Genotypic and phenotypic analysis for the typing and characterisation of veterinary and human isolates of *Staphylococcus aureus*

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Abstract

Staphylococcus aureus is a member of the skin commensal flora in humans and other mammals, as well as a recognised pathogen. Treatment of *S. aureus* infections have been complicated by the emergence of antimicrobial-resistant isolates, most notably the methicillin-resistant *S. aureus* (MRSA). This organism is a significant hospital-acquired pathogen, with community-acquired infections presently on the rise. Recently, MRSA has emerged in livestock, with isolates commonly identified as clonal complex 398 (CC398). The sudden emergence and dissemination of MRSA CC398 poses questions concerning the mechanisms mediating the success of this lineage as a livestock coloniser.

The hypothesis addressed in this work is that MRSA CC398 has become livestock-associated due to host specific or non-host related adaptations. The work aims to investigate virulence as well as antimicrobial resistance genotypic and phenotypic features of MRSA CC398 isolates through a comparative study using a panel of isolates from different *S. aureus* lineages. The analysis of virulence genotypes revealed that the CC398 lineage had the lowest content of virulence genes, with all isolates lacking accessory virulence determinants and carrying mostly core-variable genes such as adhesin and staphylococcal exotoxin-like protein genes. Keratinocyte adhesion assays demonstrated inter-lineage variation in adhesion to porcine skin cells and comparatively poor binding by CC398 isolates. MRSA CC398 isolates also demonstrated a limited capacity for biofilm formation. The antimicrobial susceptibility analysis together with investigation of antimicrobial resistance genotypes found that MRSA CC398 isolates were resistant to a number of non-β-lactam agents. This was mediated by diverse genetic resistance determinants and was also observed for MRSA isolates belonging to other CCs. A number of resistance genes were confirmed as plasmid-borne and the sequence analysis revealed carriage of novel resistance gene clusters and resistance determinants. Biological fitness analysis revealed a competitive advantage of CC398 strains over MRSA isolates of other lineages. In conclusion, MRSA CC398 isolates revealed lack of any significant virulence features. Instead the lineage demonstrated broad resistance properties, accompanied by superior biological fitness.

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- 'Identification of novel plasmid-associated tetracycline efflux protein gene *tetK2* in methicillin-resistant *Staphylococcus aureus* ST398'
- 'Comparative biological fitness analysis of methicillin-resistant *Staphylococcus aureus* strains belonging to CC8, CC22, CC30 and CC398'

Dedication

I dedicate this work to my husband for his support, patience and understanding over the course of my studies.

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Chapter 1 Introduction

1.1 *Staphylococcus aureus:* commensal and pathogen

Staphylococcus aureus is a Gram-positive bacterium, member of the Firmicutes phylum. The organism is a facultative anaerobe, with cocci-shaped cells that form clusters when viewed under a light microscope following a Gram-stain analysis. *S. aureus* has been recognised as an extremely versatile prokaryote, which can act as both a commensal organism as well as a pathogen (Lowy, 1998). The bacterium can colonize various mammalian species although humans represent its natural reservoir (Lowy, 1998). An estimated 20% of healthy adults are persistent carriers, with a considerable proportion of the population being colonized intermittently (Williams, 1963; Kluytmans, van Belkum and Verbrugh, 1997). *S. aureus* primarily colonizes the anterior nares although it can be also isolated from other sites of the body, such as pharynx, perineum, axillae and vagina (Williams, 1963; Lowy, 1998). Development of a *S. aureus* infection is commonly associated with a breach of skin or mucosal barrier (Lowy, 1998).

The organism was first described as an infectious agent in the 1880s when it was found to be associated with wound suppuration (Ogston, 1882). Prior to the discovery and widespread use of penicillin, *S. aureus* bacteraemia resulted in a 82% mortality rate (Skinner and Keefer, 1941). The pathogenesis of *S. aureus* is mediated by a wide range of virulence factors such as adhesins, extracellular enzymes and exotoxins, which facilitate tissue adhesion and invasion, immune evasion, systemic dissemination as well as induction of sepsis and toxinosis (Archer, 1998). The organism is thus a causative agent of a broad spectrum of disease in humans and can affect any organ system, most commonly causing infections of skin, soft-tissue, respiratory, bone, joint and endovascular systems (Lowy, 1998). In animals, the type of infection is largely species-specific. Lactating ruminants commonly suffer from *S. aureus*-associated mastitis, in horses and companion animals skin infections can occur whereas poultry can suffer from 'bumble foot', chondronecrosis and septic arthritis (Fitzgerald and Penadés, 2008).

1.2 Virulence factors and pathogenesis

1.2.1 Cell surface elements and adhesins

As a Gram-positive bacterium, a distinctive feature of *S. aureus* is its cell wall, of which the main component is peptidoglycan, also known as murein (Pinho, 2008).

The peptidoglycan is a polymer composed of glycan chains, which are cross-linked by peptide bridges (Pinho, 2008). It has been proposed that peptidoglycan might represent a virulence factor through endotoxin-like activity, which would induce release of cytokines, activation of complement and aggregation of platelets (Lowy, 1998). Furthermore, in most *S. aureus* isolates the cell wall is also covered by a capsule (Fournier, 2008). The production of extracellular capsular polysaccharides by bacterial pathogens promotes virulence and in *S. aureus* was shown to mediate resistance to phagocytosis by human polymorphonuclear (PMN) leukocytes (Karakawa *et al.*, 1988; O'Riordan and Lee, 2004). *S. aureus* express various surface proteins, many of which have been shown to bind host components (Fournier, 2008). The vast majority of strains produce protein A, which has been detected in a cell wall-bound form, as well as an extracellular state (Forsgren, 1970; Kronvall, Holmberg and Ripa, 1972). Protein A acts as an immune evasion molecule by binding the Fc region of IgG, which prevents recognition by the neutrophil Fc receptor and thus inhibits phagocytosis (Forsgren and Sjöquist, 1966; Foster, 2005). Protein A was also found to bind to the von Willebrand factor, a glycoprotein mediating platelet adhesion at sites of endothelial damage (Hartleib *et al.*, 2000). As such, it is now also recognized as a member of *S. aureus* family of cell wall-associated proteins that mediate a direct interaction with the host cells and other host components and are collectively known as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) (Foster and Höök, 1998; Hartleib *et al.*, 2000). As presented in table 1.1, various other cell surface proteins represent MSCRAMMs, although the group does not encompass all *S. aureus*-related adhesins, since certain elements are either extracellular or membrane-associated.

A wide range of *S. aureus* adhesion determinants have been identified (Table1.1), which includes fibronectin-, fibrinogen-, collagen, bone-, elastin- and laminin-binding proteins (Peacock *et al.*, 2002; Tristan *et al.*, 2003). The adhesins and their function have been identified mostly through *in vitro* adhesion assays and affinity chromatography using extracellular matrix proteins as substrates (Flock *et al.*, 1987; Bodén and Flock, 1989; Park *et al.*, 1991; Patti *et al.*, 1992). The specific role of the *S. aureus* adhesins in the initiation, development and maintenance of infection is still being elucidated, primarily through animal models of infection (Foster and Höök, 1998).

Table 1.1 Virulence determinants of *S. aureus*

	Determinant	Biological function	Ref
Adhesins	Spa (Protein A)	Binds Fc of IgG and von Willebrand factor, MSCRAMM	Foster and Höök, 1998
	ClfA, ClfB	Fibrinogen-binding protein (clumping factor), MSCRAMM	Foster and Höök, 1998
	Fib (Efb)	Fibrinogen-binding protein, secreted protein	Fournier, 2008
	FnbA, FnbB	Fibronectin-binding protein, MSCRAMM	Foster and Höök, 1998
	Cna	Collagen-binding protein, MSCRAMM	Foster and Höök, 1998
	Bbp	Bone sialoprotein-binding protein, MSCRAMM	Tung *et al.*, 2000
	SdrC, SdrD, SdrE	Binding ligand unknown, putative MSCRAMM	Foster and Höök, 1998
	SasG	Binding ligand unknown, putative MSCRAMM	Roche, Meehan and Foster, 2003
	EbpS	Elastin-binding protein, integral membrane protein	Downer *et al.*, 2002
	Eno	Laminin-binding protein, enolase, putative MSCRAMM	Carneiro *et al.*, 2004
	Bap	Surface protein involved in biofilm formation	Cucarella *et al.*, 2001
	PIA	Polysaccharide inter-cellular adhesin involved in biofilm formation	Cramton *et al.*, 1999
Enzymes	SspA, SspB, SspC, ScpB, SplA, SplB	Proteases	Fournier, 2008
	Nuc	Nuclease	Archer, 1998
	Lip	Lipase	Archer, 1998
	HysA	Hyaluronate lyase	Archer, 1998
Exotoxins	SEA, SEB, SEC, SED, SEE, SEG, SEH, SEI, SEJ, SEK, SEL, SEM, SEN, SEO, SEP, SEQ, SER and SEU	Enterotoxins, superantigen activity	Balaban and Rasooly, 2000; Jarraud *et al.*, 2001; Orwin *et al.*, 2001; Yarwood *et al.*, 2002; Omoe *et al.*, 2003; Letertre *et al.*, 2003; Omoe *et al.*, 200; Orwin *et al.*, 2003; Fournier, 2008
	TSST-1	Toxic shock syndrome toxin-1, superantigen activity	Fournier, 2008

Table 1.1 (continued) Virulence determinants of *S. aureus*

	Determinant	Biological function	Ref
Exotoxins	EtA, EtB	Exfoliative toxins, superantigen activity	Fournier, 2008
	EDIN-A, EDIN-B, EDIN-C	Epidermal-cell differentiation inhibitors	Franke *et al.*, 2010
	Hla, Hlb, Hld, Hlg	Haemolysins, cytolytic toxin activity	Fournier, 2008
	Hlg, LukE/D, LukS/F-PV, LukM/F-PV(83)	Leukocidins, cytolytic toxin activity	Fournier, 2008; Gravet *et al.*, 1998; Rainard *et al.*, 2003
	SET1, SET2, SET3, SET4, SET5, SET6, SET7, SET8, SET9, SET10, SET11	Staphylococcal exotoxin-like proteins	Williams *et al.*, 2000; Baba *et al.*, 2008
Other	Coa	Coagulase, binds prothrombin and leads to conversion of fibrinogen to fibrin	Fournier, 2008
	Sak	Staphylokinase, activates plasminogen into plasmin	Rooijakkers *et al.*, 2005
	CHIPS	Chemotaxis inhibitor protein	Fournier, 2008
	SCIN	Complement inhibitor protein	Fournier, 2008

Amongst the fibrinogen adhesins, which are also recognised as clumping factors, ClfA was found to promote pathogenesis of *S. aureus* in experimental endocarditis as well as to inhibit phagocytosis by human PMN, whereas ClfB was shown to play a role in adherence to cytokeratin and persistent nasal colonization (Moreillon *et al.*, 1995; Entenza *et al.*, 2000; Higgins *et al.*, 2006; O'Brien *et al.*, 2002; Wertheim *et al.*, 2008). Other fibrinogen-binding proteins have been also identified such as the extracellular Fib, also recognised as Efb, which is a secreted protein found to contribute to the pathogenesis of experimental wound infection (Palma *et al.*, 1996; Fournier, 2008). The two fibronectin-binding proteins, FnbA and FnbB were reported to mediate adhesion to human endothelial cells and subsequent internalization (Peacock *et al.*, 1999). The collagen-binding protein, Cna, was shown to contribute to the pathogenesis of osteomyelitis by promoting haematogenous spread and to mediate maintenance of experimental endocarditis (Elasri *et al.*, 2002; Hienz *et al.*, 1996).

In addition to the described *S. aureus* binding proteins, other putative adhesins have been identified from *S. aureus* genome sequences based on sequence homology, more specifically the presence of a conserved LPXTG motif within the C-terminal sorting signal (Roche *et al.*, 2003). This includes the SasG protein that binds to an unknown ligand on the surface of nasal epithelial cells and thus is likely to contribute to nasal colonization (Roche, Meehan and Foster, 2003). The bone-binding protein, Bbp, belongs to a family of structurally related Sdr proteins, which also includes SdrC, SdrD and SdrE (Tung *et al.*, 2000). However, ligand specificity has been determined only for Bbp and thus the remaining members of the Sdr class are recognized as putative cell-surface proteins (Tung *et al.*, 2000). In addition to expression of surface proteins that interact directly with the host cells, *S. aureus* pathogenesis can be also enhanced by elements that mediate cell-to-cell adhesion, such as the polysaccharide intercellular adhesin (PIA), which facilitates biofilm formation (Cramton *et al.*, 1999).

1.2.2 Exoproteins

1.2.2.1 Extracellular enzymes

S. aureus isolates may produce an array of enzymes such as proteases, nucleases, lipases and hyaluronate lyase, which have been associated with mechanisms of host tissue destruction (Archer, 1998). However, some of these

enzymes revealed a further role in the pathogenesis aimed at evasion of host defences. As demonstrated by Table 1.1, *S. aureus* can produce a range of proteases, which might mediate infection through degradation of host immunoglobulins, antimicrobial peptides and plasma proteins (Fournier, 2008). The *S. aureus* nuclease was recently shown to act as immune evasion determinant by promoting resistance to extracellular killing by activated neutrophils (Berends *et al.*, 2010). An immunomodulatory function has also been assigned to *S. aureus* lipase, which was found to induce functional alterations in human leukocytes (Rollof *et al.*, 1988).

1.2.2.2 Exotoxins

A considerable proportion of *S. aureus*-expressed exotoxins belong to a broad family of virulence factors that are referred to as toxin superantigens, which includes enterotoxins, toxic shock syndrome toxin-1 and exfoliative toxins (Fournier, 2008). Staphylococcal enterotoxins (SEs) are heat-stable gastrointestinal toxins responsible for staphylococcal food poisoning (SFP) and a wide range of SEs serotypes have been identified as demonstrated by Table 1.1 (Balaban and Rasooly, 2000). The TSST-1 exotoxin is a major cause of toxic shock syndrome (TSS) (Schlievert *et al.*, 1981). Furthermore, TSST-1 is the only staphylococcal superantigen known to induce menstrual-associated TSS (Dinges, Orwin and Schlievert, 2000). Three types of exfoliative toxins (ET) have been identified and described: ETA, ETB and ETD (Lee *et al.*, 1987; Yamaguchi *et al.*, 2002). ETs demonstrate serine protease activity, which causes epidermal separation and formation of blisters (Ladhani *et al.*, 1999). The condition is recognised as staphylococcal scalded skin syndrome (SSSS) and can be either localised, known as bullous impetigo, or affect the whole body surface (Ladhani *et al.*, 1999).

S. aureus-associated skin invasion can be also mediated by a group of staphylococcal toxins called epidermal cell differentiation inhibitors (EDINs) (Sugai *et al.*, 1990). These toxins represent a C3-transferase family of ADP-ribosyltransferases and consist of EDIN-A, -B and -C (Franke *et al.*, 2010). The EDIN toxin activity inhibits terminal differentiation of keratinocytes and endothelial cell wound repair (Sugai *et al.*, 1990; Aepfelbacher *et al.*, 1997). EDIN was also reported to induce transcellular tunnels in endothelial cells, which results in a loss of barrier function (Boyer *et al.*, 2006).

Cytotoxic molecules represent another class of staphylococcal exotoxins, which

consists of haemolysins and leukocidins. The haemolysin family is represented by four members: alpha (Hla), beta (Hlb), delta (Hld) and gamma (Hlg). The α-haemolysin is a pore-forming haemolytic toxin that can induce membrane damage in a wide range of mammalian cells (Bhakdi and Tranum-Jensen, 1991). The β-haemolysin activity towards erythrocytes is host species-restricted and its expression is particularly prevalent amongst animal isolates (Dinges, Orwin and Schlievert, 2000). The β-haemolysin toxin is also recognized as sphingomyelinase C as it degrades sphingomyelin in Mg^{2+}-dependent fashion (Wiseman, 1975). The δ-haemolysin demonstrates lytic activity towards erythrocytes and other mammalian cells (Dinges, Orwin and Schlievert, 2000). The γ-haemolysin is a bi-component toxin and thus belongs to a family of synergohymenotropic toxins, as the toxic effect is exerted by an activity of two synergistic components (Supersac, Prevost and Piemont, 1993). The γ-haemolysin consist of two class S components (HlgA, HlgC) and a single class F (HlgB) component (Prévost *et al.*, 1995). The toxin is active against macrophages, neutrophils and mammalian erythrocytes (Dinges, Orwin and Schlievert, 2000).

S. aureus can produce other two-component toxins that are leukotoxic, but lack haemolytic activity and thus are recognised as leukocidins. The most widely recognised has been the Panton-Valentine leukocidin (PVL), due to its prominent pathogenesis. PVL consists of a single class S (LukS-PV) and a single class F (LukF-PV) component (Prévost *et al.*, 1995). In addition to leukotoxicity, PVL also demonstrates necrotizing activity, which has been associated with necrotic skin infections and necrotic haemorrhagic pneumonia (Ward and Turner, 1980; Lina *et al.*, 1999b). More recently another leukocidin has been identified, designated LukE-LukD and found to demonstrate dermonecrotic activity, but weak leukotoxicity towards human blood cells (Gravet *et al.*, 1998). The synergohymenotropic toxin family includes also the LukM-LukF-PV(P83) leukocidin, which was found to be strongly active against ruminant PMN cells (Rainard *et al.*, 2003).

The most recently identified category of putative virulence determinants of *S. aureus* is a novel family of staphylococcal exotoxin-like proteins (SET) (Williams *et al.*, 2000). The SET proteins share a level of protein sequence homology with staphylococcal exotoxins and were shown to demonstrate immunostimulatory properties (Williams *et al.*, 2000). Production of multiple SET proteins during

invasive *S. aureus* infection in humans has also been demonstrated, but their exact function remains to be elucidated (Fitzgerald *et al.*, 2003).

1.2.3 Other virulence determinants

S. aureus produces a surface-associated coagulase, which binds prothrombin and leads to conversion of fibrinogen to fibrin resulting in serum coagulation (Fournier, 2008). It has been suggested that coagulase is involved in evasion of host immune defences, but its precise role in infection has not been fully determined beyond reports that it is an important factor in experimental blood-borne pneumonia and acute bacterial endocarditis (Archer, 1998; Fournier, 2008).

Other important immunomodulatory proteins include staphylokinase (Sak), chemotaxis inhibitory protein (CHIPS) and staphylococcal complement inhibitor (SCIN) (Fournier, 2008). The virulence factor Sak is an extracellular protein that activates human plasminogen into plasmin, which demonstrates proteolytic activity and has been associated with degradation of human IgG and C3b leading to inhibition of phagocytosis (Rooijakkers *et al.*, 2005). The remaining two immune evasion elements are secreted proteins that were also shown to interfere with components of the complement system (Fournier, 2008). CHIPS is known to bind to the C5a receptors on neutrophils and monocytes, which leads to chemotaxis inhibition, whereas SCIN inhibits all three complement pathways by interfering with C3b binding to bacterial cell (Fournier, 2008).

1.3 Antimicrobial resistance: an overview

Antimicrobial resistance of a bacterial pathogen is defined as an ability to remain viable or to continue replication in the presence of an antimicrobial agent at a concentration that occurs at the site of infection (Schwarz and Chaslus-Dancla, 2001). Four main mechanisms of antimicrobial resistance are recognized: enzymatic inactivation of the antimicrobial compound, target modification or acquisition of an alternate pathway, active efflux and reduced permeability (Boerlin and White, 2006). Fundamentally, resistance can be either intrinsic or acquired. An intrinsic resistance is generally either genus- or species-specific, and usually results from inaccessibility of the target site or its absence (Schwarz and Chaslus-Dancla, 2001). Traditionally, the 'antimicrobial resistance' term refers to instances of acquired tolerance to a compound due to genetic change in the bacterial cell (Cloete, 2003). Such change might involve

mutation of a native chromosomal gene, which commonly involves a gene encoding the antimicrobial target (Martinez and Baquero, 2000). More commonly the acquired resistance involves transfer of foreign genetic material that contains single or multiple determinants encoding antimicrobial resistance (Schwarz and Chaslus-Dancla, 2001).

The origins of the resistance genes have been associated with the environmental microorganisms, such as soil bacteria (Alonso, Sánchez and Martínez, 2001). Amongst the first antimicrobial compounds used therapeutically were biological substances synthesized as a product of 'intermicrobic antagonism', such as penicillin, and it was later found that their producers might also express an analogous self-protective compound (Spring, 1975; Benveniste and Davies, 1973). However, such relationships have been identified for a minority of resistance genes and thus it has been proposed that their primary physiological function in the organism of origin was other than antimicrobial resistance (Alonso, Sánchez and Martínez, 2001). This is supported by the findings that certain resistance genes might play a metabolic role in the organism, which carries them as intrinsic chromosomal elements (Macinga and Rather, 1999; Shaw *et al.*, 1992). Similarly, some resistance determinants might have evolved from chromosomal genes, which were primarily involved in cell's physiological function (Adachi *et al.*, 1992). This could have occurred in response to selective pressure of environmentally dispersed antimicrobial substances produced by other microorganisms and prior to the discovery, development and clinical use of antibiotics such as penicillin (Schwarz and Chaslus-Dancla, 2001). The subsequent horizontal transfer of resistance genes between organisms of distinct species and genera has played a critical role in the emergence of resistance amongst pathogenic bacteria (Saunders, 1984). In *S. aureus* the acquired antimicrobial resistance determinants have been predominantly associated with plasmids, transposons and chromosomal cassettes (Malachowa and DeLeo, 2010).

1.3.1 Antimicrobial resistance in *S. aureus*

Antimicrobial susceptibility represents one of the most critical features of *S. aureus* isolates since it is the susceptibility to antimicrobial compounds selected for treatment that might have a decisive influence on the disease outcome. The significance of *S. aureus* antimicrobial susceptibility status can be exemplified by the traditional approach to broadly define *S. aureus* isolates as either methicillin-susceptible (MS) or methicillin-resistant (MR) based on their susceptibility to a

representative member of the β-lactamase-resistant penicillins, such as oxacillin or cefoxitin. This also highlights the significance of β-lactam agents, which represent the antimicrobial class of choice for the treatment of staphylococcal infections (Bamberger and Boyd, 2005; Roberts and Chambers, 2005; Corey, 2009; Thwaites *et al.*, 2011). However, the emergence of resistance, first to penicillin and then to methicillin resulted in the expansion of potential lines of treatment to include other classes of antimicrobial compounds such as macrolides, tetracyclines, aminoglycosides, quinolones and glycopeptides (Lina *et al.*, 1999a; Schmitz *et al.*, 1999; Trzcinski *et al.*, 2000; Lowy, 2003). As presented by Figure 1.1, resistance to majority of these agents occurred shortly after their introduction for management of staphylococcal infections (Hunter, 1947; Gewin and Friou, 1951; Levinson *et al*, 1951; Dowling and Lepper, 1953; Kirby *et al*, 1953; Lowbury, 1960; Nakhla, 1972; Budnick and Schaefler, 1990; Lowy, 2003; Chambers and DeLeo, 2009).

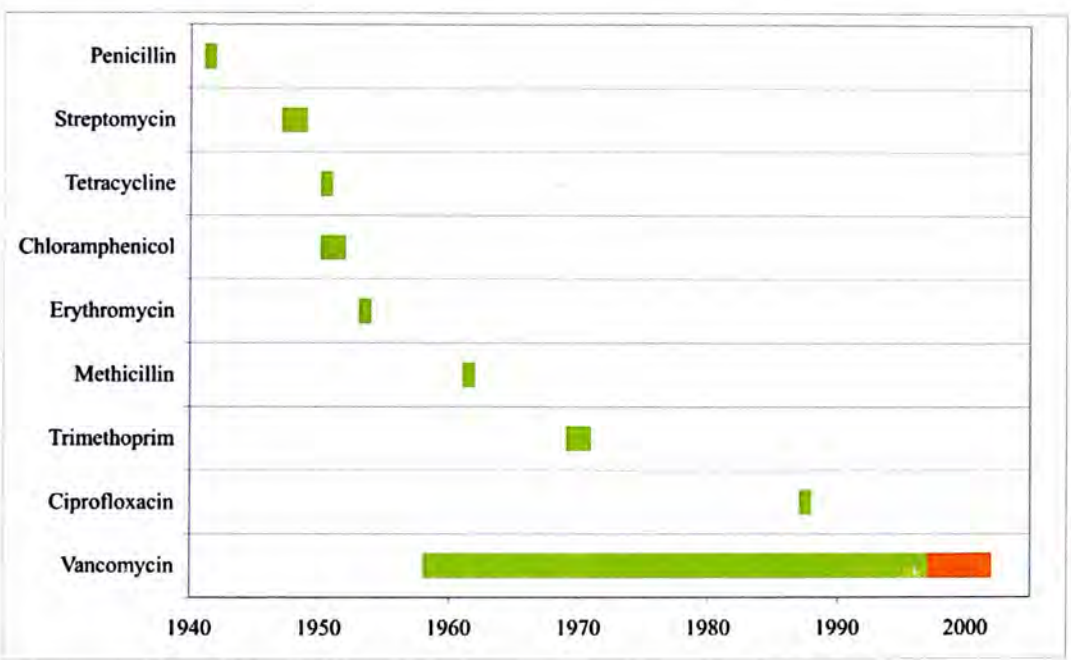


Figure 1.1 Timescale showing introduction of different antimicrobial compounds for treatment of staphylococcal infections, and the length of period until emergence of resistance in clinical isolates of *S. aureus*. The left hand-side of each bar aligns with the year of introduction whereas the righ-hand side marks year of emergence of resistance in *S. aureus*. For vancomycin, the bar shows period until emergence of the vancomycin-intermediate *S. aureus* (VISA; green bar), followed by period until emergence of the vancomycin-resistant *S. aureus* (VRSA; orange bar).

1.3.1.1 Resistance to β-lactams

β-Lactam antibiotics are a broad class of bactericidal compounds that prevent bacterial cell wall synthesis by inhibiting the activity of peptidoglycan-active enzymes called penicillin-binding proteins (PBPs) (Prescott, 2000). The PBPs mediate peptidoglycan synthesis by catalysing the transglycosylation and transpeptidation reactions, which lead to the formation of the glycosidic and peptide bonds, respectively (Pinho, 2008). The cornerstone for the development of β-lactams has been the discovery of penicillin G, a *Penicillium notatum* metabolite, by Alexander Fleming in 1929 (Bryskier, 2005b). The antibacterial activity of penicillin G in a form of growth inhibitory properties was first observed against staphylococcal cultures (Fleming, 1929). Whilst the introduction of penicillin into clinical use considerably reduced the mortality of *S. aureus*-associated infections, it was rapidly followed by the emergence of resistant isolates (Rammelkamp and Maxon, 1942). Penicillin resistance in *S. aureus* is mediated primarily by the production of extracellular enzyme designated penicillinase or β-lactamase, which hydrolyses penicillin's β-lactam ring (Bondi and Dietz, 1945; Prescott, 2000). As demonstrated by Table 1.2, in *S. aureus* the β-lactamase is encoded by the *blaZ* gene (Lowy, 2003). The β-lactamase activity and carriage of the *blaZ* was first associated with a plasmid (Peyru, Wexler and Novick, 1969; Murphy and Novick, 1979). However, it was later found that the gene is located on a transposon, which can translocate between the chromosomal and plasmid sites (Gillespie, Lyon and Skurray, 1988). The identified *blaZ* transposons include Tn*552*, Tn*4002* and Tn*4201* with the first two detected on both plasmid and the chromosome (Gillespie, Lyon and Skurray, 1988; Weber and Goering, 1988; Rowland and Dyke, 1989; Holden *et al.*, 2004).

To overcome the therapeutic challenge of an increasing prevalence of β-lactamase-producing *S. aureus* isolates, a group of semisynthetic penicillinase-resistant penicillins was developed (Bryskier, 2005b). The first synthesised compound was methicillin. Soon after its introduction the first methicillin-resistant (MR) staphylococcal isolates were identified and reported (Jevons, 1961). Resistance to methicillin can be mediated by three mechanisms: hyperproduction of the β-lactamase, modification of native PBPs and expression of an acquired PBP2a (Bryskier, 2005b; McDougal and Thornsberry, 1986; Tomasz *et al.*, 1989). However, the majority of methicillin-resistant isolates demonstrate the latter mechanism and

thus the carriage of the PBP2a genetic determinant is generally considered as the primary definition of staphylococcal methicillin resistance.

The PBP2a demonstrates a low affinity for methicillin and other β-lactams, and thus it allows continuation of cell wall synthesis in the presence of antimicrobials at a concentration that inactivates the native PBPs (Bryskier, 2005b). As such, the mechanism confers a broad-spectrum resistance to all β-lactam agents. PBP2a is encoded by a chromosomally located *mecA* gene (Ubukata *et al.*, 1989). The analysis of the surrounding chromosomal region led to observation that it also represents a foreign genetic material and it was later identified as a novel mobile element designated the staphylococcal cassette chromosome *mec* or SCC*mec* (Ito and Hiramatsu, 1998). The majority of MR *S. aureus* or MRSA isolates are considered to carry a SCC*mec* element, which always inserts into the *orfX* gene of the *S. aureus* chromosome (Malachowa and DeLeo, 2010). Several SCC*mec* types and subtypes have been identified and described, ranging in size from around 20 kilobase pairs (kbp) up to > 65 kbp (Deurenberg *et al.*, 2007; Robinson *et al.*, 2005b). The elements fundamentally consist of the *mec* gene complex and the *ccr* gene complex, and the SCC*mec* variation results from differences within the structure of those complexes, distinct *ccr* genes as well as differences in the content of the surrounding environment known as joining regions J1, J2 and J3 (International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC), 2009).

The SCC*mec* variations have been closely associated with the molecular evolution of MRSA strains demonstrating an adaptation towards a more transmissible structure (Deurenberg *et al.*, 2007). In addition to the *mecA* gene, the SCC*mec* can serve as a vector for other resistance determinants associated with transposons and plasmids that have integrated into the SCC*mec,* such as *ermA*, *spc* and *tetK* (Ito *et al.*, 2001). Recently a divergent *mecA* homologue has been identified and designated *mecC* (Garcia-Alvarez *et al.*, 2011). The gene was located on a novel SCC*mec* element and shared 70% homology with the *mecA* gene (Garcia-Alvarez *et al.*, 2011).

Table 1.2. Mechanisms of antimicrobial resistance associated with *S. aureus*

Compound	Genetic basis [a]	Mechanism of action	Genetic origin	Ref
β-Lactams	*blaZ*	Enzymatic inactivation by β-lactamase	Plasmid, transposon	Gillespie, Lyon and Skurray, 1988
	mecA, mecC	Expression of PBP2a with reduced affinity for β-lactams	SCC*mec*	Ito and Hiramatsu, 1998
Macrolides-lincosamides-streptogramins	*msrA*	Active efflux	Plasmid	Pechère, 2001
	linA	Enzymatic inactivation by lincosamide nucleotidyltransferase	Plasmid	Leclercq *et al.*, 1987
	ermA, *ermB*	Modification of the ribosomal target by methyltransferase	Transposon	Lyon and Skurray, 1987
	ermC	Modification of the ribosomal target by methyltransferase	Plasmid	Lyon and Skurray, 1987
Aminoglycosides	*aacA-aphD*	Enzymatic inactivation by acetyltransferase and phosphotransferase	Transposon	Lyon and Skurray, 1987
	aphA-3	Enzymatic inactivation by phosphotransferase	Transposon	Derbise, Dyke and El Solh, 1996
	aadE	Enzymatic inactivation by adenytransferase	Transposon	Derbise, Dyke and El Solh, 1996
	aadD	Enzymatic inactivation by adenytransferase	Plasmid	Lyon and Skurray, 1987
	spc (ant(9)-Ia)	Enzymatic inactivation by adenytransferase	Transposon	Lyon and Skurray, 1987
Tetracyclines	*tetK, tetL*	Active efflux	Plasmid	De Vries *et al.*, 2009
	tetM	Ribosomal protection	Transposon	De Vries *et al.*, 2009

[a] All determinants of resistance represent horizontally transferred genes

Table 1.2 (continued) Mechanisms of antimicrobial resistance associated with *S. aureus*

Compound	Genetic basis [a]	Mechanism of action	Genetic origin	Ref
Vancomycin	*vanA*	Expression of cell wall precursor with reduced affinity for vancomycin	Plasmid	Lowy, 2003
Fluoroquinolones	*gyrA, gyrB*	Reduced quinolone affinity for DNA gyrase	Chromosome	Takahashi *et al.*, 1998
	grlA, grlB	Reduced quinolone affinity for DNA topoisomerase IV	Chromosome	Takahashi *et al.*, 1998
	norA	Active efflux	Chromosome	Takahashi *et al.*, 1998
Chloramphenicol	*cat*	Enzymatic inactivation by chloramphenicol acetyltransferases	Plasmid	Schwarz *et al.*, 2004
	cfr	Modification of the ribosomal target by methyltransferase	Plasmid	Kehrenberg and Schwarz, 2006
	fexA	Active efflux	Transposon	Kehrenberg and Schwarz, 2005
Trimethoprim	*dfrA*	Expression of an alternate trimethoprim-resistant dihydrofolate reductase	Transposon	Rouch *et al.*, 1989
	dfrG	Expression of an alternate trimethoprim-resistant dihydrofolate reductase	MGE unknown	Sekiguchi *et al.*, 2005
	dfrK	Expression of an alternate trimethoprim-resistant dihydrofolate reductase	Plasmid, transposon	Kadlec and Schwarz, 2010
QACs	*qacAB, qacCD*	Active efflux	Plasmid	McDonnell and Russell, 1999
Cadmium and zinc	*cadA, cadC, cadD*	Active efflux	Plasmid	Lai and Weisblum, 1971
Mercury	*merA*	Activity of mercuric reductase	Plasmid	Lai and Weisblum, 1971

[a] All determinants of resistance represent horizontally transferred genes with exception of *gyrA, gyrB, grlA, grlB* and *norA* that constitute chromosomal genes that acquire mutations

1.3.1.2 Resistance to macrolides, lincosamides and streptogramins

The emergence of penicillin-resistant *S. aureus* prompted a search for other microbial fermentation metabolites with an anti-staphylococcal activity, which led to the discovery of erythromycin A, a product of *Saccharopolyspora erythraea* (Bryskier and Bergogne-Berezin, 2005). Erythromycin A is a representative of the macrolide group, which are generally bacteriostatic agents that inhibit protein synthesis by reversibly binding to the 50S subunit of the bacterial ribosome (Bryskier and Bergogne-Berezin, 2005). This antimicrobial mechanism of action is shared with lincosamides, such as lincomycin and clindamycin, and streptogramins, such as virginiamycin and quinupristin (Giguère, 2006a). The macrolide molecule contains a central 12- to 16-membered lactone ring and the size variation serves in the classification of macrolide compounds (Bryskier and Bergogne-Berezin, 2005). Erythromycin resistance is commonly associated with cross-resistance to lincosamides and streptogramin B agents, which is referred to as macrolide-lincosamide-streptogramin B (MLS_B) resistance (Vester and Douthwaite, 2001).

As demonstrated by Table 1.2, macrolide resistance in *S. aureus* has been associated with three mechanisms: modification of the ribosomal target, enzymatic inactivation and active efflux (Giguère, 2006c). Efflux-mediated resistance in *S. aureus* has been associated with a plasmid-encoded *msrA* gene, which confers resistance to 14- and 15-memberred macrolides and streptogramins (Pechère, 2001). The mechanisms of enzymatic inactivation are not well documented, but an isolate with a resistance to 14- and 16-memebered macrolides and demonstrating esterase activity has been reported (Pechère, 2001). In contrast, *S. aureus*-associated determinants of enzymatic degradation have been identified for other MLS_B compounds such as *linA*, which encodes lincosamide nucleotidyltransferase (Pechère, 2001). The majority of MLS_B resistant *S. aureus* isolates demonstrate modification of the ribosomal target, which is induced by the activity of a methyltransferase. This enzyme specifically methylates adenine residues in the 23S rRNA of the 50S ribosomal subunit, which is essential for the binding of MLS_B antimicrobials (Lai and Weisblum, 1971; Bryskier and Bergogne-Berezin, 2005). Such modification reduces the affinity between ribosome and the MLS_B compounds (Bryskier and Bergogne-Berezin, 2005).

A number of erythromycin resistance methylase (*erm*) genes have been identified in various bacterial species (Bryskier and Bergogne-Berezin, 2005). In S. *aureus* methyltransferase expression has been associated with the acquisition of *ermA*, *ermB* and *ermC* (Lyon and Skurray, 1987). The *ermA* gene is encoded by Tn*544* transposon, which integrates into the *S. aureus* chromosome in a site- and orientation-specific manner (Murphy, Huwyler and de Freire Bastos, 1985). The Tn*554* transposon also contains the spectinomycin resistance determinant, the *spc* gene (Murphy, Huwyler and de Freire Bastos, 1985). The *ermB* gene is carried by transposon Tn*551*, which can translocate to multiple chromosomal and plasmid sites (Novick *et al.*, 1979). The *ermC* determinant has been mainly associated with small, multi-copy plasmids. The first to be identified was pE194, which has become a prototype for other *ermC*-carrying plasmids of similar size (Lyon and Skurray, 1987). The expression of *erm*-mediated resistance can be constitutive or inducible (Leclercq and Courvalin, 1991). The constitutive expression confers the MLS_B resistance phenotypes, whereas the inducible expression mediates resistance only to 14- and 15-membered macrolides (Leclercq and Courvalin, 1991). The nature of expression is defined by the regulatory region located upstream of the *erm* determinant (Leclercq and Courvalin, 1991).

1.3.1.3 Resistance to aminoglycosides

Aminoglycosides are a broad class of antimicrobial agents also referred to as aminocyclitols or aminoglycosidic aminocyclitols (Dowling, 2006). The compounds can be classified into four groups of derivatives based on the aminocyclitol substitution: streptidine (streptomycin), streptamine (spectinomycin), 4,5-disubstituted deoxystreptamine moiety (neomycin) and 4,6-disubstituted deoxystreptamine moiety (gentamicin, kanamycin, amikacin, tobramycin) (Dowling, 2006). Aminoglycosides are generally bactericidal agents that interfere with protein synthesis by binding to the 30S ribosomal subunit (Dowling, 2006). The different compounds vary in their potency as well as spectrum of activity with the highest assigned to amikacin and the lowest to streptomycin (Dowling, 2006). Resistance to aminoglycosides can be mediated by three mechanisms. Target modification due to mutations of the ribosomal genes has been identified in a streptomycin-resistant isolate (Lyon and Skurray, 1987). A reduced permeability due to chromosomal mutations was found to mediate a low level resistance to most aminoglycoside

compounds (Lyon and Skurray, 1987). However, the majority of clinical isolates acquire resistance through aminoglycoside-modifying enzymes that, depending on the mechanism of catalysed reaction can be grouped into acetyltransferases (AAC), adenyltransferases (AAD) and phosphotranfserases (APH) (Shaw *et al.*, 1993).

For each group, different subclasses of aminoglycoside-modifying enzymes have been identified across various bacterial species that vary in type and site of modification as well as the conferred resistance profile and the *S. aureus*-associated aminoglycoside resistance determinants are presented in Table 1.2 (Shaw *et al.*, 1993). The aminoglycoside acetyltransferase and phosphotransferase activity in *S. aureus* is commonly associated with the acquisition of a bi-functional *aacA-aphD* resistance determinant (Lyon and Skurray, 1987). The element is carried by Tn*4001* transposon, which can be either chromosomally or plasmid located and mediates resistance to gentamicin, kanamycin and tobramycin (Rouch *et al.*, 1987; Gillespie *et al.*, 1987). *S. aureus* resistance mediated by phosphotransferase activity is related to the acquisition of the *aphA-3* determinant, which confers resistance to kanamycin and neomycin (Lyon and Skurray, 1987). The gene has been identified on various transposons, namely Tn*3854*, Tn*5404* and Tn*5405* (Udo and Grubb, 1991; Derbise, Dyke and Solh, 1995; Derbise, Dyke and El Solh, 1996). While the Tn*3854* transposon was found to be plasmid-located, the Tn*5404* and Tn*5405* elements were identified on both plasmids and the chromosome. Furthermore, the Tn*5404* and Tn*5405* transposons carry a second aminoglycoside modification determinant, an adenyltransferase-encoding *aadE* gene that mediates resistance to streptomycin only (Derbise, Dyke and El Solh, 1996). The adenyltransferase aminoglycoside modification in *S. aureus* has been commonly associated with the carriage of the *aadD* gene, which mediates resistance to kanamycin, neomycin, tobramycin and amikacin (Lyon and Skurray, 1987). The *aadD* determinant has been identified on a number of small multicopy as well as larger low-copy plasmids (Lyon and Skurray, 1987). Of significance in mediating *S. aureus* resistance to aminoglycosides has also been the Tn*554*-associated *spc* gene, which encodes a spectinomycin adenyltransferase and is also recognised as *ant(9)-Ia* (Shaw *et al.*, 1993).

1.3.1.4 Resistance to tetracyclines

Tetracyclines are broad-spectrum bacteriostatic agents that inhibit protein synthesis by binding reversibly to the 30S subunit of bacterial ribosomes (Giguère,

2006b). The class consists of natural molecules synthesized by the *Streptomyces* species such as oxytetracyline and tetracycline as well as semi-synthetic derivatives such as doxycycline and minocycline (Giguère, 2006b). Resistance to tetracyclines can be mediated by various mechanisms although in *S. aureus* it has been associated largely with efflux and ribosomal protection, as demonstrated by Table 1.2. The tetracycline active efflux in *S. aureus* is most commonly mediated by the *tetK* gene, which was found to be associated with small multicopy plasmids, such as the pT181 (Lyon and Skurray, 1987; Guay and Rothstein, 1993). The TetK protein demonstrates a level of substrate specificity and as such confers resistance to tetracycline, but not minocycline (Guay and Rothstein, 1993). The active efflux in *S. aureus* can be also conferred by acquisition of the *tetL* determinant, although it has been found at very low prevalence amongst *S. aureus* isolates (Schmitz *et al.*, 2001; De Vries *et al.*, 2009). The ribosomal protection mechanism of tetracycline resistance in *S. aureus* has been associated primarily with the *tetM* determinant. The gene was identified in several *S. aureus* chromosomally located transposons: Tn*916*-like, Tn*5801* and Tn*6014* (Kuroda *et al.*, 2001; De Vries *et al.*, 2009). The TetM protein mediates resistance to both tetracycline and minocycline by binding to the ribosome and releasing the antimicrobial molecule from its binding site (Lyon and Skurray, 1987; Connell *et al.*, 2003).

1.3.1.5 Resistance to other classes of antimicrobial compounds

Vancomycin, a glycopeptide compound that inhibits cell wall synthesis has gained clinical significance, when it was introduced for the treatment of severe *S. aureus* infections in the late 1950s (Lowbury, 1960). Although still uncommon, vancomycin-intermediate and vancomycin-resistant *S. aureus* (VISA and VRSA, respectively) isolates have occured 40 years later (Lowy, 2003). The vancomycin-intermediate phenotype results from an alteration in the peptidoglycan synthesis, which leads to vancomycin molecules being trapped in the outer layers of the cell wall (Lowy, 2003). The genetic determinant of this mechanism is not known. In contrast, vancomycin-resistance is associated with acquisition of the enterococcal *vanA* genetic element, which encodes a cell wall precursor with a reduced affinity for vancomycin (Lowy, 2003).

Fluoroquinolones, such as ciprofloxacin and enrofloxacin, are synthetic antimicrobial compounds that target topoisomerase IV and DNA gyrase, enzymes

involved in bacterial DNA synthesis (Blondeau, 2004). Resistance to fluoroquinolones in *S. aureus* is often associated with target modification due to chromosomal mutations in the DNA gyrase genes *gyrA* and *gyrB*, as well as DNA topoisomerase IV genes *grlA* and *grlB* (Takahashi *et al.*, 1998). The resistance phenotype can be also mediated by induction of multidrug resistance efflux pumps, in *S. aureus* encoded the chromosomal gene *norA* (Takahashi *et al.*, 1998).

Chloramphenicol and the derivative florfenicol are broad-spectrum antimicrobial agents that inhibit protein synthesis by binding to the 50S ribosomal subunit (Fish and Bryskier, 2005). Resistance to chloramphenicol only, is often mediated by enzymatic inactivation due to acquisition of plasmid-associated *cat* genes that encode chloramphenicol acetyltransferases (CATs) (Schwarz *et al.*, 2004). Combined resistance to both chloramphenicol and florfenicol can be conferred either by target modification or associated with efflux protein (Schwarz *et al.*, 2004). The former is related to acquisition of a plasmid-borne *cfr* gene, which mediates methylation of the 23S rRNA, a mechanism that also confers resistance to clindamcyin (Kehrenberg and Schwarz, 2006). A number of efflux determinants related to chloramphenicol and florfenicol resistance has been identified in different bacterial species, and in *S. aureus* it is represented by the *fexA* gene carried on Tn*558* transposon (Schwarz *et al.*, 2004; Kehrenberg and Schwarz, 2005).

Trimethoprim belongs to diaminopyrimidines, a class of antimicrobial agents that inhibit bacterial dihydrofolate reductase and interfere with folic acid synthesis (Prescott, 2006). Their clinical use commonly involves a combination with sulfonamides (Prescott, 2006). Resistance to trimethoprim can be related to mutational changes in the native chromosomal genes, but is most often mediated by acquisition of *dfr* genes that encode trimethoprim-resistant dihydrofolate reductases (Skold, 2001). In *S. aureus* such genes are represented by a *dfrA* located on transposon Tn*4003*, a chromosomally located *dfrG* (mobile vector unknown) and a *dfrK* gene detected on plasmid and transposon Tn*559* (Rouch *et al.*, 1989; Sekiguchi *et al.*, 2005; Kadlec and Schwarz, 2010).

1.3.1.6 Resistance to biocides

Biocides are defined as a bacteriostatic or bactericidal chemical agents, that differ from antibiotics by demonstrating a broader spectrum of activity, having less defined mode of action and generally acting non-specifically on multiple cellular

targets (McDonnell and Russell, 1999). Biocides have been commonly applied as antiseptics, disinfectants or preservatives (McDonnell and Russell, 1999). They have been widely utilised as part of infection control and prevention measures within healthcare settings (McDonnell and Russell, 1999). Similarly, biocides are common components of personal care, as well as household cleaning products (White and McDermott, 2001; Fraise, 2002). Biocides have also been heavily applied in prevention of infection among food-producing animals, which includes use of environmental and living tissue disinfectants, as well as food preservatives (Aarestrup and Hasman, 2004).

Although a wide range of chemical agent classes has been found to exert a biocidal activity, analysis of reduction in inhibitory effects against *S. aureus* has been predominantly conducted for quaternary ammonium compounds (QACs), biguanides, bisphenols, diamidines, acriflavine, ethidium bromide and heavy metal ions (McDonnell and Russell, 1999). Acquired resistance to some of these agents in *S. aureus* has been commonly associated with carriage of plasmid-located *qac* genes, namely *qacAB* and *qacCD* that encode an efflux system, as described in Table 1.2 (McDonnell and Russell, 1999). Heavy metal ions such as cadmium, mercury and arsenate have no current therapeutic applications due to high toxicity towards all organisms but can be present within the environment and thus exert selective pressure on bacteria (Lyon and Skurray, 1987). In contrast, certain inorganic ions such as zinc and copper served as livestock feed additives as well as components of animal disinfectants (Aarestrup and Hasman, 2004). In *S. aureus* resistance to cadmium and zinc can be mediated by plasmid-associated *cad* determinants, such as *cadA*, *cadC* and *cadD* that encode efflux systems (Lai and Weisblum, 1971). Resistance to mercury ions has been related to an activity of mercuric reductase encoded by an acquired *merA* gene, commonly a component of a *mer* operon (Stapleton *et al.*, 2006). Also plasmid-associated, the *mer* elements can be co-carried with the *cad* determinants (Lai and Weisblum, 1971). Similarly, arsenate resistance is most commonly plasmid-related and involves enhanced efflux and reduced uptake (Lai and Weisblum, 1971). The *qac* genes as well as heavy metal resistance determinants such as *cad* and *mer* have been associated with plasmids carrying β-lactamase genes and thus demonstrate a significant potential for a co-selection of antimicrobial and biocide resistance (Lai and Weisblum, 1971).

1.4 *S. aureus* molecular evolution

A diverse range of *S. aureus* strains have now been fully genome-sequenced, with the number still growing. The characterised genomes vary in size, between 2.7 and 3 megabase pairs (mbp), as well as genetic content, varying between approximately 2500 up to 2900 genes, with the majority demonstrating a 32.8 - 32.9% GC content (http://www.ncbi.nlm.nih.gov/genome/genomes/154). Based on the pairwise alignments, the *S. aureus* genomes demonstrate a largely conserved and collinear organization (Lindsay and Holden, 2004). A DNA microarray-based analysis revealed that 78% of the *S. aureus* genome content is shared amongst strains of divergent lineages derived from both human and animal hosts (Fitzgerald *et al.*, 2001b). This has been recognised as the core genome, in which the gene order is conserved and the individual genes share typically 98-100% homology at the protein sequence level (Lindsay and Holden, 2004). The intra-species diversity has thus been attributed mainly to the remaining 22% of genome, which is considered to constitute non-essential and strain-specific genetic material (Fitzgerald *et al.*, 2001b). Furthermore, a considerable proportion of the variation has been associated with putative virulence and antimicrobial resistance determinants, which is thought to demonstrate the considerable capacity of *S. aureus* for host or environmental niche adaptations (Fitzgerald *et al.*, 2001b). Such elements have been associated with diverse mobile genetic elements. It has therefore been concluded that horizontal gene transfer has played a pivotal role in the molecular evolution of *S. aureus* (Fitzgerald *et al.*, 2001b). However, other mechanisms such as point mutations and large-scale genome rearrangements have also been implicated (Robinson and Enright, 2004; Herron *et al.*, 2002). Such evolutionary events have been profoundly mirrored in the dynamics of *S. aureus* population structure, the lineage diversity and emergence of successful epidemic clones (Fitzgerald *et al.*, 2001b; Feil *et al.*, 2003; Robinson and Enright, 2004).

1.4.1 Mechanisms of *S. aureus* molecular evolution

1.4.1.1 Genome point mutations

A point mutation is the alteration of a DNA sequence involving a single nucleotide (Dale and Park, 2010). This can occur in a form of a base substitution where one nucleotide is replaced by another (Dale and Park, 2010). If it arises within

a coding sequence, such change can result in amino acid alteration (nonsynonymous), generation of a premature stop codon (nonsense), or have no impact on the protein sequence (synonymous) (Dale and Park, 2010). A point mutation might also involve a deletion or addition of a nucleotide, which is recognised as a frameshift mutation as it leads to alteration in the open reading frame and can also result in premature termination of translation (Dale and Park, 2010).

Point mutations are thought to exert a very subtle effect on bacterial diversification, which at most involves an alteration of an existing function and gradual niche adaptation (Lawrence, 1999). Also, in the case of *S. aureus* point mutations are considered to have a limited impact on its genome, mostly as they rarely occur in coding sequences, are synonymous or arise outside the functional regions of the protein sequence (Lindsay, 2008). This is in accordance with a general conception that nonsynonymous mutations will occur at lower frequency than the synonymous changes particularly within genes that are essential for bacterial survival as the resulting phenotype will be mostly unfavourable (Herron *et al.*, 2002). However, the bacterial chromosome can often acquire single base changes in response to the surrounding environmental conditions, which can be then categorized as adaptive mutations (Rosenberg, 2001). A prominent example of adaptive mutation in bacteria is thus a chromosomal change induced by exposure to antimicrobial agents, which as previously described, can occur in *S. aureus* isolates and confers resistance to certain classes of compounds, such as fluoroquinolones (Takahashi *et al.*, 1998).

Single base mutations have also been implicated in the alteration of *S. aureus* virulence. A point mutation in the *agr* (accessory gene regulator) locus has been implicated as a causative factor of a virulence phenotype in experimental murine pneumonia (Villaruz *et al.*, 2009). Also, a single base substitution resulting in a stop codon was found to significantly attenuate adhesion properties of fibronectin-binding proteins of *S. aureus* strain Newman (Grundmeier *et al.*, 2004). Point mutations have also been implicated in evolution and diversification of animal-derived lineages, which was observed for bovine- and poultry-associated strains (Herron *et al.*, 2002; Lowder *et al.*, 2009). The process was found to involve a high ratio of nonsynonymous to synonymous mutations in various coding sequences, which was associated with a putative loss of gene function (gene decay) (Herron *et al.*, 2002; Lowder *et al.*, 2009; Herron-Olson *et al.*, 2007).

Of particular significance have been point mutations that occur in the genes that constitute the basis for the *S. aureus* multi-locus sequence typing (MLST) scheme (Feil *et al.*, 2003). MLST involves sequence analysis of seven housekeeping genes, in which variation occurs at very low rate and is representative of strain's overall chromosomal divergence (Enright *et al.*, 2000). Such sequence changes have been occurring mostly through point mutations (Feil *et al.*, 2003). In the context of MLST loci analysis, point mutations have thus played a central role in the clonal diversification of *S. aureus* (Feil *et al.*, 2003). Also, a closer analysis of point mutation-derived diversification within the seven loci allows the determination of the ancestry and inter-lineage association of major *S. aureus* clones (Enright *et al.*, 2002).

1.4.1.2 Horizontal transfer of mobile genetic elements

Horizontal transfer of genetic material can occur through one of three fundamental mechanisms: conjugation, transformation and transduction (Saunders, 1984). The process commonly involves or is facilitated by mobile genetic elements (MGEs) (Malachowa and DeLeo, 2010). MGEs are segments of DNA carrying genes that mediate their intra- and inter-cellular movement (Frost *et al.*, 2005). It is estimated that MGEs make up around 10-20% of the *S. aureus* genome (Lindsay, 2008). The close GC content resemblance between the chromosome and a typical MGE suggests that the transfer of genetic material occurs mostly at the species level (Lindsay, 2008). The MGEs can be broadly categorized into autonomous extrachromosomal elements, a group represented solely by plasmids, and elements that must integrate into another DNA molecule, such as transposons, insertion sequences, pathogenicity islands and bacteriophages (Malachowa and DeLeo, 2010). Genome diversification through horizontal transfer differs considerably from an impact exerted by point mutations as it involves acquisition of defined and novel, often multiple genetic determinants in a single event (Lawrence, 1999). The acquired elements are not essential for bacterial survival under typical physiological conditions but might promote expansion within a specific niche and thus provide a distinct selective advantage (Lawrence, 1999; Schwarz and Chaslus-Dancla, 2001). MGEs thus commonly serve as vectors of virulence and antimicrobial resistance genes, as well as metabolic function determinants (Schwarz and Chaslus-Dancla, 2001).

Plasmids are extrachromosomal elements that undergo autonomous replication and can vary in size from < 2 kbp to > 100 kbp (Schwarz and Chaslus-Dancla, 2001).

The functional structure of plasmids is composed of a 'backbone' that encodes the replication elements and a variable number of accessory genes (Frost *et al.*, 2005). Two types of plasmids are most prevalent amongst *S. aureus* isolates (Lindsay, 2010). The first are small multicopy plasmids that carry one resistance determinant and replicate by the rolling circle mechanism (Lindsay, 2010). Another class are larger low copy plasmids that carry multiple resistance genes and replicate via the theta mechanism, and some might also transfer through conjugation, which is facilitated by the *tra* genes (Lindsay, 2010). The overall prevalence of plasmids varies amongst *S. aureus* isolates, although many carry one or more (Lindsay, 2008). Also, not all plasmids are carried in a free form as some might become integrated into the chromosome or other chromosomally located MGEs (Lindsay, 2008).

Transposons are fragments of DNA sequence that can selftransfer between other DNA molecules such, as plasmids, bacteriophages and chromosomes (Saunders, 1984). They lack function of independent replication and vary considerably in size between < 1 kbp and > 60 kbp (Schwarz and Chaslus-Dancla, 2001). Replication and integration into another DNA is mediated by the transposon-encoded transposase, with many transposases lacking site specificity resulting in multiple insertions within the same chromosome (Lindsay, 2008). Tranposons might also carry *tra* genes and thus transfer via conjugation (Lindsay, 2008). Most of the *S. aureus* sequenced strains were found to carry at least one transposon (Lindsay, 2008).

Bacteriophages are recognised as an MGE that has most profoundly contributed to the evolution and genetic diversity of *S. aureus* isolates (Goerke *et al.*, 2009; Malachowa and DeLeo, 2010). The majority of *S. aureus*-associated bacteriophages are temperate and around 45 kbp in size (Lindsay, 2008; Malachowa and DeLeo, 2010). Temperate bacteriophages can lyse bacterial cell following the infection, but often they integrate into the bacterial chromosome in a site-specific manner and enter a dormant state becoming a prophage (Lindsay, 2008; Malachowa and DeLeo, 2010). Bacteriophage acquisition and carriage has had a considerable impact on *S. aureus* pathogenicity through either positive or negative lysogenic conversion (Goerke *et al.*, 2009; Malachowa and DeLeo, 2010). The positive lysogenic conversion occurs when the bacterial cell expresses the prophage-encoded virulence determinants (Malachowa and DeLeo, 2010). In contrast, the negative lysogenic conversion involves an insertional inactivation of an intrinsic chromosomal gene following integration of the bacteriophage (Malachowa and DeLeo, 2010). Bacteriophages are widely distributed

amongst *S. aureus* genomes, with most strains carrying between one and four integrated prophage elements (Lindsay, 2008).

Another class of MGEs that have mediated the dissemination of virulence determinants amongst *S. aureus* isolates are the staphylococcal pathogenicity islands (SaPIs). The SaPIs are discrete chromosomal regions located at specific sites within the genome (Novick, 2003). They can vary in size between 15 kbp and 20 kbp, and are transferred at high frequency by certain bacteriophages (Novick, 2003). The SaPIs are related to bacteriophages, but lack genes that would mediate their horizontal transfer (Lindsay, 2010). A number of SaPIs have been identified and sequenced, and all were found to share a set of core genes encoding elements such as integrase, Rep protein and terminase (Malachowa and DeLeo, 2010). The SaPI elements are not as widely distributed as prophages amongst *S. aureus* genomes, but up to two can be carried by a single isolate (Lindsay, 2008).

Distinct from SaPIs, but also associated with virulence determinants are the staphylococcal genomic islands (GIs), such as the *v*Saα and *v*Saβ, located within specific loci of the *S. aureus* genome (Baba *et al.*, 2008). The GIs are associated with a remnant transposase gene and are considered to be non-mobile (Lindsay, 2008; Malachowa and DeLeo, 2010). However, based on the variation in the GC content between the GIs and the core genome, the regions are thought to have been acquired horizontally (Malachowa and DeLeo, 2010). In addition to putative virulence genes, the *v*Saα and *v*Saβ GIs carry copies of the *sau1hsdM* and *sau1hsdS* elements (Lindsay, 2008). The genes encode components of *S. aureus* restriction modification system, which plays a key role in the regulation of horizontal gene transfer as it recognizes and digests foreign DNA (Waldron and Lindsay, 2006). The mechanism can block the transfer of genetic material from other species as well as between different *S. aureus* lineages (Waldron and Lindsay, 2006).

The evolution of *S. aureus*, particularly in the context of antimicrobial resistance emergence and dissemination, has been also mediated by a genus-specific MGE designated staphylococcal cassette chromosome (SCC) (Ito and Hiramatsu, 1998). All SCC elements carry cassette chromosome recombinase genes that mediate integration at a specific site in the staphylococcal chromosome (International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC), 2009). Although SCC can carry diverse resistance determinants, the element

1.4.2 Host specificity

A genetic analysis of bovine-associated populations of *S. aureus* demonstrated that the dominant lineages are distinct from the prevalent human-related clones (Kapur *et al.*, 1995). As host specificity has been described for various pathogenic bacteria, the molecular typing evidence suggested that such evolutionary adaptation has also occurred within the *S. aureus* population (Kapur *et al.*, 1995). Further confirmation has been derived from identification of virulence elements that are carried mostly or exclusively by animal-associated isolates. This can be exemplified by genes encoding the LukM/F-PV(P83) leukocidin, which was found to be the most potent leukotoxin against bovine neutrophils (Barrio, Rainard and Prevost, 2006). Another example of animal-associated virulence determinant is the novel *bap* gene, which encodes a surface protein Bap involved in biofilm formation that was identified in a bovine mastitis isolate (Cucarella *et al.*, 2001). Furthermore, the host specificity of certain animal-associated lineages has been associated with carriage of novel MGEs carrying host-adapted virulence determinants. A unique pathogenicity island, designated SaPIbov was identified in a bovine *S. aureus* strain and found to carry a range of superantigen genes encoding toxins that could specifically activate bovine lymphocytes (Fitzgerald *et al.*, 2001a).

The whole genome sequence analyses of animal-associated strains have allowed a closer examination of the subject. Sequencing the genomes of strains RF122, representing a common bovine-related CC151, and ED133, of the prevalent ruminant-associated CC133, demonstrated a number of unique genetic features in comparison with genomes of human-associated strains (Herron-Olson *et al.*, 2007; Guinane *et al.*, 2010). Both strains demonstrated a high degree of genetic divergence amongst certain genes encoding surface proteins, which was linked with the process of adaptation to the tissue environment of their respective hosts (Herron-Olson *et al.*, 2007; Guinane *et al.*, 2010). The evidence of gene diversification was also observed in both strains amongst metabolic determinants. In particular the RF122 strain demonstrated differences in a number of iron metabolism genes, which was to a lower degree observed in the ED133 and was related to the variation in processes required for iron acquisition by *S. aureus* in different hosts (Herron-Olson *et al.*, 2007; Guinane *et al.*, 2010). A proportion of the genetic diversification observed in the RF122 and ED133 genomes was associated with gene decay and transformation of certain elements into

pseudogenes (Herron-Olson *et al.*, 2007; Guinane *et al.*, 2010). This process in the RF122 strain has been linked with transition into an intracellular lifestyle (Herron-Olson *et al.*, 2007).

Identification of host-specific features has not been limited to ruminant-associated lineages, as revealed by the description of genome analysis of a strain belonging to the poultry-adapted clade of ST5 (Lowder *et al.*, 2009). The process of adaptation has involved previously described mechanisms of acquisition of novel MGEs and loss of function of genes involved in pathogenesis in a human host (Lowder *et al.*, 2009). Phenotypically the analysed strain demonstrated an enhanced resistance to killing by avian neutrophils (Lowder *et al.*, 2009).

Analysis of the origin of the animal-associated lineages of *S. aureus* has thus far revealed a common theme of a host jump event involving a strain of human origin and subsequent genetic adaptation to the animal species (Guinane *et al.*, 2010; Lowder *et al.*, 2009).

1.4.3 Population structure and lineages

A number of studies have been conducted to elucidate the population structure of *S. aureus* with a consistent observation that it is highly clonal and is composed mostly of few dominant lineages (Feil *et al.*, 2003; Lindsay, 2010). Such features have been associated with the observation that the clonal diversification of *S. aureus* lineages occurs mostly by point mutation rather than recombination (Feil *et al.*, 2003). The interest in this area of staphylococcal biology was partly prompted by the increasing prevalence of methicillin-resistant *S. aureus* (MRSA) as a hospital-acquired pathogen as well as its global dissemination (Musser and Kapur, 1992). The fundamental objectives included a better understanding of variation, global distribution and relationship between MRSA genotypes, as well as association between carriage of the *mecA* gene and *S. aureus* genotypes (Musser and Kapur, 1992). Later such attention was also directed at genotypes of methicillin-susceptible *S. aureus* (MSSA) isolates with an aim to study their geographical variation and to determine if there is an association between certain genotypes and severe infections (Enright *et al.*, 2000).

S. aureus lineages are commonly described by their clonal complex (CC) or sequence type (ST), as determined by molecular typing methodology (Lindsay, 2010). Molecular epidemiology analyses of globally derived *S. aureus* isolates, associated

with carriage as well as hospital and community-derived infections, found that the most prevalent lineages were CC1, CC5, CC8, CC9, CC12, CC15, CC22, CC25, CC30, CC45 and CC51 (Chambers and DeLeo, 2009). Whilst all of the described lineages are common amongst MSSA isolates their distribution is more limited amongst MRSA clones (Deurenberg and Stobberingh, 2008). As demonstrated by Table 1.3, the hospital-acquired (HA) MRSA has been most commonly associated with CC5, CC8, CC20, CC30 and CC45 whereas the community-acquired (CA) MRSA with CC1, CC8 and CC30 (Deurenberg and Stobberingh, 2008). Furthermore, the CA-MRSA has also been associated with CC59 and CC80 demonstrating that certain lineages can become more successful following acquisition of the SCC*mec* element (Deurenberg and Stobberingh, 2008; Lindsay, 2010). Only a proportion of prevalent CCs are represented by both MSSA and MRSA isolates with the MSSA isolates recognised as the ancestral genotypes of the MRSA lineages that emerged through acquisition of the SCC*mec* (Enright *et al.*, 2000). Both MSSA and MRSA lineages demonstrate a specific geographic distribution (Deurenberg and Stobberingh, 2008). Isolates belonging to the same lineage demonstrate a considerable genome homology, regardless of their methicillin sensitivity status (Lindsay, 2010). This, however, applies mostly to their core and non-MGE associated elements of the genome (Lindsay, 2010).

Analysis of lineage distribution amongst isolates derived from invasive community- and hospital-acquired infections revealed no significant genotype variation amongst the MSSA isolates (Enright *et al.*, 2000). Such findings have allowed a conclusion that in general there is no association between certain lineages and a capacity to cause invasive disease (Grundmann *et al.*, 2002). This was later confirmed by a study that reported a lack of significant difference in the distribution of genotypes amongst isolates derived from carriers and patients with invasive disease (Feil *et al.*, 2003). The results thus provided evidence against a possibility that the *S. aureus* population carries hypervirulent clones (Feil *et al.*, 2003). However, the long-term epidemiology of *S. aureus* has been marked by events of apparently sudden emergence of particularly successful clones that demonstrated an enhanced transmissibility or pathogenicity (Enright *et al.*, 2000; Smith, Feil and Smith, 2000). Such clones would often become epidemic over a length of time, but eventually merge back into the background population (Smith, Feil and Smith, 2000). This has involved mostly MRSA clones, with shifts in the most prevalent lineages occurring

where one epidemic genotype becomes replaced by another newly emerged dominant clone (Deurenberg and Stobberingh, 2008). Some *S. aureus* epidemic episodes have also involved strains demonstrating a particular virulence genotype, such as expression of the PVL or TSST-1 (Musser *et al.*, 1990; Robinson *et al.*, 2005a). This can be exemplified by the early epidemic clone of the PVL-positive *S. aureus*, recognized as phage type 80/81, that was responsible for causing both hospital- and community-acquired infections (Robinson *et al.*, 2005a).

Table 1.3 The prevalent lineages of HA-MRSA and CA-MRSA

	CC	ST	SCC*mec*	Clone
HA-MRSA	8	250	I	Archaic
	8	247	I	Iberian
	8	8	II	Irish
	8	8	IV	UK EMRSA-2/-6
	8	239	III	Brazilian/Hungarian
	5	5	I	EMRSA-3
	5	5	II	New York/Japanese
	5	5	IV	Paediatric clone
	22	22	IV	EMRSA-15
	30	36	II	EMRSA-16
	45	45	IV	Berlin
CA-MRSA	1	1	IV	USA400
	8	8	IV	USA300
	30	30	IV	Southwest Pacific
	59	59	IV	USA1000
	80	80	IV	European

1.4.4 MRSA evolution

The molecular evolution involving emergence and dissemination of successful clones has been most notably demonstrated by the strains of MRSA (Deurenberg and Stobberingh, 2008). The epidemiology of MRSA has thus far involved three main events. After the emergence of the first MRSA isolates in the early 1960s, MRSA has disseminated worldwide to become a major hospital-associated (HA) pathogen (Musser and Kapur, 1992). A shift in MRSA epidemiology occurred when it emerged in persons with no healthcare-related predisposing risk factors and became recognised as a community-acquired (CA) MRSA (Herold *et al.*, 1998). Most recently a novel MRSA reservoir has been identified, with many reports of MRSA isolation from

food-producing animals and the clones categorised as livestock-associated (LA) MRSA (Lindsay, 2010). While such classification of MRSA isolates is based on origin and risk factors that contributed to MRSA infection or carriage, the isolates can be further grouped based on their underlying genotype (Lindsay, 2010).

Despite the clonal population structure of *S. aureus*, a level of genotypic heterogeneity exists amongst dominant lineages that represent HA, CA and LA isolates of MRSA. As such, each of those MRSA categories is represented by distinct *S. aureus* clonal complexes (Lindsay, 2010). This relates to the accepted multi-clone hypothesis that the dissemination of methicillin-resistance involved multiple independent SCC*mec* acquisition events by genotypically distinct strains (Musser and Kapur, 1992; Fitzgerald *et al.*, 2001b). It has discounted the contrasting proposal that MRSA strains evolved from a single clone that emerged from a MSSA isolate following only one occurrence of SCC*mec* horizontal transfer (Kreiswirth *et al.*, 1993). However, it has been later demonstrated that changes in the molecular epidemiology of MRSA have been mediated by a combination of factors and as such involved the emergence of single locus variants (SLV) of the pre-existing dominant MRSA lineages, as well as later transfer of the SCC*mec* into new MSSA hosts (Enright *et al.*, 2002). The highly prevalent MRSA genotypes have been commonly referred to as major MRSA clones, which relates to isolates that share the same ST and SCC*mec* type and have been derived from at least two different countries (Deurenberg *et al.*, 2007).

1.4.4.1 HA-MRSA

Analysis of early MRSA isolates revealed that the majority belonged to CC8, as revealed by Table 1.3, and that methicillin resistance was most likely first acquired by a ST250 strain (Enright *et al.*, 2002). This first MRSA carried SCC*mec* type I and has been designated the Archaic clone (Deurenberg *et al.*, 2007). The ST250-MRSA-I is also an ancestral genotype of ST247-MRSA-I, a SLV that has become recognised as the Iberian clone (Enright *et al.*, 2002). It was first identified in Spain in 1989, but has since disseminated to several other European countries as well as the USA (Oliveira, Tomasz and de Lencastre, 2002). The CC8 clones also consist of those belonging to ST8, which have emerged through multiple independent SCC*mec* transfers into the prevalent ST8-MSSA clone (Enright *et al.*, 2002). These are represented by ST8-MRSA-II, the Irish clone, and ST8-MRSA-IV, the UK EMRSA-

2/-6 (Deurenberg *et al.*, 2007). Furthermore, the CC8 includes ST239-MRSA-III, the Brazilian/Hungarian clone that has emerged through homologous recombination between ST30-MSSA and ST8-MRSA-III (Robinson and Enright, 2004; Deurenberg *et al.*, 2007). The Brazilian clone was first reported in Brazil in 1992 followed by spread to a few other South American countries, as well as Portugal and the Czech Republic (Oliveira, Tomasz and de Lencastre, 2002). The Hungarian clone derived its name from high prevalence in the Hungarian hospitals, although it was also reported in Taiwan (Oliveira, Tomasz and de Lencastre, 2002).

A number of MRSA clones have also derived from multiple *mecA* acquisitions by ST5-MSSA, involving SCC*mec* types I-IV, and giving rise to major clones such as ST5-MRSA-I: the EMRSA-3, ST5-MRSA-II: the New York/Japanese clone and ST5-MRSA-IV: the Paediatric clone (Enright *et al.*, 2002). ST5-MRSA-II became prevalent in the states of New York, New Jersey, Pennsylvania, and Connecticut and was also isolated in Tokyo (Oliveira, Tomasz and de Lencastre, 2002). Strain ST5-MRSA-IV was first isolated in a paediatric hospital in Portugal, but was later found also in Poland, USA, Argentina, and Colombia (Oliveira, Tomasz and de Lencastre, 2002).

In the UK, ST36-MRSA-II, designated EMRSA-16, has been particularly prevalent and it emerged following SCC*mec* transfer into a SLV of the prevalent ST30-MSSA (Enright *et al.*, 2002). EMRSA-16 belongs to CC30, which also includes ST30-MRSA-IV that has arisen independently of ST36-MRSA-II (Enright *et al.*, 2002). Currently the most dominant clone in the UK is ST22-MRSA-IV, which belongs to the CC22 and is recognised as the EMRSA-15 (Enright *et al.*, 2002).

A MRSA clone has also emerged from within the CC45 and has been typed as ST45-MRSA-IV (Enright *et al.*, 2002). It became prevalent in Germany and has been recognised as the Berlin clone (Enright *et al.*, 2002; Witte, Guido and Cuny, 2001).

The CC5 and CC8 have demonstrated the highest level of MRSA diversification (Robinson and Enright, 2003). In contrast the CC22, CC30 and CC45 are represented by a lower variety of MRSA clones isolated mostly from within Europe (Robinson and Enright, 2003). Figure 1.2 shows the geogrpahic distribution of MRSA lineages CC5, CC8, CC22 and CC30 across Europe.

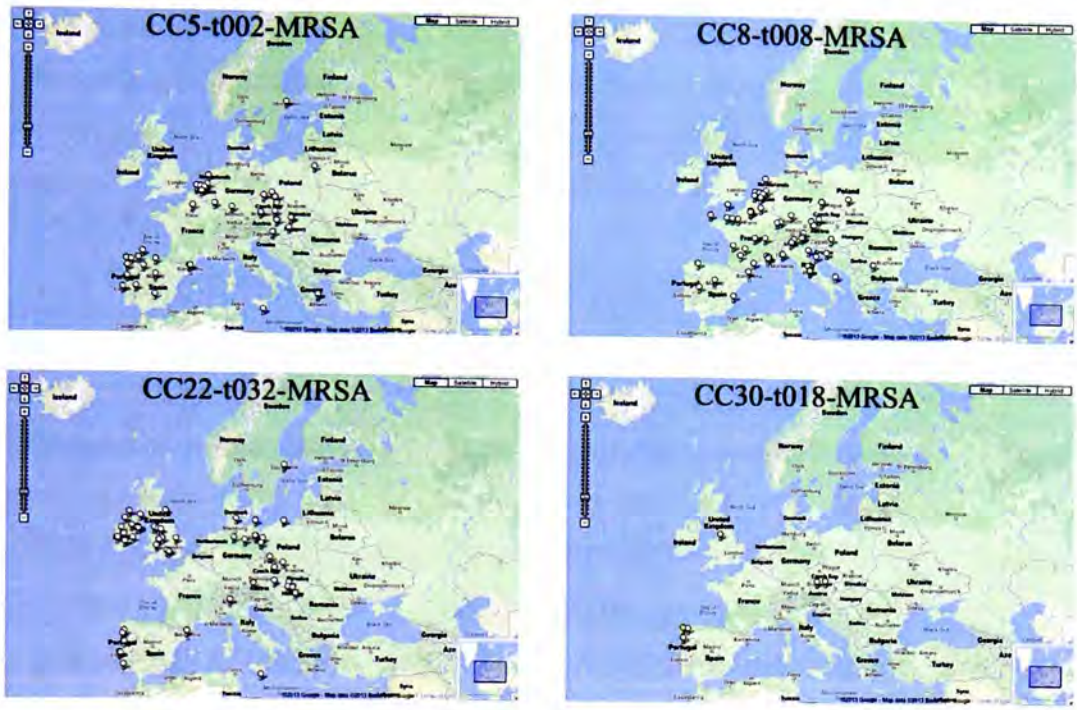


Figure 1.2 Geographic distribution of four prevalent lineages of MRSA across Europe. Pins show the locations of reference laboratories that reported isolation of MRSA belonging to CC5-t002, CC8-t008, CC22-t032 and CC30-t018 from invasive infections. Isolates were collected between Septemeber 2006 and April 2007. The map images were generated using Staphylococcal Reference Laboratories Maps tool (http://www.spatialepidemiology.net/SRL-Maps/).

1.4.4.2 CA-MRSA

The first reported isolation of CA-MRSA occurred in 1993 in Western Australia (Udo, Pearman and Grubb, 1993). CA-MRSA has now been reported worldwide but it is still comparatively low in prevalence, although it has been reported to replace the HA-MRSA within the healthcare setting in certain countries (Deurenberg and Stobberingh, 2008). The CA-MRSA clones have evolved independently from the HA-MRSA and have demonstrated a considerably lower diversification (Lindsay, 2010; Deurenberg and Stobberingh, 2008). The prevalent clones, presented in Table 1.3, have been mostly associated with SCC*mec* type IV (Deurenberg and Stobberingh, 2008). Similarly to HA-MRSA, distinct clones have become dominant within specific geographic regions. In the USA two predominant clones emerged, ST1-MRSA-IV: the USA400 and ST8-MRSA-IV: the USA300 (Lindsay, 2010; Deurenberg and Stobberingh, 2008). The European clone is

represented by ST80-MRSA-IV, whereas the Southwest Pacific by ST30-MRSA-IV (Deurenberg and Stobberingh, 2008). In Asia the CA-MRSA clone is ST59-MRSA-IV, designated USA1000 (Lindsay, 2010; Deurenberg and Stobberingh, 2008).

1.4.4.3 LA-MRSA

The first reported isolation of MRSA from animals occurred in 1972 when it was identified in milk from mastitic cows (Devriese, Vandamme and Fameree, 1972). During the three decades that followed, the incidence of MRSA infections in animals has been low with a considerable increase in the prevalence, including assymptomatic carriage, observed over the last ten years (Leonard and Markey, 2008). This has also included reports of MRSA infection and carriage in companion animals (Tomlin *et al.*, 1999; Loeffler *et al.*, 2005). It has been commonly observed that pets might act as a potential MRSA reservoir and a source of infections in humans, but the isolates predominantly represent the dominant HA-MRSA clones suggesting a human-to-animal transmission as the fundamental route (Loeffler *et al.*, 2005; Strommenger *et al.*, 2006). MRSA infections have also been reported in horses, with MRSA CC8 as a common causative agent (Cuny *et al.*, 2006).

The reports of MRSA isolation from food producing animals were still sporadic and mostly related to cases of bovine mastitis (Lee, 2003). However, the epidemiology of MRSA in livestock has changed considerably since the identification in 2005, an unusually high frequency of MRSA colonization amongst pigs on a Dutch farm as well as in pig farmers (Voss *et al.*, 2005). The pig-associated strain has been later identified as a novel MRSA ST398 lineage, which has now become recognised as the main representative of the LA-MRSA group.

1.4.4.3.1 MRSA ST398

1.4.4.3.1.1 Identification of MRSA ST398 in pigs

Amongst the studies often cited as the first to report the identification of ST398 association with pig farming, is the French investigation in 2004 that found a high prevalence of *S. aureus* amongst pig farmers (Armand-Lefevre, Ruimy and Andremont, 2005). Only six out of 44 isolates collected belonged to ST398 and while 14 *S. aureus* strains from swine infections were also investigated only four were assigned to ST398 (Armand-Lefevre, Ruimy and Andremont, 2005). Furthermore, amongst the total of 10 isolates belonging to ST398, only one human isolate was

identified as MRSA (Armand-Lefevre, Ruimy and Andremont, 2005). However, this was soon followed by a Dutch report of MRSA ST398 carriage amongst pigs and persons having direct contact with pigs, which included five out of 26 screened regional pig farmers (Voss *et al.*, 2005). The report identified an association between the pig farming and a high prevalence of MRSA amongst pig caretakers. The dissemination of MRSA ST398 in pig farms and pig populations in the Netherlands was later confirmed and the lineage was then recognized as a pig-MRSA of potentially international significance (Huijsdens *et al.*, 2006; de Neeling *et al.*, 2007). MRSA ST398 was subsequently identified in pigs in other European countries such as Denmark, Germany and Belgium (Guardabassi, Stegger and Skov, 2007; Harlizius *et al.*, 2008; Schwarz, Kadlec and Strommenger, 2008; Denis *et al.*, 2009).

These findings have prompted the European Commission to conduct a survey on the prevalence of MRSA in breeding pigs and production holdings amongst the member states as well as in Norway and Switzerland (European Food Safety Authority, 2009). The data was presented as a % of MRSA positive farms and the highest proportion of MRSA ST398 was detected in Spain (46%), Germany (43.5%) and Belgium (40%), with significantly lower prevalence in the Netherlands (12.8%). In all of these countries the isolated MRSA panel consisted of ST398 lineage only, which was also the case for Portugal (14.7%). In contrast, while a high level of MRSA positive holdings was found in Italy (34.9%), only a proportion contained ST398 (14%). Countries where MRSA was not detected on pig farms included: Bulgaria, Estonia, Ireland, Lithuania, Sweden, UK and Switzerland. However, the detection of MRSA ST398 in pigs in Switzerland has since been reported (Huber *et al.*, 2010). Furthermore, MRSA ST398 isolation from pigs has not been limited to Europe and was also reported for Canada, the USA and Korea (Khanna *et al.*, 2008; Smith *et al.*, 2009; Lim *et al.*, 2012). The occurrence of MRSA ST398 amongst pigs has been primarily associated with asymptomatic carriage although infections have also been reported, such as a case of exudative epidermitis (van Duijkeren *et al.*, 2007).

1.4.4.3.1.2 MRSA ST398 in other animal species

The initial studies on MRSA ST398 colonisation amongst swine were soon followed by reports of its isolation from other food-producing animals. It has been associated with bovine mastitis in Germany, Belgium, Switzerland and the

Netherlands (Fessler *et al.*, 2010; Vanderhaeghen *et al.*, 2010; Huber *et al.*, 2010; Tavakol *et al.*, 2012). MRSA ST398 was also identified in nasal swabs from calves in Switzerland and cows in Belgium (Huber *et al.*, 2010; Verhegghe *et al.*, 2012). Very recently it was isolated from bulk tank milk in the UK, which indicates its presence in dairy cattle and is the first report of MRSA ST398 derived from food-producing animals in this country (Paterson *et al.*, 2012). MRSA ST398 was isolated from a sheep in Denmark, but due to low carriage frequency authors proposed that the acquisition might have occurred through cross contamination through contact with pigs (Eriksson *et al.*, 2012).

Nasal carriage of MRSA ST398 was identified amongst Belgian, French and Dutch horses (Van den Eede *et al.*, 2009; van Duijkeren *et al.*, 2010). It was associated with an outbreak of post-surgical infections in horses at a Dutch veterinary teaching hospital and a horse infection in Austria (Witte *et al.*, 2007; van Duijkeren *et al.*, 2010). MRSA ST398 was also implicated in a number of infections in equine patients in a Belgian horse clinic (Hermans *et al.*, 2008). In contrast, a recent screening for the prevalence of MRSA nasal carriage amongst horses on equine farms in Belgium revealed a very low colonization frequency indicating that horses are an unlikely reservoir of MRSA ST398 (Van den Eede *et al.*, 2012).

Asymptomatic MRSA ST398 carriage in poultry has been reported in Belgium (Nemati *et al.*, 2008; Persoons *et al.*, 2009). There was also a report of a dog infection in Germany (Witte *et al.*, 2007). MRSA ST398 has been also isolated from retail meat products such as pork, chicken, rabbit and veal from Spain, pork from the USA and the Netherlands, pork and beef from Denmark and in broiler meat from Germany (Lozano *et al.*, 2009; Agersø *et al.*, 2011; O'Brien *et al.*, 2012).

1.4.4.3.1.3 MRSA ST398 in humans

The association of MRSA ST398 with pig farming was first made through investigation of MRSA carriage or infection in persons in close contact with the animals. The Dutch report by Voss *et al* (2005) came across MRSA colonization in a 6-month-old child in a family that lived on a farm and raised pigs, with MRSA subsequently isolated from the child's parents. The study by Huijsdens *et al* (2006). investigated a case of mastitis in a young mother and persistent MRSA carriage by her family, where the father was a pig-farmer.

In studies investigating occurrence of MRSA on a pig farms, the molecular

typing of isolates would commonly reveal that the MRSA ST398 strains isolated from farmers and their animals shared the same genotypic features such as *spa* type, SCC*mec* type or PFGE fingerprint (Voss *et al.*, 2005; Huijsdens *et al.*, 2006; Khanna *et al.*, 2008; Denis *et al.*, 2009). It was thus evident that MRSA cross-transfers between humans and animals (Huijsdens *et al.*, 2006). This was further confirmed by a study reporting a significant association between the presence of MRSA in pigs and colonization in humans, with no detection of MRSA in humans on farms with all pigs MRSA-negative (Khanna *et al.*, 2008). Carriage of MRSA ST398 by persons in contact with animals has been also implicated in its further transmission within farms (Pletinckx *et al.*, 2013). In a veterinary clinic setting the transmission of MRSA ST398 by the personnel has been associated with nosocomial infections in equine patients (van Duijkeren *et al.*, 2010).

The prevalence of MRSA ST398 human colonisation has been investigated to determine the potential for a long-term carriage and dissemination within population. It was reported that MRSA ST398 transfers at low frequency between persons that are colonized due to pig exposure and their non-exposed family members (Cuny *et al.*, 2009). Furthermore, MRSA ST398 colonisation amongst farmers was shown to be directly associated with the duration of exposure to animals and a decrease in prevalence in humans was observed when no animal contact occurred (Graveland *et al.*, 2011). It was thus concluded that MRSA ST398 demonstrated a low capacity for persistent human colonisation (Graveland *et al.*, 2011). This was confirmed by a recent study, which demonstrated that human carriage of MRSA ST398 following a short-term exposure to animals is transient and should be recognised as nasal contamination rather than colonisation (Frana *et al.*, 2013).

MRSA ST398 was also reported to be less transmissible within Dutch hospitals in comparison with non-ST398 MRSA (Bootsma *et al.*, 2011). In Belgium, analysis of MRSA carriage frequency in persons upon admission to hospital revealed that the prevalence of MRSA ST398 accounted for less than 2% of MRSA isolates (Vandendriessche *et al.*, 2012). In contrast, a region in the Netherlands with high density of pig farms demonstrated a 925% increase in the number of identified MRSA carriers, which was largely attributed to MRSA ST398 (Wulf *et al.*, 2012).

MRSA ST398 has been considered to demonstrate a low pathogenic potential and constitute a low risk to humans as an infectious agent. Analysis of MRSA ST398 prevalence amongst human clinical isolates in several European countries revealed

that overall the lineage represented only a minor proportion of MRSA isolates from humans (van Cleef *et al.*, 2011). The highest rates of isolation were observed for the Netherlands, Belgium, Denmark, and Austria (van Cleef *et al.*, 2011). MRSA ST398 has been mostly associated with skin and wound infections (van Cleef *et al.*, 2011). Respiratory tract infections have also occurred and the reported case studies include ventilator-associated nosocomial pneumonia, empyema and severe pneumonia in a newborn (Witte *et al.*, 2007; Lozano *et al.*, 2011b; Hartmeyer *et al.*, 2010). MRSA ST398 has been infrequently isolated from blood, which reveals lower association with severe disease in comparison with other MRSA lineages (van Cleef *et al.*, 2011). Most of the reported cases of human infection were found to be associated with direct livestock contact (Declercq *et al.*, 2008; Lozano *et al.*, 2011b; Mammina *et al.*, 2010; Lozano *et al.*, 2011a; Hartmeyer *et al.*, 2010). MRSA ST398 has been also associated with several outbreaks in the Netherlands that occurred in a hospital setting, residential and nursing care facilities (Wulf *et al.*, 2008; Fanoy *et al.*, 2009; Verkade *et al.*, 2012). Whilst demonstrating that MRSA ST398 can transmit by human-to-human route, in one instance the outbreak could not be related to livestock contact for any of the patients or the personnel (Fanoy *et al.*, 2009). There was also a report of MRSA ST398 isolation from three human patients in Scotland, where no connection with pigs or pig farming was established and no link between the individual persons was found (http://www.documents.hps.scot.nhs.uk/ewr/pdf2008/0823.pdf)

1.4.4.3.1.4 Methicillin-susceptible isolates of *S. aureus* ST398

In contrast to the epidemiology of MRSA ST398, the methicillin-susceptible isolates belonging to this lineage have demonstrated a considerably higher potential for human transmission as well as pathogenesis in the human host. Despite some studies reporting identification of MSSA ST398 in pigs, general prevalence in swine herds has not been widely investigated since the primary focus has been concentrated on MRSA carriage (Armand-Lefevre, Ruimy and Andremont, 2005; Guardabassi, Stegger and Skov, 2007; Hasman *et al.*, 2010a). However, it has now been reported by a number of studies describing carriage and infections in humans. MSSA ST398 was isolated from cases of bacteraemia in the Netherlands, whereas in France it was associated with a case of lethal necrotizing pneumonia (van Belkum *et al.*, 2008; Rasigade *et al.*, 2010). Also in France it was identified as a causative agent of several cases of bloodstream infections, as well as a newly emerged lineage associated with

infective endocarditis, with proposals that the identified MSSA ST398 isolates might represent a novel human-associated subclone (Valentin-Domelier *et al.*, 2011; Tristan *et al.*, 2012). It was also identified amongst human nasal carriage isolates in Spain (Lozano *et al.*, 2011c). MSSA ST398 was also described outside Europe and in Beijing it was identified as a predominant lineage amongst CA-MSSA isolates causing skin and soft tissue infections (Zhao *et al.*, 2012). MSSA ST398 has been prevalent within a community in New York and it was later associated with skin and soft tissue, as well as bloodstream infections (Mediavilla *et al.*, 2012; Bhat *et al.*, 2009). MSSA ST398 infections in humans have not been associated with exposure to livestock and the lineage has been defined as an animal-independent ST398 MSSA (Valentin-Domelier *et al.*, 2011; Zhao *et al.*, 2012; Uhlemann *et al.*, 2012).

1.4.4.3.2 Other LA-MRSA lineages

In recent years other MRSA lineages that are distinct from the prevalent human-associated clonal complexes have been isolated from food-producing animals. MRSA CC9 has been showing high prevalence in pigs and it has occurred as a dominant pig-associated MRSA in some Asian countries such as China, Malaysia and Taiwan (Cui *et al.*, 2009; Neela *et al.*, 2009; Lo *et al.*, 2012). An analysis of MRSA nasal colonisation amongst pork butchers in Hong Kong also found that majority of isolates to belonged to CC9 (Boost *et al.*, 2012). However, also detected was CC97, recently identified in slaughter pigs in Spain (Boost *et al.*, 2012; Gomez-Sanz *et al.*, 2010). MRSA CC97 was also detected amongst nasal carriage isolates derived from pigs in Italy (Battisti *et al.*, 2010). In Europe of particular significance has also been the CC130, recently associated with a novel variant of the *mecA* gene designated mec*C* (Garcia-Alvarez *et al.*, 2011). The MRSA CC130 was found amongst cattle and human isolates in the UK (Garcia-Alvarez *et al.*, 2011). The lineage was also identified in human clinical isolates in Denmark and Germany (Garcia-Alvarez *et al.*, 2011; Cuny *et al.*, 2011).

1.5 Hypothesis and aims

S. aureus is an evolving organism which demonstrates a considerable capacity for niche exploitation. The bacterium has demonstrated ability for host as well as environmental adaptation. While the former can be mediated by acquisition of particular virulence factors that mediate host specificity, the latter is more evident

amongst MRSA isolates and thus can be associated with carriage of antimicrobial resistance determinants. These features are reflected in the *S. aureus* population structure, with distinct lineages demonstrating a particular niche preference. As such, the *S. aureus* clonal complexes can be generally separated into either human or animal-associated, with the latter predominantly represented by cattle-related isolates. The lineages prevalent within the human population can be further subdivided into clonal complexes that are prevalent either amongst both MSSA and MRSA isolates or only within one of these groups. Furthermore, the human MRSA population consists largely of either healthcare- or community-associated lineages.

In recent years MRSA lineages have begun to emerge within animal hosts. Thus far, this has been most prominently demonstrated by the MRSA ST398 strain, which has become prevalent amongst livestock, in particular porcine populations. Whilst a number of reports have focused on describing the prevalence of MRSA ST398 on farms, in animals and in humans in contact with livestock, another important subject of research is to determine what mechanisms of biological adaptations have facilitated the success of MRSA ST398 as a porcine coloniser. Based on the apparent animal-association it might be speculated that MRSA ST398 isolates have become host-specific through acquisition of virulence factors that promote colonisation and stable carriage. However, the methicillin-resistance status of ST398 isolates might also suggest non-host related adaptations as in the case of human MRSA, which have demonstrated adaptation to the hospital environment.

The aim of this work is to characterise the genotypic and phenotypic features of MRSA ST398 in order to identify potential mediators of host specificity or other non-host associated adaptations. The host-specificity will be investigated through analysis of virulence-associated features, whereas the non-host related adaptations will be tested through evaluation of antimicrobial resistance followed by biological fitness analysis. The work will be conducted predominantly through a comparative study involving a panel of isolates belonging to MRSA ST398 and other prevalent *S. aureus* lineages, isolated from humans and animals. If identified, MRSA ST398-unique features might reveal elements that facilitate the lineage's porcine colonisation or livestock-association. In contrast, significant similarities between MRSA ST398 and other prevalent lineages of *S. aureus* might reveal elements that generally promote *S. aureus* carriage. Furthermore, the analysis will further expand the current knowledge of *S. aureus* inter-lineage diversity and variation.

In summary, the hypothesis addressed in this work is that MRSA ST398 has become livestock-associated due to specific host or other non-host associated adaptations and the objectives are:

A. To genotype a collection of *S. aureus* isolates in order to select a panel for the comparative analysis (covered in Chapter 3)
B. To analyse the virulence-associated features: carriage of virulence genes, adhesion to host cells and biofilm formation (covered in Chapter 4)
C. To analyse the antimicrobial and biocide susceptibility of isolates as well as the carriage of resistance determinants (covered in Chapters 5 and 6)
D. To determine the relative fitness of MRSA ST398 isolates in comparison with isolates of other lineages (covered in Chapter 7)

Chapter 2 Materials and methods

2.1 Reagents and chemicals

Reagents and chemicals were obtained from Sigma-Aldrich unless stated otherwise.

2.2 Bacterial isolates, storage and growth conditions

The *Staphylococcus aureus* culture collection was stored at -80° C in CryoBank™ tubes (Copan Diagnostics Inc). To generate an overnight culture, isolates were streaked on Columbia agar with 5% (v/v) sheep blood (CBA) or inoculated in tryptic soy broth (TSB) and incubated for 14-16 h at 37° C, stationery or with shaking, respectively.

Escherichia coli reference strains were stored at -80° C in cryovial tubes containing heart infusion broth with 30% (v/v) glycerol. For an overnight culture the strains were streaked on Luria-Bertani agar or inoculated in Luria-Bertani broth and incubated for 14-16 h at 37° C, stationery or with shaking, respectively.

All culture media were prepared and supplied by the Animal Health and Veterinary Laboratories Agency Biological Products Unit.

2.2.1 Human and animal isolates

A total of 134 *S. aureus* isolates of human and animal origin were characterised. The MRSA ST398 isolates (n=22) were kindly provided by Dr. Patrick Butaye from Veterinary & Agrochemical Research Centre, Brussels, Belgium. The isolates were collected in Belgium from various host species: human (pig farmers, n=4), pig (surveillance, n=5), cattle (mastitis, n=3), horse (infections various, n=3) chicken (surveillance, n=4) and rat (surveillance, n=3) between 2008 and 2010. The UK human clinical isolates (n=82) were collected in the London area from Kingston Hospital (n=12) and provided by Dr. Mike Smith, from The Royal Brompton Hospital (n=20) and provided by Mrs Maureen Chadwick, and from The Royal Marsden Hospital (n=50) and provided by Ms Jackie Kenny, between 2007 and 2009. The cattle clinical isolates (n=28) were isolated from cases of mastitis in the UK between April 2006 and September 2007. The equine clinical isolates (n=2) were collected in Northern Ireland in 2010.

A list of all isolates is presented in appendix I. A summary of all genotypic and phenotypic features of the selected strains is shown in appendix II.

2.2.2 Transformation and reference strains

Transformation experiments were conducted using the following recipient strains: *S. aureus* RN4220, *E. coli* TOP10F, *E. coli* NEB10β.*S. aureus* ATCC 29213 was used as a control strain when conducting antimicrobial minimum inhibitory concentration (MIC) testing.

MRSA NCTC 12493 and Oxford *S. aureus* NCTC 06571 were used as controls in *nuc/mecA* PCR assay.

2.3 General molecular biology methods

2.3.1 Isolation of genomic DNA

Genomic DNA was isolated using a commercial kit QIAamp (Qiagen) following the manufacturer's instructions. Briefly, isolate was inoculated on a CBA and grown overnight. Bacterial culture was inoculated into 180 µl of Gram-positive lysis buffer (20mg/ml lysozyme, 20 mM Tris·HCl pH 8.0, 2 mM EDTA, 1.2% (v/v) Triton) and incubated for 1 h at 37° C with shaking. Following the incubation, 20 µl of proteinase K solution and 200 µl of lysis buffer (AL) were added to bacterial suspension, and the tube was placed at 56° C for 1 h. The suspension was then mixed with 200 µl of ethanol and the genomic DNA was purified using Qiagen spin column in accordance with the instructions.

2.3.2 Isolation of plasmid DNA

Plasmid DNA was isolated using a commercial kit QIAprep (Qiagen) following the manufacturer's instructions with minor modifications. Isolates were inoculated in 5 ml culture broth and incubated with shaking at 37° C overnight. The cells were collected by centrifugation at 4000 rpm for 5 minutes. They were washed with 1 ml of 0.1 M phosphate buffered saline (PBS), transferred into 1.5 ml tube and collected by centrifugation at 13000 rpm for 5 minutes and the supernatant was removed. When isolating plasmid DNA from *E. coli* the manufacturer's protocol was then followed beginning with step 1: addition of 250 µl of buffer P1. When isolating plasmid DNA from *S. aureus* the cells were re-suspended in 250 µl of P1 buffer containing 10 mg/ml lysozyme and 50 µg/ml lysostaphin, then incubated for 1 h at 37° C. Further preparation was conducted as described by the manufacturer's protocol beginning with step 2: addition of 250 µl of P2 buffer.

2.3.3 Polymerase chain reaction

Polymerase chain reactions (PCR) were run using HotStar Taq DNA polymerase (Qiagen) in a 25 µl reaction volume, which contained: 1X PCR buffer, 3 mM $MgCl_2$, 200 µM of each dNTP, 0.1 mM primer, 0.5 unit of HotStar Taq DNA polymerase and 1 µl of DNA template. Unless described otherwise the following thermal cycling conditions were used: initial activation step for 15 minuets at 95° C, then 25 cycles of 1 minute denaturtion at 94° C, 1 minute annealing at 55° C and 1 minute extension at 72° C, with the final extension for 10 minute at 72° C. For each PCR experiment a negative control reaction containing no DNA template was prepared and run in concomitance with the test samples. For positive control, a recommended reference strain was used or a strain characterised in this work (confirmed to carry the gene of interest based on a preliminary PCR assay).

Amplification of large fragments (> 10 kbp) was performed with a Long Range PCR kit (Qiagen) in accordance with the manufacturer instructions. Briefly, the reaction was prepared in a 25 µl volume, which contained: 1X Long Range PCR buffer, 500 µM of each dNTP, 0.4 mM of each primer, 1 unit of Long Range PCR Enzyme Mix and 1 µl of DNA template. The following thermal cycling conditions were used: 3 minute initial denaturation at 93° C, then 25 cycles of 15 seconds denaturation at 93° C, 30 seconds annealing at 65° C and 15 minutes extension at 68° C, with the final extension for 15 minutes at 68° C.

All reactions were performed in GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems).

2.3.4 Oligonuclotides

DNA Oligonucleotides were synthesised by Sigma® Life Science. Upon receipt the oligonulcotides were re-suspended in molecular grade water for a final concentration of 100 mM and the stock solutions were stored at -20° C. Oligonucleotides are listed in Table 2.1.

Table 2.1 Oligonucleotides

Assay	Target gene	Name	Sequence 5'-3'	Size (bp)	Reference
spa typing	*spa*	spa-1113f	TAA AGA CGA TCC TTC GGT GAG C	NA	http://www.ridom.de/
		spa-1514r	CAG CAG TAG TGC CGT TTG CTT		
dru typing	*dru*	dru_F	GTTAGCATATTACCTCTCCTTGC	NA	http://dru-typing.org/site/
		dru_R	GCCGATTGTGCTTGATGAG		
MLST typing	*arcC*	arcC-Up	TTG ATT CAC CAG CGC GTA TTG TC	456	Enright *et al.*, 2000
		arcC-Dn	AGG TAT CTG CTT CAA TCA GCG		
	aroE	aroE-Up	ATC GGA AAT CCT ATT TCA CAT TC	456	
		aroE-Dn	GGT GTT GTA TTA ATA ACG ATA TC		
	glpF	glpF-Up	CTA GGA ACT GCA ATC TTA ATC C	465	
		glpF-Dn	TGG TAA AAT CGC ATG TCC AAT TC		
	gmk	gmk-Up	ATC GTT TTA TCG GGA CCA TC	429	
		gmk-Dn	TCA TTA ACT ACA ACG TAA TCG TA		
	pta	pta-Up	GTT AAA ATC GTA TTA CCT GAA GG	474	
		pta-Dn	GAC CCT TTT GTT GAA AAG CTT AA		
	tpi	tpi-Up	TCG TTC ATT CTG AAC GTC GTG AA	402	
		tpi-Dn	TTT GCA CCT TCT AAC AAT TGT AC		
	yqiL	yqiL-Up	CAG CAT ACA GGA CAC CTA TTG GC	516	
		yqiL-Dn	CGT TGA GGA ATC GAT ACT GGA AC		
SCC*mec* typing	*ccrA2-B*	b	ATTGCCTTGATAATAGCCYTCT	937	Boye *et al.*, 2007
		a3	TAAAGGCATCAATGCACAAACACT		
	ccrC	ccrCF	CGTCTATTACAAGATGTTAAGGATAAT	518	
		ccrCR	CCTTTATAGACTGGATTATTCAAAATAT		

Table 2.1 (continued) Oligonucleotides

Assay	Target gene	Name	Sequence 5'-3'	Size (bp)	Reference
SCC*mec* typing	IS*1272*	1272F1	GCCACTCATAACATATGGAA	415	Boye *et al.*, 2007
		1272R1	CATCCGAGTGAAACCCAAA		
	mecA-IS*431*	5RmecA	TATACCAAACCCGACAACTAC	359	
		5R431	CGGCTACAGTGATAACATCC		
	ccrB2	ccrB2-F	CGAACGTAATAACATTGTCG	203	Milheirico, Oliveira and de Lencastre, 2007
		ccrB2-R	TTGGCWATTTTACGATAGCC		
	SCC*mec* IVa	IVa-F	ATAAGAGATCGAACAGAAGC	278	
		IVa-R	TGAAGAAATCATGCCTATCG		
	SCC*mec* IVb and IVF	IVb-F	TTGCTCATTTCAGTCTTACC	336	
		IVb-R	TTACTTCAGCTGCATTAAGC		
	SCC*mec* IVc and IVE	IVc-F	CCATTGCAAATTTCTCTTCC	483	
		IVc-R	ATAGATTCTACTGCAAGTCC		
	SCC*mec* IVd	IVd-F	TCTCGACTGTTTGCAATAGG	575	
		IVd-R	CAATCATCTAGTTGGATACG		
	SCC*mec* IVg	IVg-F	TGATAGTCAAAGTATGGTGG	792	
		IVg-R	GAATAATGCAAAGTGGAACG		
	SCC*mec* IVh	IVh-F	TTCCTCGTTTTTTCTGAACG	663	
		IVh-R	CAAACACTGATATTGTGTCG		
	ccrC2	ccrC2-F	ATAAGTTAAAAGCACGACTCA	257	Higuchi *et al.*, 2008
		ccrC2-R	TTCAATCCTATTTTTCTTTGTG		
	ccrC8	ccrC8-F	GCATGGGTACTCAATCCA	562	
		ccrC8-R	GGTTGTAATGGCTTTGAGG		
	mecC2	mecC2-F	ATCAGTTCATTGCTCACGATATGTGTA	344	
		mecC2-R	CAATACGCCATTTGTAATAAGCTTTT		

Table 2.1 (continued) Oligonucleotides

Assay	Target gene	Name	Sequence 5'-3'	Size (bp)	Reference
nuc/mecA	*nuc*	nuc-1	TCAGCAAA TGCA TCACAAACAG	255	http://www.crl-ar.eu/
		nuc-2	CGTAAATGCACTTGCTTCAGG		
	mecA	mecA-1	GGGATCATAGCGTCATTATTC	527	
		mecA-2	AACGATTGTGACACGATAGCC		
	16S	16S-1	GTGCCAGCAGCCGCGGTAA	886	
		16S-2	AGACCCGGGAACGTATTCAC		
Adhesin genes	*bbp*	BBP-1	AACTACATCTAGTACTCAACAACAG	575	Tristan *et al.*, 2003
		BBP-2	ATGTGCTTGAATAACACCATCATCT		
	clfA	CLFA-1	ATTGGCGTGGCTTCAGTGCT	292	
		CLFA-2	CGTTTCTTCCGTAGTTGCATTTG		
	clfB	CLFB-1	ACATCAGTAATAGTAGGGGGCAAC	205	
		CLFB-2	TTCGCACTGTTTGTGTTTGCAC		
	cna	CNA-1	GTCAAGCAGTTATTAACACCAGAC	423	
		CNA-2	AATCAGTAATTGCACTTTGTCCACTG		
	eno	ENO-1	ACGTGCAGCAGCTGACT	302	
		ENO-2	CAACAGCATYCTTCAGTACCTTC		
	fib	FIB-1	CTACAACTACAATTGCCGTCAACAG	404	
		FIB-2	GCTCTTGTAAGACCATTTTCTTCAC		
	fnbB	FNBB-1	GTAACAGCTAATGGTCGAATTGATACT	524	
		FNBB-2	CAAGTTCGATAGGAGTACTATGTTC		
	fnbA	fnbA_F	CATAAATTGGGAGCAGCATCA	127	Vancraeynest, Hermans and Haesebrouck, 2004
		fnbA_R	ATCAGCAGCTGAATTCCCATT		
	ebpS	EBP-1	CATCCAGAACCAATCGAAGAC	652	
		EBP-2	CTTAACAGTTACATCATCATGTTTATCTTTG		

Table 2.1 (continued) Oligonucleotides

Assay	Target gene	Name	Sequence 5'-3'	Size (bp)	Reference
Adhesin genes	*icaA*	AF	CCTAACTAACGAAAGGTAG	1315	Ciftcil *et al.*, 2009
		AR	AAGATATAGCGATAAGTGC		
	icaD	DF	AAACGTAAGAGAGGTGG	381	
		DR	GGCAATATGATCAAGATAC		
	sasG	sasG_F	CGCGGATTCGCAGCTGAAAACAATATT	1110	Rohde *et al.*, 2007
		sasG_R	CCCAAGCTTTAATTCTGTTATTGTTTTTGG		
	sdrC	sdrC_F	ACGACTATTAAACCAAGAAC	560	Peacock *et al.*, 2002
		sdrC_R	GTACTTGAAATAAGCGGTTG		
	sdrD	sdrD_F	GGAAATAAAGTTGAAGTTTC	500	
		sdrD_R	ACTTTGTCATCAACTGTAAT		
	sdrE	sdrE_F	CAGTAAATGTGTCAAAAGA	767	
		sdrE_R	TTGACTACCAGCTATATC		
Antimicrobial resistance genes	*aadD*	aadD-1	GCAAGGACCGACAACATTTC	165	van Asselt *et al.*, 1992
		aadD-2	TGGCACAGATGGTCATAACC		
	dfrG	dfrG-F	TGCTGCGATGGATAAGAA	405	
		dfrG-R	TGGGCAAATACCTCATTCC		
	dfrK	dfrK_F	GCTGCGATGGATAATGAACAG	214	Fessler *et al.*, 201
		dfrK_R	GGACGATTTCACAACCATTAAAGC		
	ermA	ermA_F	TATCTTATCGTTGAGAAGGGATT	139	Argudin *et al.*, 2011
		ermA_R	CTACACTTGGCTTAGGATGAAA		
	ermC	ermC-F	CTTGTTGATCACGATAATTTCC	190	
		ermC-R	ATCTTTTAGCAAACCCGTATTC		
	ermT	ermT_F	ATTGGTTCAGGGAAAGGTCA	536	Fessler *et al.*, 2010
		ermT_R	GCTTGATAAAATTGGTTTTTGGA		

Table 2.1 (continued) Oligonucleotides

Assay	Target gene	Name	Sequence 5'-3'	Size (bp)	Reference
Antimicrobial resistance genes	*mecC*	mecLGA_F	TCACCAGGTTCAACYCAAAA	356	Garcia-Alvarez *et al.*, 2011
		mecLGA_R	CCTGAATCWGCTAATAATATTTC		
	spc	spc_F	ACCAAATCAAGCGATTCAAA	561	Fessler *et al.*, 2010
		spc_R	GTCACTGTTTGCCACATTCG		
	tetK	tetK-F	TCGATAGGAACAGCAGTA	169	Argudin *et al.*, 2011
		tetK-R	CAGCAGATCCTACTCCTT		
	tetL	tetL_F	TCGTTAGCGTGCTGTCATTC	267	Ng *et al.*, 2001
		tetL_R	GTATCCCACCAATGTAGCCG		
	tetM	tetM_F	GTGGACAAAGGTACAACGAG	406	Argudin *et al.*, 2011
		tetM_R	CGGTAAAGTTCGTCACACAC		
	vgaA	vgaA-1	AGTGGTGGTGAAGTAACACG	659	Argudin *et al.*, 2011
		vgaA-2	CTTGTCTCCTCCGCGAATAC		
	vgaE	vgaE-1	GAAATATGGGAAATAGAAGATGG	995	Schwendener and Perreten, 2011
		vgaE-2	TGATTCTCTAACCACTCTTC		
Heavy metal resistance genes	*cadA*	cadA-F	GTTCGATTGTAATTGGCGG	267	Takano *et al.*, 2008
		cadA-R	TTTCCTGACCATTCCGC		
	cadC	cadC-F	GAAGATAAGGTAAACAGGGCT	283	
		cadC-R	CAAGCTGTTTAACATGCTC		
	cadD	cadD-F	ATGAGGTGTATTATGATTCAAACGGT	618	Massidda *et al.*, 2006
		cadD-R	TCCTAAAATTGTTTGAATAGTGTCAT		
	czrC	czrC_F	TAGCCACGATCATAGTCATG	655	Cavaco *et al.*, 2010
		czrC_R	ATCCTTGTTTTCCTTAGTGACTT		

Table 2.1 (continued) Oligonucleotides

Assay	Target gene	Name	Sequence 5'-3'	Size (bp)	Reference
Phage integrase genes	Sa1*int*	Sa1-F	AAGCTAAGTTC GGGCACA	569	Goerke *et al.*, 2009
		Sa1-R	GTAATGTTTGGGAGCCAT		
	Sa2*int*	Sa2-F	TCAAGTAACCCGTCAACTC	640	
		Sa2-R	ATGTCTAAATG TGTGCGTG		
	Sa3*int*	Sa3-F	GAAAAACAAACGGTGCTAT	475	
		Sa3-R	TTATTGACTCTACAGGCTGA		
	Sa4*int*	Sa4-F	ATTGATATTAACGGAACTC	320	
		Sa4-R	TAAACTTATATG CGTGTGT		
	Sa5*int*	Sa5-F	AAAGATGCCAAACTA GCTG	375	
		Sa5-R	CTTGTGGTTTTGTTCTGG		
	Sa6*int*	Sa6-F	GCCATCAATTCAAGGATAG	167	
		Sa6-R	TCTGCAGCTGAGGAC AAT		
	Sa7*int*	Sa7-F	GTCCGGTAGCTAGAGGTC	214	
		Sa7-R	GGCGTATGCTTGACTGTGT		

2.3.5 DNA analysis

2.3.5.1 Gel electrophoresis

Isolated genomic or plasmid DNA and PCR amplification products were analysed by gel electrophoresis using 1X TAE buffer and 1% (w/v) agarose gel. The agarose gel with embedded DNA was stained in 1 μg/ml ethidium bromide solution and the image was captured using GeneGeneius Imaging System (Syngene).

2.3.5.2 DNA purification and quantification

DNA purification was performed either by polyethylene glycol (PEG) precipitation or by using the commercial kit QIAquick (Qiagen) and following manufacturer's instructions.

For PEG precipitation, 60 μl of 20% (w/v) PEG/2.5 M sodium chloride was added to DNA sample and mixed. The sample was centrifuged for 1 minute at 2000 rpm, incubated at room temperature for 30 minutes, and then centrifuged for 45 minutes at 13,000 rpm at 4° C. The supernatant was removed and DNA pellet was washed with 150 μl of 70% (v/v) ethanol, and then centrifuged for 10 minutes at 13,000 rpm. The wash step was performed twice. After second removal of ethanol, the pellet was air dried and re-suspended in 15 μl of molecular grade water.

Quantification of DNA was conducted using a Nanodrop ND-1000 spectrophotometer (Labtech International Ltd, Ringmer, UK).

2.3.5.3 DNA sequencing and data analysis

All DNA sequencing was performed on the ABI 3730 DNA Analyzer (Applied Biosystems) by the Animal Health and Veterinary Laboratories Agency Central Sequencing Unit.

Sequence data generated through primer walking was analysed and assembled using SeqMan (Lasergene 10; DNAStar, USA). The contigs were annotated with Rapid Annotation using Subsystem Technology (http://rast.nmpdr.org). Homology searches were performed with Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple alignments, sequence homology and cluster analysis was conducted with Geneious (Version 5.6.5; Biomatters Ltd, New Zealand) and BioNumerics (Version 6.1; Applied Maths, Belgium)

2.4 Molecular typing methods

2.4.1 SCC*mec* typing

The multiplex PCR reaction for staphylococcal cassette chromosome *mec* (SCC*mec*) typing was performed using primers (Table 2.1), primer concentrations and thermal cycling conditions described by Boye *et al* (2007). Briefly, the primer concentrations ranged from 0.08 mM to 0.25 mM, the amplification was performed over 30 cycles with 55° C annealing temperature and 1 minute extension.

Strains harbouring SCC*mec* type IV were further sub-typed by multiplex PCR using primers (Table 2.1), primer concentrations and thermal cycling conditions described by Milheirico *et al* (2007). Briefly, the primer concentrations ranged from 0.2 mM to 1.8 mM and the amplification was performed over 35 cycles with 48° C annealing temperature and 2 minutes extension.

Strains harbouring SCC*mec* type V were further sub-typed by multiplex PCR using primers (Table 2.1) described by Higuchi *et al.*(2008), and reaction conditions as described in 2.3.3.

2.4.2 *spa* typing

The PCR reaction for *spa* typing was performed using primers (Table 2.1) and thermal cycling conditions described at http://www.ridom.de. Briefly, the primer concentration was 0.1 mM and the amplification reaction was performed over 35 cycles with 60° C annealing temperature and 1.5 minute extension. The product was cleaned up by PEG precipitation and sequenced. The analysis of raw sequence data, assignment of *spa* type and cluster analysis was performed using BioNumerics software (Version 5.10; Applied Maths, Belgium). The *spa* types were clustered by creating minimum spanning tree based on a categorical coefficient. Complexes were created based on a maximum neighbour distance of 1 change and a minimum size of 2 types. The calculations were based on default settings

2.4.3 *dru* typing

The PCR reaction for *dru* typing was performed using primers (Table 2.1) and thermal cycling conditions described at http://dru-typing.org/site. Briefly, the primer concentration was 0.1 mM and the amplification reaction was performed over 30 cycles with 52° C annealing temperature and 1 minute extension time. The product

was cleaned up by PEG precipitation and sequenced. The analysis of raw sequence data and assignment of *dru* types was performed using DruID typing tool available at http://dru-typing.org/site. The *dru* types were clustered using BioNumerics (Version 6.10; Applied Maths, Belgium) by creating UPGMA dendrogram based on Pearson correlation. The calculations were based on default settings

2.4.4 Multi-locus sequence typing

The PCR reaction to determine multi-locus sequence typing (MLST) was performed using primers (Table 2.1) and thermal cycling conditions described by Enright *et al* (2000). Briefly, the primer concentration was 0.2 mM and the amplification reaction was performed over 30 cycles with 55° C annealing temperature and 1 minute extension time. The PCR fragments were cleaned up by PEG precipitation and sequenced. The analysis of raw sequence data, assignment of MLST and cluster analysis was performed using BioNumerics software (Version 5.10; Applied Maths, Belgium). The MLST types were clustered by creating minimum spanning tree based on a categorical coefficient. Complexes were created based on a maximum neighbour distance of 2 changes and a minimum size of 2 types. The calculations were based on default settings. The sequence data of *S. aureus* MLST alleles is available from http://saureus.mlst.net.

2.4.5 Macro-restriction digest pulsed field gel electrophoresis

The macro-restriction digest pulsed-field gel electrophoresis was performed in accordance with the Harmony protocol (Murchan *et al.*, 2003) with minor modifications. Bacterial cells were suspended in sodium chloride/EDTA (SE) buffer (5X stock solution: 75 mM sodium chloride, 25 mM EDTA, pH 7.5,) and the inoculum turbidity was adjusted to 3.6-4.2 MacFarland standard. The inoculum was mixed with an equal volume of 2 % (w/v) low-melting point agarose and poured into plug moulds. The plugs were incubated in Gram positive lysis buffer (0.1 M EDTA disodium salt, 1 M sodium chloride, 1mM magnesium chloride, 0.5% (v/v) sarkosyl, 0.2% (w/v) sodium deoxycholate, 6 mM tris(hydroxymethyl)aminomethane, 0.5% (w/v) Brij 58 (polyethylene glycol hexadecyl ether), pH 7.5) containing 500 μg/ml lysozyme and 1 μg/ml lysostaphin for 6 h at 37° C. The buffer was then replaced with Gram negative lysis buffer (0.5 M EDTA disodium salt, 1% (v/v) sarkosyl, 0.8 M sodium hydroxide, pH 9.5) containing 60 μg/ml proteinase K and the plugs were

incubated overnight at 56° C. The plugs were then washed five times with 1X Tris-EDTA (TE) buffer and incubated overnight with 20U of either *Sma*I (Fermentas) or *Cfr9*I (Fermentas). After the restriction enzyme digest the plugs were loaded onto a 1.2% (w/v) PFGE agarose gel. For normalization Lambda Ladder PFG Marker (NEB) was used. The gel was run for 23 h, the first block switch time was 5 to 15 seconds for 10 h, and the second block switch time was 15 to 60 seconds for 13 h. The gel was stained as described by Harmony protocol. Gel image was taken and analysedwith BioNumerics software (Version 5.10; Applied Maths, Belgium) as a fingerprint type experiment. The PFGE profiles were compared by calculating cluster analysis. The UPGMA dendrogram was created based on Jaccard similarity coefficient and a band matching tolerance of 1.3%. Other calculations were based on default settings

2.4.6 DNA microarray

2.4.6.1 DNA isolation, labelling and hybridisation

DNA microarray analysis was performed using Identibac MRSA Array-Tube (AT, STAU-PM5_5, production year: 2008; Identibac, Surrey, UK). Genomic DNA was isolated using Clondiag StaphyType Kit lysis reagents and Qiagen DNeasy kit, following the StaphyType protocol (Staphytype; Alere Technologies GmbH). In brief, the bacterial culture was suspended in 1.5 ml tube containing 200 µl Lysis Buffer A1 and lyophilized Lysis Enhancer A2, then incubated for 1 h at 37° C. DNeasy protocol was then followed beginning with step 4 of pre-treatment of Gram positive bacteria protocol: addition of 25 µl proteinase K solution and 200 µl of Buffer AL.

Amplification, labelling and array hybridisation of target DNA was performed according to the manufacturer's instructions (Identibac, Surrey, UK). In short, around 1.5 µg of the isolated DNA was amplified using primers provided by the manufacturer and labelled by incorporation of biotin-16-dUTP (Roche Diagnostics). The labelled PCR product was hybridised to the probes, after which the AT was washed successively with 2xSSC 0.01% (v/v) Triton, 2xSSC and 0.2xSSC buffers, followed by treatment with 2% (w/v) blocking solution and addition of poly-HRP streptavidin (Thermo Fisher Scientific). The AT was washed again and the Seramun green peroxidase substrate was added (Seramun). The chip images were captured using ATR03 reader (AlereTechnologies GmbH). Visualisation and analysis of hybridized probes was conducted using IconoClust software package (CLONDIAG).

2.4.6.2 Data analysis

The complete list of genes included on the array is presented by Table 2.2. The analysis of data (probe signal values) was performed in accordance with previously published methods (Monecke *et al.*, 2007b; Monecke, Slickers and Ehricht, 2008). To determine threshold for distinguishing postitve and negative hybridisation probes, the average hybridisation value of all positive control probes was calculated. A probe was considered as postitive if its signal value was above 33% of average positive control signal, negative if below 25% and ambiguous if between 25% and 33%. Certain genes are represented on the DNA microarray chip by more than one allelic variant. In the case of some *set* genes the probes for different allelic variants are cross-reactive leading to multiple positive signals. In such instance, only the allelic variant represented by the probe with the strongest signal was regarded as present, with others considered as absent. For some of the genes in certain isolates the signal was equally strong for multiple allelic variants and the positive allelic variant could not be accurately distinguished. In such case both allelic variants were reported (*set4, set7, setB1*). For *set6* the combination of positive signal probes was analysed to determine the allelic variant (Monecke *et al.*, 2007b). The formatted raw data was further analysed with BioNumerics (Version 5.10; Applied Maths, Belgium) as a character type experiment. The gene carriage profiles were compared by calculating cluster analsysis. The UPGMA dendrogram was created based on Jaccard similarity coefficient. The calculations were based on default settings

Table 2.2 Genes included on the DNA microarray platform

	Gene	Function
Species markers	*femA*	Involved in peptidoglycan synthesis
	katA	Catalase A
	gapA	Glyceraldehyde 3-phosphate dehydrogenase
	coa	Coagulase
	spa	Protein A
	sbi	IgG-binding protein
Virulence genes	*agr*	Accessory regulator gene, detection of types I, II, III and IV
	sarA	Staphylococcal accessory regulator A
	seA	Enterotoxin A, detection of variants A, B and C
	seB	Enterotoxin B
	seC	Enterotoxin C
	seD	Enterotoxin D
	seE	Enterotoxin E
	seG	Enterotoxin G
	seH	Enterotoxin H
	seI	Enterotoxin I
	seJ	Enterotoxin J
	seK	Enterotoxin K
	seL	Enterotoxin L
	seM	Enterotoxin M
	seN	Enterotoxin N
	seO	Enterotoxin O
	seQ	Enterotoxin Q
	seR	Enterotoxin R
	seU	Enterotoxin U
	seX	Enterotoxin X
	seY	Enterotoxin Y
	entCM14	Enterotoxin-like protein
	tst1	Toxic shock syndrome toxin-1
	sak	Staphylokinase
	etA	Exfoliative toxin A
	etB	Exfoliative toxin B
	etD	Exfoliative toxin D
	edinA	Epidermal cell differentiation inhibitor A
	edinB	Epidermal cell differentiation inhibitor B
	edinC	Epidermal cell differentiation inhibitor C
	hl	Putative membrane protein
	hla	Haemolysin alpha
	hlb	Haemolysin beta
	hld	Haemolysin delta
	hlgA	γ-haemolysin component A
	hl-III	Putative membrane protein

Table 2.2 (continued) Genes included on the DNA microarray platform

	Gene	Function
Virulence genes	*lukD*	LukD leukocidin
	lukE	LukE leukocidin
	lukF	γ-haemolysin component
	lukF-PV	PVL, F subunit
	lukF-PV-P83	Bovine bicomponent leukocidin, F subunit
	lukM	Bovine bicomponent leukocidin, S subunit
	lukS	γ-haemolysin component
	lukS-PV	PVL, S subunit
	lukX	Leukocidin/haemolysin toxin family protein
	lukY	Leukocidin/haemolysin toxin family protein, detection of allelic variants 1 and 2
	splA	Serine protease-like exoprotein A
	splB	Serine protease-like exoprotein B
	set1	Staphylococcal exotoxin-like protein, detection of allelic variants 1, 2 and 4
	set2	Staphylococcal exotoxin-like protein, detection of allelic variants 1, 2, 3 and 4
	set3	Staphylococcal exotoxin-like protein, detection of allelic variants 1 and 2
	set4	Staphylococcal exotoxin-like protein, detection of allelic variants 1 and 4
	set5	Staphylococcal exotoxin-like protein, detection of allelic variants 1 and 2
	set6	Staphylococcal exotoxin-like protein, detection of allelic variants 1, 2, 3 and 4
	set7	Staphylococcal exotoxin-like protein, detection of allelic variants 1 and 2
	set8	Staphylococcal exotoxin-like protein, detection of allelic variants 1 and 2
	set9	Staphylococcal exotoxin-like protein, detection of allelic variants 1 and 2
	set12	Staphylococcal exotoxin-like protein
	set21	Staphylococcal exotoxin-like protein
	setB	Staphylococcal exotoxin-like protein, detection of setB1, setB2 and setB3
	setC	Staphylococcal exotoxin-like protein

Table 2.2 (continued) Genes included on the DNA microarray platform

	Gene	Function
Antimicrobial resistance genes	*blaZ*	β-Lactamase
	blaI	β-Lactamase inhibitor
	blaR	β-Lactamase regulatory protein
	mecA	Methicillin resistance
	ermA	Macrolie-lincosamide-streptogramin B resistance
	ermB	Macrolie-lincosamide-streptogramin B resistance
	ermC	Macrolie-lincosamide-streptogramin B resistance
	msrA	Macrolide resistance, efflux pump
	linA	Lincosamide resistance
	vatA	Virginiamycin A acetyltransferase
	vatB	Acetyltransferase inactivating streptogramin A
	vga	ATP binding protein, streptogramin resistance
	vgaA	Putative ABC protein conferring resistance to streptogramin A
	vgb	Virginiamycin B hydrolase
	aacA-aphD	Aminoglycoside resistance, bifunctional enzyme Aac/Aph
	aadD	Aminoglycoside adenyltransferase
	aphA-3	3'5'-aminoglycoside phosphotransferase
	sat	Streptothricine acetyltransferase
	dfrA	Trimethoprim resistance, dihydrofolate reductase type 1
	far1	Fusidic acid resistance
	mupR	Mupirocin resistance
	tetK	Tetracycline resistance
	tetM	Tetracycline resistance
	cat	Chloramphenicol acetyltransferase
	fexA	Chloramphenicol/florfenicol exporter
	cfr	23S rRNA methyltransferase
	vanA	Vancomycin resistance
	vanB	Vancomycin resistance
	vanZ	Teicoplanin resistance

2.4.7 PCR-based detection of genes

2.4.7.1 *nuc/mecA*

The *nuc/mecA* PCR reaction was performed using primers (Table 2.1) and thermal cycling conditions described by EU Reference Laboratory (http://www.crl-ar.eu). Briefly, the primer concentration was 0.125 mM and the amplification reaction was performed over 30 cycles with 55° C annealing temperature and 1 minute extension time. The following *S. aureus* reference strains were used as controls: NCTC 12493 (*mecA* positive) and NCTC 06571 (*mecA* negative).

2.4.7.2 Adhesin genes

Previously described primers (Table 2.1) were used for detection of: *bbp, cna, eno, ebpS, fnbB, fib, clfA, clfB, fnbA, sdrC, sdrD, sdrE, icaA, icaD* and *sasG*. The analysis was conducted by running four multiplex and two simplex PCRs with amplification over 25 cycles. Multiplex PCR 1 screened for *bbp, cna, eno*, and was prepared with a final $MgCl_2$ concentration of 7 mM and run with annealing temperature of 49° C. Multiplex PCR 2 screened for: *fnbB, fib, clfA, clfB* and was prepared and run as PCR 1. Multiplex 3 screened for: *sdrD, sdrE, icaD* and was prepared with a final $MgCl_2$ concentration of 5 mM and run with annealing temperature of 50° C. Multiplex 4 screened for: *sdrC, fnbA, sasG* and was prepared with final $MgCl_2$ concentration of 5 mM and run with annealing temperature of 55° C and extension for 1.5 minute. Simplex PCR was used to screen for *icaA* and *ebpS*, with *icaA* amplified at annealing temperature of 50° C and extension time of 1.5 minute whereas *ebpS* analysed as described in section 2.3.3. The following strains, confirmed to carry the gene of interest by a preliminary PCR assay, were used as controls: 1 (*cna* and *bbp* positive), 6 (*clfA, clfB, eno, ebpS, fib, fnbA, fnbB, icaA, icaD, sasG, sdrC* and *sdrE* positive), 8 (*sdrD* positive).

2.4.7.3 Phage integrase genes

Phage integrase multiplex PCR was preformed as descried by Goerke *et al* (2009). Briefly, the primer concentration was 0.2 mM and the amplification reaction was performed over 35 cycles with 55° C annealing temperature and 45 seconds extension time.

2.4.7.4 Antimicrobial resistance genes

Previously described primers (Table 2.1) were used for detection of: *aadD, dfrG, dfr*K, *ermA*, *ermC, erm*T, *mecC, spc, tetK, tet*L, tetM, *vgaA* and *vgaE*. The analysis was conducted by running simplex PCR for each primer pair as described in section 2.3.3. The following strains confirmed to carry the gene of interest by either a preliminary PCR assay or by DNA array, were used as controls: 90 (*aadD, ermT, tetL* and *tetM* positive), 91 (*ermC* and *tetK* positive), 92 (*dfrK* positive), 95 (*dfrG* positive), 96 (*vgaA* positive), 104 (*ermA*, *spc* and *vgaE* positive) and 173 (*mecC* positive).

2.4.7.5 Heavy metal resistance genes

Previously described primers (Table 2.1) were used for detection of: *cadA*, *cadC*, *cadD* and *czrC*. The analysis was conducted by running simplex PCR for each primer pair as described in section 2.3.3. The following strains confirmed to carry the gene of interest by a preliminary PCR assay, were used as controls: 6 (*cadD* positive), 31 (*cadA* and *cadD* positive), 91 (*czrC* positive).

2.4.8 Sequence analysis of the vSaα genomic island

To amplify the *set* region of the vSaα genomic island, degenerate primers were designed based on sequence homology within genes flanking the region. The forward primer (*set*_F, 5'- CTGGAACTTTCTCAACATTTCTAACACCAATG TG) was designed based on multiple alignment and homology analysis of the following loci: SAPIG0489 (AM990992), SAB0374c (AJ938182), SAHV_0418 (AP009324), NWMN_0387 (AP009351), SAV0421 (BA000017), SAR0421 (BX571856), SAOUHSC_00382 (CP000253), SAUSA300_0394 (CP000255) and SAAV_0364 (CP001781). The reverse primer (*set*_R, 5'- CACTCGCATCCATATTC CCTCTTAAATCATTCGC) was designed based on multiple alignment and homology analysis of the following loci: SAPIG0499 (AM990992), SAB0384 (AJ938182), SAHV_0429 (AP009324), NWMN_0398 (AP009351), SAV0431 (BA000017), SAR0433 (BX571856), SAOUHSC_00397 (CP000253), SAUSA300_0405 (CP000255) and SAAV_0374 (CP001781). The PCR product was sequenced in full by primer walking.

The raw nucleotide sequences were analysed and assembled as described in 2.3.5.3. The formatted sequence data was further analysed with BioNumerics (Version 6.1; Applied Maths). The nucleotide sequence percentage similairty was

determined by UPGMA clustering based on multiple alignment. The calculations were based on default settings except for similarity calculation where 100% gap penalty was applied.

2.5 Plasmid transformation and molecular cloning

2.5.1 Preparation and transformation of electro-competent *S. aureus*

Preparation of electro-competent *S. aureus* and electroporation of plasmid DNA was conducted by following a modified method of Kraemer and Iandolo (1990) as described by McNamara (2008). *S. aureus* RN4220 strain was grown overnight in 10 ml of TSB. From the overnight culture 4 ml was transferred into 200 ml of fresh TSB, which was then incubated at 37° C with vigorous shaking until the culture reached OD_{600nm} of 0.4. The cells were then collected by centrifugation at 5000 rpm for 20 minutes at 4° C. The pellet was re-suspended in an equal volume of ice-cold 0.5 M sucrose and centrifuged. The cells were re-suspended in half volume of ice-cold 0.5 M sucrose and incubated on ice for 30 minutes. The cells were collected by centrifugation and re-suspended in a 0.1 volume of ice-cold 0.5 M sucrose and centrifuged. Finally, the pellet was re-suspended in 300 µl of ice-cold 0.5 M sucrose and 80 µl aliquots were used immediately or stored at -80° C.

For the electroporation of plasmid DNA, circa 0.5 µg of plasmid DNA was added to tube containing 80 µl of electro-competent cells, and the mixture was transferred to a 0.1 cm gap electroporation cuvette. The cuvette was electroporated with a single pulse using the following settings: 100 Ohms resistance, 25mF capacitance and 2.3 kV charging voltage. Immediately after electroporation, 920 µl of SMMP broth [5.5. parts SMM buffer (1 M sucrose, 0.04 M maleic acid, 0.04 M Magnesium Chloride, pH 6.5), 4 parts 7% (w/v) Penassay Broth, 0.5 parts 10% (w/v) Bovine Serum Albumin] was added to the cuvette and the cell suspension was transferred to a 1.5 ml tube. The cells were incubated for 1 h at 37° C with shaking, after which aliquots were plated out onto selective agar medium.

2.5.2 Preparation and transformation of chemically competent *E. coli*

Preparation and transformation of chemically competent *E. coli* was conducted in accordance with previously described method by Sambrook and Russel (2001), with minor modifications. *E. coli* strain (TOP10F or NEB10β, as described in Chapter 6) was grown overnight in 10 ml of LB. From the overnight culture 2 ml was

transferred into 100 ml of fresh LB, which was incubated at 37° C with vigorous shaking until the culture reached OD_{600nm} of 0.4. Equal volumes of culture were transferred to two polypropylene tubes, and the cells were collected by centrifugation at 3900 rpm for 10 minutes at 4° C. The pellet was re-suspended in 30 ml of ice-cold $MgCl_2$-$CaCl_2$ solution (80 mM magnesium chloride, 20mM calcium chloride) and collected by centrifugation. The cells was re-suspended in 2 ml of ice-cold 0.1 M calcium chloride and used on the same day for transformation.

To transform chemically competent *E. coli*, circa 50 ng of plasmid DNA was added to 200 µl of cell suspension in 1.5 ml tube and the mixture was incubated on ice for 30 min. The tube was then incubated for 90 seconds at 42° C in a circulating water bath. The cells were cooled for 1 minute on ice and 800 µl of LB was added. The cells were incubated for 45 minutes at 37° C, and then aliquots were plated onto selective agar medium.

2.5.3 Selection of transformants

The transformed *S. aureus* cells were selected on Tryptic Soy Agar (TSA) whereas *E. coli* cells were selected on LBA. The antimicrobial compounds used for selection and concentrations applied are described it Table 2.3. For blue/white colony selection of *E. coli* transformants the ampicillin plate was supplemented with IPTG/X-gal by spreading 50 µl of 0.1 M IPTG and 16 µl of 50 µg/ml X-gal (Promega) over the plate prior to use.

2.5.4 Restriction enzyme digest of plasmid DNA

Restriction enzyme digest of plasmid DNA was conducted in accordance with supplier's instructions (Promega). In brief, the digest reaction was prepared using circa 1 µg of plasmid DNA in a total volume of 20 µl with the following volume of individual reagents: 2 µl of 10X buffer, 0.2 µl of 10 µg/µl acetylated BSA, 0.5 µl of restriction enzyme and molecular grade water to final volume. The reaction was incubated at restriction enzyme optimal activity temperature for 4 h. If applicable, the enzyme was heat-inactivated for 15 minutes at 65° C.

Table 2.3 Antimicrobial compounds used in selection of transformed cells

Transformant species	Compound	Concentration (μg/ml)
S. aureus	penicillin	0.25
	erythromycin	10
	clindamycin	2
	tiamulin	10
	gentamicin	10
	kanamycin	10
	spectinomycin	200
	ciprofloxacin	2
	tetracycline	10
	chloramphenicol	10
	trimethoprim	10
E.coli	ampicillin	100
	kanamycin	8
	tetracycline	5
	erythromycin	200

2.5.5 Ligation

Ligation reactions were conducted using T4 DNA Ligase (Promega) in accordance with supplier's recommendations. The reaction was set up in a total volume of 10 μl, using 50 ng of vector and applying 2:1 insert vector ratio. To calculate the quantity of insert, the molar ratio was converted to mass ratio using the following formula:

$$\frac{\text{ng of vector x kb size of insert}}{\text{kb size of vector}} \quad \text{x} \quad \text{molar ratio of } \frac{\text{insert}}{\text{vector}} = \text{ng of insert}$$

The ligation reaction was incubated overnight at 15° C. The ligase was heat-inactivated for 10 minutes at 70° C. For transformation, 5 μl of the ligation mixture was used in a single reaction.

2.6 Susceptibility testing

2.6.1 Antimicrobial susceptibility testing

The antimicrobial susceptibility testing was conducted by determining the minimum inhibitory concentration (MIC) by the broth microdilution method in accordance with the CLSI guidelines (Clinical and Laboratory Standards Institute, January 2006).

The 96-well microtitre plates were prepared in-house. For each antimicrobial the concentration range to be tested was selected to include interpretive breakpoints and quality control strain's acceptable limits. Antimicrobial dilutions were prepared in Cation-adjusted Mueller-Hinton Broth (Ca-MHB) and dispensed into 96-well plate at 50 µl volume per well. The plates were used on the same day or stored at 4° C for up to 24 h. The antimicrobial tested and their dilution ranges are presented in Table 2.4.

The bacterial inoculum was prepared in Mueller-Hinton Broth to contain circa 6 x 10^5 CFU/ml and 50 µl was aliquoted into each well, resulting in a final test inoculum density of circa 5 x 10^5 CFU/ml. The plates were incubated for 16-20 h at 37 ° C for all compounds except cefoxitin, which was incubated for 24 h at 35° C.

Results were interpreted in accordance with CLSI MIC Interpretive Standards for *Staphylococcus* spp (Clinical and Laboratory Standards Institute; Clinical and Laboratory Standards Institute, January 2008).

Table 2.4 Antimicrobial compounds selected for MIC analysis

	µg/ml				
Antimicrobial	**Dilution range**	**QC range**	**Breakpoints***		
			S	I	R
penicillin	0.03 - 16	0.25 - 2	≤ 0.12	-	≥ 0.25
cefoxitin	0.12 - 16	1 - 8	≤ 4	-	≥ 8
erythromycin	0.06 - 32	0.25 - 1	≤ 0.5	1 - 4	≥ 8
tylosin	0.06 - 128	0.5 - 4	≤ 8	16	≥ 32
clindamycin	0.015 - 32	0.06 - 0.25	≤ 0.5	1 - 2	≥ 4
tiamulin	0.06 - 128	0.5 - 2	≤ 16	-	≥ 32
apramycin	0.25 - 128	2 - 8	≤ 16	-	≥ 32
gentamicin	0.03 - 64	0.12 - 1	≤ 4	8	≥ 16
kanamycin	0.25 - 128	0.12 - 1	≤ 16	32	≥ 64
spectinomycin	2 - 1024	64 - 256		NA	
vancomycin	0.06 - 32	0.5 - 2	≤ 2	4 - 8	≥ 16
teicoplanin	0.06 - 32	0.25 - 1	≤ 8	16	≥ 32
ciprofloxacin	0.03 - 16	0.12 - 0.5	≤ 1	2	≥ 4
tetracycline	0.03 - 64	0.12 - 1	≤ 4	8	≥ 16
chloramphenicol	0.25 - 128	2 - 16	≤ 8	16	≥ 32
florfenicol	0.5 - 32	2 - 8		NA	
trimethoprim	0.12 - 64	1 - 4	≤ 8	-	≥ 16

* S: susceptible; I: intermediate, where applicable otherwise '-'; R: resistant; NA: no breakpoints available

2.6.2 Biocide susceptibility testing

Biocide susceptibility testing was conducted by determining the minimum inhibitory concentration and was conducted using the broth microdilution method.

The 96-well microtitre plates were prepared in-house. The concentration ranges were prepared by two-fold dilution and selected based on previously reported *S. aureus* susceptibility ranges to tested biocides and heavy metals (Bjorland, Sunde and Waage, 2001; Brenwald and Fraise, 2003; Aarestrup and Hasman, 2004; Bjorland *et al.*, 2005; Noguchi *et al.*, 2005; Suller and Russell, 2000; Suller and Russell, 1999; Cavaco *et al.*, 2010). Dilutions were prepared in Ca-MHB. For the following compounds the Ca-MHB was adjusted to pH 5.5 to promote solubility: chlorhexidine digluconate, cadmium acetate and zinc chloride. The dilutions were dispensed into 96-well plate at 50 µl volume per well. The plates were used on the same day or stored at 4° C for up to 24 h. Biocides tested and their dilution ranges are presented in Table 2.5. The bacterial inoculum was prepared as described in section 2.6.1. The plates were incubated for 20 h at 37° C. Isolates showing comparatively elevated MIC value were reported as having reduced susceptibility.

Table 2.5 Biocide agents selected for MIC analysis

Biocide	Dilution range
Irgasan	0.002 – 4 µg/ml
acriflavine	0.25 – 128 µg/ml
chlorhexidine digluconate	0.03 – 16 µg/ml
benzalkonium chloride	0.12 – 64 µg/ml
CTAB	0.25 – 128 µg/ml
cadmium acetate	0.015 – 8 mM
zinc chloride	0.06 – 32 mM
copper sulphate	0.25 – 128 mM
hydrogen peroxide	0.00006 – 0.002 %
formaldehyde	0.00006 – 0.002 %

2.7 Biofilm formation assay

The biofilm formation assay was conducted as described by Futagawa-Saito *et al.* (2006), with minor modifications. Isolates were grown overnight in 2 ml of TSB supplemented with 0.25 % (w/v) glucose. The culture was diluted 1:200 in the same medium and 200 µl was aliquoted per well into Immuno 96 MicroWell MaxiSorp plate (Nunc). Each isolate was inoculated in triplicate. The plates were prepared in

duplicates and incubated for 24 h, each at different temperature: 25° C and 37° C. The culture was removed and wells were washed twice with 200 µl of PBS, and then stained with 100 µl of 0.1 % safranin-O (w/v) for 30 seconds. The staining solution was removed, wells were washed once with 200 µl of PBS and 100 µl of 97 % (v/v) ethanol/3 % (v/v) acetone solution was added to each well. The solution absorbance was measured at OD_{490nm} and biofilm formation potential was reported as absorbance intensity after subtracting the absorbance value of blank control well. The assay was conducted in three biological replicates.

2.8 Keratinocyte adhesion assay

2.8.1 Keratinocyte growth conditions

Cells were cultured using Kerationocyte Growth Medium (KGM; Promega) that contained 0.06 mM of calcium. The medium was supplemented with 100 U/µg per ml penicillin/streptomycin, 0.05 µg/ml amphothericin B and 8 % (v/v) calcium-depleted fetal bovine serum (FBS). FBS was depleted of calcium by Chelex treatment as described below (2.8.1.1.) Cells were grown at 37° C in a 5% (v/v) CO_2 atmosphere. Modified (calcium chloride-free and magnesium chloride-free) Dulbecco's phosphate buffered saline (DPBS) was used throughout the protocol.

2.8.1.1 Chelex treatment of fetal bovine serum

Chelex treatment of fetal bovine serum was conducted in accordance with previously described method by Hodivala-Dilke, (2002) with minor modifications. Briefly, 20 g of Chelex 100 resin (Bio-Rad) was mixed with 130 ml of distilled water. The solution's pH was adjusted to 7.35-7.4. The Chelex resin was allowed to settle and water was removed. The resin was washed twice with an equal volume of distilled water, then twice with Dulbecco`s Phosphate Buffered Saline (DPBS). In each wash the resin was stirred for 5 minutes, then allowed to settle for 30 minutes and water/DPBS was removed. On the last wash with DPBS the solution pH was stabilized to 7.35-7.4 before allowing Chelex to settle. Following the washes, 100 ml of cold FBS was added to Chelex and the solution was allowed to stir overnight at 4° C. After the treatment the Chelex was allowed to settle, the FBS was removed and passed through 0.45 µm filter to remove remaining resin followed by sterilization using a 0.22 µm filter. The FBS was stored at -20° C.

2.8.1.2 Collagen-coating of cell culture flasks and plates

Collagen (PromoCell) dilution was prepared using sterile 0.01M HCl solution. The dilution was transferred to culture flasks/24 well plates at 100 μl per cm^2/well. The flasks/plates were left to stand on a level surface for 1 h at room temperature, after which the collagen solution was removed and the flask/plate was washed three times with four times the volume of DPBS. The flask/plate was used immediately or allowed to dry and stored at 4° C. Culture flasks were coated with collagen at 5 μg/cm^2 (using 50 μg/ml dilution) whereas 24-well plates were coated at 0.5 μg/well (using 5 μg/ml dilution).

2.8.2 Isolation of keratinocytes

Ear skin fragments were obtained from two 6-week old piglets during post-mortem procedures. The skin was cleaned with a scalpel to remove hair, fat, muscle and membranous material, and then washed three times with DPBS. The fragments were cut into 1 x 0.5 cm strips and washed three times with DPBS. To allow separation of epidermis from dermis, the dissected skin was floated dermis side down in a petri dish containing 0.25% (w/v) trypsin solution supplemented with 100 U/μg per ml penicillin/streptomycin and 0.05 μg/ml amphothericin B, and incubated overnight at 4° C. The epidermis was peeled off and transferred to polypropylene tube containing 5 ml of KGM. To release keratinocytes the epidermis fragments were triturated and the cell suspension was transferred to a clean tube. The cells were collected by centrifugation for 5 minuets at 750 rpm and washed twice with KGM. The cell concentration and viability was checked by mixing an aliquot of cell suspension with equal volume of Trypan Blue, followed by a transfer of 10 μl onto Kova Glasstic cell counter (Hycor) for a cell count. The cells were seeded onto collagen-coated flasks at the density of 5 x 10^4 cells/cm^2.

2.8.3 Trypsinization of confluent cells

To trypsinize confluent cells, medium was removed from the culture flask and the cells were washed once with an equal volume of DPBS. A 1X trypsin solution was prepared from 10X stock (Gibco) using DPBS and the solution was added to the flask (80 μl per 1 cm^2). The culture was incubated at 37° C for 10 minutes and the reaction was stopped by adding an equal volume of KGM. The detached cells were transferred to polypropylene tube and cells were collected by centrifugation for 5 minutes at 750

rpm. The pellet was re-suspended in KGM and the cell count and viability was checked as described in section 2.8.2.

2.8.4 Keratinocyte adhesion assay

The keratinocyte adhesion assay was performed as described by Kintarak *et al.* (2004), with minor modifications as described below.

2.8.4.1 Cell culture

Keratinocytes were sub-cultured in 24-well plates at concentration 7 x 10^4 cells/per well and grown for 3 days to full confluence. On the day of the adhesion assay medium was removed from each well and cell monolayer was washed twice with 1 ml of DPBS. After the wash, 0.5 ml of 2% (w/v) BSA solution was added to each well and the plates were then incubated for 1 h at 37° C. The cells were washed twice with 1 ml of DPBS.

2.8.4.2 Preparation of bacterial inoculum

Isolates were grown overnight in 2 ml of TSB. Cells were collected by centrifugation for 5 minutes at 10000 rpm, and then washed once with 1 ml of DPBS and centrifuged again. The pellet was re-suspended in1 ml of DPBS and the inoculum density was adjusted to OD_{540nm} of 0.15 (*circa* 1 x 10^8 CFU/ml). Bacterial viable counts were determined by preparing serial dilutions of the inoculum and plating out on CBA by following the Miles, Misra and Irwin (1938) method.

2.8.4.3 Adhesion of bacterial isolates to keratinocytes

For each analysed isolate a 0.5 ml (*circa* 5 x 10^7 CFU per well) of the prepared inoculum was transferred into each well of the 24-well plate containing keratinocyte cell monolayer and the plate was incubated for 1 h at 37° C with shaking. The bacterial inoculum was removed and the wells were washed eight times with 1 ml of DPBS. To release adherent bacteria from the cell monolayer, 0.5 ml of lysing solution (1X trypsin – 0.1% (v/v) triton) was added to each well and the plate was incubated for 20 minutes at 37° C with shaking. Bacterial viable counts were determined by preparing serial dilutions of the suspension and plating out on CBA following the Miles, Misra and Irwin (1938) method. The data was presented as a percentage (%) of adherent bacteria as a function of total inoculum.

2.9 Competition assay

Direct competition assay of MRSA CC398 against MRSA CC8, CC22 and CC30 was performed. Two strains were selected for each lineage and both MRSA CC398 strains were competed against each representative of the other MRSA lineages. The experiment was conducted as described by Nielsen *et al.* (2012), with minor modifications. Bacterial strains were grown in 2 ml of TSB overnight at 37° C with shaking. Using the overnight culture, an inoculum with OD_{540nm} of 0.15 was prepared for each isolate. The inoculum was diluted 1:1000 (*circa* 1 x 10^5 CFU/ml) and 40 µl of each isolate was transferred to 4 ml of TSB, with final concentration of each isolate of *circa* 1 x 10^3 CFU/ml. For each isolate, bacterial viable count were determined by preparing serial dilutions of the 1 x 10^5 CFU/ml inoculum and plating out on CBA by following the Miles, Misra and Irwin (1938) method. The cultures were incubated for 24 h at 37° C with 150 rpm shaking. Two further 24 h growth cycles were performed by passaging the mixed bacterial culture: after each 24 h cycle an inoculum containing *circa* 1 x 10^5 CFU/ml was prepared (as described above) and 40 µl was used to inoculate 4 ml of fresh TSB (final inoculum circa 10^3 CFU/ml). The bacterial viable counts of both strains were determined after each 24 h growth cycle by preparing serial dilutions of the culture and plating out on antibiotic selective TSA following the Miles, Misra and Irwin (1938) method. The selective isolation was conducted using compounds and concentrations described in Table 2.6.

Table 2.6 Selective isolation of strains from mixed culture

Lineage	Compound	Concentration µg/ml
CC8	gentamicin	10
CC22	ciprofloxacin	2
CC30	ciprofloxacin	2
CC398	spectinomycin (CC8) [a]	200
	tetracycline (CC22 & CC30)	5

[a] Selection for CC398 strains differed between mixture culture containing CC8 strains and CC22/CC30 strains, as described.

For each assay three biological replicates were conducted. Based on analysis method described by Lenski *et al.* (1994), the difference in colony counts between the competing strains on each day of competition was expressed as a ratio:

$$r = \log_e(C1/C2)$$

where C1 and C2 is the number of a CC398 strain and a competitor strain CFU, respectively.

The difference in fitness defined as S_t between CC398 and the competitor strain after each cycle of competition was calculated in accordance with the following formula, as described by Nielsen *et al.* (2012):

$$S_t = \ln\left[\left(\frac{C1_t/C2_t}{C1_{t-1}/C2_{t-1}}\right)^{1/17}\right]$$

where $C1_t$ and $C2_t$ is the number of a CC398 strain and a competitor strain CFU, respectively, at a given time point t and $C1_{t-1}$ and $C2_{t-1}$ is the number of a CC398 strain and a competitor strain CFU, respectively, at a preceding time point.

2.10 Determination of growth rate

Growth rates of bacterial isolates were measured using FLUOstar OPTIMA microplate reader (BMG Labtech, version 1.32 R2) by taking absorbance measurements of inoculated cultures every 15 minutes at OD_{600nm} over 24 h incubation period.

Isolates were grown in 2 ml of TSB overnight at 37° C with shaking. The culture was diluted 1 x 10^{-5} in the same medium and 200 µl was transferred into 96-well plate. Each isolate was inoculated in triplicate. The plate was incubated at 37° C using the following settings: 97 cycles, cycle time 900 seconds, 10 flashes per well and cycle, orbital well scanning, additional shaking of 5 seconds before cycle, well diameter 3 mm. For each experiment three biological replicates were performed.

2.10.1 Comparative analysis of growth curves and growth rates with and without antimicrobial selection

Changes in growth curves and growth rates of resistant isolates when cultured with and without antibiotic were analysed following exposure to four compounds (selected based on frequency of phenotypic resistance amongst the analysed strains) at the following concentrations: cefoxitin 1µg/ml, tetracycline 8 µg/ml, erythromycin 4 µg/ml and trimethoprim 16 µg/ml. The growth curves were plotted based on blank

subtracted OD values at each time point. The growth rate (GR) in the absence and presence of antimicrobial compound was calculated between OD values of 0.1 and 0.3 in accordance with formula described by Foucault *et al.* (2009):

$$GR = [\ln(0.3) - \ln(0.1)]/(t_{0.3} - t_{0.1})$$

where $t_{0.3}$ and $t_{0.1}$ represent time points when the culture OD was measured as 0.3 and 0.1, respectively. The area under a growth curve (AUC) in the absence and presence of antimicrobial compound was estimated by applying a trapezoidal rule formula where the AUC is a sum of areas, and each of the areas is calculated as:

$$AUC\ t\text{-}t_{-1} = (OD_t\text{-}OD_{t\text{-}1})/2 \times (t\text{-}t_{-1})$$

where AUC $t\text{-}t_{-1}$ is a single area between a time point t and a preceding time point t-1, calculated based on a difference in OD values between the two time points.

Chapter 3 Isolate characterisation

3.1 Introduction

Bacterial typing methods allow differentiation of isolates within a species and thus have become essential tools in studying pathogen epidemiology, such as outbreaks of infection, nosocomial transfer and spread of more virulent or successful clones (Olive and Bean, 1999). One of the earliest *S. aureus* typing methods was the bacteriophage typing scheme that utilized the variation in susceptibility of *S. aureus* isolates to bacteriophage lysis (Enright, 2008). The technique involved the use of a series of typing phages and the result was referred to as phage pattern (Wentworth, 1963). Phage typing had been a standard *S. aureus* typing tool for many years and although the use of the method is still being reported, it is known to have considerable limitations such as isolate non-reactivity and issues with reproducibility (Tenover *et al.*, 1994; Wisniewska *et al.*, 2012).

A number of methods have since been developed that allow a more discriminatory characterisation of *S. aureus* isolates together with an investigation of *S. aureus* population structure and its genetic diversity (Grundmann *et al.*, 2002). Pulsed field gel electrophoresis (PFGE) involves separation of chromosomal DNA fragments based on size on an agarose gel by using an alternating voltage field (Enright, 2008). The cell's total DNA is digested with a restriction endonuclease of choice and the resulting banding pattern represents a unique fingerprint of the analysed isolate (Enright, 2008). This method is among the most commonly used *S. aureus* typing tools (Enright, 2008).

A widely established technique for *S. aureus* epidemiology analysis has been *spa* typing, a single-locus based method that involves sequencing of an internal fragment of the *spa* gene (Enright, 2008). The *spa* gene encodes *S. aureus*-specific protein A, and contains a polymorphic X region that consists of repeats that vary among isolates in number and sequence (Uhlen *et al.*, 1984; Shopsin *et al.*, 1999). Each recognised repeat is assigned an identifying number and *spa* type is determined based on the combination of individual repeats, which is known as repeat succession (Harmsen *et al.*, 2003; http://www.ridom.de). A considerable advantage of *spa* typing is the availability of an online database, which not only mediates interpretation of data but also provides supplementary information about individual *spa* types such as their frequency and association with sequence types and clonal complexes (http://www.ridom.de).

Typing methods based on analysis of multiple loci have also been developed such as multilocus sequence typing (MLST), which allows a more fundamental analysis of bacterial population structure by differentiating isolates on the basis of allelic variants of house-keeping genes (Maiden *et al.*, 1998). MLST of *S. aureus* entails sequencing of internal fragments of seven such genes and the sequence data can be directly analysed through an online database that allows the assignment of a allelic variant number for each gene and then determine the sequence type based on the allelic profile (Enright *et al.*, 2000; http://saureus.mlst.net). This technique has been particularly effective in studying the epidemiology of MRSA clones, both hospital- and community-acquired, and facilitated monitoring the spread and emergence of novel successful lineages (Deurenberg and Stobberingh, 2008).

Schemes have also been developed for the typing of MRSA strains based on the variations in the structure and sequence of the SCC*mec*. A number of SCC*mec* typing methods have been described that commonly involve PCR-based detection of unique molecular markers that are representative of specific SCC*mec* types (Oliveira and de Lencastre, 2002; Zhang *et al.*, 2005; Boye *et al.*, 2007). There are currently eight recognised SCC*mec* types that can be further classified into subtypes (International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC), 2009). A sequence-based method for the typing of MRSA isolates has also been developed and is based on analysis of the direct repeat unit (*dru*) region located within the *mecA* complex (Goering *et al.*, 2008). In the *dru* typing scheme, each identified repeat is assigned a numerical value and the combination of the *dru* repeats represents a specific *dru* type (Goering *et al.*, 2008). An online database is available for querying sequence data and the assignment of *dru* repeats and *dru* types (www.dru-typing.org). The method is considerably more discriminatory than the SCC*mec* typing and allows differentiation between isolates found to carry the same SCC*mec* type (Goering *et al.*, 2008; Shore *et al.*, 2010).

The described typing methods are rarely used separately. Instead the techniques are combined for a comprehensive analysis of isolates of interest. Although all can be used to determine the genetic relationship between analysed isolates and the level of clonality within a panel, they vary considerably in analytical and discriminatory properties (Malachowa *et al.*, 2005). The macro-restriction PFGE method demonstrates a high level of discriminatory power and is recognised as a gold standard for investigation of MRSA nosocomial outbreaks. The *spa* typing technique

is less discriminatory than PFGE but the development of a standardised scheme for the assignment of *spa* types and the free accessibility of SpaServer allows for an immediate comparative analysis of results in the context of previously deposited data (http://www.ridom.de). MLST analysis, although less stringent in isolate differentiation than *spa* typing, has facilitated the global analysis of *S. aureus* population and led to identification of dominant lineages (Enright *et al.*, 2002; Feil *et al.*, 2003).

S. aureus isolates (described in section 2.2.1.) were characterised by conducting PFGE, *spa* typing, MLST and SCC*mec* and *dru* typing. The aim of the work presented here was to determine isolate clonality within the analysed panel in order to select strains for further genotypic and phenotypic characterisation.

3.2 Materials and methods

Isolate genotyping and data analysis was conducted as described in sections 2.4.1 - 2.4.5 and 2.4.7.1. Macro-restriction PFGE analysis involved the use of two restriction endonucleases: *SmaI* and *Cfr9I*. Although *SmaI* is the enzyme of choice for *S. aureus*, isolates belonging to CC398 are refractory to *Sma*I digestion due to methylation of the recognition sequence 5'-CCCGGG-3' at the second cytosine (Argudin, Rodicio and Guerra, 2010). Instead, DNA from *Sma*I non-typeable isolates was digested with *Cfr9*I, a neoschizomer of *Sma*I that cuts within the same recognition sequence but at different position.

All isolates were subjected to PFGE analysis whereas *spa* typing was conducted only on isolates showing a unique PFGE clonal type (fingerprint type; based on a cut-off value of 100% and paired with visual inspection to ensure the accuracy of unique fingerprint type identification). Thus if a clonal type was assigned to more than one isolate (clonal group), a single representative was selected for *spa* typing. For each identified *spa* type, a single representative was selected for the MLST analysis. The identified sequence types were assigned to the same clonal complex if the MLST-derived allelic profiles shared five or more of the seven loci. Isolates were then assigned to a clonal complex based on the determined *spa* and sequence types, based on assumption that isolates that belong to identical PFGE clonal type would demonstrate the same *spa* type, and isolates with identical *spa* type would belong to the same sequence type.

For the purposes of this work all identified lineages will be referred to, in this and later chapters, as the assigned clonal complex (CC).

3.3 Results

3.3.1 *nuc/mecA* PCR

All analysed isolates were confirmed as *Staphylococcus aureus* based on detection of the *nuc* gene. The *mecA* gene was detected in all CC398 isolates, 45 human isolates and the two equine isolates. All 27 cattle isolates were *mecA* negative.

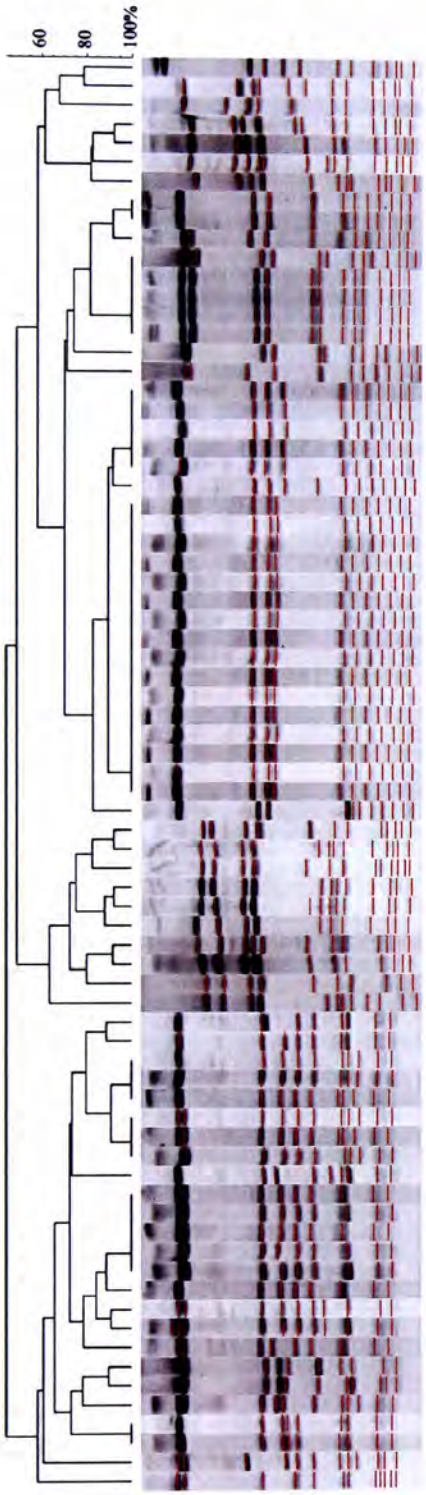
3.3.2 Analysis of isolate heterogeneity by PFGE, *spa* typing and MLST

3.3.2.1 Human and equine isolates

The macrorestriction PFGE typing of 75 human and the two equine isolates revealed 42 clonal types (Figure 3.1). Among the 42 clonal types, 24 different *spa* types were identified (Figures 3.1 and 3.2). Two novel *spa* types were detected for which the repeat succession was identified as 'unknown'. MLST analysis of each *spa* type representative identified nine different sequence types: ST5, ST8, ST9, ST15, ST22, ST30, ST36 and ST39 (Figures 3.1 and 3.7). Isolates were assigned to seven clonal complexes: CC5 (n=4), CC8 (n=6), CC9 (n=1), CC15 (n=5), CC22 (n=25), CC30 (n=33) and CC45 (n=1).

Isolates belonging to CC5, CC9 and CC15 formed a single PFGE clonal cluster with 64% identity. All isolates within that group were *mecA* negative and each represented a unique fingerprint type. The cluster complex also demonstrated considerable *spa* type diversity with the majority of selected strains displaying a unique *spa* type. The CC5 group was associated with *spa* types t002, t179 and t442 whereas CC15 strains carried t084, t228, t360 and t491. The single CC9 strain was *spa* type t8887.

Isolates belonging to CC8 co-clustered with a single representative of CC45 and all shared 62% identity. The CC8 group consisted of unique fingerprint types only and included the two equine isolates that represented the only two *mecA*-positive isolates within the cluster group. All but one human CC8 strains carried t008 whereas both equine strains were *spa* type t064. A single human strain was found to contain a novel *spa* type ('unknown').



ID	Host	*mecA*	*spa* type	MLST	CC
2	Human	-	t026	ST45	CC45
201	Horse	+	t064		CC8
200	Horse	+	t064	ST8	CC8
11	Human	-	t008		CC8
6	Human	-	t008	ST8	CC8
15	Human	-	unknown		CC8
16	Human	-	t008		CC8
1	Human	-	t122	ST30	CC30
7	Human	-			CC30
22	Human	-	t298	ST39	CC30
51	Human	-	t019	ST30	CC30
53	Human	-			CC30
54	Human	-			CC30
57	Human	-			CC30
58	Human	-			CC30
62	Human	-	t021	ST30	CC30
13	Human	-	t012		CC30
34	Human	+	t018	ST36	CC30
63	Human	+			CC30
64	Human	+			CC30
73	Human	+			CC30
74	Human	+			CC30
31	Human	+	t1675	ST36	CC30
65	Human	+	t018		CC30
82	Human	+			CC30
66	Human	+			CC30
67	Human	+			CC30
68	Human	+			CC30
69	Human	+			CC30
70	Human	+			CC30
71	Human	+			CC30
72	Human	+			CC30
75	Human	+			CC30
76	Human	+			CC30
77	Human	+			CC30
78	Human	+			CC30
79	Human	+			CC30
80	Human	+			CC30
81	Human	+			CC30
5	Human	-	t012	ST30	CC30
17	Human	-	t442	ST5	CC5
24	Human	-	t491	ST15	CC15
25	Human	-	t002	ST5	CC5
21	Human	-	t084		CC15
23	Human	-	t228	ST15	CC15
60	Human	-	t360	ST15	CC15
4	Human	-	t8887	ST9	CC9
8	Human	-	t179	ST5	CC5
19	Human	-	t442		CC5
10	Human	-	t084	ST15	CC15
47	Human	+	t032		CC22
48	Human	+	t032		CC22
33	Human	+			CC22
37	Human	+			CC22
38	Human	+	t020	ST22	CC22
29	Human	+			CC22
30	Human	+	t032		CC22
49	Human	+			CC22
46	Human	+	t032		CC22
26	Human	+	t032		CC22
41	Human	+			CC22
42	Human	+			CC22
43	Human	+			CC22
50	Human	+			CC22
36	Human	+	t032		CC22
27	Human	+	t032		CC22
35	Human	+	t032		CC22
32	Human	+	t032		CC22
28	Human	+	t032		CC22
40	Human	+	t032		CC22
39	Human	+	t379	ST22	CC22
44	Human	+	t032	ST22	CC22
45	Human	+			CC22
20	Human	-	unknown	ST22	CC22
18	Human	-	t608	ST22	CC22

Figure 3.1 UPGMA cluster analysis of PFGE profiles (gel image) of human and equine *S. aureus* isolates. The dendrogram was created as described in 2.4.5. The figure also shows a summary of key genotypic features such as carriage of *mecA* gene, *spa* type (where applicable), MLST (where applicable) and clonal complex (CC). Isolates, for which the *spa* type and MLST were not determined, represent members of a PFGE clonal group (as desicribed in 3.2)

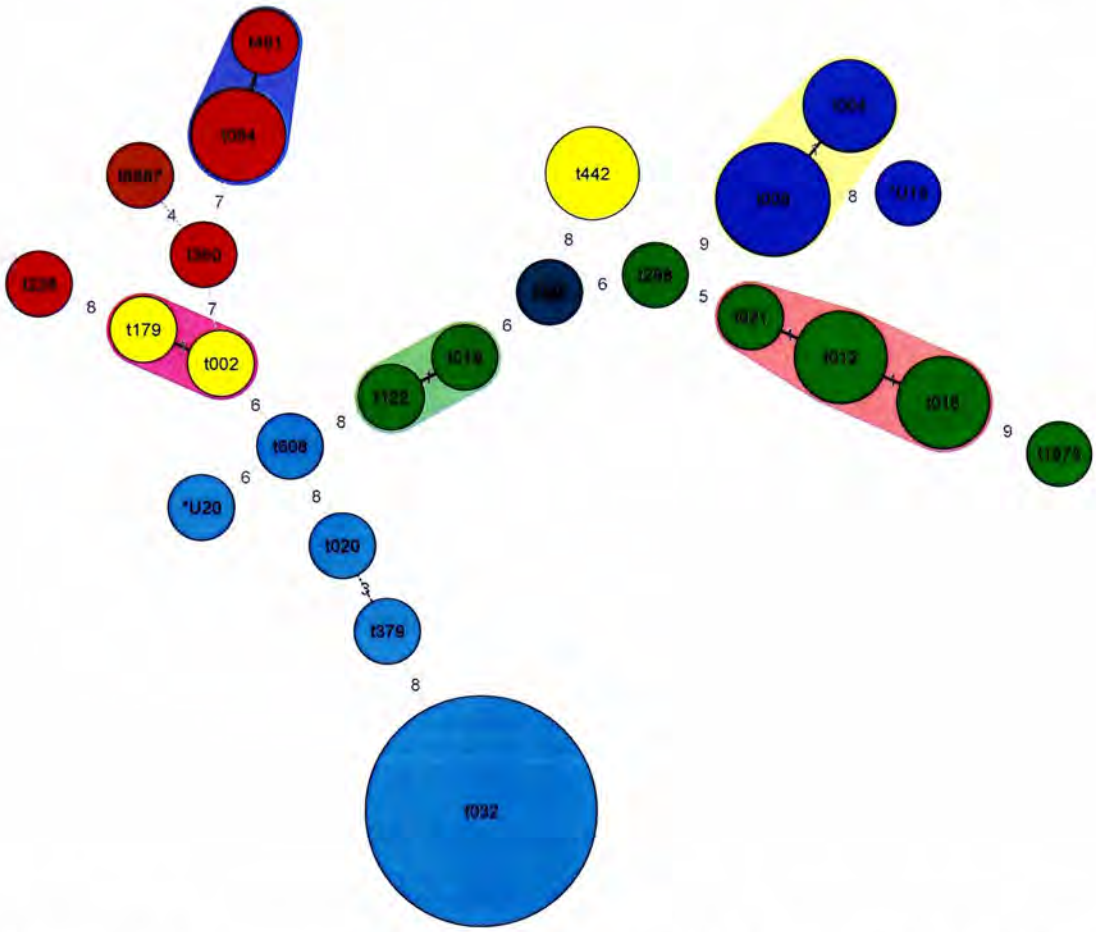


Figure 3.2 Minimum spanning tree cluster analysis of *spa* types derived from human- and equine-associated *S. aureus* isolates. The dendrogram was created as described in 2.4.2. Distance is presented in value and logarithmic branch lengths. Nodes are scaled with member count and colour grouped to represent the associated clonal complex. Further information regarding isolates that were associated with the presented *spa* types can be found in Figure 3.1. The *spa* clustering complexes were created based on a maximum neighbour distance of one and minimum size of two, and are displayed with colour background.

Legend: CC5; CC8; CC9; CC15; CC22; CC30; CC45.

Furthermore, MLST analysis of the strain revealed that it shared only five out of seven alleles with ST8, both of which demonstrated novel identity and thus the sequence type for that strain was not determined. The single CC45 strain was *spa* type t026.

The CC22 cluster group demonstrated 58% identity and consisted of 16 fingerprint types with four clonal types represented by more than one isolate. All but two of the isolates belonging to CC22 were *mecA* positive (23/25) and formed a major cluster sharing 67% identity. Also, nearly all were assigned to *spa* type t032 (12/14) with the remaining two strains being t020 and t379. The two *mecA* negative isolates demonstrated a unique fingerprint type, clustered separately and were associated with distinct *spa* types: t608 and a novel ('unknown') *spa* type.

The CC30 group constituted 44% of all analysed isolates, but consisted of only 9 clonal types that shared 70% identity. Isolates positive for the *mecA* gene (22/33) were highly clonal as only 3 unique fingerprint types could be identified among the 22 isolates. Two representatives of the largest *mecA*-positive clusters demonstrated *spa* type t018 whereas a single strain with unique fingerprint type contained t1676. Unlike other analysed clonal complexes, the *mecA* negative isolates belonging to CC30 contained clonal groups with some fingerprint types demonstrated by more than one isolate. Among the six clonal types, five *spa* types were identified: t012, t019, t021, t122 and t298. Furthermore the CC30 group contained three sequence types: ST30, ST36 (single-locus variant of ST30) and ST39 (double-locus variant of ST30).

Based on the typing results a total of 30 strains representing CC5, CC8, CC15, CC22 and CC30 were selected for further analysis (Table 3.1).

3.3.2.2 Cattle isolates

The macrorestriction PFGE typing of 26 cattle isolates revealed 16 clonal types (Figure 3.3). Among the 16 clonal types, 8 different *spa* types were identified (Figures 3.3 and 3.4). MLST analysis of each *spa* type representative identified six different sequence types: ST97, ST118, ST130, ST1074, ST1245 and ST1527 (Figures 3.3 and 3.7). Isolates were assigned to three clonal complexes: CC97 (n=9), CC130 (n=11) and CC151 (n=6).

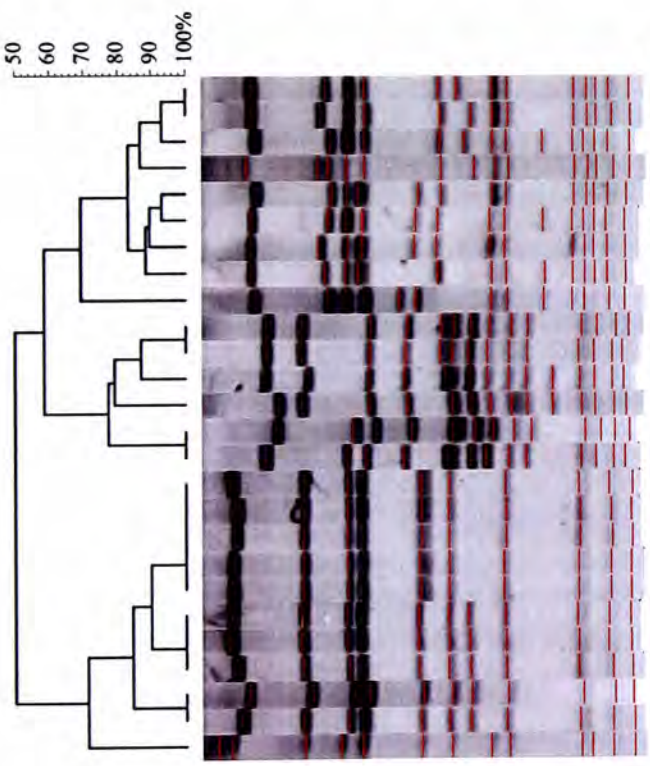
The majority of isolates belonging to CC97 represented a unique fingerprint type (8/9) that shared 69% identity. Among selected strains five different *spa* types were identified: t224, t267, t131, t359 and t521. Furthermore, the CC97 group

contained three sequence types: ST97, ST118 (single-locus variant of ST97) and ST1527 (single-locus variant of ST97).

The CC130 group consisted of three clonal groups and a single isolate with unique fingerprint type with an overall 71% identity. All representatives of the clonal groups were *spa* typed as t843 whereas the singleton carried t6220. The two identified *spa* types were associated with two different sequence types: ST1245 (single-locus variant of ST130) and ST130, respectively.

Isolates belonging to CC151 demonstrated four clonal types with an overall 77% identity. Only one *spa* type was identified: t529 and it was associated with ST1074 that varies from ST151 at a single allele.

Based on the typing results total of 16 strains representing each of the identified clonal complexes were selected for further analysis (Table 3.1).



ID	Host	*spa* type	MLST	CC
189	Cattle	t224	ST97	CC97
192	Cattle			CC97
177	Cattle	t267	ST118	CC97
174	Cattle	t131	ST1527	CC97
190	Cattle	t359	ST97	CC97
197	Cattle	t267		CC97
195	Cattle	t359		CC97
198	Cattle	t224		CC97
175	Cattle	t521	ST97	CC97
193	Cattle	t529		CC151
196	Cattle			CC151
199	Cattle	t529		CC151
194	Cattle	t529		CC151
179	Cattle	t529	ST1074	CC151
180	Cattle			CC151
182	Cattle	t843		CC130
183	Cattle			CC130
185	Cattle			CC130
187	Cattle			CC130
188	Cattle			CC130
181	Cattle	t843		CC130
184	Cattle			CC130
186	Cattle			CC130
178	Cattle	t843	ST1245	CC130
191	Cattle			CC130
173	Cattle	t6220	ST130	CC130

Figure 3.3 UPGMA cluster analysis of PFGE profiles (gel image) of cattle *S. aureus* isolates. The dendrogram was created as described in 2.4.5. The figure also shows a summary of key genotypic features such as *spa* type (where applicable), MLST (where applicable) and clonal complex (CC). Isolates, for which the *spa* type and MLST were not determined, represent members of a PFGE clonal group (as desicribed in 3.2)

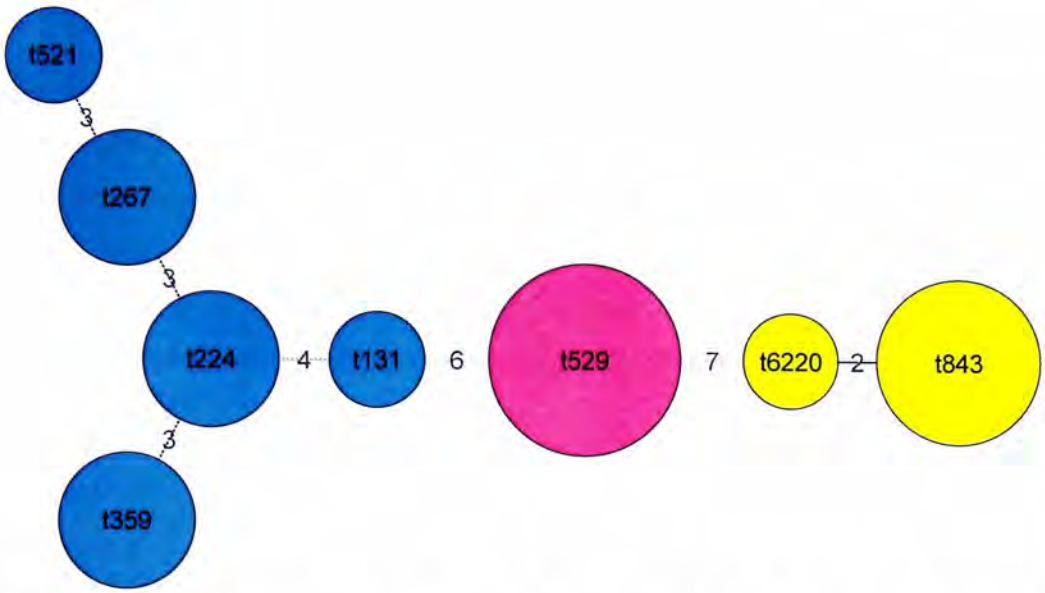
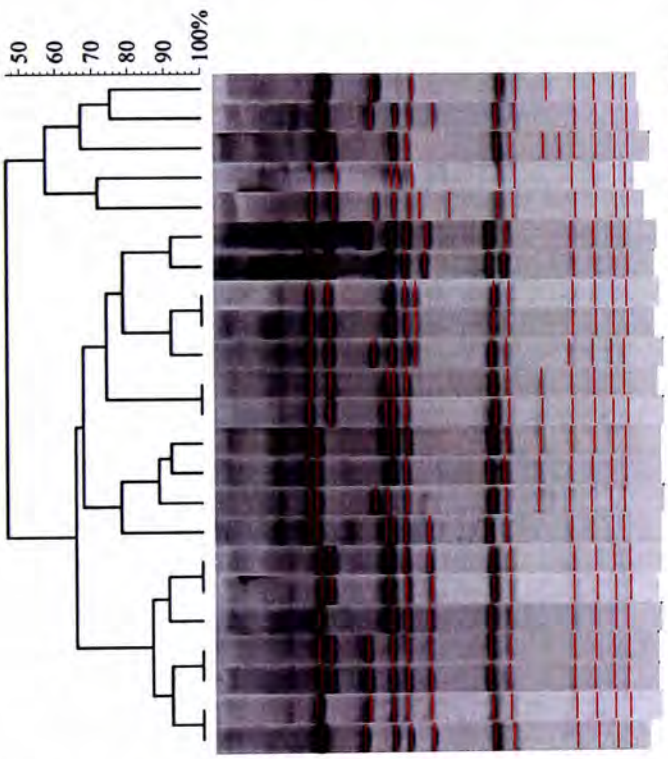


Figure 3.4 Minimum spanning tree cluster analysis of *spa* types derived from cattle-associated *S. aureus* isolates. The dendrogram was created as described in 2.4.2. Distance is presented in value and logarithmic branch lengths. Nodes are scaled with member count and colour grouped to represent the associated clonal complex. Further information regarding isolates that were associated with the presented *spa* types can be found in Figure 3.3.

Legend: CC97; CC130; CC151.

3.3.2.3 MRSA CC398 isolates

The PFGE typing revealed 18 different clonal types. The majority of the fingerprint types were represented by a single isolate, whereas five were shared by a clonal group consisting of two isolates only. Four clonal groups consisted of isolates derived from different hosts. The majority of strains (10/18) were found to be *spa* type t011 (Figure 3.5). The remaining detected *spa* types were: t034, t567, t1451 and t4872. Each of the *spa* types was confirmed to be associated with CC398 (Figures 3.5 and 3.6). Based on the typing results total of 18 strains were selected for further analysis (Table 3.1)



ID	Host	*spa* type	MLST	CC
91	Chicken	t011		CC398
92	Chicken	t011		CC398
95	Human	t034	ST398	CC398
90	Chicken	t011	ST398	CC398
93	Chicken	t567	ST398	CC398
101	Horse	t1451	ST398	CC398
96	Human	t034		CC398
110	Pig	t567		CC398
97	Human			CC398
107	Cattle	t567		CC398
100	Horse			CC398
108	Pig	t011		CC398
103	Rat	t011		CC398
104	Rat	t4872	ST398	CC398
106	Cattle	t011		CC398
105	Cattle	t011		CC398
109	Pig	t011		CC398
112	Pig			CC398
102	Rat	t011		CC398
98	Human			CC398
99	Horse	t011		CC398
111	Pig	t034		CC398
94	Chicken			CC398

Figure 3.5 UPGMA cluster analysis of PFGE profiles (gel image) of MRSA CC398 isolates. The dendrogram was created as described in 2.4.5. The figure also shows a summary of key genotypic features such as *spa* type and MLST (where applicable). Isolates, for which the *spa* type and MLST were not determined, represent members of a PFGE clonal group (as desicribed in 3.2)

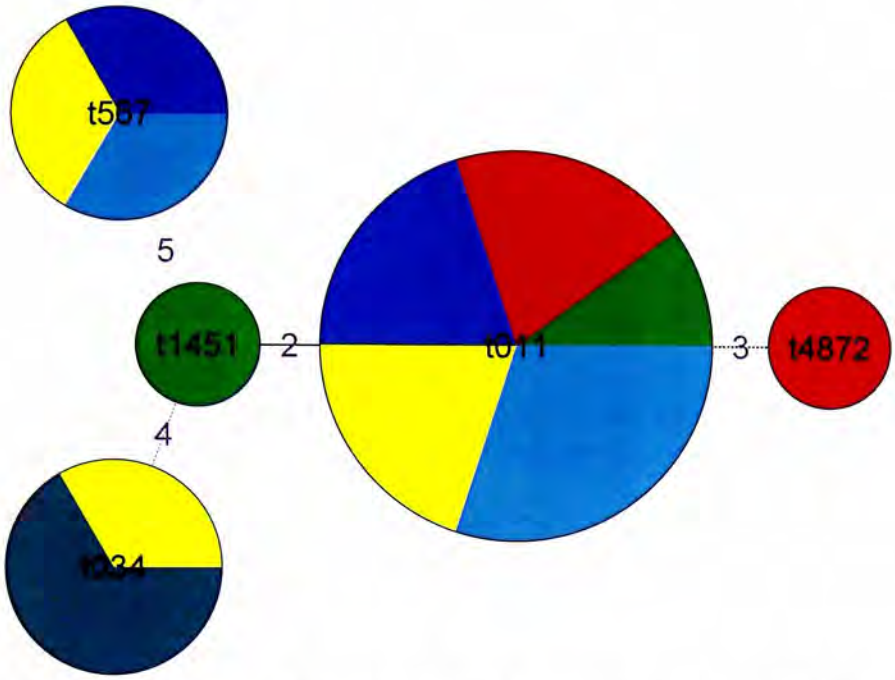


Figure 3.6 Minimum spanning tree cluster analysis of *spa* types derived from MRSA CC398 isolates.The dendrogram was created as described in 2.4.2. Distance is presented in value and logarithmic branch lengths. Nodes are scaled with member count and pie chart colour grouped to represent the associated host species. Further information regarding isolates that were associated with the presented *spa* types can be found in Figure 3.5.

Legend: Horse; Rat; Cattle; Pig; Chicken; Human.

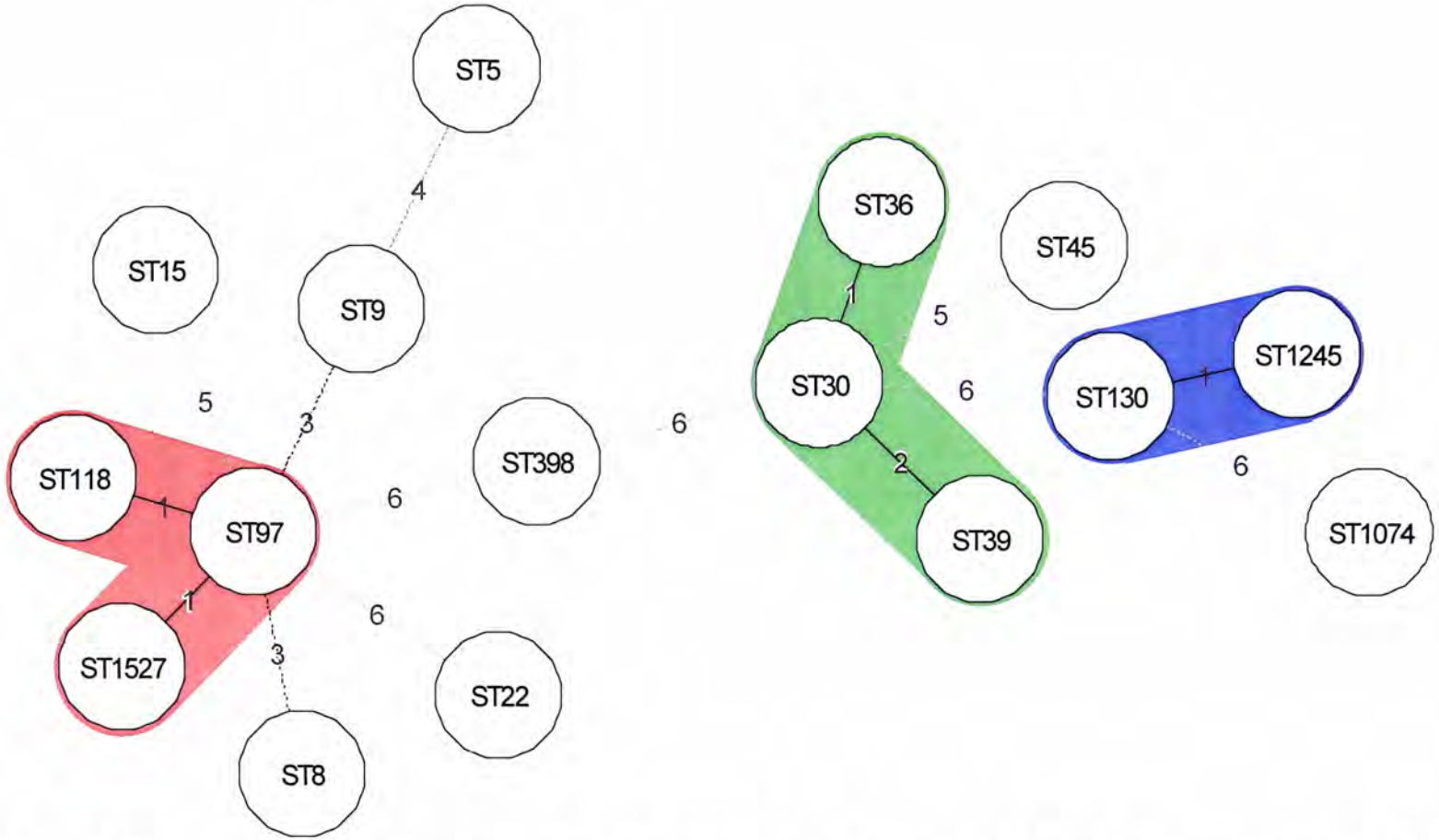


Figure 3.7 Minimum spanning tree (categorical coefficient) cluster analysis of all identified sequence types (ST). The dendrogram was created as described in 2.4.4. Distance is presented in value and logarithmic branch lengths. MLST clustering complexes were created based on a maximum neighbour distance of two and minimum size of two, and are displayed in colour.

3.3.3 SCC*mec* and *dru* typing of selected isolates

The *mecA*-positive strains that were selected for further characterisation based on the typing results were also analysed by SCC*mec* and *dru* typing (Table 3.1). the majority of strains carried SCC*mec* type IV (20/29), which included all non-CC398 and half of CC398 strains. Among the remaining CC398 strains, most were found to carry SCC*mec* type V 5C2&5, whereas three strains were non-typeable with the applied method. The sub-type of the SCC*mec* type IV was determined for all but two strains (Figure 3.8).

A total of 11 *dru* types were identified, which included two 'unknown' (novel) types (Figure 3.8). The most common *dru* type was dt10a (9/29), followed by dt10q (6/29). In some instances strains carrying the same SCC*mec* type, such as V 5C2&5, demonstrated *dru* type variation. However, it was also observed that the same *dru* type was identified among strains of distinct SCC*mec* types. For instance strains with *dru* type dt10a demonstrated three distinct sub-types of SCC*mec* type IV. Furthermore, strains that displayed *dru* type dt10a belonged to three separate lineages, namely CC8, CC22 and CC398.

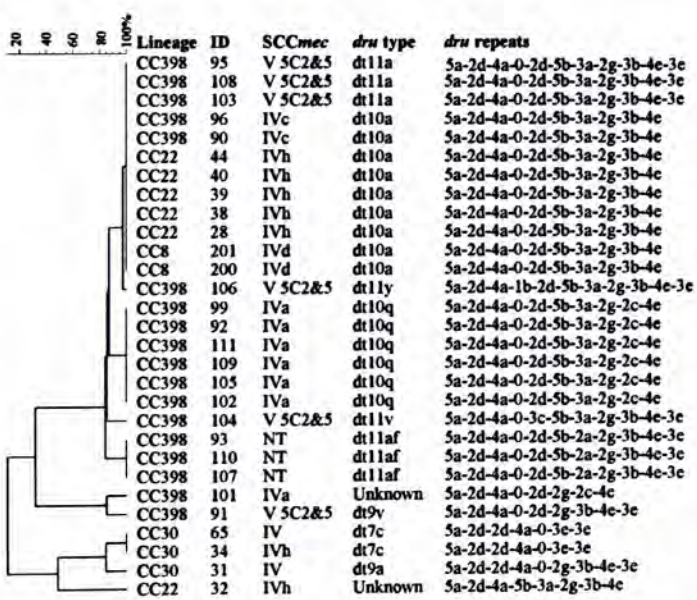


Figure 3.8 UPGMA cluster analysis of *dru* types identified among MRSA strains. The dendrogram was created as described in 2.4.3. The figure shows the SCC*mec* type of each of the analysed strains, followed by the identified *dru* type and the associated *dru* repeat succession.

Table 3.1 Summary of genotypic features of analysed *S. aureus* strains [a]

No	ID	Host	*spa* type	ST [b]	CC [c]	*mecA* [d]	SCC*mec* [e]	*dru* [f]
1	25	Human	t002	5	5	-	-	-
2	8	Human	t179	5	5	-	-	-
3	17	Human	t442	5	5	-	-	-
4	19	Human	t442	-	5	-	-	-
5	6	Human	t008	8	8	-	-	-
6	11	Human	t008	-	8	-	-	-
7	16	Human	t008	-	8	-	-	-
8	200	Horse	t064	8	8	+	IVd	dt10a
9	201	Horse	t064	-	8	+	IVd	dt10a
10	10	Human	t084	15	15	-	-	-
11	21	Human	t084	-	15	-	-	-
12	23	Human	t228	15	15	-	-	-
13	60	Human	t360	15	15	-	-	-
14	24	Human	t491	15	15	-	-	-
15	38	Human	t020	22	22	+	IVh	dt10a
16	44	Human	t032	22	22	+	IVh	dt10a
17	28	Human	t032	-	22	+	IVh	dt10a
18	32	Human	t032	-	22	+	IVh	unknown
19	40	Human	t032	-	22	+	IVh	dt10a
20	39	Human	t379	22	22	+	IVh	dt10a
21	18	Human	t608	22	22	-	-	-
22	20	Human	unknown	22	22	-	-	-
23	5	Human	t012	30	30	-	-	-
24	13	Human	t012	-	30	-	-	-
25	34	Human	t018	36	30	+	IVh	dt7c
26	65	Human	t018	-	30	+	IV	dt7c
27	51	Human	t019	30	30	-	-	-
28	62	Human	t021	30	30	-	-	-
29	1	Human	t122	30	30	-	-	-
30	31	Human	t1675	36	30	+	IV	dt9a
31	174	Cattle	t131	1527	97	-	-	-
32	189	Cattle	t224	97	97	-	-	-
33	198	Cattle	t224	-	97	-	-	-

[a] isolates selected for further characterisation based on PFGE analysis results thus each ID represents a unique PFGE clonal type (as described in 3.2)

[b] '-' not determined

[c] determined or presumptive (based on *spa* type)

[d] '-' absent, '+' present

[e] '-' not applicable, NT: non-typeable

[f] '-' not applicable

Table 3.1. (continued) Summary of genotypic features of analysed strains [a]

No	ID	Host	*spa*	ST [b]	CC [c]	*mecA* [d]	SCC*mec* [e]	*dru* [f]
34	177	Cattle	t267	118	97	-	-	-
35	197	Cattle	t267	-	97	-	-	-
36	190	Cattle	t359	97	97	-	-	-
37	195	Cattle	t359	-	97	-	-	-
38	175	Cattle	t521	97	97	-	-	-
39	173	Cattle	t6220	130	130	-	-	-
40	178	Cattle	t843	1245	130	-	-	-
41	181	Cattle	t843	-	130	-	-	-
42	182	Cattle	t843	-	130	-	-	-
43	179	Cattle	t529	1074	151	-	-	-
44	193	Cattle	t529	-	151	-	-	-
45	194	Cattle	t529	-	151	-	-	-
46	199	Cattle	t529	-	151	-	-	-
47	90	Chicken	t011	398	398	+	IVc	dt10a
48	91	Chicken	t011	-	398	+	V 5C2&5	dt9v
49	92	Chicken	t011	-	398	+	IVa	dt10q
50	99	Horse	t011	-	398	+	IVa	dt10q
51	102	Rat	t011	-	398	+	IVa	dt10q
52	103	Rat	t011	-	398	+	V 5C2&5	dt11a
53	105	Cattle	t011	-	398	+	IVa	dt10q
54	106	Cattle	t011	-	398	+	V 5C2&5	dt11y
55	108	Pig	t011	-	398	+	V 5C2&5	dt11a
56	109	Pig	t011	-	398	+	IVa	dt10q
57	95	Human	t034	398	398	+	V 5C2&5	dt11a
58	96	Human	t034	-	398	+	IVc	dt10a
59	111	Pig	t034	-	398	+	IVa	dt10q
60	101	Horse	t1451	398	398	+	IVa	unknown
61	104	Rat	t4872	398	398	+	V 5C2&5	dt11v
62	93	Chicken	t567	398	398	+	NT	dt11af
63	107	Cattle	t567	-	398	+	NT	dt11af
64	110	Pig	t567	-	398	+	NT	dt11af

[a] isolates selected for further characterisation based on PFGE analysis results thus each ID represents a unique PFGE clonal type (as described in 3.2)

[b] '-' not determined

[c] determined or presumptive (based on *spa* type)

[d] '-' absent, '+' present

[e] '-' not applicable, NT: non-typeable

[f] '-' not applicable

3.4 Discussion

Methods routinely used in the *S. aureus* genotyping were applied to determine the genotypic background and heterogeneity of a panel of human- and animal-associated isolates, including the MRSA CC398 group. The overall aim was to select strains for further characterisation involving comparative analysis of MRSA CC398 against strains of the remaining clonal complexes. The main selection criterion applied was PFGE genotype so that all the selected isolates demonstrated a unique fingerprint type. Furthermore, the comparator panel (non-CC398) was selected to include clonal complexes that were represented by at least four strains and consisted of: CC5, CC8, CC15, CC22, CC30, CC97, CC130 and CC151.

All CC5 isolates identified in this study were *mecA*-negative although the lineage has been previously associated predominantly with major clones of MRSA such as UK EMRSA-3, New York/Japan and Paediatric (Deurenberg *et al.*, 2007). Two of the *spa* types detected among CC5 strains: t002 and t179 were previously reported as related to the described clones (Deurenberg *et al.*, 2007). However, the MSSA CC5 isolates were previously also identified as common among community acquired (CA) isolates causing skin and soft tissue infections in the UK and other countries (Larsen *et al.*, 2008). CC5 is therefore recognized as one of the endemic MSSA lineages from which MRSA had originated (Deurenberg and Stobberingh, 2008). Similarly to CC5, all CC15 isolates described in this study lacked the *mecA* gene but unlike the CC5, the lineage is predominantly MSSA-associated and as such it has been reported to represent a successful lineage among community- and hospital-acquired (HA) MSSA (Deurenberg and Stobberingh, 2008; Nulens *et al.*, 2008; Donker *et al.*, 2009; Argudin *et al.*, 2009). CC22 and CC30 represent the two dominant HA-MRSA lineages that have become epidemic in the UK hospitals (Johnson *et al.*, 2001). Strains identified as ST22-SCC*mec* IV are representative of the UK EMRSA-15 clone, with t032 known as the most prevalent *spa* type (Deurenberg *et al.*, 2007). The two *mecA*-positive strains identified as ST36-t018 are partly representative of the UK EMRSA-16, but were found to carry the SCC*mec* type IV instead of type II (Deurenberg *et al.*, 2007). CC8 is a successful lineage of both CA- and HA-MRSA, although in this study the human-associated isolates of CC8 were exclusively *mecA*-negative (Deurenberg *et al.*, 2007). MRSA CC8 is also recognized

as globally predominant lineage among MRSA isolates from horses (Moodley *et al.*, 2006; Walther *et al.*, 2009; Tokateloff *et al.*, 2009).

All cattle related isolates analysed in this study were assigned to common bovine-associated lineages: CC97, CC130 and CC151, with CC97 and CC151 in particular often reported as causative agents of bovine mastitis (Smith *et al.*, 2005; Jorgensen *et al.*, 2005; Rabello *et al.*, 2007; Ikawaty *et al.*, 2009; Hasman *et al.*, 2010b; Hata *et al.*, 2010; Delgado *et al.*, 2011). Recently, isolates belonging to CC97 were also found in pigs and a MRSA clone of CC97 was isolated from humans including both healthy carriers, as well as cases of hospital-acquired infections (Battisti *et al.*, 2010; Gomez-Sanz *et al.*, 2010; Udo *et al.*, 2011; Schuenck *et al.*, 2009).

The PFGE analysis revealed a varying level of heterogeneity among the identified clonal complexes. It has been previously observed that the MSSA population is considerably more heterogeneous than the population of MRSA (Strommenger *et al.*, 2008). In accordance with this, the majority of *mecA*-negative human-associated strains demonstrated a considerable *spa* type diversity that was particularly prominent among strains belonging to CC5 and CC15. In contrast, the majority of *mecA*-positive strains of CC22 was found to carry a single dominant *spa* type, t032, whereas isolates belonging to CC30 demonstrated a significant PFGE pulsotype clonality. The results demonstrate the particularly highly clonal population structure of MRSA isolates, which consists mostly of epidemic lineages (Deurenberg *et al.*, 2007).

The clonality of cattle-associated isolates varied between the lineages. Isolates belonging to CC97 demonstrated both PFGE and *spa* type heterogeneity whereas CC130 and CC151 were comparatively less diverse. Although all three clonal complexes are known to be bovine-related, their frequency of detection among *S. aureus* from cattle varies, with CC97 reported more often (Hata *et al.*, 2010; Rabello *et al.*, 2007; Smith *et al.*, 2005). The higher prevalence might suggest that CC97 has become a bovine-associated lineage earlier than CC130 or CC151, which would allow for the development of intra-lineage diversity.

The MRSA CC398 group consisted of both single unique fingerprint types as well as clonal groups but overall the panel was heterogeneous with 18 strains out of 23 selected for further characterisation. Based on PFGE % identity for isolates belonging to the same clonal complex, the CC398 demonstrated the highest level of

variation. The identification of three clonal groups that consisted of isolates from different sources suggests that the particular clone was transferred between the different host species. For instance identical clones were isolated from pig and chicken, as well as pig and horse. Whilst the possibility of a direct transfer between the animals cannot be excluded, the strains might have been cross-transferred by a colonised human as previous studies have reported that persons working at farms represent a likely vector of MRSA CC398 transmission and dissemination (Khanna *et al.*, 2008; van Duijkeren *et al.*, 2008). The predominant *spa* type among the MRSA CC398 strains was t011, which is in agreement with previous reports (van Belkum *et al.*, 2008; Van den Eede *et al.*, 2009; Harlizius *et al.*, 2008; Huijsdens *et al.*, 2009).

The *mecA*-positive strains included in this study were additionally characterised by SCC*mec* typing with type IV identified among the majority of strains. SCC*mec* type IV is considered to be widely disseminated due to its relatively small size and it is commonly harboured by CA-MRSA strains (Kluytmans-Vandenbergh and Kluytmans, 2006). According to previously published studies the predominant SCC*mec* element among MRSA CC398 strains was type V (de Neeling *et al.*, 2007; van Duijkeren *et al.*, 2008; Lewis *et al.*, 2008; Smith *et al.*, 2009), which has been identified among only a proportion of MRSA CC398 strains analysed in this study. Also, three strains within the CC398 panel were identified as SCC*mec* non-typeable, which is consistent with the previously described limitations of the PCR-based SCC*mec* typing techniques.

The most common *dru* type amongst the analysed MRSA strains was dt10a, which was detected in the majority of CC22 MRSA strains, as well as both CC8 and two CC398. This is in agreement with previous studies that reported dt10a as a prevalent *dru* type amongst MRSA CC22 isolates, which carry SCC*mec* type IV (Shore *et al.*, 2010; Creamer *et al.*, 2012). In contrast, the majority of MRSA CC398 strains with SCC*mec* type IV displayed *dru* type dt10q, as previously reported (Fessler *et al.*, 2010; Monecke *et al.*, 2013). Amongst the remaining MRSA CC398 strains, some carried *dru* type dt11a, which appears to be associated with SCC*mec* type V (Fessler *et al.*, 2010; Monecke *et al.*, 2013). Interestingly, the three MRSA CC398 strains non-typable for the SCC*mec* type by PCR-based method, were all found to carry *dru* type dt11af. This might suggest the better suitability of *dru* typing for subtyping of MRSA isolates based on the SCC*mec* carriage. However, the identification of the same *dru* type amongst strains carrying distinct SCC*mec* sub-

types poses the question of the *dru* typing specificity in distinguishing between the distinct SCC*mec* cassettes. As a single locus-based method, *dru* typing might in some instances lack the power of differentiation amongst MRSA isolates. This might be the case if the rate of genetic changes that occur within the variable region of the *mecA* complex is not mirrored by changes across the entire region of SCC*mec* cassette.

Isolates selected on the basis of the described genotypic analysis were further comparatively characterised using more specialist approaches, which involved: screening for the carriage of virulence and adhesin genes; carriage of phage-associated genes; sequence analysis of the vSaα genomic island; investigation of a potential to adhere to porcine skin cells and to form biofilm; analysis of antimicrobial and biocide resistance genotypes and phenotypes.

Chapter 4 Analysis of virulence genotypes and phenotypes

4.1 Introduction

The ability of *S. aureus* to cause a diverse spectrum of disease is mediated by a wide range of virulence-associated factors (Archer, 1998). Comparative genomic analyses have revealed that certain putative virulence determinants, such as α-haemolysin or nuclease genes are highly conserved and constitute a part of the core genome of *S. aureus* (Lindsay and Holden, 2004; Holden and Lindsay, 2008). However, a significant proportion of virulence genes are variably detected between isolates (Holden and Lindsay, 2008). Collectively these genes are components of the *S. aureus* accessory genome (Lindsay and Holden, 2004). Some are components of putatively mobile elements, most notably the *S. aureus* genomic islands, such as vSaα and vSaβ (Baba *et al.*, 2002). A greater proportion of the accessory virulence genes are associated with mobile genetic elements such as plasmids, bacteriophages and pathogenicity islands, and thus can be acquired by strains through horizontal gene transfer (Malachowa and DeLeo, 2010).

Investigation of virulence gene carriage among *S. aureus* isolates often constitutes a fundamental part of descriptive studies, as due to the plasticity of the accessory genome, the epidemiology of virulence genotypes needs to be monitored (Holden *et al.*, 2004; Argudín *et al.*, 2013). The heterogeneous distribution of virulence factors within *S. aureus* populations is not entirely random and association between certain lineages and virulence determinants has been shown to exist (Peacock *et al.*, 2002; Lindsay *et al.*, 2006). Nevertheless, the accessory virulence genes can be suddenly lost or acquired (Moore and Lindsay, 2001). The analysis of virulence gene content can be limited to only few determinants, although more comprehensive studies of virulence gene carriage in *S. aureus* are commonly conducted, which allows investigation of associations between virulence genotype and infection type or clinical origin of isolate (Nashev *et al.*, 2004; Becker *et al.*, 2003; Tristan *et al.*, 2003; Ferry *et al.*, 2005). In addition to the analysis of virulence gene carriage, isolates are often concomitantly subjected to *agr* class typing and designation (Moore and Lindsay, 2001; Jarraud *et al.*, 2002; Peacock *et al.*, 2002). The Agr (accessory gene regulator) system encoded by the *agr* locus regulates expression of virulence genes in *S. aureus* and four classes of *agr* are recognised (Ji, Beavis and Novick, 1997; Jarraud *et al.*, 2000; Fournier, 2008). The *agr* genes belong to the core variable genome as the system can be identified in all isolates with variable class distribution (Lindsay *et al.*,

2006). Generally, *agr* type carriage has been found to be homogenous among isolates belonging to the same clonal complex (Robinson *et al.*, 2005b; Monecke *et al.*, 2007a).

Detection of genes is usually conducted by PCR, using primers designed to specifically identify each individual gene. To facilitate a more cost and time efficient screening method several multiplex PCR typing schemes have been developed (Monday and Bohach, 1999; Tristan *et al.*, 2003; Martín, González-Heviac and Mendozaa, 2003). However, a truly robust screening of gene carriage has been facilitated with the development of DNA microarray methodology. This involves use of a glass chip (microarray) that has been printed with DNA probes, specific for selected genes. The capacity to detect genes is limited by the number of probes on the microarray. *S. auerus*-specific microarrays developed in the last few years vary in their analytical scope, from platforms that allow detection of all relevant virulence and antimicrobial resistance determinants to highly comprehensive microarrays containing probes against all open reading frames identified across seven *S. aureus* genomes (Monecke *et al.*, 2007a; Witney *et al.*, 2005). DNA microarrays have facilitated a rapid and high throughput screening of bacterial genome content. In the case of *S. aureus*, a number of DNA microarray-based studies have been conducted that contributed to the elucidation of *S. aureus* genomic diversity, evolution of epidemic strains, host specificity and relationship between isolates from different host species (Saunders *et al.*, 2004; Monecke *et al.*, 2007a; Monecke *et al.*, 2007b; Herron-Olson *et al.*, 2007; Sung, Lloyd and Lindsay, 2008).

The ability to attach to host cells is an essential property of *S. aureus* isolates that mediates both asymptomatic colonisation as well as infection (Elasri *et al.*, 2002; Corrigan, Miajlovic and Foster, 2009). Thus isolates are often screened for the carriage of genes encoding host cell adherence-associated elements known as microbial surface components recognizing adhesive matrix molecules (MSCRAMM) (Tristan *et al.*, 2003). When investigating the capacity of *S. aureus* to adhere to host cells, the genotypic analysis can be complemented with *in vitro* adhesion assays. The presence of MSCRAMM molecules can be examined by conducting specific binding assays using purified matrix molecules, whereas the potential for adhesion to host cells can be investigated with cell or tissue adhesion assay (Vancraeynest, Hermans and Haesebrouck, 2004; Kintarak *et al.*, 2004; Cho *et al.*, 2001). Although the latter investigates the ability to adhere through undefined ligands, it has been utilised in

studying *S. aureus* host specificity by determining the capacity of strains for attachment to cells isolated from different host species (Mcewan, 2000; Uhlemann *et al.*, 2012). Some of the adhesin genes are known to encode proteins that mediate biofilm formation by *S. aureus*, more specifically the *ica* locus and *bap* (Cramton *et al.*, 1999; Cucarella *et al.*, 2001). As such these elements are commonly included when investigating the carriage of virulence determinants and *S. aureus* isolates can be assessed for *in vitro* for biofilm formation (Vasudevan *et al.*, 2003).

The analysed *S. aureus* strains were screened for the carriage of virulence-associated determinants including superantigen, exotoxin, adhesin, *set* and *agr* genes, followed by sequence analysis of the *set* region. Due to the significance of bacteriophages in mediating horizontal transfer of virulence determinants between *S. aureus* isolates, the strains were also screened for presence of phage integrase genes. The analysis of virulence phenotype involved examining the potential to attach to porcine keratinocytes and to form biofilms.

4.2 Materials and methods

All strains (n=64) described in Table 3.1 have been included in the analysis of virulence genotypes. The virulence genes screened for are presented in Table 2.2, with the adhesion genes listed in section 2.4.7.2. Analysis of virulence gene carriage was conducted as described in sections 2.4.6 and 2.4.7.2. The screening of bacteriophage integrase genes was performed as described in section 2.4.7.3. A proportion of strains was selected for keratinocyte adhesion and biofilm formation assays (n=44), which consisted of representatives of all analysed clonal complexes: CC5 (n=4), CC8 (n=4), CC15 (n=4), CC22 (n=4), CC30 (n=4), CC97 (n=4), CC130 (n=4), CC151 (n=4) and CC398 (n=12). For clonal complexes consisting of more than four strains, where applicable the selection was based on *spa* type diversity. An additional selective criterion for the CC398 group was host species variation and strains from chicken, horse, cattle, pig and human were included. The biofilm formation assay was conducted as described in section 2.7, whereas the keratinocyte adhesion assay was performed in accordance with section 2.8. The sequence analysis of the vSaα genomic island was conducted on a single representative of each analysed clonal complex (with an exception of CC8, as described in section 4.3.3) and performed as described in section 2.4.8. PCR amplification of the analysed region of the vSaα was performed by Long-Range PCR as described in section 2.3.3 followed

by sequencing of the fragment by primer walking. The analysis of the sequence data was conducted as described in section 2.3.5.3. The raw assembled sequence data is provided in a FASTA file format (Electronic Appendix I).

4.3 Results

4.3.1 Carriage of virulence genes

4.3.1.1 Superantigen genes

The carriage of enterotoxin genes is presented in Figure 4.1. Enterotoxin gene *seX* was detected in all analysed strains, while *seY* was identified in all analysed clonal complexes (CC) with 7 strains from various lineages (CC8, CC15, CC97 and CC398) lacking the gene. Enterotoxin A gene *seA* was detected at low prevalence in three CCs: CC5 (n=1), CC8 (n=2), CC30 (n=5). All but one strain encoded the *seA-var1* gene with a single positive strain from CC5 carrying the *seA-var3* gene variant. The *seB+seK+seQ* eneterotoxin gene cluster was detected in a single CC8 strain, with another CC8 strain carrying the *seK+seQ* genes. Furthermore, several strains belonging to various lineages were positive for *seB* only: CC5 (n=1), CC22 (n=5), CC97 (n=3). The *seD+seJ+seR* gene cluster was identified in all but one CC5 strain and no other CC group, whereas the *seC+seL* genes were present in 9 strains belonging to CC22 (n=5), CC30 (n=1) and CC97 (n=3). The *egc* cluster: *seG+seI+seM+seN+seO+seU* was prevalent among strains belonging to CC5, CC22, CC30 and CC151, but absent from all other CC groups. The *entCM14* gene, which encodes an enterotoxin-like protein, was present in all CC151 strains, but in no other lineages analysed. Finally, the *tst* gene was variably detected in several CC: CC8 (n=1), CC22 (n=2), CC30 (n=4), and CC97 (n=3). The *tst* gene can be carried on a pathogenicity island that also contains the *seC+seL* genes (Fitzgerald *et al.*, 2001a). In this study four of the *tst*-positive strains (three CC97 and one CC22) also carried the *seC+seL* gene cluster. All strains were negative for *seE* and *seH* genes.

As demonstrated in Figure 4.1 all strains belonging to CC15, CC130 and CC398 carried no accessory enterotoxin genes. The analysis of superantigen genotypes revealed that CC398 strains formed a heterogeneous cluster together with strains of other CCs that also lacked accessory enterotoxin genes. The overall profile similarity for all strains was 19%.

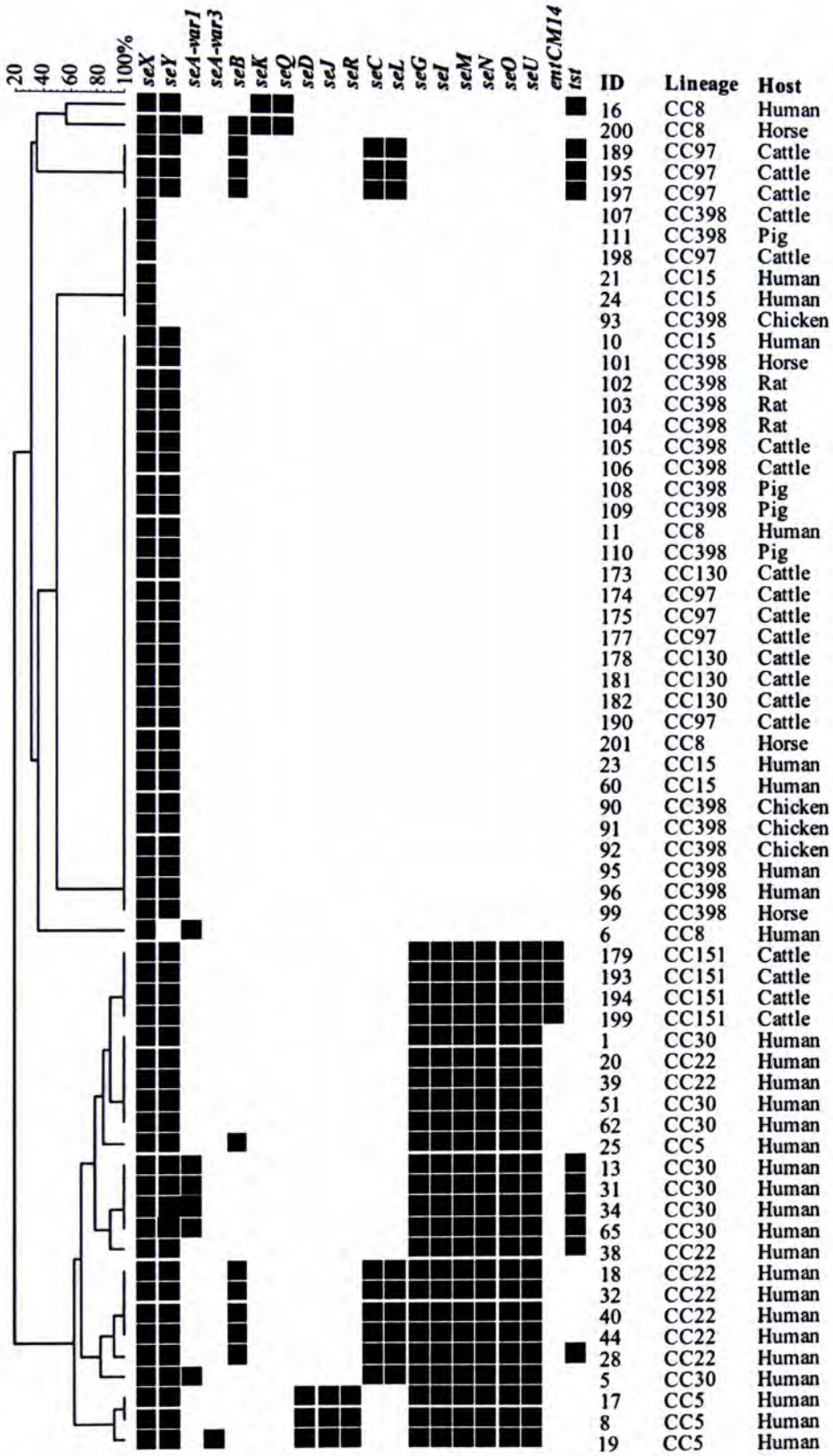


Figure 4.1 UPGMA dendrogram displaying clustering of superantigen gene profiles of the analysed *S. aureus* panel. Black box – positive; white/blank – negative.

4.3.1.2 The *agr* locus and *set* genes

The carriage of *set* genes and *agr* type are presented in Figure 4.2. Strains belonging to the same lineage displayed an identical accessory gene regulator (*agr*) type: CC8, CC22, CC97 and CC398 harboured *agrI*; CC5, CC15 and CC151 displayed *agrII*; whereas CC30 and CC130 contained *agrIII*.

The *set* genes encode Staphylococcal exotoxin-like proteins. The inter-lineage variation for *set* carriage can be defined by two factors: presence/absence of a particular gene and the allelic variant. The following genes: *set1*, *set4*, *set5* and *set7* were detected in all CCs, but the allelic variants differed between lineages. In the case of the *set4* gene, the true positive variant could not be identified due to strong cross-reactivity of the probes and thus the strains appear to carry both *set4-var1* and *set4-var2* gene variants. As revealed in Figure 4.2, the majority of the remaining *set* genes were highly prevalent and absent in only one of the analysed lineages: *set3* (not detected in CC130), *set6* (not detected in CC97), *set8* (not detected in CC398), *set9* (not detected in CC151). For *set6*, the majority of strains was found to carry one of four of the previously described allelic variants. Strains belonging to CC15 and CC151 displayed a distinct combination of positive probes, different for both lineages, and were termed *var5* and *var6*, respectively. Three *set* genes were less common: *set2* (detected in CC5, CC8, CC15, CC30, CC151 and CC398), *set12* (detected in CC5, CC8, CC15, CC97, CC130 and CC151) and *set21* (detected in CC8 and CC15).

The microarray platform also contains probes for the detection of *setB* gene cluster, which consists of *setB3*, *setB2* and *setB1*. The complete cluster was detected in all CC groups except CC22. Another gene encoding enterotoxin-like protein, *setC* was also highly prevalent and could be detected in all strains apart from those belonging to CC30 group. The comparison of *set* genotypes demonstrated a considerable level of variation between the analysed lineages. The overall profile similarity was 21% with strains belonging to the same CC forming a homogenous cluster. The CC398 lineage appeared most closely related to the CC30 group with 56% similarity. The two lineages shared allelic variants for *set2*, *set3*, *set7*, *set9*, *setB3* and *setB2*.

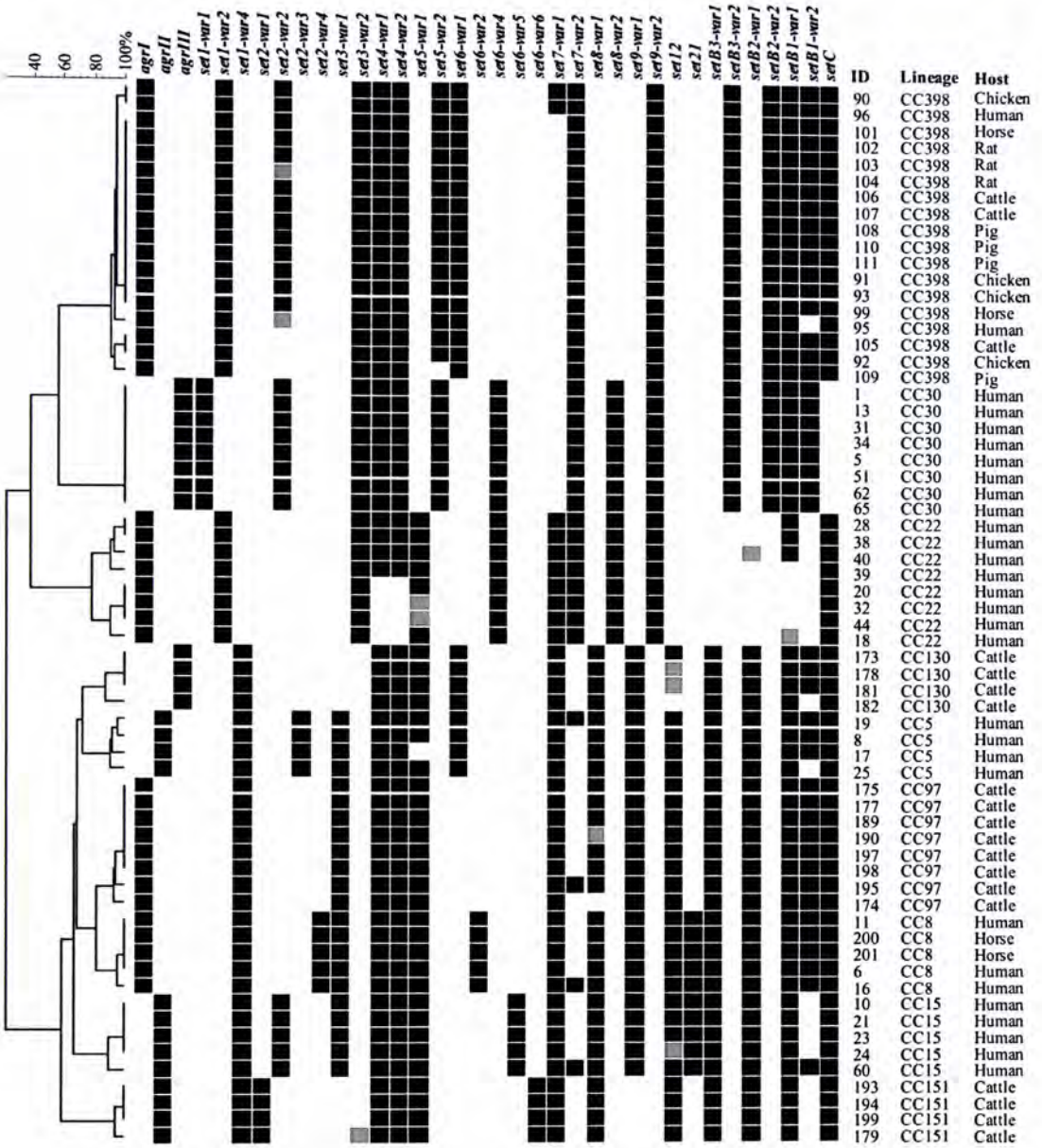


Figure 4.2 UPGMA dendrogram displaying clustering of *agr* and *set* gene profiles of the analysed *S. aureus* panel.. Black box – positive; grey – ambiguous; white/blank – negative.

4.3.1.3 Leukocidin, haemolysin and other virulence genes

The carriage of leukocidin, haemolysin and other virulence genes is presented in Figure 4.3. All strains carried the *lukF/S*, *hlgA*, *hl*, *hla* and *hld* genes. All strains were also positive for the *lukY* gene, with the *lukY-var1* variant detected for all strains except those belonging to CC30. In this group all strains carried the gene variant *lukY-var2*. The *lukX* gene was detected in all CC groups, but not all analysed strains. This was particularly prominent within the CC398 group as the *lukX* gene was detected in less than half of the analysed strains (n=7). The *lukD/E*, *splA* and *splB* genes, known to reside together on an allelic variant of the vSaβ genomic island (Baba *et al.*, 2008) were all detected in strains belonging to CC5, CC8, CC15 (one strain *lukE* negative), CC97 and CC151.The genes except for *lukE* were also detected among CC130 strains. The *lukF-PV-P83+lukM* gene cluster was detected only in CC151 strains, although the *lukF-PV-P83* gene only was variably detected amongst strains belonging to CC5, CC8 and CC97. The PVL leukocidin gene locus *lukF/S-PV* was present in three strains, two belonging to CC30 and one to CC15.

An intact *hlb* gene (not disrupted by *hlb*-converting bacteriophage) was present in all strains belonging to CC130 and CC151. It was also detected in the majority of CC97 strains and in more than half of CC398 strains (n=11). Amongst the *hlb*-negative strains the *sak* gene was detected in over 60% (22/36), mainly those belonging to CC5, CC22 and CC30. The *hl-III* haemolysin gene was also variably detected and it was found in all strains belonging to CC5, CC8, CC15, CC30 and CC398, as well as majority of CC97 strains. Finally the *edinB* gene was identified in all CC130 strains but no other lineage. All strains were negative for the carriage of *etA, etB, etD, edinA* and *edinC* genes.

Cluster analysis of genotype similarity based on the carriage of leukocidin, haemolysin and other described virulence genes revealed an overall 55% similarity. All CC398 strains formed a homogenous cluster, which was most closely related to the CC22 cluster at 70% similarity. Strains belonging to CC398, CC22 and CC30 shared 64% similarity forming a major distinct cluster.

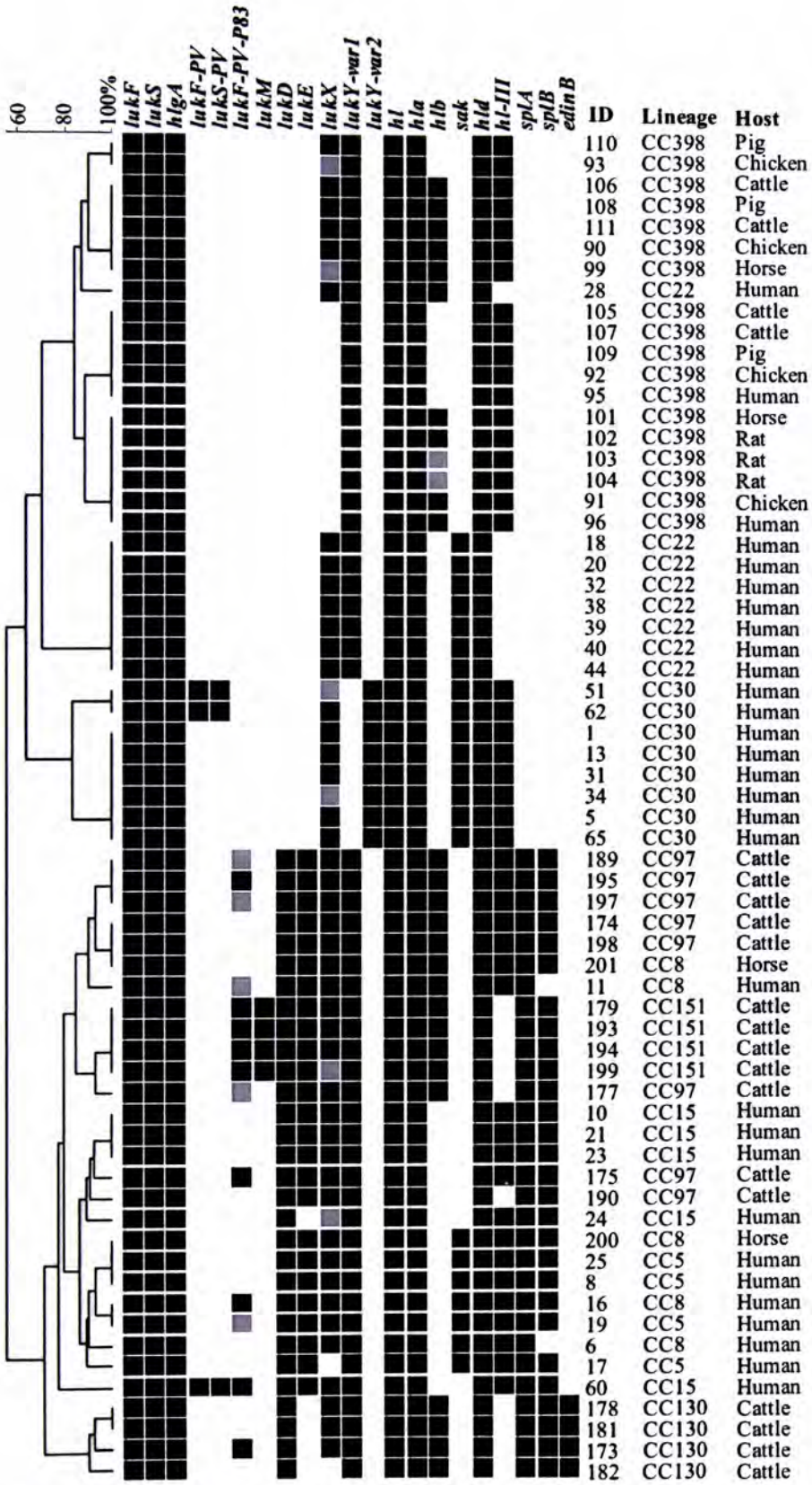


Figure 4.3 UPGMA dendrogram displaying clustering of leukocidin, haemolysin and other virulence gene profiles of the analysed *S. aureus* panel. Black box – positive; grey – ambiguous; white/blank – negative.

4.3.1.4 Adhesin genes

The carriage of adhesin genes is presented in Figure 4.4. The genes: *clfB*, *ebpS*, *fnbA*, *icaA* and *icaD* were identified in all strains. The following genes were highly prevalent, but not detected in all analysed lineages: *clfA* (not detected in CC130), *eno* (not detected in CC398), *fib* (not detected in CC22), *sdrC* (not detected in CC5 and CC22) and *sdrE* (not detected in CC30 and CC151). Adhesin genes that were less common among the analysed clonal complexes were: *bbp* (detected in CC30 and CC151), *cna* (detected in CC22, CC30 and CC398), *fnbB* (detected in CC8, CC15, CC97 and CC398), *sasG* (detected in CC5, CC8 and CC97) and *sdrD* (detected in CC5, CC8, CC15, CC22 and CC97). All strains were negative for the *bap* gene. The cluster analysis of adhesin genotypes revealed an overall 62% similarity for all analysed strains. The majority of analysed lineages, including the CC398 group formed a homogenous cluster. The CC398 cluster shared 69% similarity with the nearest cluster group, which was formed of CC5, CC8, CC15, CC97 and CC130.

4.3.1.5 Statistical analysis of association between virulence determinants and clonal complexes

Clustering of strains based on the identified virulence genotypes revealed that the distribution of analysed virulence determinants was mostly lineage associated, with all or the majority of strains within each lineage carrying highly homogenous sets of the virulence elements. The statistical significance of distributions of individual genes was therefore investigated using contingency table analysis and Chi-square test (0.001 alpha level).

Only genes found to be variably distributed among analysed clonal complexes were tested thus determinants found in all lineages were excluded. The distribution of all tested genes is presented in Table 4.1 together with the calculated p values. The CC398 group contained a considerably higher number of strains than the remaining clonal complex groups and it was anticipated that the statistical analysis might be biased with a single lineage accounting for 28% of all analysed strains leading to either false positive or false negative errors. Thus the analysis was repeated after excluding the CC398 strains. It was observed that for some of the genes the p value change was significant enough to alter the data interpretation (moved over or below the 0.001 alpha level).

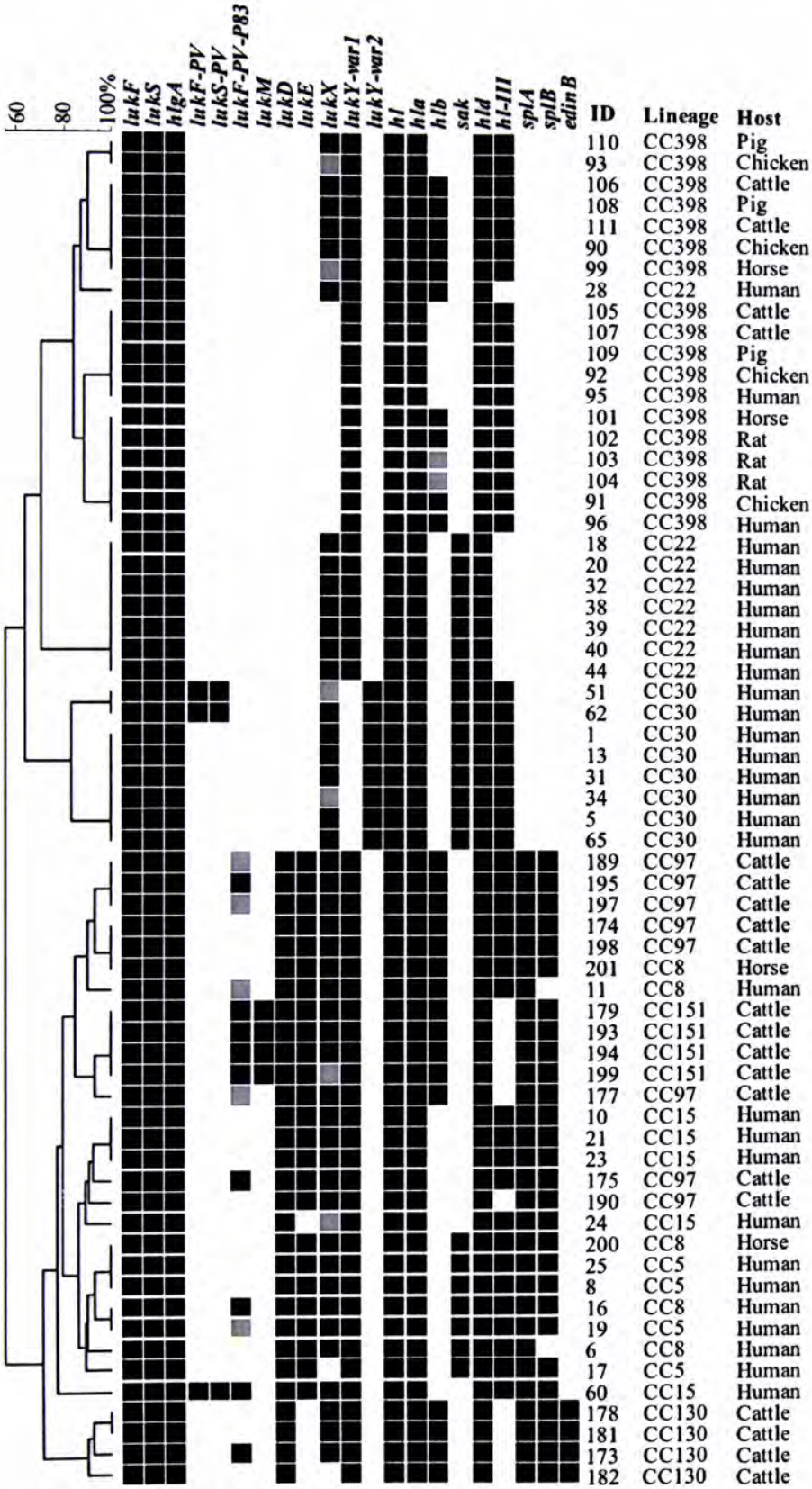


Figure 4.4 UPGMA dendrogram displaying clustering of adhesin gene profiles of the analysed *S. aureus* panel. Black box – positive; white/blank – negative.

Table 4.1 Distribution of detected genes amongst analysed clonal complexes and statistical significance of association. The values in each lineage column show: number of strains from the lineage that were included in the analysis, followed by a number of strains from this lineage that was positive for the corresponding virulence gene.

Lineage	CC5	CC8	CC15	CC22	CC30	CC97	CC130	CC151	CC398	*p* value [a]
No. of strains	4	5	5	8	8	8	4	4	18	
seA-var1	0	2	0	0	5	0	0	0	0	< 0.001*
seA-var3	1	0	0	0	0	0	0	0	0	0.059
seB	1	1	0	5	0	3	0	0	0	0.010
seC+L	0	0	0	5	1	3	0	0	0	0.005
seD+J+R	3	0	0	0	0	0	0	0	0	< 0.001
egc	4	0	5	8	8	0	0	0	0	< 0.001*
seK	0	1	0	0	0	0	0	0	0	0.160
seQ	0	1	0	0	0	0	0	0	0	0.160
entCM14	0	0	0	0	0	0	0	4	0	< 0.001
tst	0	1	0	2	4	3	0	0	0	0.070
agrI	0	5	0	8	0	8	0	0	18	0.002**
agrII	4	0	5	0	0	0	0	4	0	< 0.001
agrIII	0	0	0	0	8	0	4	0	0	< 0.001
set1-var1	0	0	0	0	8	0	0	0	0	< 0.001
set1-var2	0	0	0	8	0	0	0	0	18	< 0.001
set1-var4	4	5	5	0	0	8	4	4	0	< 0.001*
set2-var1	0	0	0	0	0	0	0	4	0	< 0.001
set2-var2	0	0	5	0	8	0	0	0	15	< 0.001
set2-var3	4	0	0	0	0	0	0	0	0	< 0.001
set2-var4	0	5	0	0	0	0	0	0	0	< 0.001
set3-var1	4	5	5	0	0	8	0	0	0	< 0.001*
set3-var2	0	0	0	8	8	0	0	1	18	< 0.001
set5-var1	3	5	5	8	0	8	4	4	0	0.001
set5-var2	0	0	0	0	8	0	0	0	17	< 0.001
set6-var1	4	0	0	0	0	0	4	0	18	< 0.001
set6-var2	0	5	0	0	0	0	0	0	0	< 0.001
set6-var4	0	0	0	8	8	0	0	0		< 0.001
set6-var5	0	0	5	0	0	0	0	0	0	< 0.001
set6-var6	0	0	0	0	0	0	0	4	0	< 0.001
set8-var1	4	5	4	0	0	7	4	4	0	< 0.001*
set8-var2	0	0	0	8	8	0	0	0	0	< 0.001
set9-var1	4	5	5	0	0	8	4	0	0	< 0.001*
set9-var2	0	0	0	8	8	0	0	0	18	< 0.001

[a] *p* value caluculated based on contingency table analysis and Chi-square test

* *p* value change to > 0.001 when CC398 group removed from analysis

** *p* value change to < 0.001 when CC398 group removed from analysis

Table 4.1 (continued) Distribution of detected genes amongst analysed clonal complexes and statistical significance of association. The values in each lineage column show: number of strains from the lineage that were included in the analysis, followed by a number of strains from this lineage that was positive for the corresponding virulence gene.

Lineage	CC5	CC8	CC15	CC22	CC30	CC97	CC130	CC151	CC398	*p* value[a]
No. of strains	4	5	5	8	8	8	4	4	18	
set12	4	5	5	0	0	8	3	4	0	< 0.001*
set21	0	5	5	0	0	0	0	0	0	< 0.001
setB2-var1	4	5	5	1	0	8	4	4	0	< 0.001*
setB2-var2	0	0	0	0	8	0	0	0	18	< 0.001
setB3-var1	4	5	5	0	0	8	4	4	0	< 0.001*
setB3-var2	0	0	0	0	8	0	0	0	18	< 0.001
setC	4	5	5	8	0	8	4	4	18	0.433
hlb	0	2	0	1	0	6	4	4	11	0.021
hlIII	4	5	5	0	8	6	0	0	18	0.044
lukD	4	5	5	0	0	8	4	4	0	< 0.001*
lukE	4	5	4	0	0	8	0	4	0	< 0.001*
splA	4	5	5	0	0	8	4	4	0	< 0.001*
splB	4	3	5	0	0	8	4	4	0	< 0.001*
lukD/E+splA/B	4	3	4	0	0	8	0	4	0	< 0.001*
lukF/S-PV	0	0	1	0	2	0	0	0	0	0.154
lukF-PV-P83	1	2	1	0	0	5	1	4	0	0.001
lukF/M-PV-P83	0	0	0	0	0	0	0	4	0	< 0.001
lukY-var1	4	5	5	8	0	8	4	4	18	0.433
lukY-var2	0	0	0	0	8	0	0	0	0	< 0.001
edinB	0	0	0	0	0	0	4	0	0	< 0.001
sak	4	3	0	7	8	0	0	0	0	< 0.001*
bbp	0	0	0	0	8	0	0	4	0	< 0.001
clfA	4	5	5	8	8	8	0	4	18	0.788
cna	0	0	0	8	8	0	0	0	18	< 0.001
eno	4	5	5	8	8	8	4	4	0	0.652
fib	4	5	5	0	8	8	4	4	18	0.362
fnbB	0	5	5	0	0	8	0	0	18	0.003**
sasG	4	5	0	0	0	8	0	0	0	< 0.001
sdrC	0	5	5	0	8	8	4	4	18	0.129
sdrD	4	3	5	8	0	8	0	0	0	< 0.001
sdrE	4	5	4	7	0	7	4	0	18	0.168

[a] *p* value caluculated based on contingency table analysis and Chi-square test

* *p* value change to > 0.001 when CC398 group removed from analysis

** *p* value change to < 0.001 when CC398 group removed from analysis

After collating both data sets together (with and without CC398 isoolates) consistent statistical association with the underlying clonal complex was detected for the following genes / gene complexes ($p < 0.001$): *seD+R+J*, *entCM14*, *agrII*, *agrIII*, *set1-var*, *set1-var2*, *set2-var1*, *set2-var2*, *set2-var3*, *set2-var4*, *set3-var2*, *set5-var2*, *set6-var1*, *set6-var2*, *set6-var4*, *set6-var5*, *set6-var6*, *set8-var2*, *set9-var2*, *set21*, *setB-var2*, *lukF-PV+lukM*, *lukY-var2*, *edinB*, *cna*, *bbp* and *sasG*. The majority of genes for which analysis without the CC398 group caused a significant shift in the *p* value were found to loose their statistically valid clonal complex association. Only two genes, namely *agrI* and *fnbB* gained statistically significant association with corresponding lineages after removing CC398 cluster from analysis.

4.3.2 Analysis of bacteriophage integrase gene carriage

The bacteriophage integrase multiplex PCR, based on detection of Sa*int* types, was developed by Goerke *et al.* (2009) to investigate the prevalence of prophages amongst *S. aureus* isolates. As described by the authors, the integrase genes are conserved providing a high level of bacteriophage discriminatory power and the integrase type is closely correlated with the virulence gene content of the respective bacteriophage (Goerke *et al.*, 2009). The scheme distinguishes between seven types of integrase genes, from Sa*int1* to Sa*int7*. The distribution of the genes is presented in Table 4.2. The lowest prevalence of prophage integrase genes was detected within the CC15 and CC130 groups with only a single positive strain in each lineage. In contrast, considerable prophage diversity was observed amongst strains belonging to CC22 and CC30, as each lineage revealed the presence of five distinct integarse gene types. The highest prevalence was observed for the Sa*int3* and Sa*int6* integrase genes. The Sa*int3* was detected in strains belonging to CC5, CC8, CC22, CC30 and CC97, whereas the Sa*int6* was identified in CC22, CC30, CC97, CC151 and CC398. Interestingly, the CC398 group revealed the presence of two other integrase gene types, namely the Sa*int1* and Sa*int2* although Sa*int6* was the most prevalent.

Table 4.2 Distribution of bacteriophage integrase genes amongst analysed lineages. The values in each lineage column show: number of strains from the lineage that were included in the analysis, followed by a number of strains from this lineage that were positive for the corresponding integrase gene.

Lineage	CC5	CC8	CC15	CC22	CC30	CC97	CC130	CC151	CC398
No. of strains	4	5	5	8	8	8	4	4	18
Sa*int1*	0	0	0	2	0	0	0	0	5
Sa*int2*	0	0	1	1	1	0	0	0	8
Sa*int3*	3	3	0	4	4	3	0	0	0
Sa*int4*	0	0	0	0	0	0	0	0	0
Sa*int5*	0	2	0	0	3	1	1	0	0
Sa*int6*	0	0	0	3	1	3	0	4	11
Sa*int7*	0	1	0	1	2	0	0	2	0

4.3.3 Sequence analysis of the *set* region of the vSaα genomic island

The analysis of virulence gene carriage revealed that the MRSA CC398 strains share a number of *set* genes with the CC30 lineage. To investigate further the level of *set* homology between the MRSA CC398 and other analysed clonal complexes selected strains were further investigated, which involved sequence analysis of the chromosomal region containing the *set* genes. PCR amplification of the *set* region in three representatives of each clonal complex revealed that the *set* fragment was homologus in size amongst strains belonging the same lineage, with the exception of CC8. Thus for the sequence analysis a single representative of each clonal complex was selected, with the exception of CC8, for which two strains were included for the analysis. As such the analysis panel consisted of the following strains: 19-CC5, 6-CC8, 200-CC8, 10-CC15, 28-CC22, 5-CC30, 175-CC97, 173-CC130, 179-CC151 and 90-CC398.

As demonstrated by Figure 4.5 the *set* region structure was largely conserved amongst the analysed strains with homologous gene order and orientation. The analysed *set* region sequences ranged in size between 8079 base pairs (bp) and 11604 bp. The variation in size was associated with differences in the *set* gene content between the analysed strains. Fundamentally, all strains contained genes *set6*, *set7*, *set8*, *set3*, *set1*, *set5* and *set4* with genes *set9*, *set12* and *set21* detected amongst some but not all strains. As such, strain 28-CC22 was the only one that demonstrated the presence of all the shared *set* genes and no other elements whereas strains 6-CC8 and

10-CC15 revealed carriage of all described *set* genes. Interestingly, some discrepancies with the results of microarray *set* gene analysis were observed. This involved the *set3*, *set6* and *set8* genes, which based on the DNA microarray results, were described as absent in all strains belonging to CC130, CC97 and CC398, respectively. Furthermore, the *set9* gene was detected by DNA microarray in all strains except for those belonging to CC151, whereas the sequence analysis revealed that this gene, in addition to 179-CC151, was also absent in 28-CC22, 175-CC97 and 200-CC8. In fact, the lack of *set9* was identified as the key variable feature between the strains 6-CC8 and 200-CC8. While the DNA microarray analysis included the *set2* gene, this determinant is located downstream of the analysed *set* region and is separated from the described *set* genes by the *hsdM* determinant and as such was not detected within the amplified section of the vSaα genomic island.

To analyse nucleotide sequence homology of the *set* genes from investigated strains, the sequence percentage similarity was determined by cluster analysis based on multiple sequence alignments. As revealed by Figure 4.6, the complete *set* sequence fragments shared 65% identity. The 90-CC398 strain shared the highest level of homology with the *set* region from the 5-CC30 strain, which is in accordance with the *set* cluster analysis described in 4.3.1.2. Homology analysis of individual *set* genes demonstrated that the 90-CC398 strain most closely resembled the equivalent in the 5-CC30 strain for the following genes: *set3*, *set5*, *set7*, *set8* and *set9* with the level of sequence similarity varying from 87% up to 99%.

For the genes *set1* and *set4*, the 90-CC398 strain shared most homology with the 28-CC22 strain although both formed a sequence homology cluster with the 5-CC30. Interestingly, in case of the *set6* gene, the 90-CC398 strain shared most identity with the 19-CC5 strain and was distinct from both 5-CC30 and 28-CC22.

A comparative analysis of two strains from the same clonal complex, namely the 6-CC8 and 200-CC8, revealed that the strains shared 100% sequence identity for most of the *set* genes, which included: *set1*, *set4*, *set5*, *set6*, *set7* and *set12*. Some variation was observed in the sequence of the *set3* gene (~99% identity) whereas the *set8* gene was considerably distinct between the two strains as demonstrated by 89% sequence identity.

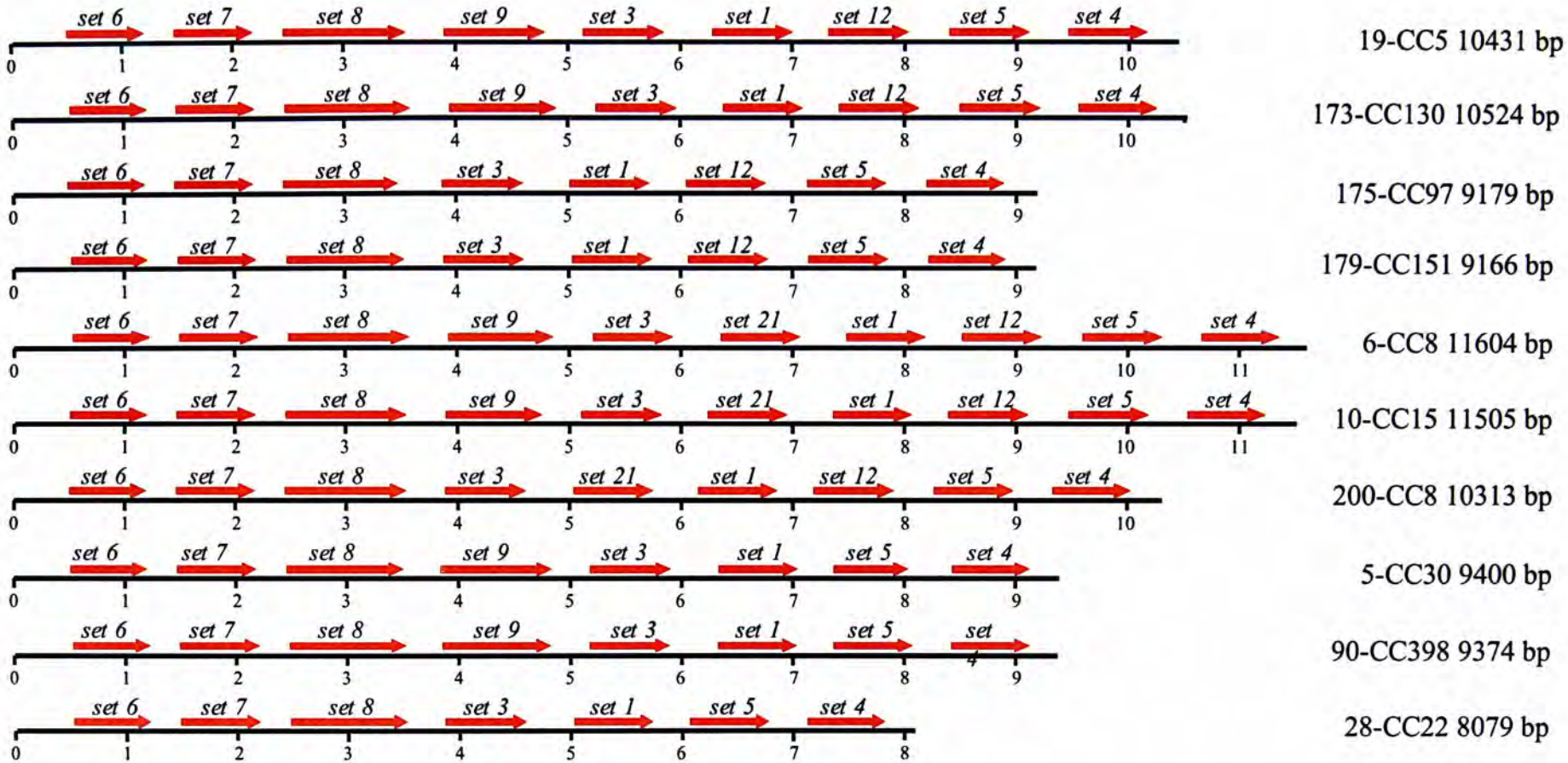


Figure 4.5 Schematic genetic structure of the vSaα genomic island *set* region from the analysed strains. The red arrows represent coding sequences with the gene name shown above. The region length scale is presented in kilobase pairs. The corresponding strain, lineage as well as exact size in base pairs (bp) is shown on the right.

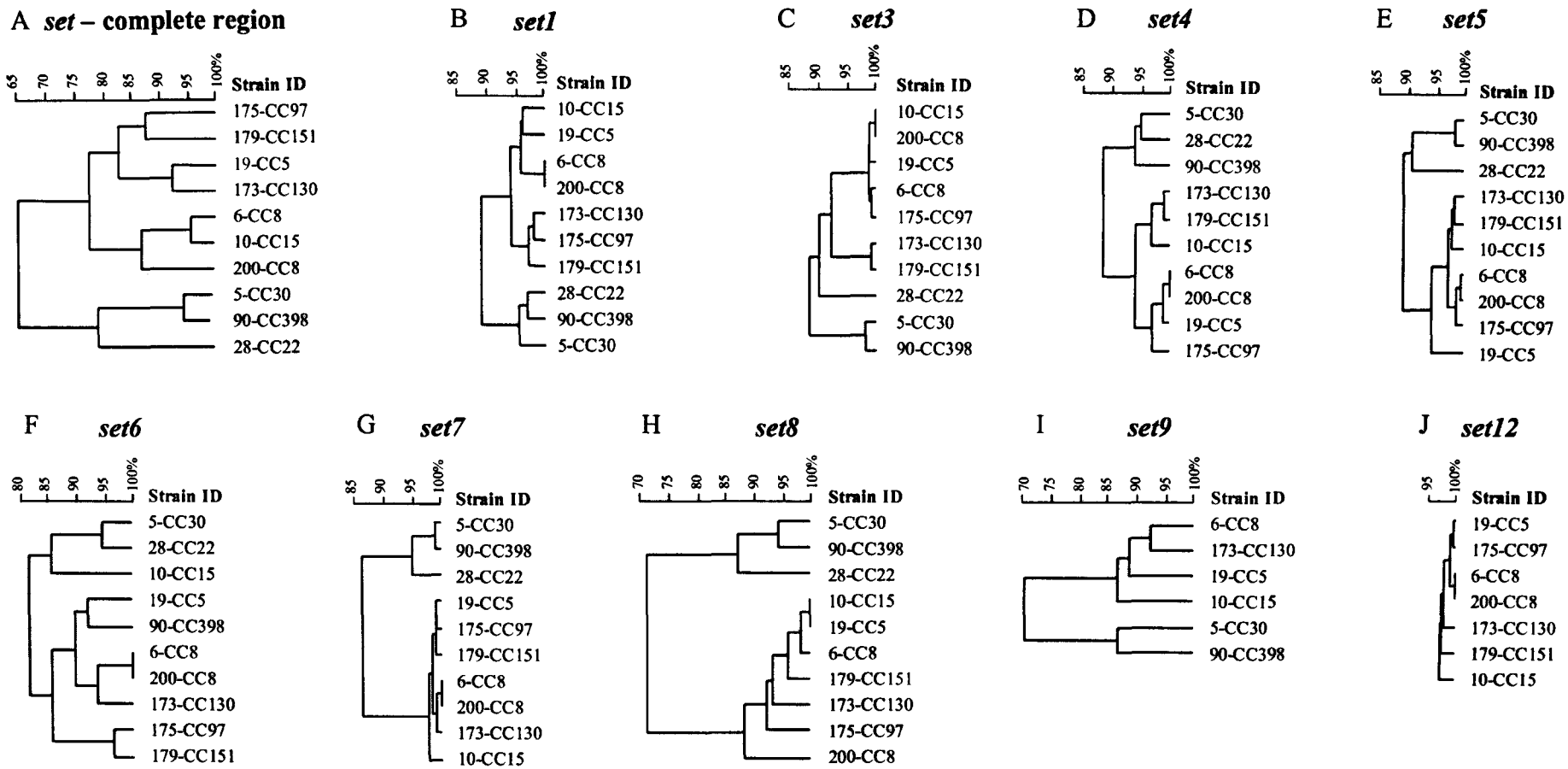


Figure 4.6 Nucleotide sequence identity analyses of the *set* region and *set* genes from the analysed strains. The UPGMA clustering was calculated based on multiple alignment of nucleotide sequences, as described in 2.4.8. Individual figures show A: complete *set* region, B: *set1* gene, C: *set3* gene, D: *set4* gene, E: *set5* gene, F: *set6* gene, G: *set7* gene, H: *set8* gene, I: *set9* gene, J: *set12* gene

4.3.4 Porcine keratinocyte adhesion assay

The adhesion assay was conducted using keratinocyte cell culture derived from two different animals and the two cell cultures will be referred to as P1 and P2. The analysed strains were found to vary considerably in their potential to adhere to keratinocytes. Capacity for adhesion to P1 among all the strains varied from 0.04% to 21%, whereas the adhesion to P2 was in the range of between 0.05% and 14%. The variation could be observed at both inter- and intra-lineage level. Based on the average adhesion rates of strains belonging to the same lineage (Figure 4.7) the highest capacity for adhesion to P1 was observed for CC22 (15%) and CC30 strains (10%), followed by considerably lower CC97 (2.5%) and CC398 (1.3%) strains. Variation in average adhesion between CC398 and other clonal complexes was statistically significant ($p < 0.05$), only with CC22 and CC151 (Table 4.3).

The highest average rates of adhesion to P2 (Figure 4.7) were observed for strains beloning to CC22 (9%) and CC30 (8%) followed by CC398 strains, but with considerably lower value (1.4%). The remaining clonal complexes displayed adhesion rates of 1% or less.Variation in average adhesion between CC398 and other clonal complexes was statistically significant ($p < 0.05$) with CC5, CC15, CC22 and CC151 (Table 4.3). Comparative analysis of adhesion capacity of all strains within lineage (Figures 4.8) showed considerable differences between the highest and the lowest value, which was particularly prominent for strains belonging to CC97 and CC398. Furthermore, as demonstrated by Figure 4.8, most strains revealed a statistically significant variation in their capacity to attach to P1 and P2.

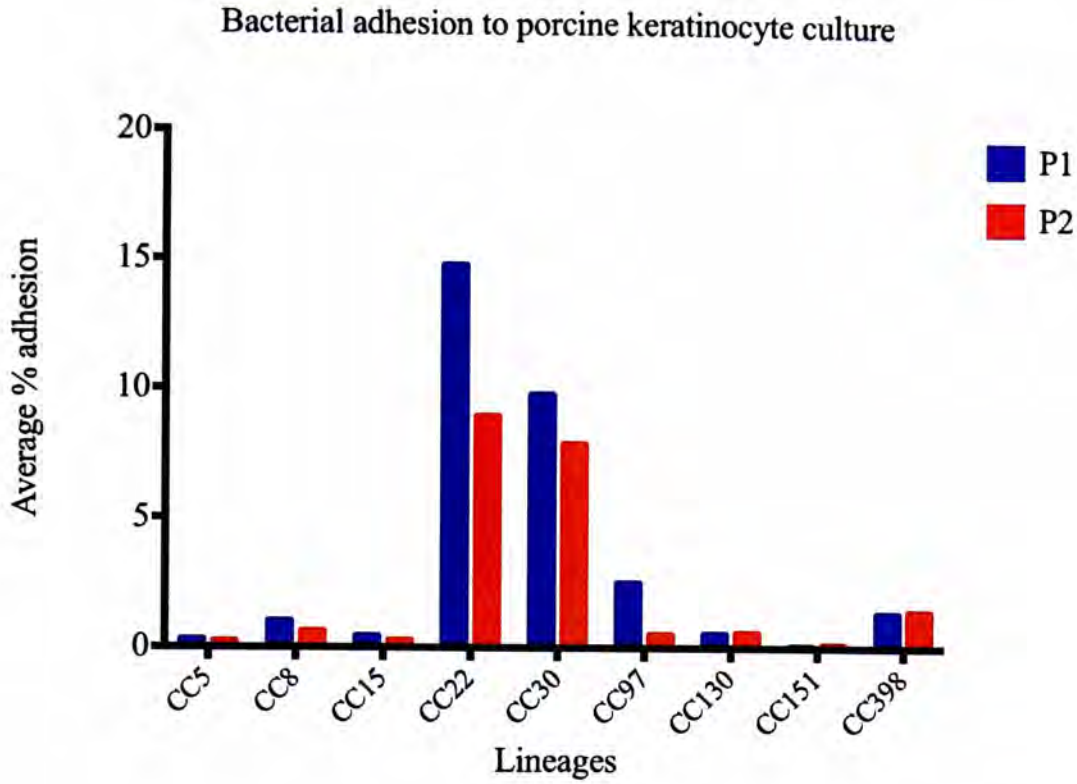


Figure 4.7 Comparative representation of bacterial adhesion to porcine keratinocyte cultures P1 and P2, demonstrated as average % adhesion of strains belonging to the same lineage

Table 4.3 Average adhesion values to porcine keratinocyte cultures P1 and P2 for each analysed clonal complex

	CC5	CC8	CC15	CC22	CC30	CC97	CC130	CC151	CC398
P1									
Average[a]	0.30	1.01	0.44	14.79	9.80	2.50	0.54	0.07	1.33
SEM[b]	0.03	0.38	0.12	3.12	4.07	2.31	0.10	0.02	0.50
p value[c]	0.06	0.62	0.11	0.02*	0.13	0.65	0.14	0.03*	
P2									
Average	0.22	0.61	0.28	8.94	7.87	0.51	0.58	0.12	1.40
SEM	0.04	0.17	0.09	2.15	2.53	0.33	0.23	0.04	0.46
p value	0.03*	0.13	0.03*	0.04*	0.08	0.14	0.13	0.02*	

[a] Mean of adhesion values for all strains within the lineage

[b] Standard error of mean

[c] Based on *t* test of statistical significance of variation in average adhesion between the CC398 and the corresponding clonal complex. Statistically significant *p* values (< 0.05) are marked with *

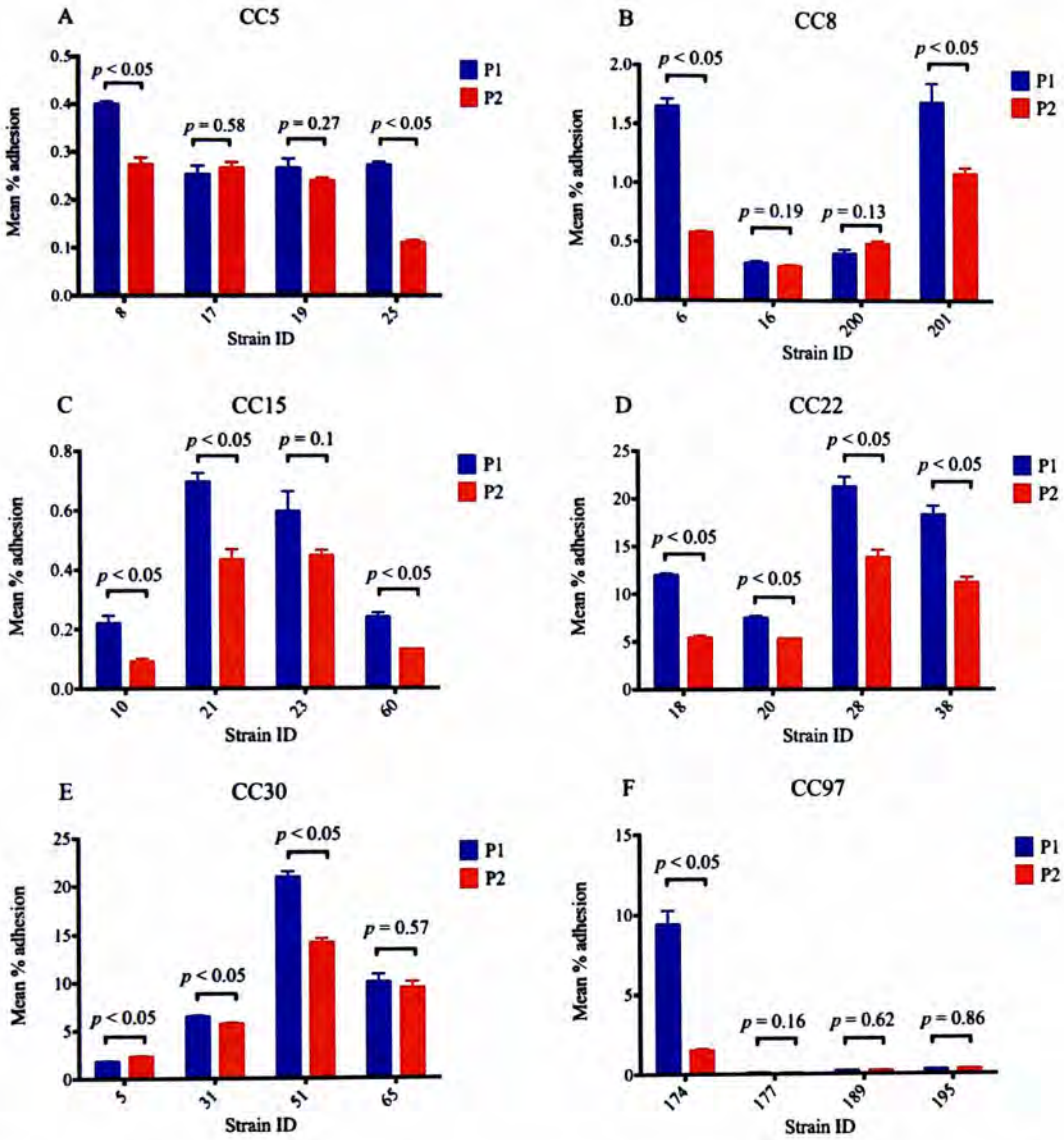


Figure 4.8 Comparative representation of bacterial adhesion to porcine keratinocyte cultures P1 and P2. Demonstrated as mean % adhesion of each strain within the analysed lineages: A - CC5, B - CC8, C - CC15, D - CC22, E- CC30 and F - CC97. Error bars are based on three biological replicates and represent SEM; the *p* value was calculated with *t* test. Continued on next page.

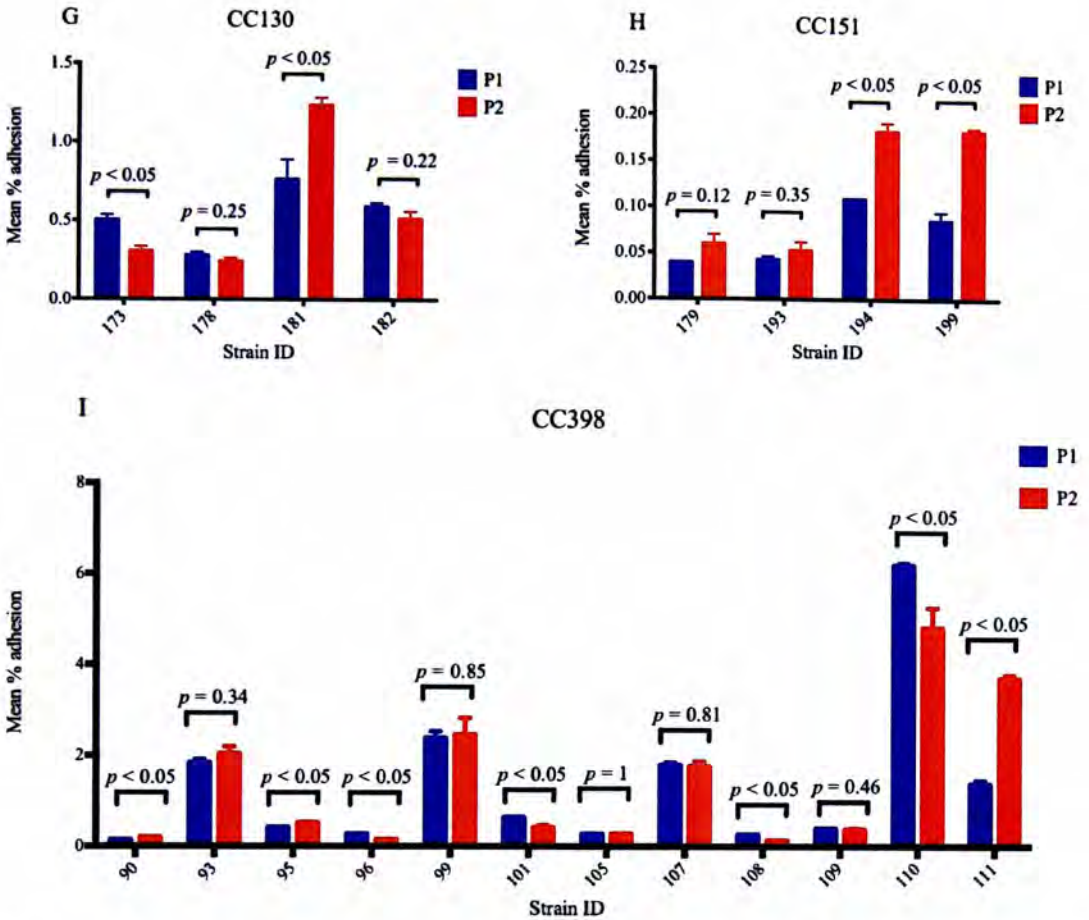


Figure 4.8 (continued) Comparative representation of bacterial adhesion to porcine keratinocyte cultures P1 and P2. Demonstrated as mean % adhesion of each strain within the analysed lineages: G - CC130, H - CC151 and I - CC398. Error bars are based on three biological replicates and represent SEM; the *p* value was calculated with *t* test.

4.3.5 Biofilm formation

Biofilm formation was assessed at 37° C and 25° C and the analysis involved those strains that were selected for the keratinocyte adhesion assay. In accordance with the method described by Mack *et al.* (2000) an OD_{490nm} value of 0.1 was applied as an arbitrary threshold for calling strains biofilm producers. The biofilm formation OD_{490nm} values at 37° C ranged from 0.017 to 1.336, with 32 strains (73%) identified as biofilm formers, which included all strains belonging to CC5, CC8, CC15, CC130 and all but one strain from CC22 and CC97 (Table 4.4). Among the strains that did not demonstrate biofilm formation, the majority (8/12) belonged to the CC398 group. The mean of OD_{490nm} values was calculated for all strains belonging to the same clonal complex for the inter-lineage comparative analysis. The CC30 was found to demonstrate the highest biofilm activity, whereas CC398 revealed the lowest. However, a considerable intra-lineage variation in potential to form biofilm could be observed. Standard error of mean (SEM) in OD_{490nm} values was calculated for each clonal complex group and it was observed that a high mean value was associated with a high SEM, demonstrating that enhanced capacity for biofilm formation was mostly strain-specific rather than a clonal complex feature. The CC30 group revealed both the highest mean and SEM values with a single strain showing the strongest biofilm activity among all analysed strains, that was twice the value of the second most prominent biofilm former within the whole panel. After CC30 the second highest mean value was observed for CC130 and the third for CC97.

The biofilm formation OD_{490nm} values at 25° C ranged from 0.001 to 1.306 (Table 4.4). A considerable drop in the number of biofilm producers could be observed in comparison to 37° C, as only 10 strains (23%) demonstrated an OD_{490nm} value of > 0.1. Within the CC130 group all but one strain remained biofilm formers, followed by CC8 (2/4), CC30 (1/4) and CC97 (1/4). Biofilm production was also detected for three out of 12 CC398 strains. Among the strains that revealed biofilm formation at 25° C, four demonstrated at least 50% drop in the value when compared with activity at 37° C, three showed comparable values at the two temperatures and further three had higher OD_{490nm} values at 25° C (at least 25% increase). The highest mean of lineage OD_{490nm} values was observed for strains belonging to CC30, which again was associated with a particularly high biofilm OD_{490nm} of a single strain and a high SEM value.

Table 4.4 Biofilm formation OD_{490nm} values of each analysed strain at 37° C and 25° C

		37° C			25° C		
Lineage	ID	OD_{490nm} [a,b]	Mean [c]	SEM [d]	OD_{490nm}	Mean	SEM
CC5	8	0.197	0.176	0.026	0.095	0.069	0.020
CC5	17	0.101			0.012		
CC5	19	0.222			0.068		
CC5	25	0.185			0.099		
CC8	6	0.250	0.214	0.043	0.117	0.171	0.078
CC8	16	0.128			0.068		
CC8	200	0.160			0.098		
CC8	201	0.319			0.402		
CC15	10	0.160	0.148	0.014	0.047	0.075	0.011
CC15	21	0.148			0.095		
CC15	23	0.109			0.068		
CC15	60	0.177			0.088		
CC22	18	0.097	0.162	0.025	0.035	0.058	0.008
CC22	20	0.174			0.075		
CC22	28	0.215			0.066		
CC22	38	0.162			0.057		
CC30	5	1.336	0.515	0.278	1.306	0.356	0.317
CC30	31	0.362			0.050		
CC30	51	0.145			0.068		
CC30	65	0.215			0.001		
CC97	174	0.467	0.236	0.082	0.206	0.080	0.042
CC97	177	0.096			0.054		
CC97	189	0.148			0.030		
CC97	195	0.234			0.029		
CC130	173	0.170	0.403	0.095	0.059	0.258	0.121
CC130	178	0.622			0.167		
CC130	181	0.463			0.610		
CC130	182	0.359			0.196		
CC151	179	0.080	0.099	0.008	0.044	0.038	0.008
CC151	193	0.118			0.041		
CC151	194	0.107			0.054		
CC151	199	0.092			0.015		

[a] Blank subtracted

[b] Shaded field if OD_{490nm} value greater than 0.1

[c] Average of OD_{490nm} values of all strains within clonal complex

[d] Standard error of the mean

Table 4.4 (continued) Biofilm formation OD_{490nm} values of each analysed strain at 37° C and 25° C

		37° C			25° C		
Lineage	ID	OD_{490nm} [a,b]	Mean [c]	SEM [d]	OD_{490nm}	Mean	SEM
CC398	90	0.035	0.082	0.017	0.026	0.077	0.012
CC398	93	0.182			0.155		
CC398	95	0.156			0.134		
CC398	96	0.017			0.066		
CC398	99	0.067			0.049		
CC398	101	0.087			0.122		
CC398	105	0.091			0.092		
CC398	107	0.144			0.074		
CC398	108	0.023			0.044		
CC398	109	0.017			0.042		
CC398	110	0.126			0.083		
CC398	111	0.043			0.040		

[a] Blank subtracted

[b] Shaded field if OD_{490nm} value greater than 0.1

[c] Average of OD_{490nm} values of all strains within clonal complex

[d] Standard error of the mean

4.4 Discussion

In this work a panel of strains representing nine different clonal complxes was investigated for carriage of a wide range of virulence factors that included superantigen, leukocidin, haemolysin, staphylococcal exotoxin-like protein (Set) and adhesin genes. To investigate further the level of *set* gene homology, the chromosomal region containing the *set* gene cluster was amplified and sequenced in selected representatives of each lineage. Strains were also screened for the presence of bacteriophage-associated integrase genes. Furthermore, a proportion of the strains was investigated for capacity to adhere to porcine keratinocytes, as well as biofilm formation activity.

The comprehensive analysis of virulence-associated gene carriage revealed heterogeneity mainly at the inter-lineage level with strains that belonged to the same clonal complex found to display an identical or similar virulence genotype. A proportion of the detected genes were carried by all analysed strains. Amongst the variably detected virulence determinants, the majority appeared to be associated with an underlying clonal complex. The statistical analysis of the distribution of these elements found that some but not all were indeed significantly associated with the

corresponding lineage. The lack of association was observed mainly for determinants that were very rare or highly prevalent, which has been previously interpreted as lack of association due to lack of power and a type II error (Peacock *et al.*, 2002). It can be speculated that the genes found to be highly prevalent, but still not carried by all clonal complexes represent determinants that have been relatively stable and conserved across the majority of *S. aureus* genomes, but loss or decay occurred at very low rates in only few lineages.

The different classes of genes were analysed separately and this led to observation that there is a variable level of intra-lineage virulence genotype homogeneity depending on the gene group. Clustering based on the carriage of the adhesin and *set* genes was largely clonal complex-related, such that main clusters were formed by strains belonging to the same lineage. Some dispersion of strains could be observed with leukocidin / haemolysin genotype clustering and finally the distribution of superantigen genes resulted in mainly heterogeneous clusters. As the superantigen genes are commonly carried on mobile genetic elements such as plasmids, bacteriophages and pathogenicity islands, their distribution is largely governed by horizontal gene transfer (Omoe *et al.*, 2003). Only the *seD+R+J* gene cluster and the *entCM14* gene were found to be significantly associated with the underlying clonal complex. Interestingly, the *tst* gene was found to lack lineage association, despite being previously reported as significantly related to a clonal complex, more specifically the CC30 (Peacock *et al.*, 2002).

The comparative analysis of virulence gene carriage across all strains included in this study revealed that the CC398 strains feature a limited content of virulence determinants. This was particularly evident among genes encoding superantigens and leukocidins. Analysis of both gene categories separately demonstrated that in each group certain other lineages, in addition to the CC398 strains, also showed a lack of a large proportion of genes. However, the CC398 was the only lineage amongst the analysed clonal complexes that lacked any significant accessory virulence determinants from both gene categories. The minimal virulence gentoype of CC398 isolates has been previously observed and later confirmed by the whole genome analysis of an MRSA CC398 strain (Kadlec *et al.*, 2009; Fessler *et al.*, 2010; Schijffelen *et al.*, 2010). The majority of virulence genes that were detected in CC398 strains analysed in this work, were common amongst all analysed clonal complexes as

they represent the core virulence genes of *S. aureus* and only few were found to be variably carried by strains of other lineages. The latter included *hlb*, *cna* and *fnbB*.

An intact *hlb*, which encodes a β-haemolysin, was detected in the majority of CC398 strains as well as in cattle-associated strains belonging to CC97, CC130 and CC151. The gene is chromosomally located, but it might become inactivated by insertion of *hlb*-converting phages that carry immune evasion genes such as *sak*, *chp* and *scn* (Coleman *et al.*, 1986; Coleman *et al.*, 1989; Carroll, Cafferkey and Coleman, 1993; van Wamel *et al.*, 2006). Although these elements can provide the strain with a selective advantage, they encode human-specific molecules (van Wamel *et al.*, 2006). As such, the carriage of staphylokinase gene *sak* is prevalent amongst human strains whereas the presence of an intact *hlb* is common amongst cattle isolates (Monecke *et al.*, 2007b; Delgado *et al.*, 2011). Furthermore *hlb* has been shown to be actively expressed in bovine isolates of *S. aureus* (Aarestrup *et al.*, 1999). In this study all strains carrying an intact *hlb* were negative for the *sak* gene, confirming that the presence of an intact *hlb* gene and carriage of *sak* is commonly antagonistic. Interestingly, some strains belonging to CC398, CC97 and CC15 carried a disrupted *hlb* but were *sak*-negative, which might indicate insertion of a different phage. The carriage of an intact *hlb* was not significantly associated with underlying clonal complexes, most likely caused by non-uniform distribution of the gene amongst positive clonal complexes, ranging from a single representative to all strains.

Two of the adhesion genes detected amongst the CC398 strains displayed limited prevalence in other clonal complexes: *fnbB* and *cna*. The *fnbB* gene is a homologue of *fnbA* and both genes encode fibronectin-binding protein. Although *fnbA* is highly prevalent and was identified in all analysed strains, the *fnbB* gene was only detected in CC398, CC8, CC15 and CC97. It has been shown that the presence of both *fnbA* and *fnbB* can significantly enhance biofilm formation in MRSA isolates (O'Neill, Humphreys and O'Gara, 2009). Furthermore, the cooperative function of *fnbA*- and *fnbB*-expressed molecules was shown to be significant for the development of systemic infection (Shinji *et al.*, 2011). The *cna* gene was detected in only two other lineages, namely CC22 and CC30. The *cna* gene was previously reported as prevalent amongst pandemic CA-MRSA isolates belonging to ST30 (Otsuka *et al.*, 2006). It was also suggested that the gene might play a role in facilitating stable colonisation (Nashev *et al.*, 2004). In a recent study of mastitis-associated isolates, the *cna* gene was reported to be more common in bovine than human isolates (Delgado *et*

al., 2011). In the work described here, the *cna* gene was found to be significantly associated with the underlying lineages.

Analysis of the overall virulence genotypes revealed a varying level of similarity in virulence profile between the CC398 lineage and other clonal complexes based on the % identity of the formed clusters. The comparison of superantigen genotypes revealed that the lack of enterotoxin genes was not a unique feature of CC398 as the same profile was identified for all strains belonging to CC15 and CC130, originating from humans and cattle, respectively. Comparing genes associated with leukocidins, haemolysins and serine proteases shows that CC398 strains most closely resemble the CC22 and CC30 groups, as all three groups lack *lukD/lukE*, *splA* and *splB* genes. These three lineages also displayed similar *set* gene profiles. Interestingly, both CC22 and CC398 lineages were found to carry CC30-specific allelic variants of some of the *set* genes. The high level of homology between *set* genes carried by CC30 and CC398 strains was also demonstrated by *set* region sequence analysis. The exact function of *set*-encoded exotoxin-like proteins remains to be established. However, it has been demonstrated that the *set* products can stimulate human peripheral blood mononuclear cells to secrete proinflammatory cytokines (Williams *et al.*, 2000). Furthermore it has been shown that proteins encoded by different variants of the same *set* gene can differ significantly in their immunogenic capacity (Williams *et al.*, 2000). The carriage of a particular allelic variant can therefore have impact on host-pathogen interaction. Identification of *set* markers that are shared between CC398 and other successful MRSA lineages such as CC30 and CC22 can provide candidate genes for further investigation of individual *set* genes and their role in host colonisation and infection.

Certain interesting clonal complex-virulence gene associations could be observed for lineages other than CC398. The *lukF-PV-P83/lukM* locus, which encodes a bicomponent leukotoxin that is highly active against bovine neutrophils, was previously reported to be associated only with cattle isolates related to the RF122 strain (Monecke, Slickers and Ehricht, 2008). In this study these genes were also detected only amongst the CC151 strains and were absent in other cattle-associated strains belonging to CC97 and CC130. Furthermore, the carriage of the entire *lukF-PV-P83/lukM* locus was significantly associated with the CC151 lineage. However, this relationship diminished when carriage of only *lukF-PV-P83* was taken into account since the gene was variably detected amongst strains other than CC151. The

enterotoxin gene cluster *egc*, was detected in all CC5, CC22, CC30 and CC151 strains. This cluster has been previously reported to be more common amongst carriage isolates rather than invasive isolates (van Belkum *et al.*, 2006; Nashev *et al.*, 2004). In this study such association could not be investigated, instead the *egc* cluster appeared to be prevalent amongst specific *S. aureus* lineages. However, the statistical analysis deemed the *egc* cluster distribution as not significantly associated with the underlying clonal complexes. The results also indicated that the allelic variant of staphylococcal genomic island vSaβ carrying *splA*, *splB*, *lukD* and *lukE* might be prevalent in CC5, CC8, CC15, CC97, CC130 and CC151, although it was only for CC5, CC97 and CC151 that all strains analysed carried all four genes. Similarly to *egc*, however, the genes were not significantly related to their underlying clonal complexes, individually as well as a cluster. Another intriguing finding was detection of the *setC* gene in all strains except for those belonging to CC30, which can be interpreted as an outcome of carrying the SaPI4 pathogenicity island that was found to displace the *setC* gene in the MRSA252 strain (Monecke, Slickers and Ehricht, 2008).

The analysis of bacteriophage-associated gene carriage revealed that prophages were prevalent amongst the majority of clonal complexes. Strains belonging to CC398 demonstrated high frequency of Sa*int6*, followed by Sa*int2* and Sa*int1*. This is in accordance with the description of MRSA CC398 S0385 strain whole genome content that revealed the presence of two baceriophages φSa6S0385 and φSa2S0385, which were found to lack any known virulence determinants (Schijffelen *et al.*, 2010). Common amongst other analysed clonal complexes was the Sa*int3* integrase gene type, which was previously reported as the most prevalent prophage group detected in *S. aureus* (Goerke *et al.*, 2009). The Sa*int3* prophage type has also been identified as frequent carrier of the immune evasion genes *sak*, *chp* and *scn* (Goerke *et al.*, 2009). Interestingly, in the work described here the Sa*int3* was particularly prevalent amongst lineages that contained *sak*-positive strains and was absent in all strains belonging to CC15, CC130, CC151 and CC398, which were uniformly *sak*-negative. The results of this analysis confirm that bacteriophgaes are prevalent mobile elements amongst *S. aureus* isolates, however, their contribution to the accessory virulence genotype varies between strains and lineages.

The keratinocyte adhesion assay demonstrated a variable capacity for attachment to porcine skin cells amongst the analysed strains. Although a considerable intra-lineage variation was observed for some clonal complexes, the

overall highest rates of adhesion were demonstrated by strains belonging to CC22 and CC30. The CC398 strains were attaching at comparatively higher rates than the remaining six clonal complexes, but still the rates of attachment observed for CC22 and CC30 were considerably higher. Although these findings would need to be confirmed by an *in vivo* approach, the data suggests that a high prevalence of MRSA CC398 amongst pig herds might have been driven by factors other than host specificity. In fact, a recently reported study demonstrated that MRSA CC398 strains lacked the ability to preferentially attach to porcine skin cells in comparison to human-derived cells (Moodley *et al.*, 2012). However, in a different study a livestock-associated MRSA CC398 revealed reduced capacity for attachment to human keratinocytes in comparison to human-associated MSSA CC398 strain, although the strains displayed no significant variation in attachment to porcine keratinocytes (Uhlemann *et al.*, 2012). The variation in capacity to attach to human keratinocytes was correlated with a considerable variation in the composition of adhesin genes between the human MSSA CC398 and the livestock-associated MRSA CC398 strain, as determined by whole genome sequence analysis. In this study, strains were investigated for either presence or absence of a range of adhesin genes, rather than sequence composition. Comparative analysis revealed that the two clonal complexes with the highest capacity for keratinoycte adhesion, CC22 and CC30, carried a single variable factor that was absent in all other lineages except for CC398, namely the *cna* gene. As described in previous paragraphs, a recent study suggested that the *cna* adhesin gene might promote stable colonisation (Nashev *et al.*, 2004). However, if the *cna* determinant was involved in mediating the enhanced binding of CC22 and CC30 strains to keratinocytes, comparable rates of adhesion would be also expected for CC398 strains. Thus the mechanism that promoted the high rates of keratinocytes binding observed for CC22 and CC30 strains cannot be defined with the current data and further analysis would need to be conducted to clarify the results.

The biofilm formation assay revealed that most strains were biofilm formers at 37° C. Although the highest average biofilm activity was observed for the CC30 group, it was significantly enhanced by a single strain with extremely high biofilm formation. More uniform and still comparatively high biofilm activity was demonstrated by CC97 and CC130. Detection of enhanced biofilm formation among strains associated with cases of bovine mastitis is in agreement with the reports that biofilm formation plays an important role in the development of disease (Melchior,

Vaarkamp and Fink-Gremmels, 2006; Fox, Zadoks and Gaskins, 2005). Interestingly, strains belonging to CC151 did not show significant capacity for biofilm formation. Finally, the comparatively lowest average and lack of biofilm formation amongst majority of CC398 strains can be correlated with reports that the lineage demonstrates an overall low pathogenic potential.

At 25° C a considerable proportion of strains lost the biofilm phenotype and it could be speculated that those strains found to still form biofilm at lower temperature might be more likely to persist in the environment. Bacterial biofilm formation on abiotic structures has been identified as a significant factor mediating the prolonged contamination of hospital surfaces and equipment (Smith and Hunter, 2008). Outside the hospital settings, environmental contamination by biofilm forming organisms has also been detected on surfaces in food-processing plants and on milking equipment on dairy farms (Shi and Zhu, 2009; Latorre *et al.*, 2010). In accordance with these reports, the majority of strains belonging to the cattle-associated CC130 lineage analysed in this work, were found to form biofilms at 25° C. The lack of biofilm activity at 25° C by MRSA CC398 strains correlates with the results of biofilm formation analysis at 37° C. Dewaele *et al* (2011), reported recently environmental contamination by MRSA isolates, presumably belonging to CC398, within pig farms. The observed here poor ability of MRSA CC398 to form biofilm at lower temperature, might suggest that its occurrence on farm surfaces is mediated by non-biofilm related mechanisms such as MRSA shedding by colonised animals (Szabo *et al*, 2012).

The strains were next analysed for their carriage of antimicrobial resistance determinants as well as antimicrobial susceptibility phenotypes, including sensitivity to biocides.

Chapter 5 Analysis of antimicrobial and biocide resistance phenotypes and genotypes

5.1 Introduction

Antimicrobial susceptibility testing is an essential routine procedure performed by clinical microbiology laboratories, as it ensures that the bacterial pathogen is sensitive to agents used empirically for management of the infection (Jorgensen and Ferraro, 2009). Continuous local monitoring of antimicrobial susceptibility among clinical isolates also reveals trends in the epidemiology of antimicrobial resistance, which over the last couple of decades have been expanded into national and global antimicrobial resistance surveillance studies (Masterton, 2008). Emergence and increased prevalence of antimicrobial resistance among bacterial pathogens has been generally associated with settings where the antimicrobial compounds are in common use (Van den Bogaard, London and Stobberingh, 2000). In addition to human medicine, antimicrobial agents are also widely used in the treatment of bacterial infections in animals and have various applications in prophylaxis (Werckenthin *et al.*, 2001). In concomitance with the therapeutic use, antimicrobials also served as growth promoters in food-producing animals, a practice that was banned in the European Union in 2006, but still occurs in the United States (Castanon, 2007; Wells *et al.*, 2013).

The need for the surveillance of antimicrobial resistance among isolates derived from animals has several underlying benefits. As with antimicrobial use in humans, the appropriate selection of antimicrobial agent for treatment of bacterial infection is mediated by susceptibility analysis, which promotes successful treatment and limits the impacts on animal welfare (Gentilini *et al.*, 2000). Furthermore, antimicrobial-resistant isolates of animal origin represent a health risk for humans due to possible transmission to humans via foodborne route or direct contact with an animal (Piddock, 1996). Also, animal isolates may constitute reservoirs of resistance determinants for human-associated strains (Aarestrup *et al.*, 2000). Surveillance of antimicrobial resistance has been conducted on isolates mostly from the following two categories of host: companion animals such as cats, dogs, and horses; and livestock such as cattle, swine and poultry (Aarestrup *et al.*, 2009; Dierikx *et al.*, 2012). High prevalence of antimicrobial resistance has been in particular associated with the latter, with extensive farming settings recognised as a considerable reservoir of resistant bacteria (Van den Bogaard, London and Stobberingh, 2000).

Of lesser significance than antimicrobial resistance, but also clinically relevant is reduced susceptibility of bacterial pathogens to biocidal agents (Fraise, 2002). The

importance of this can be related to the vital role of disinfectant use in both healthcare settings, and for cleansing and disinfection of livestock and animal holdings (Rodgers *et al.*, 2001; Bjorland, Sunde and Waage, 2001; Fraise, 2002). The main consequences of reduced susceptibility to biocides among bacterial pathogens have been associated with co-selection of antimicrobial resistance as well as environmental persistence (Carson *et al.*, 2008; Langsrud *et al.*, 2003). A concomitant reduced susceptibility to biocides and multiple antimicrobial resistance among nosocomial isolates has been observed (Fraise, 2002). The analysis of biocide susceptibility has thus been often focused on hospital-associated pathogens with an identified antimicrobial resistance phenotype, such as MRSA strains (Fraise, 2002; Noguchi *et al.*, 2005).

A number of methods have been developed to determine *in vitro* bacterial susceptibility to antimicrobial compounds such, as disk diffusion, broth and agar dilution as well as antimicrobial gradient method such as Etest (Jorgensen and Ferraro, 2009). Antimicrobial susceptibility testing has been widely standardised and should be performed in accordance with the developed guidelines, such as BSAC Methods for Antimicrobial Susceptibility Testing or CLSI Methods for Dilution Antimicrobial Susceptibility Tests (Clinical and Laboratory Standards Institute, January 2006; Andrews, 2010). Biocide sensitivity is normally determined through MIC analysis using either agar or broth dilution (Suller and Russell, 2000; Aarestrup and Hasman, 2004; Bjorland *et al.*, 2005). However, when investigating the activity of biocides against bacterial isolates, no standardised protocols are available.

Analysis of antimicrobial resistance can also entail a genotypic approach of screening for the carriage of resistance determinants. If resistance genes are carried on MGEs, the genotypic analysis is based on the detection of the gene or its absence. Screening is most often conducted by PCR and, as described for virulence determinants (section 4.1), a number of multiplex PCR schemes have been developed to allow time and cost efficient screening (Ng *et al.*, 2001; Strommenger *et al.*, 2003). The most robust analysis can be achieved with a DNA microarray, as described previously (section 4.1). In case of resistance mediated by a mutation in an intrinsic chromosomal gene, the genotypic analysis would normally require downstream characterisation of PCR products, such as RFLP and sequencing to confirm presence of point mutations (Takahashi *et al.*, 1998).

Previously selected strains (Table 3.1) were tested for antimicrobial and biocide susceptibility followed by analysis of antimicrobial and biocide resistance gene carriage.

5.2 Materials and methods

All strains (n=64) described in Table 3.1 have been included in the analysis. Antimicrobial and biocide susceptibility testing was performed as described in section 2.6. The resistance genes that were analysed by DNA microarray are presented in Table 2.2 whereas the genes that were screened for by PCR are listed in sections 2.4.7.4 and in 2.4.7.5. The analysis of the resistance genotypes was conducted as described in sections 2.4.6, 2.4.7.4 and 2.4.7.5

5.3 Results

5.3.1 Antimicrobial susceptibility phenotypes

The antimicrobial susceptibility testing results are presented in Table 5.1. The majority of analysed strains displayed penicillin resistance (n=57), with penicillin-susceptible strains consisting mostly of strains belonging to CC151. All strains belonging to CC398 and CC130 displayed cefoxitin resistance (in further description referred to as methicillin resistance), which was also detected among CC22 (n=6), CC30 (n=3) and both equine CC8 strains. Resistance to the non-β-lactam agents was observed mostly among methicillin-resistant (MR) strains, with the exception of CC130 strains that were uniformly susceptible to all other tested compounds. Resistance to tetracycline was detected in all CC398, as well as in both equine CC8 strains. Interestingly, three methicillin-susceptible (MS) strains of various lineages were also tetracycline-resistant. Gentamicin and kanamycin resistance was observed only among MRSA strains, which consisted of CC398 (n=9), CC22 (n=1), CC8 (n=2) and CC30 (n=2) strains. In addition two strains belonging to CC30 and CC398 revealed elevated kanamycin MICs but were gentamicin susceptible. Considerably lower lineage heterogeneity was observed among spectinomycin resistant strains, which consisted mostly of CC398 strains (n=10), as well as those belonging to CC30 (n=4) and CC97 (n=1). Resistance to erythromycin-clindamycin was most prevalent amongst, although no limited to, MRSA strains. It was observed in the majority of CC398 strains (n=13), as well as in most MRSA strains belonging to CC22 (n=5) and CC30 (n=4). The clindamycin constitutive resistance was observed mostly in strains

belonging to CC398, whereas in majority of strains of other lineages the resistance was inducible. Furthermore, tylosin resistance was observed in all strains displaying constitutive, but not inducible clindamycin resistance, which consisted of CC398 (n=12) and CC30 (n=2) strains. The CC398 group also demonstrated high prevalence of trimethoprim resistance (n=13), which was sparsely detected amongst other lineages such as CC5 (n=1), CC8 (n=2), CC22 (n=2) and CC30 (n=2). Resistance phenotypes identified exclusively in strains belonging to CC398 included chloramphenicol-florfenicol (n=3) and tiamulin resistance (n=4). Of comparatively lower prevalence in CC398 strains was resistance to ciprofloxacin (n=3). In contrast it was observed in all MR strains belonging to CC22 (n=6) and CC30 (n=3). All strains were susceptible to apramycin, vancomycin and teicoplanin.

MIC values above the resistance breakpoint varied considerbly among strains for some of the analysed compounds. For instance, the MIC for penicillin-resistant strains ranged from 0.25 to >16 μg/ml and for cefoxitin-resistant strains it was between 8 and > 32 μg/ml. On the other hand, the same MIC values for all or vast majority of resistant strains were observed for kanamycin, spectinomycin, clindamycin, erythromycin, tylosin, chloramphenicol/florefenicol and tiamulin.

Table 5.1 Antimicrobial susceptibility testing results [a, b]

		MIC (μg/ml)																	
Lineage	ID	PEN	CEF	GEN	KAN	APR	SPE	VAN	TEI	CLI	CLIi	ERY	TYL	CHL	FLO	CIP	TET	TIA	TRI
CC5	8	**2**	4	0.25	2	4	32	0.5	0.5	0.06	-	0.25	0.5	8	8	0.25	0.25	0.5	2
CC5	17	**1**	4	0.5	2	4	32	1	1	0.06	-	0.12	1	4	4	**8**	0.25	0.5	**>64**
CC5	19	**0.25**	2	0.25	2	4	32	0.5	1	0.06	-	0.12	0.5	8	4	0.12	0.25	0.5	2
CC5	25	≤ 0.03	4	0.5	2	4	32	0.5	1	0.06	-	0.12	0.5	8	4	0.25	0.25	0.5	2
CC8	6	**1**	4	0.5	1	4	32	0.5	1	0.03	-	0.12	0.5	4	4	≤ 0.03	**32**	0.5	1
CC8	11	**1**	4	0.5	1	8	32	0.5	0.5	0.06	-	0.12	0.5	8	4	0.25	0.25	0.5	1
CC8	16	**1**	2	0.25	1	2	16	0.5	1	0.06	-	0.12	0.5	8	4	0.25	0.12	0.5	2
CC8	200	**>16**	**8**	**>64**	**>128**	4	32	1	1	0.03	+	**>32**	0.5	8	4	0.25	**64**	0.5	**>64**
CC8	201	**>16**	**16**	**64**	**>128**	8	16	0.5	1	0.06	-	0.12	0.5	4	4	0.12	**64**	0.5	**>64**
CC15	10	**16**	2	0.25	1	4	32	1	1	0.06	-	0.12	0.5	4	4	0.06	0.12	0.25	1
CC15	21	**0.5**	4	0.25	2	4	32	1	1	0.06	-	0.12	1	4	4	0.25	0.25	0.5	2
CC15	23	**16**	4	0.5	2	4	32	0.5	1	0.06	-	0.12	1	8	4	0.25	0.25	0.5	2
CC15	24	**8**	4	0.25	1	2	16	1	1	0.03	-	0.12	0.5	4	4	0.25	0.12	0.25	2
CC15	60	**16**	2	0.25	1	2	16	1	1	0.06	-	0.25	1	8	8	0.12	**32**	0.5	2
CC22	18	**1**	4	0.25	2	4	32	0.5	0.5	0.06	-	0.12	0.5	4	4	**4**	0.25	0.5	**32**
CC22	20	**1**	2	0.25	2	4	32	0.5	1	0.06	-	0.12	1	8	4	0.12	0.25	0.5	2
CC22	28	**16**	**8**	**16**	**>128**	2	8	0.5	1	0.06	+	**>32**	0.5	8	8	**8**	0.25	0.5	1
CC22	32	**16**	**16**	0.25	1	2	32	0.5	1	0.06	+	**>32**	0.5	8	8	**>16**	0.12	0.5	1
CC22	38	**16**	**8**	0.25	1	4	32	0.5	1	0.06	+	**>32**	0.5	8	8	**>16**	0.25	0.5	**16**
CC22	39	**>16**	**8**	0.25	2	4	32	0.5	0.5	0.06	+	**>32**	0.5	8	8	**>16**	0.25	0.5	1
CC22	40	**16**	**8**	0.25	0.5	2	32	0.5	1	0.06	+	**>32**	0.5	8	8	**>16**	0.25	0.5	1
CC22	44	**4**	**8**	0.25	1	4	32	0.25	0.25	0.06	-	0.12	0.5	8	8	**>16**	0.25	0.5	1

[a] PEN - penicillin; CEF - cefoxitin; GEN - gentamicin; KAN - kanamycin; APR - apramycin; SPE - spectinomycin; VAN - vancomycin; TEI - teicoplanin; CLI - clindamycin; CLI_i – clindamycin inducible; ERY - erythromycin; TYL - tylosin; CHL - chloramphenicol; FLO - florfenicol; CIP - ciprofloxacin; TET - tetracycline; TIA - tiamulin; TRI - trimethoprim

[b] red font highlights MIC values that are equal to or above resistance breakpoint

Table 5.1 (continued) Antimicrobial susceptibility testing results [a, b]

		MIC (µg/ml)																	
Lineage	ID	PEN	CEF	GEN	KAN	APR	SPE	VAN	TEI	CLI	CLIi	ERY	TYL	CHL	FLO	CIP	TET	TIA	TRI
CC30	1	**1**	2	0.25	1	4	32	0.5	1	0.12	-	0.12	1	8	8	0.12	0.12	0.5	1
CC30	5	**16**	4	0.25	1	4	**>1024**	0.5	1	0.06	+	**>32**	0.5	4	4	0.12	0.25	0.5	1
CC30	13	0.12	2	0.25	1	4	32	0.5	1	0.06	-	0.12	0.5	8	8	0.12	0.12	0.5	1
CC30	31	**>16**	**16**	**32**	**>128**	2	**>1024**	1	0.5	0.06	+	**>32**	1	8	4	**>16**	0.25	0.5	2
CC30	34	**>16**	**>32**	0.25	**64**	2	**>1024**	0.5	1	**>32**	+	**>32**	**>128**	8	8	**>16**	0.12	0.25	**64**
CC30	51	**>16**	2	0.25	1	4	32	1	1	0.06	-	0.25	0.5	8	8	0.12	0.25	0.5	2
CC30	62	**16**	2	0.25	1	4	32	1	2	0.06	-	0.12	1	8	8	0.25	**32**	0.5	1
CC30	65	**>16**	**>32**	**32**	**128**	4	**>1024**	0.5	1	**>32**	+	**>32**	**>128**	8	4	**>16**	0.25	0.25	**>64**
CC97	174	**0.5**	2	0.12	0.5	2	32	0.5	1	0.06	-	0.12	1	4	4	0.25	0.25	0.5	2
CC97	175	**2**	4	0.12	0.5	4	32	0.5	1	0.06	-	0.12	1	8	4	0.12	0.5	0.5	2
CC97	177	≤ 0.03	4	0.25	1	4	32	0.5	1	0.06	-	0.12	1	8	4	0.25	0.5	0.5	4
CC97	189	**2**	4	0.25	2	4	32	1	1	0.06	-	0.12	1	8	8	0.5	0.25	0.5	2
CC97	190	**1**	4	0.25	1	2	32	0.5	2	0.25	-	0.25	1	8	8	0.25	0.25	0.5	1
CC97	195	**2**	4	0.25	1	2	**>1024**	0.5	0.5	0.06	+	**>32**	0.5	8	4	0.12	0.5	0.5	2
CC97	197	**1**	4	0.12	1	4	32	0.5	1	0.06	-	0.25	1	8	8	0.25	0.5	0.5	2
CC97	198	**4**	4	0.25	1	8	32	0.5	1	0.12	-	0.12	0.5	8	4	0.12	0.5	0.5	4
CC130	173	**1**	**8**	0.25	2	4	32	0.5	2	0.06	-	0.25	1	8	4	0.12	0.25	0.5	2
CC130	178	**0.5**	**8**	0.25	1	4	32	0.5	1	0.06	-	0.25	1	8	4	0.25	0.25	0.5	2
CC130	181	**0.25**	**8**	0.25	2	8	32	0.5	1	0.06	-	0.12	0.5	8	4	0.12	0.25	0.5	2
CC130	182	**1**	**8**	0.12	1	2	32	0.5	1	0.12	-	0.25	1	8	4	0.25	0.25	0.5	2
CC151	179	≤ 0.03	4	0.12	1	4	32	1	0.5	0.03	-	0.12	0.5	8	4	0.06	0.25	0.5	1
CC151	193	≤ 0.03	4	0.12	1	2	32	0.5	2	0.03	-	0.25	0.5	8	4	0.06	0.25	0.5	1
CC151	194	≤ 0.03	4	0.12	1	2	32	0.5	0.5	0.06	-	0.12	0.5	8	4	0.06	0.25	0.5	2
CC151	199	≤ 0.03	4	0.12	1	2	32	0.5	0.5	≤ 0.015	-	0.12	0.5	8	4	0.06	0.25	0.25	2

[a] PEN - penicillin; CEF - cefoxitin; GEN - gentamicin; KAN - kanamycin; APR - apramycin; SPE - spectinomycin; VAN - vancomycin; TEI - teicoplanin; CLI - clindamycin; CLI_i – clindamycin inducible; ERY - erythromycin; TYL - tylosin; CHL - chloramphenicol; FLO - florfenicol; CIP - ciprofloxacin; TET - tetracycline; TIA - tiamulin; TRI - trimethoprim

[b] red font highlights MIC values that are equal to or above resistance breakpoint

Table 5.1 (continued) Antimicrobial susceptibility testing results [a, b]

		MIC (µg/ml)																	
Lineage	ID	PEN	CEF	GEN	KAN	APR	SPE	VAN	TEI	CLI	CLIi	ERY	TYL	CHL	FLO	CIP	TET	TIA	TRI
CC398	90	16	16	64	>128	8	32	0.5	1	>32	+	>32	>128	8	8	0.25	64	0.5	>64
CC398	91	16	8	0.25	2	4	>1024	0.5	1	>32	+	>32	>128	64	>32	0.12	64	0.5	1
CC398	92	16	8	32	>128	4	>1024	1	1	>32	+	>32	>128	8	8	0.12	32	0.5	>64
CC398	93	16	8	0.12	1	4	>1024	0.5	1	>32	+	>32	>128	64	>32	0.12	32	0.5	1
CC398	95	>16	16	0.5	1	4	>1024	1	1	>32	+	>32	>128	64	>32	>16	64	>128	>64
CC398	96	>16	16	0.25	32	4	64	0.5	2	>32	+	>32	>128	8	8	0.25	>64	0.5	>64
CC398	99	8	8	32	>128	4	>1024	0.5	1	0.12	-	0.25	0.5	8	4	0.12	32	0.5	>64
CC398	101	16	16	64	>128	4	64	0.5	1	0.12	-	0.25	0.5	8	8	0.12	64	0.5	>64
CC398	102	8	8	16	128	4	>1024	0.5	1	>32	+	>32	>128	8	8	0.12	32	0.25	>64
CC398	103	8	8	0.25	2	4	64	0.5	0.5	>32	+	>32	>128	8	8	8	64	0.5	>64
CC398	104	4	8	32	>128	8	>1024	0.5	1	>32	+	>32	>128	8	8	0.12	>64	>128	1
CC398	105	>16	8	16	>128	8	32	0.5	1	>32	+	>32	>128	8	8	0.12	32	0.25	>64
CC398	106	8	8	0.25	1	4	32	0.5	1	16	-	0.25	0.5	8	8	8	64	128	>64
CC398	107	16	8	0.25	1	4	>1024	0.5	1	0.12	-	0.25	0.5	8	4	0.25	32	0.5	1
CC398	108	8	8	0.25	2	4	32	0.5	1	>32	+	>32	>128	8	8	1	64	0.5	>64
CC398	109	16	16	32	>128	8	>1024	0.5	1	>32	+	>32	>128	8	8	0.25	32	0.25	>64
CC398	110	16	8	0.12	1	4	>1024	0.5	1	0.06	-	0.12	0.5	8	4	0.25	32	0.5	0.5
CC398	111	16	8	16	>128	8	>1024	0.5	1	8	+	>32	0.5	8	8	0.12	32	>128	>64

[a] PEN - penicillin; CEF - cefoxitin; GEN - gentamicin; KAN - kanamycin; APR - apramycin; SPE - spectinomycin; VAN - vancomycin; TEI - teicoplanin; CLI - clindamycin; CLI_i – clindamycin inducible; ERY - erythromycin; TYL - tylosin; CHL - chloramphenicol; FLO - florfenicol; CIP - ciprofloxacin; TET - tetracycline; TIA - tiamulin; TRI - trimethoprim

[b] orange font highlights MIC values that represent an intermediate status whereas red font highlights MIC values that are equal to or above resistance breakpoint

5.3.2 Biocide susceptibility phenotypes

The biocide susceptibility testing results are presented in Table 5.2. MIC values for cadmium acetate ranged from ≤ 0.015 to 2 mM, with the majority of strains (n=50) displaying a MIC of ≤ 0.06 mM. The remaining strains displayed a comparatively elevated cadmium acetate MIC of 0.05 - 2 mM. Reduced susceptibility to cadmium was particularly prevalent among strains belonging to CC30 (n=6), but was also observed for CC398 (n=5) and CC22 (n=3) strains. The MICs for zinc chloride varied from ≤ 0.06 to 1 mM, but for most strains (n=51) the value was ≤ 0.25 mM. The MIC of 0.5 - 1 mM was observed among all but one strain with higher tolerance to cadmium and no other strains. The MIC of copper sulphate for majority of strains (n=54) was 8 mM although a single strain showed an increased susceptibility and MIC of 2 mM. The remaining nine strains demonstrated MIC of 16 mM and consisted of CC22 (n=3), CC30 (n=3) and CC398 (n=3) strains. All but one strain with reduced susceptibility to copper sulphate also had an elevated MIC for sodium acetate and zinc chloride. MIC values for acriflavine were for majority of strains (n=45) in the range of 4 - 8 μg/ml. A considerably lower MIC of a 0.5 μg/ml was observed for a single strain. Furthermore, 18 strains demonstrated acriflavine MICs of 16 - 64 μg/ml, which consisted primarily of strains belonging to CC22 (n=5), CC30 (n=7) and CC97 (n=5). The MICs of benzalkonium chloride varied from ≤ 0.12 to 8 μg/ml, although most strains (n=57) displayed MIC of 0.5 - 2 μg/ml. Five strains from CC22 (n=3) and CC30 (n=2) had benzalkonium chloride MIC of 4 μg/ml and a single CC97 strain displayed MIC of 8 μg/ml.

The MIC range for chlorhexidine digluconate was between ≤ 0.03 and 0.25 μg/ml with majority of strains (n=60) demonstrating a MIC of 0.12 - 0.25 μg/ml. As such, no strain exhibited reduced susceptibility to this biocide compound. Considerable MIC variation was observed for CTAB with values ranging from ≤ 0.25 up to 16 μg/ml. However, for most of the analysed strains (n=48) the MIC was ≤ 2 μg/ml. Strains demonstrating CTAB MIC of ≥ 4 μg/ml were considered to have reduced susceptibility to this compound and consisted of CC22 (n=5), CC30 (n=4), CC97 (n=5) and CC130 (n=2) strains. The MIC of formaldehyde for the vast majority of strains (n=60) was 0.0005% (v/v), whereas for hydrogen peroxide most strains (n=61) demonstrated MIC of 0.00025% (v/v). Irgasan MIC values ranged from ≤ 0.002 up to 4 μg/ml although nearly all strains demonstrated MIC of ≤ 0.06 μg/ml.

Table 5.2 Biocide susceptibility testing results [a, b]

		MIC									
		mM			μg/ml					% (v/v)	
Lineage	ID	Cd	Zn	Cu	ACR	BCH	CHD	CTAB	IRG	FORM	HPER
CC5	8	0.03	0.12	8	8	0.5	0.12	1	0.5	0.0005	0.00025
CC5	17	0.06	0.25	8	8	0.5	0.12	1	0.0075	0.0005	0.00025
CC5	19	0.03	0.12	8	8	0.5	0.06	1	0.03	0.0005	0.00025
CC5	25	0.03	0.12	8	8	0.5	0.12	1	0.015	0.0005	0.00025
CC8	6	0.03	≤ 0.06	8	8	0.5	0.12	0.5	**4**	0.0005	0.00025
CC8	11	0.06	0.12	8	8	0.5	0.12	1	**4**	0.0005	0.00025
CC8	16	0.06	0.25	8	8	1	0.25	2	0.0075	0.0005	0.00025
CC8	200	0.06	0.25	8	8	2	0.25	2	0.015	0.0005	0.00025
CC8	201	0.03	0.12	8	8	2	0.5	2	0.0075	0.0005	0.00025
CC15	10	0.06	0.12	8	4	0.5	0.06	1	0.0075	0.0005	0.00025
CC15	21	≤ 0.015	0.12	8	8	0.5	0.06	1	0.0075	0.0005	0.00025
CC15	23	0.06	0.12	8	8	1	0.12	1	0.015	0.0005	0.00025
CC15	24	0.12	0.12	8	8	0.5	0.12	1	0.015	0.001	0.00025
CC15	60	0.12	0.25	8	8	0.5	0.12	1	0.0075	0.0005	0.00025
CC22	18	≤ 0.015	≤ 0.06	8	0.5	≤ 0.12	≤ 0.03	≤ 0.25	0.015	0.0005	0.00012
CC22	20	**2**	**1**	**16**	8	1	0.12	2	0.03	0.0005	0.00025
CC22	28	≤ 0.015	≤ 0.06	8	8	1	0.25	2	0.12	0.0005	0.00025
CC22	32	**2**	**1**	**16**	**64**	**4**	0.25	**8**	0.015	0.0005	0.00025
CC22	38	≤ 0.015	≤ 0.06	8	**32**	2	0.25	**8**	0.0075	0.0005	0.00012
CC22	39	**1**	**1**	**16**	**64**	**4**	0.25	**16**	0.0075	0.0005	0.00012
CC22	40	≤ 0.015	≤ 0.06	8	**32**	**4**	0.25	**16**	0.015	0.0005	0.00025
CC22	44	≤ 0.015	≤ 0.06	8	**32**	2	0.25	**16**	0.0075	0.0005	0.00025

[a] Cd – cadmium acetate, Zn - zinc chloride; Cu – copper sulphate; ACR - acriflavine; BCH – benzalkonium chloride; CHD - chlorhexidine digluconate; CTAB - cetyltrimethylammonium bromide; IRG - irgasan; FORM - formaldehyde; HPER – hydrogen peroxide

[b] bold red font highlights MIC values interpreted to represent reduced susceptibility to the corresponding compound

Table 5.2 (continued) Biocide susceptibility testing results [a, b]

		MIC									
		mM			µg/ml					% (v/v)	
Lineage	ID	Cd	Zn	Cu	ACR	BCH	CHD	CTAB	IRG	FORM	HPER
CC30	1	**1**	0.25	8	8	0.5	0.12	2	0.00375	0.0005	0.00025
CC30	5	**1**	**1**	8	**16**	1	0.25	2	0.00375	0.0005	0.00025
CC30	13	**1**	**0.5**	**16**	**16**	2	0.25	**4**	0.0075	0.001	0.00025
CC30	31	**1**	**0.5**	**16**	**16**	**4**	0.25	**16**	0.00375	0.001	0.00025
CC30	34	**2**	**0.5**	8	**16**	2	0.25	**16**	0.0075	0.0005	0.00025
CC30	51	0.06	0.12	8	**16**	1	0.12	2	0.0075	0.0005	0.00025
CC30	62	0.06	0.25	8	**32**	1	0.25	2	0.0075	0.0005	0.00025
CC30	65	**1**	**0.5**	**16**	**16**	**4**	0.25	**16**	0.00375	0.001	0.00025
CC97	174	0.03	≤ 0.06	8	8	1	0.25	2	0.0075	0.0005	0.00025
CC97	175	≤ 0.015	0.25	8	8	1	0.25	2	0.0075	0.0005	0.00025
CC97	177	0.06	0.25	8	**16**	2	0.25	**4**	0.00375	0.0005	0.00025
CC97	189	0.06	0.25	8	**32**	2	0.25	**4**	0.00375	0.0005	0.00025
CC97	190	0.06	0.25	8	**16**	2	0.25	**8**	≤ 0.001875	0.0005	0.00025
CC97	195	≤ 0.015	0.25	8	8	1	0.25	2	0.00375	0.0005	0.00025
CC97	197	0.06	0.25	8	**16**	**8**	0.12	**8**	0.0075	0.0005	0.00025
CC97	198	0.03	0.25	8	**16**	2	0.12	**8**	≤ 0.001875	0.0005	0.00025
CC130	173	≤ 0.015	≤ 0.06	8	8	1	0.12	2	0.00375	0.0005	0.00025
CC130	178	≤ 0.015	0.12	8	8	2	0.12	**8**	0.015	0.0005	0.00025
CC130	181	≤ 0.015	0.25	8	8	1	0.25	2	0.00375	0.0005	0.00025
CC130	182	≤ 0.015	0.12	8	**16**	2	0.25	**4**	0.0075	0.0005	0.00025
CC151	179	≤ 0.015	0.12	8	4	1	0.12	2	0.015	0.0005	0.00025
CC151	193	≤ 0.015	0.12	8	4	1	0.12	1	0.015	0.0005	0.00025
CC151	194	≤ 0.015	0.12	8	4	1	0.25	1	0.0075	0.0005	0.00025
CC151	199	≤ 0.015	0.25	2	4	1	0.12	1	0.0075	0.0005	0.00025

[a] Cd – cadmium acetate, Zn - zinc chloride; Cu – copper sulphate; ACR - acriflavine; BCH – benzalkonium chloride; CHD - chlorhexidine digluconate; CTAB - cetyltrimethylammonium bromide; IRG - irgasan; FORM - formaldehyde; HPER – hydrogen peroxide

[b] bold red font highlights MIC values interpreted to represent reduced susceptibility to the corresponding compound

Table 5.2 (continued) Biocide susceptibility testing results [a, b]

		MIC									
		mM			µg/ml					% (v/v)	
Lineage	ID	Cd	Zn	Cu	ACR	BCH	CHD	CTAB	IRG	FORM	HPER
CC398	90	0.06	0.25	8	8	2	0.25	2	0.015	0.0005	0.00025
CC398	91	**1**	**1**	**16**	8	1	0.25	2	0.0075	0.0005	0.00025
CC398	92	≤ 0.015	0.12	8	8	1	0.12	2	0.015	0.0005	0.00025
CC398	93	≤ 0.015	0.12	8	8	1	0.12	2	0.0075	0.0005	0.00025
CC398	95	**1**	**1**	8	8	2	0.25	2	0.03	0.0005	0.00025
CC398	96	≤ 0.015	0.12	**16**	8	2	0.12	2	0.06	0.0005	0.00025
CC398	99	≤ 0.015	0.25	8	8	1	0.12	2	0.015	0.0005	0.00025
CC398	101	0.06	0.12	8	8	1	0.12	2	0.0075	0.0005	0.00025
CC398	102	≤ 0.015	0.25	8	8	1	0.25	2	0.0075	0.0005	0.00025
CC398	103	**1**	**1**	8	4	1	0.12	2	0.03	0.0005	0.00025
CC398	104	0.03	0.25	8	8	1	0.12	2	0.015	0.0005	0.00025
CC398	105	0.06	0.25	8	8	1	0.12	2	0.015	0.0005	0.00025
CC398	106	**1**	**1**	8	8	1	0.25	2	0.015	0.0005	0.00025
CC398	107	≤ 0.015	0.12	8	8	1	0.25	2	0.03	0.0005	0.00025
CC398	108	**0.5**	**1**	**16**	8	1	0.12	2	0.015	0.0005	0.00025
CC398	109	≤ 0.015	0.25	8	8	1	0.25	2	0.03	0.0005	0.00025
CC398	110	≤ 0.015	0.12	8	8	1	0.25	2	0.015	0.0005	0.00025
CC398	111	≤ 0.015	0.25	8	8	1	0.25	2	0.015	0.0005	0.00025

[a] Cd – cadmium acetate, Zn - zinc chloride; Cu – copper sulphate; ACR - acriflavine; BCH – benzalkonium chloride; CHD - chlorhexidine digluconate; CTAB - cetyltrimethylammonium bromide; IRG - irgasan; FORM - formaldehyde; HPER – hydrogen peroxide

[b] bold red font highlights MIC values interpreted to represent reduced susceptibility to the corresponding compound

5.3.3 Antimicrobial and biocide resistance genotypes

The resistance genotypes are presented in Figure 5.1. The *blaZ* gene was detected in the majority of analysed strains except for all CC130 and CC151 strains, as well as single strains belonging to CC5 and CC97. The *mecA* gene was identified in all CC398 strains, as well as strains belonging to CC8 (n=2, both equine), CC22 (n=6) and CC30 (n=3). All CC130 strains carried the *mecA* homologue - *mecC*. The *aacA-aphD* gene was the most common aminoglycoside resistance gene and it was prevalent among CC398 strains (n=9) but was also detected in various other lineages: CC8 (n=2), CC22 (n=1) and CC30 (n=2). The *aadD* gene was less frequent and found in strains belonging to CC8 (n=1), CC30 (n=3) and CC398 (n=4) with all but two of these strains also carrying the *aacA-aphD* determinant. One strain belonging to CC8 carried the *aphA* gene (also *aacA-aphD*-positive). The erythromycin resistance gene *ermA* was mainly detected in strains belonging to CC30 (n=4), but was also identified amongst CC398 (n=2) and CC97 (n=1) strains. All *ermA*-positive strains carried also the spectinomycin resistance gene *spc*, suggesting the insertion of Tn*554* transposon. More common among CC398 strains was the *ermC* gene (n=10), also detected in CC22 (n=4) and CC8 (n=1). The *ermT* gene was detected by PCR among CC398 strains only (n=4).

In accordance with previous reports, the *tetM* element was strongly associated with the CC398 lineage as all analysed strains carried the gene. It was not detected in any other lineage except CC8 (n=2). The *tetK* gene was not as prevalent among CC398 strains (n=5), but it was detected in three other clonal complexes: CC8 (n=1), CC15 (n=1) and CC30 (n=1). Screening for *tetL* by PCR revealed the presence of the gene in strains belonging to CC398 (n=4) and CC8 (n=1). The trimethoprim resistance genes were prevalent mainly among CC398 strains, with more than half of strains carrying the *dfrK* gene (n=10), which was not detected in any other lineage. The *dfrA* gene was identified in CC8 (n=2), CC30 (n=1) and CC398 (n=2) strains. Strains were also screened for the carriage of *dfrG*, which was detected in a single strain belonging to CC398 and a single CC5 strain. The chloramphenicol-florfenicol resistance gene *fexA* was detected among three CC398 strains only. The streptogramin resistance genes *vgaA* and *vgaE* were detected in CC398 only, each in two different strains. The streptothricin resistance gene *sat* was detected in a single CC8 strain, with a low probe signal observed for some CC398 strains.

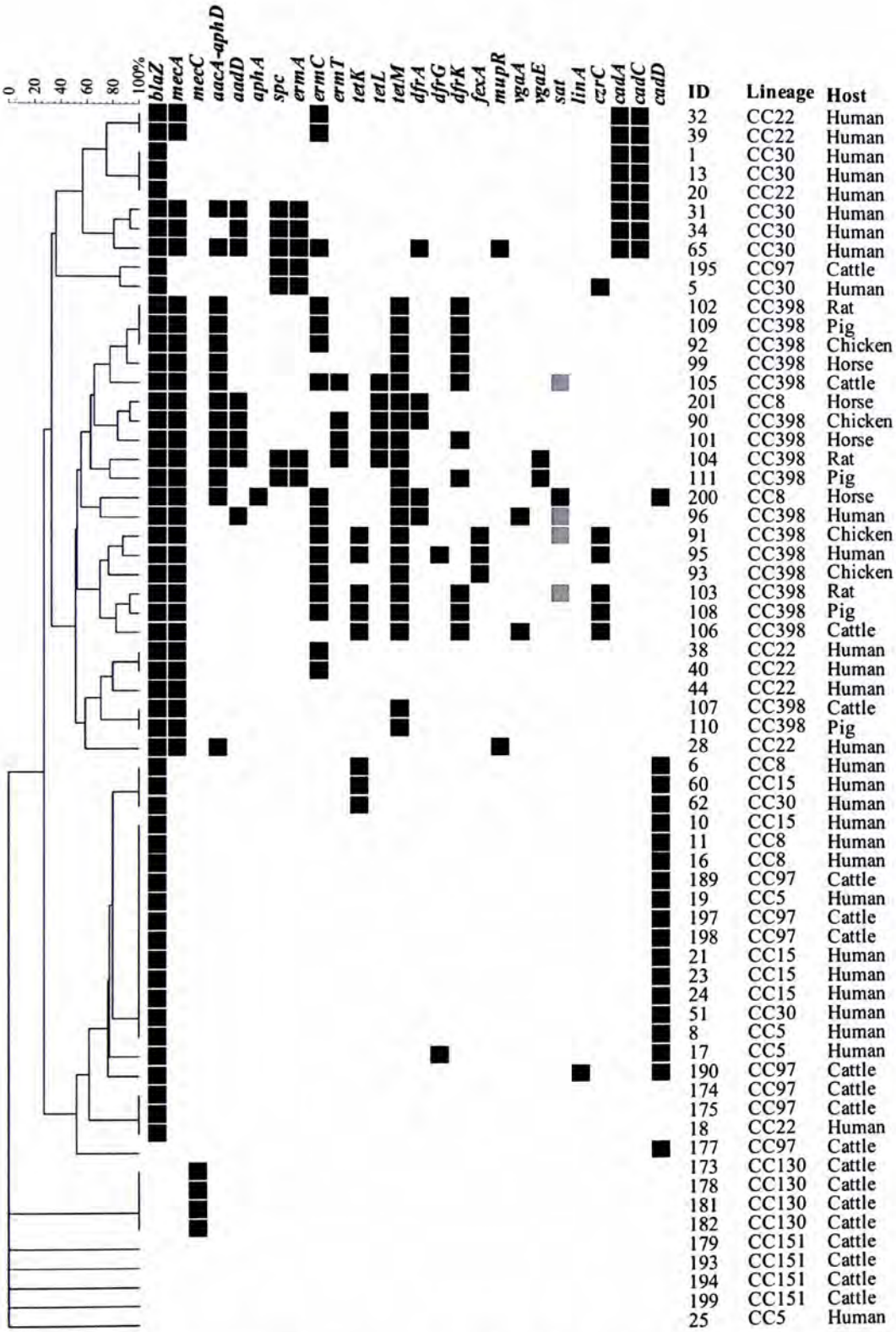


Figure 5.1 UPGMA dendrogram displaying clustering of antimicrobial and heavy metal resistance gene profiles of the analysed *S. aureus* panel. Black box – positive; grey – ambiguous; white/blank – negative.

The mupirocin resistance determinant *mupR* was found in two strains each from different lineage, CC22 and CC30. Finally, the *linA* resistance gene was detected in one strain belonging to CC97. All strains were negative for the carriage of *ermB*, *msrA*, *vatA*, *vatB*, *vanA*, *vanB*, *vanZ*, *far1*, *cat*, *cfr* and *vgb* genes.

Strains were screened by PCR for the carriage of metal and biocide resistance genes. The cadmium and zinc resistance gene *czrC* that was recently found to be associated with the SCC*mec* element from MRSA CC398 (Cavaco *et al.*, 2010) was detected in six strains. It included all but one of the CC398 strain carrying the SCC*mec* type V (n=5), as well as a single MSSA strain belonging to CC30. The *cadA* and *cadC* genes, which mediate resistance to cadmium and zinc, were both detected in strains belonging to CC22 (n=3) and CC30 (n=5). A considerably larger proportion of strains carried the *cadD* gene, which included strains belonging to CC5 (n=3), CC8 (n=3), CC15 (n=5), CC30 (n=2) and CC97 (n=5).

Clustering of resistance genotypes based on profile similarity, as shown in Figure 5.1, demonstrated a considerable intra- and inter-lineage heterogeneity. As such, the cluster formation was mainly non-clonal complex specific. Strains belonging to CC398 were dispersed into two major clusters that contained strains of other lineages.

5.3.4 Correlation between resistance phenotypes and genotypes

The detected antimicrobial resistance phenotypes largely corresponded with the carriage of a relevant resistance determinant for the majority of tested antimicrobial compounds, where the resistance is known to be associated with a mobile genetic element. As such, the mechanism of resistance to ciprofloxacin was not investigated. The mechanism of resistance to spectinomycin was not identified for majority of spectinomycin-resistant strains belonging to CC398, with only two out of 10 carrying the *spc* gene. Also the mechanisms of resistance to tiamulin were not determined for a single CC398 strain. Furthermore, a single CC22 strain displayed resistance to erythromycin and inducible clindamycin resistance, but was negative for all *erm* genes included in the analysis. Lack of resistance phenotype despite carriage of resistance determinant was less common but also observed. A single CC398 strain carrying *ermT* gene was erythromycin and clindamycin susceptible, and a single *blaZ*-positive CC30 strain was susceptible to penicillin. Finally, a single CC398 strain that contained the *aadD* gene demonstrated a reduced susceptibility to kanamycin, but the

MIC was significantly lower in comparison to other *aadD*-positive strains, and the strain was categorised as kanamycin-intermediate.

Reduced susceptibility to metals, observed for some strains, largely correlated with the carriage of *czrC*, *cadA* and *cadC* determinants. However, none of the strains positive for the *cadD* gene demonstrated increased tolerance to either cadmium of zinc.

5.4 Discussion

The prevalence of antimicrobial resistance varied considerably among the analysed strains. While a proportion of strains were susceptible to all analysed antimicrobial compounds and carried none of the resistance determinants that were screened for, some were found to be resistant to a wide range of antimicrobial agents. However, the vast majority of strains was resistant to pencillin. Over the decades, penicillin has been the drug of choice for treatment of staphylococcal infections and an estimated 90% of human *S. aureus* isolates are now resistant to this compound (Chambers, 2001; Olsen, Christensen and Aarestrup, 2006). In this study, only one human nosocomial MSSA strain was *blaZ*-negative. β-Lactam agents are still the preferred choice for the management of MSSA infections, which often involves use of beta-lactamase-resistant penicillins or cephalosporins (Bamberger and Boyd, 2005; Roberts and Chambers, 2005; Corey, 2009; Thwaites *et al.*, 2011).

Other than frequency, a considerable variation was also observed among strains that did carry resistance determinants. As such, only limited homogeneity could be observed among resistance genotype profiles of strains belonging to the same clonal complex. This, however, is not surprising, since it has already been observed by Monecke *et al.* (2008) that the carriage of antibiotic resistance determinants is largely non-lineage specific due to the frequently promiscuous nature of mobile genetic elements that mediate their transfer. Furthermore, analysis described in this work demonstrated that in general carriage of multiple resistance determinants was associated with the *mecA*-positive genotype. Such correlation has been previously reported although one study found that while the majority of MSSA isolates was susceptible to agents other than β-lactams, resistance to erythromycin was an exception (Diekema *et al.*, 2001). Multiple-drug resistance (resistance to ≥ 3 non-β-lactam agents) is common amongst MRSA, in particular the hospital-associated isolates (Chambers, 2001; Styers *et al.*, 2006). This reflects the widely recognized

ability of *S. aureus* to adapt quickly under the selective pressure of antimicrobial agents (de Lencastre, Oliveira and Tomasz, 2007).

The antimicrobial susceptibility patterns observed for CC398 strains revealed a high frequency of resistance to antimicrobial compounds that are used exclusively or at high rates in veterinary medicine. Tetracyclines are amongst antimicrobial compounds most commonly used for management of infection in food producing animals (Schwarz and Chaslus-Dancla, 2001). The high prevalence of tetracycline resistance in MRSA CC398 strains, also observed in this study, was immediately associated with the widespread use of this antimicrobial class in pig husbandry (de Neeling *et al.*, 2007). Although the use of antibiotics for growth promotion has been banned in Europe, the practice is still implemented in the United States and tetracyclines are among the drugs of choice (Aarestrup and Jenser, 2007). While macrolide resistance was observed for the majority of MRSA strains, resistance to tylosin was only prevalent among CC398 strains. The drug was introduced to veterinary medicine only and has been widely used in livestock (Schwarz and Chaslus-Dancla, 2001; McEwen and Fedorka-Cray, 2002). Similar to tetracyclines, the applications of tylosin have involved both treatment as well as growth promotion, with the latter now limited to the United States only (Aarestrup and Jenser, 2007). Other antimicrobial compounds that are restricted in application to veterinary medicine include apramycin, florfenicol, tiamulin and spectinomycin. Whilst all strains analysed in this study were apramycin susceptible, a MRSA CC398 strain was recently reported to carry a novel apramycin resistance gene, *apmA* - the first such gene to be reported in Gram-positive cocci (Fessler, Kadlec and Schwarz, 2011). The majority of CC398 strains analysed in this work demonstrated spectinomycin resistance, and a small proportion also revealed resistance to florfenicol and tiamulin. This further illustrates the selective pressures that have been shaping the resistance genotype of this lineage. It has been observed that many of the compounds to which MRSA CC398 strains acquire resistance are not used primarily for the treatment of staphylococcal infections (Fessler, Kadlec and Schwarz, 2011). Since MRSA CC398 is often a colonizing agent, the multiple-drug resistance of this lineage is not a consequence of failed therapeutic management of MRSA infections, rather a by-product of extensive use of antimicrobial therapy against a wide range of bacterial pathogens.

The analysis of resistance genotypes by DNA microarray and PCR revealed the highest level of determinant diversity among genes encoding resistance to aminoglycosides, macrolides, tetracycline and trimethoprim. This diversity revealed some level of association between certain clonal complexes and specific resistance determinants. For instance, the *ermA* gene was most prevalent among CC30 strains, whereas the *ermC* was mainly detected in strains belonging to CC22 and CC398. Although the *ermA* gene is often reported as the most common macrolide resistance determinant among *S. aureus*, some studies have found the *ermC* gene prevalent (Schmitz *et al.*, 2000a; Schmitz *et al.*, 2000b). Also, the *ermA* gene was reported as more common among MRSA isolates, whereas *ermC* as predominant amongst MSSA isolates (Schmitz *et al.*, 2000a; Schmitz *et al.*, 2000b). Such associations were not, however, observed for strains analysed in this study as only two MSSA strains were found to carry a macrolide resistance determinant, *ermA* in both. However, lack of *ermB* detection among the analysed strains is in accordance with other studies reporting that the gene is infrequently found in *S. aureus* (Schmitz *et al.*, 2000a; Schmitz *et al.*, 2000b). Amongst tetracycline resistance genes analysed in this study, the most common was *tetM* although it was predominantly found in CC398 strains. The *tetK* element, while less common, was identified across a wider clonal complex panel. Interestingly, all non-CC398 strains carrying the *tetK* gene were MSSA strains, while all human MRSA strains analysed in this study were *tet*-negative. This is in agreement with the observation that the UK epidemic clones of EMRSA-15 and EMRSA-16 are generally tetracycline susceptible (Speller *et al.*, 1997). The *tetL* element has thus far been uncommon among *S. aureus* isolates (Schmitz *et al.*, 2001), but was detected here in CC398 and CC8 MRSA strains. Unlike the *erm* elements, carriage of multiple *tet* determinants by a single strain was observed and, as reported previously, strains with two *tet* genes demonstrated higher tetracycline MIC value than those with a single element only (Trzcinski *et al.*, 2000; Schmitz *et al.*, 2001).

A high prevalence and diversity of antimicrobial resistance determinants was a general feature of all MRSA strains analysed in this study, but it was particularly prominent among the CC398 group. Other reports of uniform carriage of *tetM* gene among MRSA CC398 strains indicate that while not unique it constitutes a characteristic feature of the MRSA CC398 genetic profile (Witte *et al.*, 2007; Denis *et al.*, 2009; Hallin *et al.*, 2011; Argudin *et al.*, 2011). Although a proportion of CC398 strains also carried the *tetK* and *tetL* elements, their prevalence was considerably

lower than previously reported and, unlike in other studies, none of the strains carried a combination of all three elements *tetM+tetK+tetL* (Kadlec *et al.*, 2009; Fessler *et al.*, 2010; Argudin *et al.*, 2011). On the other hand, the distribution of trimethoprim resistance genes is in agreement with other reports that *dfrK*, a novel resistance determinant first identified in MRSA CC398 strain, is prevalent and has been associated thus far mainly with the CC398 lineage (Kadlec *et al.*, 2009; Kadlec and Schwarz, 2009a; Argudin *et al.*, 2011). Other resistance determinants that were identified exclusively amongst CC398 strains included the *vga* elements. As demonstrated in this analysis, the *vga* genes are currently of comparatively infrequent occurrence but identification of *vgaE,* another novel resistance determinant recently identified in MRSA CC398 strain (Schwendener and Perreten, 2011), suggests dissemination of *vga* elements within the CC398 lineage and thus their frequency is likely to increase.

The major resistance genotype cluster that contained all but two CC398 strains also included the two MRSA CC8 strains, suggesting that the resistance profiles of CC398 were most comparable to the equine MRSA CC8 strains. The CC8 strains shared the *aacA-aphD, aadD, ermC, tetL* and *dfrA* resistance genes with the CC398 strains and both carried *tetM*. This suggests that the animal-associated MRSA isolates share a common resistance gene pool amongst each other to a greater degree than with the human strains. Evaluation of a larger collection of animal *S. aureus* strains, both MSSA and MRSA would be necessary to further substantiate this finding. Moreover, the diversity and high prevalence of *tet* genes might not be a unique feature of MRSA CC398, but rather a common characteristic of animal-associated MRSA.

Cattle-associated strains demonstrated the lowest prevalence of antimicrobial resistance, in both phenotype and genotype. The CC151 was the only lineage analysed in this study that was represented by susceptible strains only. However, while relatively uncommon, antimicrobial resistance in cattle-related *S. aureus* does occur although it is often limited to the carriage of the *blaZ* determinant as demonstrated by the CC97 lineage. Other than penicillin, some studies have found a low prevalence of resistance to erythromycin, clindamycin and gentamicin (Gentilini *et al.*, 2000; Pitkala *et al.*, 2004). Despite the global spread of MRSA, its prevalence amongst cattle has been very low with many studies reporting the lack of MRSA among bovine *S. aureus* (Gentilini *et al.*, 2000; De Oliveira *et al.*, 2000; Pitkala *et al.*, 2004). Often, isolates found to demonstrate an elevated MIC to oxacillin lacked the *mecA* gene and

the apparent resistance phenotype was associated with β-lactamase hyper-production (De Oliveira *et al.*, 2000). Recently a novel *mecA* homologue, designated *mecC* was identified amongst bovine isolates (Garcia-Alvarez *et al.*, 2011). In this study all CC130 were found to carry the gene and while the lineage was previously reported as a predominant carrier of the determinant it was also reported in *S. aureus* isolates belonging to CC151, ST425 and ST1943 (Garcia-Alvarez *et al.*, 2011). The lineage variation suggests that the spread of *mecC* gene is, in addition to clonal dissemination of *mecC*-positive CC130 isolates, also mediated by the horizontal transfer of the SCC*mec* element. Whilst this would indicate that the prevalence of *mecC*-positive *S. aureus* isolates among cattle is likely to increase, some sceptisism can be expressed regarding the clinical significance of these MRSA isolates. In contrast to *mecA* carrying strains described here, the CC130 strains were susceptible to all non-beta-lactam agents tested. Such finding provokes conclusion that the *mecC*-positive isolates do not, at present, pose a threat to either animals or humans, in a manner comparable to the MRSA isolates carrying the *mecA* gene. This in particular relates to the *mecA*-positive HA- and LA-MRSA isolates that commonly display a multiple antimicrobial resistance phenotype, and thus represent a considerable therapeutic challenge.

The analysis of biocide susceptibility demonstrated that whilst variation in MIC values between different strains can be observed, strains with apparent reduced susceptibility most often demonstrated between 2- and 4-fold increase in the MIC. The relatively small MIC differences represent an obstacle in the accurate identification of isolates with increased tolerance to biocides (Noguchi *et al.*, 2005). Furthermore, none of the strains carried the transferable biocide resistance determinants that were screened for in this analysis. As such, some of the strains were regarded as having reduced tolerance to biocides only on the basis of comparatively elevated MIC. This was the case for majority of human MRSA strains, which is in agreement with previous reports that nosocomial MRSA strains are particularly prone to acquisition or development of enhanced biocide tolerance due to widespread use of disinfectants in the healthcare environment (Vali *et al.*, 2008).

Biocides are also widely used in veterinary medicine, which was recognized as a risk factor for emergence of animal-associated isolates with reduced susceptibility to biocides (Aarestrup and Hasman, 2004). In cattle, biocides are components of veterinary medicine used for teat and hoof disinfection (Aarestrup and Hasman, 2004;

Bjorland *et al.*, 2005). Interestingly, a relatively high prevalence of increased tolerance to some of the tested biocides agents was observed among cattle-associated strains, particularly the CC97 lineage. Other studies reported a high frequency of disinfectant resistance genes among staphylococci originating from cattle, demonstrating that dissemination of biocide resistance determinants is not limited to human nosocomial isolates (Bjorland *et al.*, 2005).

There are currently no reports of reduced disinfectant susceptibility among MRSA CC398 isolates. In this study all analysed strains demonstrated comparable levels of susceptibility to all tested biocide compounds. Some strains, however, displayed reduced susceptibility to metals, due to the carriage of *czrC* element, which can be associated with the use of copper sulphate and zinc chloride as animal feed additives (Aarestrup and Hasman, 2004). Although detected at low frequency in this study it was found to be highly prevalent among Danish MRSA CC398 isolates of both human and animal origin (Cavaco *et al.*, 2010). Furthermore, the physical association between the *czrC* and SCC*mec* V element is likely to mediate a co-selection of methicillin and metal resistance, which might further enhance dissemination of the *czrC* element (Cavaco *et al.*, 2010).

Finally, a number of strains from various lineages were found to carry the *cadD* resistance determinant, but failed to display cadmium resistance phenotype. The *cadD* element shares no significant similarly with *cadA* or *cadC* and constitutes a part of the *cadDX* operon (Crupper *et al.*, 1999). The *cadX* gene was previously found to be inactive, which was associated with a low level resistance to cadmium (Crupper *et al.*, 1999). Presence of *cadD* gene, but lack of elevated cadmium MIC in all *cadD*-positive strains might suggest further deterioration of the *cadDX* operon amongst the analysed strains.

In summary, the MRSA CC398 strains displayed considerable antimicrobial resistance genotype heterogeneity. This feature was not, however, strictly restricted to the CC398 lineage. High content of antimicrobial resistance genes could be also observed in MRSA strains belonging to CC8 and CC30, with MRSA CC22 displaying a comparatively lower prevalence of resistance determinants. Furthermore, the antimicrobial resistance genotype of the equine MRSA CC8 shared a considerable similarity with the CC398 strains.

To characterise further the determinants of antimicrobial resistance amongst the analysed panel of MRSA CC398, the strains were investigated for carriage of resistance-associated plasmids, with an aim of determining, which of the identified resistance genes are plasmid-encoded as well as to conduct a follow-up analysis of the discrepancies between resistance genotypes and phenotypes that were observed in this chapter.

Chapter 6 Analysis of plasmid-associated antimicrobial resistance amongst MRSA CC398 strains

6.1 Introduction

The horizontal transfer of antimicrobial resistance genes in staphylococci has been predominantly associated with plasmids, transposons and staphylococcal cassette chromosome (SCC) elements (Malachowa and DeLeo, 2010). Plasmids are thought to have played a prominent role in the emergence of resistance to a wide range of antimicrobial agents (Werckenthin *et al.*, 2001). Unlike other mobile genetic elements, plasmids do not normally integrate into the bacterial chromosome and remain as extra-chromosomal elements (Schwarz and Chaslus-Dancla, 2001). This feature is thought to have allowed plasmids to become particularly efficient vectors of accessory genetic material, as chromosomal integration is more likely to reduce fitness of the host cell (Lyon and Skurray, 1987). Furthermore, plasmids not only mediate transfer and acquisition of plasmid-borne elements, but also transposon-associated resistance genes (Werckenthin *et al.*, 2001). A number of staphylococcal resistance genes have been described as plasmid-related (Malachowa and DeLeo, 2010). It has also been determined that the dissemination of the same resistance determinants amongst different species of staphylococci is mediated by structurally related plasmids (Werckenthin *et al.*, 2001). However, homologous plasmids have also been identified in bacilli demonstrating that plasmid-associated transfer of antimicrobial resistance can occur not only at inter-species, but also at the inter-genus level (Schwarz *et al.*, 1996).

Plasmid carriage is generally an unstable genotypic feature and its analysis has limited application in molecular typing aimed at differentiation between resistant isolates (Werckenthin *et al.*, 2001). However, it gives an insight into the nature and transferability of resistance properties (Werckenthin *et al.*, 2001). The autonomous nature of plasmid elements within a bacterial cell has been utilized in the development of methods for the analysis of plasmid DNA. As it is separate from the bacterial chromosome, the plasmid DNA can be isolated and subjected to further characterisation. A number of methods have been developed for the isolation of plasmid DNA, which are aimed at separation of plasmid DNA from the chromosomal material and downstream isolation as mostly purified plasmid DNA (Sambrook and Russell, 2001). General characterisation of plasmid DNA carriage involves methods such as plasmid profiling, whereas rapid analysis of plasmid structure can be achieved with restriction analysis (Werckenthin *et al.*, 2001). However, the most critical feature

of plasmids, and other mobile genetic elements, is their function within the host cell. As vectors of accessory genes, acquisition of plasmids is expected to provide the host cell with a selective advantage (Schwarz and Chaslus-Dancla, 2001). To investigate this, the host cell and plasmid DNA can be subjected to genetic manipulation (McNamara, 2008). Inducing loss of plasmids, known as plasmid curing is one of the approaches and is often applied with an aim of analyzing how removal of the plasmid affects previously determined metabolic and/or phenotypic features of the host strain (Neve, Geis and Teuber, 1988). Alternatively the isolated plasmid DNA can be transformed into a competent recipient strain, which can then be analysed for the newly acquired phenotypic and genotypic characteristics (McNamara, 2008).

A number of studies recently described the identification of novel plasmid elements in MRSA CC398 strains. This has included the pKKS2187 plasmid carrying a novel trimethoprim resistance gene designated *dfrK*, pKKS825 carrying a novel ABC transporter gene called *vgaC* and pKKS25 that encoded a resistance gene cluster and contained a novel insertion sequence element IS*Sau10* (Kadlec and Schwarz, 2009a; Kadlec and Schwarz, 2009b; Kadlec and Schwarz, 2010). Furthermore, the whole genome sequence analysis of a MRSA CC398 strain revealed the presence of three plasmids, two of which carried a single determinant of resistance (Schijffelen *et al.*, 2010). These reports demonstrate that plasmids represent important vectors of antimicrobial resistance carriage among MRSA CC398 isolates. The genotypic analysis of antimicrobial resistance described in the previous chapter (Chapter 5, section 5.3) revealed that the investigated MRSA CC398 strains carry a heterogeneous panel of resistance determinants, consisting of both common, as well as less prevalent genes.

To gain further insight into the characteristics of antimicrobial resistance among the MRSA CC398 strains, an analysis of resistance-associated plasmid carriage was conducted. The experiment involved isolation of plasmid DNA, transformation into a recipient strain, identification and characterisation of transformed cells that acquired the resistance phenotype followed by sequence analysis of plasmids that were confirmed to carry a resistance determinant.

6.2 Materials and methods

Plasmid DNA was isolated as described in section 2.3.2 from 18 MRSA CC398 strains (plasmid donors, Table 6.1). The isolated plasmid DNA was transformed by

electroporation into *S. aureus* RN4220 strain as described in section 2.5.1. Transformed colonies were selected as described in section 2.5.3, Table 6.1 presents the panel of antimicrobial agents chosen for the selection of transformants for each plasmid donor strain. In general, it consisted of antimicrobial agents that the donor strain displayed resistance to. The antimicrobial susceptibility analysis of transformed cells was conducted as described in section 2.6.1.

The detection of resistance determinants was performed by PCR as described in section 2.3.3, using primers specific for *aadD*, *ermA*, *ermC*, *ermT*, *tetL*, *tetM*, *vgaA* and *vgaE* (Table 2.1). If the same resistance determinant was associated with plasmids of comparable size among various strains, the plasmids were analysed by restriction digest as described in 2.5.4.

Sequencing of plasmid elements was performed by primer walking. Plasmids under 10 kilobase pairs (kbp) in size carrying a known determinant of resistance were fully sequenced from within the resistance gene using previously described PCR detection primers for the initial sequencing run. In case of larger plasmids (over 10 kb) or plasmids carrying an unidentified determinant of resistance a restriction digest library was prepared using pUC19 plasmid as a vector. Restriction enzyme digest and preparation of recombinant DNA were performed as described in sections 2.5.4 and 2.5.5. The recombinant DNA was transformed into either *E. coli* TOP10F or *E. coli* NEB10β by chemical transformation as described in section 2.5.2. The transformed cells were selected as described in section 2.5.3. The vector-inserted restriction fragments from selected transformants were sequenced in full by primer walking. The DNA sequence analysis was conducted as described in section 2.3.5.3. The raw assembled sequence data is provided in a FASTA file format (Electronic Appendix I).

Multiple alignment of amino acid sequences were generated with Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/).

Table 6.1 MRSA CC398 strains used in the analysis (plasmid donors) and the list of antimicrobial agents used for selection of transformed cells

Strain ID	Resistance phenotype [a, b]	Resistance genotype [c]
90	PEN, GEN, KAN, ERY, CLI, TET, TMP	*blaZ, aacA-aphD, aadD, ermT, tetM, tetL, dfrA,*
91	PEN, SPE, ERY, CLI, CHL, TET	*blaZ, ermC, fexA, tetM, tetK*
92	PEN, GEN, KAN, SPE, ERY, CLI, TET, TMP	*blaZ, aacA-aphD, ermC, tetM, dfrK*
93	PEN, SPE, ERY, CLI, CHL, TET	*blaZ, ermC, tetM, fexA*
95	PEN, SPE, ERY, CLI, CIP, CHL, TET, TIA, TMP	*blaZ, ermC, fexA, tetM, tetK, dfrG*
96	PEN, KAN, ERY, CLI, TET, TMP	*blaZ, aadD, ermC, tetM, dfrA*
99	PEN, GEN, KAN, SPE, TET, TMP	*blaZ, aacA-aphD, tetM, dfrK*
101	PEN ,GEN, KAN, TET, TMP	*blaZ, aacA-aphD, aadD, tetM, ermT, tetL, dfrK*
102	PEN, GEN, KAN, SPE, ERY, CLI, TET, TMP	*blaZ, aacA-aphD, ermC, tetM, dfrK*
103	PEN, ERY, CLI, CIP, TET, TMP	*blaZ, ermC, tetM, tetK, dfrK*
104	PEN ,GEN, KAN, SPE, ERY, CLI, TET, TIA	*blaZ, aacA-aphD, aadD, spc, ermA, ermT, tetM, tetL, vgaE*
105	PEN, GEN, KAN, ERY, CLI, TET, TMP	*blaZ, aacA-aphD, ermC, ermT, tetM, tetL, dfrK*
106	PEN, CLI, CIP, TET, TIA, TMP	*blaZ, tetM, tetK, vgaA, dfrK*
107	PEN, SPE, TET	*blaZ, tetM*
108	PEN, ERY, CLI, TET, TMP	*blaZ, ermC, tetM, tetK, dfrK*
109	PEN, GEN, KAN, SPE, ERY, CLI, TET, TMP	*blaZ, aacA-aphD, ermC, tetM, dfrK*
110	PEN, SPE, TET	*blaZ, tetM*
111	PEN, GEN, KAN, SPE, ERY, CLI, TET, TIA, TMP	*blaZ, aacA-aphD, spc, ermA, tetM, vgaE, dfrK*

[a] the selected antimicrobial agents represent all classes of antimicrobial compounds to which the isolate was found to be resistant (Chapter 5)

[b] PEN - penicillin; GEN - gentamicin; KAN - kanamycin; SPE - spectinomycin; VAN - vancomycin; CLI - clindamycin; ERY - erythromycin; CHL - chloramphenicol; CIP - ciprofloxacin; TET - tetracycline; TIA - tiamulin; TRI - trimethoprim

[c] resistance genes detected in the corresponding isolate; underlined are determinants previously associated with plasmids (as described in Chapter 1 - 1.2.1)

6.3 Results

The isolated plasmid DNA was analysed by gel electrophoresis (Figure 6.1). Plasmid DNA bands were observed for all analysed strains.

6.3.1 Transformations

The outline of transformation results is presented in Table 6.2. In summary, erythromycin- and clindamycin-resistant transformed colonies were observed for all but one plasmid donor strain. Spectinomycin-resistant transformants were generated for 8 out of 11 donor strains. All transformed cultures were screened on tetracycline plates and resistant colonies were generated with plasmid DNA from five donor strains. Tiamulin-resistant transformants were observed for one strain only. Altogether, at least one transformed strain was generated for all plasmid donor strains except strain 111.

The resistant transformed strains were designated using the ID of the donor strain and abbreviated name of the selective medium, for instance transformant generated with plasmid DNA from strain 90 and displaying erythromycin resistance was designated 90 ERY. No transformed cultures were observed on penicillin, gentamicin, kanamycin, chloramphenicol, ciprofloxacin or trimethoprim plates.

6.3.2 Transformant analysis

Plasmid DNA was extracted from each transformed strain and analysed by gel electrophoresis (Figure 6.2).

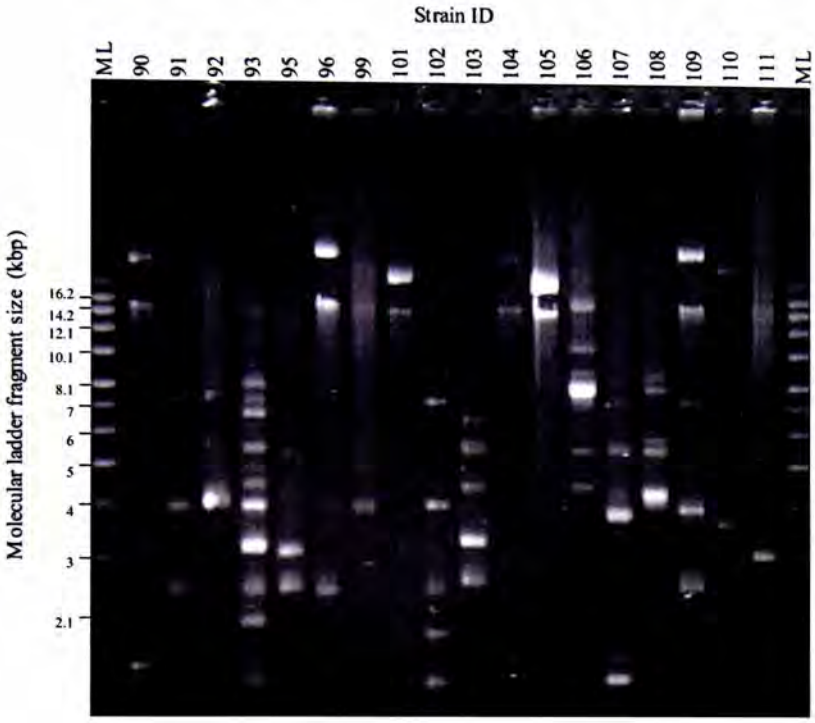


Figure 6.1 Plasmid profiles of all analysed MRSA CC398 strains. The gel lanes are labelled as: 'ML' - molecular ladder (Supercoiled 2–16 kbp; Sigma D5292), strain ID 90 - 111.

Table 6.2 Summary of transformation results for each plasmid donor strain [a]

Strain ID	Transformants	No colonies
90	ERY, CLI, TET	PEN, GEN, KAN, TMP
91	SPE, ERY, CLI	PEN, CHL, TET
92	SPE, ERY, CLI	PEN, GEN, KAN, TET, TMP
93	SPE, ERY, CLI, TET	PEN, CHL
95	ERY, CLI	PEN, SPE, CIP, CHL, TET, TIA, TMP
96	ERY, CLI, TET	PEN, KAN, TMP
99	SPE	PEN, GEN, KAN, SPE, TET, TMP
101	TET	PEN, GEN, KAN, TMP
102	SPE, ERY, CLI	PEN, GEN, KAN, TET, TMP
103	ERY, CLI	PEN, CIP, TET, TMP
104	CLI	PEN, GEN, KAN, SPE, ERY, TET, TIA
105	ERY, CLI, TET	PEN, GEN, KAN, TMP
106	CLI, TIA	PEN, CIP, TET, TMP
107	SPE	PEN, TET
108	ERY, CLI, SPE	PEN, TET, TMP
109	SPE, ERY, CLI	PEN, GEN, KAN, TET, TMP
110	SPE	PEN, TET
111		PEN, GEN, KAN, SPE, ERY, CLI, TET, TIA, TMP

[a] Antimicrobial name abbreviation description provided in Table 6.1

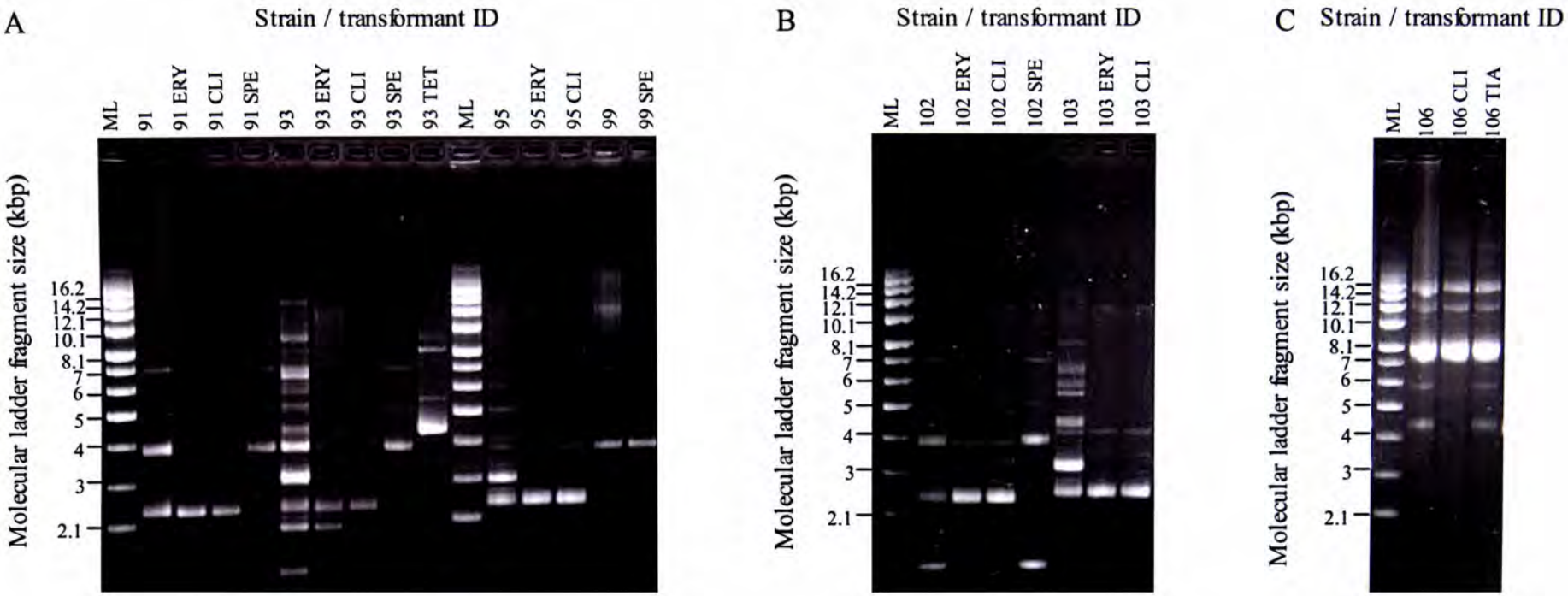


Figure 6.2 Plasmid profiles of plasmid DNA donors and the transformed strains. The figures A - C show plasmid profile of donor strains (A: 91, 93, 95, 99; B: 102, 103; C: 106) and their corresponding transformants (strain ID with suffix indicating the resistance phenotype: CLI – clindamycin, ERY – erythromycin, SPE – spectinomycin, TET – tetracycline, TIA - tiamulin). The gel lanes are labelled as: 'ML' - molecular ladder (Supercoiled 2−16 kb; Sigma D5292); strain and transformant ID.

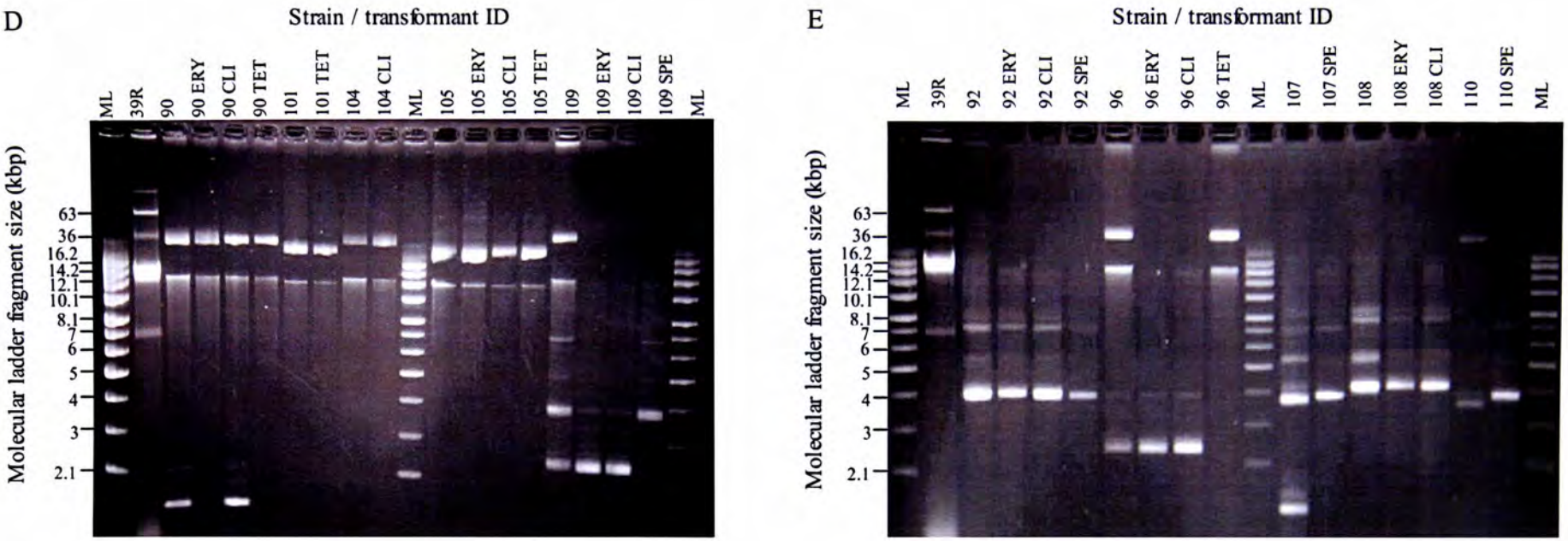


Figure 6.2 (continued) Plasmid profiles of plasmid DNA donors and the transformed strains. The figures D - E show plasmid profile of donor strains (D: 90, 101, 104, 105, 109; E: 92, 96, 107, 108, 110) and their corresponding transformants (strain ID with suffix indicating the resistance phenotype: CLI – clindamycin, ERY – erythromycin, SPE – spectinomycin, TET – tetracycline, TIA - tiamulin). The gel lanes are labelled as: 'ML' - molecular ladder (Supercoiled 2−16 kb; Sigma D5292); 39R - *E. coli* plasmid sizing reference strain; strain and transformant ID.

6.3.2.1 Antimicrobial susceptibility testing

For each donor strain, the transformed strains were tested against a panel of antimicrobial compounds that was used for the selection of transformants for that particular donor (Table 6.1). The results are presented in Table 6.3. All transformed strains were confirmed to display resistance to the corresponding selective agent. Furthermore, some were found to also display resistance to other antimicrobial compounds. All SPE transformants were found to be resistant to spectinomycin only and no other analysed agent. All ERY and CLI transformants were resistant to both erythromycin and clindamycin. The 92 ERY and 92 CLI transformants both also displayed resistance to spectinomycin, although the 92 SPE was found to be erythromycin and clindamycin susceptible. The cross-resistance observed for 92 ERY and 92 CLI might have thus been mediated by the transfer of more than one plasmid. A proportion of ERY and CLI transformants were also resistant to tetracycline, with the TET transformants of the same donor strain found to be resistant to both erythromycin and clindamycin, confirming the linkage. Other TET transformants were found to be resistant to tetracycline only. Some of the transformants displayed reduced susceptibility to kanamycin, which included the transformed strains resistant to erythromycin, clindamycin and tetracycline or tetracycline only. Finally, the 106 CLI and 106 TIA transformants were resistant to both clindamycin and tiamulin.

6.3.2.2 Detection of resistance determinants

The transformed strains were screened by PCR for the presence of resistance determinants carried by the donor strain (Table 6.1) in order to identify underlying mechanism of resistance phenotype and to confirm transfer of a resistance-associated mobile element. The results are presented in Table 6.4. The *ermC* gene was detected in the majority of ERY and CLI transformants, with the remaining carrying the *ermT* determinant. The majority of TET transformants contained the *tetL* gene, with two carrying an undetermined mechanism of resistance. All transformed strains that displayed reduced susceptibility to kanamycin carried the *aadD* gene. The analysis revealed a co-carriage of *ermT*, *tetL* and *aadD* genes in all 90 (ERY, CLI, TET), as well as 101 TET and 104 CLI transformants. The carriage of *ermT*+*tetL* was observed for all 105 transformants (ERY, CLI, TET) that were in addition found to be positive for *ermC*. Both 106 (CLI, TIA) transformed strains carried the *vgaA* gene.

Table 6.3 Antimicrobial susceptibility testing of transformed strains [a, b, c]

	MIC µg/ml										
ID	PEN	GEN	KAN	SPE	CLI	ERY	CHL	CIP	TET	TIA	TMP
90 ERY	0.12	0.25	8		>32	>32			64		2
90 CLI	0.12	0.25	8		>32	>32			64		2
90 TET	0.12	0.25	8		>32	>32			64		2
91 SPE	0.12			>1024	0.12	0.25	4		0.12		
91 ERY	0.12			32	>32	>32	4		0.12		
91 CLI	0.12			32	>32	>32	4		0.12		
92 SPE	0.12	0.25		>1024	0.12	0.25			0.12		2
92 ERY	0.12	0.25		>1024	>32	>32			0.12		2
92 CLI	0.12	0.25		>1024	>32	>32			0.12		2
93 SPE	0.12			>1024	0.12	0.25	4		0.12		
93 ERY	0.12			32	>32	>32	4		0.12		
93 CLI	0.12			32	>32	>32	8		0.12		
93 TET	0.12			32	0.12	0.25	4		16		
95 ERY	0.06			32	>32	>32	4	0.25	0.12	0.12	2
95 CLI	0.06			32	>32	>32	8	0.25	0.12	0.12	2
96 ERY	0.06		2		>32	>32			0.12		2
96 CLI	0.12		2		>32	>32			0.12		2
96 TET	0.12		16		0.12	0.25			64		2
99 SPE	0.12	0.25		>1024					0.25		2
101 TET	0.12	0.25	16						64		2
102 SPE	0.12	0.25	2	>1024	0.12	0.25			0.12		2
102 ERY	0.12	0.5	4	32	>32	>32			0.12		2
102 CLI	0.12	0.25	2	32	>32	>32			0.12		2
103 ERY	0.12				>32	>32		0.25	0.25		2
103 CLI	0.12				>32	>32		0.25	0.25		2
104 CLI	0.12	0.25	8	32	>32	>32			64	0.25	
105 ERY	0.12	0.25	2		>32	>32			64		2
105 CLI	0.12	0.25	2		>32	>32			64		2
105 TET	0.12	0.25	2		>32	>32			64		2
106 CLI	0.12				32			0.25	4	64	2
106 TIA	0.12				16			0.12	0.25	64	2
107 SPE	0.12			>1024					0.25		
108 ERY	0.12				>32	>32			0.25		2
108 CLI	0.12				>32	>32			0.25		2
109 SPE	0.12	0.25	2	>1024	0.12	0.25			0.25		2
109 ERY	0.12	0.25	2	32	>32	>32			0.25		2
109 CLI	0.12	0.25	2	32	>32	>32			0.25		2
110 SPE	0.12			>1024					0.25		

[a] Antimicrobial name abbreviation description provided in Table 6.1

[b] Red font highlights values that are equal to or above the resistance breakpoint

[c] Grey area indicates that the strain was not analysed for the MIC of corresponding antimicrobial compound

Table 6.4 Analysis of antimicrobial resistance genotype of transformed strains [a]

ID	*ermA*	*ermC*	*ermT*	*tetL*	*tetM*	*aadD*	*vgaA*	*vgaE*
90 ERY			+	+	-	+		
90 CLI			+	+	-	+		
90 TET			+	+	-	+		
91 ERY		+						
91 CLI		+						
92 ERY		+						
92 CLI		+						
93 ERY		+						
93 CLI		+						
93 TET					-			
95 ERY		+						
95 CLI		+						
96 ERY		+						
96 CLI		+						
96 TET					-	+		
101 TET			+	+	-	+		
102 ERY		+						
102 CLI		+						
103 ERY		+						
103 CLI		+						
104 CLI	-		+	+		+		-
105 ERY		+	+	+	-			
105 CLI		+	+	+	-			
105 TET		+	+	+	-			
106 CLI							+	
106 TIA							+	
108 ERY		+						
109 ERY		+						
109 CLI		+						

[a] Grey area indicates that the strain was not analysed for carriage of the corresponding antimicrobial resistance gene

6.3.3 Plasmid sequence analysis

The transformed plasmid DNA was further characterised by conducting sequence analysis. If the resistance determinant was identified by PCR, the objective of the analysis was to determine the genetic background of the mobile element. In the case of plasmids isolated from 96 TET, 93 TET and all SPE transformants the primary aim was to identify the genetic determinant responsible for the phenotypic resistance.

6.3.3.1 ERY/CLI transformants carrying *ermC*

ERY/CLI transformants that were found to have acquired the *ermC* gene carried either a 2.3-kbp (n=7) or a 4-kbp (n=2) plasmid. All 2.3-kbp *ermC*-carrying plasmids were analysed by *Mbo*I+*Hha*I double restriction digest. As demonstrated by Figure 6.3, the plasmids revealed a level of variation in their restriction fragment patterns. Primer-walking sequencing was performed on three selected representatives of the 2.3-kbp plasmid (91 ERY, 96 ERY, 103 ERY) and both 4-kbp plasmids (92 ERY, 108 ERY). The annotated schematic of determined plasmid sequence is presented in Figure 6.4.

Plasmids from 91 ERY, 96 ERY and 103 ERY were designated pDJ91E, pDJ96E and pDJ103E respectively. The pDJ91E was 2369 base pairs (bp), pDJ96E was 2350 bp, whereas pDJ103E measured 2512 bp in length. Thus the variation in restriction fragment pattern between the sequenced plasmids, presented in Figure 6.3, corresponded with differences in plasmid size. The three plasmids shared 95% identity and all consisted of two open reading frames (ORFs). The first ORF encoded a replication protein whereas the second ORF encoded an rRNA adenine N-6-methyltransferase (EC 2.1.1.48). Alignment of pDJ91E, pDJ96E and pDJ103E demonstrated that the plasmids were identical across the coding sequence regions (Figure 6.4). The BLAST analysis of nucleotide sequence revealed that the plasmid sequences were highly homologues with a number of previously identified *ermC*-carrying plasmids including pNE131 (NC_001390) from *S. epidermidis* and SAP078B (NC_013305) from *S. aureus*.

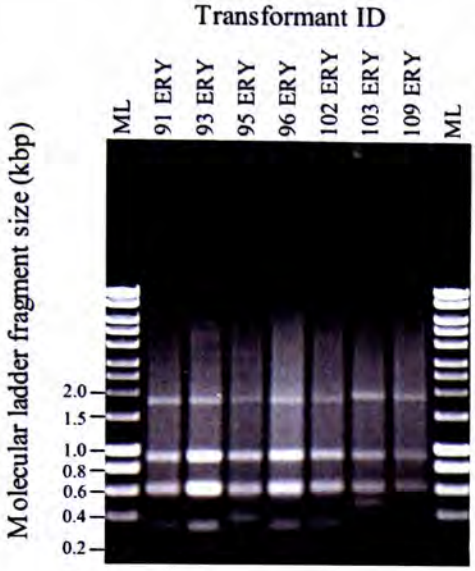


Figure 6.3 *Mbo*I+*Hha*I restriction digest analysis of plasmid DNA from ERY transformants carrying the *ermC* gene. The gel lanes are labeled as: 'ML' - molecular ladder (Hyperladder I; Bioline BIO-33053); transformant ID.

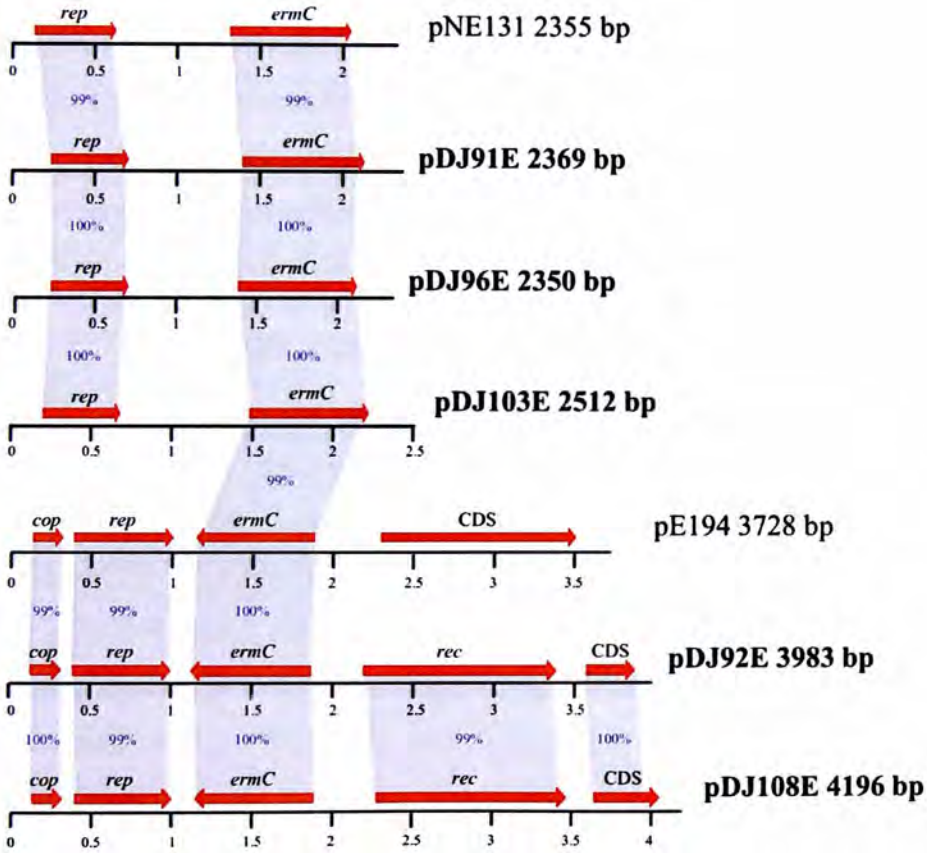


Figure 6.4 Schematic representation of *ermC*-carrying plasmids identified in this work (bold) pDJ91E, pDJ96E, pDJ103E, pDJ92E and pDJ108E as well as plasmids pNE131 and pE194. The red arrows represent the coding DNA sequences with the name shown above (elements of unknown function are referred to as CDS). Homologous genes are indicated by grey shading with the % sequence identity displayed within.

Plasmids from 92 ERY and 108 ERY were designated pDJ92E and pDJ108E, respectively, and were 3983 bp and 4196 bp in length. The plasmids shared 99% identity and consisted of five ORFs. The first two loci constituted a *cop-rep* region that encoded Cop and Rep proteins, respectively. Around 150 bp downstream was located the *ermC* gene coding for a 244-aa rRNA adenine N-6-methyltransferase (EC 2.1.1.48). The fourth ORF was found to encode plasmid recombination enzyme and was designated *rec* gene. The last ORF represented a hypothetical protein of unknown function. Alignment analysis of pDJ92E and pDJ108E revealed that the plasmids were nearly identical across regions containing coding sequences with exception of the gene encoding the hypothetical protein, which was 119 bp longer at the 3' end in pDJ108E (Figure 6.4). The plasmids were found to share over 90% identity with *S. aureus* pE194 plasmid (NC_005908), over 75% of sequence length, which comprised the *cop-rep* region and *ermC*. The *rec* element of pDJ92E and pDJ108E was only partly homologues with the corresponding region in pE194.

pDJ91E and pDJ92E sequence alignment analysis revealed that the plasmids were largely distinct across majority of the sequence and shared only the *ermC* gene together with around a 200-bp region upstream of the determinant's coding sequence.

6.3.3.2 106 CLI/TIA transformants carrying *vgaA*

The 106 transformants, CLI and TIA, were found to both carry an 8-kbp plasmid that contained the *vgaA* determinant and thus it was concluded that the transformed strains acquired the same mobile element. The 106 CLI transformant was selected for plasmids analysis, which was designated pDJ106V and fully sequenced by primer-walking. The annotated schematic of pDJ106V is presented in Figure 6.5. The plasmid was 7750 bp in length and contained six ORFs. The first ORF was identified as a *rep* element that encoded a 154-aa replication protein. The second ORF was identified as the *vgaA* gene that encoded a 524-aa ABC transporter ATP-binding protein. The protein sequence shared nearly 100% identity with previously identified VgaA elements from *Staphylococcus hominis* (ZP_04060074), *S. aureus* (YP_002332257) and *S. epidermidis* (YP_006962147). Further downstream was positioned a second *rep* gene. The fourth ORF was found to encode a conserved hypothetical protein. Based on protein homology search and the identity of the neighbouring genetic elements it was designated as Mob-like protein.

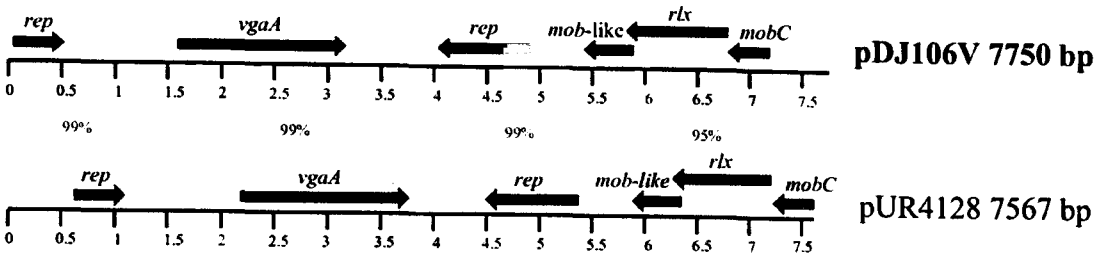


Figure 6.5 Schematic representation of the *vgaA*-carrying plasmid pDJ106V identified in this work (bold) and plasmid pUR4128. The red arrows represent the coding DNA sequences with the name shown above. Homologous genes are indicated by grey shading with the % sequence identity displayed within.

Next to and overlapping the *mob*-like element was a relaxase/mobilization gene designated *rlx* that also overlapped the downstream element and the sixth ORF identified as a *mob* gene. The entire pDJ106V sequence shared 99% identity with recently reported *S. aureus* plasmids pUR4128 (JQ861960) and pUR2355 (JQ312422), both originating from MRSA CC398 strains.

6.3.3.3 93 TET transformant

A plasmid of approximately 4.5 kbp in size and designated pDJ93T was found to mediate tetracycline resistance through an unidentified mechanism (Table 6.4). The transformant was negative for the carriage of the *tetM* gene, the only tetracycline resistance determinant identified in strain 93, the plasmid DNA donor. To determine the sequence of pDJ93T, a restriction digest library of the plasmid was created following *Xba*I digestion. The enzyme was found to cut once within the plasmid sequence and thus the plasmid was sequenced by primer-walking of the vector-inserted linearised plasmid.

Sequence analysis revealed that pDJ93T was 4,440 bp in length and contained three open reading frames (Figure 6.6). The First ORF encoded a 314-aa replication initiation protein. The next ORF was identified as *tetK* gene and as such coded for a 459-aa tetracycline resistance protein, which shared nearly 100% identity with previously described Tet*K* proteins from various *S. aureus* strains (ZP_13506406, NP_040465, ZP_06859673) and *S. epidermidis* (ZP_06283689). The third ORF encoded a 413-aa plasmid recombination enzyme. The full plasmid sequence was found to share a nearly 100% identity with a number of *S. aureus* plasmids such as pT181 (CP000045), SAP094A (GQ900443) and pS0385-1 (AM990993).

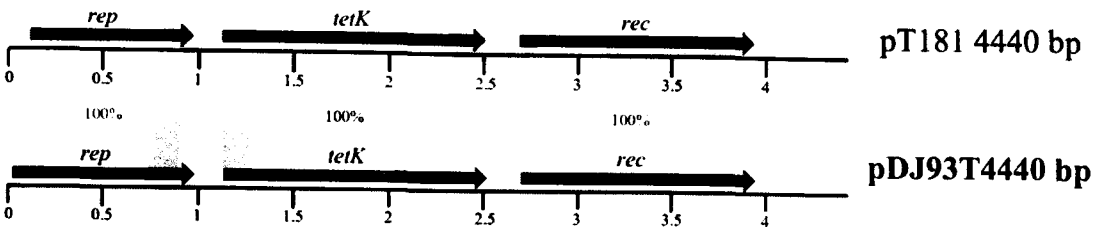


Figure 6.6 Schematic representation of the *tetK*-carrying plasmid pDJ93T identified in this work (bold) and plasmid pT181. The red arrows represent the coding DNA sequences with the name shown above. Homologous genes are indicated by grey shading with the % sequence identity displayed within.

6.3.3.4 SPE transformants

All transformants that demonstrated resistance to spectinomycin were found to carry a plasmid of approximately 4 kbp. The plasmid DNA from all SPE transformants was analysed by *Alu*I restriction digest. A level of variation in the restriction fragment pattern was observed among the analysed plasmids indicating that while comparable in size the plasmids from different strains were not 100% identical by nucleotide sequence (Figure 6.7). To identify the genetic mechanism of resistance, 91 SPE was selected as a single representative for sequence analysis. Plasmid restriction digest library was created following *Xba*I digestion. The enzyme was found to cut twice within the plasmid sequence. The digested fragments were approximately 3.8 kbp and 0.2 kbp in size. The 3.8-kbp fragment was sequenced in full by primer walking and primers were designed to close the gap represented by the 0.2-kbp restriction fragment.

The sequenced plasmid, designated pDJ91S, was 3,928 bp in length and consisted of three open reading frames (Figure 6.8). The first ORF, designated *rep*, coded for a 322-aa replication initiation protein. The next ORF was located 25 bp downstream and encoded a novel spectinomycin adenyltransferase that was 257 aa in length. The gene was designated *aad9A* and BLAST analysis of the nucleotide sequence found no significant similarities. The protein sequence demonstrated a 51% identity over 77% of the length with a spectinomycin adenyltransferase AAD9, from *Enterococcus faecalis* (AAA16527). Aad9A protein also shared 48% sequence identity with the recently described Spw protein from *S. aureus* (AFU35063) and 45%

with the *S. aureus* Aad9 (CAA26428). Multiple protein sequence alignment analysis further revealed the regions of amino acid sequence homolgy between Aad9A and the described spectinomycin adenyltransferases (Figure 6.9). The third ORF of pDJ91S was identified as a *rec* and coded for a 405-aa plasmid recombination protein. The *rec* gene shared 76% over 85% of its sequence with the nearest identity match. The full plasmid sequence demonstrated around 85% homology with previously identified plasmids, mostly from *S. aureus* and only over the *rep* and *rec* regions (Figure 6.8). A considerable proportion of the mobile elements that were found to share identity with the pDJ91S plasmid and that were of comparable size, varied from pDJ91S primarily by carrying a different resistance gene such as *tet* or *cat*. This included a chloramphenicol resistance plasmid pKH7 (U38429) and a tetracycline resistance plasmid SAP094A (GQ900443), both from *S. aureus*.

To confirm that the *aad9A* gene was carried by the remaining SPE transformants as well as the corresponding plasmid donor strains, primers were designed to screen for the presence of the determinant by PCR (aad9A_F: 5'-CATGAAAATGAAAATTGGTCTTATCC-3'; aad9A_R: 5'-CCTGTTTCATAAGT TACGATC-3'; denature for 30 seconds at 94° C, anneal for 30 seconds at 60° C, amplify for 1 minute at 72° C, total of 25 cycles). An expected product of 317 bp in size was generated for other SPE transformant strains and eight of the MRSA CC398 strains: 91, 92, 93, 99, 102, 107, 109 and 110.

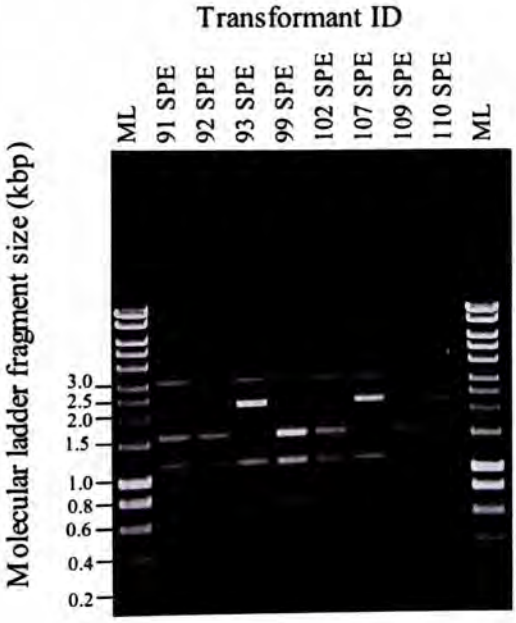


Figure 6.7 *Alu*I restriction digest analysis of plasmid DNA from SPE transformants. The gel lanes are labelled as: 'ML' - molecular ladder (Hyperladder I; Bioline BIO-33053); transformant ID

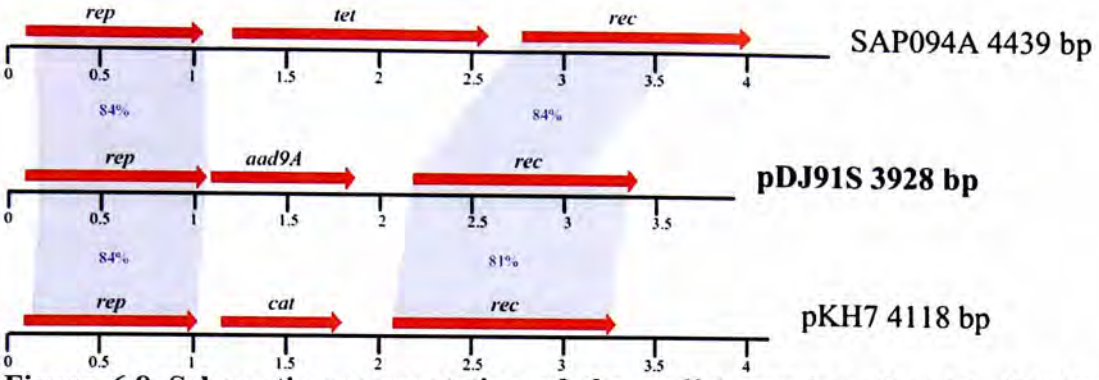


Figure 6.8 Schematic representation of the *aad9A*-carrying plasmid pDJ91S identified in this work (bold) as well as plasmids SAP094A and pKH7. The red arrows represent the coding DNA sequences with the name shown above. Homologous genes are indicated by grey shading with the % sequence identity displayed within.

```
Spw_AFU35063      --------------------------MLIGVYLYGSAVMGGLRMNSDVDILVITNQSLSE  34
Aad9_CAA26428     MSNLINGKIPNQAIQTLKIVKDLFGSSIVGVYLFGSAVNGGLRINSDVDVLVVVNHSLPQ  60
Aad9A_pDJ91S      -----MEEPNKQIDNVLIELKRLFSKDLLGVYLYGSYVKGGLKKDSDVDFLVIINRDMTK  55
Aad9_AAA16527     MRRI-YLNTYEQINKVKKILRKHLKNNLIGTYMFGSGVESGLKPNSDLDFLVVVSEPLTD  59
                                                ::*.*::** * .**: :**:*.**: .. : .

Spw_AFU35063      KTRRNLTNRLMLISGKIGNIKDMRPLEVTVINQKDIVPWHFPPKYEFMYGEWLREQFEKG  94
Aad9_CAA26428     LTRKKLTERLMTISGKIGNTDSVRPLEVTVINRSEVVPWQYPPKREFIYGEWLRGEFENG  120
Aad9A_pDJ91S      EEKRILISKIMPISKEIGEDTSLKYIELTVLNYHENENWSYPPIEEFIYGEWLREDYLNY  115
Aad9_AAA16527     QSKEILIQKIRPISKKIGDKSNLRYIELTIIIQQEMVPWNHPPKQEFIYGEWLQELYEQG  119
                    :. * .::  ** :**:  .:: :*:*::   :   * .**  **:*****:  : :

Spw_AFU35063      EIPESTYDPDLAILLAQLRKNSINLLGPKA-TEVIEPVPMTDIRKAIKESLPGLIASING  153
Aad9_CAA26428     QIQEPSYDPDLAIVLAQARKNSISLFGPDS-SSILVSVPLTDIRRAIKDSLPELIEGIKG  179
Aad9A_pDJ91S      FIPEKNNNIDLTILLYQAKLSSISIYGENNINNLIPDVPFIDLQKAIKESSKELIKDFYG  175
Aad9_AAA16527     YIPQKELNSDLTIMLYQAKRKNKRIYGNYDLEELLPDIPFSDVRRAIMDSSEELIDNYQD  179
                   * :   : **:*:* * : ..  : *     .::  :*: *:::** :*   **

Spw_AFU35063      DERNVILTLARMWLTASTGEIRSKDLAAEWAIPQL----PDEHATLLNKAREAYLGECVD  209
Aad9_CAA26428     DERNVILTLARMWQTVTTGEITSKDVAAEWAIPLL----PKEHVTLLDIARKGYRGECDD  235
Aad9A_pDJ91S      DETNVILTLCRMIVTYETGKFYSKDLAGSMIIENLSENLSIEENNLISLAISSYKNGNSV  235
Aad9_AAA16527     DETNSILTLCRMILTMDTGKIIPKDIAGNAVAESS----PLEHRERILLAVRSYL-GENI  234
                  ** * ****.**  *  **::  **:*..             *.   :  *  .*

Spw_AFU35063      KWEGMES--EVAELVNHMKKSIESSLNIQLPFRIV   242
Aad9_CAA26428     KWEGLYS--KVKALVKYMKNSIETSLN--------   260
Aad9A_pDJ91S      DWELFPVKSVIKKLYAYLNYKL-------------   257
Aad9_AAA16527     EWTNEMVNLTI----NYLNNRLKKL----------   255
                  .*        :     :::  :
```

Figure 6.9. Multiple alignment of amino acid sequences of the novel Aad9A protein from MRSA CC398 strain and three homologous spectinomycin adenyltransferases: Spw from *S. aureus*, Aad9 from *S. aureus* and Aad9 from *E. faecalis*. The alignment was created as described in 6.2. The left column shows protein ID in a format: name_accession number (except for Aad9A, instead the associated plasmid name shown). The alignment symbols show: '*' residue in the column is identical in all sequences in the alignment; ':' conserved substitutions observed; '.'semi-conserved substitutions observed; '-' sequence gap.

6.3.3.5 ERY, CLI and TET transformants carrying *ermT*, *tetL* and *aadD*

Transformants 90 ERY, 90 CLI, 90 TET, 101 TET and 104 CLI were found to have acquired a *circa* 20-kbp plasmid and were carrying the *ermT*, *tetL* and *aadD* resistance genes. A plasmid of comparable size was also identified in transformants 105 ERY/CLI/TET that carried *ermC*, *ermT* and *tetL* genes. The 90 ERY, 90 CLI and 90 TET transformants displayed identical phenotypic and genotypic resistance profiles and thus it was concluded that transformed strains acquired the same plasmid. This was also true for the 105 transformants. For the analysis a single representative of the 90 and 105 transformants was selected: 90 ERY and 105 ERY, and the plasmids were designated pDJ90E and pDJ105E, respectively. The plasmids from 101 TET and 104 CLI were named pDJ101T and pDJ104C, respectively. The analysis involved sequencing of restriction fragments that contained the described resistance genes. The plasmid DNA library was created following *Eco*RI digestion (Figure 6.10) and using *E. coli* NEB10β for the transformation. Plasmid DNA was isolated form selected transformants and analysed on gel electrophoresis to confirm presence of an insert. In addition, plasmid DNA was screened by PCR to confirm the presence of the resistance gene.

The *Eco*RI fragments of the analysed plasmids that were found to carry resistance genes are described in Table 6.5. Each fragment was sequenced in full by primer walking. For each plasmid, PCR primers were designed to amplify a plasmid region between the identified digest fragments in order to determine their proximity within the plasmid. In all, the identified *Eco*RI fragments were found to be adjacent and the amplified PCR fragments were sequenced to close the gap. As such, for each plasmid the analysed resistance region represents two neighbouring *Eco*RI fragments. The complete resistance regions of each analysed plasmid are described in Table 6.5. A schematic structure of each resistance region fragment is presented in Figure 6.11. An alignment analysis revealed that the fragments were highly homologous over some regions but not the entire sequence, with the exception of 101T_*Eco*RI and 104C_*Eco*RI that shared nearly 96% identity across the full sequence. The resistance genes were flanked by insertion sequence elements suggesting integration into a plasmid rather than structurally conserved association.

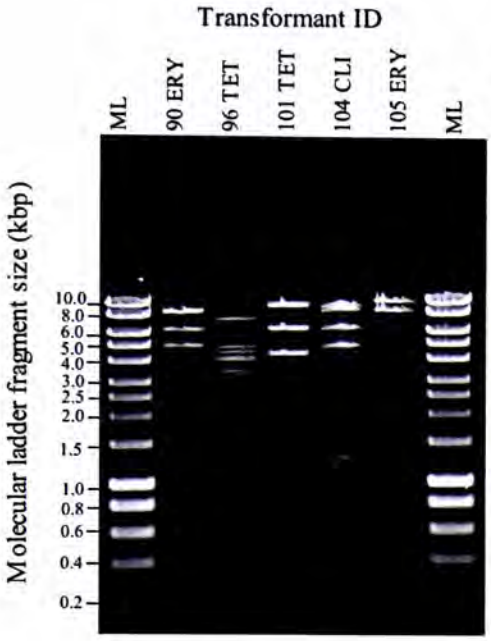


Figure 6.10 *Eco*RI restriction digests of plasmid DNA from 90 ERY, 96 TET, 101 TET, 104 CLI and 105 ERY conducted to prepare plasmid DNA library. The gel lanes are labelled as: 'ML' - molecular ladder (Hyperladder I; Bioline BIO-33053); transformant ID.

Table 6.5 *Eco*RI fragments of plasmids pDJ90E, pDJ101T, pDJ104C and pDJ105E found to carry resistance genes (listed). Also shown is the description of the complete resistance region analysed (composed of the corresponding *Eco*RI fragments).

Plasmid	**Identified *Eco*RI fragments**		**Complete resistance region analysed**	
	Size (kbp)	Resistance genes	Size (bp)	Name
pDJ90E	5	*aadD, tetL*	13,877	90E_*Eco*RI
	9	*ermT*		
pDJ101T	4.5	*aadD, ermT*	10,237	101T_*Eco*RI
	6	*tetL*		
pDJ104C	5	*aadD, ermT*	10,772	104C_*Eco*RI
	6	*tetL*		
pDJ105E	8.5	*ermT*	19,317	105E_*Eco*RI
	10.5	*tetL*		

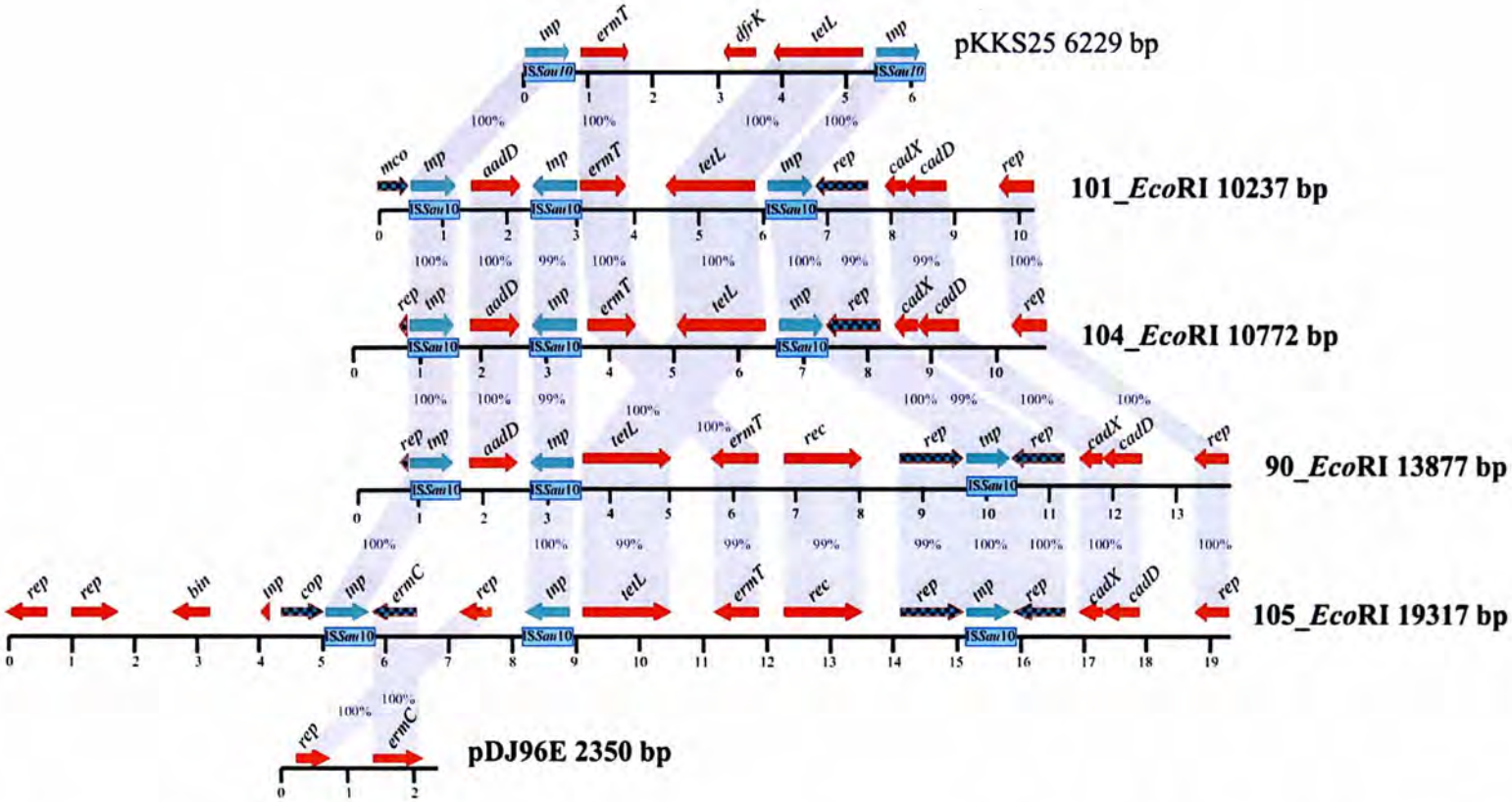


Figure 6.11 Schematic representation of *Eco*RI restriction fragments 90_*Eco*RI, 101_*Eco*RI, 104_*Eco*RI and 105_*Eco*RI identified in this work (bold) and plasmid pKKS25. The red arrows represent the coding DNA sequences with the name shown above. Genes that were putatively truncated by the insertion of the IS*Sau10* element are marked as . Homologous genes are indicated by grey shading with the % sequence identity displayed within.

The 5' end part was identical for the 90E_*Eco*RI and 104C_*Eco*RI and contained a gene coding for a putative truncated replication protein. In the 101T_*Eco*RI, the region differed as it contained a *mco* gene encoding a multi-copper oxidase. The sequence downstream of *rep* in 90E_*Eco*RI and 104_*Eco*R, and downstream of *mco* in 101T_*Eco*RI was nearly identical in all three fragments over an approximately 2.7-kbp region. It contained three ORFs: a kanamycin resistance gene *aadD* flanked by a 793-bp insertion sequence IS*Sau10*, recently identified in a pKKS25 plasmid (FN390947) isolated from MRSA CC398 (Kadlec and Schwarz, 2010). The two copies of IS*Sau10* were positioned in opposite direction and carried a 16-bp inverted repeat (5'-GGTTCTGTTGCAAAGT-3') located at both ends. BLAST analysis of this region demonstrated that the *aadD* gene was previously found to be associated with a different insertion sequence element IS257 in plasmid SAP069A from *S. aureus* (GQ900422).

Downstream of the IS*Sau10* second copy right terminal repeat was located a region containing the *ermT* and *tetL* genes, at the end of which was located a third IS*Sau10* element. As such, the entire resistance gene region was flanked by two IS*Sau10* elements of the same orientation with a third IS*Sau10* located within and separating *aadD* from the remaining resistance determinants.

The 105E_*Eco*RI differed considerably from other fragments at the 5' end region located upstream of the *ermT* and *tetL* genes. The first two ORFs of the 105E_*Eco*RI coded for two separate replication proteins, which were oriented in the opposite directions. Since the 5' end of the first ORF overlapped with the beginning of the 105E_*Eco*RI sequence, it most likely represented only a proportion of this *rep* carried on the pDJ105E. Further downstream was located a gene encoding an invertase enzyme that mediates site-specific DNA recombination. The protein sequence was highly homologous with several invertase enzymes including BinR (ZP_04839235) associated with the *Tn*552 transposon (Rowland and Dyke, 1989). The next ORF represented a putative transposase gene *tnp*, as it was only 120 bp in length and demonstrated no more then 66% identity with similar elements. Around 180 bp downstream was located a copper transloctaing-ATPase gene *cop*. The gene was homologous with various other copper-exporting ATPases from various staphylococci such as *S. epidermidis* (YP_189983), *Staphylococcus warneri* (ZP_12295272) and *S. aureus* (ZP_11452984). The 3' end of *cop* overlapped with the 5' site of the first IS*Sau10* element in 105E_*Eco*RI, demonstrating that integration of

this mobile element occurred within the *cop* sequence. The 3' end of IS*Sau10* also overlapped with the neighbouring coding sequence identified as the erythromycin resistance determinant *ermC*. This is in contrast with the results described in 6.3.3.1 where the *ermC* was reported as primarily associated with small, single resistance determinant plasmids. Furthermore, downstream of the *ermC* was located a *rep* element and the region was largely homologous with the pDJ91E, pDJ96E and pDJ103E plasmids. Also, the *ermC-rep* fragment of 105E_*Eco*RI was flanked by the IS*Sau10* element, which suggests that the *ermC*-associated plasmid has become integrated into pDJ105E via transposase-associated mechanism. Downstream and inclusive of the second IS*Sau10* element, the remaining 11-kbp sequence of 105E_*Eco*RI fragment was fully homologous with the corresponding region of 90E_*Eco*RI, which contained the *tetL* and *ermT* genes.

The *tetL- ermT* section of 90E_*Eco*RI and 105E_*Eco*RI was approximately 6 kbp in size and it consisted of 4 ORFs. The first coding sequence was the *tetL* resistance determinant that encoded a 458-aa tetracycline resistance protein. The entire sequence was fully homologous with previously reported *tetL* genes from various organisms such as *Streptococcus agalactiae* (NP_040422), *Streptococcus suis* (YP_003028728) and *Enterococcus faecium* (ZP_19536324). Around 640 bp downstream was located the *ermT* gene orientated in the opposite direction indicating a convergent transcription. The gene encoded a 244-aa rRNA adenine N-6-methyltransferase. The protein sequence was identical to previously reported ErmT elements from various organisms such as *Streptococcus bovis* (ZP_07467525), *Streptococcus pyogenes* (YP_001716198) and *Lactobacillus* sp. (NP_862543). The third ORF was identified as a *mob* element encoding a 403-aa mobilisation protein that shared 86% identity with a *mob* product of pK214 plasmid (NC_009751) from *Lactobacillus lactis*, the nearest homology match. Analysis of the nucleotide sequence revealed that the gene was previously identified solely among the lactococci and lactobacilli. Around 600 bp downstream was located the fourth ORF that coded for a putatively truncated replication protein.

In 101T_*Eco*RI and 104C_*Eco*RI the region containing *ermT* and *tetL* genes differed from 90E_*Eco*RI and 105E_*Eco*RI. It was approximately 3 kbp in size and consisted of the resistance genes only lacking the *mob* and *rep* elements identified in 90E_*Eco*RI and 105E_*Eco*RI. Furthermore the entire section located between the IS*Sau10* elements was inverted thus the first coding sequence at the 5' end was the

ermT gene, followed by the *tetL* determinant. An *ermT* truncation was detected in the 101T_*Eco*RI, which, as defined by an alignment with the 104C_*Eco*RI sequence, consisted of the first 40 bp of the coding sequence and a 123-bp fragment immediately upstream of the start codon. This finding justifies the previously observed lack of phenotypic resistance to erythromycin displayed by the strain 101 (Chapter 5, Table 5.1) despite the carriage of the *ermT* element, which was also observed for the 101 TET transformant. Excluding the described truncation, the region containing *ermT* and *tetL* genes was nearly fully homologous in 101T_*Eco*RI and 104C_*Eco*RI.

Downstream of the third IS*Sau10* element all four analysed plasmid fragments shared a nearly 99% sequence identity. The section was approximately 3.4 kbp in length and contained four ORFs. The first ORF coded for a 280-aa replication protein and the nucleotide sequence demonstrated a 21-bp overlap with the upstream IS*Sau10* element. A cadmium resistance operon was located further downstream and consisted of the *cadX* and *cadD* elements (*cadXD*). The genes encode a transcriptional regulator and a cadmium resistance transporter protein, respectively. Both elements were found to be highly homologous with previously described CadX and CadD proteins from various staphylococcal species. Interestingly, the highest nucleotide sequence homology, 98%, was shared with the chromosomally located *cadXD* operon in *Tetragenococcus halophilus* (AP012046). Finally, the last 53 bp (approximately) represented part of a *rep* element.

The fragments were analysed for an overall sequence homology, which revealed that the analysed regions of pDJ90E, pDJ101T, pDJ104E and pDJ105E were mosaics composed of elements of previously described plasmids as presented for 90_*Eco*RI by Figure 6.12. Overall, the highest level of nucleotide sequence homology was with the pKKS25 plasmid (FN390947), which involved the *ermT-tetL* resistance region and the IS*Sau10* elements.

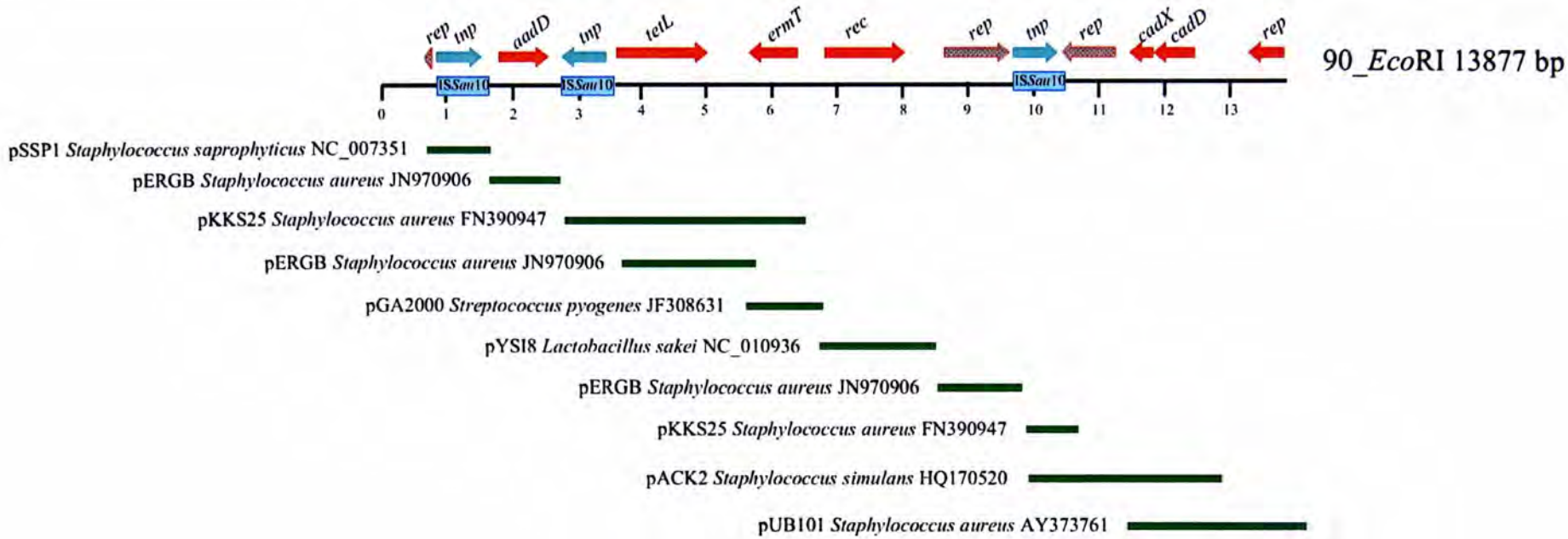


Figure 6.12 The schematic of annotated 90_*Eco*RI restriction fragment with regions of homology (green line) between the analysed sequence and previously described plasmids

6.3.3.6 96 TET transformant

The 96 TET transformant carried a *circa* 30-kbp plasmid, designated pDJ96T that mediated resistance to tetracycline and kanamycin. The transformant was confirmed to contain the *aadD* kanamycin resistance determinant, but was negative for *tetM* (the only *tet* element carried by the donor strain). The plasmid DNA was digested with *Eco*RI and the fragments were inserted into a pUC19 vector, which was transformed into *E. coli* NEB10β strain (Figure 6.10). The transformed cells were screened on selective agar containing ampicillin with either kanamycin or tetracycline. The kanamycin resistant transformant carried a vector with a 4,965-bp *Eco*RI fragment (96K_*Eco*RI), whereas the tetracycline resistant transformant carried a vector with a 3,527-bp *Eco*RI fragment (96T_*Eco*RI). Primers were designed based on the sequence periphery of each *Eco*RI fragment and PCR was set up to amplify a region between 96K_*Eco*RI and 96T_*Eco*RI. In contrast to *Eco*RI fragments described in 6.3.3.5, the analysed restriction fragments of pDJ96T were not located in close proximity to each other but at an estimated 10-kbp distance. Thus the fragments were analysed separately and sequence of the joining region was not determined.

Four ORFs were identified within the 96K_*Eco*RI fragment as presented in Figure 6.13. The first element was a *rep* gene whereas the remaining two ORFs represented resistance determinants: the previously described *aadD* gene and a bleomycin resistance gene *bleO*. The *aadD* gene was 99% identical to kanamycin resistance determinants identified in 90E_*Eco*RI, 101T_*Eco*RI and 104C_*Eco*RI. The bleomycin resistance gene coded for a 132-aa protein, which was identical to previously described BleO element from *S. aureus* (NP_370558). The fourth coding sequence was identified as a *rec* gene. The protein sequence shared the highest level of homology with a plasmid recombination enzyme from *E. faecalis* (ZP_18108675). The 3' end of the *rec* element overlapped with the end sequence of 96K_*Eco*RI suggesting that the identified ORF most likely constituted only a part of the complete *rec* gene present on the pDJ96T. The complete nucleotide sequence of 96K_*Eco*RI, except for the initial 600 bp fragment at the 5' end upstream of the *rep* gene, was highly homologous with a 4.5-kbp plasmid pUB110 (M19465) from *S. aureus*, suggesting that the region might represent a smaller plasmid integrated within the pDJ96T.

The 96T_*Eco*RI fragment contained three ORFs and the first was identified as *hsdS* gene, which coded for the S subunit of the type I restriction modification enzyme (Figure 6.14). The 299-aa protein shared a 72% identity over 98% of its sequence with a restriction modification element from *S. warneri* (ZP_12295024), which was its closest identity match. The second ORF coded for a novel 452-aa tetracycline resistance protein, which shared 74% identity with the tetracycline efflux protein TetK from *S. aureus* (BAK53120) and 62% with tetracycline efflux protein TetL, also from *S. aureus* (AEW23139). . The identified protein was thus designated TetK2. Multiple protein sequence alignment analysis further revealed the regions of amino acid sequence homolgy between TetK2 and the described tetracycline efflux proteins (Figure 6.15). The last ORF of the 96T_*Eco*RI fragment encoded a hypothetical protein, which contained a conserved domain of unknown function, designated DUF1541. The complete nucleotide sequence of 96T_*Eco*RI did not reveal any overall homologies when screened by BLAST.

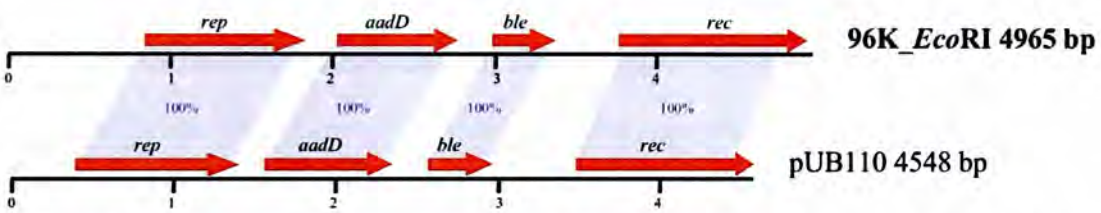


Figure 6.13 Schematic representation of the 96K_*Eco*RI fragment identified in this work (bold) and plasmid pUB110. The red arrows represent the coding DNA sequences with the name shown above. Homologous genes are indicated by grey shading with the % sequence identity displayed within.

Figure 6.14 Schematic representation of the 96T_*Eco*RI fragment identified in this work. The red arrows represent the coding DNA sequences with the name shown above (elements of unknown function referred to as CDS).

```
TetL_AEW23139     MNTSYSQSNLRHNQILIWLCILSFFSVLNEMVLNVSLPDIANDFNKPPASTNWVNTAFML 60
TetK2_pDJ96T      -----NKIKSLSYNILFWLCILSFFSVLNEMVLNVSLPDIAISFNTSPAITNWVNTAYML 55
TetK_BAK53120     MFSLYKKFKGLFYSVLFWLCILSFFSVLNEMVLNVSLPDIANHFNTTPGITNWVNTAYML 60
                       .: :    .:*:*************************  **. *. *******:**

TetL_AEW23139     TFSIGTAVYGKLSDQLGIKRLLLFGIIINCFGSVIGFVGHSFFSLLIMARFIQGAGAAAF 120
TetK2_pDJ96T      TFSIGTAVYGKLSDQVSIKKLLILGIILSCLGSLIGFFGHKHFLILILGRLIQGIGSAAF 115
TetK_BAK53120     TFSIGTAVYGKLSDYINIKKLLIIGISLSCLGSLIAFIGHNHFFILIFGRLVQGVGSAAF 120
                  ************** : **:**::** :.*:**:*.*.**..* :**:.*::** *:***

TetL_AEW23139     PALVMVVVARYIPKENRGKAFGLIGSIVAMGEGVGPAIGGMIAHYIHWSYLLLIPMITII 180
TetK2_pDJ96T      PSLVMVVVSRNITKAKQGKAFGFIGSIVALGEGIGPSIGGVVTHYIHWSYLLIIPIFTLI 175
TetK_BAK53120     PSLIMVVVARNITRKKQGKAFGFIGSIVALGEGLGPSIGGIIAHYIHWSYLLILPMITIV 180
                  *:*:****:* * : ::*****:******:***:**:***:::*********::*::*::

TetL_AEW23139     TVPFLMKLLKKEVRIKGHFDIKGIILMSVGIVFFMLFTTSYSISFLIVSVLSFLIFVKHI 240
TetK2_pDJ96T      TIPFLNKIMEPGESQKGDLDILGILLMSISIISFMLFTTSYKWFYLITFVIFFIIFIKHI 235
TetK_BAK53120     TIPFLIKVMVPGKSTKNTLDIVGIVLMSISIICFMLFTTNYNWTFLILFTIFFVIFIKHI 240
                  *:*** *::       *  :** **:***:.*: ******.*.  :**  .: *:**:***

TetL_AEW23139     RKVTDPFVDPGLGKNIPFMIGVLCGGIIFGTVAGFVSMVPYMMKDVHQLSTAEIGSVIIF 300
TetK2_pDJ96T      VKVTHPFIDPALRKNPSFIFGLISGALIFATVAGFISMVPYMMKALYHINAATIGNNVIL 295
TetK_BAK53120     SRVSNPFINPKLGKNIPFMLGLFSGGLIFSIVAGFISMVPYMMKTIYHVNVATIGNSVIF 300
                   :*:.**::* * **  *::*::.*.:**. ****:******** :::::..* **. :*:

TetL_AEW23139     PGTMSVIIFGYIGGILVDRRGPLYVLNIGVTFLSVSFLTASFLLETTSWFMTIIIVFVLG 360
TetK2_pDJ96T      PGTISVIIFGYIGGYLVDKKGALFVFVIGSLFISISFLVIAFFVELNLWVTTISFIFVMG 355
TetK_BAK53120     PGTMSVIVFGYFGGFLVDRKGSLFVFILGSLSISISFLTIAFFVEFSMWLTTFMFIFVMG 360
                  ***:***:***:** ***::* *:*: :*   :*:***. :*::* . *. *: ::**:*

TetL_AEW23139     GLSFTKTVISTIVSSSLKQQEAGAGMSLLNFTSFLSEGTGIAIVGGLLSIPLLDQRLLPM 420
TetK2_pDJ96T      GLSFTKTVISTIVSSSLSHEEVGSGMSLLNFTSFLSEGTGIIIMGGLLSTQFLNYNFLSE 415
TetK_BAK53120     GLSFTKTVISKIVSSSLSEEEVASGMSLLNFTSFLSEGTGIAIVGGLLSLQLINRKLVLE 420
                  **********.******..:*...***************** *:*****  ::: .::

TetL_AEW23139     EVDQSTYLYSNLLLLFSGIIVISWLVTLNVYKHSQRDF-      458
TetK2_pDJ96T      FITFSTNLYSNILIGCTIIIALCCLLTFILFNRTVKQ--      452
TetK_BAK53120     FINYSSGVYSNILVAMAILIILCCLLTIIVFKRSEKQFE      459
                   :  *: :***:*:  : :* :. *:*: ::::: ::
```

Figure 6.15. Multiple alignment of amino acid sequences of the novel TetK2 protein from MRSA CC398 strain and two homologous tetracycline efflux proteins from *S. aureus*: TetL and TetK. The alignment was created as described in 6.2. The left column shows protein ID in a format name_accession number (exept for TetK2, instead the associated plasmid name shown). The alignment symbols show: '*' residue in the column is identical in all sequences in the alignment; ':' conserved substitutions observed; '.'semi-conserved substitutions observed; '-'sequence gap.

6.4 Discussion

It has been previously observed that resistance genes in staphylococci are commonly plasmid-associated and that the structural analysis of these elements gives an insight into the heterogeneity of mobile elements that serve in the dissemination of resistance amongst *S. aureus* strains (Werckenthin *et al.*, 2001). The work presented here confirmed that proportion of resistance determinants carried by the analysed MRSA CC398 strains were plasmid-borne. The identified plasmids ranged in size from *circa* 2.3 kbp up to 30 kbp. The strains were therefore found to carry two types of plasmids commonly detected in *S. aureus*: small carrying single-resistance gene and larger with multiple resistance determinants (Malachowa and DeLeo, 2010). Based on the sequence analysis the identified plasmids could be broadly classified as representing one of the following: 1. prevalent resistance-associated plasmids that are common in staphylococci; 2. novel MRSA CC398-associated plasmids that were recently identified; 3. mosaics of previously identified and novel genetic elements.

The *ermC* element was common among MRSA CC398 strains and found to be associated mostly with one of two distinct plasmids. The smaller element of 2.3 kbp in size, represented by pDJ91E, pDJ96E and pDJ103E, was the most common as it was carried by seven out of ten *ermC*-positive strains. The complete plasmid sequence was nearly identical to a number of previously described *ermC*-carrying elements. The pDJ91E and pDJ96E plasmids demonstrated a high level of homology with a 2355-bp pNE131 from *S. epidermidis* that was reported in 1986 (Lampson and Parisi, 1986). This and the high prevalence of the 2.3-kbp *ermC*-positive plasmids among MRSA CC398 strains, indicate that the element has been very successful at dissemination within the staphylococcal population over the decades.

The second identified *ermC*-carrying plasmid type was 4 kbp in size and demonstrated a considerably lower prevalence as it was detected in two strains only. The sequence analysis of both pDJ92E and pDJ108E revealed that the plasmid most closely resembled a 3728-bp pE194 from *S. aureus*. It was reportedly first isolated in 1976 and has been since well characterised (Iordanescu, 1976; Horinouchi and Weisblum, 1982; Villafane *et al.*, 1987). Detection of this element suggests that, similarly to the previously described *ermC* plasmid vector, it has been maintained within staphylcococcal populations over the last three decades. However, based on the results of this analysis, it might have been less successful at horizontal transfer than

the pNE131. Furthermore, pDJ92E and pDJ108E do not share a complete homology with pE194, with novel coding sequences detected within the identified plasmids. This might suggest a level of structural instability of the pE194 mobile element.

For a single strain, namely 105, the *ermC* resistance gene was found to be located on a larger multiple-resistance determinant plasmid, as demonstrated by the analysis of the 105E_*Eco*RI restriction fragment. Such structural association has been reported previously as in the case of a 37,136-bp plasmid pUSA03 that was identified in *S. aureus* USA300_FPR3757, an epidemic clone of CA-MRSA (Diep *et al.*, 2006). Similarly to pDJ105E, the *ermC*-containing region of pUSA03 was flanked by transposon elements suggesting that a small *ermC*-carrying plasmid has become inserted into a larger mobile element.

Only one strain was confirmed to carry a *tetK*-associated plasmid. The results of sequence analysis of this mobile element were in agreement with the observation that the *tetK* gene is commonly located on small plasmids that fall into a 4.3 to 4.7 kbp size range (Werckenthin *et al.*, 2001). The described pDJ93T plasmid was identical to pT181 plasmid, which has been recognised as a prototype of *tetK*-carrying plasmids, with pT181 homologs identified across a wide range of staphylococcal species (Werckenthin *et al.*, 2001). Interestingly, the *tetK* gene was not indentified in the pDJ93T-carrying strain during antimicrobial resistance genotype analysis (Chapter 5, Figure 5.1). Following the identification and sequence analysis of pDJ93T, the genomic DNA from the donor strain was screened by PCR for the presence of the *tetK* gene, however with negative results. This discrepancy might have had resulted from a low copy number of the DNA template although it was not further investigated. Interestingly, none of the remaining *tetK*-positive strains revealed carriage of a *tetK*-associated plasmid. The transformation experiment using plasmid DNA from these strains with selection for tetracycline resistant transformants was repeated but no colonies were observed. It has been thus concluded that the *tetK* determinant among these strains is likely to be chromosomally located. Although considered of rare occurrence, this would not be a novel feature since a *tetK*-carrying plasmid was previously found integrated into a chromosome and larger plasmid through IS*257*-mediated recombination (Stewart *et al.*, 1994; Werckenthin, Schwarz and Roberts, 1996).

The analysis of *Eco*RI restriction fragments of larger plasmids demonstrated that for the majority the resistance determinants were located in close proximity

forming a resistance gene cluster. This was observed for pDJ90E, pDJ101T, pDJ104C and pDJ105E. Only in pDJ96T, the *Eco*RI restriction fragments were analysed as separate regions due to considerable physical separation across the plasmid sequence. The sequence homology analysis of these resistance-cluster *Eco*RI fragments revealed that the regions shared a level of similarity with previously described resistance plasmids across some sections, but not the entire sequence. The results described in this work revealed a novel resistance cluster consisting of *aadD*, *ermT* and *tetL* genes. In previous reports the genes were found in either *aadD-tetL* or *ermT-tetL* association (Kadlec and Schwarz, 2010; de Gopegui *et al.*, 2012), but all three have not yet been described on a single mobile element. The pDJ105E plasmid lacked *aadD*, but contained an integrated *ermC*-carrying plasmid within the location that corresponded to the *aadD* region of the other analysed plasmids. It has been previously observed that presence of additional resistance determinants on *tetL*-carrying plasmids can be associated with events such as co-integration formation and recombination with other resistance plasmids (Schwarz *et al.*, 1996). Analysis of only a partial plasmid sequence described here did not allow the determination of a potential structural plasmid backbone. As such no conclusion was drawn on the structural association between identified resistance determinants and the remaining plasmid sequence. That is to say, it was not determined, which of the resistance genes might represent native elements of that particular plasmid and which might have become inserted as foreign elements. However, the highly mosaic structure of the described plasmid fragments, as determined by homology analysis, suggests that the investigated mobile elements have likely undergone various recombination and/or co-integration events. These might constitute important mechanisms that mediate the accumulation of resistance determinants on a single plasmid. As previously observed by Schwarz *et al.* (1996), acquisition of multiple resistance determinants through transfer of individual resistance plasmids might be blocked by plasmid incompatibility or host-specific restriction mechanisms. However, structural integration into existing plasmids or chromosome allows such restrictions to be overcome (Schwarz *et al.*, 1996).

Of particular importance in mediating dissemination of resistance determinants among MRSA CC398 strains might be the IS*Sau10* insertion sequence. This mobile element was recently identified in pKKS25 plasmid, which was carried by a porcine MRSA CC398 strain and contained a *ermT-dfrK-tetL* resistance gene cluster (Kadlec and Schwarz, 2010). All *Eco*RI plasmid restriction fragments, except for those

derived from pDJ96T, shared a considerable level of homology with pKKS25, which included the presence of IS*Sau10*, located on each end of the resistance clusters. However, while pKKS25 was found to carry two copies of IS*Sau10*, the plasmid resistance regions analysed in this work demonstrated three. In all, two copies of IS*Sau10* were identified flanking the *ermT-tetL* fragment, while a third was located upstream of the *aadD* gene in pDJ90E, pDJ101T, pDJ104C and upstream of the integrated *ermC* plasmid in pDJ105E. The 16-bp inverted repeat sequence of IS*Sau10* was found to overlap with neighbouring coding sequences at one or both termini demonstrating homology that might have mediated the integration event leading to truncation of the corresponding gene. The IS*Sau10* insertion sequence was implicated in formation of pKKS25 plasmid resistance gene cluster (Kadlec and Schwarz, 2010). The results described in this work provide further evidence that IS*Sau10* might indeed contribute significantly to generation of plasmids carrying multiple antimicrobial resistance determinants. A similar role has been attributed to other previously described insertion sequence elements such as IS257, which was associated with integration of small resistance-carrying plasmids into larger plasmid elements (Werckenthin, Schwarz and Roberts, 1996).

Only a proportion of resistance genes previously identified among the analysed MRSA CC398 strains (Chapter 5), were associated with a plasmid carriage. As such, the analysis results are largely in agreement with a previously described association between certain resistance determinants and particular mobile elements. For instance, genes such as *tetM*, *ermA*, *fexA* and *aacA-aphD* are carried by transposons and thus are recognised as chromosomally located (Malachowa and DeLeo, 2010). In contrast, other elements such as *ermC*, *aadD* and *tetL* have been recognised as mostly plasmid-associated (Malachowa and DeLeo, 2010), which was confirmed by the work presented here. However, some exceptions to the previously reported MGE-resistance determinant associations have also been identified. An example of this can be the above described apparent chromosomal location of the mainly plasmid-related *tetK* gene among all but one of the *tetK*-positive strains. Another exception has been the *dfrK* gene, which was first identified as a plasmid-borne novel resistance determinant in a MRSA CC398 isolate (Kadlec and Schwarz, 2009a). It was later also identified on the pKKS25 plasmid as a structural component of the above described resistance cluster, containing *ermT* and *tetL* genes (Kadlec and Schwarz, 2010). Interestingly, the plasmid resistance cluster described here did not

carry the *dfrK* determinant, which was identified in a large proportion of the analysed MRSA CC398 strains. However, in another study the *dfrK* gene was described as a component of a novel Tn*559* transposon, which was identified within the chromosome of a porcine MSSA CC398 strain (Kadlec and Schwarz, 2010). The Tn*559* element was integrated into the chromosomal *radC* gene, at a site known as Att554, which has been previously recognised as an integration site for the Tn*554* transposon (Kadlec and Schwarz, 2010). The Att554 chromosomal location was recently reported as an integration site for another novel transposon Tn*6133* carrying *spc-ermA-vgaE* resistance cluster, which was identified in a porcine MRSA CC398 strain (Schwendener and Perreten, 2011). To briefly investigate whether the *dfrK* gene is chromosomally located in the analysed MRSA CC398 strains, a PCR was set up using a primer combination specific for the *drfK* gene and the chromosomal Att554 site (Appendix III). In all *dfrK*-positive strains an amplification of approximately 800-bp fragment occurred when *dfrK*_F and *att554*_R primers were used (Appendix III). Although association with Tn559 was not confirmed, the amplicon size corresponded with the approximate physical distance between the *dfrK* and *radC* gene of Tn*559* that was described by Kadlec *et al.* (2010). Similar analysis was also conducted for the *fexA* and *vgaE* genes, previously associated with Tn*558* and Tn*6133*, respectively (Kehrenberg and Schwarz, 2005; Schwendener and Perreten, 2011). PCR analysis using *fexA*_R/*att554*_R primers resulted in amplification of a 2.4-kbp fragment in all *fexA*-positive strains, whereas a *vgaE*_1/*att554*_R primer combination allowed amplification of a 2.5-kbp PCR fragment in one of the two *vgaE*-positive strains (Appendix III).

Of considerable significance amongst the described findings is the identification of novel resistance determinants, namely the spectinomycin resistance gene *aad9A* and tetracycline resistance gene *tetK2*. The most recent identification of a novel spectinomycin resistance gene, designated *aadA14* was in *P. multocida* (Kehrenberg *et al.*, 2005). The same study also reported that a number of other *P. multocida* isolates that displayed spectinomycin resistance, but tested negative for the *aadA14* gene and other known *aadA* elements as well the as *spc* gene. A recent study on the prevalence of MRSA in slaughter pigs in Switzerland reported a 70% prevalence of spectinomycin resistance among identified MRSA isolates, with 3 isolates that belonged to CC398 carrying an unidentified mechanism of resistance (*spc*-negative) (Overesch *et al.*, 2011). Collectively, such reports together with this

study suggest the existence of a pool of novel spectinomycin adenyltranferase genetic determinants, with MRSA CC398 isolates constituting a likely reservoir of such factors. Furthermore, the considerably high prevalence of the *aad9A* determinant among the described strains of MRSA CC398, particularly in comparison with the distribution of the *spc* gene, might suggest high rates of horizontal transfer of the associated plasmid. The *spc* gene has been associated with the Tn*554* transposon or more recently with the Tn*6133*, as described in the previous paragraph (Schwendener and Perreten, 2011). However, the emergence of other Att554 site-specific transposons such as the *dfrK*-positive Tn*559* might have resulted in a reduced prevalence of Tn*554* among MRSA CC398 isolates due to unavailability of the integration site. Indeed, all but two strains carrying the *aad9A*-positive plasmid, demonstrated presence of either *dfrK* or *fexA* gene that were, as described previously, confirmed as chromosomally located at the Att554 site. This could partly justify the emergence and dissemination of the described novel spectinomycin resistance gene.

Identification of a novel tetracycline resistance gene can be related with a common observation that this antimicrobial agent is among the most frequently used in food-producing animals leading to considerable selective pressure on the livestock-associated skin or mucosal microbiota, such as MRSA CC398 (Schwarz and Chaslus-Dancla, 2001; Price *et al.*, 2012). This can exemplified by the homogenous distribution of the *tetM* gene among MRSA CC398 strains as well as co-carriage of additional tetracycline resistance determinants, such as *tetK* and *tetL*. The relatively short sequence of the 96T_*Eco*RI restriction fragment that was found to contain the novel *tet* determinant, renders it difficult to speculate on potential origins of the gene. However, an unusual feature was the presence of an *hsdS* gene homologue upstream of the *tetK2* determinant. The *hsdS* gene constitutes a component of bacterial type I restriction modification system, an important mechanism aimed at protecting the host cell from potentially harmful foreign DNA (Murray, 2000; Waldron and Lindsay, 2006). Although not considered as essential to bacterial survival, the type I restriction modification system was identified in a number of bacterial species (Murray, 2000). Its components are predominantly chromosomally encoded although plasmid-associated *hsdS* genes have been described in *L. lactis* (Murray, 2000). Here, the presence of the *hsdS* on a plasmid might represent a remnant of a recombination event between the plasmid and a bacterial chromosome. Thus the native carrier of this *hsdS* element might have also acted as donor of the novel tetracycline resistance

determinant. A full pDJ96T plasmid sequence analysis could further reveal if such event have occurred.

In summary, the results presented in this work demonstrate that the previously identified considerable antimicrobial resistance genotype heterogeneity among MRSA CC398 strains (Chapter 5) is accompanied by structural diversity of resistance-carrying plasmids. The identification of novel resistance determinants and structural heterogeneity of plasmid-associated resistance clusters described here, add further significance to the resistance-related accessory genotype of MRSA CC398 strains. Based on these findings, it might be speculated that the acquisition and carriage of resistance-associated MGEs by this lineage is an important factor promoting its success as livestock coloniser.

Chapter 7 Competition and antimicrobial resistance fitness analysis

7.1 Introduction

Biological fitness of bacterial strains is commonly investigated and discussed in the context of development or acquisition and expression of antimicrobial resistance, which can induce a biological cost (Wichelhaus *et al.*, 2002). The cost of resistance is defined as a reduced biological fitness of the bacterial host in the absence of antimicrobial compound (Sander *et al.*, 2002). The processes that lead to a reduction in biological fitness can be distinguished based on the mechanisms of resistance acquisition. As such, target alteration caused by chromosomal mutations might affect physiological processes that are normally expressed or regulated by the mutated gene, whereas acquisition of accessory determinants can be associated with the burden of replication and maintenance of the mobile element (Lenski, 1998; Andersson and Levin, 1999). In *S. aureus,* fitness cost due to acquired antimicrobial resistance has been identified for the carriage of SCC*mec* and methicillin resistance, mutations in the elongation factor G and fusidic acid resistance, and mutations in the RNA polymerase and rifampicin resistance (Nagaev *et al.*, 2001; Wichelhaus *et al.*, 2002; Ender *et al.*, 2004).

Experimental *in vitro* assessment of the biological cost of resistance is commonly conducted through measuring and comparing the relative rates of growth of the resistant and susceptible strains of bacteria (Andersson and Levin, 1999). Such approach would often involve generation of resistant mutants, which are then compared against the susceptible isogenic strain (Wichelhaus *et al.*, 2002; Sander *et al.*, 2002; Besier *et al.*, 2005). The biological fitness of the resistant strain in relation to the susceptible parent can then be measured by conducting a monoculture with quantification and comparison of the doubling times during the exponential growth (Andersson and Levin, 1999). Another approach is to perform a mixed culture competition assay, which involves selective plating to determine viable cell counts of the inoculated strains (Andersson and Levin, 1999). Fundamentally, such comparative analysis is conducted in the absence of antimicrobial pressure. In contrast, the burden of resistance can be also assessed by comparing growth rates in the absence and presence of the respective antimicrobial compound, in particular if the resistance is mediated by an accessory determinant (Foucault, Courvalin and Grillot-Courvalin, 2009).

The biological cost of antimicrobial resistance is considered as one of the decisive factors that govern its long-term stability and subsequent dissemination (Foucault, Courvalin and Grillot-Courvalin, 2009). Thus it has been speculated that the understanding of the fitness cost of specific resistance phenotypes and/or genotypes might provide clues for development of potential intervention measures (Andersson, 2006). However, this has been challenged by observations that not all resistance genotypes result in a measurable cost of resistance (Lenski, 1998; Andersson and Levin, 1999). In addition, bacteria can adapt and overcome the biological cost of acquired resistance (Nagaev *et al.*, 2001). Consequently it can be also postulated that such stabilisation of resistance carriage will occur at varying rates among different strains, depending on the underlying genetic background.

In line with the above thought and considering the antimicrobial resistance status of MRSA CC398 lineage, growth fitness of strains was investigated. The analysis involved a pairwise competition assay of selected MRSA CC398 strains against MRSA representatives of other lineages that were previously described in this work, namely CC8, CC22 and CC30. The aim was to assess if MRSA CC398 features a selective advantage when competed directly against other successful lineages of MRSA. Also, growth rates of all strains that were previously identified as resistant to cefoxitin, erythromycin, tetracycline and trimethoprim (Chapter 5) were measured in the presence of the respective antimicrobial compound and compared with kinetics of replication in a non-selective medium. The compounds for the analysis were selected based on high prevalence of resistance within the CC398 group, although the analysis included resistant strains of all other analysed lineages. The objective of this work was to determine if exposure to the described antimicrobial agents leads to variable rates of growth reduction amongst resistant strains, and if so whether the MRSA CC398 demonstrate a comparatively enhanced ability to overcome the selective pressure.

7.2 Materials and methods

The competition assay and data analysis were conducted as described in section 2.9. The comparative analysis of growth rates in the absence and presence of antimicrobial compounds and the data analysis were performed as described in section 2.10. The strains that were selected for these analyses are described in Table 7.1.

Table 7.1 Strains selected for growth competition assay (*) and growth rate analysis in presence of erythromycin, cefoxitin, tetracycline and trimethoprim [a]

		Erythromycin		Cefoxitin		Tetracycline		Trimethoprim	
ID	Lineage	MIC [b]	Genotype [c]	MIC	Genotype	MIC	Genotype	MIC	Genotype
17	CC5							>64	*dfrG*
6	CC8					32	*tetK*		
200*	CC8	>32	*ermC*	8	*mecA*	64	*tetM*	>64	*dfrA*
201*	CC8			16	*mecA*	64	*tetM, tetL*	>64	*dfrA*
60	CC15					32	*tetK*		
18	CC22							32	unknown
28*	CC22	>32	unknown	8	*mecA*				
32	CC22	>32	*ermC*	16	*mecA*				
38*	CC22	>32	*ermC*	8	*mecA*			16	unknown
39	CC22	>32	*ermC*	8	*mecA*				
40	CC22	>32	*ermC*	8	*mecA*				
44	CC22			8	*mecA*				
5	CC30	>32	*ermA*						
31*	CC30	>32	*ermA*	16	*mecA*				
34	CC30	>32	*ermA*	>32	*mecA*			64	unknown
62	CC30					32	*tetK*		
65*	CC30	>32	*ermA, ermC*	>32	*mecA*			>64	*dfrA*
195	CC97	>32	*ermA*						
173	CC130			8	*mecC*				
178	CC130			8	*mecC*				
181	CC130			8	*mecC*				
182	CC130			8	*mecC*				
90	CC398	>32	*ermT*	16	*mecA*	64	*tetM, tetL*	>64	*dfrA*
91	CC398	>32	*ermC*	8	*mecA*	64	*tetK, tetM*		
92	CC398	>32	*ermC*	8	*mecA*	32	*tetM*	>64	*dfrG*
93*	CC398	>32	*ermC*	8	*mecA*	32	*tetK, tetM*		
95	CC398	>32	*ermC*	16	*mecA*	64	*tetK, tetM*	>64	*dfrK*
96	CC398	>32	*ermC*	16	*mecA*	>64	*tetK2, tetM*	>64	*dfrA*
99	CC398			8	*mecA*	32	*tetM*	>64	*dfrK*
101	CC398			16	*mecA*	64	*tetM, tetL*	>64	*dfrK*
102	CC398	>32	*ermC*	8	*mecA*	32	*tetM*	>64	*dfrK*
103	CC398	>32	*ermC*	8	*mecA*	64	*tetK, tetM*	>64	*dfrK*
104	CC398	>32	*ermA, ermT*	8	*mecA*	>64	*tetM, tetL*		
105	CC398	>32	*ermC, ermT*	8	*mecA*	32	*tetM, tetL*	>64	*dfrK*
106	CC398			8	*mecA*	64	*tetK, tetM*	>64	*dfrK*
107*	CC398			8	*mecA*	32	*tetM*		
108	CC398	>32	*ermC*	8	*mecA*	64	*tetK, tetM*	>64	*dfrK*
109	CC398	>32	*ermC*	16	*mecA*	32	*tetM*	>64	*dfrK*
110	CC398			8	*mecA*	32	*tetM*		
111	CC398	>32	*ermA*	8	*mecA*	32	*tetM*	>64	*dfrK*

[a] For each compound the analysis of growth rates was performed with strains, for which the MIC and genotype are presented; shaded boxes - the strain was not analysed with the corresponding agent.

[b] Minimum inhibitory concentration as determined and described in chapter 5 (Table 5.1)

[c] Carriage of antimicrobial resistance genes associated with the resistance phenotype as determined and described in chapter 5 (Figure 5.1)

7.3 Results

7.3.1 Competition assay

The relative fitness of two MRSA CC398 strains, 93 and 107, was assessed by competitive growth with each of the following strains: 28 (CC22), 38 (CC22), 31 (CC30), 65 (CC30), 200 (CC8) and 201 (CC8), in the absence of antimicrobial compounds. As presented by Figures 7.1 and 7.2, both MRSA CC398 strains outgrew all competing strains, as defined by the selective CFU counts with the $\log_E x$, where x represents the ratio of 93 and 107 to the competing strain, > 0 after each cycle of competition. The overall outcome of the competitions was reproducible although some variation between the different biological replicates in the ratio values at corresponding time points was observed, as demonstrated by Figures 7.1 and 7.2.

All assays demonstrated a consistent trend of gradual increase in the $\log_e x$ value, indicating a continuous shift in the strain ratio to the advantage of the MRSA CC398 strains. The $\log_E x$ values after the last cycle of competition ranged from 1.46 up to 3.89 for strain 93-CC398, and from 2.19 up to 3.90 for strain 107-CC398 (Table 7.2). It was also observed that in general the final ratio of either MRSA CC398 strain to the competing strain was comparable for strains belonging to the same CC. On average, the highest final ratio of MRSA CC398 strains, both for 93 and 107, was detected after competition against the CC30 strains, followed by strains belonging to CC8 and then CC22.

The difference in fitness, S_t, between MRSA CC398 and the competing strains after each cycle of competition was calculated and is presented in Table 7.3. All values are > 0, indicting that MRSA CC398 strains demonstrated a higher level of fitness at each sampling point in the competition assay. In general, the values show a drop in S_t following the first cycle of competition. Furthermore, a proportion demonstrated a consistent decrease in S_t with the final competition cycle, S_3, representing the lowest difference in fitness. However, the drop in the S_t value was statistically significant (p value < 0.05) only for minority of competitions, as presented by Table 7.3.

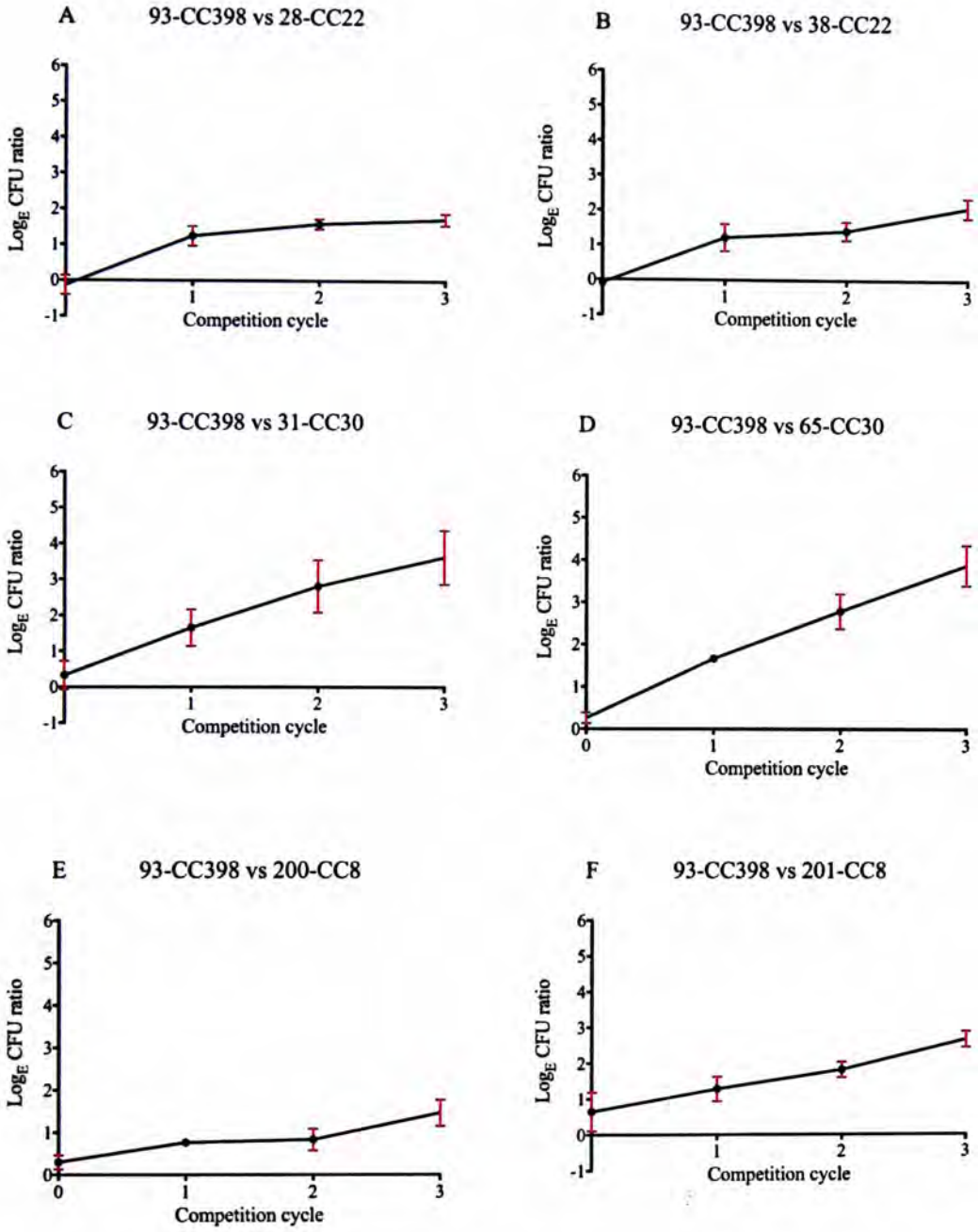


Figure 7.1 Log$_E$ CFU ratio of strain 93-CC398 to the competing strains (A: 28-CC22, B: 38-CC22, C: 31-CC30, D: 65-CC30, E: 200-CC8, F: 201-CC8) over three cycles of the competition assay. The error bars represent standard error of mean (SEM) and are based on three biological replicates.

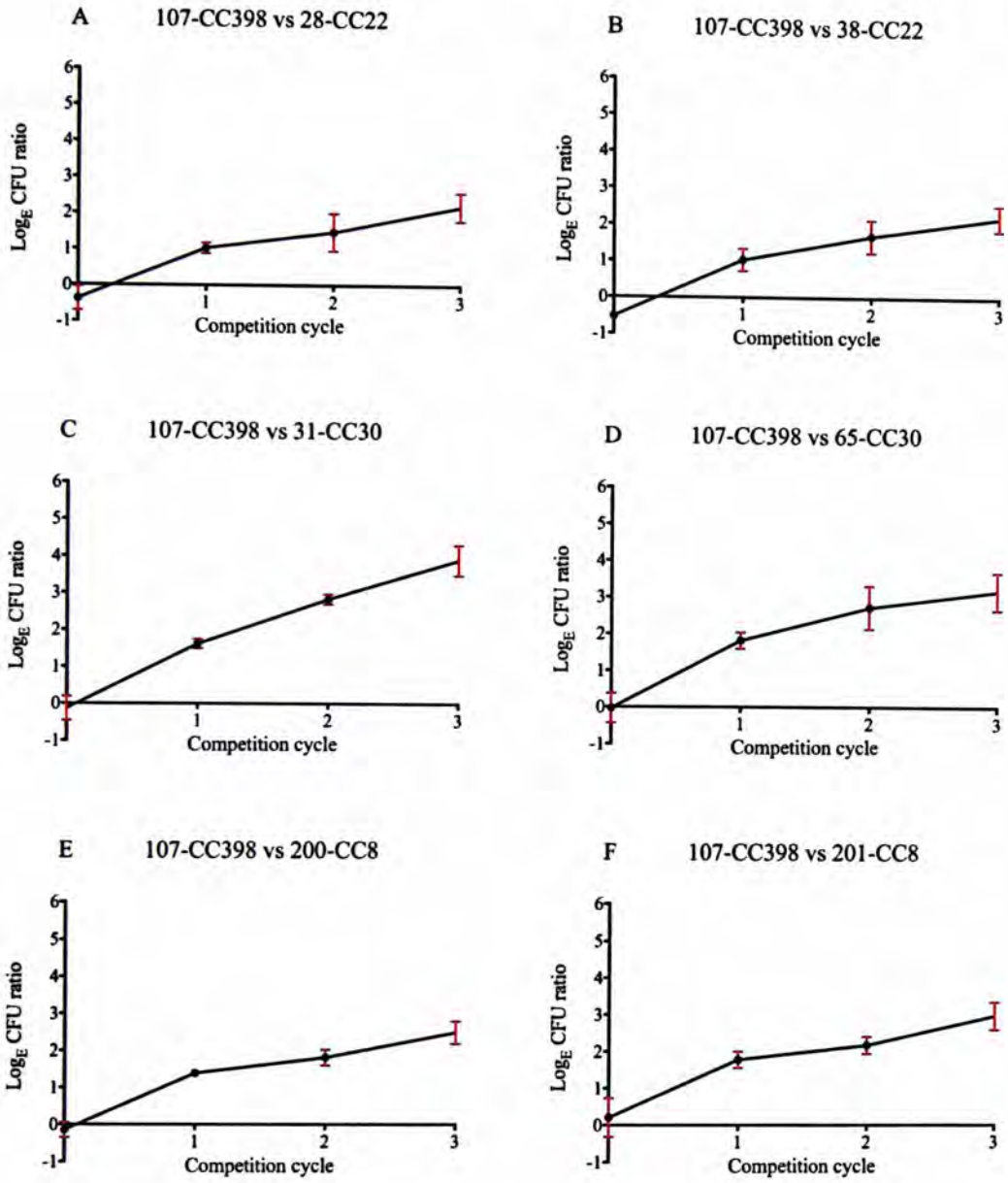


Figure 7.2 Log$_E$ CFU ratio of strain 107-CC398 to the competing strains (A: 28-CC22, B: 38-CC22, C: 31-CC30, D: 65-CC30, E: 200-CC8, F: 201-CC8) over three cycles of the competition assay. The error bars represent standard error of mean (SEM) and are based on three biological replicates.

Table 7.2 The $\log_E$ ratio of CFU after the last cycle of competition (C_3)

Strain ID	C_3 ratio 93-CC398		C_3 ratio 107-CC398	
	Mean [a]	SEM [b]	Mean	SEM
28-CC22	1.72	0.16	2.19	0.39
38-CC22	2.09	0.28	2.20	0.35
31-CC30	3.62	0.75	3.90	0.41
65-CC30	3.89	0.48	3.17	0.51
200-CC8	1.46	0.31	2.49	0.30
201-CC8	2.68	0.23	2.98	0.38

[a] Mean value of three biological replicates

[b] Standard error of mean.

Table 7.3 Difference in fitness (S_t) between the CC398 strains and the competitors after each cycle of competition

Strain ID	S_t	93-CC398			107-CC398		
		Mean [a]	SEM [b]	*p* value [c]	Mean	SEM	*p* value
28-CC22	S_1	0.081	0.026	**< 0.05**	0.082	0.021	0.183
	S_2	0.020	0.009		0.026	0.023	
	S_3	0.008	0.002		0.042	0.013	
38-CC22	S_1	0.077	0.018	0.103	0.091	0.016	**< 0.05**
	S_2	0.012	0.012		0.039	0.011	
	S_3	0.039	0.002		0.030	0.008	
31-CC30	S_1	0.078	0.052	0.596	0.103	0.026	0.317
	S_2	0.068	0.013		0.072	0.015	
	S_3	0.048	0.004		0.063	0.024	
65-CC30	S_1	0.083	0.008	0.127	0.102	0.016	0.053
	S_2	0.066	0.022		0.053	0.026	
	S_3	0.064	0.005		0.026	0.023	
200-CC8	S_1	0.027	0.015	0.566	0.090	0.013	**< 0.05**
	S_2	0.004	0.017		0.025	0.010	
	S_3	0.037	0.004		0.040	0.008	
201-CC8	S_1	0.037	0.047	0.812	0.093	0.041	0.377
	S_2	0.032	0.008		0.023	0.010	
	S_3	0.050	0.018		0.047	0.022	

[a] Mean value of three biological replicates

[b] Standard error of mean

[c] The significance of difference between S_1 and S_3 values, statistically significant *p* values (< 0.05) are highlighted in bold

7.3.2 Growth rates in the presence of antimicrobial compound

The growth curves of all analysed strains are presented in Figure 7.3. In general, for each antimicrobial compounds tested a statistically significant ($p < 0.05$) drop in growth rate (GR) was observed for a proportion, but not all analysed strains. Whilst the majority of strains demonstrated a considerable GR drop in the presence of cefoxitin and tetracycline, this was less frequent following exposure to erythromycin and trimethoprim. A significant drop in area under the curve (AUC) value occurred frequently upon exposure to all of the antimicrobial agents tested. In some instances the growth rate in the presence of antimicrobial compounds was comparable with the values when no selective pressure was applied, however, there was a considerable drop in the AUC value. This was associated with a comparatively longer lag phase and/or early growth plateau. Also, the variation in growth rates between selective and non-selective conditions in some instances appeared correlated with a resistance genotype as described below for erythromycin and tetracycline.

7.3.2.1 Erythromycin

A total of 24 erythromycin-resistant strains were analysed, which included CC398 (n=13) and strains belonging to CC8 (n=1), CC22 (n=5), CC30 (n=4) and CC97 (n=1). Statistically significant reductions (p value < 0.05) in GRs, ranging from 7.7% to 54.5 %, were observed for 11 strains, which included the single CC8, all but one CC22, two CC30, two CC398 and the single CC97 strain (Table 7.4). Amongst the remaining strains the majority demonstrated a marginal drop in growth rates whereas eight MRSA CC398 strains showed higher growth rate values following exposure to erythromycin than in the absence of antimicrobial induction. A statistically significant drop in the AUC value, ranging from 3.8% to 95%, was observed for 18 strains (Table 7.4). This included all strains that demonstrated a significant reduction in the GR. The highest drop in the AUC value was observed for strain 5-CC30, which remained in the lag phase for most of the incubation period and entered the exponential phase only after around 20 h, as presented by Figure 7.3 (M). A considerably longer lag phase (>10 h) was also observed for strains 31-CC30, 195-CC97 and 111-CC398, as revealed by Figure 7.3 (N, R, AN). Overall the four strains (5-CC30, 31-CC30, 195-CC97 and 111-CC398) demonstrated the highest % drop in the GR (> 20%) and the AUC (> 50%) values. Interestingly, all four demonstrated an

ermA-positive genotype and absence of other erythromycin resistance determinants, which was revealed by only one other strain of the analysed panel (Table 7.1).

7.3.2.2 Cefoxitin

A total of 33 cefoxitin-resistant strains were analysed, which included all CC398 strains (n=18) as well as strains belonging to CC8 (n=2), CC22 (n=6), CC30 (n=3) and CC130 (n=4). Statistically significant reductions in GRs, ranging from 6.6% to 56.3%, were observed for 27 strains, which included a single CC8, all but one CC22, all but one CC30, 15 strains belonging to CC398 and all CC130 strains (Table 7.5). A significant drop in the AUC value, ranging from 10.3% to 68.1%, was observed for all strains (Table 7.5). A proportion of strains appeared to enter an early growth plateau. For instance, strains 200-CC8, 201-CC8, 28-CC22, 173-CC130, 178-CC130, 181-CC130, 182-CC130, 93-CC398, 107-CC398 and 110-CC398 entered the stationery growth phase at an OD of around 0.4 - 0.5, as presented by Figure 7.3 (C, D, G, S, T, U, V, Z, AJ, AM), which represents a two-fold lower turbidity in comparison with growth in non-selective conditions.

7.3.2.3 Tetracycline

A total of 23 tetracycline-resistant strains were analysed, which included all CC398 strains (n=18) as well as strains belonging to CC8 (n=3), CC15 (n=1) and CC30 (n=1). A statistically significant drop in GRs, ranging from 14.1% to 51.5%, was observed for 16 strains: all but one CC8, the single CC15 and ten CC398 strains (Table 7.6). Amongst the remaining strains, five CC398 strains demonstrated a higher GR when grown in the presence of tetracycline in comparison with growth in a non-selective medium. Significant reductions in the AUC values, which ranged from 4.8% to 56.6%, were observed for 20 strains (Table 7.6). The CC398 strains that appeared to have a higher GR in the presence of tetracycline than with no selective pressure, demonstrated a marginal drop in the AUC. A single CC8 strain with a comparable GR in the presence and absence of tetracycline, also revealed a nearly identical AUC values. For the majority of strains that were found to show a significant decrease in the GR and AUC values, the reduction appeared to be associated with an extended lag phase, with the pattern of exponential growth resembling the non-selective conditions, as demonstrated by Figure 7.3 (B, C, E, P, Y, Z, AC, AE, AJ, AL, AM, AN). Furthermore, all strains demonstrating the highest % drop in the GR (> 25%) and the

AUC (> 25%) values revealed a single *tet* determinant genotype (carriage of *tetK* or *tetM* only) while the remaining analysed strains featured either a concomitant carriage of *tetK*+*tetM* or *tetL*+*tetM* (Table 7.1)

7.3.2.4 Trimethoprim

A total of 20 trimethoprim-resistant strains were analysed, which consisted of CC398 strains (n=13) as well as strains belonging to CC5 (n=1), CC8 (n=2), CC22 (n=2) and CC30 (n=2). Amongst these strains only four demonstrated a statistically significant drop in the GR values, ranging from 0.1% to 0.2% (Table 7.7). This included the single CC5, one CC8, one CC22 and one CC30 strain. The majority of remaining strains revealed a GR comparable to the growth in a non-selective medium. Considerable reductions in the AUC values were observed for eight strains and ranged from 4.3% to 88.5% (Table 7.7). The strain with the highest drop in the AUC, namely 38 belonging to CC22, appeared to undergo an extended lag phase, with the exponential growth observed only after around 18 h, as demonstrated by Figure 7.3 (I). As revealed by Table 7.1, this strain also presented the lowest MIC value amongst trimethoprim-resistant strains and represented one of three strains with an unknown determinant of resistance. With the exception of strains 18, 38 and 201, the growth patterns in the presence of trimethoprim amongst the analysed strains closely resembled the non-selective conditions, as presented by Figure 7.3 (C, O, Q, W, Y, AA, AB, AC, AD, AE, AF, AH, AI, AK, AL, AN).

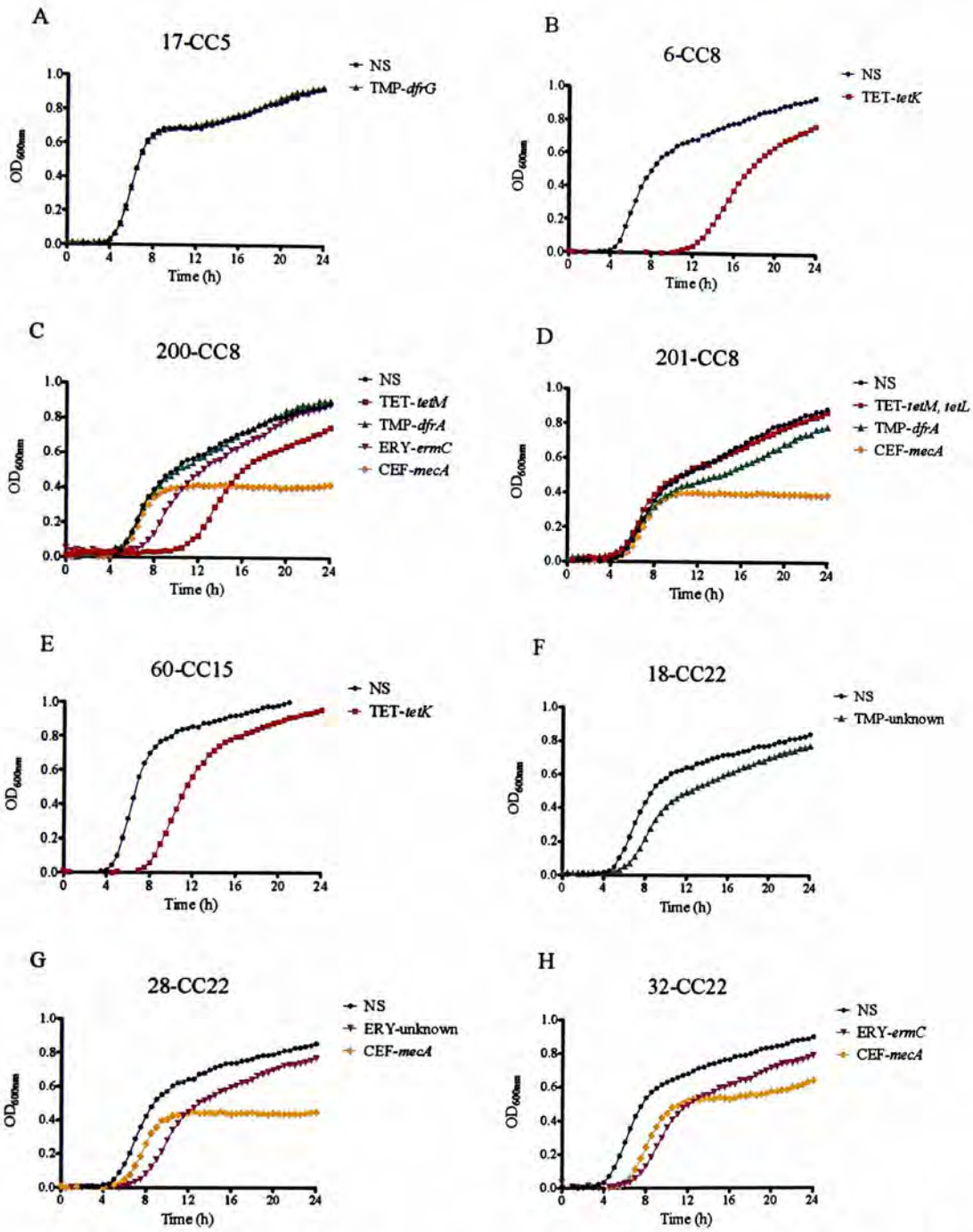


Figure 7.3 Growth curves of analysed strains (A: 17, B: 6, C: 200, D: 201, E: 60, F: 18, G: 28, H: 32) in the presence and absence of antimicrobial agents determined by measuring OD_{600nm} over a 24 h incubation period. NS - non-selective, ERY - erythromycin, CEF - cefoxitin, TET - tetracycline, TMP - trimethoprim. For each strain, included in the figure legend is also the genetic mechanism of resistance to the corresponding compound. Continued on next page.

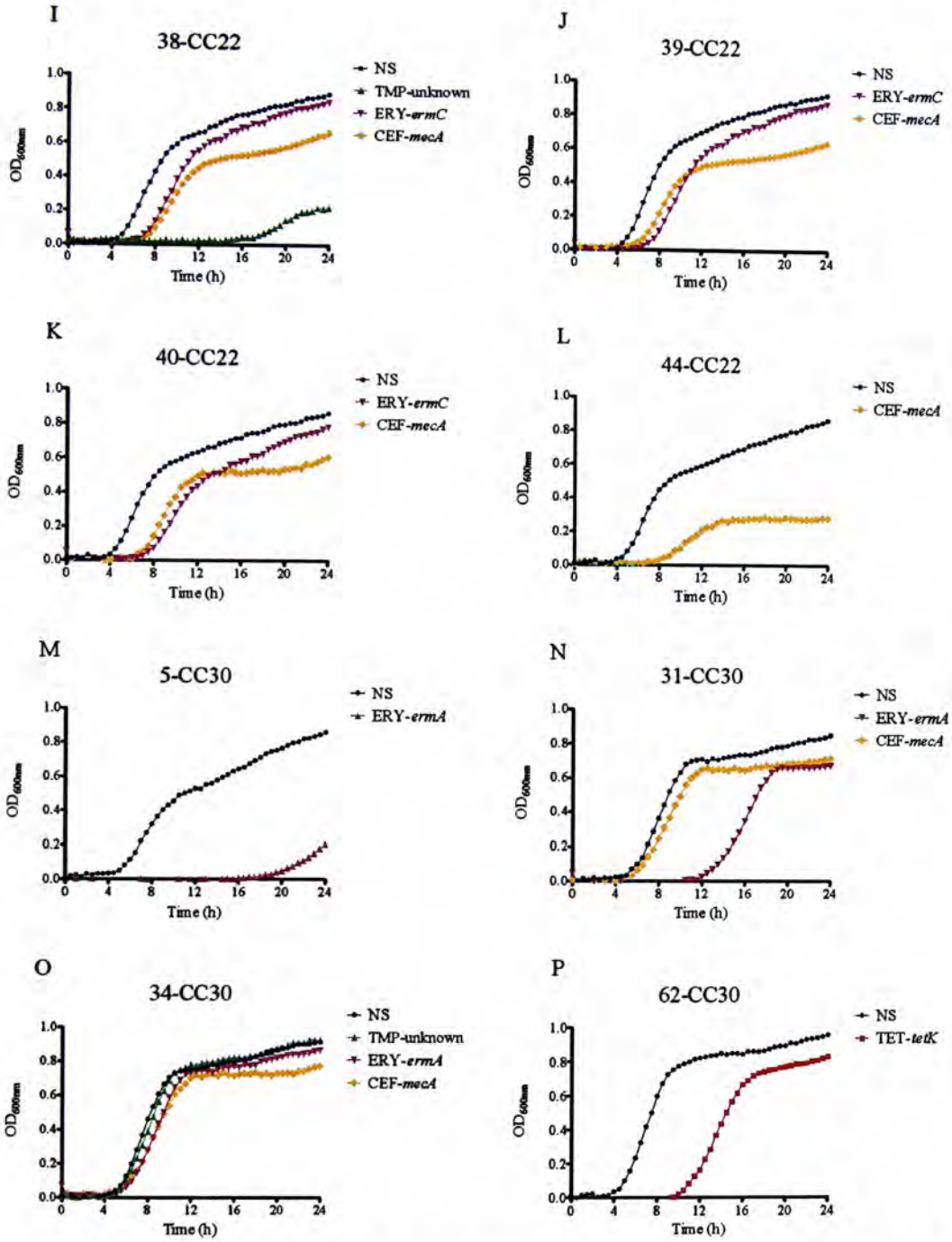


Figure 7.3 (continued) Growth curves of analysed strains (I: 38, J: 39, K: 40, L: 44, M: 5, N: 31, O: 34, P: 62) in the presence and absence of antimicrobial agents determined by measuring OD_{600nm} over a 24 h incubation period. NS - non-selective, ERY - erythromycin, CEF - cefoxitin, TET - tetracycline, TMP - trimethoprim. For each strain, included in the figure legend is also the genetic mechanism of resistance to the corresponding compound. Continued on next page.

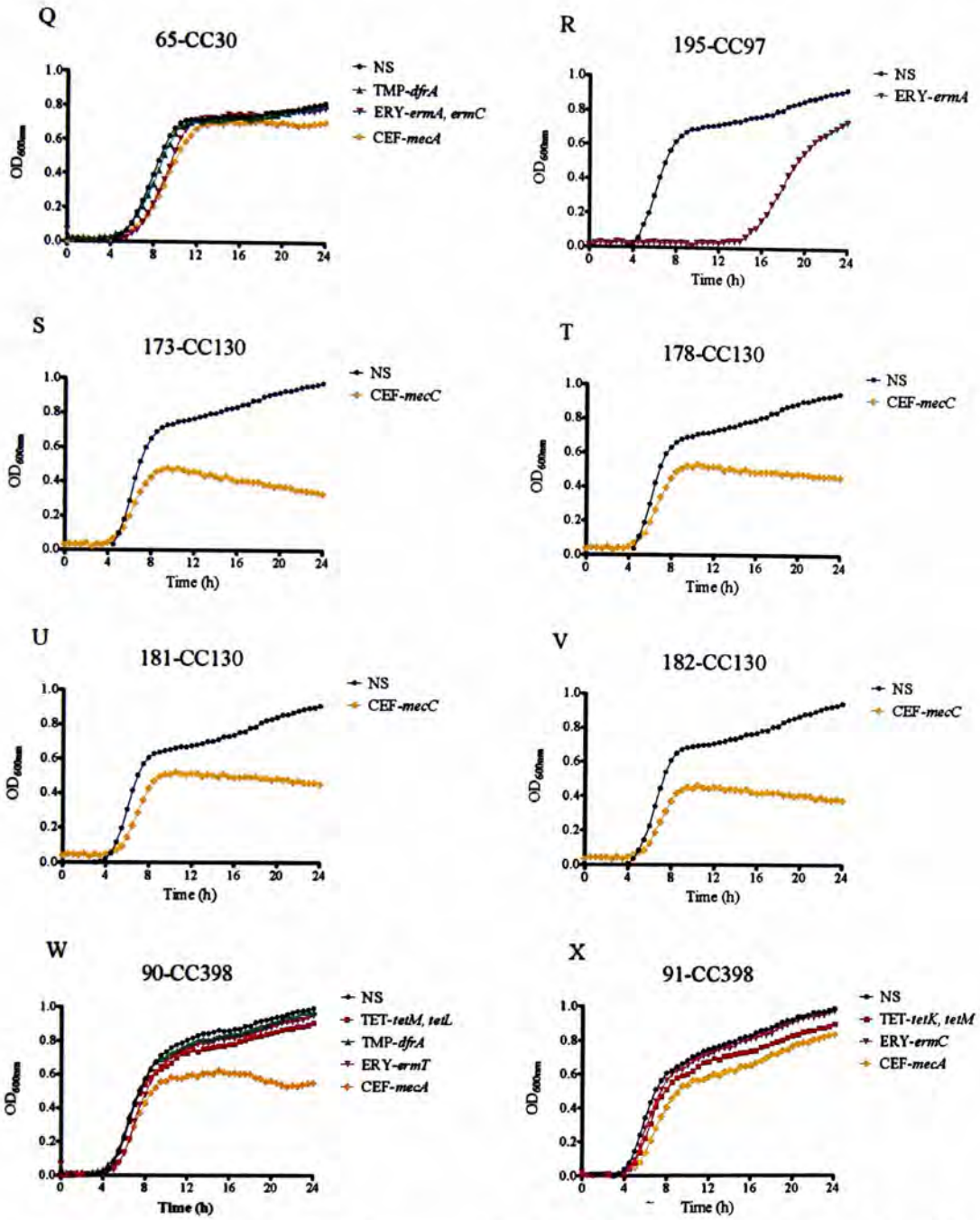


Figure 7.3 (continued) Growth curves of analysed strains (Q: 65, R: 195, S: 173, T: 178, U: 181, V: 182, W: 90, X: 91) in the presence and absence of antimicrobial agents determined by measuring OD_{600nm} over a 24 h incubation period. NS - non-selective, ERY - erythromycin, CEF - cefoxitin, TET - tetracycline, TMP - trimethoprim. For each strain, included in the figure legend is also the genetic mechanism of resistance to the corresponding compound. Continued on next page.

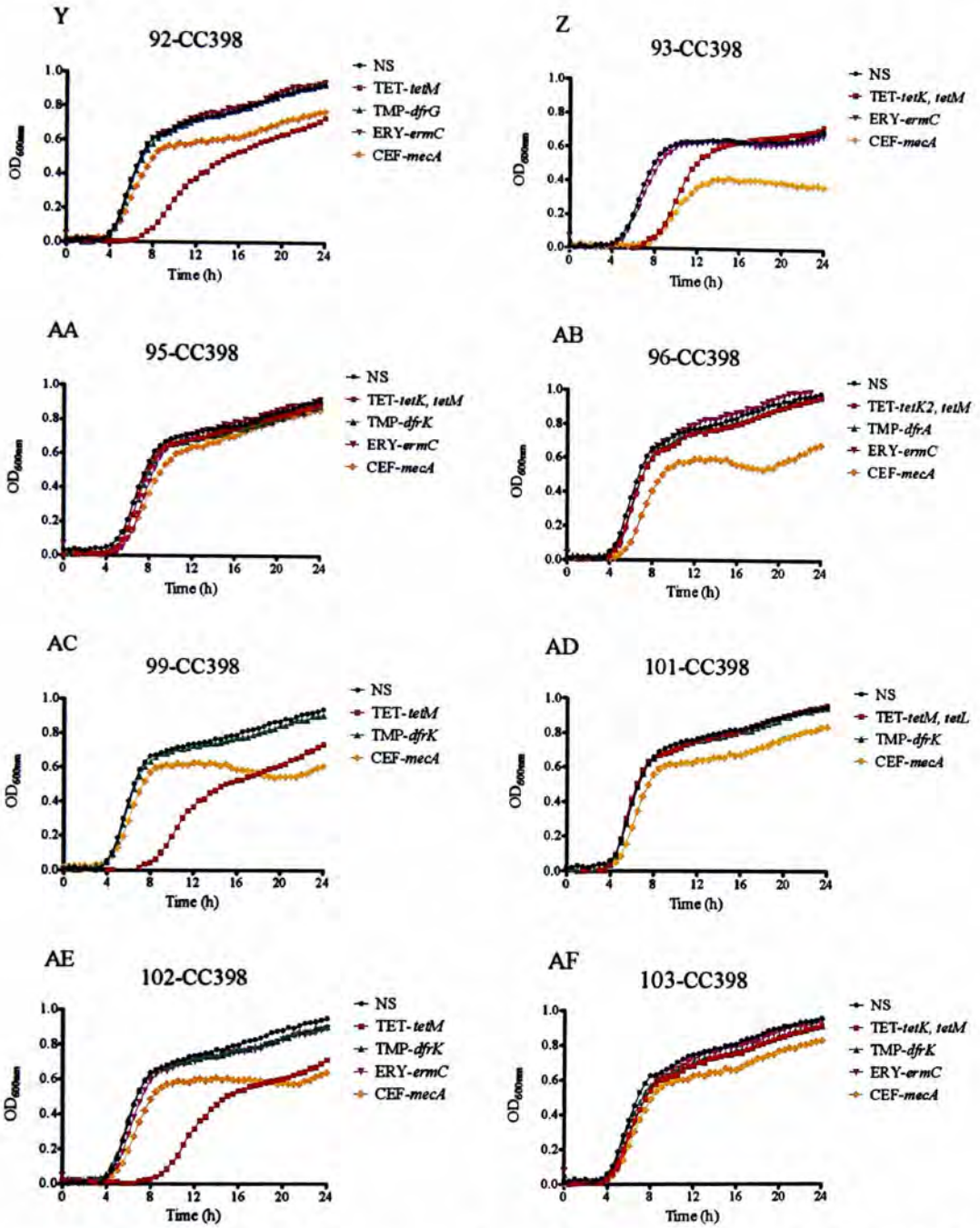


Figure 7.3 (continued) Growth curves of analysed strains (Y: 92, Z: 93, AA: 95, AB: 96, AC: 99, AD: 101, AE: 102, AF: 103) in the presence and absence of antimicrobial agents determined by measuring OD_{600nm} over a 24 h incubation period. NS - non-selective, ERY - erythromycin, CEF - cefoxitin, TET - tetracycline, TMP - trimethoprim. For each strain, included in the figure legend is also the genetic mechanism of resistance to the corresponding compound. Continued on next page.

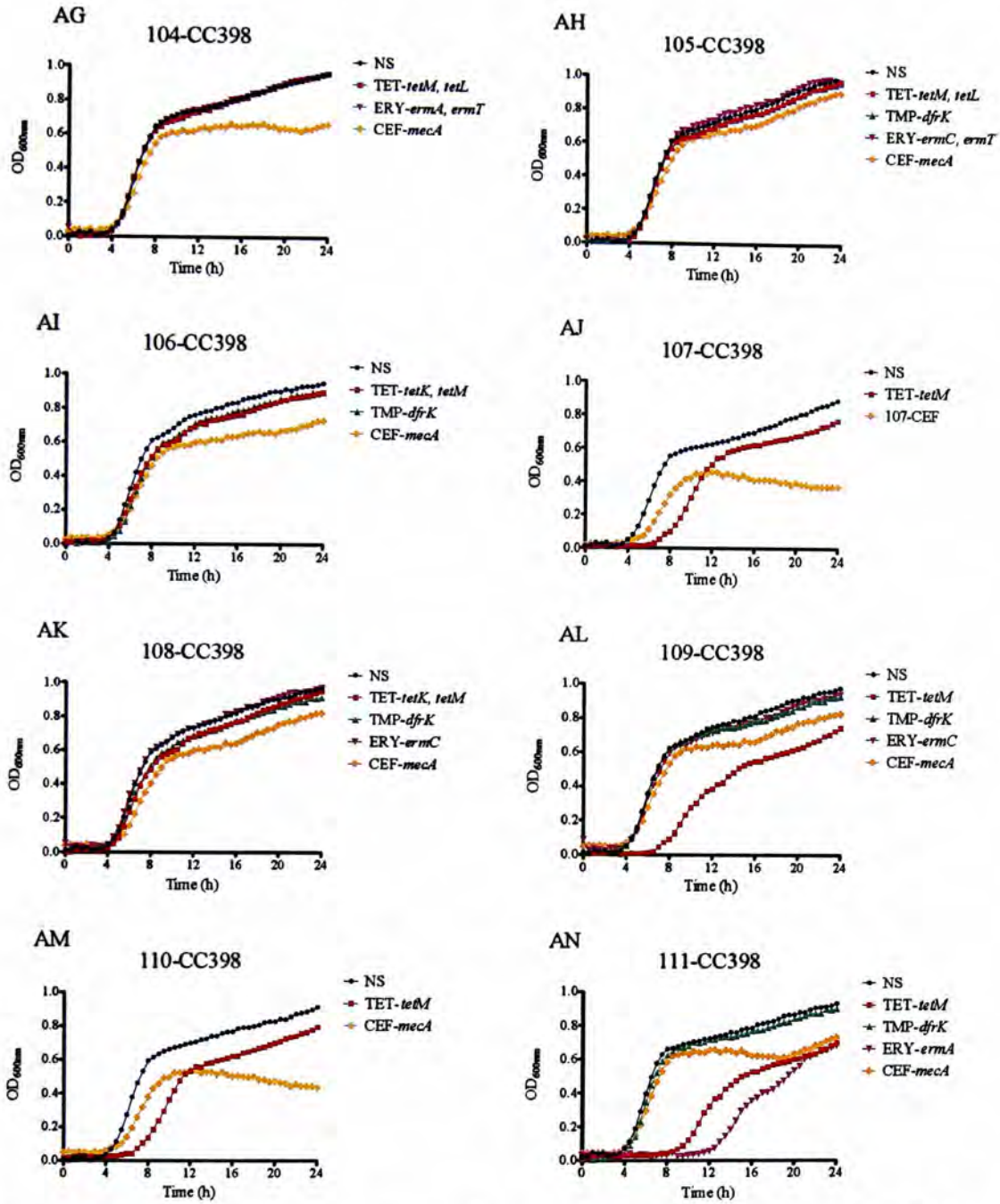


Figure 7.3 (continued) Growth curves of analysed strains (AG: 104, AH: 105, AI: 106, AJ: 107, AK: 108, AL: 109, AM: 110, AN: 111) in the presence and absence of antimicrobial agents determined by measuring OD_{600nm} over a 24 h incubation period. NS - non-selective, ERY - erythromycin, CEF - cefoxitin, TET - tetracycline, TMP - trimethoprim. For each strain, included in the figure legend is also the genetic mechanism of resistance to the corresponding compound.

Table 7.4 Growth rates and area under a curve of analysed strains when grown in a non-selective medium (NS) and in the presence of 4 μg/ml erythromycin (ERY)

		Growth rates (GR)						Area under curve (AUC)					
		NS		ERY		Difference [c]		NS		ERY		Difference	
ID	Lineage	Mean[a]	SEM[b]	Mean	SEM	%	*p* value	Mean	SEM	Mean	SEM	%	*p* value
5	CC30	0.56	0.06	0.25	0.04	54.5	**< 0.05**	10.85	0.36	0.55	0.35	95.0	**< 0.05**
28	CC22	0.74	0.01	0.70	0.03	5.7	0.13	12.07	0.20	8.81	0.23	27.0	**< 0.05**
31	CC30	0.70	0.05	0.53	0.02	25.1	**< 0.05**	12.17	0.34	5.49	0.06	54.9	**< 0.05**
32	CC22	0.81	0.01	0.67	0.05	16.9	0.06	13.25	0.24	9.42	0.13	28.9	**< 0.05**
34	CC30	0.81	0.02	0.69	0.04	14.0	**< 0.05**	13.36	0.19	12.14	0.22	9.1	**< 0.05**
38	CC22	0.71	0.03	0.63	0.04	11.1	0.11	12.59	0.15	10.32	0.36	18.0	**< 0.05**
39	CC22	0.84	0.06	0.68	0.02	19.2	**< 0.05**	13.33	0.17	10.42	0.14	21.8	**< 0.05**
40	CC22	0.75	0.02	0.62	0.02	16.9	**< 0.05**	12.46	0.23	8.72	0.21	30.0	**< 0.05**
65	CC30	0.74	0.01	0.69	0.01	7.7	**< 0.05**	12.17	0.16	11.34	0.24	6.8	**< 0.05**
90	CC398	1.03	0.04	0.89	0.02	14.0	**< 0.05**	14.69	0.19	13.52	0.06	7.9	**< 0.05**
91	CC398	0.99	0.06	0.98	0.05	1.0	0.45	14.55	0.26	13.91	0.08	4.4	0.06
92	CC398	0.95	0.07	1.03	0.02	-8.2	0.19	14.05	0.36	14.18	0.08	-1.0	0.37
93	CC398	0.82	0.01	0.78	0.06	4.4	0.30	11.21	0.04	10.70	0.04	4.5	**< 0.05**
95	CC398	0.82	0.03	0.92	0.05	-12.3	0.08	13.52	0.05	12.88	0.08	4.7	**< 0.05**
96	CC398	0.99	0.04	1.06	0.09	-6.9	0.27	14.95	0.05	15.01	0.10	-0.4	0.32
102	CC398	1.00	0.06	0.92	0.07	8.4	0.21	14.25	0.20	13.42	0.14	5.8	**< 0.05**
103	CC398	0.97	0.02	0.97	0.04	0.5	0.46	14.53	0.03	13.75	0.08	5.3	**< 0.05**
104	CC398	1.00	0.08	0.97	0.04	3.3	0.37	14.55	0.12	14.43	0.61	0.9	0.43
105	CC398	0.90	0.06	1.01	0.05	-12.5	0.12	14.36	0.12	14.56	0.10	-1.4	0.13
108	CC398	0.93	0.07	0.89	0.03	4.5	0.32	14.29	0.15	14.45	0.17	-1.1	0.26
109	CC398	1.00	0.05	0.86	0.02	14.3	**< 0.05**	14.56	0.17	14.01	0.13	3.8	**< 0.05**
111	CC398	0.96	0.04	0.66	0.06	31.2	**< 0.05**	14.40	0.07	5.40	1.09	62.5	**< 0.05**
195	CC97	0.92	0.03	0.59	0.12	35.5	**< 0.05**	13.80	0.11	4.69	0.68	66.0	**< 0.05**
200	CC8	0.75	0.01	0.59	0.04	21.6	**< 0.05**	11.74	0.05	10.20	0.36	13.2	**< 0.05**

[a] Mean of three biological replicates

[b] Standard error of mean

[c] Difference between the NS and ERY values expressed as (NS-ERY)/NS x 100 (%); *p* value calculated with t test, statistically significant (< 0.05) *p* values highlighted in bold

Table 7.5 Growth rates and area under a curve of analysed strains when grown in non-selective medium (NS) and in the presence of 1 μg/ml cefoxitin (CEF)

		Growth rates (GR)						Area under curve (AUC)					
		NS		CEF		Difference[c]		NS		CEF		Difference[c]	
ID	Lineage	Mean[a]	SEM[b]	Mean	SEM	%	*p* value	Mean	SEM	Mean	SEM	%	*p* value
28	CC22	0.74	0.01	0.69	0.02	6.2	0.10	10.85	0.36	0.55	0.35	95.0	**< 0.05**
31	CC30	0.70	0.05	0.57	0.01	19.2	0.05	12.07	0.20	8.81	0.23	27.0	**< 0.05**
32	CC22	0.81	0.01	0.75	0.03	6.6	**< 0.05**	12.17	0.34	5.49	0.06	54.9	**< 0.05**
34	CC30	0.81	0.02	0.61	0.03	24.9	**< 0.05**	13.25	0.24	9.42	0.13	28.9	**< 0.05**
38	CC22	0.71	0.03	0.58	0.02	18.1	**< 0.05**	13.36	0.19	12.14	0.22	9.1	**< 0.05**
39	CC22	0.84	0.06	0.66	0.04	21.4	**< 0.05**	12.59	0.15	10.32	0.36	18.0	**< 0.05**
40	CC22	0.75	0.02	0.68	0.00	9.5	**< 0.05**	13.33	0.17	10.42	0.14	21.8	**< 0.05**
44	CC22	0.70	0.04	0.31	0.04	56.3	**< 0.05**	12.46	0.23	8.72	0.21	30.0	**< 0.05**
65	CC30	0.74	0.01	0.56	0.03	24.6	**< 0.05**	12.17	0.16	11.34	0.24	6.8	**< 0.05**
90	CC398	1.03	0.04	0.84	0.03	18.7	**< 0.05**	14.69	0.19	13.52	0.06	7.9	**< 0.05**
91	CC398	0.99	0.06	0.74	0.01	24.8	**< 0.05**	14.55	0.26	13.91	0.08	4.4	0.06
92	CC398	0.95	0.07	0.73	0.01	23.4	**< 0.05**	14.05	0.36	14.18	0.08	-1.0	0.37
93	CC398	0.82	0.01	0.48	0.02	40.6	**< 0.05**	11.21	0.04	10.70	0.04	4.5	**< 0.05**
95	CC398	0.82	0.03	0.69	0.04	16.7	**< 0.05**	13.52	0.05	12.88	0.08	4.7	**< 0.05**
96	CC398	0.99	0.04	0.89	0.00	10.6	0.06	14.95	0.05	15.01	0.10	-0.4	0.32
99	CC398	1.00	0.07	0.80	0.03	20.5	**< 0.05**	14.25	0.20	13.42	0.14	5.8	**< 0.05**
101	CC398	1.03	0.06	0.96	0.05	6.8	0.21	14.53	0.03	13.75	0.08	5.3	**< 0.05**
102	CC398	1.00	0.06	0.80	0.07	19.8	0.06	14.55	0.12	14.43	0.61	0.9	0.43
103	CC398	0.97	0.02	0.84	0.05	13.4	**< 0.05**	14.36	0.12	14.56	0.10	-1.4	0.13
104	CC398	1.00	0.08	0.79	0.05	21.8	**< 0.05**	14.29	0.15	14.45	0.17	-1.1	0.26
105	CC398	0.90	0.06	0.68	0.01	23.8	**< 0.05**	14.56	0.17	14.01	0.13	3.8	**< 0.05**
106	CC398	0.94	0.03	0.67	0.02	28.7	**< 0.05**	14.40	0.07	5.40	1.09	62.5	**< 0.05**
107	CC398	0.81	0.04	0.57	0.01	29.7	**< 0.05**	13.80	0.11	4.69	0.68	66.0	**< 0.05**
108	CC398	0.93	0.07	0.65	0.08	29.7	**< 0.05**	11.74	0.05	10.20	0.36	13.2	**< 0.05**
109	CC398	1.00	0.05	0.65	0.01	34.9	**< 0.05**	14.56	0.17	12.45	0.22	14.5	**< 0.05**
110	CC398	0.88	0.05	0.56	0.03	36.3	**< 0.05**	13.48	0.12	8.73	0.14	35.2	**< 0.05**
111	CC398	0.96	0.04	0.72	0.02	25.1	**< 0.05**	14.40	0.07	11.73	0.22	18.6	**< 0.05**
173	CC398	1.10	0.07	0.72	0.04	34.4	**< 0.05**	14.60	0.02	7.55	0.29	48.3	**< 0.05**
178	CC398	1.03	0.07	0.73	0.07	28.6	**< 0.05**	14.06	0.06	8.90	0.29	36.7	**< 0.05**
181	CC398	1.11	0.02	0.70	0.04	37.1	**< 0.05**	13.33	0.19	8.75	0.22	34.4	**< 0.05**
182	CC398	1.00	0.02	0.63	0.02	37.1	**< 0.05**	13.62	0.21	7.54	0.09	44.6	**< 0.05**
200	CC8	0.75	0.01	0.67	0.01	11.0	**< 0.05**	11.74	0.05	7.02	0.18	40.2	**< 0.05**
201	CC8	0.64	0.00	0.62	0.04	3.2	0.32	11.26	0.48	6.74	0.08	40.1	**< 0.05**

[a] Mean of three biological replicates

[b] Standard error of mean

[c] Difference between the NS and CEF values expressed as (NS-CEF)/NS x 100 (%); *p* value calculated with t test, statistically significant (< 0.05) *p* values highlighted in bold

Table 7.6 Growth rates and area under a curve of analysed strains when grown in non-selective medium (NS) and in the presence of 8 μg/ml tetracycline (TET)

		Growth rates (GR)						Area under curve (AUC)					
		NS		TET		Difference [c]		NS		TET		Difference	
ID	Lineage	Mean[a]	SEM[b]	Mean	SEM	%	*p* value	Mean	SEM	Mean	SEM	%	*p* value
6	CC8	0.88	0.03	0.55	0.07	37.9	**< 0.05**	13.29	0.24	5.76	0.28	56.6	**< 0.05**
60	CC15	1.17	0.10	0.87	0.02	25.2	**< 0.05**	15.88	0.27	11.66	0.13	26.6	**< 0.05**
62	CC30	0.83	0.06	0.58	0.03	30.6	**< 0.05**	14.68	0.24	7.90	0.29	46.2	**< 0.05**
90	CC398	1.03	0.04	0.90	0.07	12.6	0.09	14.69	0.19	13.38	0.39	8.9	**< 0.05**
91	CC398	0.99	0.06	0.85	0.05	13.7	0.08	14.55	0.26	12.83	0.01	11.8	**< 0.05**
92	CC398	0.95	0.07	0.46	0.02	51.5	**< 0.05**	14.05	0.36	7.52	0.21	46.5	**< 0.05**
93	CC398	0.82	0.01	0.67	0.03	17.4	**< 0.05**	11.21	0.04	8.93	0.09	20.3	**< 0.05**
95	CC398	0.82	0.03	0.94	0.02	-14.5	**< 0.05**	13.52	0.05	12.86	0.17	4.9	**< 0.05**
96	CC398	0.99	0.04	1.02	0.05	-3.0	0.34	14.95	0.05	14.10	0.25	5.7	**< 0.05**
99	CC398	1.00	0.07	0.57	0.02	42.9	**< 0.05**	14.41	0.22	7.62	0.22	47.1	**< 0.05**
101	CC398	1.03	0.06	1.14	0.03	-10.8	0.10	14.86	0.15	14.69	0.19	1.2	0.26
102	CC398	1.00	0.06	0.63	0.01	37.0	**< 0.05**	14.25	0.20	7.94	0.32	44.3	**< 0.05**
103	CC398	0.97	0.02	0.83	0.05	14.8	**< 0.05**	14.53	0.03	13.27	0.05	8.6	**< 0.05**
104	CC398	1.00	0.08	1.02	0.03	-1.2	**< 0.05**	14.55	0.12	14.36	0.26	1.3	0.28
105	CC398	0.90	0.06	0.94	0.06	-5.1	0.30	14.36	0.12	13.66	0.23	4.8	**< 0.05**
106	CC398	0.94	0.03	0.81	0.03	14.1	**< 0.05**	14.47	0.17	13.21	0.12	8.7	**< 0.05**
107	CC398	0.81	0.04	0.56	0.06	31.5	**< 0.05**	12.66	0.10	8.86	0.07	30.1	**< 0.05**
108	CC398	0.93	0.07	0.83	0.07	10.6	0.19	14.29	0.15	13.20	0.13	7.6	**< 0.05**
109	CC398	1.00	0.05	0.50	0.04	49.8	**< 0.05**	14.56	0.17	8.29	0.19	43.1	**< 0.05**
110	CC398	0.88	0.05	0.51	0.02	41.4	**< 0.05**	13.48	0.12	9.69	0.13	28.1	**< 0.05**
111	CC398	0.96	0.04	0.56	0.02	41.1	**< 0.05**	14.40	0.07	7.62	0.03	47.1	**< 0.05**
200	CC8	0.75	0.01	0.49	0.04	34.7	**< 0.05**	11.74	0.05	6.86	0.12	41.6	**< 0.05**
201	CC8	0.64	0.00	0.65	0.02	-1.2	0.36	11.26	0.48	11.34	0.12	-0.7	0.44

[a] Mean of three biological replicates

[b] SEM - standard error of the mean

[c] Difference between the NS and TET values expressed as (NS-TET)/NS x 100 (%); *p* value calculated with t test, statistically significant (< 0.05) *p* values highlighted in bold

Table 7.7 Growth rates and area under a curve of analysed strains when grown in non-selective medium (NS) and in the presence of 16 µg/ml trimethoprim (TMP)

		Growth rates (GR)						Area under curve (AUC)					
		NS		TMP		Difference [c]		NS		TMP		Difference	
ID	Lineage	Mean[a]	SEM[b]	Mean	SEM	%	*p* value	Mean	SEM	Mean	SEM	%	*p* value
17	CC5	1.09	0.02	0.97	0.04	0.1	**< 0.05**	13.75	0.18	14.00	0.15	-1.8	0.18
18	CC22	0.74	0.02	0.58	0.02	0.2	**< 0.05**	11.98	0.29	9.68	0.45	19.2	**< 0.05**
34	CC30	0.81	0.02	0.78	0.02	0.0	0.21	13.36	0.19	13.33	0.43	0.2	0.48
38	CC22	0.71	0.04	0.30	0.14	57.5	0.10	12.59	0.15	1.45	0.44	88.5	**< 0.05**
65	CC30	0.74	0.01	0.59	0.03	0.2	**< 0.05**	12.17	0.16	12.02	0.49	1.2	0.40
90	CC398	1.03	0.04	0.95	0.02	0.1	0.07	14.69	0.19	14.48	0.11	1.4	0.20
92	CC398	0.95	0.07	0.97	0.04	0.0	0.41	14.05	0.36	13.91	0.54	1.0	0.42
95	CC398	0.82	0.03	0.87	0.07	-0.1	0.29	13.52	0.05	12.73	0.05	5.9	**< 0.05**
96	CC398	0.99	0.04	0.90	0.04	0.1	0.08	14.95	0.05	14.41	0.24	3.6	0.07
99	CC398	1.00	0.07	0.97	0.04	0.0	0.34	14.41	0.22	13.98	0.15	3.0	0.10
101	CC398	1.03	0.06	1.07	0.01	0.0	0.28	14.86	0.15	14.58	0.19	1.9	0.16
102	CC398	1.00	0.06	0.95	0.05	0.1	0.26	14.25	0.20	13.90	0.27	2.4	0.18
103	CC398	0.97	0.02	0.88	0.04	0.1	0.08	14.53	0.03	13.66	0.23	6.0	**< 0.05**
105	CC398	0.90	0.06	0.98	0.07	-0.1	0.21	14.36	0.12	13.92	0.45	3.1	0.22
106	CC398	0.94	0.03	0.94	0.06	0.0	0.49	14.47	0.17	13.26	0.33	8.3	**< 0.05**
108	CC398	0.93	0.07	0.90	0.03	0.0	0.36	14.29	0.15	13.30	0.30	6.9	**< 0.05**
109	CC398	1.00	0.05	0.88	0.02	0.1	0.06	14.56	0.17	14.08	0.26	3.3	0.10
111	CC398	0.96	0.04	0.94	0.02	0.0	0.39	14.40	0.07	13.79	0.20	4.3	**< 0.05**
200	CC8	0.75	0.01	0.69	0.03	0.1	0.06	11.74	0.05	11.93	0.30	-1.6	0.30
201	CC8	0.64	0.00	0.50	0.02	0.2	**< 0.05**	11.26	0.48	9.84	0.08	12.6	**< 0.05**

[a] Mean of three biological replicates

[b] SEM - standard error of the mean

[c] Difference between the NS and TMP values expressed as (NS-TMP)/NS x 100 (%); *p* value calculated with t test, statistically significant (< 0.05) *p* values highlighted in bold

7.4 Discussion

In accordance with the observation that acquisition of antimicrobial resistance can lead to reduction in the biological fitness of the bacterial host (Wichelhaus *et al.*, 2002), it can be expected that such biological cost would affect all MRSA strains. In fact, acquisition of the SCC*mec* element has been shown to cause a reduction in bacterial growth rate (Ender *et al.*, 2004). This is considered to result mostly from the extensive size of the chromosomally integrated SCC*mec*, as well as utilisation of the novel PBP-2a as a cell wall structural element (Ender *et al.*, 2004). However, it has also been observed that bacteria can adapt in response to the biological cost of antimicrobial resistance (Nagaev *et al.*, 2001). For instance, it was found that while acquisition of plasmid-associated antimicrobial resistance might lead to a preliminary reduction in fitness, the bacterial cell can quickly adapt and overcome such fitness cost to an extent where the plasmid-bearing strain is out-competing the plasmid-free isogenic counterpart (Bouma and Lenski, 1988). Also, the process of adaptation to the biological cost of resistance was shown to involve the selection of resistant clones that suffer the lowest level of fitness cost (Wichelhaus *et al.*, 2002; Sander *et al.*, 2002). It can therefore be speculated that the acquisition and carriage of a variety of antimicrobial resistance determinants by MRSA CC398 lineage resulted in a development of fitness cost coping mechanisms that also provided the strains with a competitive advantage.

In the case of MRSA strains and adaptations to reduce the biological cost of SCC*mec*, it has been observed that a low level of methicillin resistance, recognised as heterogeneous resistance, is accompanied by elevated rates of growth in comparison with highly resistant strains, also referred to as homogenous resistance (Ender *et al.*, 2004). In particular, the heterogeneous methicillin resistance has been associated with CA-MRSA isolates, which were found to display a significantly lower doubling times when compared against homogenously resistant HA-MRSA strains (Okuma *et al.*, 2002). Authors further observed that such a feature was likely to promote the success of CA-MRSA strains as colonising agents in a non-healthcare setting. All MRSA CC398 strains analysed in this study displayed a low level of cefoxitin resistance, with the MIC values of either 8 μg/ml or 16 μg/ml (Table 7.1). However, this was also observed amongst the majority of the remaining MRSA strains. As such, amongst the strains selected for the competition assay analysis, only the 65-CC30 strain

demonstrated MIC of > 32 μg/ml. However, the analysis of growth rates following exposure to cefoxitin revealed a level of variation in the degree of strain growth rate and area under a curve reduction. More specifically, amongst the strains that were subjected to the mixed culture competition assay, the MRSA CC398 strains demonstrated the highest % drop in the GR as well as AUC values. As presented by Table 7.5, strains 93-CC398 and 107-CC398 revealed a 40.6% and 29.7% drop in the GR values, respectively, whereas the next highest reduction in the GR measured at 24.6% was demonstrated by strain 65-CC30. Further work would be required to determine if an association between growth rates in the presence of cefoxitin and competitive fitness exists. However, the results suggest that the apparent enhanced fitness of MRSA CC398 strains might be related to an adaptation towards the cost of SCC*mec* carriage.

While the aim of the analysis of the growth rate in the presence of antimicrobials was to investigate whether the MRSA CC398 demonstrate a comparatively enhanced ability to overcome the selective pressure, no apparent correlation was observed between GR/AUC reduction and an underlying lineage. Instead, an association appeared between the relative growth rates in the presence of the selected antimicrobial compound and the resistance genotype of the analysed strain. As such, the majority of erythromycin resistant strains that carried the *ermA* gene only, demonstrated a considerably longer lag phase in comparison to strains carrying either *ermC* only or *ermA* coupled with *ermC* or *ermT*. Amongst the causative factors behind variation in the response to erythromycin exposure might be the copy number of the described resistance genes within the bacterial cell. Although multiple copies of the *ermA*-carrying transposon Tn*554* within a single *S. aureus* genome have been previously identified, most often it has been limited to two such entities (Baba *et al.*, 2008). In contrast, the *ermC* gene is commonly carried on small multiple copy plasmids whereas the *ermT* gene has been associated with larger plasmids (Werckenthin *et al.*, 2001; Kadlec and Schwarz, 2010). Thus it might be speculated that these determinants provide the bacterial cell with a more efficient protection from erythromycin, as their expression would be enhanced by presence of higher gene copy number in comparison with the *ermA* gene.

Correlation between an underlying resistance genotype and differential growth curve response was also observed for tetracycline. However, in contrast to *erm*

determinants, rather than association with a particular resistance gene, a comparatively lower reduction in growth rate was observed in strains carrying dual as oppose to a single *tet* gene. As demonstrated by Table 7.1, this was mostly applicable to CC398 strains, with a single CC8 strain also carrying two tetracycline resistance genes. The multiple *tet* determinant carriage was associated with presence of the *tetM* gene together with either *tetK* or *tetL* genes. A single CC398 strain carried a *tetM* gene and a novel tetracycline resistance determinant identified in this work and designated *tetK2*, as described previously (Chapter 6). The tetracycline resistance genes are broadly classified in accordance with the underlying mechanism of resistance as encoding either efflux mechanism or ribosomal protection proteins (Ng *et al.*, 2001). While the *tetK* and *tetL* genes encode the former, the *tetM* element is related to the latter (Werckenthin *et al.*, 2001). As such, the co-carriage of *tetM* together with either *tetK* or *tetL* genes might be associated with a complementary mechanism of resistance against tetracycline.

The results have provided a preliminary analysis of MRSA CC398 strains comparative growth fitness when subjected to mixed culture competition assay. While the data suggests that the lineage is comparatively more fit than other analysed MRSA strains *in vitro*, further studies would need to be conducted to investigate if this is also true within an environment or a host. The MRSA CC398 lineage has not demonstrated a uniform capacity for enhanced tolerance of antimicrobial selective pressure. However, an apparent association between the degree of growth rate reduction and the underlying resistance genotype was observed. As such, it has revealed that majority of the MRSA CC398 strains have acquired resistance determinants that mediate a more efficient protection against the activity of the analysed antimicrobial compounds.

Chapter 8 General discussion and future work

Molecular epidemiological studies of *S. aureus* from humans have shown that the population consists of several prevalent lineages such as CC5, CC8, CC22 and CC30 that dominate as the hospital-acquired MRSA and CC1, CC59 and CC80 that are common among the community-acquired MRSA (Deurenberg and Stobberingh, 2008). The existence of dominant lineages among animal-associated isolates has also been identified and can be exemplified by strains causing mastitis in cattle, which are often represented by CC97, CC126, CC130 and CC151 (Rabello *et al.*, 2007; Monecke *et al.*, 2007b; Garcia-Alvarez *et al.*, 2011). *S. aureus* lineages associated with other animal species have also been described such as CC5 which is highly prevalent among poultry and CC8 that is commonly isolated from horses (Lowder *et al.*, 2009; Walther *et al.*, 2009). The MRSA CC398 emerged as primarily a pig-associated lineage as it has been found highly prevalent among pig herds in several countries in Europe and North America (de Neeling *et al.*, 2007; Khanna *et al.*, 2008; Harlizius *et al.*, 2008; Smith *et al.*, 2009). However, the lineage has been also isolated from other animal species such as cattle, poultry and horses (Van den Eede *et al.*, 2009; Persoons *et al.*, 2009; Fessler *et al.*, 2010). Furthermore, transfer of MRSA CC398 between animals and humans has been reported and the strain has been also isolated from persons with no history of livestock contact (Huijsdens *et al.*, 2006; Lewis *et al.*, 2008; Fanoy *et al.*, 2009).

The aim of work presented here was to investigate whether the MRSA CC398 strains demonstrate any unique genotypic or phenotypic features, when compared against a panel of *S. aureus* strains belonging to eight prevalent *S. aureus* clonal complexes, which would represent potential factors mediating the apparent pig-association. Molecular evolution towards development of host-specificity has been described for certain lineages of *S. aureus* (Herron-Olson *et al.*, 2007; Guinane *et al.*, 2010). However, the results of this work lead to a conclusion that the success of MRSA CC398 as a coloniser of swine and other livestock, might have been mediated by non-host related factors. Instead the high prevalence of MRSA CC398 amongst food-producing animals might be associated with adaptations towards the selective pressure of the antimicrobial use in livestock. This is evident in the acquisition by MRSA CC398 strains of a broad range of antimicrobial resistance determinants. It can thus be proposed that the success of MRSA CC398 has been largely facilitated by the horizontal transfer of resistance-associated MGEs, which is supported by the results presented in Chapter 6. However, the high frequency of antimicrobial resistance gene

carriage does not truly distinguish the MRSA CC398 strains from other lineages of MRSA, as demonstrated in Chapter 5 (Figure 5.1). Another important feature of MRSA CC398 identified in this work is the biological fitness advantage when competed in a non-selective medium against MRSA strains of other clonal complexes.

8.1 MRSA CC398 and host non-specificity

MRSA CC398 has been referred to as livestock-associated MRSA, with a particular predisposition to colonise swine (Verhegghe *et al.*, 2012). This has compelled the question of whether MRSA CC398 represents a host-adapted lineage and what molecular markers mediate the host specificity. The comparative analysis of virulence gene carriage described in this work revealed that the lineage does feature a unique genetic background different from both human and cattle-associated CCs (Chapter 4). Amongst the most distinctive features of MRSA CC398 strains analysed in this study has been the lack of MGE-associated virulence determinants (4.3.1.1). Apart from the absence of plasmid, bacteriophage or SaPI-associated genes, the allelic variant of the chromosomally intrinsic vSaβ also lacked a set of virulence elements such as *splA*, *splB* and *lukD/E* (4.3.1.3). It thus resembles the process of niche adaptation that in *S. aureus* has been associated with the loss of virulence determinants involved in human disease and has been previously observed in lineages such as the poultry-specific clade of ST5 and the bovine-specific CC133 (Lowder *et al.*, 2009; Guinane *et al.*, 2010). It has now been demonstrated by Price *et al.* (2012) that the LA-MRSA CC398 has indeed originated from a human-associated MSSA and thus the lack of a number of significant virulence genes might have resulted from their progressive loss.

The work presented here did not identify amongst MRSA CC398 strains any unique molecular marker that could represent putative determinants of host specificity. A proportion of strains demonstrated the presence of an intact *hlb* gene, a feature that has been previously recognised as a common property of animal isolates of *S. aureus* (Monecke *et al.*, 2007b). However it was not applicable to all analysed MRSA CC398 strains (4.3.1.3). An earlier study involving a whole genome analysis of a MRSA CC398 strain identified two novel putative virulence determinants (Schijffelen *et al.*, 2010). The elements were located within a novel pathogenicity island, designated SaPI5, and represented homologues of *scn* and *vwb* genes encoding staphylococcal complement inhibitor and von Willebrand factor-binding protein,

respectively (Schijffelen *et al.*, 2010). The vWbp has been implicated in host specificity of ruminant-associated *S. aureus* strains, but the function has not been determined for a porcine host (Guinane *et al.*, 2010). Also, screening for the carriage of SaPI5 amongst a wider panel of MRSA CC398 revealed that it is not present in all isolates (Schijffelen *et al.*, 2010). Finally, a recent study on MGE distribution amongst MRSA CC398 isolates from pigs and humans reported that the SaPI5 was prevalent, but not associated with pig strains and thus is unlikely to mediate pig specificity (McCarthy *et al.*, 2011).

The process of *S. aureus* lineage diversification towards adaptation to an animal host has been described to involve in *S. aureus* gene decay of various adhesin determinants (Herron-Olson *et al.*, 2007). The genotypic analysis presented in this work revealed that the MRSA CC398 strains carry a number of surface protein genes (4.3.1.4). However, further characterisation involving sequence analysis of the entire coding sequence would be essential for a comprehensive investigation of variation in adhesin determinants between MRSA CC398 and other analysed lineages. Evidence of gene decay in various surface proteins was already reported by a recent study, which compared genetic features of a LA-MRSA CC398 with a human-related MSSA CC398 strain (Uhlemann *et al.*, 2012). The latter has been associated with high rates of human-to-human transmission and demonstrated a distinct composition of adhesin genes as well as significantly higher rates of adhesion to human keratinocytes (Uhlemann *et al.*, 2012). In the work presented here, a cell culture-based *in vitro* adhesion assay revealed that MRSA CC398 strains lacked an enhanced capacity for attachment to porcine keratinocytes in comparison with other lineages (4.3.4). This might have resulted from a loss of function amongst certain surface protein genes, as described in the study by Uhlemann *et al.* (2012).

Although LA-MRSA CC398 has been referred to as pig-specific strain there is still insufficient evidence to confirm that it has indeed become adapted to specifically colonise the porcine host. In an earlier study on virulence and antimicrobial resistance determinants amongst MRSA CC398 isolates from pigs, authors proposed that the broad host tropism is indicative of a limited host specificity of this *S. aureus* lineage (Kadlec *et al.*, 2009). Furthermore, it has been classified as an extended-host-spectrum genotype (EHSG) due to an apparent ease to transfer between different host species (Walther *et al.*, 2009).

8.2 MRSA CC398 success mediated by non-host specific adaptations

The significance of MRSA CC398 as a novel prevalent lineage relates to the livestock-association combined with the carriage of the *mecA* gene. Analysis of each of these aspects individually, reveals that only the latter can be correlated with other defining or verifying features. A comparison of CC398 with MRSA strains of other lineages described in this work, demonstrated a homologous clustering based on antimicrobial resistance genotypes (Chapter 5). As such, MRSA CC398 strains were found to resemble other *mecA*-positive strains in the type and frequency of antimicrobial resistance gene carriage. It has been accepted that emergence of multiple drug resistant bacteria, such as the HA-MRSA, can be correlated with the use of antimicrobial agents and the selective pressure within the healthcare environment (Hawkey, 2008). This has been further confirmed by the observation that CA-MRSA isolates typically demonstrate susceptibility to non-β-lactam agents and have been categorised as non-multi-drug-resistant MRSA (Hiramatsu *et al.*, 2002; Okuma *et al.*, 2002).

In addition to human healthcare settings, antimicrobial agents are also used for the prophylaxis and control of bacterial infections in food-producing animals (Schwarz and Chaslus-Dancla, 2001). Tetracycline and broad-spectrum cephalosporins have been specifically implicated in the selection of MRSA CC398 (Price *et al.*, 2012). However, it has also been observed that as a livestock coloniser, MRSA CC398 is under a selective pressure of a wider range of antimicrobials, many of which are not applied as means of controlling staphylococcal infections (Fessler, Kadlec and Schwarz, 2011). This is evident in not only a multiple drug resistance phenotype, but also carriage of novel determinants of resistance, as demonstrated by the work presented in this thesis (Chapter 6). Based on the current findings it appears that the success of MRSA CC398 as a livestock coloniser has been thus far mediated by the adaptations to the external environment rather than development of host-specificity. This can be further supported by studies that demonstrated a positive correlation between antimicrobial use and MRSA CC398 colonisation of livestock as well as transmission rates at a farm level (Graveland *et al.*, 2010; Broens *et al.*, 2012). An *in vivo* pig colonisation model has also shown that supplementing feed with tetracycline or zinc increases MRSA CC398 nasal colonisation (Moodley, Nielsen and Guardabassi, 2011).

8.3 Significance of MGEs for MRSA CC398 lineage

The absence of MGE-associated virulence genes amongst MRSA CC398 strains can not be associated with a limited capacity for horizontal gene transfer as demonstrated by the broad diversity of acquired resistance determinants (Chapter 5). It has been previously observed that the main limiting factor of microarray-based analysis is the heterogeneity of the platform, with probes designed mostly using gene sequences derived from genomes of human isolates (Sung, Lloyd and Lindsay, 2008). As such, screening for animal specific genetic determinants carried on MGEs, would first require a broad identification of such elements within the genetic pool of animal *S. aureus*. The sequence analysis of plasmids from MRSA CC398 strains that was described in this work has revealed a unique structural organisation as well as presence of novel genetic determinants (Chapter 6). Since sequence analysis of larger plasmids was limited to the identified resistance region, it can be presumed that complete sequencing would reveal further heterogeneity. Also, not all isolated plasmids were associated with the carriage of resistance determinants and consequently those were excluded from the sequence analysis.

The molecular population structure studies have shown that *S. aureus* clonal diversification occurs mostly through point mutations (Feil *et al.*, 2003). The whole genome comparative analyses of human and animal strains further revealed that genetic divergence of intrinsic chromosomal genes in the animal-associated lineages has mostly involved loss of function (Herron-Olson *et al.*, 2007; Guinane *et al.*, 2010). In contrast, the expression of animal-specific virulence factors has been predominantly associated with carriage of novel MGEs (Fitzgerald *et al.*, 2001a; Guinane *et al.*, 2010). Furthermore, it has been previously proposed that MGEs might constitute the main determinants of variation between human and animal strains (Sung, Lloyd and Lindsay, 2008). Although MGEs of MRSA CC398 strains have been mostly vectors of antimicrobial resistance, their diversity and high prevalence suggests an important role in the prevalence of this lineage. Also, while the analysis of MGEs amongst MRSA CC398 strains was focused in this work predominantly on plasmids, preliminary data confirming carriage of other elements, such as transposons and prophages has also been presented (Chapters 4 and 6).

In the light of the above-described findings, it would be worthwhile to suggest that the future investigations of MRSA CC398 strains should be focused on the

identification and description of carried MGEs. Although screening for prevalence of mobile determinants that have been previously associated with MRSA CC398 isolates allows monitoring the epidemiology of resistance in this lineage, it gives an incomplete overview of the true diversity of its MGEs. As demonstrated by other studies as well as findings that are presented in this work (Chapter 6), MRSA CC398 strains have a considerable capacity for acquisition of novel determinants of resistance (Kadlec and Schwarz, 2009a; Kadlec and Schwarz, 2009b; Schwendener and Perreten, 2011). This might eventually be replaced or accompanied by transfer of novel virulence determinants as persistent carriage of MRSA CC398 by swine and other livestock animals might induce more prominent host adaptation. Such a scenario can be supported by recent reports that MRSA CC398 isolated from humans is demonstrating an enhanced virulence phenotype and genotype such as carriage of the PVL genes (Welinder-Olsson *et al.*, 2008; Schmidt *et al.*, 2013). Carriage of immune evasion cluster (IEC) genes has also been recently reported for MRSA CC398 isolates from calves (Haenni *et al.*, 2011). Also, MGE-mediated diversification of MRSA CC398 has been demonstrated for human-derived isolates carrying a number of phage-related virulence determinants such as *pvl*, *chp*, *scn* and *sak* (Stegger *et al.*, 2010).

8.4 Enhanced biological fitness phenotype of MRSA CC398

The conclusion that the dissemination of MRSA CC398 amongst livestock has been mediated primarily or solely by the acquisition of multiple resistance determinants still does not resolve the matter of why this particular genotype has become the prevalent LA-MRSA. Despite the early emergence of MRSA, its global dissemination and lineage heterogeneity, reports of its isolation from food producing animals have been uncommon until less then a decade ago (Leonard and Markey, 2008). This might reflect the changes in the livectok microbiota surveillance practices, and represent a consequence of a recent increase in the frequency of studies on MRSA carriage among animals. However, it might also suggest that a specific genotypic background is vital for stable colonisation of livestock and persistence within its environment. This notion can be further supported by the presence of another lineage of multiple drug resistant MRSA clone in animals, namely the equine MRSA CC8 (Walther *et al.*, 2009). The first reported isolation of MRSA from an equine patient was in 1996 in Japan and the PFGE typing revealed that the isolates were distinct

from the predominant human-associated MRSA (Anzai *et al.*, 1996; Shimizu *et al.*, 1997). Later, the equine MRSA isolates in Europe were identified as CC8 and reported to demonstrate multiple drug resistance (O'Mahony *et al.*, 2005; Cuny *et al.*, 2006). However, the MRSA CC8 has not spread amongst food-producing animals and thus it is not recognised as a LA-MRSA. Furthermore, there is evidence that MRSA CC398 might be replacing MRSA CC8 lineage in an equine clinic setting. A recent study has reportted an increase in the frequency of MRSA CC398 isolation from clinical equine samples at a Dutch Veterinary Microbiological Diagnostic Centre, with the prevalence exceeding that of MRSA CC8 isolates (van Duijkeren *et al.*, 2010). More recently MRSA CC398 has been also reported to replace a previously dominant strain of borderline-oxacillin-resistant *S. aureus* ST1 as a causative agent of equine infections and an equine clinic personnel coloniser (Sieber *et al.*, 2011).

A single most significant feature of MRSA CC398 strains identified by this work that might have contributed to its success as a livestock coloniser is the comparative biological fitness advantage (Chapter 7). A recent study has reported that a significant fitness cost associated with multiple drug resistance has most likely contributed to the decline of a dominant MRSA clone that was epidemic during 1965-75 (Nielsen *et al.*, 2012). This shows that carriage and expression of antimicrobial resistance can exert a considerable burden on *S. aureus* to a degree that will influence the long-term persistence of a strain. However, it has also been observed that bacteria can adapt to the stress of antimicrobial resistance (Spratt, 1996). Furthermore, such adaptations might also involve selection of a clone that suffers the lowest level of fitness cost (Sander *et al.*, 2002). As demonstrated by the molecular epidemiology of MRSA, a dominant lineage will often subside and be followed by an emergence of a novel epidemic clone (Deurenberg and Stobberingh, 2008). MRSA CC398 might thus represent a particularly fit MRSA clone that has developed a high level of adaptation to the burden of carriage and expression of high number of resistance genes. This might constitute an essential property in the ability to stably colonise food-producing animals due to the broad use of antimicrobial compounds in livestock farming.

8.5 Future work

The analysis of adhesin gene carriage revealed that MRSA CC398 strains carry a number of MSCRAMM determinants and the *in vitro* keratinocyte adhesion assay demonstrated a level of capacity for binding to host skin cells. To further verify

the potential of MRSA CC398 strains for adhesion to host proteins, an *in vitro* binding assay could be conducted using individual substrates such as fibrinogen or fibronectin. This would allow determination as to whether the carriage of an adhesin gene is associated with a phenotypic function of the expressed MSCRAMM. Such work could be further supplemented with a sequence analysis of the investigated adhesin genes, which would reveal any mutational changes such as premature stop codons and could thus be correlated with the observed adhesion phenotype.

Further work would be required to confirm the findings of the mixed culture competition assay as the investigation was conducted with only a small number of representative strains. Verification of the results by a more comprehensive analysis would provide grounds to study the mechanisms of MRSA CC398 enhanced biological fitness. One possible factor can be already proposed and that is the observed low content of virulence determinants. If the antimicrobial resistance confers a fitness cost, a similar correlation can be expected for the carriage of MGE-associated virulence genes. It has already been observed that the biological fitness cost of virulence-associated determinants is one of key factors that influence the frequency of virulence-gene carriage (Peacock *et al.*, 2002). Thus the low prevalence of virulence genes amongst MRSA CC398 strains might mediate a selective advantage of comparatively higher biological fitness. This could be investigated by conducting the growth competition experiment using MRSA CC398 that has acquired phage-related IEC cluster or PVL. Alternatively, the virulence determinants can be introduced into a naive MRSA CC398 strain by bacteriophage transduction, which would allow direct assessment of virulence gene carriage influence by comparing phenotypic features of the wild type and the transduced isogenic strain.

While the work presented in this thesis did not involve animal swabbing for isolate collection, future work aimed at the characterisation of animal-associated strains could be accompanied by active skin microflora sampling and analysis. Thus far, the analysis of livestock-associated *S. aureus* has been focused mostly on isolates derived from cattle, which allowed identification of the prevalent *S. aureus* lineages together with characterisation of their genotypic features (Smith *et al.*, 2005; Rabello *et al.*, 2007; Monecke *et al.*, 2007b). However, little information is still available on the skin microflora of swine since the surveillance studies have been largely focused on identification of MRSA isolates only (de Neeling *et al.*, 2007; Khanna *et al.*, 2008; Harlizius *et al.*, 2008). Although isolation of MSSA CC398 from swine was

previously reported, only one study described it as a common colonising lineage of *S. aureus* in pigs (Armand-Lefevre, Ruimy and Andremont, 2005; Guardabassi, Stegger and Skov, 2007; Hasman *et al.*, 2010a). Knowledge of MSSA CC398 swine colonisation frequency would add valuable information to the deliberations on whether the CC398 lineage is truly host-adapted or whether the high prevalence has been mostly associated with the acquisition of the SCC*mec* and other resistance determinants. However, the analysis of swine skin microflora could be expanded beyond surveillance to identify MSSA CC398 and involve a broader aim of determining what methicillin-susceptible *S. aureus* lineages are associated with swine colonisation. Such lineages could be further investigated and methods such as whole genome sequencing could be applied to determine whether *S. aureus* colonisation of pigs is mediated by host-specific virulence determinants.

To conclude, the work presented here did not provide any evidence in support of the hypothesis that the high prevalence of MRSA CC398 lineage amongst livestock has been mediated by the development of host specificity. Instead, the results suggest that the success of MRSA CC398 as a livestock coloniser might have been associated with adaptation towards the high selective pressure of antimicrobial use in food-producing animals. Furthermore, the MRSA CC398 strains were found to demonstrate a superior biological fitness when competed against representatives of other successful MRSA lineages, which might have also contributed to the emergence and dissemination of MRSA CC398 as a livestock-associated lineage although further work would be required to confirm this.

In retrospect, it can be also concluded that the DNA array methodology applied here for the analysis of virulnence and resistance gene carriage was a limiting factor in identification of significant genetic traits amongst characterised strains, in particular the MRSA CC398 panel. As presented in chapter 6, the sequencing of the fragments or complete mobile genetic elements derived from MRSA CC398 strains, revealed presence of novel coding sequences, which wouldn't be otherwise identified. Considering that amongst the work aims was identification of MRSA CC398-unique genotypic features, it can be decided that the whole genome sequencing of the analysed bacterial strains would likely be a more suitable approach.

The specific scientific contributions of the thesis presented here include further expansion of the current knowledge of *S. aureus* inter-lineage diversity and variation,

identification of novel plasmid elements and novel plasmid-borne resistance determinants as well as a greater understanding of the MRSA CC398 biology in particular a comparative capacity for adhesion to porcine keratinocytes and comparative biological fitness.

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Appendices

Electronic Appendix I. Chapters 4 and 6 raw assembled sequence data in FASTA file format.

Appendix I. Table of all *S. aureus* isolates included in the preliminary analysis that was aimed at seletion of strains for further characteristion, as described in 2.2.1 and chapter 3.

No	ID	Host	MS/MR[a]	No	ID	Host	MS/MR
1	1	Human	MS	51	51	Human	MS
2	2	Human	MS	52	52	Human	MS
3	3	Human	MS	53	53	Human	MS
4	4	Human	MS	54	54	Human	MS
5	5	Human	MS	55	55	Human	MS
6	6	Human	MS	56	56	Human	MS
7	7	Human	MS	57	57	Human	MS
8	8	Human	MS	58	58	Human	MS
9	9	Human	MS	59	59	Human	MS
10	10	Human	MS	60	60	Human	MS
11	11	Human	MS	61	61	Human	MS
12	12	Human	MS	62	62	Human	MS
13	13	Human	MS	63	63	Human	MR
14	14	Human	MS	64	64	Human	MR
15	15	Human	MS	65	65	Human	MR
16	16	Human	MS	66	66	Human	MR
17	17	Human	MS	67	67	Human	MR
18	18	Human	MS	68	68	Human	MR
19	19	Human	MS	69	69	Human	MR
20	20	Human	MS	70	70	Human	MR
21	21	Human	MS	71	71	Human	MR
22	22	Human	MS	72	72	Human	MR
23	23	Human	MS	73	73	Human	MR
24	24	Human	MS	74	74	Human	MR
25	25	Human	MS	75	75	Human	MR
26	26	Human	MR	76	76	Human	MR
27	27	Human	MR	77	77	Human	MR
28	28	Human	MR	78	78	Human	MR
29	29	Human	MR	79	79	Human	MR
30	30	Human	MR	80	80	Human	MR
31	31	Human	MR	81	81	Human	MR
32	32	Human	MR	82	82	Human	MR
33	33	Human	MR	83	90	Chicken	MR
34	34	Human	MR	84	91	Chicken	MR
35	35	Human	MR	85	92	Chicken	MR
36	36	Human	MR	86	93	Chicken	MR
37	37	Human	MR	87	94	Chicken	MR
38	38	Human	MR	88	95	Human	MR
39	39	Human	MR	89	96	Human	MR
40	40	Human	MR	90	97	Human	MR
41	41	Human	MR	91	98	Human	MR
42	42	Human	MR	92	99	Horse	MR
43	43	Human	MR	93	100	Horse	MR
44	44	Human	MR	94	101	Horse	MR
45	45	Human	MR	95	102	Rat	MR
46	46	Human	MR	96	103	Rat	MR
47	47	Human	MR	97	104	Rat	MR
48	48	Human	MR	98	105	Bovine	MR
49	49	Human	MR	99	106	Bovine	MR
50	50	Human	MR	100	107	Bovine	MR

[a] MS – methicillin susceptible, MR – methicillin resistant

Appendix I (continued). Table of all *S. aureus* isolates included in the preliminary analysis that was aimed at seletion of strains for further characteristion, as described in 2.2.1 and chapter 3.

No	ID	Host	MS/MR[a]
101	108	Pig	MR
102	109	Pig	MR
103	110	Pig	MR
104	111	Pig	MR
105	112	Pig	MR
106	173	Cattle	MS
107	174	Cattle	MS
108	175	Cattle	MS
109	176	Cattle	MS
110	177	Cattle	MS
111	178	Cattle	MS
112	179	Cattle	MS
113	180	Cattle	MS
114	181	Cattle	MS
115	182	Cattle	MS
116	183	Cattle	MS
117	184	Cattle	MS
118	185	Cattle	MS
119	186	Cattle	MS
120	187	Cattle	MS
121	188	Cattle	MS
122	189	Cattle	MS
123	190	Cattle	MS
124	191	Cattle	MS
125	192	Cattle	MS
126	193	Cattle	MS
127	194	Cattle	MS
128	195	Cattle	MS
129	196	Cattle	MS
130	197	Cattle	MS
131	198	Cattle	MS
132	199	Cattle	MS
133	200	Equine	MR
134	201	Equine	MR

[a] MS – methicillin susceptible, MR – methicillin resistant

Appendix II. Table of all genotypic and phenotypic features of the selected *S. aureus* strains [a].

No	ID	Host	*spa*	ST	CC	SCC*mec*	*dru*	Resistance phenotype	Resistance genes	Virulence genes			
										Superantigens	Exotoxin-like proteins	Adhesins	Other
1	1	Human	t122	30	30	-	-	PEN	*blaZ, cadA, cadC*	*seX, seY, seG, seI, seM, seN, seO, seU*	*set1, set2, set3, set4, set5, set7, set8, set9, setB*	*bbp, clfA, clfB, cna, eno, ebpS, fib, fnbA, icaA, icaD, sdrC*	*lukF, lukS, hlgA, lukX, lukY, hl, hla, sak, hld, hl-III*
2	5	Human	t012	30	30	-	-	PEN, SPE, CLIi, ERY	*blaZ, spc, ermA, czrC*	*seX, seY, seA, seC, seL, seG, seI, seM, seN, seO, seU*	*set1, set2, set3, set4, set5, set7, set8, set9, setB*	*bbp, clfA, clfB, cna, eno, ebpS, fib, fnbA, icaA, icaD, sdrC*	*lukF, lukS, hlgA, lukX, lukY, hl, hla, sak, hld, hl-III*
3	6	Human	t008	8	8	-	-	PEN, TET	*blaZ, tetK, cadD*	*seX, seA*	*set1, set2, set3, set4, set5, set6, set7, set8, set9, set12, set21, setB, setC*	*clfA, clfB, eno, ebpS, fib, fnbA, fnbB, icaA, icaD, sasG, sdrC, sdrE*	*lukF, lukS, hlgA, lukD, lukE, lukX, lukY, hl, hla, sak, hld, hl-III, splA*
4	8	Human	t179	5	5	-	-	PEN	*blaZ, cadD*	*seX, seY, seD, seJ, seR, seG, seI, seM, seN, seO, seU*	*set1, set2, set3, set4, set5, set6, set7, set8, set9, set12, setB, setC*	*clfA, clfB, eno, ebpS, fib, fnbA, icaA, icaD, sasG, sdrD, sdrE*	*lukF, lukS, hlgA, lukD, lukE, lukX, lukY, hl, hla, sak, hld, hl-III, splA, splB*
5	10	Human	t084	15	15	-	-	PEN	*blaZ, cadD*		*set1, set2, set3, set4, set5, set6, set7, set8, set9, set12, set21, setB, setC*	*clfA, clfB, eno, ebpS, fib, fnbA, fnbB, icaA, icaD, sdrC, sdrD, sdrE*	*lukF, lukS, hlgA, lukD, lukE, lukX, lukY, hl, hla, hld, hl-III, splA, splB*
6	11	Human	t008	-	8	-	-	PEN	*blaZ, cadD*	*seX, seY*	*set1, set2, set3, set4, set5, set6, set7, set8, set9, set12, set21, setB, setC*	*clfA, clfB, eno, ebpS, fib, fnbA, fnbB, icaA, icaD, sasG, sdrC, sdrE*	*lukF, lukS, hlgA, lukF-PV-P83, lukD, lukE, lukX, lukY, hl, hla, hlb, hld, hl-III, splA*
7	13	Human	t012	-	30	-	-	-	*blaZ, cadA, cadC*	*seX, seY, seA, seG, seI, seM, seN, seO, seU, tst*	*set1, set2, set3, set4, set5, set7, set8, set9, setB*	*bbp, clfA, clfB, cna, eno, ebpS, fib, fnbA, icaA, icaD, sdrC*	*lukF, lukS, hlgA, lukX, lukY, hl, hla, sak, hld, hl-III*
8	16	Human	t008	-	8	-	-	PEN	*blaZ, cadD*	*seX, seY, seK, seQ, tst*	*set1, set2, set3, set4, set5, set6, set7, set8, set9, set12, set21, setB, setC*	*clfA, clfB, eno, ebpS, fib, fnbA, fnbB, icaA, icaD, sasG, sdrC, sdrD, sdrE*	*lukF, lukS, hlgA, lukF-PV-P83, lukD, lukE, lukX, lukY, hl, hla, sak, hld, hl-III, splA, splB*
9	17	Human	t442	5	5	-	-	PEN, CIP, TRI	*blaZ, dfrG, cadD*	*seX, seY, seD, seJ, seR, seG, seI, seM, seN, seO, seU*	*set1, set2, set3, set4, set6, set7, set8, set9, set12, setB, setC*	*clfA, clfB, eno, ebpS, fib, fnbA, icaA, icaD, sasG, sdrD, sdrE*	*lukF, lukS, hlgA, lukD, lukE, lukY, hl, hla, sak, hld, hl-III, splA, splB*
10	18	Human	t608	22	22	-	-	PEN. CIP, TRI	*blaZ*	*seX, seY, seB, seC, seL, seG, seI, seM, seN, seO, seU*	*set1, set3, set5, set6, set7, set8, set9, setC*	*clfA, clfB, cna, eno, ebpS, fnbA, icaA, icaD, sdrD, sdrE*	*lukF, lukS, hlgA, lukX, lukY, hl, hla, sak, hld*

[a] list of abbreviations is provided on page 288

Appendix II. Table of all genotypic and phenotypic features of the selected *S. aureus* strains [a].

No	ID	Host	*spa*	ST	CC	SCC*mec*	*dru*	Resistance phenotype	Resistance genes	Virulence genes			
										Superantigens	Exotoxin-like proteins	Adhesins	Other
11	19	Human	t442	-	5	-	-	PEN	*blaZ, cadD*	*seX, seY, seA, seD, seJ, seR, seG, seI, seM, seN, seO, seU*	*set1, set2, set3, set4, set5, set6, set7, set8, set9, set12, setB, setC*	*clfA, clfB, eno, ebpS, fib, fnbA, icaA, icaD, sasG, sdrD, sdrE*	*lukF, lukS, hlgA, lukF-PV-P83, lukD, lukE, lukX, lukY, hl, hla, sak, hld, hl-III, splA, splB*
12	20	Human	UN	22	22	-	-	PEN	*blaZ, cadA, cadC*	*seX, seY, seG, seI, seM, seN, seO, seU*	*set1, set3, set5, set6, set7, set8, set9, setC*	*clfA, clfB, cna, eno, ebpS, fnbA, icaA, icaD, sdrD*	*lukF, lukS, hlgA, lukX, lukY, hl, hla, sak, hld*
13	21	Human	t084	-	15	-	-	PEN	*blaZ, cadD*	*seX*	*set1, set2, set3, set4, set5, set6, set7, set8, set9, set12, set21, setB, setC*	*clfA, clfB, eno, ebpS, fib, fnbA, fnbB, icaA, icaD, sdrC, sdrD, sdrE*	*lukF, lukS, hlgA, lukD, lukE, lukX, lukY, hl, hla, hld, hl-III, splA, splB*
14	23	Human	t228	15	15	-	-	PEN	*blaZ, cadD*	*seX, seY*	*set1, set2, set3, set4, set5, set6, set7, set8, set9, set12, set21, setB, setC*	*clfA, clfB, eno, ebpS, fib, fnbA, fnbB, icaA, icaD, sdrC, sdrD, sdrE*	*lukF, lukS, hlgA, lukD, lukE, lukX, lukY, hl, hla, hld, hl-III, splA, splB*
15	24	Human	t491	15	15	-	-	PEN	*blaZ, cadD*	*seX*	*set1, set2, set3, set4, set5, set6, set7, set8, set9, set12, set21, setB, setC*	*clfA, clfB, eno, ebpS, fib, fnbA, fnbB, icaA, icaD, sdrC, sdrD*	*lukF, lukS, hlgA, lukD, lukX, lukY, hl, hla, hld, hl-III, splA, splB*
16	25	Human	t002	5	5	-	-	-	-	*seX, seY, seB, seG, seI, seM, seN, seO, seU*	*set1, set2, set3, set4, set5, set6, set7, set8, set9, set12, setB, setC*	*clfA, clfB, eno, ebpS, fib, fnbA, icaA, icaD, sasG, sdrD, sdrE*	*lukF, lukS, hlgA, lukD, lukE, lukX, lukY, hl, hla, sak, hld, hl-III, splA, splB*
17	28	Human	t032	-	22	IVh	dt10a	PEN, CEF, GEN, KAN, CLIi, ERY, CIP	*blaZ, mecA, aacA-aphD, mupR*	*seX, seY, seB, seC, seL, seG, seI, seM, seN, seO, seU, tst*	*set1, set3, set4, set5, set6, set7, set8, set9, setC*	*clfA, clfB, cna, eno, ebpS, fnbA, icaA, icaD, sdrD, sdrE*	*lukF, lukS, hlgA, lukX, lukY, hl, hla, hlb, hld*
18	31	Human	t1675	36	30	IV	dt9a	PEN, CEF, GEN, KAN, SPE, CLIi, ERY, CIP	*blaZ, mecA, aacA-aphD, aadD, spc, ermA, cadA, cadC*	*seX, seY, seA, seG, seI, seM, seN, seO, seU, tst*	*set1, set2, set3, set4, set5, set7, set8, set9, setB*	*bbp, clfA, clfB, cna, eno, ebpS, fib, fnbA, icaA, icaD, sdrC*	*lukF, lukS, hlgA, lukX, lukY, hl, hla, sak, hld, hl-III*
19	32	Human	t032	-	22	IVh	UN	PEN, CEF, CLIi, ERY, CIP	*blaZ, mecA, ermC, cadA, cadC*	*seX, seY, seB, seC, seL, seG, seI, seM, seN, seO, seU*	*set1, set3, set5, set6, set7, set8, set9, setC*	*clfA, clfB, cna, eno, ebpS, fnbA, icaA, icaD, sdrD, sdrE*	*lukF, lukS, hlgA, lukX, lukY, hl, hla, sak, hld*

[a] list of abbreviations is provided on page 288

Appendix II. Table of all genotypic and phenotypic features of the selected *S. aureus* strains [a].

No	ID	Host	*spa*	ST	CC	SCC*mec*	*dru*	Resistance phenotype	Resistance genes	Virulence genes			
										Superantigens	Exotoxin-like proteins	Adhesins	Other
20	34	Human	t018	36	30	IVh	dt7c	PEN, CEF, KAN, SPE, CLI, ERY, TYL, CIP, TRI	*blaZ, mecA, aadD, spc, ermA, cadA, cadC*	*seX, seY, seA, seG, seI, seM, seN, seO, seU, tst*	*set1, set2, set3, set4, set5, set7, set8, set9, setB*	*bbp, clfA, clfB, cna, eno, ebpS, fib, fnbA, icaA, icaD, sdrC*	*lukF, lukS, hlgA, lukX, lukY, hl, hla, sak, hld, hl-III*
21	38	Human	t020	22	22	IVh	dt10a	PEN, CEF, CLIi, ERY, CIP, TRI	*blaZ, mecA, ermC*	*seX, seY, seG, seI, seM, seN, seO, seU, tst*	*set1, set3, set4, set5, set6, set7, set8, set9, setC*	*clfA, clfB, cna, eno, ebpS, fnbA, icaA, icaD, sdrD, sdrE*	*lukF, lukS, hlgA, lukX, lukY, hl, hla, sak, hld*
22	39	Human	t379	22	22	IVh	dt10a	PEN, CEF, CLIi, ERY, CIP	*blaZ, mecA, ermC, cadA, cadC*	*seX, seY, seG, seI, seM, seN, seO, seU*	*set1, set3, set4, set5, set6, set7, set8, set9, setC*	*clfA, clfB, cna, eno, ebpS, fnbA, icaA, icaD, sdrD, sdrE*	*lukF, lukS, hlgA, lukX, lukY, hl, hla, sak, hld*
23	40	Human	t032	-	22	IVh	dt10a	PEN, CEF, CLIi, ERY, CIP	*blaZ, mecA, ermC*	*seX, seY, seB, seC, seL, seG, seI, seM, seN, seO, seU*	*set1, set3, set4, set5, set6, set7, set8, set9, setC*	*clfA, clfB, cna, eno, ebpS, fnbA, icaA, icaD, sdrD, sdrE*	*lukF, lukS, hlgA, lukX, lukY, hl, hla, sak, hld*
24	44	Human	t032	22	22	IVh	dt10a	PEN, CEF, CIP	*blaZ, mecA*	*seX, seY, seB, seC, seL, seG, seI, seM, seN, seO, seU*	*set1, set3, set5, set6, set7, set8, set9, setC*	*clfA, clfB, cna, eno, ebpS, fnbA, icaA, icaD, sdrD, sdrE*	*lukF, lukS, hlgA, lukX, lukY, hl, hla, sak, hld*
25	51	Human	t019	30	30	-	-	PEN	*blaZ, cadD*	*seX, seY, seG, seI, seM, seN, seO, seU*	*set1, set2, set3, set4, set5, set7, set8, set9, setB*	*bbp, clfA, clfB, cna, eno, ebpS, fib, fnbA, icaA, icaD, sdrC*	*lukF, lukS, hlgA, lukF-PV, lukS-PV, lukX, lukY, hl, hla, sak, hld, hl-III*
26	60	Human	t360	15	15	-	-	PEN, TET	*blaZ, tetK, cadD*	*seX, seY*	*set1, set2, set3, set4, set5, set6, set7, set8, set9, set12, set21, setB, setC*	*clfA, clfB, eno, ebpS, fib, fnbA, fnbB, icaA, icaD, sdrC, sdrD, sdrE*	*lukF, lukS, hlgA, lukF-PV, lukS-PV, lukF-PV-P83, lukD, lukE, lukY, hl, hla, hld, hl-III, splA, splB*
27	62	Human	t021	30	30	-	-	PEN, TET	*blaZ, tetK, cadD*	*seX, seY, seG, seI, seM, seN, seO, seU*	*set1, set2, set3, set4, set5, set7, set8, set9, setB*	*bbp, clfA, clfB, cna, eno, ebpS, fib, fnbA, icaA, icaD, sdrC*	*lukF, lukS, hlgA, lukF-PV, lukS-PV, lukX, lukY, hl, hla, sak, hld, hl-III*

[a] list of abbreviations is provided on page 288

Appendix II. Table of all genotypic and phenotypic features of the selected *S. aureus* strains [a].

No	ID	Host	*spa*	ST	CC	SCC*mec*	*dru*	Resistance phenotype	Resistance genes	Virulence genes			
										Superantigens	Exotoxin-like proteins	Adhesins	Other
28	65	Human	t018	-	30	IV	dt7c	PEN, CEF, GEN, KAN, SPE, CLI, ERY, TYL, CIP, TRI	*blaZ, mecA, aacA-aphD, aadD, spc, ermA, ermC, dfrA, mupR, cadA, cadC*	*seX, seY, seA, seG, seI, seM, seN, seO, seU, tst*	*set1, set2, set3, set4, set5, set7, set8, set9, setB*	*bbp, clfA, clfB, cna, eno, ebpS, fib, fnbA, icaA, icaD, sdrC*	*lukF, lukS, hlgA, lukX, lukY, hl, hla, sak, hld, hl-III*
29	90	Chicken	t011	398	398	IVc	dt10a	PEN, CEF, GEN, KAN, CLI, ERY, TYL, TET, TRI	*blaZ, mecA, aacA-aphD, aadD, ermT, tetL, tetM, dfrA*	*seX, seY*	*set1, set2, set3, set4, set5, set6, set7, set9, setB, setC*	*clfA, clfB, cna, ebpS, fib, fnbA, fnbB, icaA, icaD, sdrC, sdrE*	*lukF, lukS, hlgA, lukX, lukY, hl, hla, hlb, hld, hl-III*
30	91	Chicken	t011	-	398	V 5C2&5	dt9v	PEN, CEF, SPE, CLI ERY, TYL, CHL, FLO, TET	*blaZ, mecA, ermC, tetK, tetM, fexA, sat, czrC*	*seX, seY*	*set1, set2, set3, set4, set5, set6, set7, set9, setB, setC*	*clfA, clfB, cna, ebpS, fib, fnbA, fnbB, icaA, icaD, sdrC, sdrE*	*lukF, lukS, hlgA, lukY, hl, hla, hlb, hld, hl-III*
31	92	Chicken	t011	-	398	IVa	dt10q	PEN, CEF, GEN, KAN, SPE, CLI, ERY, TYL, TET, TRI	*blaZ, mecA, aacA-aphD, ermC, tetM, dfrK*	*seX, seY*	*set1, set3, set4, set5, set6, set7, set9, setB, setC*	*clfA, clfB, cna, ebpS, fib, fnbA, fnbB, icaA, icaD, sdrC, sdrE*	*lukF, lukS, hlgA, lukY, hl, hla, hld, hl-III*
32	93	Chicken	t567	398	398	NT	dt11af	PEN, CEF, SPE, CLI, ERY, TYL , CHL, FLO, TET	*blaZ, mecA, ermC, tetK, tetM, fexA*	*seX*	*set1, set2, set3, set4, set5, set6, set7, set9, setB, setC*	*clfA, clfB, cna, ebpS, fib, fnbA, fnbB, icaA, icaD, sdrC, sdrE*	*lukF, lukS, hlgA, lukX, lukY, hl, hla, hld, hl-III*

[a] list of abbreviations is provided on page 288

Appendix II. Table of all genotypic and phenotypic features of the selected *S. aureus* strains [a].

No	ID	Host	*spa*	ST[b]	CC[c]	SCC*mec*[e]	*dru*[f]	Resistance phenotype	Resistance genes	Virulence genes			
										Superantigens	Exotoxin-like proteins	Adhesins	Other
33	95	Human	t034	398	398	V 5C2&5	dt11a	PEM, CEF, SPE, CLI, ERY, TYL, CHL, FLO, CIP, TET, TIA, TRI	*blaZ, mecA, ermC, tetK, tetM, dfrG, fexA, czrC*	*seX, seY*	*set1, set2, set3, set4, set5, set6, set7, set9, setB, setC*	*clfA, clfB, cna, ebpS, fib, fnbA, fnbB, icaA, icaD, sdrC*	*lukF, lukS, hlgA, lukY, hl, hla, hld, hl-III*
34	96	Human	t034	-	398	IVc	dt10a	PEN, CEF, KAN, CLI, ERY, TYL, TET, TRI	*blaZ, mecA, aadD, ermC, tetM, dfrA, vgaA, sat*	*seX, seY*	*set1, set2, set3, set4, set5, set6, set7, set9, setB, setC*	*clfA, clfB, cna, ebpS, fib, fnbA, fnbB, icaA, icaD, sdrC, sdrE*	*lukF, lukS, hlgA, lukY, hl, hla, hlb, hld, hl-III*
35	99	Horse	t011	-	398	IVa	dt10q	PEN, CEF, GEN, KAN, SPE, TET, TRI	*blaZ, mecA, aacA-aphD, tetM, dfrK*	*seX, seY*	*set1, set2, set3, set4, set5, set6, set7, set9, setB, setC*	*clfA, clfB, cna, ebpS, fib, fnbA, fnbB, icaA, icaD, sdrC, sdrE*	*lukF, lukS, hlgA, lukX, lukY, hl, hla, hlb, hld, hl-III*
36	101	Horse	t1451	398	398	IVa	UN	PEN, CEF, GEN, KAN, TET, TRI	*blaZ, mecA, aacA-aphD, aadD, ermT, tetL, tetM, dfrK*	*seX, seY*	*set1, set2, set3, set4, set5, set6, set7, set9, setB, setC*	*clfA, clfB, cna, ebpS, fib, fnbA, fnbB, icaA, icaD, sdrC, sdrE*	*lukF, lukS, hlgA, lukY, hl, hla, hlb, hld, hl-III*
37	102	Rat	t011	-	398	IVa	dt10q	PEN, CEF, GEN, KAN, SPE, CLI, ERY, TYL, TET, TRI	*blaZ, mecA, aacA-aphD, ermC, tetM, dfrK*	*seX, seY*	*set1, set2, set3, set4, set5, set6, set7, set9, setB, setC*	*clfA, clfB, cna, ebpS, fib, fnbA, fnbB, icaA, icaD, sdrC, sdrE*	*lukF, lukS, hlgA, lukY, hl, hla, hlb, hld, hl-III*
38	103	Rat	t011	-	398	V 5C2&5	dt11a	PEN, CEF, CLI, ERY, TYL, CIP, TET, TRI	*blaZ, mecA, ermC, tetK, tetM, dfrK, sat, czrC*	*seX, seY*	*set1, set2, set3, set4, set5, set6, set7, set9, setB, setC*	*clfA, clfB, cna, ebpS, fib, fnbA, fnbB, icaA, icaD, sdrC, sdrE*	*lukF, lukS, hlgA, lukY, hl, hla, hlb, hld, hl-III*

[a] list of abbreviations is provided on page 288

Appendix II. Table of all genotypic and phenotypic features of the selected *S. aureus* strains [a].

No	ID	Host	*spa*	ST	CC	SCC*mec*	*dru*	Resistance phenotype	Resistance genes	Virulence genes			
										Superantigens	Exotoxin-like proteins	Adhesins	Other
39	104	Rat	t4872	398	398	V 5C2&5	dt11v	PEN, CEF, GEN, KAN, SPE, CLI, ERY, TYL, TET, TIA	*blaZ, mecA, aacA-aphD, aadD, spc, ermA, ermT, tetL, tetM, vgaE*	*seX, seY*	*set1, set2, set3, set4, set5, set6, set7, set9, setB, setC*	*clfA, clfB, cna, ebpS, fib, fnbA, fnbB, icaA, icaD, sdrC, sdrE*	*lukF, lukS, hlgA, lukY, hl, hla, hlb, hld, hl-III*
40	105	Cattle	t011	-	398	IVa	dt10q	PEN, CEF, GEN, KAN, CLI, ERY, TYL, TET, TRI	*blaZ, mecA, aacA-aphD, ermC, ermT, tetL, tetM, dfrK, sat*	*seX, seY*	*set1, set3, set4, set5, set6, set7, set9, setB, setC*	*clfA, clfB, cna, ebpS, fib, fnbA, fnbB, icaA, icaD, sdrC, sdrE*	*lukF, lukS, hlgA, lukY, hl, hla, hld, hl-III*
41	106	Cattle	t011	-	398	V 5C2&5	dt11y	PEN, CEF, CLI, CIP, TET, TIA, TRI	*blaZ, mecA, tetK, tetM, dfrK, vgaA, czrC*	*seX, seY*	*set1, set2, set3, set4, set5, set6, set7, set9, setB, setC*	*clfA, clfB, cna, ebpS, fib, fnbA, fnbB, icaA, icaD, sdrC, sdrE*	*lukF, lukS, hlgA, lukX, lukY, hl, hla, hlb, hld, hl-III*
42	107	Cattle	t567	-	398	NT	dt11af	PEN, CEF, SPE, TET	*blaZ, mecA, tetM*	*seX*	*set1, set2, set3, set4, set5, set6, set7, set9, setB, setC*	*clfA, clfB, cna, ebpS, fib, fnbA, fnbB, icaA, icaD, sdrC, sdrE*	*lukF, lukS, hlgA, lukY, hl, hla, hld, hl-III*
43	108	Pig	t011	-	398	V 5C2&5	dt11a	PEN, CEF, CLI, ERY, TYL, TET, TRI	*blaZ, mecA, ermC, tetK, tetM, dfrK, czrC*	*seX, seY*	*set1, set2, set3, set4, set5, set6, set7, set9, setB, setC*	*clfA, clfB, cna, ebpS, fib, fnbA, fnbB, icaA, icaD, sdrC, sdrE*	*lukF, lukS, hlgA, lukX, lukY, hl, hla, hlb, hld, hl-III*
44	109	Pig	t011	-	398	IVa	dt10q	PEN, CEF, GEN, KAN, SPE, CLI, ERY, TYL, TET, TRI	*blaZ, mecA, aacA-aphD, ermC, tetM, dfrK*	*seX, seY*	*set1, set3, set4, set6, set7, set9, setB, setC*	*clfA, clfB, cna, ebpS, fib, fnbA, fnbB, icaA, icaD, sdrC, sdrE*	*lukF, lukS, hlgA, lukY, hl, hla, hld, hl-III*
45	110	Pig	t567	-	398	NT	dt11af	PEN, CEF, SPE, TET	*blaZ, mecA, tetM*	*seX, seY*	*set1, set2, set3, set4, set5, set6, set7, set9, setB, setC*	*clfA, clfB, cna, ebpS, fib, fnbA, fnbB, icaA, icaD, sdrC, sdrE*	*lukF, lukS, hlgA, lukX, lukY, hl, hla, hld, hl-III*

[a] list of abbreviations is provided on page 288

Appendix II. Table of all genotypic and phenotypic features of the selected *S. aureus* strains [a].

No	ID	Host	*spa*	ST	CC	SCC*mec*	*dru*	Resistance phenotype	Resistance genes	Virulence genes			
										Superantigens	Exotoxin-like proteins	Adhesins	Other
46	111	Pig	t034	-	398	IVa	dt10q	PEN, CEF, GEN, KAN, SPE, CLI, ERY, TET, TIA, TRI	*blaZ, mecA, aacA-aphD, spc, ermA, tetM, dfrK, vgaE*	*seX*	*set1, set2, set3, set4, set5, set6, set7, set9, setB, setC*	*clfA, clfB, cna, ebpS, fib, fnbA, fnbB, icaA, icaD, sdrC, sdrE*	*lukF, lukS, hlgA, lukX, lukY, hl, hla, hlb, hld, hl-III*
47	173	Cattle	t6220	130	130	-	-	PEN, CEF	*mecC*	*seX, seY*	*set1, set4, set5, set6, set7, set8, set9, set12, setB, setC*	*clfB, eno, ebpS, fib, fnbA, icaA, icaD, sdrC, sdrE*	*lukF, lukS, hlgA, lukF-PV-P83, lukD, lukX, lukY, hl, hla, hlb, hld, splA, splB, edinB*
48	174	Cattle	t131	1527	97	-	-	PEN	*blaZ*	*seX, seY*	*set1, set3, set4, set5, set7, set9, set12, setB, setC*	*clfA, clfB, eno, ebpS, fib, fnbA, fnbB, icaA, icaD, sasG, sdrC, sdrD, sdrE*	*lukF, lukS, hlgA, lukD, lukE, lukX, lukY, hl, hla, hlb, hld, hl-III, splA, splB*
49	175	Cattle	t521	97	97	-	-	PEN	*blaZ*	*seX, seY*	*set1, set3, set4, set5, set7, set8, set9, set12, setB, setC*	*clfA, clfB, eno, ebpS, fib, fnbA, fnbB, icaA, icaD, sasG, sdrC, sdrD*	*lukF, lukS, hlgA, lukF-PV-P83, lukD, lukE, lukX, lukY, hl, hla, hld, hl-III, splA, splB*
50	177	Cattle	t267	118	97	-	-	-	*cadD*	*seX, seY*	*set1, set3, set4, set5, set7, set8, set9, set12, setB, setC*	*clfA, clfB, eno, ebpS, fib, fnbA, fnbB, icaA, icaD, sasG, sdrC, sdrD, sdrE*	*lukF, lukS, hlgA, lukF-PV-P83, lukD, lukE, lukX, lukY, hl, hla, hlb, hld, splA, splB*
51	178	Cattle	t843	1245	130	-	-	PEN, CEF	*mecC*	*seX, seY*	*set1, set4, set5, set6, set7, set8, set9, set12, setB, setC*	*clfB, eno, ebpS, fib, fnbA, icaA, icaD, sdrC, sdrE*	*lukF, lukS, hlgA, lukD, lukX, lukY, hl, hla, hlb, hld, splA, splB, edinB*
52	179	Cattle	t529	1074	151	-	-	-	-	*seX, seY, seG, sel, seM, seN, seO, seU, entCM14*	*set1, set2, set3, set4, set5, set6, set7, set8, set12, setB, setC*	*bbp, clfA, clfB, eno, ebpS, fib, fnbA, icaA, icaD, sdrC*	*lukF, lukS, hlgA, lukF-PV-P83, lukM, lukD, lukE, lukX, lukY, hl, hla, hlb, hld, splA, splB*
53	181	Cattle	t843	-	130	-	-	PEN, CEF	*mecC*	*seX, seY*	*set1, set4, set5, set6, set7, set8, set9, set12, setB, setC*	*clfB, eno, ebpS, fib, fnbA, icaA, icaD, sdrC, sdrE*	*lukF, lukS, hlgA, lukD, lukX, lukY, hl, hla, hlb, hld, splA, splB, edinB*
54	182	Cattle	t843	-	130	-	-	PEN, CEF	*mecC*	*seX, seY*	*set1, set4, set5, set6, set7, set8, set9, setB, setC*	*clfB, eno, ebpS, fib, fnbA, icaA, icaD, sdrC, sdrE*	*lukF, lukS, hlgA, lukD, lukY, hl, hla, hlb, hld, splA, splB, edinB*

[a] list of abbreviations is provided on page 288

Appendix II. Table of all genotypic and phenotypic features of the selected *S. aureus* strains [a].

No	ID	Host	*spa*	ST	CC	SCC*mec*	*dru*	Resistance phenotype	Resistance genes	Virulence genes			
										Superantigens	Exotoxin-like proteins	Adhesins	Other
55	189	Cattle	t224	97	97	-	-	PEN	*blaZ, cadD*	*seX, seY, seB, seC, seL, tst*	*set1, set3, set4, set5, set7, set8, set9, set12, setB, setC*	*clfA, clfB, eno, ebpS, fib, fnbA, fnbB, icaA, icaD, sasG, sdrC, sdrD, sdrE*	
56	190	Cattle	t359	97	97	-	-	PEN	*blaZ, linA, cadD*	*seX, seY*	*set1, set3, set4, set5, set7, set8, set9, set12, setB, setC*	*clfA, clfB, eno, ebpS, fib, fnbA, fnbB, icaA, icaD, sasG, sdrC, sdrD, sdrE*	*lukF, lukS, hlgA, lukD, lukE, lukX, lukY, hl, hla, hld, splA, splB*
57	193	Cattle	t529	-	151	-	-	-	-	*seX, seY, seG, sel, seM, seN, seO, seU, entCM14*	*set1, set2, set4, set5, set6, set7, set8, set12, setB, setC*	*bbp, clfA, clfB, eno, ebpS, fib, fnbA, icaA, icaD, sdrC*	*lukF, lukS, hlgA, lukF-PV-P83, lukM, lukD, lukE, lukX, lukY, hl, hla, hlb, hld, splA, splB*
58	194	Cattle	t529	-	151	-	-	-	-	*seX, seY, seG, sel, seM, seN, seO, seU, entCM14*	*set1, set2, set4, set5, set6, set7, set8, set12, setB, setC*	*bbp, clfA, clfB, eno, ebpS, fib, fnbA, icaA, icaD, sdrC*	*lukF, lukS, hlgA, lukF-PV-P83, lukM, lukD, lukE, lukX, lukY, hl, hla, hlb, hld, splA, splB*
59	195	Cattle	t359	-	97	-	-	PEN, SPE, CLIi, ERY	*blaZ, spc, ermA*	*seX, seY, seB, seC, seL, tst*	*set1, set3, set4, set5, set7, set8, set9, set12, setB, setC*	*clfA, clfB, eno, ebpS, fib, fnbA, fnbB, icaA, icaD, sasG, sdrC, sdrD, sdrE*	*lukF, lukS, hlgA, lukF-PV-P83, lukD, lukE, lukX, lukY, hl, hla, hlb, hld, hl-III, splA, splB*
60	197	Cattle	t267	-	97	-	-	PEN	*blaZ, cadD*	*seX, seY, seB, seC, seL, tst*	*set1, set3, set4, set5, set7, set8, set9, set12, setB, setC*	*clfA, clfB, eno, ebpS, fib, fnbA, fnbB, icaA, icaD, sasG, sdrC, sdrD, sdrE*	*lukF, lukS, hlgA, lukF-PV-P83, lukD, lukE, lukX, lukY, hl, hla, hlb, hld, hl-III, splA, splB*
61	198	Cattle	t224	-	97	-	-	PEN	*blaZ, cadD*	*seX*	*set1, set3, set4, set5, set7, set8, set9, set12, setB, setC*	*clfA, clfB, eno, ebpS, fib, fnbA, fnbB, icaA, icaD, sasG, sdrC, sdrD, sdrE*	*lukF, lukS, hlgA, lukD, lukE, lukX, lukY, hl, hla, hlb, hld, hl-III, splA, splB*
62	199	Cattle	t529	-	151	-	-	-	-	*seX, seY, seG, sel, seM, seN, seO, seU, entCM14*	*set1, set2, set4, set5, set6, set7, set8, set12, setB, setC*	*bbp, clfA, clfB, eno, ebpS, fib, fnbA, icaA, icaD, sdrC*	*lukF, lukS, hlgA, lukF-PV-P83, lukM, lukD, lukE, lukX, lukY, hl, hla, hlb, hld, splA, splB*

[a] list of abbreviations is provided on page 288

Appendix II. Table of all genotypic and phenotypic features of the selected *S. aureus* strains [a].

No	ID	Host	*spa*	ST	CC	SCC*mec*	*dru*	Resistance phenotype	Resistance genes	Virulence genes			
										Superantigens	Exotoxin-like proteins	Adhesins	Other
63	200	Horse	t064	8	8	IVd	dt10a	PEN, CEF, GEN, KAN, CLIi, ERY, TET, TRI	*blaZ, mecA, aacA-aphD, aphA, ermC, tetM, dfrA, sat, cadD*	*seX, seY, seA, seB, seK, seQ*	*set1, set2, set3, set4, set5, set6, set7, set8, set9, set12, set21, setB, setC*	*clfA, clfB, eno, ebpS, fib, fnbA, fnbB, icaA, icaD, sasG, sdrC, sdrD, sdrE*	*lukF, lukS, hlgA, lukD, lukE, lukX, lukY, hl, hla, sak, hld, hl-III, splA, splB*
64	201	Horse	t064	-	8	IVd	dt10a	PEN, CEF, GEN, KAN, TET, TRI	*blaZ, mecA, aacA-aphD, aadD, tetL, tetM, dfrA*	*seX, seY*	*set1, set2, set3, set4, set5, set6, set7, set8, set9, set12, set21, setB, setC*	*clfA, clfB, eno, ebpS, fib, fnbA, fnbB, icaA, icaD, sasG, sdrC, sdrD, sdrE*	*lukF, lukS, hlgA, lukD, lukE, lukX, lukY, hl, hla, hlb, hld, hl-III, splA, splB*

[a] list of appendix II abbreviations: 'NT' – non-typeable; 'UN' – unknown; 'ST' – sequence type; 'CC' – clonal comples; PEN - penicillin; CEF - cefoxitin; GEN - gentamicin; KAN - kanamycin; SPE - spectinomycin; CLI - clindamycin; CLI_i – clindamycin inducible; ERY - erythromycin; TYL - tylosin; CHL - chloramphenicol; FLO - florfenicol; CIP - ciprofloxacin; TET - tetracycline; TIA - tiamulin; TRI - trimethoprim

Appendix III. PCR analysis of chromosomal location of *dfrK*, *vgaE* and *fexA* genes at the Att554 site. Isolates 92, 99, 101, 102, 103, 105, 106, 108, 109 and 111 were screened using *dfrK*_F (Table 2.1) and *att554*_R (5' - CCCGCTTCTACAAGACTGG – 3', as described by Schwendener *et al*. 2011) primers, isolate 104 was screened using *vgaE*_1 (Table 2.1) and *att554*_R primers whereas isolates 91, 93 and 95 were screened with *fexA*_R (5' – CGCATCTGAGTAGGACATAGCGTC – 3', as described by Argudin *et a.*, 2011) and *att554*_R primers. The PCR was performed as described in section 2.2.3 with a 2-minute extension time. The gel lanes are labeled as: 'ML' - molecular ladder (DirectLoad Step Ladder 50 bp; Sigma D3812); strain ID with resistance genotype.