the culture was split into 2 × 50 ml aliquots. IPTG to 1 mM (500 μl of sterile 100 mM IPTG) was added to one of the 50 ml aliquots. The other culture served as the uninduced control. At 3 h post-induction 1-ml samples were taken and the OD_{600} values recorded. The cells were harvested and resuspended in PBS and pelleted at maximum speed twice using a benchtop microfuge (12,300 × *g*). At this stage the samples were frozen and maintained at –20 °C. In order to obtain approximately equal loadings of total protein on the SDS-PAGE gel, the cells were resuspended in 1 × SDS sample buffer such that for each OD of 0.2, 20 μl SDS sample buffer was used. Samples were boiled for 5 min immediately before loading 10 μl of the sample onto the gel. Prestained molecular weight markers were included on every gel (Prestained Protein Marker, low range, New England Biolabs) which included protein bands of the following masses 175 kDa, 83 kDa, 62 kDa, 47.5 kDa, 32.5 kDa, 25 kDa, 16.5 kDa and 6.5 kDa.

7.2.2.2.Sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

The discontinuous SDS system of Laemmli (1970) was utilised for SDS-PAGE. Gels were electrophoresed at a constant voltage of 120 V for 2 h. Samples were resolved through separating gels consisting of 12 % (w/v) polyacrylamide with stacking gels consisting of 5 % (w/v) polyacrylamide. Tris-glycine electrophoresis buffer [25 mM Tris, 250 mM glycine (pH 8.3), 0.1 % (w/v) SDS] was added to the gel tank prior to application of samples to the wells. Separated samples were stained using Coomassie brilliant blue (250 mg Coomassie brilliant blue, 90 ml methanol:H_2O (1:1 v/v), 10 ml glacial acetic acid) for a minimum of 4 h. Excess dye was removed from the gel by soaking in destain solution (methanol-acetic acid solution described above but omitting the dye) for several hours with frequent changing of the destain solution.

7.2.2.3. Transfer of separated proteins to membranes.

After electrophoresis was completed the separating gel was immersed in transfer buffer [25mM Tris-HCl (pH 8.3), 192 mM glycine, 20 % (v/v) methanol] to equilibrate for 30 min with gentle shaking. The separated polypeptides were transferred electrophoretically onto Polyvinylidene Difluoride (PVDF) filters (Millipore) which had been soaked in 100 % methanol for 15 s, distilled H_2O for 2 min and equilibrated in transfer buffer as before. Transfer was completed at a constant voltage of 19 V for 1 h using a wet system (Bio-Rad). Afterwards any proteins remaining in the gel were stained with Coomassie brilliant blue. Transfer of proteins to the PVDF membrane (Millipore) was confirmed by staining the membrane in Ponceau S solution [0.1 % (w/v) Ponceau S in 5 % (v/v) acetic acid] for 5 min and destaining in several changes of distilled water.

7.2.2.4. Western immunoblotting using bovine serum.

Following immobilisation of the separated proteins on PVDF (Millipore) filters, the filters were blocked and then probed with polyclonal sera from cows with mastitis (Table 7.2). Blocking typically took place overnight at 4 °C in blocking solution consisting of 5 % (w/v) dried milk (Marvel) in PBS with gentle shaking. Incubation with a 1:300 dilution of bovine sera in 5 % (w/v) dried milk (Marvel) in PBS then followed overnight at 4 °C with shaking. Unbound antibody was removed by washing the membrane three times for 20 min in PBS. Protein A coupled to horseradish peroxidase (Sigma), diluted 1:500 in 5 % (w/v) dried milk in PBS was incubated with the membrane for 1 h with shaking. The membrane was then washed three times for 20 min in PBS buffer. The blot was then visualized by developing in the dark using the LumiGLO Reserve™ chemiluminescent substrate (Cell Signalling Technologies), and finally exposed to X-Omat autoradiographic film (Kodak).

Importantly, it should be noted that several different protocols were tried. Several different membranes were used. Extensive optimisation was necessary in order to generate results for Western immunoblotting using the bovine sera as poor reproducibility was a problem.

7.3. Results.

7.3.1. Cloning of genomic *S. aureus* fragments.

All five SAg and SAg-like genes (*seg*, *sei*, *selm*, *seln* and *selo*) from bovine strain RF122 were successfully cloned into pET22b. The nucleotide sequences of the genes from the bovine strain were aligned by ClustalW and the translated proteins were subjected to BlastP. An example of an alignment of a PCR amplified pET22b plasmid with an insert is shown in Figure 7.7.

The *egc*-encoded proteins from RF122 were not identical to the *egc*-encoded proteins described by Jarraud *et al.* (2001) (Accession number: AF285760). The SEI, SElN, and SElO proteins from strain RF122 had amino-acid similarities of 97%, 97% and 90% to the strain A900322 *egc*-encoded proteins, respectively (Fig 7.8). The proteins were analysed by BlastP to assess their similarity to *egc*-encoded variants in the NCBI database. The SElO protein from strain RF122 had 98% and 90% amino-acid identities to SElO variants with accession numbers YP_041295 (Holden *et al.*, 2004) and NP_372354 (Kuroda *et al.*, 2001), respectively. The SElN protein from strain RF122 had 98% and 96% amino-acid identities to SElN variants with accession numbers NP_372349 (Kuroda *et al.*, 2001) and YP_041291 (Holden *et al.*, 2004), respectively. The SEI protein from strain RF122 had amino-acid identities of 96%, 97%, 97%, 96% and 96% to SEI variants with accession numbers YP_041293 (Holden *et al.*, 2004), AAX11330 (Blaiotta *et al.*, 2005, direct submission to NCBI database), NP_372352 (Kuroda *et al.*, 2001), BAB85991 (Omoe, *et al.*, 2002) and AAP78526 (Blaiotta *et al.*, 2004).

The genes encoding the SEG and SElM proteins from strain RF122 contained a number of terminator codons that altered the frame and disabled the translation of the recombinant protein. The *selm* gene from strain RF122 had an extra T at position 473 of the cloned gene. The *seg* gene from strain RF122 had base pair deletion of an A at position 499 of the cloned gene.

The SEI, SElN and SElO proteins from strain RF122 therefore represent novel bovine-associated variants.

```
SElO_RF122_         CSVNNAYANEENPKIEDLCKKSSVDDIALHNIDKDYMTNRFTINESPVLTTEKFLDFDLL 60
SElO_A900322_       CSVNNAYANEEDPKIESLCKKSSVDPIALHNINDDYINNRFTTVKSIVSTTEKFLDFDLL 60
                    ***********:****.******** ******:.**:.****  :* * ***********

SElO_RF122_         FKNFTWLDGKSAEFKDLKVEFSSSEISKEYFGKTVDIYGVYYKAHCHGEHQVKTACTYGG 120
SElO_A900322_       FKSINWLDGISAEFKDLKVEFSSSAISKEFLGKTVDIYGVYYKAHCHGEHQVDTACTYGG 120
                    **.:.**** ************** ****::*********************.******* 

SElO_RF122_         VTPHENNKLNEPKEIGVAVYKDNVNVNTFIVTTDKKKVTAQELDIKVRTKLNNVYKLYDR 180
SElO_A900322_       VTPHENNKLSEPKNIGVAVYKDNVNVNTFIVTTDKKKVTAQELDIKVRTKLNNAYKLYDR 180
                    *********.***:****************************************.****** 

SElO_RF122_         MTSDVQKGYIKFHSHSEHKESFYYDLFYIKGNLPDQCLQIYNDNKTIDSSDYHIDVYLFT 240
SElO_A900322_       MTSDVQKGYIKFHSHSEHKESFYYDLFYIKGNLPDQYLQIYNDNKTIDSSDYHIDVYLFT 240
                    ********************************** ********************** 
```

```
SEI_RF122_          QGDIGVGNLRNFYTKYDYIDLKGVTDKNLPTANQLEFSTGPNDLISESNNWDEISKFKGK 60
SEI_A900322_        QGDIGVGNLRNFYTKHDYIDLKGVTDKNLPIANQLEFSTGTNDLISESNNWDEISKFKGK 60
                    ***************:************** *********.****************** 

SEI_RF122_          KLDIFGIDYNGPCKSKYMYGGATLSGQYLNSARKIPINLWVNGKHKTISTDKIATNKKLV 120
SEI_A900322_        KLDIFGIDYNGPCKSKYMYGGATLSGQYLNSARKIPINLWVNGKHKTISTDKIATNKKLV 120
                    ************************************************************ 

SEI_RF122_          TAQEIDVKLRRYLQEEYNIYGHNNTGKGKEYGYKSKFYSGFNKGKVLFHLNDEKSFSYDL 180
SEI_A900322_        TAQEIDVKLRRYLQEEYNIYGHNNTGKGKEYGYKSKFYSGFNNGKVLFHLNNEKSFSYDL 180
                    ****************************************:********:******** 

SEI_RF122_          FYTGDGVPVSFLKIYEDNKIIESEKFHLDVEISYVDSN 218
SEI_A900322_        FYTGDGLPVSFLKIYEDNKIIESEKFHLDVEISYVDSN 218
                    ******:******************************* 
```

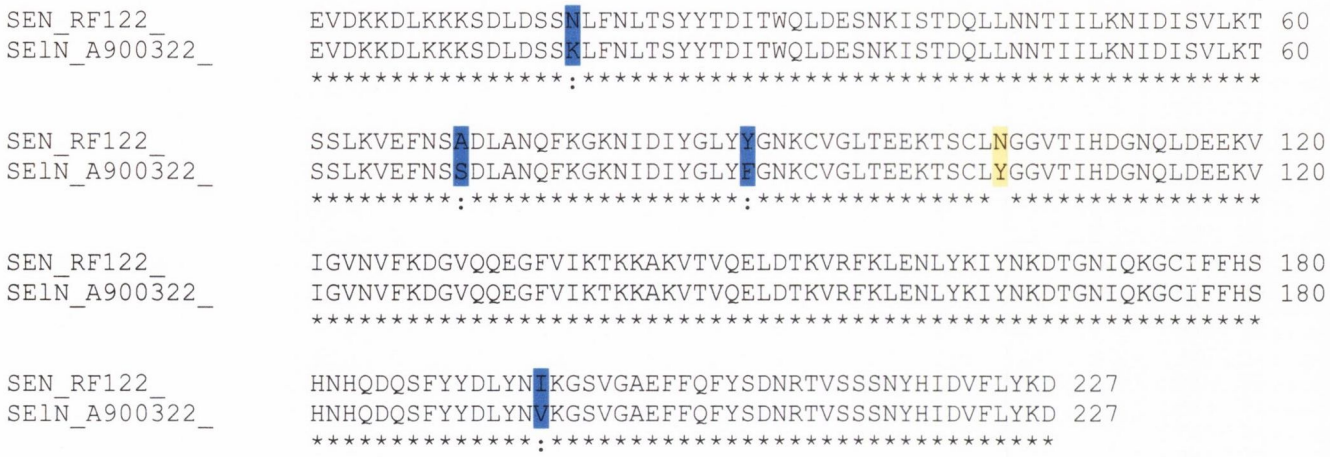


Fig. 7.8. ClustalW-generated alignments of SElO, SEI and SElN proteins from bovine strain RF122 and human clinical strain A900322.

Functionally conserved or semi-conserved residues are indicated below the alignment by : or . and are highlighted in blue. Identical residues are indicated below the alignment by *. Dissimilar residues are highlighted in yellow. The SEI, SElN, and SElO proteins from strain RF122 had amino-acid similarities of 97%, 97% and 90%, respectively, to the *egc*-encoded proteins from strain A900322.

7.3.2. Induction and expression of the SET (SSL) proteins.

Expression of recombinant SET (SSL) protein by the strains provided by Dr. J. Ross Fitzgerald was observed following induction as described in the Methods section. High levels of expression were observed in every strain except that expressing SET8/SSL3 that did not produce an induced band upon SDS-PAGE analysis (Fig. 7.9). In the study of Fitzgerald *et al.* (2003), the SET14 (SSL10), SET12 (SSL8), SET1 (SSL7), SET9 (SSL4), SET8 (SSL3) and SET7 (SSL2) proteins were reactive with at least one convalescent patient's serum. Only the SET8 (SSL3) protein was reactive with almost all of the control human sera tested. In the current study four of the *E. coli* strains expressing recombinant SET (SSL) proteins were investigated, namely, SET13 (SSL9), SET12 (SSL8), SET10 (SSL5) and SET1 (SSL6).

7.3.3. Induction and expression of the SAg and SAg-like proteins.

In the case of the SAg and SAg-like proteins the levels of expression observed were much lower than those observed for the SET/SSL constructs and expression of recombinant SEI, SEI, SElN and SElO was observed. No expression of the SEG and SElM proteins was observed. Inductions at both 37 °C and 28 °C were carried out to see if lower temperatures would increase the levels of recombinant protein. However, no major difference in the levels of expression was observed (Fig 7.10). As a result all samples used for Western immunoblots were derived from cultures induced at 37 °C.

7.3.4. Western immunoblotting using bovine serum.

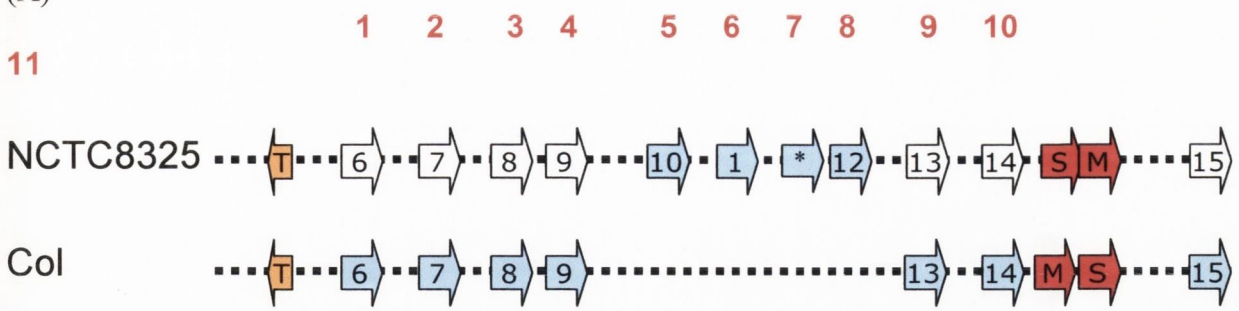
To determine if the SET/SSL, SAg and SAg-like proteins were expressed during the course of mastitis infection and stimulated an antibody response, Western immunoblot analysis of *E. coli* lysates expressing recombinant proteins was carried out using bovine sera obtained from 8 cows with or without mastitis (Table 7.2). Figure 7.11 (A) shows an SDS-PAGE gel used to transfer *E. coli* lysate proteins to a PVDF membrane. High amounts of recombinant proteins remained in the SDS-PAGE gel post transfer. Figure 7.11 (B) shows developed X-ray films of a representative immunoblotted membrane. Table 7.3 shows the results of the Western immunoblotting experiments. Sera from all four cows with mastitis reacted with the SET1/SSL6 protein. Serum from one cow (2487) reacted with the SET10/SSL5 protein. Sera from two cows (2487 and 2480) reacted with the SET12/SSL8

Fig. 7.9. Schematic of the *set* (*ssl*) locus of strains COL and NCTC8325 and SDS-PAGE of strain BL21 expressing recombinant SET (SSL) proteins.

Panel (A) shows the *set* (*ssl*) locus of strains COL and NCTC8325. The old *set* nomenclature is shown in the arrows, with the new *ssl* nomenclature over the locus in red text. These strains were used as templates to generate pET21b vectors with cloned *set* (*ssl*) genes. The genes marked in blue were used in PCR to generate inserts.

Panel (B) shows SDS-PAGE of strain BL21 expressing recombinant SET (SSL) proteins. The SET (SSL) proteins all migrated at a mass of approximately 25 kDa with the exception of the SET9 (SSL4) and SET8 (SSL3) proteins. The SET9 (SSL4) protein migrated at twice its predicted molecular weight (arrow on gel) suggesting that it formed dimers, whilst an induced band was not obvious for the SET8 (SSL3) protein.

(A)


1 2 3 4 5 6 7 8 9 10
11
NCTC8325
T 6 7 8 9 10 1 * 12 13 14 S M 15
Col
T 6 7 8 9 13 14 M S 15

(B)

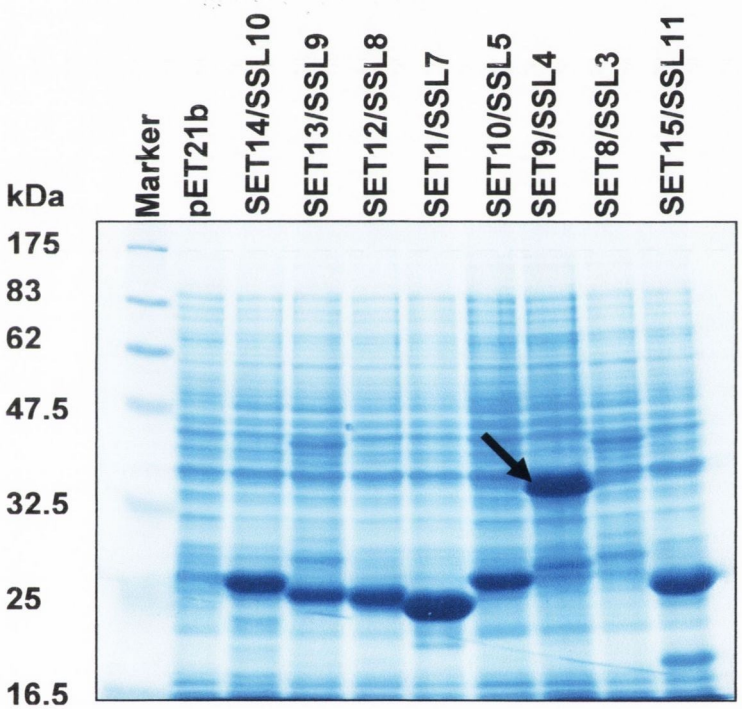

kDa
175
83
62
47.5
32.5
25
16.5
Marker
pET21b
SET14/SSL10
SET13/SSL9
SET12/SSL8
SET1/SSL7
SET10/SSL5
SET9/SSL4
SET8/SSL3
SET15/SSL11

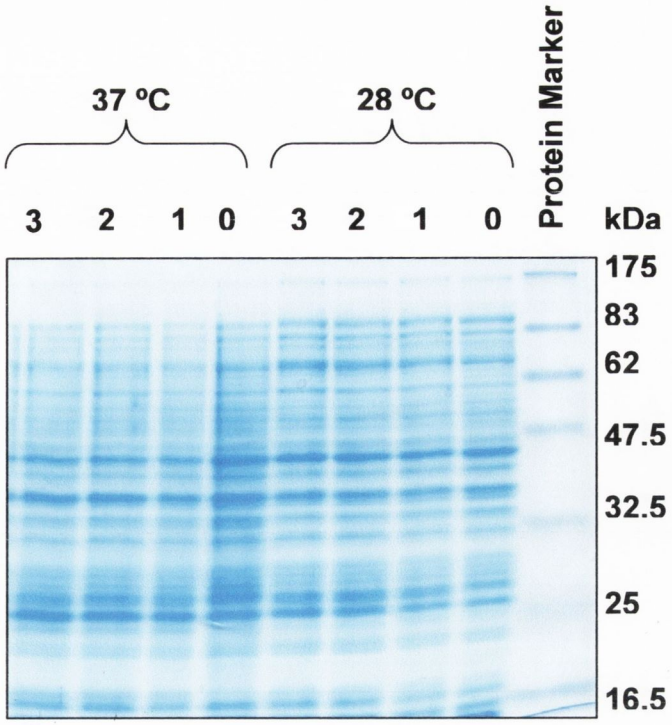
Fig. 7.10. SDS-PAGE of induced strain pET*seg* and pET*sei* recombinant plasmids.

Due to the fact that the *pelB* leader sequence in plasmid pET22b directs recombinant proteins to the periplasm and could cause toxic effects, induction was performed at both 37 °C and 28 °C. Numbers above the lanes indicate hours post induction.

Panel (A) shows the expression from strain BL21 bearing recombinant plasmid pET*seg*. No visible induced band was observed for this strain. This is probably due to the single base pair deletion present in the *seg* gene used for this construct.

Panel (B) shows the expression from strain BL21 bearing recombinant plasmid pET*sei*. An induced band of approximately 29 kDa was observed for this strain. Slightly higher levels of expression were observed at 3 h post induction at 37 °C than at 28 °C.

(A)


37 °C
28 °C
Protein Marker
3 2 1 0 3 2 1 0
kDa
175
83
62
47.5
32.5
25
16.5

(B)

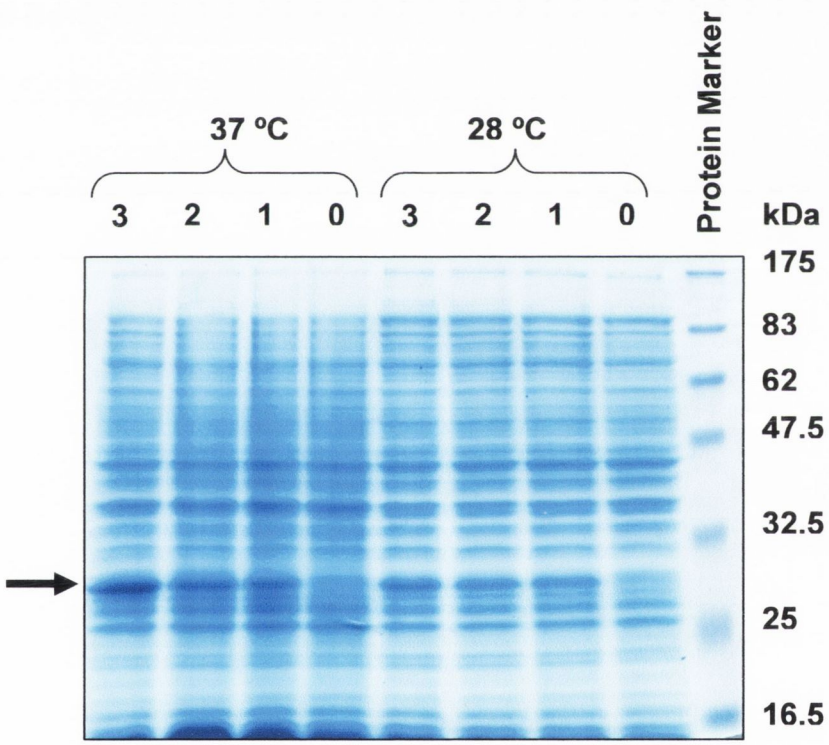

37 °C
28 °C
Protein Marker
3 2 1 0 3 2 1 0
kDa
175
83
62
47.5
32.5
25
16.5

Table 7.2. Somatic cell counts (SCCs) of quarter milk samples from cows, sera from which were used in Western immunoblotting experiments.

Cow	Right Front	Right Hind	Left Front	Left Hind	*S. aureus*
2521	2000/ml	23000/ml	16000/ml	2000/ml	Positive
2242	4000/ml	71000/ml	445000/ml	407000/ml	Positive
2480	236000/ml	220000/ml	62000/ml	1843000/ml	Positive
2487	40000/ml	2145000/ml	17000/ml	124000/ml	Positive
4204	28000/ml	30000/ml	13000/ml	16000/ml	Negative
4227	11000/ml	3000/ml	6000/ml	16000/ml	Negative
4232	9000/ml	19000/ml	9000/ml	15000/ml	Negative
4217	13000/ml	12000/ml	14000/ml	18000/ml	Negative

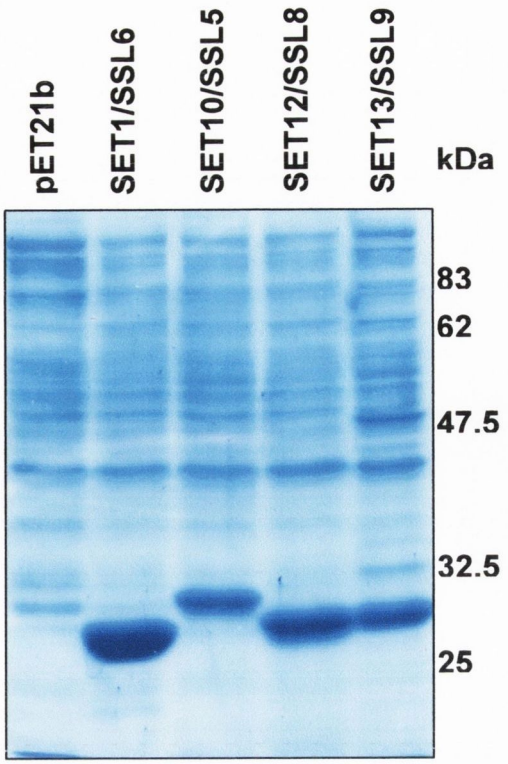
The normal SCC in milk is generally below 200,000 cells per ml, but may be below 100,000 in first lactation animals or in well-managed herds. An SCC above 250,000 - 300,000 is considered abnormal and nearly always is an indication of bacterial infection causing inflammation of the udder. Samples with SCC > 200,000 cells/ml are underlined. Milk samples from cows 2521, 2242, 2480, 2487 were cultured and *S. aureus* colonies were obtained. DNA was extracted and subjected to PCR for *set/ssl* and superantigen genes and to RAPD typing as described in Chapter 4.

Fig. 7.11. Western immunoblot analysis of lysates of *E. coli* expressing recombinant SET (SSL) proteins.

Panel (A) shows an SDS-PAGE gel of *E. coli* lysates expressing recombinant SET (SSL) proteins that were subjected to wet-transfer and stained post-transfer with Coomassie brilliant blue. It is interesting to note the high amounts of recombinant SET (SSL) proteins that remained in the SDS-PAGE gel.

Panel (B) shows X-ray films (Fluorograms) of a PVDF membrane to which the *E. coli* lysate proteins were transferred. The membrane was subjected to Western immunoblotting using serum from cow 2487 that had active mastitis infection when this serum sample was taken. Film (i) was exposed for 2 min and film (ii) for 3 min. The longer the exposure, the higher the background. The reactive band corresponding to the SET10 (SSL5) protein was clearly observed at both exposure times; however, reactive bands corresponding to the other proteins were weak at 2 min became clearer after a 3-min exposure.

(A)


pET21b
SET1/SSL6
SET10/SSL5
SET12/SSL8
SET13/SSL9
kDa
83
62
47.5
32.5
25

(B)

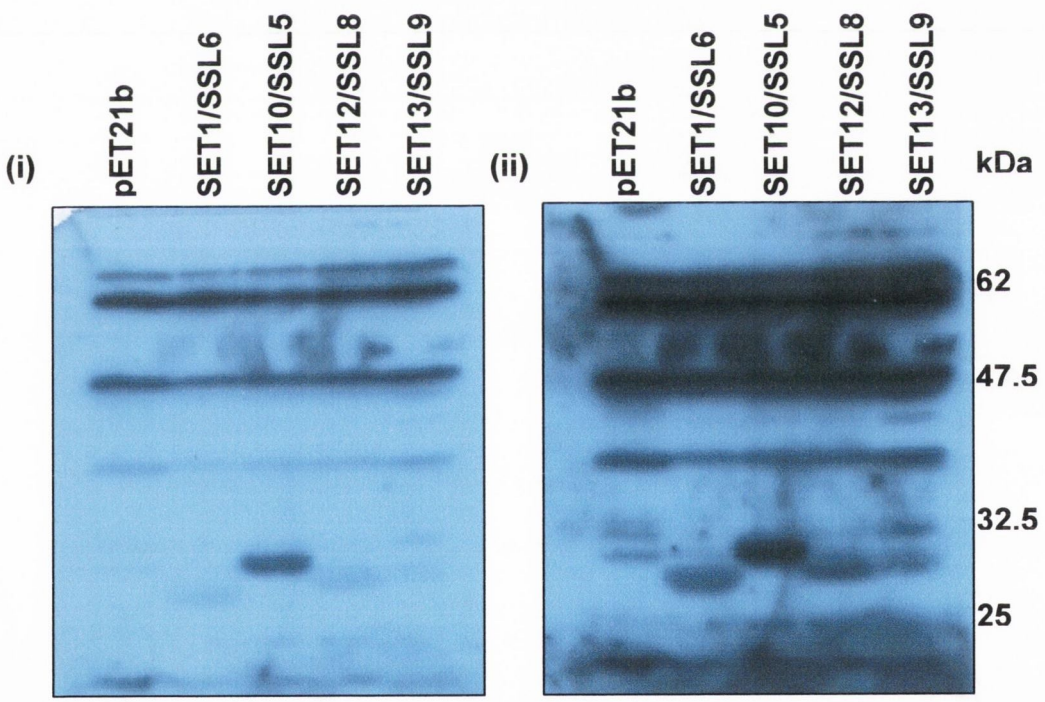

(i)
pET21b
SET1/SSL6
SET10/SSL5
SET12/SSL8
SET13/SSL9
(ii)
pET21b
SET1/SSL6
SET10/SSL5
SET12/SSL8
SET13/SSL9
kDa
62
47.5
32.5
25

Table 7.3. Immunoreactivity of recombinant SET (SSL) proteins, and SEI, SEIN, and SEIO protein with bovine sera by Western blot analysis.

Cow Serum	Clinical Status	SET1 (SSL6)	SET10 (SSL5)	SET12 (SSL8)	SET13 (SSL9)	SEI	SEM	SEN	SEO
2521	Mastitis	+	–	–	+	–	–	–	–
2242	Mastitis	+	–	–	–	–	–	–	–
2480	Mastitis	+	–	+	–	–	–	–	–
2487	Mastitis	+	+	+	+	–	–	–	–
4204	Control	–	–	–	–	–	–	–	–
4227	Control	–	–	–	–	–	–	–	–
4232	Control	–	–	–	–	–	–	–	–
4217	Control	–	–	–	–	–	–	–	–

+ or – indicate positive or negative reactivity

protein. Sera from two cows (2521 and 2487) reacted with the SET13/SSL9 protein. None of the serum samples from the four cows without mastitis reacted with the SET/SSL proteins.

The SAg and SAg-like proteins failed to react with any of the serum samples, suggesting that these proteins were either very weakly and not detectably immunogenic or were not expressed at all during infection (Fig. 7.12).

7.4. Discussion.

A large number of studies have looked at the functions and levels of anti-staphylococcal antibodies in healthy and infected individuals. The ability of human serum immunoglobulins to inactivate *S. aureus* toxins has been shown by *in vitro* assays (Darenberg *et al.*, 2004; Gauduchon *et al.*, 2004). Serological assays using bacterial lysates or recombinant protein have given insight into the prevalence of antibodies to certain *S. aureus* proteins (Casolini *et al.*, 1998; Colque-Navarro *et al.*, 1998; Colque-Navarro *et al.*, 2000, Dryla *et al.*, 2005). In some cases antibodies to surface-associated proteins as well as secreted toxins such as SAgs have been shown to fulfil a protective role for the host (Fattom *et al.*, 1996; Lee *et al.*, 1997; Flock, 1999; Josefsson *et al.*, 2001; LeClaire *et al.*, 2002; Hall *et al.*, 2003; Hu *et al.*, 2003). Investigation of the *in vivo* expression of *S. aureus* proteins using an individual's serum remains a useful way of identifying proteins that elicit an immune response and that might possess potential as vaccine components. In the case of the bovine host, an alternative approach would be to screen milk for anti-staphylococcal antibodies rather than sera. This could prove to be a more practical method for screening cows for the presence of anti-staphylococcal antibodies.

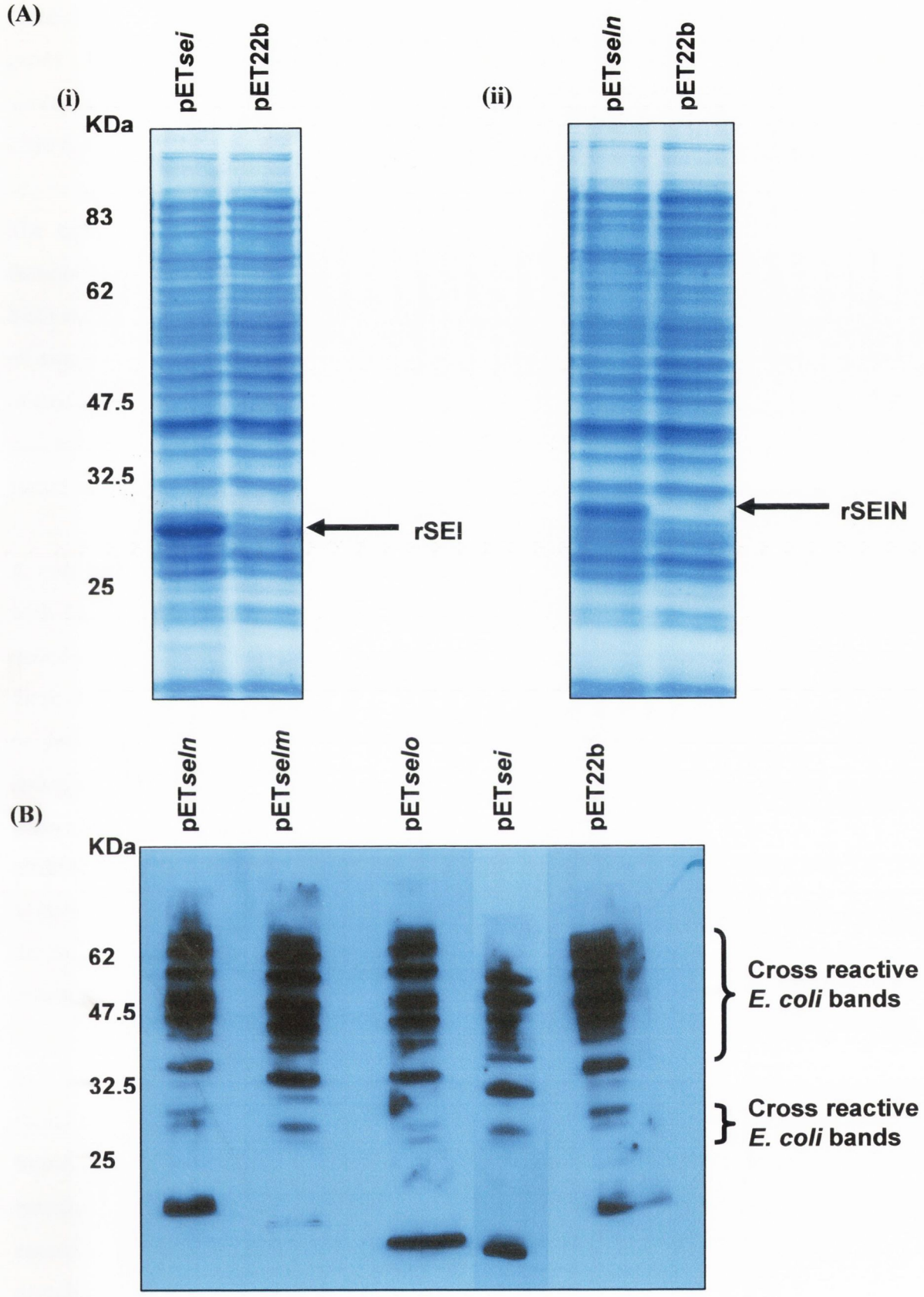
Little or no data are available on the prevalence of anti-staphylococcal antibodies in animals such as cows, goats, sheep, rabbits and chickens. Examining sera from animals could indicate which *S. aureus* proteins are highly expressed and immunogenic and potentially useful in immunoprophylaxis.

With this in mind, the current study attempted to investigate the specificities of antibodies in sera from eight cows with or without mastitis towards SAg, SAg-like and SET (SSL) proteins. The four cows with mastitis had high SCCs and *S. aureus* was cultured from the

Fig. 7.12. Western immunoblot analysis of *E. coli* lysates expressing recombinant SAg and SAg-like proteins.

Panel (A) (i) shows an SDS-PAGE gel of *E. coli* lysates expressing recombinant SEI protein, and (ii) shows an SDS-PAGE gel of *E. coli* lysates expressing recombinant SElN protein. In the case of the SAg and SAg-like proteins, the PVDF filters were strained with Ponceau S and the lanes of the gels were cut into strips to facilitate easier processing of multiple samples and different sera samples.

Panel (B) shows X-ray films of strips of PVDF membrane to which the *E. coli* lysates proteins were transferred. The strips of membrane was subjected to western immunoblotting using serum from cow 2242 that had active mastitis infection when this serum sample was taken. Cross-reactive bands were seen in every strip implying that the serum was reacting to *E. coli* proteins in the lysates. No reactive band to the SAg and SAg-like proteins was observed.


(A)
(i)
pETsei
pET22b
KDa
83
62
47.5
32.5
25
rSEI
(ii)
pETseln
pET22b
rSEIN
(B)
pETseln
pETselm
pETselo
pETsei
pET22b
KDa
62
47.5
32.5
25
Cross reactive
E. coli bands
Cross reactive
E. coli bands

quarter milk samples taken from these four cows. These *S. aureus* isolates were found to contain *set* (*ssl*) genes. However, not all the *S. aureus* strains contained SAg and SAg-like genes. The *S. aureus* isolates from cow 2487 encoded no SAg or SAg-like genes. The *S. aureus* isolates from cows 2521 and 2242 possessed the *seh* gene and the isolates from cow 2480 had the *egc* locus (the *seg*, *sei*, *selm*, *seln* and *selo* genes).

The *egc* locus genes (*seg*, *sei*, *selm*, *seln* and *selo*) from the bovine strain RF122 were demonstrated to encode novel bovine-associated variants. However, the genes encoding SEG and SElM from strain RF122 were found to contain deletion and insertion mutations of single residues that rendered the protein out of frame. This could mean that these proteins were defective and not expressed by strain RF122. The transcription of the *seg* and *selm* genes and the expression of the SEG and SElM proteins by strain RF122 warrant future investigation.

E. coli lysates containing recombinant SEI, SElM, SElN and SEO proteins failed to react with the bovine sera from all eight cows. Even cow 2480 that was infected by a strain encoding the *egc* locus (*seg*, *sei*, *selm*, *seln* and *selo*) did not react. This implies that either these proteins are not immunogenic in the cow or that they are expressed at very low levels or not expressed at all *in vivo*. This was unexpected based on the results of the mitogenicity assays in Chapter 3 which suggested that the culture supernatants of animal-associated strains encoding the *egc* locus (*seg*, *sei*, *selm*, *seln* and *selo*) did cause the proliferation of T-cells suggesting that these proteins were expressed. However, these assays used PBMCs derived from humans not animals. It would be interesting to compare the mitogenicity of supernatant fractions from animal-associated strains for PBMCs from humans and animals to see if they were the same or different.

In a study by Holtfreter *et al.* (2004), the *egc*-encoded SAg and SAg-like proteins were found to be mitogenic in an assay similar to the one used herein in Chapter 3. They also found that only about 10 % of human serum samples inhibited T-cell activation by *egc*-encoded SAg and SAg-like proteins whereas 32–46 % of the sera neutralised classical enterotoxins suggesting very low levels of serum antibodies to the *egc*-encoded SE and SEl proteins. This agrees with the study of Banks *et al.* (2003) who found that the levels of serum antibodies to recombinant SEG and SEI proteins were much lower than levels to SEA, SEB or SEC classic enterotoxins by ELISA. Also Omoe *et al.* (2002) demonstrated

in vitro secretion of very low levels of the SEG and SEI proteins in comparison to the SEH protein by *seg*-, *seh*- and *sei*-encoding strains.

It could be concluded that the lack of an immune response to these proteins suggests that these proteins are not important in terms of virulence, since they do not appear to be expressed at very high levels by strains.

The SET (SSL) proteins on the other hand were found to react with antibodies in the sera of the cows with mastitis. These proteins had been found to be immunogenic in studies using human-derived sera. However, this is the first study to investigate the expression of these proteins by *S. aureus* in cows. The SET1 (SSL6) protein was the most reactive. In comparison, this protein only reacted with 4 of 19 human samples tested in the study of Fitzgerald *et al.* (2003). These proteins may represent novel targets in vaccine development as they do not exhibit the hallmarks of SAgs. Although more studies are needed to evaluate the role and function of these proteins *in vivo,* it is clear that they represent a family of immuno-modulatory proteins, the genes of which are present in every strain regardless of the host it is derived from. Already applications of these proteins are being developed such as their use as chaperones in vaccine development. The SSLs can selectively bind to antigen-presenting cells without causing any apparent inflammation or activation. It has been proposed that the SSLs could be used in a complex to target an antigen of interest or DNA encoding an antigen, thereby improving an immune response. This application has already been patented (UK Patent 0412419.4 3rd June 2004: http://www.uclbiomedica.com/downloads/Tech-Licence_PDFs/BennyChain57.pdf).

The serum samples from all the cows reacted with *E. coli* proteins in the lysates. *E. coli* is an important cause of mastitis in cows (Bradley, 2002), along with other infections such as bovine diarrhoea. Cows also carry EHEC (Entero Haemorrhagic *E. coli*) (Smith *et al.*, 2002). It is likely that the cows had been infected with *E. coli* previously. Even the cows without mastitis reacted with *E. coli* proteins despite being in their first lactation and not having had mastitis. It is likely that these cows carry or have been infected by *E. coli* and thus have antibodies to the organism.

Chapter 8

General Discussion

8.1. SAg and SAg-like genes in animal-associated *S. aureus*.

At the outset of this project little data were available on the occurrence and significance of the SAgs, and in particular of the newly identified SAgs and SAg-like proteins such as SEG-SEI, SElJ-SElR, SElU, in strains of *S. aureus* from animal infection (Akineden *et al.*, 2001; Larsen *et al.*, 2002). It was thought that some of these novel SEs and SEls might contribute to the persistence of *S. aureus* in sub-clinical mastitis due to their immuno-modulatory effects. Because of the putative significance of these enterotoxins and enterotoxin-like proteins for public health and food safety, a greater knowledge of their prevalence and an efficient means of screening for their genes was needed (Wieneke *et al.*, 1993; Mead *et al.*, 1999; Shimizu *et al.*, 2000; Chen *et al.*, 2004).

In order to investigate the occurrences of the SAg and SAg-like genes in strains of *S. aureus* of animal origin, multiplex PCR assays were adapted and developed that screened for the *sea-see*, *seg-selo* and *selq* genes as well as the *tst* gene. The genes encoded by the complete *egc* cluster were found to be present in 42.7 % of the SAg-gene positive animal-associated strains with a further 10 % of SAg-gene positive strains encoding the *egc* cluster lacking the *seg* gene. The three genes associated with the bovine pathogenicity island (SaPIbov) were found in 40 % of SAg-gene positive animal-associated strains with a further 6.4 % encoding the *sec* and *tst* genes only. Novel SAg and SAg-like genes were present at a much higher frequency than those encoding classical enterotoxins.

Mitogenicity assays, which test for superantigenic activity, revealed that strains containing novel SAg and SAg-like genes were capable of stimulating the proliferation of T-cells, a hallmark of superantigenicity. The mitogenicity assays also suggested that some strains from animals may harbour genes for as yet unidentified SAgs or the *selp*, *selr* and *selu* genes, the latter two of which were identified during the present study (Kuroda *et al.*, 2001; Letertre *et al*, 2003a; Omoe *et al*, 2003). The *selr* gene localises to a plasmid that encodes the *sed* and *selj* genes. Accordingly *sed-selj*-positive isolates would be anticipated to also harbour the *selr* gene. The *selu* gene has only been reported in some strains that contain the *egc* cluster. Thus, it is likely that some of the animal strains analysed in the present study may contain the *selu*, *selr* and *selp* genes. These genes should be tested for in future studies of animal associated *S. aureus*.

The multiplex PCR used herein was developed from that described by Monday & Bohach (1999) with the addition of primers to amplify more newly described SE- or SEl-encoding genes. To ensure reliability and reproducibility primers were divided into three multiplex reactions plus sets of primers for the *seb* and *sek* genes individually. However, the multiplex PCR 3 containing the primers for the *sei, selm, seln, selo* and *selq* genes did not always detect the *seln* gene. The *seln* gene was not amplified by this multiplex PCR in 25% of *egc*-positive strains but only by rechecking of these strains with the *seln* primers alone. In the present study 31.3% (10 of 32 isolates) of the *egc* cluster-bearing strains lacked the *seg* gene even after retesting by PCR with the *seg* primers alone and probing genomic DNA from *seg*-gene negative (by PCR) strains with DIG-labelled probes to the *seg* gene in a dot blotting experiment. All PCR reactions included primers for the 16S rRNA gene as an internal positive control.

Becker *et al.* (2004), in a follow-up study to one in which the *seg* and *sei* genes had already been screened for (Becker *et al.,* 2003), used a multiplex PCR to detect the *selm, seln* and *selo* genes in 429 *S. aureus* isolates from anterior nares and blood. Whereas 55% of strains possessed both the *seg* and *sei* genes, the *selm, seln* and *selo* genes were found in 46.6%, 66.9% and 52.2% of isolates, respectively. In combination with the *seg* and *sei* genes, only 78.4% of *selm*-positive, 80.8% of *seln*-positive and 78.6% of *selo*-positive strains had these combinations of three genes. Moreover only 35% of strains had the complete *egc* cluster of genes. In a study of 69 isolates from staphylococcal food-poisoning and 97 human nasal isolates, Omoe *et al.* (2005) detected the complete *egc* locus in 63 strains (38.0%) and *egc*-cluster-associated genes in various combinations or alone in a further 13 strains (7.8%). The latter authors used primers for the *femA* or *femB* gene as an internal positive control.

In neither of these two latter studies were strains rechecked using individual primers or by Southern blotting for *egc*-located genes when strains appeared to lack one or more gene of the *egc* locus. The results presented herein for the *seg* and *seln* genes together with the complex combinations of genes suggesting incomplete *egc* clusters in the studies of Becker *et al.* (2003, 2004) and Omoe *et al.* (2005) emphasise the need to perform confirmatory checks with individual sets of primers and gene probes in Southern blotting, and to include internal positive control primers to ensure the quality and quantity of DNA examined in multiplex reactions.

Commercially available reversed passive latex agglutination (RPLA) kits, such as SET-RPLA and TST-RPLA (Oxoid, Basingstoke, UK), only allow screening of *S. aureus* for

the production of the classical enterotoxins that are known to play a role in staphylococcal food-poisoning and the TSST-1 protein. As well as the current study, a number of groups have designed rapid methods that screen for novel SAg and SAg-like genes (Sergeev *et al.* 2004; Letertre *et al.* 2003b; Letertre *et al.* 2003c). Molecular typing methods along with multiplex PCR for SE and SE-like genes have been used to investigate the epidemiology of foodborne outbreaks of *S. aureus* (Martin *et al.*, 2004; Jorgensen *et al.*, 2005).

It is hoped that these methods, along with the multiplex PCRs developed herein will aid future studies into the prevalence of the SAg and SAg-like genes in *S. aureus* strains from other sources, such as human nasal isolates or food-poisoning isolates, and into the potential a role for these novel proteins in the pathogenicity of *S. aureus*.

8.2. SAg genotypes and toxin-gene encoding mobile genetic elements.

A number of related staphylococcal pathogenicity islands have been described and reviewed by Novick (2003b). These include SaPI1 (contains the *tst* gene and two novel SAg-like genes), SaPIm1 and SaPIn2 (contains the *sec1* gene and a SAg-like gene), SaPI2 (no SAg and SAg-like genes), SaPI3 (contains the *seb* gene and two SAg-like genes) and SaPI4 (contains the *sec1* gene and three SAg-like genes). The SaPIbov (contains the *sec*-bovine gene, the *tst* gene and a SAg-like gene, *sell*) and was identified in a bovine strain RF122. The SaPIbap pathogenicity island encodes the *bap* gene, is associated with bovine *S. aureus* strains, and contains no SAg and SAg-like genes.

Herein the *sec*, *tst* and *sell* genes together were found in 18 (18.2 %) of strains from cows, 16 (41 %) of strains from goats and 10 (43.5 %) of strains from sheep. This combination of genes is indicative of bovine pathogenicity island SaPIbov (Fitzgerald *et al.*, 2001). The *sec* and *tst* genes without *sell*, were found in 1 bovine strain (1 %), 2 goat strains (5 %) and 4 sheep strains (17.4 %). In order to investigate the possible presence of these genes upon pathogenicity islands, sequencing of the *sec* gene was performed on a small number of *sec*-gene-positive animal strains that were either positive or negative for the *sell* gene. Two of the goat (one $sell^+$ and one $sell^-$) and two of the sheep strains (both $sell^+$) encoded the *sec*-ovine variant. This could mean that these animal strains contain a novel pathogenicity island containing the *sec*-ovine gene in place of the *sec*-bovine gene along with the *tst* gene and with or without the *sell* gene. This island may represent a novel SaPIov. It would be interesting to sequence both the *tst* and *sell* genes to see if they represent ovine variants.

In the case of the bovine strains, that encoded both the *tst* gene and the *sell* genes, it would be expected that they encode the *sec*-bovine gene. Strain RF122 did encode the *sec*-bovine gene but strain RF283 encoded the *sec*-ovine gene. This implies that a bovine strain possesses a *sec*-containing pathogenicity island other than SaPIbov.

These results emphasise that there may be a range of novel pathogenicity islands present in animal-associated *S. aureus*. These novel islands could mediate horizontal gene transfer of such genes as the *sec*-ovine gene to bovine-associated strains and *vice versa* and potentially to human-associated *S. aureus* strains. It also implies that the identification of SAg and SAg-like genes to the level of their variants should be done in future studies of animal-associated *S. aureus* in order to gain an insight into the true prevalence of variants of SAg and SAg-like genes.

Omoe *et al.* (2005) described many novel combinations of SAg and SAg-like genes including those found in the current study. The *sec, sell* and *tst* genes with or without other SAg and SAg-like genes were found in 2 of 97 human nasal isolates. The *sec* and *tst* genes only combination was not found in any strain. The combination of the *sec* and *sell* gene with or without other SAg and SAg-like genes was found in 5 of 97 nasal isolates. Many novel combinations of the genes found in the *egc* locus were identified among the food-poisoning and human nasal isolates. The authors listed suspected genomic islands and plasmids that could potentially encode the SAg and SAg-like genes, but the actual locations of novel combinations of SAg and SAg-like genes were not investigated. However, negative PCR results were not rechecked using primers for individual genes or by Southern blotting as in the present study, casting doubt on the identity of putative genomic islands and plasmids.

8.3. The novel SET (SSL) proteins.

The family of SET (SSL) proteins share structural and sequence similarity to the SEs and TSST-1. Animal-associated strains were tested for the presence of a number of *set* (*ssl*) genes. All strains tested, were found to contain a locus of *set* (*ssl*) genes. In Chapter 3, a number of the strains that were tested for *set* (*ssl*) genes were included in the mitogenicity assays. Mitogenic activity was found to be unrelated to the presence of the *set*/*ssl* genes. These data agree with previous studies of *S. aureus* strains of human origin that were found

to be mitogenic or non-mitogenic irrespective of the presence of a *set* (*ssl*) locus of genes (Holtfreter *et al.*, 2004). This work, although confirming the presence of a *set* (*ssl*) locus in strains of animal origin gives no further insight into the possible roles of these proteins *in vivo*. A model for the proposed roles of the SAg and SAg-like proteins and the SET/SSL proteins in the mastitis is shown in Fig. 8.1.

8.4. *In vivo* expression of SAg, SAg-like and SET (SSL) proteins.

The *in vivo* expression of the SAg and SAg-like proteins as well as the SET (SSL) proteins was investigated using sera from cows with or without mastitis. In the absence of commercial kits to detect antibodies, a Western immunoblot approach was taken that used *E. coli* lysates containing recombinant SAg, SAg-like and SET (SSL) proteins to detect antibodies in the animal sera thus demonstrating *in vivo* expression. The approach showed that the SET (SSL) proteins reacted with antibodies in the bovine sera samples, implying the *in vivo* expression of the proteins. Although a role for these proteins has not been identified to date, the current PCR and Western immunoblot data suggest that the *set* (*ssl*) genes present in animal *S. aureus* strains are expressed in the cow. These proteins warrant further investigation to see if they are virulence factors and/or immunogens that could be utilised in anti-staphylococcal therapy.

The SAg and SAg-like proteins, the genes of which were found to be highly prevalent in *S. aureus* strains of animal origin, were not found to react with the bovine serum samples, implying a lack of detectable antibodies. This could mean that the proteins are not expressed *in vivo* or are expressed at very low levels or that antibodies are not produced to these proteins. It might be concluded that the lack of an immune response to these proteins suggests that these proteins are not important in terms of virulence, since they do not appear to be expressed at high enough levels to elicit an immune response. However, these SAgs may still exert biological effects at very low levels that might contribute to bacterial persistence within the udder.

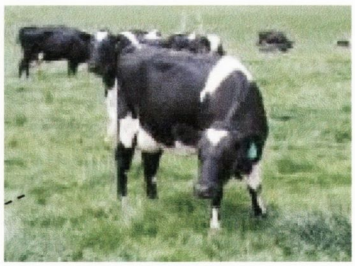
Due to problems with reproducibility, the results of the Western immunoblotting experiments could not be verified more than once for all cow serum samples. However, this work represents the first attempt to investigate the presence of antibodies to staphylococcal SAg, SAg-like and SET/SSL proteins in *S. aureus* strains from animals. If the recombinant proteins had been purified, a more controllable approach such as an

Fig. 8.1. Proposed model of the roles of the SAg and SAg-like proteins and the SET (SSL) proteins in mastitis.

S. aureus produces an array of virulence factors that could be of importance in mastitis infection (reviewed in Chapter 1). Protein A binds host IgG which is thought to mask *S. aureus* from the immune response and to prevent phagocytosis. *S. aureus* from bovine mastitis infection produce capsular polysaccharide that is also anti-phagocytic. Various MSCRAMMs are expressed by *S. aureus* that could facilitate binding to udder tissue proteins.

The SAg and SAg-like proteins are superantigens and can form trimolecular complexes with the T-cell receptor (TCR) on T cells and the MHC-II molecule on antigen-presenting cells (APCs). This results in cytokine release, massive proliferation of T cells and inflammation. Eventually this process overwhelms the immune response, causing shock. It is possible that antibodies to SAg and SAg-like proteins in the bovine udder could bind to the proteins, reducing their superantigenic activity. In the case of SEC, the type-2 cytokines released are proposed to interfere with immune clearance, thus contributing to bacterial persistence (Ferens & Bohach, 2000).

The SET (SSL) proteins have been shown to bind complement C5 and IgA and to impede serum killing of bacteria (Langley *et al.*, 2005). It is possible that the activity of SET (SSL) proteins could prevent the complement cascade and thus contribute to bacterial survival and persistence. In Chapter 7 herein antibodies to the SET (SSL) proteins were detected in bovine serum. If present in the bovine udder, these could bind to the SET (SSL) proteins reducing their ability to bind to host proteins.



Bovine Udder

IgG binds to Protein A - masking from immune response

Protein A

Capsule
Multiple serotypes

Impede phagocytosis

Adhesins
MSCRAMMs

S. aureus

Udder tissue

Novel SAg and SAg-like proteins

Anti-SAg Abs

Form complexes with TCRs on T cells and MHC-II molecules on APCs

SSL proteins

Anti-SSL Abs

Bind complement C5 and bind IgA, → inhibiting serum killing of bacteria

May prevent the complement cascade, contributing to bacterial survival

Can bind to dendritic cells, → causing T cell proliferation

May contribute to persistent infection

Cause IL-2, IFNγ, IL-12 release by T cells, and TNFα & IL-1β by the APCs

Massive proliferation of T-cells; Deletion of T-cell subsets, eventual anergy of reactive cells

Massive inflammation; Shock

In the case of SEC, type 2 cytokines (IL-4, IL-10) are released

Interference with immune clearance → causes immunosuppression

Persistent infection

ELISA-based protocol could have been used instead. Also absorption of the bovine sera with *E. coli* lysates could have been used to reduce the cross-reactive *E. coli* antibodies. However, the process of absorption effectively dilutes the sera and can remove reactive antibodies as well.

8.5. Population structure of animal-associated *S. aureus*.

Various molecular typing techniques have been applied to the study of *S. aureus* from animals but few groups have utilised novel sequence typing methods such as MLST, *sas* typing and spa typing. The current work is the first study to investigate strains of *S. aureus* from cows, goats, sheep, rabbits and chickens using MLST, *sas* typing and *spa* typing. RAPD typing and *agr* typing were also employed. This work gave insight into the usefulness, reproducibility, typeability and resolution of MLST for the investigation of animal-associated *S. aureus*.

Animal-associated *S. aureus* were found to have a clonal population structure, with a small number of clonal types causing the majority of infection. The major MLST ST, ST133, was found in 28 of the 123 strains analysed, and this MLST ST was only found in strains from cows, goats and sheep. ST133 had not been found in the human *S. aureus* population. Using eBURST ST133 was revealed to be the founder of a clonal complex that contained other animal-associated STs and thus may represent an ancestral animal-specific ST. Many novel MLST STs were identified that may represent animal-specific clonal types. Also some of the predominant MLST STs had previously been identified in the human *S. aureus* population. In particular, the chicken strains were found to be of ST5, a highly prevalent MRSA-associated ST.

Other methods used were *sas* typing and *spa* typing. Both of these methods were found to have higher discriminatory powers than MLST. *spa* typing was the method with the highest resolution. The *sas* typing method had not been previously applied to *S. aureus* from cows, goats, sheep, rabbits and chickens and as a result many novel *sas* alleles and *sas* STs were identified. The *spa* typing method also revealed the presence of novel *spa* short sequence repeats and *spa* repeat profiles in animal-associated *S. aureus*.

The combination of either MLST or *sas* typing with *spa* typing has great potential for the analysis of *S. aureus* from animals.

8.6. Potential applications of typing methods examined herein.

The various typing methods investigated herein were all successful in discriminating clonal types. However the applicability of these methods to other settings and studies remains to be seen. MLST is being widely used to look at the global epidemiology of MRSA clones and their evolution. *sas* typing can also be used for studies of global epidemiology and evolution of clones. Owing to the cost and the need for a certain degree of technical ability these methods may not always be suitable for a clinical setting such as a hospital lab for routine diagnostics. However it is now no longer enough to identify the species of bacterium causing an infection, knowing the genotype of an organism is more and more necessary.

Sequence typing methods such as MLST or *sas* typing, although discriminatory, could mask differences between genotypes such as the presence of accessory genes or pathogenicity islands. The associated cost and inability of MLST and *sas* typing to identify accessory genes remain a disadvantage of both methods. PFGE, although laborious, still remains a very suitable choice for hospital and reference laboratories, in particular with regard to outbreak analysis as it generates a profile of the whole genome of a strain including its accessory genetic information. RAPD typing, used herein, could be an alternative to PFGE. However, this method was not found to be the most reproducible of those used and the comparison of banding patterns on gels is frustratingly subjective.

Analysis of the *spa* gene is an interesting alternative, involving the sequencing of a single locus instead of several. It was the method found herein to be the most useful, simple to implement and most discriminatory. In general the presence of a particular *spa* repeat profile can be used to potentially predict the clonal complex of a *S. aureus* isolate. Databases of *spa* repeat profiles are commercially available. These are costly and beyond the means of most clinical settings. However this method would be very suitable for use in coordinated surveillance facilities for monitoring the spread of clonal types globally.

8.7. Future work.

A commercially available kit could be generated to test for all the known SAg and SAg-like proteins. Currently RPLA kits screen for the classical enterotoxins and TSST-1; these could be adapted and expanded to screen for the newly described SAg and SAg-like

proteins. This could be used to screen foodstuffs from food-poisoning outbreaks where the presence of the classical enterotoxins, known for their ability to cause food-poisoning, have been ruled out. The potential role of novel and newly described SAg and SAg-like proteins in food-poisoning could be assessed. In the study of Omoe *et al.* (2005), food-poisoning associated strains were comprehensively screened for SAg and SAg-like genes by multiplex PCR. Forty-two strains from staphylococcal food-poisoning were found to contain combinations of novel SAg and SAg-like proteins in the absence of classical SEs. This implies that the at least some of the novel SAg and SAg-like protein may play a role in staphylococcal food-poisoning.

Many different methods have been applied to the study of *S. aureus* populations. The sequence typing methods were found herein to be highly successful in analysing animal *S. aureus*. In future studies of animal-associated *S. aureus*, these methods would be a suitable alternative to gel-based methods such as PFGE and RAPD typing. Their use would facilitate the generation of databases of the *S. aureus* genetic types involved in animal infections. Database formation allows the easy comparison of studies between laboratories allowing investigators to share and combine their results.

8.8. Concluding remarks.

Animal-associated *S. aureus* populations are still an important area of *S. aureus* research. Due to the recent emergence of methicillin-resistant strains of *S. aureus* in the animal population (Lee *et al.*, 2003, Weese *et al.*, 2005a), the population dynamics of *S. aureus* from animals continues to warrant investigation.

There is evidence that inter-species transmission of MRSA occurs. The same strain of MRSA has been identified in horses and their human contacts (Seguin *et al.*, 1999). There has been a report of persistent human MRSA infections, that were sourced to the nares of the household dog (Manian *et al.*, 2003). MRSA infection following surgery on a dog resulted in the death of the animal (http://www.pets-mrsa.com). MRSA has also been identified in cows, chickens, cats and dogs (Lilenbaum *et al.*, 1998; Gortel *et al.*, 1999; Lee, 2003). Futhermore methicillin-resistant *S. intermedius* strains have been isolated from pyoderma infections in dogs that were transferable to their owners (Gortel *et al.*, 1999; Guardabassi *et al.*, 2004; Kania *et al.*, 2004). These studies suggest that MRSA is

no longer restricted to humans and is now emerging as an important zoonotic and veterinary disease.

The results of the molecular genetic typing of *S. aureus* from cows, goats sheep, rabbits and chickens completed herein should be of value to researchers involved in animal *S. aureus* epidemiology.

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