CHARACTERISATION OF COAGULASE-NEGATIVE STAPHYLOCOCCI ASSOCIATED WITH ENDOCARDITIS

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SUMMARY

Coagulase-negative staphylococci are major aetiological agents of prosthetic valve endocarditis and an occasional cause of native valve disease. It is currently unclear how this group of usually avirulent microorganisms produces an infection associated with high rates of morbidity and mortality.

The aim of this thesis was to investigate whether there are specific genotypes and/or phenotypes of coagulase-negative staphylococci with a propensity to cause infective endocarditis and to investigate any identified virulence factors as markers of infection.

In this study, strains of endocarditis-related coagulase-negative staphylococci were genotyped by determining their macrorestriction genomic profile using pulsed-field gel electrophoresis. The strains were also investigated for phenotypic characteristics that predisposed the microorganisms to infect heart valves. By comparing coagulase-negative staphylococcal strains recovered from endocarditis patients with isolates from other significant infections (prosthetic device–related osteomyelitis and catheter-associated sepsis), no specific genotype or phenotype with a predilection to cause endocarditis was identified. However, the majority of the endocarditis-associated and other infection strains expressed the potential virulence factors lipase and esterase. Another approach to the investigation of virulence determinants used patient's serum to screen a *Staphylococcus epidermidis* NCTC 11047 genomic DNA library for cellular and secreted staphylococcal products that were expressed *in vivo*. The characterisation of two clones, which reacted with serum collected from a *S. epidermidis*-related endocarditis patient identified a staphylococcal pyruvate dehydrogenase complex E2 subunit and a novel secreted protein with homology to a *Staphylococcus aureus* staphyloxanthin biosynthesis protein and a secreted protein of unknown function described in *Staphylococcus carnosus*. Investigation of the secreted protein previously undetected in *S. epidermidis*, termed staphylococcal secretory antigen (SsaA), identified a potential marker of *S. epidermidis*-related endocarditis.

Key words: virulence factors; pulsed-field gel electrophoresis; molecular cloning; SsaA; immune response.

For Ian

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ABBREVIATIONS

aa	amino acid
AAP	adhesion-associated protein
ANOVA	one-way analysis of variance
API	Analytical Profile Index
ATCC	American Type Culture Collection
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BDH	Bispebjerg Hospital, Denmark
BHI	brain heart infusion
BHIA	brain heart infusion agar
bp	base pairs
BSA	bovine serum albumin
CABG	coronary artery by-pass graft
CAPD	continuous ambulatory peritoneal dialysis
CAPS	3[cyclohexylamino]-propane sulphonic acid
cfu	colony forming unit
CHEF	contour-clamped homogeneous electric field
CNS	coagulase-negative staphylococci
CVC	central venous catheter
Da	daltons
DTT	dithiothreitol
DF	deferoxamine mesylate
DMF	N,N-dimethylformamide
DMSO	dimethyl sulphoxide
ECM	extracellular matrix
EDTA	ethylenediamine-tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ES	EDTA, sarcosyl
ESP	EDTA, sarcosyl, proteinase K
FAME	fatty acid modifying enzyme
FPLC	fast protein liquid chromatography
h	hour
HH	Hope Hospital, Salford
HHB	Heartlands Hospital, Birmingham
IE	infective endocarditis
IPTG	isopropyl-1-thio-β-D-galactopyranoside
IVDA	intravenous drug abuse
IVET	*in vivo* expression technology
kb	kilo-base pairs
kDa	kilodaltons
LB	Luria-Bertani
min	minute
M_r	relative molecular mass
MSCRAMM	microbial surface components recognising adhesion matrix molecules
M_w	molecular weight
na	nucleic acid
NBT	nitroblue tetrazolium
NBTE	non-bacterial thrombotic endocarditis

NCTC	National Collection of Type Cultures
NET-100	Tris-buffered saline EDTA
NVE	native valve endocarditis
NZY	NZ amine-yeast extract agar
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDH	pyruvate dehydrogenase complex
PFGE	Pulsed-field gel electrophoresis
pfu	plaque-forming unit
pI	isoelectric point
PIA	polysaccharide intercellular adhesin
PRT	serum from a patient with *Staphylococcus epidermidis* endocarditis
PS/A	polysaccharide/adhesin
PVDF	polyvinyldifluoridine
PVE	prosthetic valve endocarditis
QEH	Queen Elizabeth Hospital, Birmingham
RAPD	randomly amplified polymorphic DNA
RBC	red blood cells
ROH	Royal Orthopaedic Hospital, Birmingham
rpm	revolutions per minute
SAA	slime-associated antigen
sarcosyl	N-lauroyl-sarcosine
SDS	sodium dodecyl sulphate
SM	Tris-buffered saline, magnesium sulphate, gelatin
SSP	staphylococcal surface protein
TAE	Tris acetate EDTA
TBE	Tris borate EDTA
TBS	Tris-buffered saline
TBST	Tris-buffered saline and Tween 20
TE	Tris EDTA
TEMED	N,N,N'N'-tetramethyl-ethylenediamine
Tris	Tris [hydroxymethyl] aminomethane
TSA	tryptone soya agar
TSB	tryptone soya broth
UPGMA	unweighted pair group method of arithmetic averages
UV	ultra violet
X-gal	5-brom-4-chloro-3-indolyl-β-D-galactopyranoside

CHAPTER 1: INTRODUCTION

1.1 STRUCTURE OF THE HEART

Venous blood enters the right atrium (the first of four chambers that form the heart) from the superior and inferior vena cavae and flows on through the tricuspid valve into the right ventricle (Griepp, 1988). Ventricular systole (the period of contraction during the cardiac cycle) forces the blood out through the pulmonary valve and into the pulmonary arteries. Oxygenated blood returns to the left atrium and flows via the mitral valve into the left ventricle (the fourth chamber of the heart). From here blood is ejected through the aortic valve into the aorta (figure 1.1).

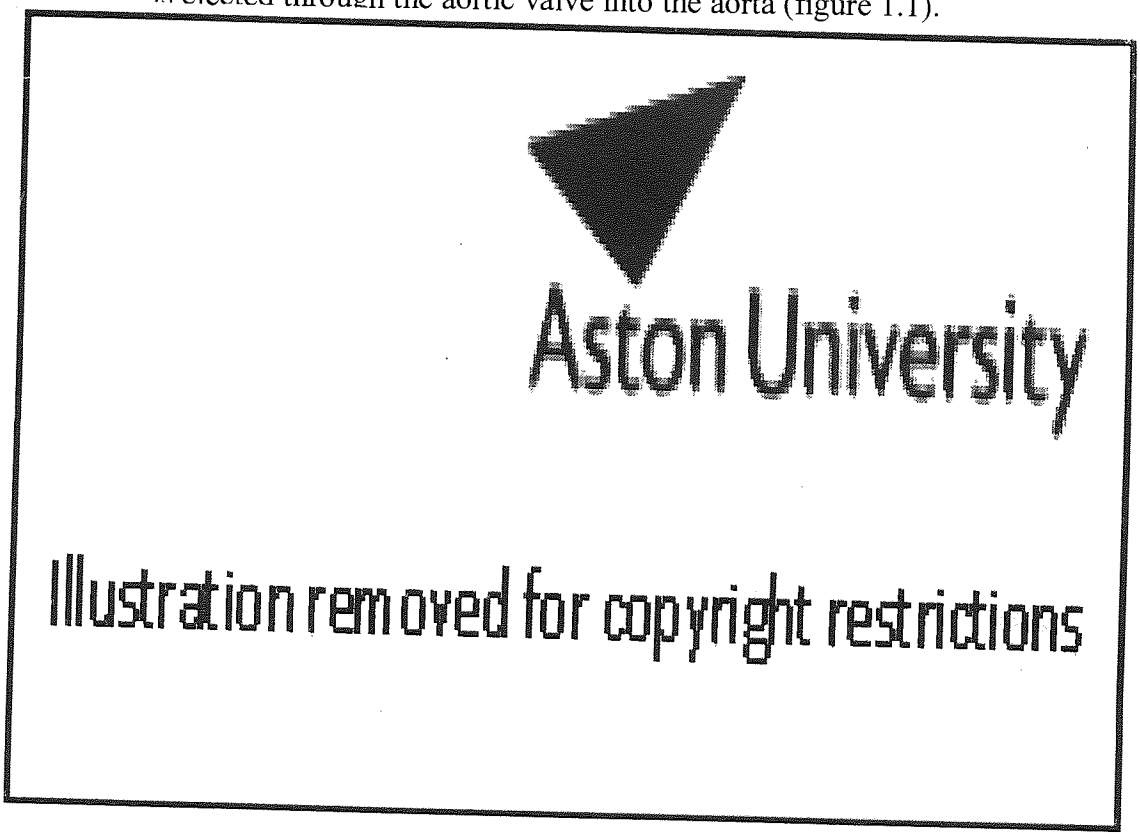


Figure 1.1. Structure of the heart and blood flow through the heart chambers. Reproduced from Guyton, 1992.

The atrioventricular valves (tricuspid and mitral) prevent blood flowing back from the ventricles into the atria during systole (Guyton, 1992). Similarly, the semilunar valves (aortic and pulmonary) prevent back-flow from the arteries into the ventricles during diastole (the period of relaxation during the cardiac cycle). The opening and

closing of all the valves is a passive process entirely dependent on a pressure gradient (Guyton, 1992). The atrioventricular valves are composed of a dense ring of fibrous connective tissue and thin delicate projections of connective tissue referred to as the valve leaflets (the mitral has only two leaflets and the tricuspid has three) (Griepp, 1988). Almost no back-flow is required to ensure closure of these valves. The papillary muscles, attached to the atrioventricular valves via the chordae tendineae fibres, contract with the ventricle walls and pull the valve inwards towards the ventricle. This movement prevents the pressure exerted on the valves during systole from forcing the leaflets backward into the atria (Guyton, 1992). By comparison the semilunar valves, composed of a ring of connective tissue and three valve leaflets, are subjected to much greater pressure at the end of systole which causes them to snap to a closed position (Guyton, 1992). The diameter of the opening of these valves is less than that of the atrioventricular valves, which causes the high-pressured flow of blood. Rapid valve closure and high fluid velocity subject these two valves to much greater mechanical stress than either the tricuspid or mitral endure (Guyton, 1992).

The surfaces of the heart valves and the interior of the cardiac chambers are covered with a thin endothelial layer (Griepp, 1988). The basal surface of these endothelial cells lies upon an extracellular matrix called the basement membrane composed of type IV collagen, laminin, fibronectin, heparan sulphate proteoglycans and glycoproteins (Griepp and Robbins, 1988). Together these components provide a rigid structure and specific receptors for other membrane constituents. Below the basement membrane lies the subendothelial layer; connective tissue composed of extracellular fibres of elastin and collagen, and muscle cells. An amorphous ground substance of fibre precursors, proteoglycans, glycoproteins and other secreted substances fill the spaces between cells and fibres (Griepp and Robbins, 1988). Together the endothelium, basement membrane, and subendothelial layer form the endocardium. The endocardium, myocardium (cardiac muscle) and epicardium (fat and nerve cells, lymphatic and blood vessels, elastic and collagen fibres) form the heart wall (Griepp, 1988).

1.2 INFECTIVE ENDOCARDITIS

Infective endocarditis (IE) denotes microbial infection of the endothelial lining of the heart (Durack, 1986).

Classically, IE has been divided into two forms of disease; acute and subacute (Scheld and Sande, 1990). Acute IE defines patients that present with pyrexia and other systemic involvement. Without treatment death occurs within days to less than six weeks. Typical aetiological agents include *Staphylococcus aureus* and more rarely *Streptococcus pyogenes*, *Streptococcus pneumoniae* and *Neisseria gonorhoeae*. Subacute IE follows a slow, indolent course characterised by low-grade pyrexia, night sweats, weight loss and other non-specific systemic manifestations. Unless treatment is received, death occurs 3 months or more after the onset of infection. Typically this form of the infection is caused by α-haemolytic streptococci (viridans streptococci) (Scheld and Sande, 1990). Currently, it is considered more appropriate to define IE by the origin of infection such as intravenous drug abuse (IVDA), associated organism or the valve infected; native valve endocarditis (NVE) or prosthetic valve endocarditis (PVE).

1.2.1 Pathogenesis of endocarditis

1.2.1.1 Tissue trauma

Trauma to the endothelial layer is usually the first stage in the development of IE. During the 1950s, Lepeschkin demonstrated that the greater the mechanical stress normally exerted on a heart valve, the higher the frequency at which it becomes infected (table 1.1) (Bansal, 1995). This suggests that mechanical stress alone is sufficient to damage valvular endothelium.

In addition to mechanical stress, abnormal blood flow can induce tissue damage. Haemodynamic turbulence generated by congenital defects (for example patent ductus arteriosus, ventricular septal defect, coarctation of the aorta and tetralogy of Fallot) or acquired valvular insufficiency (as exemplified by mitral valve prolapse or calcification of the mitral valve annulus) cause constant trauma to the endothelial layer (Livornese and Korzeniowski, 1992). Alternatively, direct damage to the tissue may emanate from infections such as rheumatic fever and syphilis (Harris, 1992).

Table 1.1. The correlation of IE involvement with mechanical stress for each heart valve established by Lepeschkin

Heart valve	Rate of infection* (%)	Resting pressure exerted on the closed valve (mm Hg)
mitral	86	116
aortic	55	72
tricuspid	19.6	24
pulmonic	1.1	5

* rate of infection is based on data collected during the 1950s (Bansal, 1995). Recent studies have indicated that the aortic has replaced the mitral as the most frequently affected valve (Dyson, Barnes and Harrison, 1999).

1.2.1.2 Development of a sterile vegetation

Trauma exposes connective tissue containing collagen fibres, which promote the deposition of platelets and fibrin and thus the formation of non-bacterial thrombotic endocarditis (NBTE) (Livornese and Korzeniowski, 1992). These NBTE lesions, also referred to as sterile vegetations, usually form where blood flows from a high-pressure source to a position of low pressure (Rodbard, 1963). For example, a vegetation develops on the ventricular surface of the aortic valve when a high velocity regurgitant stream passes from the aorta back through the incompetent valve into the left ventricle in diastole (figure 1.2) (Rodbard, 1963).

Additionally, a turbulent regurgitant stream of blood is capable of damaging the cardiac chamber endothelium and generating a jet lesion, as exemplified by MacCallum's patch (figure 1.2). Infected vegetations do occasionally evolve at these extra-valvular sites (Durack, 1986).

Figure 1.2. A diagram illustrating the high velocity streams in mitral and aortic insufficiency and sites of endocarditic lesions. A high velocity regurgitant stream passes through the orifice of an incompetent aortic valve into a low-pressure sink (the left ventricle in diastole) (arrow 1). Vegetations develop on the ventricular surface of the aortic valve. A vegetation may develop on the jet lesion on the chordae tendineae of the anterior leaflet of the mitral valve. Regurgitation from the high-pressure source of the left ventricle during systole into the left atrium (arrow 2) allows vegetations to develop on the atrial surface of the mitral valve. A vegetation may also develop on the jet lesion on the atrial endocardium formed by the regurgitant stream from the mitral valve. Vegetations are indicated in red. Reproduced from Rodbard, 1963.

1.2.1.3 Bacterial colonisation of the vegetation

The NBTE lesion forms an ideal site for bacterial colonisation should bacteraemia occur. Transient bacteraemia will result following trauma to any mucosal surface with a high bacterial count, for example the oropharynx, gastrointestinal or genitourinary tracts (Livornese and Korzeniowski, 1992). Dental work is a common source of bacteraemia and has frequently been linked with the onset of disease,

particularly viridans streptococcal IE (Livornese and Korzeniowski, 1992). Bacteraemia-related coagulase-negative staphylococci (CNS) are generally thought to originate from the skin flora, however, there is evidence to prove that the oral mucosa also serves as a reservoir (Kennedy *et al.*, 2000). Once seeded into the blood stream certain bacteria such as *S. aureus* and viridans streptococci will readily adhere to the platelet-fibrin matrix (table 1.2).

Table 1.2. Microbiology of NVE in drug abusing and non-drug abusing patients

Organism	Non- IVDA (%)	IVDA (%)
viridans streptococci	50	10
enterococci	5	8
other streptococci	5	2
S. aureus	20	57
coagulase-negative staphylococci	5	3
Gram-negative coccobacilli	4	2
Gram-negative bacilli	2	5
fungus	1	5
polymicrobial and other	3	3
culture negative	5	5

IVDA, intravenous drug abuse (Bansal, 1995).

Following attachment of the microorganisms to the NBTE lesion a layer of platelets and fibrin rapidly covers the bacterial cells and an infected vegetation develops (Scheld and Sande, 1990). The vegetations vary in colour (red, brown, grey, or tan) and size (microscopic to masses of several centimetres in diameter sufficient to block the valve orifice) (figure 1.3) (McFarland, 1992). Depending on the aetiological agent a few lymphocyte, neutrophil, mononuclear and plasma cells may penetrate the valvular mass (McFarland, 1992). In this protected environment bacterial counts can achieve 10^9 to 10^{10} microorganisms per gram of tissue (Scheld and Sande, 1990). In cases of subacute IE the presence of a NBTE lesion is generally required before the infection can become established, whereas in acute episodes the virulent pathogens are thought to be able to either attach to a sterile vegetation or directly invade the healthy cells of the endocardium (Bansal, 1995). Other IE-associated pathologies may include valve ring abscesses, rupture of the chordae tendineae, septal rupture or infection of the myocardium (McFarland, 1992).

Figure 1.3. The post-mortem specimen of a patient with *Staphylococcus lugdunensis*-related IE. Two vegetations are located on the pulmonary artery. The larger of the two vegetations (v) measures 12mm in its greatest dimension. The smaller of the two masses measures 10mm in its greatest dimension and is located around the orifice of the patent ductus arteriosus (arrow). Reproduced from Leung *et al.*, 1998.

1.2.2 Clinical manifestations and complications associated with endocarditis

The clinical manifestations outlined below are not a comprehensive list but an overview of some of those commonly or characteristically associated with IE. Certain presentations are more frequently observed with particular aetiological agents whilst others are associated primarily with the duration of infection, for example chronic infection.

1.2.2.1 Systemic presentation

Subacute IE presents clinically as an indolent systemic disease. Low-grade pyrexia, cardiac murmur, chills, rigors, night sweats, general malaise, anorexia, fatigue, asthenia, headaches, anaemia, myalgias, arthralgias and back pain are all typical manifestations of this form of the disease (Durack, 1986). The general symptoms of

acute IE are similar to those of subacute IE but are of increased severity and the progression of the disease more rapid (Durack, 1986).

1.2.2.2 Heart

Congestive heart failure, destruction of the conduction paths resulting in heart block, aneurysms, perforations, and myocardial infarcts and haemorrhages are life threatening complications that can arise with IE (McFarland, 1992).

1.2.2.3 Embolization of the vegetation

Emboli from sterile or infected vegetations affect approximately one third to one half of patients, although it is thought that many cases are undetected, and may involve almost any organ; brain, lung, coronary artery, spleen, extremities, gut and eye (Durack, 1986).

1.2.2.4 Lungs

Respiratory complications are particularly associated with right sided IE and include lung abscesses, pulmonary infarcts and pneumonia (McFarland, 1992).

1.2.2.5 Skin

Petechiae and splinter haemorrhages (linear subungual haemorrhages) arise due to circulating immune complexes (McFarland, 1992). Osler's nodes (painful erythematous nodules in the skin of the extremities, generally in the pads of fingers or toes) and Janeway lesions (painless haemorrhagic, macular plaques on the palms or soles) (figure 1.4) are produced by microembolisation of the infected vegetation (McFarland, 1992).

Figure 1.4. A Janeway lesion on the finger of a patient diagnosed with IE. Reproduced from (adam.com).

1.2.2.6 Eyes

Roth spots are pale/white retinal lesions surrounded by an area of haemorrhage that are thought to be small infarcts (McFarland, 1992).

1.2.2.7 Kidneys

Renal involvement presents in the majority of IE patients; petechiae, glomerulonephritis, infarcts and abscesses are examples of the manifestations that are attributed to circulating immune complexes and emboli (McFarland, 1992).

1.2.2.8 Spleen

Splenomegaly is a common presentation of subacute IE and results from the chronic stimulation of the reticuloendothelial system by persistent, long-term bacteraemia and circulating immune complexes (McFarland, 1992).

1.2.3 Epidemiology of endocarditis

It is estimated that there are two cases per 100,000 population per year in England and Wales (Harris, 1992). In the pre-antibiotic era IE had a mortality rate

approaching 100% (Gold, 1992). Even today with the vast array of antimicrobial agents available and advanced medical techniques there is still an overall mortality rate approaching 12% to 19% (Dyson, Barnes and Harrison, 1999; Sandre and Shafran, 1996).

The epidemiology and clinical presentations of IE have changed considerably during the last century due to a number of factors:

- In the pre-antibiotic era the incidence of heart damage due to syphilis and rheumatic fever was considerably greater (Harris, 1992).
- A number of reviews have recently identified the aortic as the most commonly affected valve and not the mitral, which historically was the most frequent vegetation site. This observation is probably due to the reduced incidence of rheumatic disease, which typically affects the mitral leaflets (Dyson, Barnes and Harrison, 1999; Sandre and Shafran, 1996).
- The ability to perform open-heart surgery has allowed congenital defects to be corrected and damaged heart valves to be replaced, so reducing the number of patients at risk from NVE but increasing the number of individuals predisposed to PVE (Durack, 1986).
- Medical advances have contributed to an increase in nosocomial IE. Intracardiac pressure-monitoring catheters, ventriculoatrial shunts and cardiac surgery can produce iatrogenic damage to the endocardium, whilst the extensive increase in catheterization of patients has provided a portal of entry to both pathogenic and opportunistic organisms (Harris, 1992).
- The recreational use of intravenous drugs has been implicated in a significant proportion of right-sided NVE. Of sixty-four patients attending a Californian medical centre, who were classified as at risk of IE, thirty had clinical evidence of the syndrome and of those, in excess of 50% were intravenous drug users (Bayer, 1993).
- The mean age of patients has increased. The increasing age of the population, and the associated degeneration of cardiac tissue related to the ageing process (for example, calcification of the leaflets), has created a new "at risk" population. The decline in rheumatic fever in the developed world and the ability to surgically correct congenital defects and replace damaged valves has reduced the number of

young individuals contracting IE and, therefore, has also contributed to the increased mean age of patients (Bayer, 1993; Scheld and Sande, 1990).

The cumulative effect of these and other subtle changes has altered the clinical presentation of IE; an increased median age of patients, a rise in the ratio of male to female patients (1.2 to 3:1 overall increasing to 2:1 to 8:1 in the 60 and over age group), a rise in the proportion of acute cases, fewer patients developing the classic signs of infection such as Osler's nodes, the proportion of streptococcal cases has fallen slightly, and the extent of cases due to unusual organisms, such as fungi, has increased marginally (Bansal, 1995; Harris, 1992; Scheld and Sande, 1990; Durack, 1986).

1.2.4 Prosthetic valve endocarditis

PVE describes the infection of a prosthetic heart valve and the surrounding cardiac tissue (Douglas and Cobbs, 1992). In the United States alone approximately 60,000 valves are replaced annually and of these it is estimated that between 1% and 4% will become infected during their life span (Vongpatanasin, Hillis and Lange, 1996; Douglas and Cobbs, 1992). PVE is traditionally divided into two groups; early PVE which denotes infection occurring within 60 days of valve implantation, and late PVE which refers to the onset of disease more than 60 days after valve insertion (Block *et al.*, 1970). These groupings are designed to distinguish between surgically-acquired infection (nosocomial) and transient bacteraemia derived (community-acquired) IE. However, a number of studies have indicated that it would be appropriate to extend the definition of early PVE to those infections occurring within 12 months of surgery (Calderwood *et al.*, 1985; Archer, Vishniavsky and Stiver, 1982). Calderwood *et al.* (1985) demonstrated that 84% of CNS that caused PVE within 12 months of device implantation were resistant to methicillin, whereas only 30% of isolates associated with infection after 12 months were resistant to the antimicrobial. Moreover, Archer *et al.* (1982) demonstrated that a cluster of PVE infections, associated with an suspected outbreak strain, were presenting up to 13 months post-operatively (average of 5.3 months).

There are two basic types of prosthetic heart valve; mechanical devices and bioprosthetic implants. Mechanical valves are made primarily of carbon alloys or metal and are classified by their design, for example bileaflet-tilting disc, single-tilting-disc or caged-ball valves (Vongpatanasin, Hillis and Lange, 1996). Bioprosthetic valves are either preserved human aortic valves (homografts) or porcine or bovine tissue attached to a metal support (heterograft) (Vongpatanasin, Hillis and Lange, 1996). There are a number of advantages and disadvantages to both types of prosthesis. Mechanical valves are highly durable (lasting 20 to 30 years) but are thrombogenic (Vongpatanasin, Hillis and Lange, 1996). Also Ivert *et al.* (1984) and Calderwood *et al.* (1985) independently identified an increased risk of early PVE with mechanical compared to bioprosthetic implants, but no difference in risk of infection was found during follow-up at 5 years. In contrast to mechanical devices, bioprostheses have a low thrombo-embolic potential and a reduced risk of early PVE but between 10% and 20% of homografts and 30% of heterografts require replacement after 10 to 15 years (Vongpatanasin, Hillis and Lange, 1996).

The microorganisms causing early PVE (table 1.3) are markedly different from those isolated from NVE (table 1.2). In NVE viridans streptococci and *S. aureus* together account for 70% of cases, whereas in early PVE they are attributable for only 23% of infections. The predominant organisms associated with early PVE are CNS and Gram-negative bacilli, bacteria typically associated with nosocomial infection (table 1.3). The type of organism associated with late PVE more closely reflects those causing infection of native valves with two notable exceptions: viridans streptococci have a lower incidence and CNS have an increased frequency (tables 1.2 and 1.3).

Table 1.3. Microbiology of PVE

Organism	**Early PVE (%)**	**Late PVE (%)**
viridans streptococci	8	30
enterococci	2	6
S. aureus	15	10
coagulase-negative staphylococci	33	29
Gram-negative bacteria	17	10
fungus	10	5
diphtheroids	8	3
others	2	2
culture negative	5	5

PVE, prosthetic valve endocarditis (Bansal, 1995).

Infection of a prosthetic valve generally involves both the device and the tissue surrounding the implant (the valve annulus and myocardium) (Karchmer and Gibbons, 1994). Partial dehiscence of the valve causes regurgitant blood flow and dysfunction of the valve. Myocardial abscess associated with artificial aortic valves can interrupt the conduction system leading to heart block, whereas prosthetic mitral valve IE is often complicated by orifice obstruction (Karchmer and Gibbons, 1994). In other respects the clinical manifestations of PVE are similar to those of NVE. There is considerable variation in the reported incidence of mortality for PVE but two recent reviews identified a rate of either 16% or 25% (Dyson, Barnes and Harrison, 1999; Sandre and Shafran, 1996).

1.2.5 Diagnosis of endocarditis

IE is notoriously difficult to diagnose with certainty because none of the symptoms is pathognomonic. Many of the manifestations such as weight loss, pyrexia, splenomegaly, anaemia and fatigue are equally as prevalent in other chronic diseases as they are in IE. Cardiac presentations including heart murmur may be equally non-specific.

If IE is suspected (the patient has an undiagnosed pyrexia lasting for in excess of seven days and a detectable heart murmur) a number of factors have to be established or assessed:

- Is the patient considered at risk for IE? For example, having a known cardiac abnormality or an IVDA.
- A persistent bacteraemia should be identified by the recovery of the same microorganism from multiple blood cultures taken at different times, ideally three over a 24h period and preferably before antimicrobial therapy is instigated. Is the isolated organism commonly associated with IE?
- Confirm that there is no extracardiac site of infection.
- Are there any other symptoms that are associated with IE?
- Has echocardiography detected any underlying valvular heart disease?

To aid diagnosis of IE a number of criteria have been established. Pelletier and Petersdorf proposed the first of these in 1977. The scheme proved to be specific but not very sensitive (von Reyn and Arbeit, 1994). Von Reyn *et al.* suggested modifications to the earlier scheme to improve both specificity and sensitivity - the von Reyn criteria (von Reyn and Arbeit, 1994). Finally in 1994, Durack *et al.* proposed further modifications, which included for the first time echocardiography in the criteria and consideration of IVDA (Durack, Lukes and Bright, 1994) (appendix 1). This scheme, which became known as the Duke criteria, was shown to be an improvement to previously existing diagnosis aids (Olaison and Hogevik, 1996; Bayer *et al.*, 1994). The Duke criteria have undergone only minor modifications and currently remain in use (Li *et al.*, 2000a; Lamas and Eykyn, 1997).

1.2.6 Treatment of endocarditis

If IE is strongly suspected antimicrobial therapy will generally be instigated empirically after blood culture samples have been taken. Streptococci, enterococci or staphylococci are the most common cause of both NVE and PVE. Therefore, a combination of a penicillin (or vancomycin if staphylococcal infection is suspected) with an aminoglycoside (generally gentamicin) is recommended by the working party of the British Society for Antimicrobial Chemotherapy (1998). On establishing the aetiological agent, therapy may be modified as appropriate.

For a number of patients surgery is necessary during active IE, for example in cases where there is persistent sepsis and recurrent emboli (Bansal, 1995). Infection of bioprostheses is initially treated with antimicrobials unless there is severe valvular dysfunction, which then requires surgery. Mechanical valve–associated IE generally necessitates immediate surgical intervention (Bansal, 1995).

1.3 THE GENUS *STAPHYLOCOCCUS*

The genus *Staphylococcus* is defined as:

Gram-positive cocci (0.5 to 1.5μm in diameter) in clusters, non-motile, non-sporing, facultatively anaerobic, usually catalase-positive and oxidase-negative (Holt *et al.*, 1994).

1.3.1 Classification of staphylococci

Historically, the genus was considered to consist of two groups, according to their ability to coagulate citrated plasma. The term coagulase-positive staphylococci became synonymous with the pathogenic species *S. aureus* whilst the CNS were collectively known as *Staphylococcus albus, Staphylococcus epidermidis albus* or *Staphylococcus epidermidis* (Kloos, 1996). This latter group was considered to consist of commensal or saprophytic organisms that were incapable of pathogenesis, and, as such, were disregarded. The genus remained ill-defined until, in the 1960s, Baird-Parker attempted to phenotype the taxon (Baird-Parker, 1965). Also at this time other species began to be assigned to CNS following the work of Kloos and Schleifer (Schleifer and Kloos, 1975; Kloos and Schleifer, 1975).

With the development of molecular typing methods, such as 16S rRNA analysis, DNA-DNA hybridisation and chromosomal analysis by pulsed-field gel electrophoresis (PFGE), new species continue to be described (Kloos, 1996; Freney *et al.*, 1988). Furthermore, the taxonomy of the staphylococci has moved to the identification of subspecies on the basis of DNA-binding values, for example *Staphylococcus capitis* subsp. *capitis* and *S. capitis* subsp. *ureolyticus* (Bannerman and Kloos, 1991). There are currently over thirty-two recognised species within the CNS taxon (Huebner and Goldmann, 1999). The molecular biology techniques that have been used in the speciation of the genus also have proved valuable in the epidemiological investigation of pathogenic staphylococci.

1.3.2 Epidemiological investigation of the coagulase-negative staphylococci strains

Epidemiological studies of CNS are notoriously difficult to evaluate. Traditional typing methods such as biotyping, antibiogram and bacteriophage typing have low discrimination. Strains of *S. epidermidis* exhibit too few distinct biotypes to have any significant discriminatory power (Geary *et al.*, 1997; Toldos *et al.*, 1997; Hedin, 1996). Equally the epidemiological value of antibiograms is low for nosocomial strains of CNS that have, by definition, been acquired within an environment that acts as a reservoir for antibiotic resistance genes (Geary *et al.*, 1997; Toldos *et al.*, 1997; Vandenesch *et al.*, 1993a). Similarly bacteriophage typing is of limited value

since many clinical strains of CNS are non-typable (Geary *et al.*, 1997). The advent of molecular methods such as restriction endonuclease analysis of genomic DNA, ribotyping, plasmid analysis, DNA-DNA hybridisation and randomly amplified polymorphic DNA (RAPD) analysis have provided potentially valuable tools for the evaluation of the relatedness of staphylococcal isolates (Olive and Bean, 1999; Kluytmans *et al.*, 1998; Geary *et al.*, 1997; Riou *et al.*, 1997; Kloos and Bannerman, 1994; Lina *et al.*, 1992). Of these techniques PFGE is currently considered to be the "gold standard" method for the epidemiological study of CNS (Olive and Bean, 1999; Bannerman *et al.*, 1995).

1.3.2.1 Pulsed-field gel electrophoresis

PFGE of the entire staphylococcal chromosome following cleavage with a rare-cutting restriction endonuclease generates a profile of discrete bands (approximately ten to twenty bands) and subsequent analysis of this macrorestriction pattern allows the relatedness of strains to be evaluated (Tenover *et al.*, 1995; Birren and Lai, 1993). The advantages of this technique, which prompt it to be a valuable tool in staphylococcal epidemiology, arise through the analysis of the entire bacterial genome. Other molecular techniques including plasmid analysis and RAPD sample only a small proportion of the bacterial genome. The high levels of discrimination, reproducibility and stability of the macrorestriction analysis technique have addressed many of the problems encountered with traditional typing methods.

PFGE of macrorestriction fragments has been used in the epidemiological study of various microorganisms, including *Escherichia coli, Nocardia farcinica, Acinetobacter baumannii* and *Serratia marcescens* with a high degree of success (Blümel *et al.*, 1998; Aarestrup *et al.*, 1997; Shi *et al.*, 1997; Sader *et al.*, 1996). Similarly, the application of macrorestriction analysis has led to a clearer understanding of CNS epidemiology. Before genotyping it was not possible to determine accurately the source of nosocomial CNS infection. Macrorestriction analysis has demonstrated that infection-associated CNS can be endogenous in origin, derived from the skin of the attending medical staff or acquired from the environment (Frebourg, Cauliez and Lemeland, 1999; Livesley *et al.*, 1998; Nouwen *et al.*, 1998; Sloos *et al.*, 1998; Burnie *et al.*, 1997; Boyce *et al.*, 1990). Furthermore,

a number of PFGE studies have demonstrated the presence of endemic strains within individual medical units, particularly neonatal intensive care units, and that in many cases, the strain persisted within the unit for several years (Kluytmans *et al.,* 1998; Nouwen *et al.,* 1998; Sloos *et al.,* 1998; Vermont *et al.,* 1998; Burnie *et al.,* 1997; Neumeister *et al.,* 1995; Huebner *et al.,* 1994).

1.3.3 Coagulase-negative staphylococci as commensal organisms

CNS are ubiquitous within the environment having been isolated from air, dust, soil, water, food and numerous plants and animals (Kloos, 1996). The genus is the major group of bacteria to inhabit human skin, with populations reaching approximately 10^4 to10^6 cfu/cm^2 in areas with a high number of pilosebaceous and sweat glands and regions of the epidermis and mucus membranes adjacent to body orifices (Kloos, 1996). A number of CNS species are recognised as indigenous to man: *S. auricularis, S. capitis, S. caprae, S. cohni, S. epidermidis, S. haemolyticus, S. hominis, S. lugdunensis, S. pasteuri, S. saccharolyticus, S. saprophyticus, S. schleiferi, S. simulans, S. warneri* (Kloos, 1996). The predominant species *S. epidermidis* is ubiquitous on the human cutaneous surface, comprising 65% to 90% of all recovered staphylococci (Archer, 1990). *S. hominis* being the second most prevalent CNS species, is detected at considerably lower numbers (Kloos, 1996). The bacterial population may be transient, temporarily resident or resident depending on the site and the species (Kloos, 1996).

1.3.4 Coagulase-negative staphylococci as pathogens

The pathogenic potential of CNS was recognised by several groups in the 1960s, but it was not until the 1970s that their role in infection became widely accepted (Kloos and Bannerman, 1994). Since that time the number of reported CNS-related infections has continued to increase. During the period 1980 to 1989, the incidence of CNS-associated nosocomial bacteraemia in the United States rose from 9% to 27% (Peters, von Eiff and Herrmann, 1995). In a recent survey of forty-nine American hospitals, carried out over a 3 year period, CNS were the most common pathogens isolated in all units, except obstetrics, and accounted for 250,000 nosocomial infections annually, almost one third (32%) of all nosocomial

bacteraemia infections (Edmond *et al.*, 1999). In the United Kingdom, CNS accounted for 4194 cases during 1999 (16.0% of notified bacteraemia episodes caused by Gram-positive bacteria) (provisional data) (Public Health Laboratory Service, 2000). This annual figure has increased gradually over the period 1990 to 1999 from 2172 cases annually to 4194 (Public Health Laboratory Service, 2000).

The emergence of this relatively "new" pathogen has coincided with new medical techniques. In the 1940s the plastic intravascular catheter was introduced (Elliott, 1988), the 1950s saw the first artificial heart valve (Vongpatanasin, Hillis and Lange, 1996) and during the 1960s, the arrival of the indwelling peritoneal catheter (Spencer, 1988). In the 1970s parenteral nutrition became widespread (Lewis and Sherertz, 1997). Improvements in medical biomaterials have allowed long-term catheterization. Additionally, there is an increase in the number of seriously ill patients with severe immunodepression, medical procedures are increasingly invasive and there is an ageing society. Together these patients form an "at-risk" population that did not exist a few decades ago:

- Patients with severe immune depression, for example neonates and bone marrow transplant patients (Burnie *et al.*, 1997; Neumeister *et al.*, 1995; Lina *et al.*, 1995; Hübner and Kropec, 1995).
- Patients with implanted medical devices, as exemplified by central venous catheters (CVC), continuous ambulatory peritoneal dialysis (CAPD) catheters, artificial joints and prosthetic cardiac devices such as heart valves (Huebner and Goldmann, 1999; Tunney *et al.*, 1999; Michalopoulos and Geroulanos, 1996; Karchmer and Gibbons, 1994; Goldmann and Pier, 1993; von Graevenitz and Amsterdam, 1992; Elliott, 1988).

In addition to the morbidity and mortality associated with these infections, it is estimated that CNS nosocomial bacteraemia has a mortality rate of 21% (Edmond *et al.*, 1999), there is also an immense financial expense to the Health Service in terms of drug budget and extended hospital stay. The cost of short-term use catheter infections has been estimated to be between 5 and 7 million pounds per annum in the United Kingdom alone (Moss and Elliott, 1997).

1.3.4.1 Coagulase-negative staphylococcal native valve endocarditis

At the Massachusetts General Hospital and Mayo Clinic, CNS were attributable for only 2% of cases during the periods 1944 to 1958 and 1951 to 1957, respectively. In more recent years both institutions saw an increase to 5% for 1975 to 1983 and 4% for 1970 to 1979, respectively (Karchmer, 1992). Since CNS rarely infect native heart valves, the incidence at both institutes has remained relatively low (Archer, 1990); however, the increase observed in the 1970s and 1980s reflects the recognition of these microorganisms as pathogens. A more recent report has indicated that the incidence of NVE caused by CNS is actually increasing; 10.4% of IE cases presenting over the period 1977 to 1989 compared with no cases seen between 1968 to 1976 (Etienne and Eykyn, 1990). The authors were unable to explain this apparent increase of infection over a 2-decade period.

When these microorganisms do attack the native structures there is generally an underlying abnormality such as a congenital defect or an acquired abnormality as exemplified by mitral valve prolapse (Baddour, Phillips and Bisno, 1986). *S. epidermidis* is by far the most frequently isolated species of the CNS group from NVE, although other species have been reported; *S. haemolyticus*, *S. hominis*, *S. lugdunensis*, *S. warneri*, *S. saprophyticus*, *S. cohnii* and *S. capitis* (Al-Rashdan, Bashir and Khan, 1998; Kessler, Kimbrough and Jones, 1998; Leung *et al.*, 1998; Buttery *et al.*, 1997; Lessing *et al.*, 1996; Karchmer, 1992; Archer, 1990; Etienne and Eykyn, 1990). The majority of infections involving native valves are community-acquired. Generally the source of the bacterium remains unknown, however, there are a few documented cases of CNS-related NVE following vasectomy, dental treatment, and wound infections (Kessler, Kimbrough and Jones, 1998; Lessing *et al.*, 1996; Etienne and Eykyn, 1990).

As observed with IE, in general the clinical manifestations of NVE caused by CNS are highly variable. Infection usually follows a subacute form with the most common presentations being pyrexia, malaise, anorexia, heart murmur and weight loss (Karchmer, 1992). Although generally regarded as the avirulent members of the genus, an aggressive pathogenic phenotype is expressed during episodes of NVE. Indeed, the ferocity of *S. lugdunensis* infection has equalled that normally observed with *S. aureus* (Vandenesch *et al.*, 1993c). Complications such as conduction

abnormalities and congestive heart failure are not uncommon and a mortality rate of 36% has been observed (Karchmer, 1992; Etienne and Eykyn, 1990).

1.3.4.2 Coagulase-negative staphylococcal prosthetic valve endocarditis

Though the majority of episodes of PVE are caused by *S. epidermidis* other CNS species, including *S. schleiferi* and *S. lugdunensis,* have also been reported (Leung *et al.,* 1999; Vandenesch *et al.,* 1993c; Karchmer, 1992). It is estimated that CNS are responsible for 33% of early PVE cases and 29% of late PVE infections (table 1.3) (Bansal, 1995). As outlined in section 1.2.4, it is likely that CNS associated with early PVE are nosocomial in origin whilst late PVE is related to a community-acquired transient bacteraemia. The clinical manifestations of early PVE differ depending on the type of implant *in situ.* Infection of a mechanical device is primarily located on the valve annulus (Karchmer, 1992). The resulting necrosis of the sewing ring causes dehiscence, regurgitant blood flow, ring abscesses and possible conduction problems. Conversely, bacteria associated with bioprosthetic implants are confined principally to the valvular leaflets. Pathogenesis related to these infections generally present as valvular incompetence due to the rigidity of the leaflets caused by the vegetation (Karchmer, 1992). Late PVE manifestations are similar to those associated with left-sided NVE caused by CNS (Karchmer, 1992).

1.3.4.3 Diagnosis of coagulase-negative staphylococcal endocarditis

Whilst the diagnosis of IE can present certain difficulties to a clinician, IE caused by CNS can be particularly problematic:

- The clinical presentation can be even more variable than observed in IE caused by other aetiologies (Karchmer, 1992).
- CNS frequently contaminate blood samples (Kloos and Bannerman, 1994).
- In cases of PVE, and, in particular, valve ring infections, bacterial seeding of the blood is thought not to be continuous and, therefore, bacteria will only be isolated from a few of the blood culture samples (Archer, 1997).

- Cardiac imaging by conventional echocardiography is limited in its ability to detect vegetations if certain types of prosthetic device are *in situ* (Vered *et al.*, 1995).

1.3.4.4 Treatment of staphylococcal endocarditis

As soon as staphylococci are identified as the aetiological agent combination therapy of gentamicin and either vancomycin or flucloxacillin, depending on the sensitivity profile of the microorganism, should be instigated (Working party of the British Society for Antimicrobial Chemotherapy, 1998). The inclusion of the aminoglycoside with a glycopeptide is thought to aid a more rapid defervescence and clearance of bacteraemia. Rifampicin, which may be given in combination with vancomycin, may be instigated for difficult cases (Working party of the British Society for Antimicrobial Chemotherapy, 1998). Treatment of NVE and PVE is the same except duration is extended from the four weeks, which is usually adequate for NVE, to up to six weeks for PVE.

1.3.5 The molecular basis of staphylococcal pathogenicity

The continuous expression of a full complement of genes by a cell would be a waste of cellular energy and consume valuable metabolic components unnecessarily. In a competitive environment of limited nutrients, inefficient cells would be rapidly out-competed. Therefore, regulatory systems have evolved in which genes essential to cell survival and multiplication are expressed constitutively ("housekeeping" genes) whilst others are expressed only as required (accessory genes) (Projan and Novick, 1996). In order for these control systems to function the cell has to be able to detect changes in the external environment and then respond appropriately. Some bacteria respond to environmental signals and others to a specific host product (Dunny and Leonard, 1997; Mekalanos, 1992). Staphylococci possess iron-regulated operons, such as the ABC transporter (Hill *et al.*, 1998), and a cell density (quorum) sensing system which regulates accessory gene expression (Ji, Beavis and Novick, 1995).

1.3.5.1 Regulation of bacterial virulence

Iron repression

Whilst iron is an essential metabolite, it is also central in the control of a number of bacterial virulence genes (Trivier and Courcol, 1996; Litwin and Calderwood, 1993; Williams and Griffiths, 1992). Indeed, iron acquisition by *Neisseria gonorrhoeae* has been demonstrated to be essential to virulence (Nau Cornelissen *et al.*, 1998). Exocellular polysaccharide slime, a potential virulence factor associated with CNS infections, is regulated by iron limitation such as encountered within the host environment (Deighton and Borland, 1993). Under these iron-restricted conditions *S. epidermidis* express a number of iron-regulated proteins, for example a 42kDa transferrin-binding receptor on the cell surface and a 32kDa lipoprotein (SitC) (Modun *et al.,* 1998; Modun, Kendall and Williams, 1994; McDermid *et al.*, 1993; Wilcox *et al.*, 1991). The latter of these bacterial proteins is encoded by the *sitABC* operon, which is regulated by SirR (an iron-dependant repressor) (Cockayne *et al.,* 1998; Hill *et al.,* 1998). Although the products and function of *sitABC* are not thoroughly understood, SitC is believed to be involved in iron transport (Cockayne *et al.,* 1998). Moreover, homology between this operon and a group of solute–binding proteins specific for metals has been described (Dintilhac *et al.,* 1997).

Quorum sensing

The global regulatory quorum-sensing system *agr* (accessory gene regulation) has been shown to be central in controlling the synthesis of exocellular and cell surface-associated proteins in staphylococci (Vuong, Götz and Otto, 2000). Although much of the *agr* work has focused on the *S. aureus* gene (Recsei *et al.,* 1986), a homologue has been identified in both *S. epidermidis* and *S. lugdunensis* (van Wamel *et al.,* 1998; Vandenesch *et al.,* 1993b). The *agr* locus has two promoters (P2 and P3) which generate the transcripts RNAII and RNAIII, respectively (figure 1.5) (Novick and Muir, 1999). The RNAII transcript encodes four proteins: AgrA, AgrB, AgrC and AgrD. AgrB and AgrD combine to form a thiolactone containing peptide pheromone (Ji, Beavis and Novick, 1995). The pheromone precursor peptide is encoded by AgrD, which requires processing by AgrB prior to secretion (Novick and Muir, 1999). The secreted pheromone accumulates in the extracellular milieu where it functions as a ligand for AgrC signal receptors and, therefore, mediates cell to cell communication. It has a conserved cysteine (five residues from the C-terminus)

linked to the carboxy group of the mature protein via a thiolactone bond (Novick and Muir, 1999). AgrA and AgrC are the response regulator and transmembrane signal-dependent histidine kinase, respectively. Together they form a two component regulatory system. The pheromone binds to the AgrC signalling pathway and, in conjunction with SarA, activates RNAII and RNAIII transcription (figure 1.5) (Novick and Muir, 1999). The RNAIII transcript, which is maximally produced during the post-exponential phase (van Wamel *et al.,* 1998), generally up-regulates transcription of exocellular protein genes and down-regulates the transcription of surface-associated protein genes (Vuong, Götz and Otto, 2000). The precise mechanism of expression control by RNAIII remains unknown. Additionally, as observed with *S. aureus* and *S. epidermidis,* but not *S. lugdunensis*, there is δ-toxin mRNA within the RNAIII transcript (*hld*) (van Wamel *et al.,* 1998; Vandenesch *et al.,* 1993b; Janzon, Lofdahl and Arvidson, 1989).

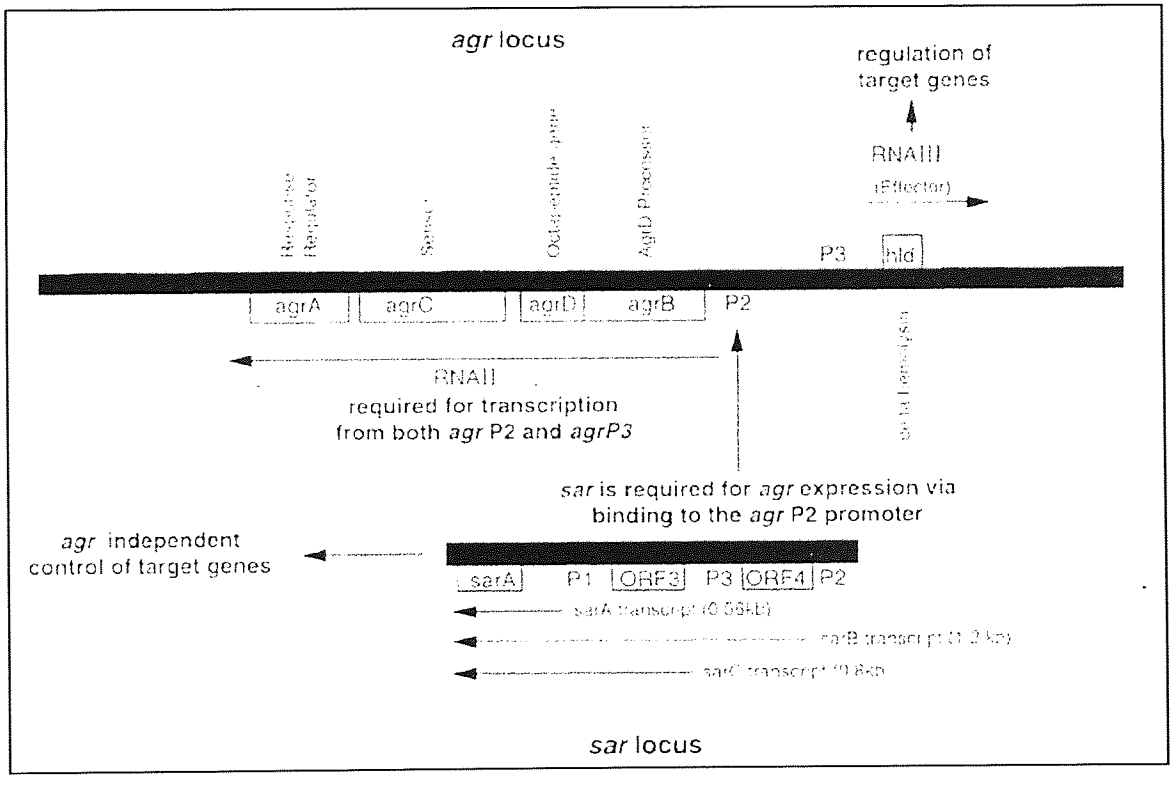


Figure 1.5. The staphylococcal *agr* and *sar* loci. The *agr* promoters P2 and P3 generate the RNAII and RNAIII transcripts, respectively. RNAII encodes AgrA, B, C, and D. An autoinducer is formed by the processing of AgrD by AgrB. This autoinducer binds to the AgrC two component regulatory system and, in conjunction with SarA, activates transcription of the P2 and P3 promoters. Reproduced from Projan and Novick, 1996.

The *sar* locus (staphylococcal accessory regulator), identified in both *S. aureus* and *S. epidermidis*, is composed of three overlapping transcripts (Fluckiger, Wolz and Cheung, 1998; Cheung *et al.,* 1992). In *S. epidermidis* the SarA promoter, if not all three, is maximally transcribed during the post-exponential phase, unlike the *S. aureus* homologue, which is most abundant during the early-exponential phase (Fluckiger, Wolz and Cheung, 1998; Bayer, Heinrichs and Cheung, 1996). It is currently thought that the temporally controlled SarA DNA binding protein binds directly to the P2 promoter of *agr* during the late-exponential phase of growth and acts as a transcription factor for RNAIII (figure 1.5) (Fluckiger, Wolz and Cheung, 1998; Heinrichs, Bayer and Cheung, 1996).

The temporal expression of *agr* and *sar* is based on a quorum sensing system and is responsible for the differential expression of exoproteins (such as lipase and haemolysins, which are expressed during the post-exponential phase of growth) and surface proteins (for example the collagen binding receptor, which is repressed during the post-exponential phase). Using animal models, mutation in the *S. aureus agr* and/or *sar* loci has been demonstrated to attenuate virulence (Booth *et al.,* 1997; Nilsson *et al.,* 1996). In *S. epidermidis*, *agr* has been shown to be involved in the expression of both a protease and a mature lipase during the post-exponential phase of growth (Vuong, Götz and Otto, 2000).

1.3.6 Staphylococcal virulence factors

The pathogenic capacity of *S. aureus* was recognised by Ogston in the late 19th century when he described the organism as an aetiological agent of furuncles and abscesses (Easmon and Goodfellow, 1990). This organism produces a plethora of surface-bound and secreted virulence determinants. Virulence factors or determinants can be defined as "all those factors contributing to infection as well as disease, with the exception of 'housekeeping' functions that are required for efficient multiplication on non-living substrates" (Mekalanos, 1992). Although CNS are less virulent than *S. aureus*, they are capable of expressing a number of potential virulence factors (table 1.4). Amongst these virulence factors are adherence receptors for host extracellular matrix proteins, such as fibronectin and collagen, which are collectively termed microbial surface components recognising adhesive matrix

molecules (MSCRAMMs) (Pattie *et al.*, 1994a). Other determinants potentially allow evasion of host defence systems, for example products that interfere with or inhibit phagocytosis. Finally, an array of secreted factors, such as cytolytic toxins and tissue-degrading enzymes are thought to be involved in tissue invasion (Lambe *et al.*, 1990). A number of these potential CNS virulence factors are discussed in more detail in sections 1.3.6.1 to 1.3.6.7.

Table 1.4. Possible virulence determinants of CNS

Proposed Virulence Factor	**Reference**
Factors potentially associated with attachment	
Fibronectin binding receptor	Valentin-Weigand *et al.*, 1993 Paulsson *et al.*, 1992 Switalski *et al.*, 1983
Fibrinogen binding receptor	Pei *et al.*, 1999 Nilsson *et al.*, 1998
Vitronectin binding receptor	Paulsson *et al.*, 1992 Paulsson and Wadström, 1990 Lundberg *et al.*, 1997
Laminin binding receptor	Paulsson *et al.*, 1992
Collagen binding receptor	Paulsson *et al.*, 1992 Paulsson and Wadström, 1990 Watts *et al.*, 1990
Biofilm/ adhesion/ polysaccharide slime	Hussain *et al.*, 1997 Heilmann *et al.*, 1997 Veenstra *et al.*, 1996 Mack *et al.*, 1992 Christensen *et al.*, 1990 Tojo *et al.*, 1988 Christensen *et al.*, 1982
von Willebrand factor	Li *et al.*, 2000b
Haemagglutinin	Rupp *et al.*, 1995 Rupp and Archer, 1992
Heparin/ heparinized surfaces	Paulsson *et al.*, 1994

Factors potentially associated with evasion of host defences	
Enterotoxin A, B or C	Udo *et al.*, 1999 Crass and Bergdoll, 1986
Fatty acid modifying enzyme (FAME)	Chamberlain and Brueggemann, 1997 Long *et al.*, 1992
Toxic shock syndrome toxin-1 (TSST-1)	Udo *et al.*, 1999 Butt *et al.*, 1998 Crass and Bergdoll, 1986
Lipase (including esterase)	Götz *et al.*, 1998 Molnàr *et al.*, 1994 Farrell *et al.*, 1993 Lambe *et al.*, 1990
Iron restriction (transferrin-binding proteins and environmental signal)	Modun *et al.*, 1994 Deighton and Borland, 1993
Factors potentially associated with tissue invasion	
Protease activity (non-specific protease and elastase)	Zhang and Maddox, 2000 Watts *et al.*, 1990 Janda, 1986 Varadi and Saqueton, 1968
DNase	Molnàr *et al.*, 1994 Lambe *et al.*, 1990
Urease	Schäfer and Kaltwasser, 1994 Gatermann *et al.*, 1989
Cytolytic toxins (haemolysins δ and β)	Butt *et al.*, 1998 Molnàr *et al.*, 1994 Lambe *et al.*, 1990 Hébert, 1990 Scheifele and Bjornson, 1988 Gemmell, 1987

Although factors are placed within distinct groupings, determinants are not necessarily limited to a single pathogenic role. For example the extracellular polysaccharide slime substance is a proposed adherence factor but evidence also suggests that it is capable of impeding phagocytosis and promoting antimicrobial resistance (Christensen, Baldassarri and Simpson, 1994).

1.3.6.1 Proteinase activity

Proteinases are central to survival, multiplication and pathogenicity of the bacterium within the host (Finlay and Falkow, 1997; Patrick and Larkin, 1995). These enzymes mediate tissue degradation, attenuation of the host defence mechanisms and modulation of the immune system (Travis, Potempa and Meada, 1995). The various functions are summarised in figure 1.6.

Elastin is a fibrous macroprotein usually present in tissues subject to stretching such as skin, arteries and lung (Goldberg and Rabinovitch, 1988). Evidence suggests that the *S. epidermidis* elastase, like the *S. aureus* equivalent (Potempa *et al.*, 1988), is a cysteine proteinase enzyme (Sloot *et al.*, 1992).

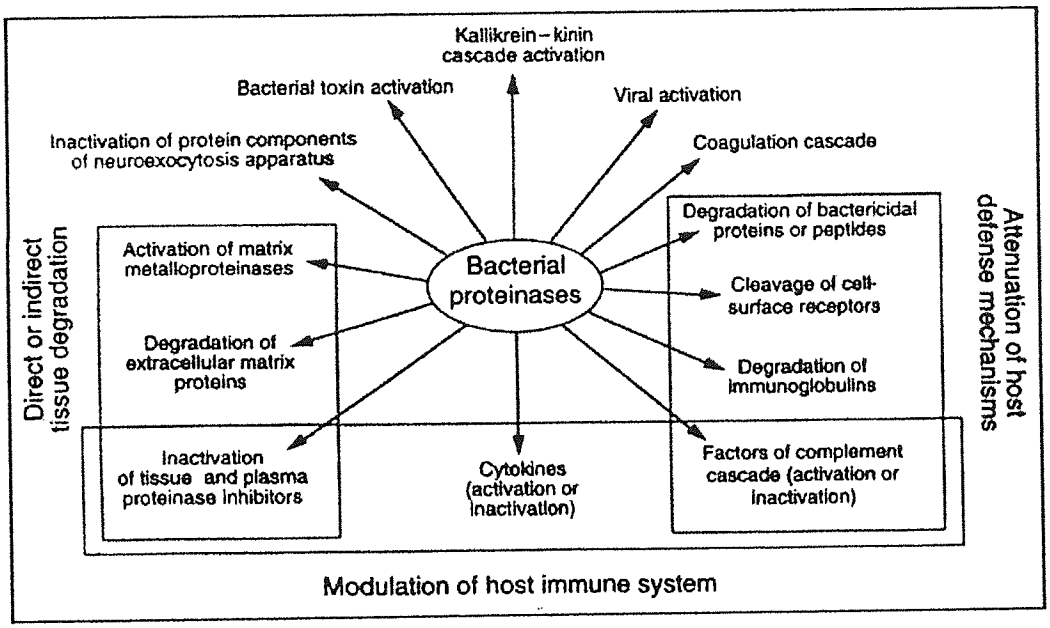


Figure 1.6. Multiple functions of bacterial proteinases in infection. Reproduced from Travis, Potempa and Meada, 1995.

1.3.6.2 Lipase and esterase

The secretion of "true" lipases (glycerol ester hydrolase) and esterases (lipolytic activity on water-soluble substrates) is a common phenomenon of both environmental and pathogenic microorganisms (Jaeger *et al.*, 1994; Smeltzer, Hart and Iandolo, 1992). *S. aureus* and CNS have been shown to secrete lipases and esterases, which expressed no substrate specificity, during the post exponential phase

of the growth cycle (Vuong, Götz and Otto, 2000; Farrell, Foster and Holland, 1993; Rollof and Normark, 1992; Saggers and Stewart, 1968). Currently the physiological roles of these enzymes remain unknown but, like iron scavenging mechanisms, lipase may have both nutritional and virulence functions.

1.3.6.3 Extracellular polysaccharide slime layer

The elaboration by CNS of an extracellular mucoid substance in association with an implanted shunt was first described in 1972 (Bayston and Penny, 1972). This secreted substance, commonly referred to as "slime" (Christensen *et al.*, 1982), soon became associated with device-related CNS infection and pathogenicity. For the last 20 years, various groups have investigated the slime phenomenon and, although numerous components have been identified, the definitive composition and regulation of this slime substance remains elusive. Although the term "slime" remains in common use, it is more appropriate to refer to the material as biofilm. Due to the extensive amount of work published covering this particular area the reader is referred to a review article covering early publications (Hussain, Wilcox and White, 1993), whilst more recent work will briefly be discussed.

Biofilm formation may be divided into two stages: a) rapid initial attachment to the surface, b) cell proliferation and expression of a polysaccharide intercellular adhesin (PIA). The *ica* operon encodes PIA, a linear β 1,6-linked glucosaminoglycan involved in cell accumulation (Mack *et al.*, 1996; Gerke *et al.*, 1998). Isolates that carry the *ica* operon and express PIA have the capacity to form a biofilm, unlike their *ica*$^-$, PIA$^-$ counterparts (Heilmann *et al.*, 1996). The regulation of this operon is complex and may involve three or more other gene loci (Mack *et al.*, 2000). Additionally, phase variation of PIA appears to be controlled by the insertion and excision of an insertion sequence element (Ziebuhr *et al.*, 1999). There is a growing body of evidence demonstrating that PIA expression and biofilm formation are essential virulence factors (Rupp *et al.*, 1999a; Rupp *et al.*, 1999b).

A number of other biofilm components have been described. Polysaccharide/adhesin (PS/A) originally reported by Tojo *et al.* (1988) has recently been identified as sharing the β 1,6-linked glucosamine backbone described in PIA (McKenney *et al.*,

1998). Furthermore, both PIA and PS/A are encoded by the *ica* operon (McKenney *et al.*, 1998). Christensen *et al.* (1990) reported a slime-associated antigen (SAA) composed of primarily N-acetyl-glucosamine (Baldassarri *et al.*, 1996; Christensen *et al.*, 1990). This antigen is exclusively produced by *S. epidermidis* strains and is associated with thick biofilm formation (Ammendolia *et al.*, 1999). Other bacterial products associated with slime and biofilm formation include:

- AtlE, an autolysin that mediates the initial attachment of the cells to a polymer surface (Heilmann *et al.*, 1997).
- Adhesion-associated protein (AAP), a 140kDa extracellular protein associated with the accumulation stage of biofilm formation (Hussain *et al.*, 1997).
- An immunogenic, slime-associated 20kDa polysaccharide (Karamanos *et al.*, 1997).
- Two staphylococcal surface proteins (SSP-1 and SSP-2), which may form a linear fimbrial structure, thought to initiate irreversible cell binding to the polymer surface (Veenstra *et al.*, 1996; Timmerman *et al.*, 1991).
- Wall-derived teichoic acid (Hussain *et al.*, 1992).

1.3.6.4 Adherence to a polymer surface

The accumulation of bacteria onto a solid surface, generally referred to as adhesion or adherence, initially involves a number of physico-chemical interactions between the bacterial cell surface and the foreign substratum. When the cell comes into close contact with the substratum, through the action of forces such as van der Waal's, hydrophobicity, and electrostatic forces, irreversible adhesion occurs (figure 1.7) (Allison, 1993; Rupp, 1996). MSCRAMMs and extracellular polysaccharide slime components such as AtlE, PS/A, SSP-1 and SSP-2 have been proposed to mediate attachment to a polymer surface (McKenney *et al.*, 1998; Heilmann *et al.*, 1997; Veenstra *et al.*, 1996; Patti *et al.*, 1994a). Subsequently a biofilm of multi-layered cells, held together by an intercellular polysaccharide antigen, embedded within an amorphous extracellular layer develops (Rupp, 1996).

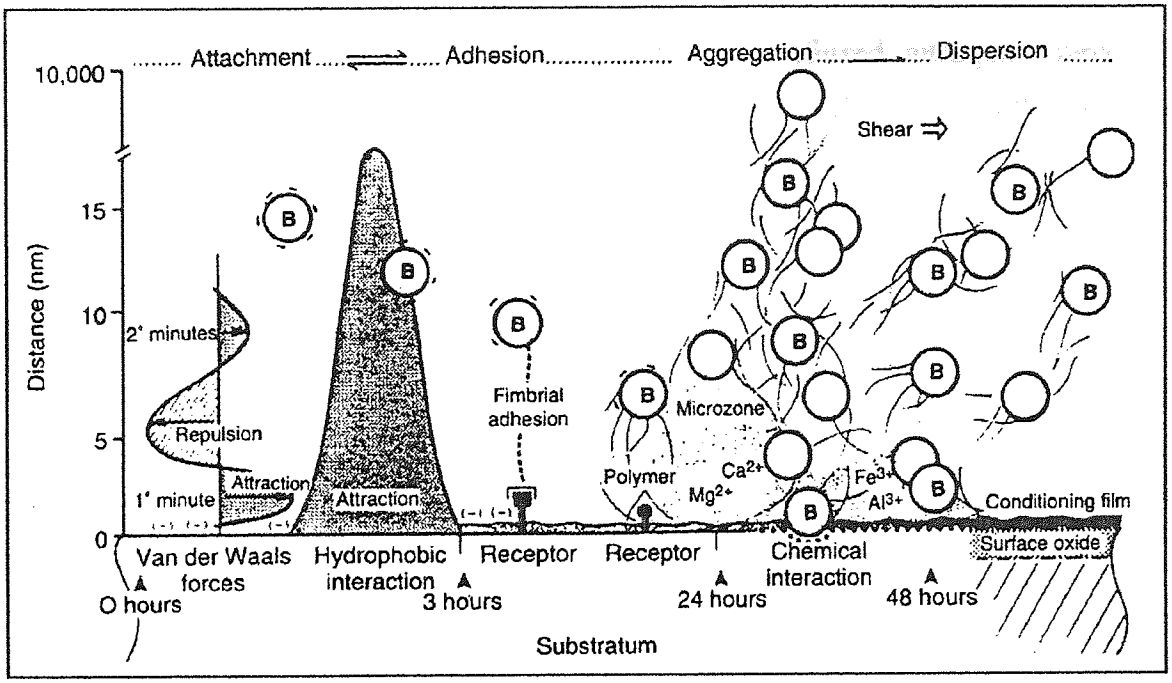


Figure 1.7. The events associated with bacterial adherence to a biomaterial in relation to time, distance from biomaterial, and stage of adherence. Non-specific physical forces mediate the initial attachment stage. Adhesion involves the interaction between specific adhesin and receptors. Finally the cells aggregate resulting in bacterial macrocolonies on the surface of the biomaterial. These cells are firmly attached to the biomaterial and to each other. Extracellular polysaccharide covers the cells creating a protective microenvironment. Bacterial seeding of the bloodstream occurs as cells disaggregate or are washed from the surface. B, bacterial cells. Reproduced from Gristina, 1987.

1.3.6.5 Urease

Numerous bacteria are capable of producing urease (urea amidohydrolase), a nickel metalloenzyme that catalyses the hydrolysis of urea using the following reaction;

$$\text{urea} + \text{water} \xrightarrow{\text{urease}} \text{ammonia} + \text{carbamate}$$

$$\text{carbamate} \xrightarrow{\text{spontaneous reaction}} \text{ammonia} + \text{carbonic acid}$$

A net pH increase occurs during the reaction (Mobley, Island and Hausinger, 1995).

In many microorganisms urease activity generates a preferred nitrogen source, however expression of the enzyme has been associated with virulence (Collins and D'Orazio, 1993). Pathogenesis has been correlated with the ureolytic activity of *Proteus mirabilis* (urolithiasis and renal pathology) and *Helicobacter pylori* (gastritis and peptic ulceration) (Mobley, Island and Hausinger, 1995; Smoot *et al.,* 1990). In staphylococcal species a urease gene cluster has been identified in isolates of *S. aureus*, *S. epidermidis*, *S. xylosus* and *S. saprophyticus*, but only in the latter species has a pathogenic role been elucidated (Mobley, Island and Hausinger, 1995; Gatermann and Marre, 1989a). The constitutively expressed *S. saprophyticus* urease was shown to contribute to cystopathogenicity during urinary tract infection in an animal model (Collins and D'Orazio, 1993; Gatermann and Marre, 1989b).

1.3.6.6 DNase

Although the ability to produce DNase is generally associated with coagulase-positive members of the genus, coagulase-negative species such as *S. caprae* are equally capable of generating nuclease active enzymes (Shuttleworth *et al.,* 1997). Release of DNA into the immediate environment of the bacterium increases viscosity. It has been postulated that this aids tissue invasion by the bacteria (Patrick and Larkin, 1995).

1.3.6.7 Cytolytic toxins

The elaboration of cytolytic toxins, frequently referred to as haemolysins, can be of benefit to an invading microorganisms in several ways:

- Provision of essential nutrients, such as iron (Lebek and Gruenig, 1985).
- Lysis of host cells aids tissue invasion.
- Destruction of components of the immune system allows the pathogen to circumvent the host's defence mechanisms (Patrick and Larkin, 1995).

S. aureus produces at least three cytolytic toxins: α, β, and δ-haemolysins (Projan and Novick, 1996; Jordens, Duckworth and Williams, 1989). Subunits of the α-toxin combine on a cell membrane to generate a pore leading to cell death (Jonas *et al.,*

1994). The β-lysin acts as a sphingomyelinase and the δ-haemolysin appears to form channels within the membrane (Projan and Novick, 1996; Titball, 1993). As observed with many extracellular proteins, cytolytic toxins are regulated by the *agr* locus and are expressed during the late exponential phase of growth (van Wamel *et al.*, 1998; Cheung and Ying, 1994).

1.3.7 Investigating pathogenicity *in vivo*

It is now widely accepted that the expression of virulence determinants is stimulated and regulated by the conditions encountered in the host, such as nutrient starvation, stress, pH, oxygen tension and iron availability (section 1.3.5.1) (Mekalanos, 1992). Microbial pathogenicity should be investigated in conditions that simulate those of the host environment.

Advances in molecular biology techniques have allowed the interaction between pathogen and host to be investigated both *in vivo* and *in situ*. Gene fusion, subtractive hybridisation, *in vivo* expression technology (IVET), signature tagged mutagenesis and differential fluorescence induction are strategies developed for the investigation of bacterial virulence genes (Handfield and Levesque, 1999; Chopra *et al.*, 1997). A number of these methodologies have been applied to the investigation of *S. aureus* virulence (Lowe, Beattie and Deresiewicz, 1998; Mei *et al.*, 1997). Another method of investigating *in vivo* gene expression is to utilise the immune response of a patient to a specific pathogen to screen for *in vivo* expressed gene products. Lowe *et al.* (1995) and Xu *et al.* (1997) both used patient serum to screen the genomic libraries of two *Enterococcus faecalis* strains for cellular and secreted products that were expressed *in vivo* and induced an immune response within the patient. An advantage of this method is that it not only identifies *in vivo* expression, but it also demonstrates interaction with the immune system. This is important if the product is to be considered as a marker of infection.

With the advent of IVET type methodologies the traditional definition of a virulence factor (for example toxins or adhesins) is being continually redefined and expanded to include various determinants that are expressed specifically during the host-pathogen interaction and potentially includes factors that allow the survival and

persistence of a bacterium. Bacterial products that aid the procurement of iron would traditionally be considered as purely metabolic in function, however the contribution to pathogenicity is now widely accepted (Bullen and Griffiths, 1999).

1.3.8 Serodiagnosis of staphylococcal infection

The first serodiagnostic test for staphylococcal infection detected anti-α-toxin antibody (anti-staphylolysin). In serious *S. aureus* infections the anti-staphylolysin antibody levels may be elevated, however the absence of antibody does not exclude infection. The serious deficiencies of this test have limited its use in diagnostic laboratories (Easmon, 1990). Early efforts to develop a routine serodiagnostic test for staphylococcal infection focused primarily on cellular antigens and wall teichoic acid in particular. Crowder and White (1972) attempted to distinguish *S. aureus* IE from other *S. aureus* infections, such as bacteraemia without clinical evidence of cardiac involvement, by the measurement of anti-wall teichoic acid antibodies levels in serum. They detected wall teichoic acid precipitins in patients with *S. aureus* and CNS-related IE as well as a number of patients with other *S. aureus* infections though precipitins were not detected in IE due to other organisms (Crowder and White, 1972). Whilst other groups continued to develop this idea, background levels of anti-wall teichoic acid antibody in the general population, inter-laboratory variability (sensitivity of the assay in patients with *S. aureus*-associated IE ranged from 61% to 100%) and low reproducibility limited progress (Julander *et al.*, 1983; West *et al.*, 1983; Jackson, Sottile and Aguilar-Torres, 1978). Eventually an anti-wall teichoic acid antibody based commercial kit, ENDO STAPH, was produced (Wheat *et al.*, 1984). Despite favourable reports the kit failed to become widely employed. Following the limited success of wall teichoic acid as a serological marker of infection, other cellular antigens were investigated, including peptidoglycan and lipoteichoic acid (Wergeland *et al.*, 1984; Verbrugh *et al.*, 1983). Again background levels of antibody, low sensitivity and cross-reactivity limited the success of tests based on these antigens. Attention then moved from cellular to exocellular antigens and the development of tests to distinguish serious staphylococcal infection from uncomplicated septicaemia (Christensson, Fehrenbach and Hedström, 1985; Espersen *et al.*, 1986). Antibody to a number of exocellular *S. aureus* antigens was demonstrated to distinguish *S. aureus* osteomyelitis from bone infections of other

aetiologies (Lambert *et al.*, 1996; Lambert *et al.*, 1992; Krikler and Lambert, 1992). As a progression of this work, an enzyme-linked immunosorbent assay (ELISA) based on the detection of anti-lipid S (a secreted short chain form of lipoteichoic acid) antibody has been developed at Aston University and the Queen Elizabeth Hospital (QEH), Birmingham (Lambert *et al.*, 2000; Elliott *et al.*, 2000).

There is currently no widely employed serological test for CNS infection in use in diagnostic laboratories. The availability of such a test would:

- Allow earlier diagnosis.
- Allow directed antimicrobial therapy to be instigated earlier.
- Confirm or reject a diagnosis based on inconclusive laboratory findings (such as the recovery of a single CNS isolate from a blood culture).
- Aid diagnosis in circumstances where recovery of an organism is difficult (for example if antibiotics were given prior to taking a blood sample).
- Be of significant benefit to both the patient and the National Health Service.

1.3.8.1 The lipid S ELISA

Lipid S is a novel short chain glycolipid closely related to the cellular lipoteichoic acid produced by most Gram-positive pathogenic organisms (Lambert *et al.*, 2000) (figure 1.8).

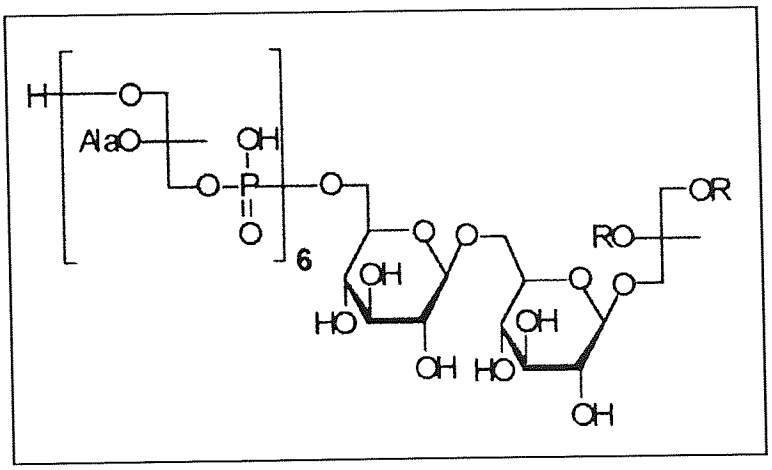


Figure 1.8. Structure of lipid S from *S. epidermidis*. R, ester-linked fatty acids; Ala, ester-linked D-alainine. Reproduced from Lambert *et al.*, 2000.

This soluble intermediate component of lipoteichoic acid biosynthesis is secreted by the *Staphylococcus* and, therefore, the host immune system is continually exposed to the antigen, particularly during chronic infections such as CNS-associated IE (Lambert *et al.,* 2000). An ELISA to detect and quantitate anti-lipid S antibody has been developed jointly at Aston University and the QEH, Birmingham (Elliott *et al.,* 2000; Rafiq *et al.,* in press, 2000). Sera from patients with a significant Gram-positive bacterial infection have anti-lipid S IgG antibody titres of 20,000 to >400,000, whilst patients with infections caused by other microorganisms and healthy individuals have background level titres of <20,000. The test is capable of distinguishing between patients with Gram-positive coccal endocarditis and those without; a sensitivity of 82% and specificity of 92% has been observed (Connaughton *et al.,* 1999; Connaughton *et al.,* 1998; Elliott *et al.,* 1997).

1.4 AIMS AND OBJECTIVES OF THE STUDY

CNS are a major cause of PVE and occasional aetiological agents of NVE. It is not precisely understood how this group of commensal bacteria produces infections associated with high rates of morbidity and mortality. It is unclear whether there are particular virulence determinants that predispose the microorganism to cause IE or indeed if there are particular characteristics essential to the infection. A number of potential virulence factors have been proposed, but with perhaps the exception of the extracellular slime substance/intracellular adhesins, their role in pathogenicity is unclear. None of the proposed virulence factors investigated in previous studies has been found to be essential in the pathogenicity of CNS-related IE.

The aims of this study were to:

- Ascertain whether there are specific CNS genotypes with a predilection to cause IE.
- To identify any virulence characteristics that are primarily expressed by IE-associated strains.
- To assess any identified virulence factors as markers of infection.

In the following chapters of this thesis these aims will be investigated. In Chapter 3 clinical isolates of CNS obtained from a range of significant infections; IE, CVC-

associated septicaemia, CAPD-related peritonitis, and osteomyelitis related to internally fixed bone fractures and prosthetic hip joints were genotyped by PFGE. The relatedness of isolates between patients and between specific infection types was investigated.

In Chapter 4 the CNS strains collected from various infections were investigated for the expression of toxins and enzymes which are potential virulence factors. The organisms were evaluated for any correlation between production of the potential virulence determinant and the associated infection or related genotype.

In Chapter 5 the range of potential virulence factors investigated was extended to include MSCRAMMs. The organisms were again evaluated for any correlation between expression of the MSCRAMM and the associated infection or related genotype.

In Chapter 6 the immune response of a patient with active *S. epidermidis* IE was exploited to screen a genomic DNA library of *S. epidermidis* NCTC 11047 for cell-associated and secreted bacterial products that are expressed *in vivo*.

In Chapter 7 the antigenic cell-associated and secreted staphylococcal products, which stimulated an immune response in an IE patient, were investigated for any function that may contribute to the pathogenicity of the microorganisms.

In Chapter 8 the immune response instigated against an identified potential virulence factor was measured by an ELISA technique and was evaluated as a serodiagnostic marker of CNS-associated IE.

CHAPTER 2- BACTERIAL STRAINS, SERA, GROWTH MEDIA AND REAGENTS

2.1 BACTERIAL STRAINS

2.1.1 Coagulase-negative staphylococci

A total of 130 CNS isolates were recovered from 89 patients with clinical presentations of infection: CVC-associated septicaemia; CAPD-related peritonitis; IE; osteomyelitis related to prosthetic hip joints and internally fixed bone fractures (table 2.1). All patients with suspected IE were assessed using the Duke criteria (section 1.2.5, appendix 1). Only those patients classified as either Duke definite or possible IE cases were included in the study (appendix 2).

Table 2.1. Origin of CNS included in the study

Type of infection	Associated medical centre	Number of patients included in study	Total number of isolates examined
CVC	QEH	15	27
CAPD	QEH	24	25
IE	QEH[1]	13	36
Internally fixed bone fracture	HH[2]	17	17
Prosthetic hip	ROH	20	25
Total		**89**	**130**

QEH, isolates recovered from clinical specimens by the Clinical Microbiology Laboratory, Queen Elizabeth Hospital (QEH), Birmingham; QEH[1], isolates recovered by the Clinical Microbiology Laboratory, QEH from clinical specimens taken from patients referred to QEH from the West Midlands region; ROH, isolates recovered by the Clinical Microbiology Laboratory, QEH from clinical specimens taken from patients at the Royal Orthopaedic Hospital, Birmingham; HH[2] patients referred to Hope Hospital, Salford from the north-west region. The internally fixed bone fracture strains were supplied by Prof. D. R. Marsh, Dept. of Orthopaedics, The Queen's University of Belfast, and J. Elliott, Dept. of Microbiology, Hope Hospital, Salford.

2.1.2 Speciation of coagulase-negative staphylococci

The clinical isolates were biotyped using the API STAPH Analytical Profile Index (bioMérieux, France), following the manufacturer's instructions, and accordingly assigned to a species. A number of the API profiles required additional tests:

- *Novobiocin sensitivity*

A bacterial suspension of the test strain was inoculated onto half of an iso-sensitest agar plate. The second half of the plate was inoculated with a novobiocin sensitive control strain; *S. epidermidis* NCTC 11047 (Holt *et al.,* 1994). A 5μg novobiocin disc (Oxoid, UK) was positioned on the interface between the two strains and the plate incubated at 37°C for 18h. A zone of inhibition around the test isolate equal in size to that of the control strain indicated sensitivity to the antibiotic, whereas a zone reduced in size denoted antibiotic resistance.

- *Turanose acidification*

Sterile carbohydrate acidification broth [1% (w/v) peptone, 0.5% (w/v) NaCl, 18μg/ml phenol red, pH 7.4] was supplemented with 1% (w/v) filter sterilised turanose solution and dispensed in 3ml aliquots. A turanose broth was inoculated with 30μl of an 18h tryptone soya broth (TSB) culture of the bacterial strain. Positive control strain (*S. saprophyticus* ROH4), negative control strain (clinical strain of *S. lugdunensis* LON2) (Holt *et al.,* 1994), and test strain inoculated broths, and an un-inoculated control broth were incubated at 37°C for 18h. Turanose acidification was indicated by a yellow/orange coloration whilst negative samples remained red (Isenberg, 1992).

- *β-glucosidase assay*

Phosphate buffer [0.067M Na_2HPO_4, 0.067M KH_2PO_4, pH 8.0] was supplemented with 0.1% (w/v) β-glucopyranoside. The solution was filter sterilised and dispensed into 0.5ml aliquots. Aliquots of the β-glucopyranoside substrate were inoculated directly with three to five colonies taken from a brain-heart infusion agar (BHIA) plate of the test strain, the positive control (clinical isolate of *S. lugdunensis* LON2) and the negative control strain (*S. capitis* CAPD11a) and incubated at 37°C for 18h. (Holt *et al.,* 1994). A yellow coloration of the initially colourless substrate indicated β-glucosidase activity (Isenberg, 1992).

A few bacterial strains gave either a low discrimination profile or a profile that was not recognised by the API database (version 3.1). Isolates that repeatedly failed to

identify were confirmed to be tube-coagulase negative, catalase positive, Gram-positive cocci. These strains were referred to as CNS.

- *Gram's stain*

Morphology and Gram's reaction were evaluated by the standard microbiological Gram's stain.

- *Catalase production*

Fifty µl of 6% (v/v) hydrogen peroxide was combined with a bacterial colony on a glass slide. The liberation of oxygen from the hydrogen peroxide, denoted by the immediate release of bubbles, indicated the presence of the bacterial catalase enzyme.

- *Tube-coagulase test*

A bijou bottle containing 0.5ml of brain-heart infusion broth (BHI) was inoculated with two to three colonies of the test strain and combined with an equal volume of human plasma (Blood Bank, QEH). The suspension was incubated at 37°C for 4h and then transferred to 20°C for a further 16h. At incubation times 1, 4, and 20h the broth-plasma suspension was observed for coagulation; indicated by the formation of a plasma clot (Duguid, 1989). *S. epidermidis* NCTC 11047 and a fresh broth-plasma were included as negative controls. *S. aureus* NCTC 6571 provided a positive control.

2.1.3 Additional bacterial strains

- *Staphylococcus*: the type strain *S. epidermidis* NCTC 11047; *S. aureus* NCTC 6571 (Oxford strain); *S. aureus* ATCC 12598 (Cowan I); *S. hominis* K ATCC 35982 (SP-2); *S. epidermidis* ATCC 35984 (RP62A); *S. carnosus* TM300 supplied by Prof. F. Götz, Universität Tübingen, Germany; one clinical isolate of *S. lugdunensis* (LON2); five clinical strains of *S. aureus* isolated at QEH.
- *Streptococcus*: *S. bovis* NCTC 11436; *S. milleri* NCTC 10708; *S. mutans* NCTC 10449; *S. salivarius* NCTC 8618; *S. sanguis* NCTC 7863; clinical strains of

S. gordonii, *S. mitis*, ß-haemolytic streptococcus groups A and G and *S. pneumoniae*.

- *Enterococcus*: *E. faecium* NCTC 7171; *E. faecalis* var. *zymogenes* NCTC 5957.
- *Micrococcus*: laboratory strain of *M. luteus*.
- *Kocuria*: laboratory strain of *K. rosea* (previously known as *Micrococcus rosea*).
- *Propionibacterium*: Six clinical strains of *P. acnes* supplied by Dr. S. Patrick, Department of Microbiology and Immunobiology, School of Clinical Medicine, The Queen's University of Belfast.
- *Bacillus*: a laboratory strain of *B. megaterium*.
- Gram-negative laboratory strains: *Escherichia coli* CIII; *Pseudomonas aeruginosa* PAO1; two *Enterobacter cloacae*; one *Citrobacter diversus*; one *Serratia* species; one *Klebsiella pneumoniae*.

2.1.4 Maintenance and culture of bacterial strains

All bacterial strains were maintained on either Columbia agar base supplemented with 5% (v/v) defibrinated horse blood or BHIA and sub-cultured every four to six weeks. The strains were cultivated in Luria-Bertani (LB) broth [1% (w/v) NaCl, 1% (w/v) tryptone, 0.5% (w/v) yeast extract, pH 7.0], TSB or BHI broth at 37°C for 18h with aeration at 200rpm. The *Streptococcus* species were grown on horse blood agar plates in a CO_2 enriched atmosphere. The *P. acnes* were cultivated on horse blood agar plates in anaerobic conditions and grown in 20ml of LB broth in static universal containers both at 37°C for 72h. All isolates were stored at -70°C in TSB supplemented with 10% (v/v) glycerol.

2.2 SERA

Serum samples were collected from patients with various bacterial infections (n = 61) and control patients with no clinical evidence of infection in the last six months (n = 10) (table 2.2).

Table 2.2. Origin of sera used

Infection Type	Organism	Number of sera	Source
CAPD-peritonitis	*S. epidermidis*	4	QEH
CAPD-peritonitis	*S. haemolyticus*	1	QEH
CAPD-peritonitis	CNS	1	QEH
CAPD-peritonitis	*Enterococcus faecalis*	1	HHB
CAPD-peritonitis	*Streptococcus sanguis*	1	HHB
CVC-sepsis	*S. epidermidis*	5	QEH
CVC-sepsis	*S. epidermidis* and *S. lugdunensis*	1	QEH
Gram-negative sepsis	Gram-negative organisms	10	QEH
IE	*S. aureus*	4	QEH
IE	*S. epidermidis*	3	QEH
IE	*S. simulans*	1	QEH
IE	*S. hominis*	1	QEH
IE	*S. warneri*	1	QEH
IE	*S. epidermidis, S. haemolyticus* and *Enterococcus* species	1	QEH
IE	CNS, *Enterococcus* species and *Enterobacter* species	1	QEH
IE	CNS	5	QEH
IE	*Enterococcus faecalis*	7	BHD
IE	*Streptococcus mitior*	1	HHB
IE	Group G streptococcus	1	HHB
IE	Nutritionally variant streptococcus	1	HHB
Nephrostomy fluid	*Enterococcus faecalis*	1	HHB
Osteomyelitis	*S. aureus*	1	HHB
Osteomyelitis	*Staphylococcus* species	1	HHB
Osteomyelitis	*Enterococcus faecalis*	1	HHB
Prosthetic hip osteomyelitis	*S. aureus*	2	ROH
Prosthetic hip osteomyelitis	Un-speciated CNS	4	ROH
Bone fracture- no infection	-	6	ROH
Coronary artery-by pass graft (CABG)- no infection	-	4	QEH

QEH, Queen Elizabeth Hospital, Birmingham; ROH, Royal Orthopaedic Hospital, Birmingham; HHB, Heartlands Hospital, Birmingham; BHD, Bispebjerg Hospital, Denmark.

Serum collected from a patient with *S. epidermidis* IE, confirmed as definite by the Duke criteria (appendix 1), was designated serum PRT and used for screening the

S. epidermidis NCTC 11047 genomic DNA library and the development of the SsaA ELISA. *E. coli*/lambda phage reactive antibodies were absorbed from PRT by *E. coli*/phage lysate treatment (section 6.2.4.2).

Serum collected from a control patient was designated serum LLD and used in the development of the SsaA ELISA.

All sera used for western blotting were diluted 1:400 in Tris-buffered saline supplemented with bovine serum albumin (TBS-BSA) [20mM Tris-HCl (pH 7.5), 150mM NaCl, 1% (w/v) BSA] and stored at -20°C. All sera used in the ELISAs were diluted 1:400 in Tris-buffered saline Tween 20 (TBS-Tween) [10mM Tris-HCl (pH 7.4), 150mM NaCl, 0.3% (v/v) Tween 20].

2.3 REAGENTS AND MEDIA

All reagents used in this study were standard laboratory grade chemicals purchased from Sigma Chemical Company, USA, except where stated.

All growth media components were purchased from Oxoid Ltd, UK, except where stated otherwise.

CHAPTER 3- PULSED-FIELD GEL ELECTROPHORESIS

3.1 INTRODUCTION

The aim of this section of the study was to investigate the hypothesis that specific CNS genotypes are associated with IE. This was investigated by the analysis of macrorestriction patterns generated by PFGE of the chromosome of CNS isolates following cleavage with a rare cutting restriction endonuclease.

3.2 MATERIALS AND METHODS

3.2.1 Bacterial Isolates

A total of 130 CNS isolates were recovered from 89 patients with clinical presentations of one of the following infections; CVC-associated septicaemia, CAPD-related peritonitis, IE, prosthetic hip joint-associated osteomyelitis or internally fixed bone fracture-related osteomyelitis. The CNS were genotyped by the PFGE technique (table 3.1). The type strain *S. epidermidis* NCTC 11047 was included in the analysis as a reference organism.

Table 3.1. Origin of the individual CNS strains identified by pulsed-field gel electrophoresis

Type of infection	Associated medical centre	Number of patients included in study	Total number of isolates examined	Number of individual strains identified by PFGE
CVC	QEH	15	27	17
CAPD	QEH	24	25	25
IE	QEH[1]	13	36	14
Internally fixed bone fracture	HH	17	17	17
Prosthetic hip	ROH	20	25	21
Total		**89**	**130**	**94**

QEH, patients admitted to Queen Elizabeth Hospital, Birmingham; QEH[1], patients referred to QEH from the West Midlands region; HH, patients referred to Hope Hospital from the north-west region; ROH, patients admitted to Royal Orthopaedic Hospital, Birmingham.

3.2.2 Preparation of chromosomal DNA

Chromosomal DNA was prepared essentially by the method described by Lina *et al.* (1992). Bacterial cells were cultivated in 20ml of BHI broth at 37°C with aeration at 200rpm for 18h. The cells were recovered from 1ml of the culture by centrifugation (11,400*g* × 5min) and re-suspended to a concentration of 20mg wet weight cells/ml in NET-100 [0.1M Na_2EDTA (pH 8.0), 0.1M NaCl, 0.01M Tris (Tris [hydroxymethyl] aminomethane)-HCl (pH 8.0)]. An equal volume of low melting point chromosomal grade agarose (BioRad, USA) [0.9% (w/v) chromosomal grade agarose in NET-100] cooled to 65°C was gently mixed with the cell suspension and the molten agarose-bacterial mixture dispensed into an ice cooled block mould (BioRad). The agarose blocks (three blocks (8mm × 15mm) were prepared for each bacterial strain) were placed into a bijou bottle containing 3ml of lysis solution [6mM Tris-HCl (pH 7.6), 100mM Na_2EDTA (pH 8.0), 1M NaCl, 0.5% (w/v) N-lauroyl-sarcosine (sarcosyl), 1mg/ml lysozyme, 6.6 units/ml lysostaphin] and incubated at 37°C for 24h. The lysis solution was replaced by 3ml of ESP solution [0.5M Na_2EDTA (pH 9.0), 1% (w/v) sarcosyl, 0.15% (w/v) proteinase K] and incubated at 50°C for 48h to remove cellular debris. The blocks, drained of ESP, were washed four times with continuous agitation over an 8h period in TE buffer [10mM Tris-HCl (pH 8.0), 1mM Na_2EDTA (pH 8.0)] (Maslow, Slutsky and Arbeit, 1993). The prepared agarose blocks, containing intact bacterial chromosomes, were stored in TE buffer at 4°C (Birren and Lai, 1993).

3.2.3 Restriction endonuclease digestion of chromosomal DNA

A 1 × 1 × 9mm sliver of an agarose block was suspended in 0.2ml of restriction endonuclease buffer (prepared with sterile double distilled water) and equilibrated at 4°C for 15min. The buffer was replaced with a fresh restriction endonuclease buffer preparation supplemented with 50 units of the restriction endonuclease *Sma*I (Boehringer Mannheim, Germany) or *Sst*II (GibcoBRL, UK) (Pantucek *et al.*, 1996; Snopková *et al.*, 1994). The restriction digest was incubated at 4°C for 15min before transfer to 25°C (*Sma*I restrictions) or 37°C (*Sst*II digests) for 18h. The restriction endonuclease suspension was replaced with 0.2ml ES solution [0.5M Na_2EDTA (pH 9.0) supplemented with 1% (w/v) sarcosyl] and incubated at 50°C for 15min to

inactivate residual enzyme activity. The sliver was then held in 1ml of TE buffer for 15min to ensure the removal of the ES solution. Once drained, the restriction endonuclease digested agarose sliver was subjected to PFGE directly or maintained without drying at 4°C for a maximum of 3 days.

3.2.4 Resolution of macrorestriction fragments by pulsed-field gel electrophoresis

Prepared DNA slivers were loaded into a 1% (w/v) agarose gel (molecular biology grade agarose, BioRad) prepared with 0.5 × TBE [0.45mM Tris-borate, 1mM Na_2EDTA (pH 8.0)]. A bacteriophage lambda (λ) concatamer ladder (BioRad) sliver was incorporated as a DNA size standard. The slivers were sealed into the wells with 0.5% (w/v) molecular biology grade agarose prepared with 0.5 × TBE. The macrorestriction fragments were separated by PFGE performed in a contour-clamped homogeneous electric field (CHEF) electrophoresis system (CHEF-DR III system, BioRad) under the following conditions: a 1-50s ramped switch interval with a voltage gradient of 6V/cm at an angle of 120° was applied over a 24h period. A Tris-borate running buffer [0.5 × TBE] was maintained at 10°C.

3.2.5 Visualisation of resolved DNA fragments

On completion of electrophoresis the gel was stained in 0.5μg/ml ethidium bromide with gentle agitation for 30min, destained in distilled water for a minimum of 1h and the DNA visualised using a ultra violet (UV) light scanner (UVP products, UK) (Maslow, Slutsky and Arbeit, 1993).

3.2.6 Evaluation of pulsed-field gel electrophoresis profile reproducibility

Each isolate was restriction endonuclease digested in duplicate to increase the accuracy of the analysis. A restriction digest of the reference strain *S. epidermidis* NCTC 11047 was included on twelve separate gels for each enzyme. The multiple reference strain band profiles were analysed with the Phoretix 1D Advanced gel analysis computer program (Phoretix International, UK) and the data evaluated

statistically using one-way analysis of variance (one-way ANOVA) (InStat, GraphPad Software Inc.) to assess the reproducibility of the method.

3.2.7 Single patient isolates

The criteria of Tenover *et al.* (1995) for the analysis of macrorestriction profiles were developed for use in the outbreak situation but may equally be applied to isolates recovered from a single patient over a short time period, as described in this study. Multiple isolates from single patients were individually subjected to PFGE and analysed according to the criteria to identify clones and conversely to indicate where two isolates were dissimilar (Tenover *et al.*, 1995). The criteria suggested for the interpretation of PFGE patterns:

- Zero band difference patterns were considered to be indistinguishable isolates.
- Two or three band differences indicated the organisms to be closely related.
- Four to six band differences in the profiles were indicative of possibly related isolates.
- Seven or more band differences in the patterns demonstrated that the two isolates were different (Tenover *et al.*, 1995).

3.2.8 Dendrogram analysis

Interpretation of the macrorestriction fragment patterns, generated in duplicate, was performed using the Phoretix 1D Advanced gel analysis computer program together with supporting visual assessment. The size of the profile bands was evaluated by positional comparison to the bands of the λ phage DNA size standard. Only bands ≥30kb were included in the analysis. Completed gel analysis data was transferred to the Phoretix 1D Database program (Phoretix International) to allow strain comparison by calculation of the Dice coefficient:

$$\text{Dice coefficient} = \frac{2h}{a+b}$$

where a is the total number of bands in isolate A, b is the total number of bands in isolate B, and h is the total number of bands shared by A and B (Dice, 1945).

The Dice coefficient is a measure of the amount of association between both the two isolates. Isolates were clustered by the unweighted pair group method of arithmetic averages (UPGMA) to permit the construction of a dendrogram.

3.3 RESULTS

3.3.1 Macrorestriction fragment profiles

Typical restriction fragment profiles using *Sma*I are shown in figure 3.1.

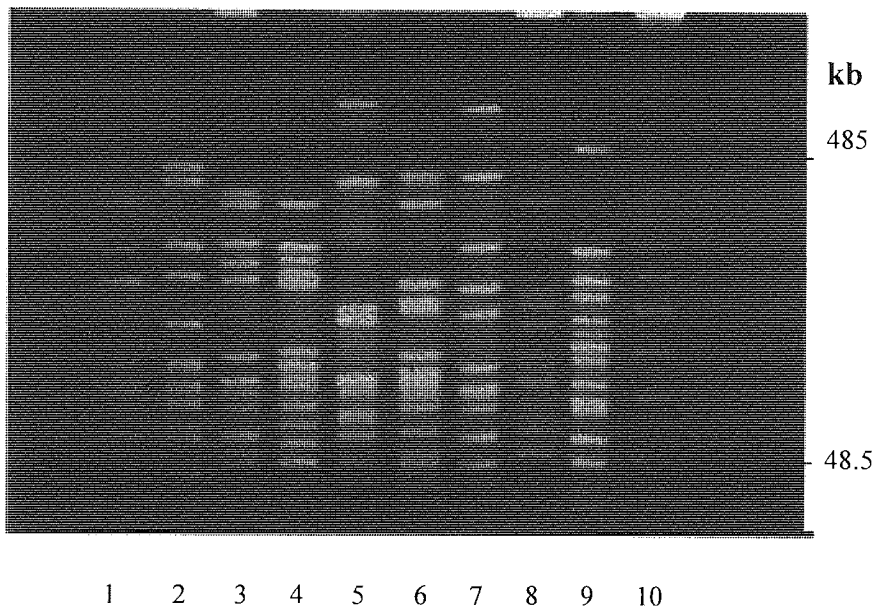


Figure 3.1. Macrorestriction fragment profiles generated by PFGE of *Sma*I restricted CNS. Lane 1, *S. epidermidis* NCTC 11047; lanes 2,3,4,6,7,9, *S. epidermidis*; lane 5, *S. saprophyticus*; lane 8, *S. caprae*; lane 10, bacteriophage λ concatamer DNA size ladder. All the clinical strains (lanes 2 to 9) were isolated from patients with prosthetic hip joint-associated osteomyelitis.

*Sma*I restriction endonuclease digestion of CNS yielded profiles of five to eighteen bands ranging in size from 30kb to 708kb whereas *Sst*II produced five to seventeen bands of 30kb to 724kb in size. For certain species the patterns generated were heterogeneous, for example profiles of *S. epidermidis* varied from seven to eighteen bands (figure 3.1). Other species could be identified by their macrorestriction pattern as exemplified by the *Sst*II digestion profile of *S. simulans* (figure 3.2), which yielded fourteen to sixteen closely positioned bands in the range 30kb to 190kb. Similarly, the *S. lugdunensis Sma*I profile had a characteristic pattern of only five to eight bands in the range 30kb to 666kb (data not shown).

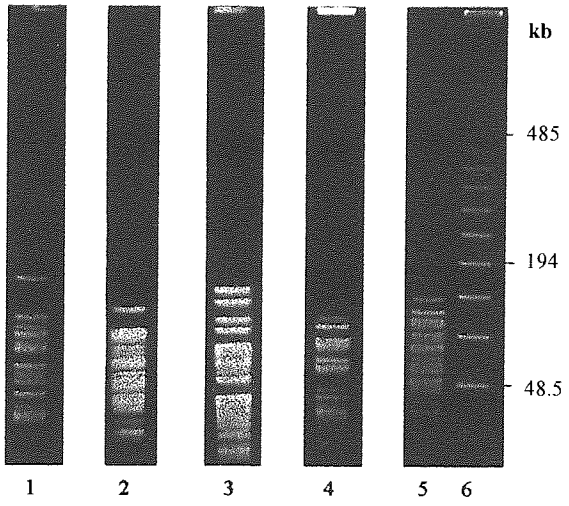


Figure 3.2. Restriction endonuclease digestion of *S. simulans* by *Sst*II. Lanes 1 to 5, clinical strains of *S. simulans*; lane 6, λ phage DNA sizing ladder.

3.3.2 Analysis of the macrorestriction profiles

The macrorestriction profile of each isolate was analysed using the Phoretix 1D Advanced gel analysis software (figure 3.3). The Phoretix Database software used the data obtained from all the strains to construct a dendrogram.

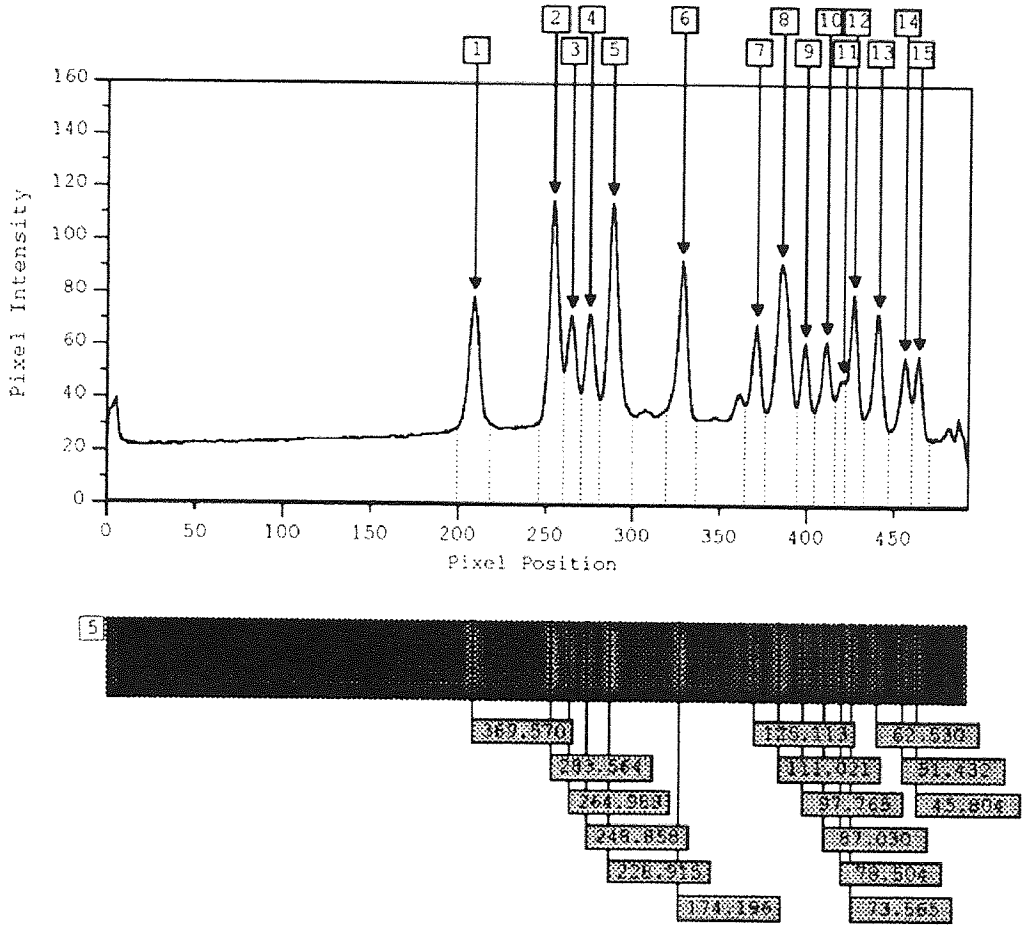


Figure 3.3. Analysis of a *S. epidermidis* strain using the Phoretix 1D Advanced gel analysis software. Pixel intensity of a gel image identified bands of DNA. Positional comparison of detected bands with those of a λ phage DNA standard allowed the size of the DNA fragments in a test sample to be estimated. The size of the fragments (bp) is indicated in the flagged boxes.

3.3.3 Assessment of the pulsed-field gel electrophoresis technique

3.3.3.1 Reproducibility of the macrorestriction profiles

The reproducibility of the macrorestriction profiles (analysed by the Phoretix 1D Advanced gel analysis computer programme) was determined by one way ANOVA of the multiple PFGE profiles (n = 12) of the type strain *S. epidermidis* NCTC 11047. With either restriction endonuclease (*Sma*I or *Sst*II) variance between the gels

was not significantly greater than expected by chance, confirming the reproducibility of the technique (p>0.9999).

3.3.3.2 Ability of the dendrogram to cluster related strains

A dendrogram generated with all the *Sma*I restricted isolates clustered all except one of the multiple *S. epidermidis* NCTC 11047 digests in two closely positioned clusters (Dice coefficient of 0.75). All multiple isolates (considered indistinguishable by Tenover's criteria) were similarly clustered (dendrogram not shown).

3.3.4 Multiple isolates from single patients

Of the 130 isolates recovered from 89 patients, 36 were identified as related strains by the application of Tenover's criteria (table 3.1) i.e. they were multiples of an individual strain (Tenover *et al.*, 1995). For example, four CNS isolates were recovered from two blood culture specimens taken from an IE patient over a 4 day period (table 3.2).

Table 3.2. Microbiological history of an IE patient

Day blood culture taken	Isolates recovered from specimen	Speciation of CNS
1	CNS × 1	*S. epidermidis* × 1
3	*Enterococcus* species	-
4	CNS × 3, *Enterococcus* species	*S. epidermidis* × 2, *S. haemolyticus* × 1

On day 1, a *S. epidermidis* isolate was recovered from this patient. On day 4, three organisms were isolated from a single specimen, two *S. epidermidis* isolates which were subsequently identified as the same strain (by PFGE) and a *S. haemolyticus* strain (figure 3.4).

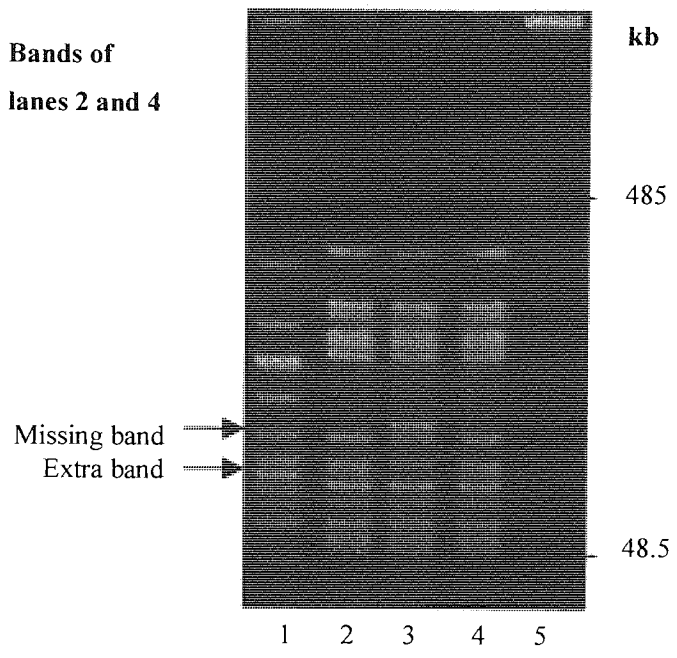


Figure 3.4. The discrimination of four CNS strains isolated from two blood cultures taken from an IE patient over a 4 day period. Lane 1, *S. haemolyticus* isolated on day 4; 2, *S. epidermidis* isolated on day 4; 3, *S. epidermidis* isolated on day 1; 4, *S. epidermidis* isolated on day 4; 5, λ phage sizing ladder.

PFGE analysis and the application of Tenover's guidelines to these samples demonstrated that the *S. epidermidis* isolates (lanes 2, 3 and 4, figure 3.4) were of a single strain despite a two-band difference indicative of a genetic event such as DNA deletion (Tenover *et al.,* 1995; Lina *et al.,* 1993). The profiles provided evidence for a *S. epidermidis* infection in this patient, who had a complicated and otherwise inconclusive microbiological history. For the majority of patients with multiple isolates the restriction profiles of the individual isolates were identical, confirming the diagnosis of a CNS infection.

3.3.5 Relatedness of all strains - dendrograms

The PFGE restriction band profiles of the 94 individual strains (table 3.1) (which included only a single representative of multiple identical isolates recovered from individual patients) were compared by calculation of the Dice coefficient, clustered by UPGMA and this relatedness visualised in the form of dendrograms (figures 3.5a and 3.5b).

Figure 3.5a

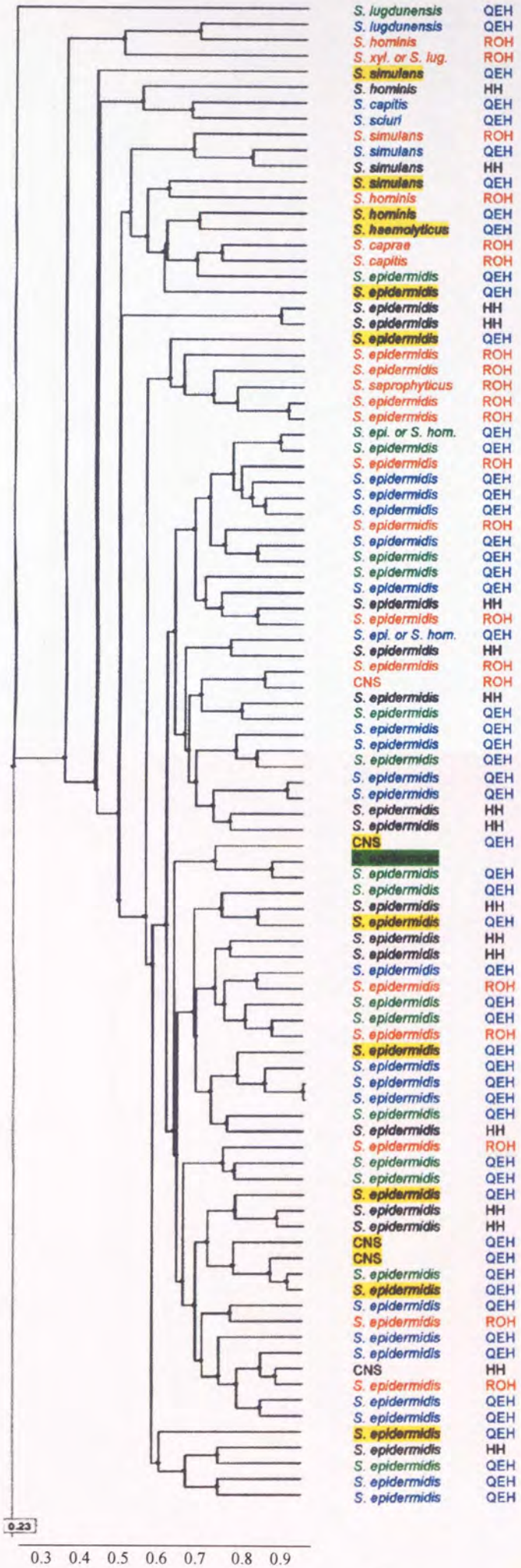
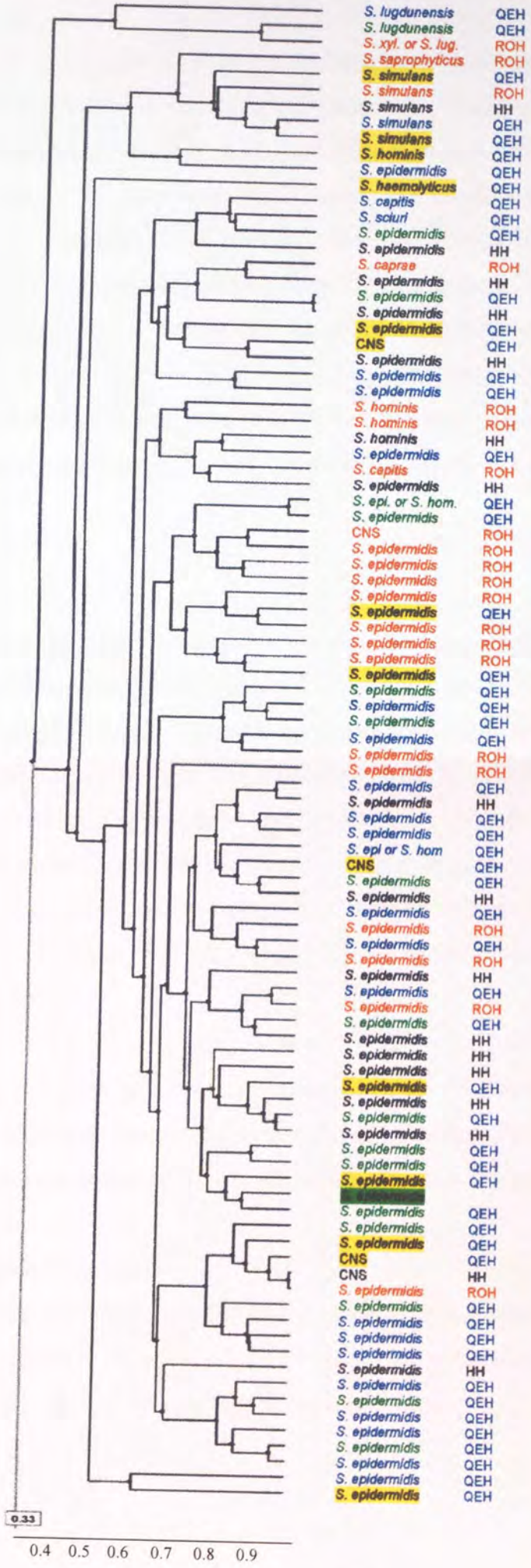

S. lugdunensis QEH
S. lugdunensis QEH
S. hominis ROH
S. xyl. or S. lug. ROH
S. simulans QEH
S. hominis HH
S. capitis QEH
S. sciuri QEH
S. simulans ROH
S. simulans QEH
S. simulans HH
S. simulans QEH
S. hominis ROH
S. hominis QEH
S. haemolyticus QEH
S. caprae ROH
S. capitis ROH
S. epidermidis QEH
S. epidermidis QEH
S. epidermidis HH
S. epidermidis HH
S. epidermidis QEH
S. epidermidis ROH
S. epidermidis ROH
S. saprophyticus ROH
S. epidermidis ROH
S. epidermidis ROH
S. epi. or S. hom. QEH
S. epidermidis QEH
S. epidermidis ROH
S. epidermidis QEH
S. epidermidis QEH
S. epidermidis QEH
S. epidermidis ROH
S. epidermidis QEH
S. epidermidis QEH
S. epidermidis QEH
S. epidermidis QEH
S. epidermidis HH
S. epidermidis ROH
S. epi. or S. hom. QEH
S. epidermidis HH
S. epidermidis ROH
CNS ROH
S. epidermidis HH
S. epidermidis QEH
S. epidermidis QEH
S. epidermidis QEH
S. epidermidis QEH
S. epidermidis QEH
S. epidermidis QEH
S. epidermidis HH
S. epidermidis HH
CNS QEH
S. epidermidis QEH
S. epidermidis QEH
S. epidermidis HH
S. epidermidis QEH
S. epidermidis HH
S. epidermidis HH
S. epidermidis QEH
S. epidermidis ROH
S. epidermidis QEH
S. epidermidis QEH
S. epidermidis ROH
S. epidermidis QEH
S. epidermidis QEH
S. epidermidis QEH
S. epidermidis QEH
S. epidermidis QEH
S. epidermidis HH
S. epidermidis ROH
S. epidermidis QEH
S. epidermidis QEH
S. epidermidis QEH
S. epidermidis HH
S. epidermidis HH
CNS QEH
CNS QEH
S. epidermidis QEH
S. epidermidis QEH
S. epidermidis QEH
S. epidermidis ROH
S. epidermidis QEH
S. epidermidis QEH
CNS HH
S. epidermidis ROH
S. epidermidis QEH
S. epidermidis QEH
S. epidermidis QEH
S. epidermidis HH
S. epidermidis QEH
S. epidermidis QEH
S. epidermidis QEH
0.23
0.3 0.4 0.5 0.6 0.7 0.8 0.9

Figure 3.5b


S. lugdunensis QEH
S. lugdunensis QEH
S. xyl. or S. lug. ROH
S. saprophyticus ROH
S. simulans QEH
S. simulans ROH
S. simulans HH
S. simulans QEH
S. simulans QEH
S. hominis QEH
S. epidermidis QEH
S. haemolyticus QEH
S. capitis QEH
S. sciuri QEH
S. epidermidis QEH
S. epidermidis HH
S. caprae ROH
S. epidermidis HH
S. epidermidis QEH
S. epidermidis HH
S. epidermidis QEH
CNS QEH
S. epidermidis HH
S. epidermidis QEH
S. epidermidis QEH
S. hominis ROH
S. hominis ROH
S. hominis HH
S. epidermidis QEH
S. capitis ROH
S. epidermidis HH
S. epi. or S. hom. QEH
S. epidermidis QEH
CNS ROH
S. epidermidis ROH
S. epidermidis ROH
S. epidermidis ROH
S. epidermidis ROH
S. epidermidis QEH
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S. epidermidis QEH
S. epidermidis ROH
S. epidermidis ROH
S. epidermidis QEH
S. epidermidis HH
S. epidermidis QEH
S. epidermidis QEH
S. epi or S. hom QEH
CNS QEH
S. epidermidis QEH
S. epidermidis HH
S. epidermidis QEH
S. epidermidis ROH
S. epidermidis QEH
S. epidermidis ROH
S. epidermidis HH
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S. epidermidis ROH
S. epidermidis QEH
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S. epidermidis QEH
S. epidermidis QEH
S. epidermidis QEH
S. epidermidis QEH
CNS QEH
CNS HH
S. epidermidis ROH
S. epidermidis QEH
S. epidermidis QEH
S. epidermidis QEH
S. epidermidis QEH
S. epidermidis HH
S. epidermidis QEH
S. epidermidis QEH
S. epidermidis QEH
S. epidermidis QEH
S. epidermidis QEH
S. epidermidis QEH
S. epidermidis QEH
S. epidermidis QEH
0.33
0.4 0.5 0.6 0.7 0.8 0.9

Figure 3.5. Dendrograms based on the Dice coefficient of macrorestriction profiles: a) *Sma*I restriction endonuclease generated fragments; b) *Sst*II restriction endonuclease generated fragments. The horizontal axis is the Dice coefficient. In the first column: CVC-associated sepsis strains are illustrated in green; CAPD-related peritonitis strains are in blue; prosthetic hip joint-associated osteomyelitis isolates are shown in red; internally fixed bone fracture-related osteomyelitis strains are illustrated in black; IE strains are shown in black with yellow highlight; the type strain *S. epidermidis* NCTC 11047 is highlighted with green. In column two: patients treated at QEH, Birmingham, are shown in blue; patients located at the ROH, Birmingham, are depicted in red; patients referred to HH, Salford, are illustrated in black.

There were no obvious clusters illustrated in the dendrograms, however, visual analysis of the macrorestriction profiles clustered in the *Sma*I dendrogram revealed thirteen small clusters of two to five isolates with identical or similar profiles. Some of these similar stains were recovered from patients with the same type of infection, but others were isolated from epidemiologically unrelated patients with different types of sepsis. There was no clear clustering of strains on the basis of infection, associated medical unit or geographical location within either of the dendrograms, nevertheless, there were a number of interesting points. In the *Sma*I based dendrogram:

- All the non-*S. epidermidis* strains (except for the *S. saprophyticus*) were segregated in outlying clusters with a Dice coefficient of <0.5.
- All the strains within the outlying clusters with a Dice coefficient of <0.5 were species other than *S. epidermidis* (except for two strains).

In the *Sst*II dendrogram:

- The strains in the outlying clusters with a Dice coefficient of <0.51 belonged to the species *S. lugdunensis*, *S. saprophyticus*, *S. simulans*, *S. haemolyticus* and one strain each of *S. hominis* and *S. epidermidis*.

- Incorporated within the main body of the dendrogram were all the *S. epidermidis* strains (except one), the *S. hominis* strains (except one), *S. capitis*, *S. caprae* and *S. sciuri*.

In both dendrograms:

- All the strains, which failed to identify with the API system (listed as CNS) were located within the main body of the dendrogram containing the *S. epidermidis* strains. This suggests that these CNS strains are *S. epidermidis*.
- The strain identified as *S. xylosus* or *S. lugdunensis* was located with the *S. lugdunensis* cluster. This suggests that this strain was indeed *S. lugdunensis*, however, there were no *S. xylosus* strains in the analysis for comparison.
- Similarly, both the strains that were speciated as *S. epidermidis* or *S. hominis* were located within the main body of *S. epidermidis* strains and not with the *S. hominis* isolates implying that both belonged to the *S. epidermidis* taxon.

The omission of species other than *S. epidermidis* (which represented only a small proportion of the isolates) from the dendrogram also failed to identify any clustering of related *S. epidermidis* strains with the various groups (data not shown).

3.4 DISCUSSION

Macrorestriction patterns are an estimate of the genetic relatedness of isolates and, therefore, can be equated to the molecular evolution of strains within a taxon. For a technique that measures genetic variability to be successful there has to be genomic heterogeneity within the isolates under investigation. The genetic diversity within and between CNS species, particularly *S. epidermidis* isolates, was confirmed by the macrorestriction profiles and utilised in the evaluation of strain relatedness. This relatedness was measured by comparing the distribution of rare cutting restriction endonuclease sites within the genome. Choosing an enzyme that infrequently cleaves the chromosome was dependant on a number of factors including the nucleotide composition of the genome and DNA methylation. Since the staphylococcal genome has a low GC nucleotide content (35% to 37%) (Iandolo, Bannantine and Stewart, 1996) cleavage with restriction endonucleases which cut at GC rich sites e.g. *Sma*I ($^{5'}$CCC↓GGG$^{3'}$) or *Sst*II ($^{5'}$CCGC↓GG$^{3'}$) yielded a sufficient number of bands to allow interpretation whilst maintaining a high level of discrimination. The number of bands produced by either *Sma*I or *Sst*II restriction endonuclease digestion (five to eighteen) was consistent with other staphylococcal studies (Snopková *et al.*, 1994; Lina *et al.*, 1992; Poddar and McClelland, 1991). If a particular taxon possessed limited genetic variability, as indicated by the *S. simulans* profiles, the chance of unrelated strains having similar genotypes greatly increased. In these instances analysis with two restriction endonucleases increased the level of discrimination further. Additionally, the use of two enzymes could accommodate strains that were untypable with one of the restriction endonucleases used. In this study three isolates recovered for a patient clinically diagnosed with CVC-sepsis failed to cleave with *Sma*I, possibly due to DNA methylation. The problem was overcome by restriction with the second enzyme, *Sst*II.

CNS, which are ubiquitous within the environment and on the skin of both the patients and attending medical staff, frequently contaminate specimens. Therefore, assessing the clinical significance of CNS isolates from single samples can be difficult (Hedin, 1996; Zaidi *et al.*, 1996). The availability of multiple specimens greatly aids interpretation since the repeated recovery of the same organism almost always indicates sepsis. Biotyping and antibiograms of staphylococci may

demonstrate when two isolates are dissimilar, but their ability to determine that two organisms are the same is greatly reduced by their low level of discrimination. Molecular techniques overcome many of the limitations of traditional typing methods and allow the relatedness of strains to be more fully evaluated than previously (Villari, Sarnataro and Iacuzio, 2000; Toldos *et al.*, 1997). The combined use of macrorestriction profiling and Tenover's criteria for the genotyping of CNS can greatly aid diagnosis. In the case of one IE patient examined in detail here (figure 3.4) the microbiological history was complicated. Mixed isolates of CNS and an *Enterococcus* species were isolated from some blood samples but not others. The clinical diagnosis indicated possible IE but the blood cultures suggested contaminated samples. The application of PFGE to macrorestriction fragments proved that two of the three samples contained the same strain of *S. epidermidis* and provided compelling evidence of a *S. epidermidis* infection. The *Enterococcus* species and *S. haemolyticus* isolate were either contaminating organisms or genuine isolates contributing to a polymicrobial infection. There have been a number of reported case histories where PFGE of macrorestriction fragments has aided the clinical management of patients with multiple blood culture isolates by differentiating genuine infecting microorganisms from contaminating isolates (Toldos *et al.*, 1997; Lina *et al.*, 1995; Breen and Karchmer, 1994).

The ability of macrorestriction patterns to delineate strain relatedness was applied not only to patients with multiple isolates but also to the epidemiological investigation of CNS-related IE. The IE isolates, recovered from thirteen patients referred to QEH over a three year period (appendix 2), and isolates recovered from patients with other CNS-associated infections were genotyped and assessed for relatedness. The similarity of fragment profiles was scored by the Dice coefficient, similar profiles clustered by the average linkage method (UPGMA) and finally similarity depicted hierarchically in a dendrogram (Logan, 1994). The use of dedicated software packages (Phoretix 1D Advanced and Phoretix Database) for the analysis removed bias from strain to strain comparisons and increased the number of macrorestriction patterns that could be analysed. Though the *Sma*I and *Sst*II dendrograms were not identical, recurring patterns of association occurred throughout both. For example, eight strains within a cluster of nine *S. epidermidis* isolates (restriction digested with *Sst*II) were also located together within a cluster of fifteen strains digested with the

restriction endonuclease *Sma*I. Additionally, the segregation of *S. epidermidis* and non-*S. epidermidis* species provided further evidence for the high level of discrimination attainable and the stability of the technique for epidemiological studies. The low representation of individual species other than *S. epidermidis* limited the potential of the procedure to segregate fully species on the basis of their PFGE pattern. However, the clustering of both *S. simulans* and *S. lugdunensis* strains indicated that species segregation was achievable with increased representation. Indeed, the comparison of a CNS macrorestriction profile with a library of patterns could potentially be used as a means of speciation.

The aim of this chapter was to investigate the hypothesis that specific CNS genotypes were associated with IE. In a previous study, two epidemiologically unrelated community acquired *S. epidermidis* NVE isolates were shown to have identical PFGE profiles (Lina *et al.*, 1992). It was suggested that the pattern might be associated with a pathogenic CNS with a propensity to cause IE. However, in the work described in this thesis identical profiles were also shared between epidemiologically unrelated isolates. For example, a CNS isolate recovered from the soft tissue of an internally fixed bone fracture-related osteomyelitis patient (Salford) was identical to the restriction endonuclease pattern, both *Sma*I (one band difference) and *Sst*II (identical band profile), of a prosthetic hip-associated osteomyelitis strain (Birmingham). Since identical profiles were randomly shared by a small number of epidemiologically unrelated strains it most probably reflected limited genetic diversity within the species.

In a number of studies the antibiotic policy of a medical unit appeared to be the selective pressure responsible for the persistence of a few resistant clones (Villari, Sarnataro and Iacuzio, 2000; Vermont *et al.*, 1998; Nouwen *et al.*, 1998; Burnie *et al.*, 1997; Neumeister *et al.*, 1995). Alternatively, relatedness between strains was traced to a common source in an out-break situation (Boyce *et al.*, 1990). In this study it was demonstrated that there was no apparent clustering of the IE-associated CNS strains. Neither was there any correlation by other specific infection type, by associated medical unit (as exemplified by the patients located on the CAPD unit) nor by medical centre (for example QEH patients). It is, therefore, unlikely that specific genotypes with an identifiable propensity to cause infection are commonly

associated with IE in the non-outbreak situation. Neither is there any evidence for a previously unrecognised source of CNS associated with IE.

The hypothesis that a specific CNS phenotype is associated with IE will be investigated in Chapters 4 and 5.

CHAPTER 4- POTENTIAL VIRULENCE FACTORS OF COAGULASE-NEGATIVE STAPHYLOCOCCI

4.1 INTRODUCTION

S. aureus express a number of characterised enzymes and toxins that have been associated with pathogenicity. In contrast, CNS elaborate few of these factors and rarely has the potential of the product as a virulence determinant been clarified. CNS-associated IE is the cause of much morbidity and mortality and yet it remains unclear what virulence factors allow these usually avirulent microorganisms to become aggressive pathogens. The aim of this section of the study was to investigate the hypothesis that specific CNS phenotypes are associated with IE.

4.2 MATERIALS AND METHODS

4.2.1 Inoculum preparation

All CNS isolates representative of a single strain were assessed for their ability to produce potential virulence factors *in vitro*.

The bacterial isolates were cultivated in 20ml TSB (except for the cytolytic toxin assays for which the bacteria were cultivated in 20ml BHI broth) at 37°C for 18h with aeration at 200rpm. Bacterial cells and culture supernatant were prepared as required for each assay (see specific assay sections). One-quarter of a BHIA plate was inoculated with each strain to confirm the purity of the bacterial culture. Every isolate was screened by duplicate or triplicate assays performed on separate occasions.

4.2.2 Proteinase activity

4.2.2.1 Non-specific proteinase activity

The large bore end of a sterile glass Pasteur pipette was used to cut eight equidistant wells into a skimmed milk agar plate [1% (w/v) agar no. 1 (LabM, UK), 1% (w/v) skimmed milk]. For each bacterial strain, two wells were inoculated with 75µl of

culture supernatant recovered from 1ml of bacterial culture (11,400*g* × 5min). The agar plates were incubated at 37°C for 18h. A zone of clearing around the well indicated proteinase activity. Supernatant from *P. aeruginosa* PAO1 was initially incorporated as a positive control and sterile TSB broth as a negative control. The proteinase-positive bone fracture-associated isolate *S. epidermidis* NU1 and the proteinase-negative clinical strain *S. lugdunensis* LON2 later replaced the original controls.

4.2.2.2 Elastase activity by qualitative plate method

Bacterial cultures, diluted 1:100 with fresh broth, were streaked onto an elastin-agar plate [BHIA supplemented with 0.3% (w/v) bovine neck ligament elastin] (Janda, 1986). The inoculated plates were incubated at 37°C for 2 days before being transferred to room temperature for a further 19 days. The cultures were observed on days 3, 5, 7, 11, 14, and 21 for elastin degradation indicated by a zone of clearing around the bacterial colonies. *P. aeruginosa* PAO1 was included as a positive control and the elastase-negative *S. epidermidis* LON2 as a negative control.

4.2.2.3 Elastase activity by quantitative elastin-Congo red method

The reaction mixture consisted of 5mg of elastin-Congo-red, 1ml of Tris-malate buffer [0.1M Tris-malate buffer (pH 7.0) supplemented with 1mM $CaCl_2$] and 0.5ml of culture supernatant, harvested at (11,400*g* × 15min), combined in a 5ml bijou. The reaction bijoux were continuously rolled for 20h at 37°C and the reaction was terminated by the addition of 1ml of sodium phosphate buffer [0.7M Na_2HPO_4/ NaH_2PO_4 (pH 6.0)] (Bjorn, Sokol and Iglewski, 1979). Particulate elastin-Congo-red was removed by centrifugation (11,400*g* × 8min) and the reaction supernatant retained. An Anthos 2001 (Anthos Labtec Instruments) microtitre plate reader, initially referenced with air, was used to read the absorbance at 492nm of 0.2ml of the reaction supernatant dispensed in duplicate into a flat-bottomed microtitre plate (Immulon 2, Dynatech, Germany). *P. aeruginosa* PAO1 was initially included as the positive control strain; later being replaced by the elastase-positive *S. epidermidis*

NU1. Sterile TSB broth and *S. epidermidis* NCTC 11047 were included as negative controls.

4.2.3 Lipase/esterase activity

4.2.3.1 Evaluation of non-specific lipase/esterase activity

Wells were cut into glycerol tributyrate agar plates [1.5% (w/v) agar no. 1 (LabM), 1% (v/v) glycerol tributyrate] as previously outlined (section 4.2.2.1) (Molnàr *et al.*, 1994). For each bacterial strain, two wells were inoculated with 75µl of culture supernatant recovered from 1ml of bacterial culture (11,400*g* × 5min). The plates were incubated in air at room temperature for 18h. A zone of clearing around the well indicated non-specific lipase/esterase activity. Supernatant recovered from a culture of *P. aeruginosa* PAO1 was included as a positive control and fresh TSB broth was incorporated as a negative control. *S. epidermidis* NU1 and *S. lugdunensis* LON2 later replaced these controls, respectively.

4.2.3.2 Lipase activity

The basal components of olive oil agar were combined and autoclaved [1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl, 1.3 % (w/v) agar no. 1]. Whilst stirring vigorously the olive oil [final concentration of 2.5% (v/v) dry heat sterilised olive oil (J Sainsbury Plc., UK)] and rhodamine B [final concentration of 0.001% (w/v) filter sterilised aqueous solution of rhodamine B] were added to the autoclaved agar pre-cooled to 60°C (Kouker and Jaeger, 1987). Bacterial cultures, diluted 1:100 in fresh TSB, were streaked onto an olive oil agar plate and incubated at 37°C for 18h. Strains negative after this period of incubation were returned for a further 18h. Lipase activity was assessed by the observation of the bacterial colonies under UV excitation. Lipase expressing colonies fluoresced. *P. aeruginosa* PAO1 was initially used as a positive control; later being replaced by *S. epidermidis* NU1. *E. coli* C III, which was initially included as a negative control, was replaced by the lipase-negative *S. lugdunensis* LON2.

4.2.3.3 Esterase assay

Bacterial cultures, diluted 1:100 with fresh TSB, were streaked onto a Tween 80 agar plate [tryptone soya agar (TSA) supplemented with 1% (v/v) Tween 80, 0.5% (w/v) NaCl, 10μg/ml $CaCl_2$] (Barrow and Feltham, 1991). The inoculated plates were incubated at 37°C for 48h followed by incubation at room temperature for a further 12 days. On days 1, 2, 3, 5, 7, 11 and 14 the area around the bacterial streak was observed for esterase activity indicated by a halo of precipitated calcium chloride. The positive control was initially provided by *P. aeruginosa* PAO1 and later replaced by *S. epidermidis* NU1. *S. lugdunensis* LON2 provided the negative control.

4.2.4 Extracellular polysaccharide slime layer

4.2.4.1 Congo-red agar method

The bacterial culture, diluted 1:100 with sterile TSB, was inoculated onto one-quarter of a Congo-red agar plate [BHI broth supplemented with 5%(w/v) sucrose, 1% (w/v) agar no. 1, 0.8mg/ml Congo-red] (Freeman, Falkiner and Keane, 1989). The basal medium was autoclaved and cooled to 55°C prior to the addition of the Congo-red solution (autoclaved separately). The inoculated plates were incubated at 37°C for 18h before being transferred to room temperature for a further 18h. Following incubation the colonial phenotype of each strain was observed. The slime producing *S. epidermidis* ATCC 35984 (RP62A) and non-slime producing *S. hominis* ATCC 35982 (SP-2) strains were included as control isolates.

4.2.4.2 Agglutination by concanavalin A

Bacterial cells were harvested from 1ml of the broth culture (11,400*g* × 4min). The pellet of cells was gently washed in 1ml of phosphate buffered saline (PBS) (Oxoid) with care not to destroy the polysaccharide layer, if present, and resuspended in 0.5ml of PBS. Twenty μl of the bacterial suspension was combined with an equal volume of concanavalin A [1% (w/v) concanavalin A prepared in PBS] on a glass slide. This slide was rotated for 1min or until agglutination occurred (Ludwicka *et al.*, 1984). Concanavalin A-PBS solution formed the negative control and a bacterial cell-PBS suspension confirmed the absence of auto-agglutination.

4.2.5 Adherence of coagulase-negative staphylococci to polystyrene

An 18h broth culture was diluted 1:100 with fresh TSB and dispensed, in 0.2ml aliquots, into eight wells of a 96-well flat-bottomed polystyrene microtitre plate. Each microtitre tray contained ten test strains, one control strain (*S. epidermidis* 023) and one column of wells inoculated with sterile TSB, which served as a negative control. The plate was sealed with a sealing strip (Titertek plate sealers) and incubated at 37°C for 21h. With the plate held at a slight angle and the pipette tip placed against the side of the well the culture was completely aspirated. This technique was maintained throughout the assay to ensure minimal disruption to the biofilm. The plate was washed three times with 0.2ml of PBS to remove non-adherent cells. To fix the biofilm, 0.2ml of Bouin's solution [0.9% (w/v) picric acid, 5% (v/v) acetic acid, 9% (v/v) formaldehyde] was dispensed into each well and the plate maintained at room temperature for 30min (Wilcox, 1994). The fixative was aspirated from the wells and then completely removed by gentle washing with running tap water. The biofilm was stained for 10min at room temperature with 0.2ml of filtered crystal violet; 20ml of crystal violet solution [10% (w/v) crystal violet in 95% (v/v) ethanol] combined with 80ml of 1% (w/v) aqueous solution of ammonium oxalate (Barrow and Feltham, 1991). Excess stain was removed by aspiration with a pipette followed by washing under running tap water until the water ran clear. The plate was tapped dry on an absorbent material. Finally, the crystal violet was leached from the stained cells, by the addition of 0.2ml of 50% (v/v) ethanol, to create a solution of even optical density and thus improve the accuracy of the spectrophotometric reading. The optical density of each well at 595nm was measured using the microtitre plate reader (Christensen *et al.*, 1985).

4.2.6 Urease activity

Production of a urease enzyme was determined by native polyacrylamide gel electrophoresis (PAGE) using a modified Mobley *et al.* (1987) method.

An 11% (w/v) separating gel (80 x 70 x 1mm) (table 4.1) was cast using the PAGE gel casting apparatus (BioRad).

Table 4.1. Composition of the native PAGE separating and stacking gels, sample buffer and electrode buffer used to detect urease activity in culture supernatant

	Separating gel 11% (w/v)	**Stacking gel 5% (w/v)**	**Sample buffer**	**Electrode buffer (pH 8.0)**
Acrylamide stock I	5ml	-	-	-
Acrylamide stock II	-	2.5ml	-	-
1.5M Tris-HCl (pH 8.8)	6ml	-	-	-
0.5M Tris-HCl (pH 6.8)	-	3.75ml	2.5ml	-
Distilled water	8ml	8ml	5ml	1L
TEMED (N,N,N^{1}N^1-tetramethyl-ethylenediamine)	50µl	40µl	-	-
10% (w/v) ammonium persulphate - freshly prepared	70µl	50µl	-	-
Glycerol	-	-	2.5ml	-
5% (w/v) bromophenol blue	-	-	0.2ml	-
Tris	-	-	-	3g
Glycine	-	-	-	14.4g

Acrylamide stock I - 44% (w/v) acrylamide and 0.8% (w/v) Bis(N,N^1,- methylene-bis-acrylamide) (Severn Biotech Ltd, UK).
Acrylamide stock II - 30% (w/v) acrylamide and 0.8% (w/v) Bis(N,N^1, -methylene-bis-acrylamide).

A 15µl protein sample, prepared by combining equal volumes of sample buffer (table 4.1) and culture supernatant recovered by centrifugation (11,400g × 8min), was loaded into the appropriate well of the gel. Urease producing *S. epidermidis* NU1 and

non-urease producing *S. epidermidis* NCTC 11047 were included with every gel as positive and negative controls, respectively. A Jack Bean urease, resuspended in PBS (1mg/ml), which was a mixture of hexamer (molecular weight (M_w) 545kDa) and trimer (M_w 272kDa) was run in one lane to indicate the approximate size of the CNS urease. Native-PAGE was conducted at 200V until the dye front reached the lower edge of the gel, using a Mini-PROTEAN® II Cell (BioRad). On completion of electrophoresis the gel was washed three times in distilled water with agitation over a 1h period to remove the electrode buffer. The gel was then transferred to a 2% (w/v) urea solution, pre-warmed to 37°C, for 30min with constant agitation. Subsequently the gel was placed in a 0.002% (w/v) aqueous solution of cresol red until bands of urease activity were visible (Mobley, Jones and Penner, 1987).

4.2.7 DNase activity

A bacterial culture, diluted 1:100 with sterile TSB, was spot inoculated, using a nichrome bacteriological loop (Medical Wire and Equipment Ltd, UK) onto the surface of a DNase agar plate (Oxoid). The plates were incubated at 37°C for 18h. *S. aureus* NCTC 6571 was included as a positive control and *S. epidermidis* NCTC 11047 as a negative control. Subsequently the plate was flooded with 1M HCl and left to stand for 2min. A zone of clearing around the bacterial colonies indicated DNase activity.

4.2.8 Detection of extracellular cytolytic toxins

4.2.8.1 Haemolysis of horse erythrocytes

Defibrinated horse red blood cells (RBC) were suspended in PBS and recovered by centrifugation (400*g* × 4min). This wash procedure was repeated once more and the cells subsequently resuspended in PBS to a final 2% (v/v) concentration (Jordens, Duckworth and Williams, 1989). Culture supernatant was recovered from 1ml of a bacterial culture in BHI broth by centrifugation (11,400*g* × 5min). Using a flat-bottomed 96-well polystyrene microtitre tray, 0.2ml of the supernatant was dispensed into the first well of the first row. The second sample was dispensed into the first well of the second row and so forth for the first column. Into the wells of column 2 to

column 6 inclusive, 0.1ml of PBS was dispensed. From well 1, 0.1ml of the supernatant was transferred to well 2, mixed thoroughly with the diluent and 0.1ml transferred to the next well. This method of 2-fold serial dilution was performed between adjacent wells up to and including well 6, from which the excess 0.1ml was discarded. The microtitre plate was incubated at 37°C for 15min. Finally 0.1ml of the prepared homogeneous 2% (v/v) RBC suspension was dispensed into each well and the plate incubated at 37°C for 1h. Haemolysis was recorded by measuring the absorbance at 570nm and the plate was then incubated for a further 1h at 4°C. Haemolysis was again assessed as previously outlined. Peritonitis-associated strains *S. sciuri* CAPD17 and *S. simulans* CAPD24 were included as positive and negative control strains, respectively.

To determine the absorbance of haemolysed RBC a 1:100 dilution of horse erythrocytes in PBS was dispensed (0.2ml into each well) into 16 wells of a flat-bottomed microtitre tray. With distilled water as the diluent a 1:100 dilution of horse RBC was prepared, similarly dispensed and the absorbance of the two sets of RBC measured. The average absorbance of the haemolysed RBC (diluted in distilled water) was compared to that of non-haemolysed cells (diluted in PBS).

4.2.8.2 Haemolysis of sheep erythrocytes

The presence in the culture supernatant of cytolytic toxins active against sheep RBC was assessed using the procedure outlined in section 4.2.8.1. A clinical strain of *S. aureus* was included as the positive control and *S. simulans* CAPD24 as a haemolysis negative strain.

4.2.9 Antibiograms

Although not strictly considered to be a virulence factor, the sensitivity of each bacterial strain against a panel of antimicrobial agents was assessed by the disc diffusion method (Scott, 1989). A bacterial suspension of the control strain *S. aureus* NCTC 6571, prepared by diluting an 18h culture 1:100 with sterile TSB, was inoculated onto the outer 1cm ring of a Mueller-Hinton agar plate using a spiral

plater. A suspension of the test strain, similarly prepared was inoculated onto the remaining section of the plate. The antibiotic discs (penicillin 1 unit, erythromycin 5μg, gentamicin 10μg, vancomycin 5μg, fusidic acid 10μg, ciprofloxacin 1μg, all manufactured by Oxoid) were placed on the interface between the control and the test strain, equidistantly separated. The plate was incubated at 37°C for 18h.

Sensitivity to methicillin was assessed by a similar method. As recommended by the manufacturer, each prepared bacterial strain was line inoculated onto a salt agar plate [Columbia agar supplemented with 2% (w/v) NaCl]. Each plate was inoculated with four test strains and a positive control (a clinical strain of methicillin resistant *S. aureus*) and negative control strain (*S. aureus* NCTC 6571). A methicillin strip (25μg) was placed onto the plate perpendicular to the line inoculations and incubated at 30°C for 18h.

4.2.10 Statistical analysis of potential virulence factors

The significance of the differences between the observed frequencies of each potential virulence factor for the different CNS associated infection types was investigated by the application of Fisher's exact test and two-sided *p* value using the InStat (GraphPad Software Inc.) computer program (Kanji, 1993).

4.3 RESULTS

The cumulative results for each individual strain are listed in appendix 3.

4.3.1 Proteinase activity

The culture supernatant recovered from each strain was assessed for proteinase activity by a skimmed milk agar plate method. A zone of clearing (extending up to 5mm from the edge of the well) around the well indicated non-specific proteinase activity of the culture supernatant (figure 4.1, table 4.2).

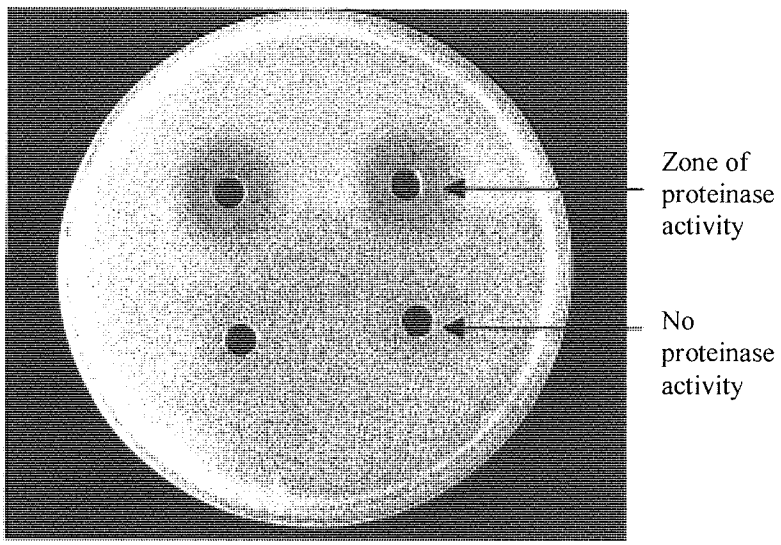


Figure 4.1. Assessment of proteinase activity by the skimmed milk agar method.

All the CNS strains were assessed for their ability to degrade elastin suspended in agar. A zone of clearing extending up to 5mm from the bacterial colony indicated elastase activity (figure 4.2, table 4.2).

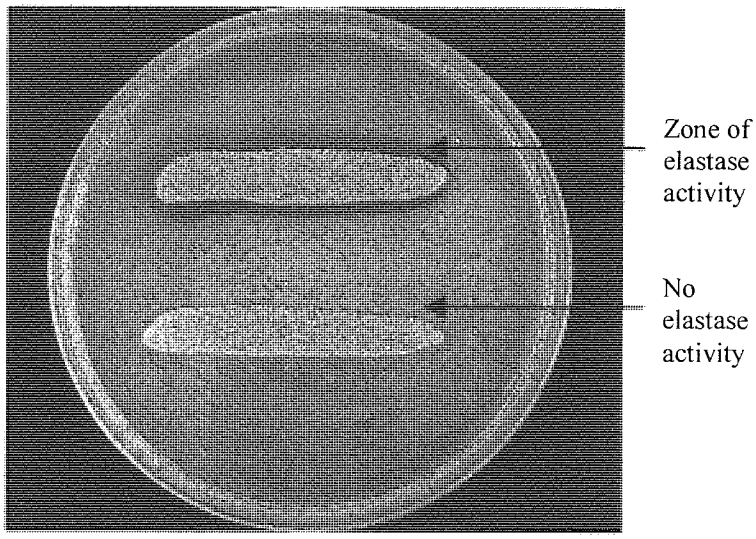


Figure 4.2. Elastase activity assessed by the elastin-agar plate method.

Digestion of the elastin component of the insoluble Congo-red impregnated elastin complex released soluble Congo-red into the reaction mixture. On termination of the reaction and removal of particulate elastin-Congo-red, the intensity of the red coloration, assessed by absorbance at 492nm, was directly proportional to elastase activity (figure 4.3, table 4.2).

An elastase positive and negative value range was determined by assessment of the numerous results obtained for the positive and negative CNS control strains generating value ranges of 0.081-0.321 (n = 13) and 0.042-0.083 (n = 13), respectively. Thus an absorbance at 492nm of $\geq$0.084 was considered to be positive, $\leq$0.080 negative, and 0.081-0.083 intermediate (+/-) for elastase activity.

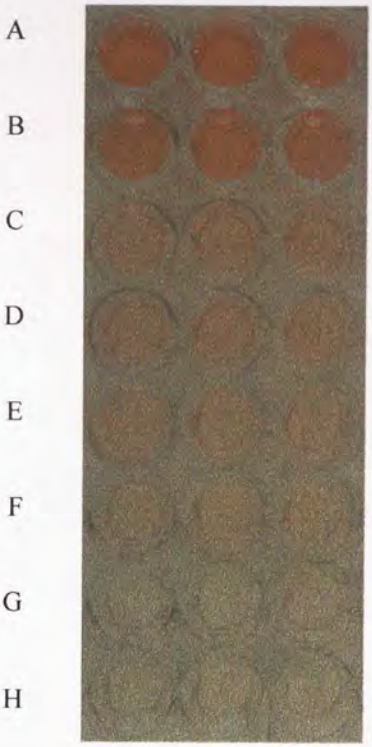


Figure 4.3. Elastase activity assessed by the elastin-Congo-red method. Elastase activity was quantitated by the release of soluble Congo-red from the insoluble elastin-Congo-red complex. Each row contains 0.2ml of the reaction mixture recovered from the assay of a single bacterial strain. The strains of rows A to H exhibited decreasing levels of elastase activity.

Table 4.2. The percentage of CNS strains expressing proteinase activity

Infection	**Strains expressing the virulence determinant (%)**					
	Non-specific proteinase		**Elastase (Agar plate method)**		**Elastase (Elastin-Congo red assay**	
CVC sepsis n = 17	14	(82)	6	(35)	7	(41)
CAPD peritonitis n = 25	20	(80)	4	(16)	11	(44)
Bone fractures n = 17	8	(47)	5	(29)	8	(47)
Hip prostheses n = 21	8	(38)	3	(14)	8	(38)
IE n = 14	8	(57)	1	(7)	6	(43)

Using Fisher's exact test no significant association was found between the observed frequencies of proteinase activity (non-specific and elastase activity) and the infection type except for:

- non-specific proteinase activity of prosthetic hip-associated and CAPD peritonitis-associated strains CNS (p = 0.0204 and p = 0.0324, respectively), considered significant. Whilst the majority of CAPD did express proteinase activity, few of the hip joint strains displayed any activity.

4.3.2 Lipase/esterase activity

Initially lipase/esterase activity of the culture supernatant was assessed by a non-specific method - glycerol tributyrate agar assay. A zone of clearing around the wells of the glycerol tributyrate agar plate (extending up to 5mm from the well edge) indicated lipase and/or esterase activity (table 4.3).

The hydrolysis of lipid (olive oil) by lipase activity in the presence of rhodamine B produced a fluorescent product (Kouker and Jaeger, 1987). Therefore, on exposure to UV light lipase-producing staphylococci fluoresced. By inspection of the lipase plates under UV light, staphylococcal lipase activity produced fluorescent pink/orange colonies whereas lipase-negative strains were white/pale pink (figure 4.4, table 4.3).

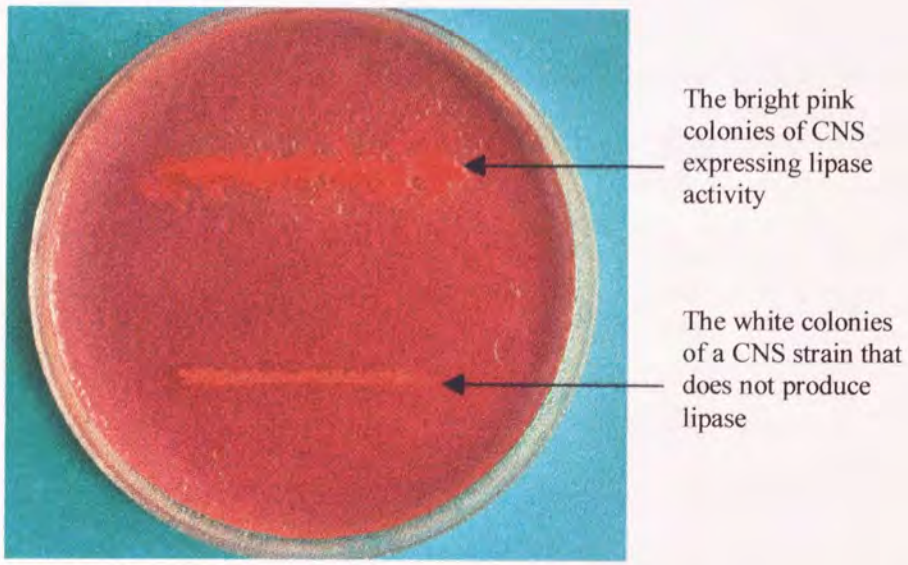


Figure 4.4. Lipase activity assessed by the olive oil agar method. Although these isolates are not shown under UV excitation, the lipase positive and negative strains displayed markedly different phenotypes. Lipase positive colonies had a bright pink appearance whilst lipase negative colonies were white.

The hydrolysis of Tween 80 by staphylococcal esterase instigated the precipitation of calcium chloride from the agar. This presented as a white halo of precipitate around the bacterial colonies of esterase producing strains (figure 4.5, table 4.3).

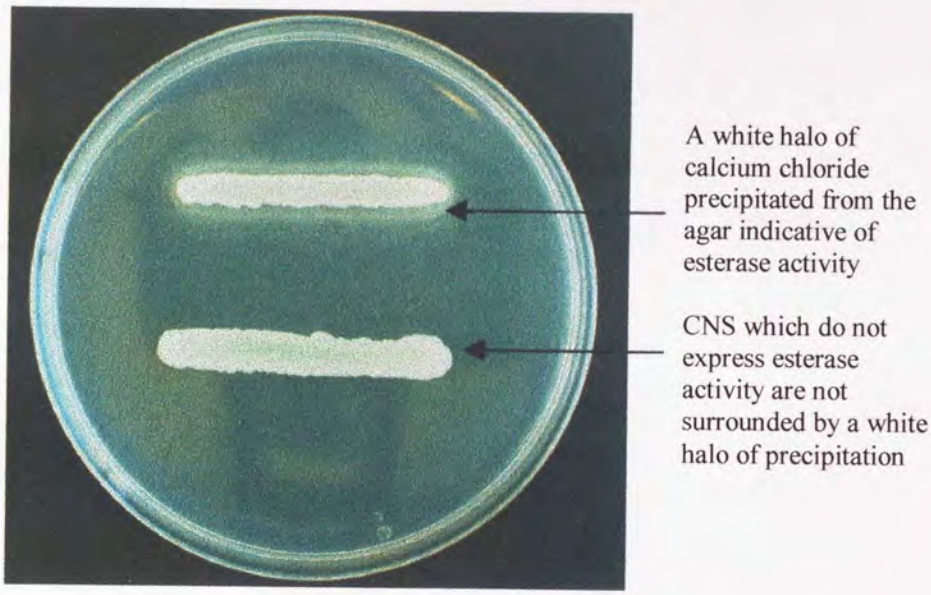


Figure 4.5. Esterase activity assessed by the Tween 80-agar method. Calcium chloride precipitated from the Tween 80 agar by esterase activity created a white halo around the bacterial colonies.

Table 4.3. The percentage of CNS strains expressing lipase and/or esterase activity

Infection	**Strains expressing the virulence determinant (%)**					
	Non-specific lipase/esterase		**Lipase**		**Esterase**	
CVC sepsis n = 17	15	(88)	16	(94)	13	(76)
CAPD peritonitis n = 25	24	(96)	24	(96)	23	(92)
Bone fractures n = 17	17	(100)	15	(88)	15	(88)
Hip prostheses n = 21	20	(95)	20	(95)	19	(90)
IE n = 14	12	(86)	12	(86)	12	(86)

Using Fisher's exact test no significant association was found between the observed frequencies of lipase and/or esterase activity and the infection type.

4.3.3 Extracellular polysaccharide layer

Extracellular polysaccharide layer (slime) producing staphylococcal colonies cultivated on Congo-red agar plates developed a characteristic dry black appearance compared to non-slime producing colonies which had a moist, red/brown presentation (figure 4.6, table 4.4) (Freeman, Falkiner and Keane, 1989).

Figure 4.6. Comparison of slime producing and non-slime producing strains of CNS grown on Congo-red agar. a, the dry, black colonies of a slime producing CNS; b, the moist red/brown colonies of a non-slime producing CNS.

The second method used to evaluate the presence of an extracellular polysaccharide layer required mixing a staphylococcal suspension with the lectin concanavalin A (Ludwicka *et al.*, 1984). Agglutination resulted in the formation of clearly visible aggregates within the suspension. Strains that failed to agglutinate the concanavalin A suspension within 1min were recorded as negative. Strains that produced a weak reaction were scored 1 and those that produced a strong, rapid reaction were scored 2 (table 4.4). One bacterial strain that auto-agglutinated in PBS was not assigned a result.

Table 4.4. CNS strains producing an extracellular polysaccharide layer (slime)

Infection	**Strains expressing the virulence determinant (%)**			
	Slime (Congo-red method)		**Slime (concanavalin A method)**	
CVC sepsis n = 17	9	(53)	7	(41)
CAPD peritonitis n = 25	8	(32)	16	(64)
Bone fractures n = 17	4	(34)	12	(59)
Hip prostheses n = 21	4	(19)	7	(33)
IE n = 14	6	(25)	6	(43)

Using Fisher's exact test no significant association was found between the observed frequencies of extracellular polysaccharide layer production (Congo-red agar and concanavalin A methods) and the infection type.

4.3.4 Adherence to polystyrene

The ability of the CNS strains to attach to polystyrene and form an adherent biofilm was assessed by a microtitre plate method.

The results of each test batch were only accepted if the absorbance of the control strain fell within a defined range (absorbance at 595nm of 0.085-0.125). This range was derived from the majority of twenty-nine repeated assay results obtained for the control strain.

For each isolate eight absorbance values were obtained during the assay. The highest and/or lowest values were not included if another value did not fall within a 15%

range to exclude anomalous results. The mean value of the accepted absorbance results was calculated and the final recorded absorbance (mean value of the repeated assay values) for each strain determined.

The final absorbance values for all the CNS strains were divided into four groups. The strains in the first group, which did not express a discernible ability to adhere to the polystyrene surface, were assigned a value of 0 (non-adherent). Isolates in the second group were assigned a value of 1 (weakly adherent) and the third group a value of 2 (adherent). Finally the strains with the highest absorbance values scored a 3 (strongly adherent) (table 4.5).

Table 4.5. The percentage of CNS strains expressing various adherence capabilities

Infection	**Strains expressing the virulence determinant (%)**							
	Not adherent		**Weakly adherent**		**Adherent**		**Strongly adherent**	
CVC sepsis n = 17	5	(29)	3	(18)	6	(35)	3	(18)
CAPD peritonitis n = 25	7	(28)	10	(40)	6	(24)	2	(8)
Bone fractures n =17	11	(65)	1	(6)	4	(24)	1	(6)
Hip prostheses n = 21	8	(38)	6	(29)	7	(29)	0	(0)
IE n = 14	4	(29)	6	(43)	2	(14)	2	(14)

Using Fisher's exact test no significant association was found between the observed frequencies of adherent CNS (adherent strains included all the isolates assigned 1,2, or 3 values) and the infection type except:

- The inability of bone fracture strains not to adhere to polystyrene (p = 0.0132), considered significant.

4.3.5 Urease activity

Native-PAGE separated the culture supernatant proteins whilst retaining enzymic activity. Urea was hydrolysed to ammonia by the urease enzyme within the acrylamide leading to the formation of a localised pH increase. After 10min in cresol red (a pH indicator solution) the gel developed red bands on a pale yellow background. These bands indicated areas of increased pH and thus the location of urease activity (figure 4.7).

In addition to the urease negative and positive staphylococcal controls, a Jack Bean urease was incorporated into one gel to confirm the specificity of the test and to indicate the general size of the staphylococcal enzyme. Of the eight staphylococcal urease enzymes separated in the gel shown in figure 4.7, all were smaller than the Jack Bean urease; less than M_w 272kDa. The number of enzymically active bands detected varied from one to three per strain. The samples were scored; no visible band (0), weakly visible band (1), clearly visible band (2), strong, clearly visible band (3) (table 4.6).

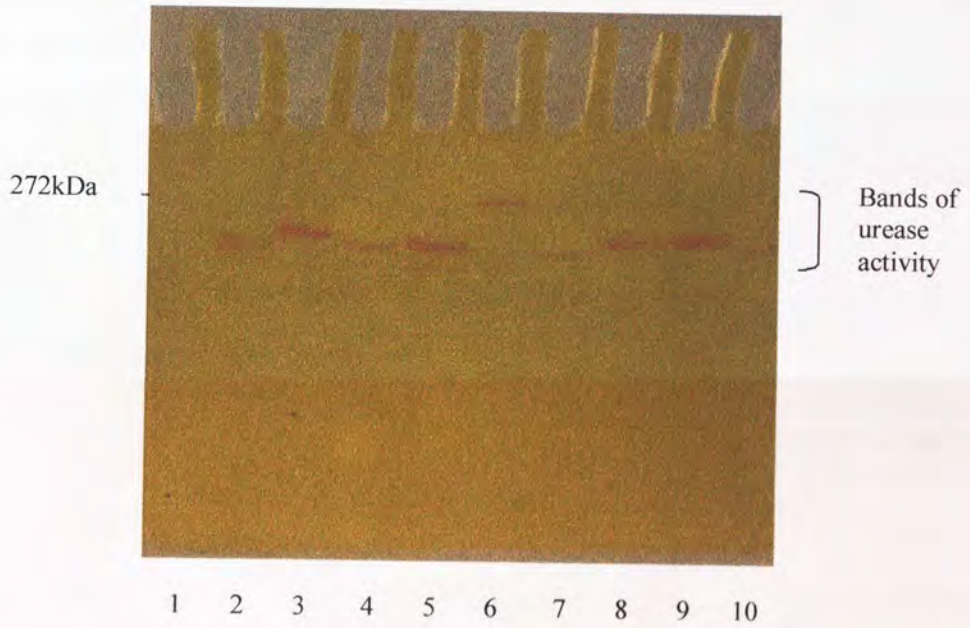


Figure 4.7. Detection of urease activity by a native-PAGE method. Lane 1 contains supernatant from a urease negative CNS, lanes 2,3,4,5,7,8,9,10 contain supernatant from urease producing CNS, and lane 6 contains Jack Bean urease. The dominant band of lane 6 is 272kDa.

Table 4.6. The percentage of CNS strains expressing urease activity

Infection	**Strains expressing the virulence determinant (%)**	
	Urease activity	
CVC sepsis n = 17	16	(94)
CAPD peritonitis n = 25	19	(76)
Bone fractures n = 17	11	(65)
Hip prostheses n = 21	11	(52)
IE n = 14	8	(57)

Using Fisher's exact test no significant association was found between the observed frequencies of urease activity and the infection type except for:

- Urease activity by CVC sepsis-associated strains of CNS (p = 0.0180), considered significant.

4.3.6 DNase activity

DNase activity was assessed by the DNase agar method. The addition of 1M HCl to the surface of a DNase agar plate precipitated polymerised DNA, resulting in the plate becoming opaque. However, in areas of DNase activity the hydrolysed DNA was not precipitated resulting in a clear zone (figure 4.8). The DNase activity result was recorded as negative (0), reduced zone of DNase activity (1), comparable zone size (2), and increased zone size (3) in comparison to the positive control *S. aureus* NCTC 6571 (table 4.7).

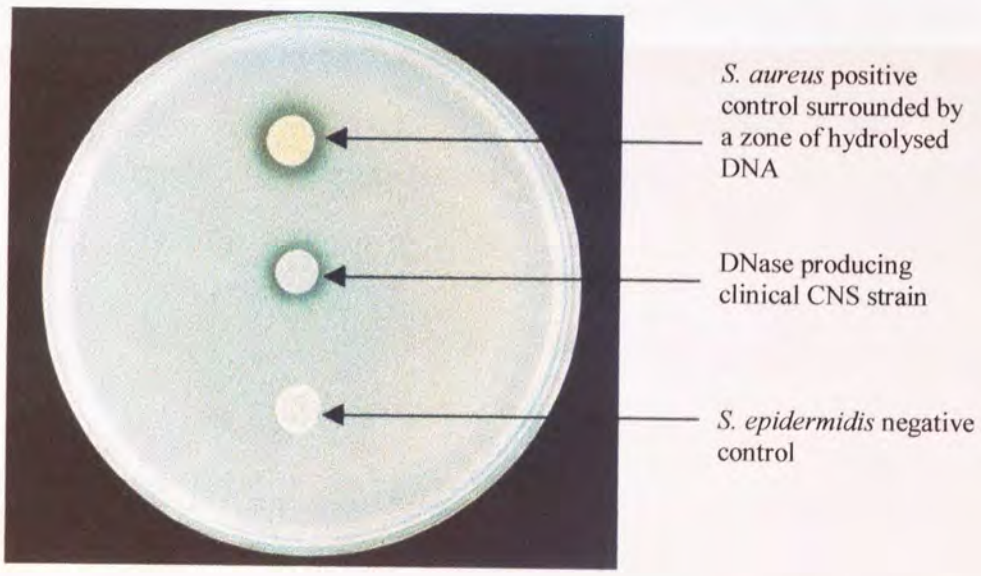


Figure 4.8. DNase activity was assessed by the DNase agar method. A zone of hydrolysed DNA around the bacterial colonies indicated DNase activity.

Table 4.7. The percentage of CNS strains expressing DNase activity

Infection	**Strains expressing the virulence determinant (%)**	
	DNase activity	
CVC sepsis n = 17	0	(0)
CAPD peritonitis n = 25	2	(8)
Bone fractures n = 17	0	(0)
Hip prostheses n = 21	1	(5)
IE n = 14	2	(14)

Using Fisher's exact test no significant association was found between the observed frequencies of DNase production and the infection type.

4.3.7 Cytolytic toxins

The average absorbance at 570nm of haemolysed RBC (diluted in distilled water) was 0.062 in contrast to a mean of 0.732 for the non-haemolysed cells (diluted in PBS). Therefore, a sample with an absorbance of <0.100 was recorded as haemolysed whilst a value of ≥ 0.100 indicated no detectable haemolytic activity (figure 4.9, table 4.8). The reciprocal of the highest supernatant dilution with an absorbance of <0.100 was recorded as the haemolytic titre.

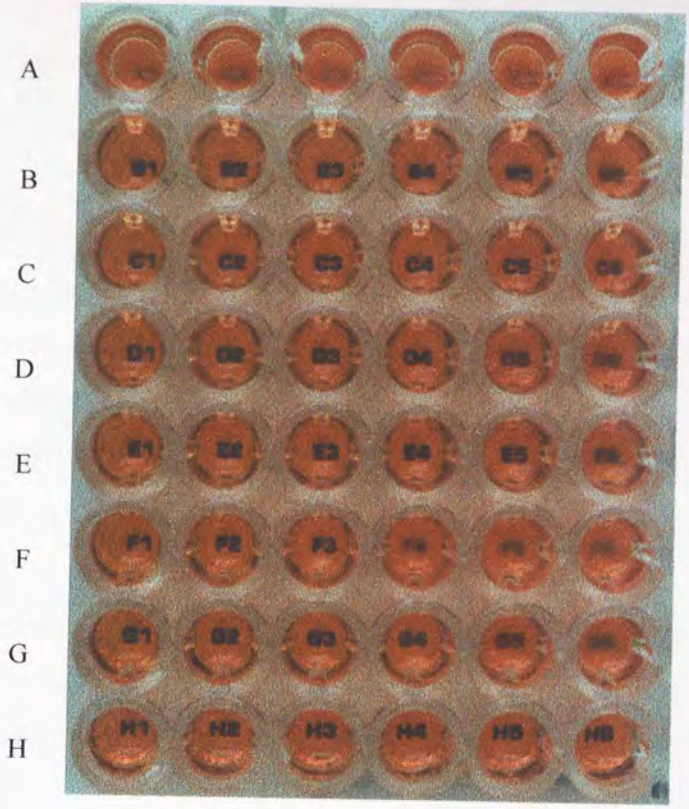


Figure 4.9. Haemolysis of horse erythrocytes by culture supernatant assessed by a microtitre tray method. Rows A-G each contain the culture supernatant, serially diluted across the plate from left to right, of seven CNS. Row A has expressed no observable haemolysis. Row H contains haemolysed RBC, diluted 1:100 with distilled water. A numbered card has been placed under the microtitre plate to aid the detection of haemolysis in the illustrated figure.

Table 4.8. The percentage of CNS strains producing haemolytic toxins

Infection	**Strains expressing the virulence determinant (%)**			
	Haemolysis of horse erythrocytes		**Haemolysis of sheep erythrocytes**	
CVC sepsis n = 17	1	(6)	0	(0)
CAPD peritonitis n = 25	4	(16)	0	(0)
Bone fractures n = 17	2	(12)	1	(6)
Hip prostheses n = 21	6	(29)	2	(10)
IE n = 14	2	(14)	0	(0)

Using Fisher's exact test no significant association was found between the observed frequencies of haemolysis (of horse or sheep erythrocytes) and the infection type.

4.3.8 Antibiograms

Each strain was tested for sensitivity to seven antibiotics by the disc diffusion method. The zone of inhibition of the test strain around the antimicrobial disc was compared to that of the control strain. Test strain zones that were larger or equal to that of the control strain indicated sensitivity, whereas growth up to the disc or zones smaller than that of the control denoted resistance to the antibiotic.

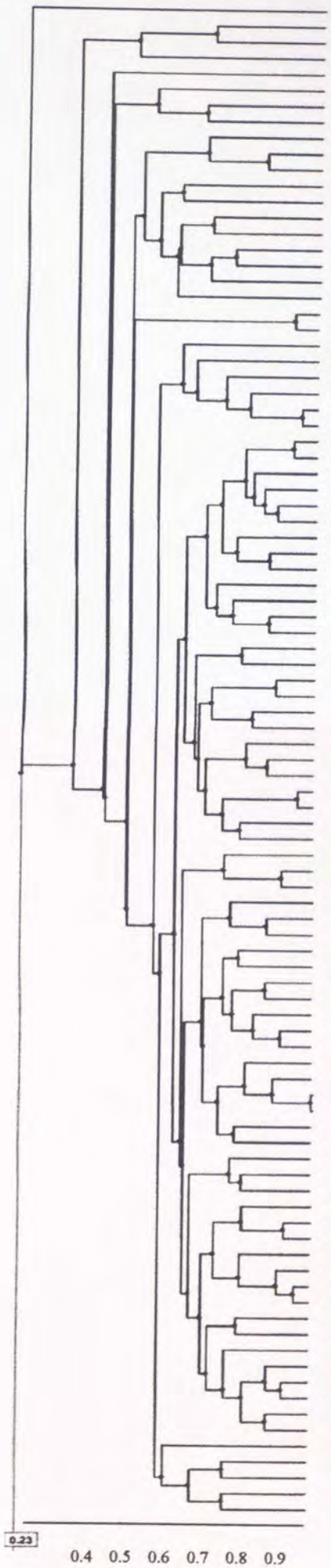
In the absence of any clear pattern of sensitivity between strains associated with a particular infection (appendix 4) each isolate was scored by the number of agents against which resistance was expressed (table 4.9).

Table 4.9. The number of antibiotics against which the strains were resistant

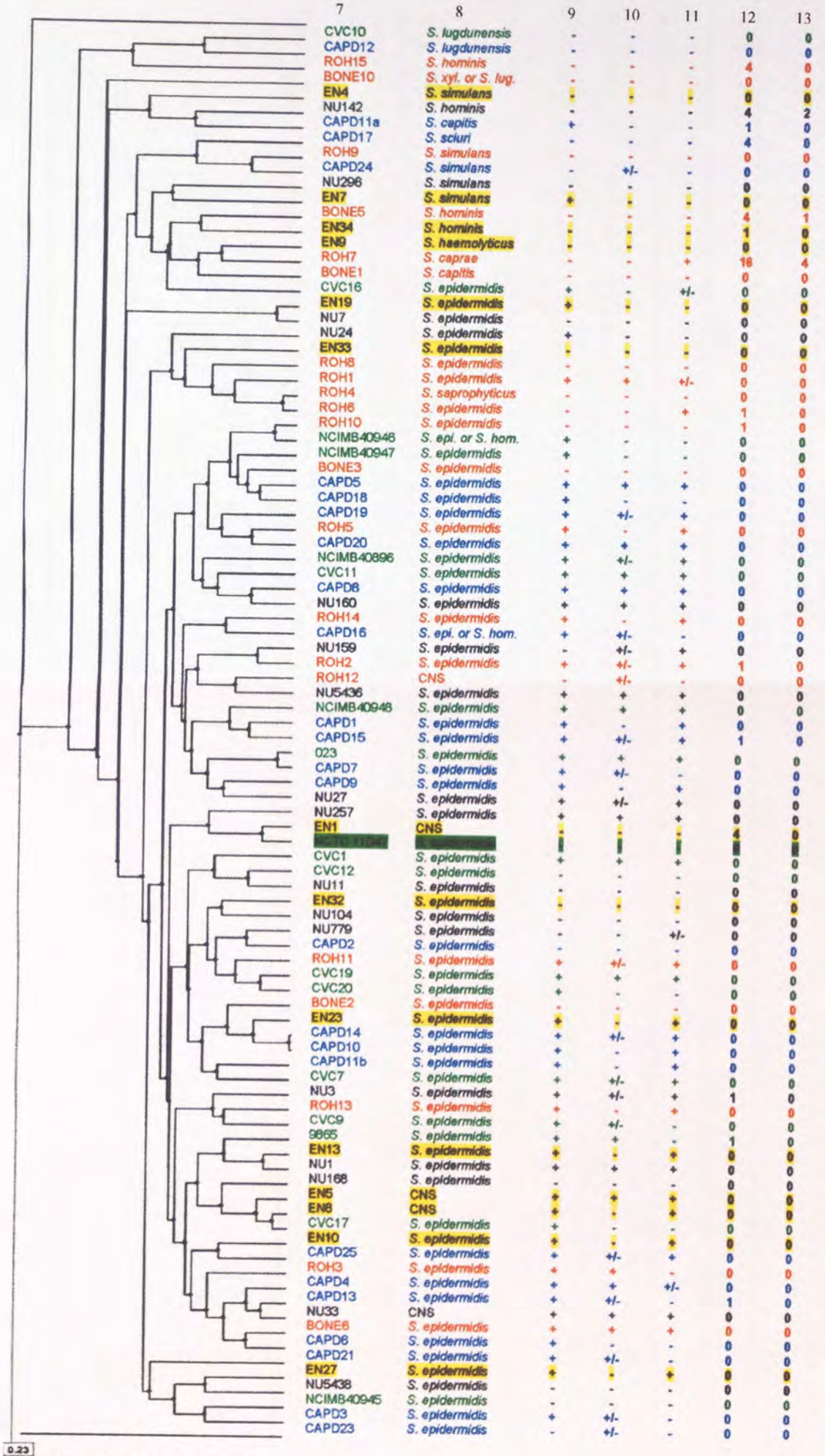
Infection	**The number of antibiotics against which the strains were resistant**								
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**Mean**
CVC sepsis n = 17	1	2	2	2	2	6	2	0	3.6
CAPD peritonitis n = 25	4	2	4	6	3	2	4	0	3.0
Bone fractures n = 17	5	1	2	8	1	0	0	0	1.9
Hip prostheses n = 21	2	6	4	5	1	3	0	0	2.3
IE n = 14	4	3	2	2	1	2	0	0	1.9

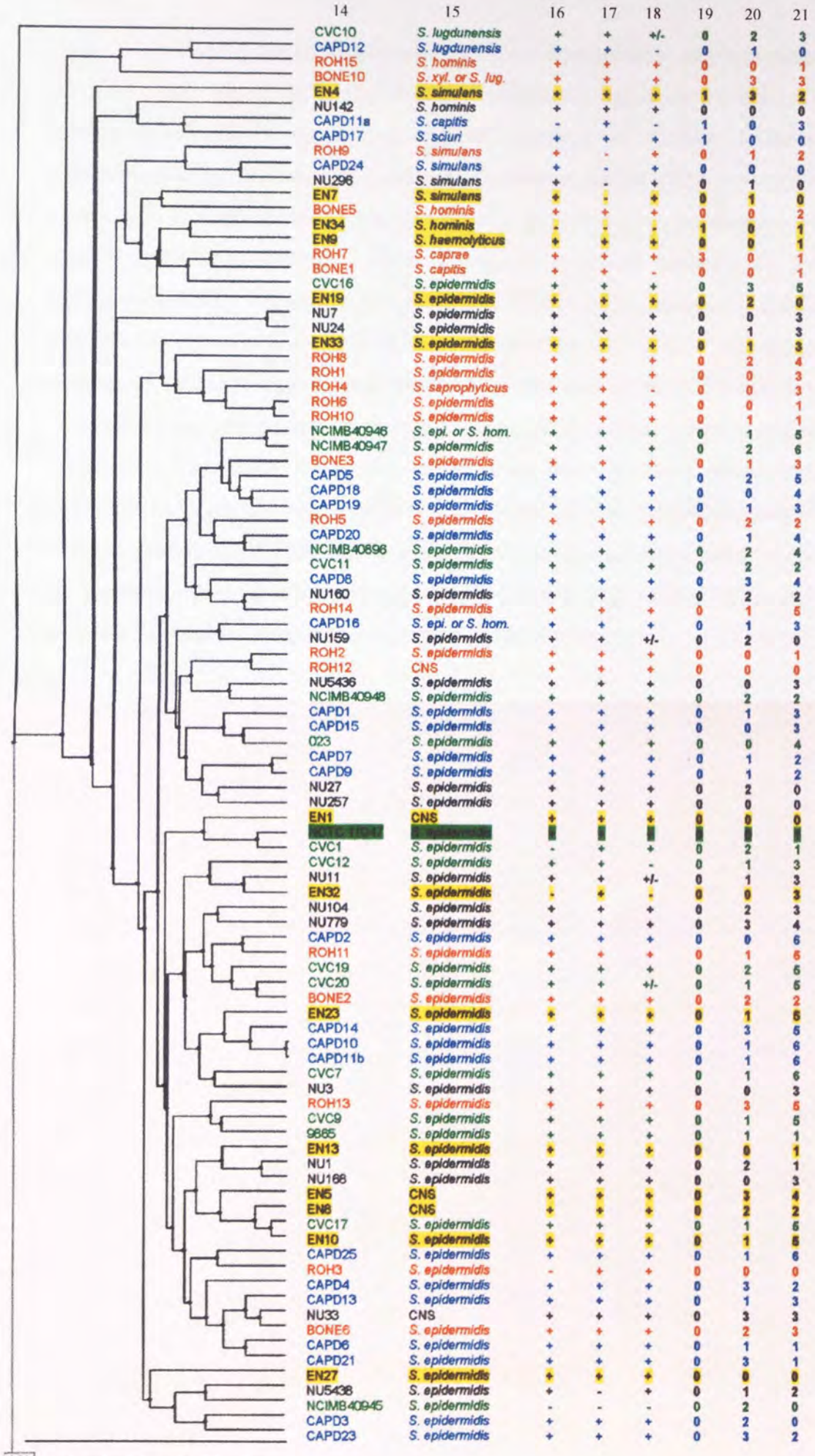
4.3.9 The association of specific phenotypes with infection

In addition to the statistical analysis of each group of strains the results were transposed onto the *Sma*I generated dendrogram described in chapter 3 (figure 4.10). There was no clustering of strains with similar phenotypes.



1	2	3	4	5	6
CVC10	*S. lugdunensis*	QEH	2	-	0
CAPD12	*S. lugdunensis*	QEH	2	-	2
ROH15	*S. hominis*	ROH	0	-	0
BONE10	*S. xyl. or S. lug*	ROH	2	+	0
EN4	*S. simulans*	QEH	0	-	0
NU142	*S. hominis*	HH	2	-	1
CAPD11a	*S. capitis*	QEH	1	-	0
CAPD17	*S. sciuri*	QEH	2	-	2
ROH9	*S. simulans*	ROH	0	-	0
CAPD24	*S. simulans*	QEH	0	-	0
NU296	*S. simulans*	HH	0	-	0
EN7	*S. simulans*	QEH	0	-	0
BONE5	*S. hominis*	ROH	0	-	2
EN34	*S. hominis*	QEH	3	-	2
EN9	*S. haemolyticus*	QEH	2	-	2
ROH7	*S. caprae*	ROH	2	-	-
BONE1	*S. capitis*	ROH	2	-	0
CVC16	*S. epidermidis*	QEH	2	+	0
EN19	*S. epidermidis*	QEH	0	-	2
NU7	*S. epidermidis*	HH	0	-	1
NU24	*S. epidermidis*	HH	0	-	2
EN33	*S. epidermidis*	QEH	1	+	2
ROH8	*S. epidermidis*	ROH	2	-	1
ROH1	*S. epidermidis*	ROH	1	-	0
ROH4	*S. saprophyticus*	ROH	0	-	0
ROH6	*S. epidermidis*	ROH	2	-	0
ROH10	*S. epidermidis*	ROH	1	-	0
NCIMB40946	*S. epi. or S. hom*	QEH	0	+	2
NCIMB40947	*S. epidermidis*	QEH	0	-	2
BONE3	*S. epidermidis*	ROH	0	-	2
CAPD5	*S. epidermidis*	QEH	0	+	0
CAPD18	*S. epidermidis*	QEH	1	-	0
CAPD19	*S. epidermidis*	QEH	1	+	0
ROH5	*S. epidermidis*	ROH	1	+	1
CAPD20	*S. epidermidis*	QEH	2	+	0
NCIMB40896	*S. epidermidis*	QEH	3	+	0
CVC11	*S. epidermidis*	QEH	1	-	2
CAPD8	*S. epidermidis*	QEH	1	-	2
NU160	*S. epidermidis*	HH	0	+/-	2
ROH14	*S. epidermidis*	ROH	1	-	1
CAPD16	*S. epi. S. hom.*	QEH	3	+	2
NU159	*S. epidermidis*	HH	0	+/-	0
ROH2	*S. epidermidis*	ROH	1	-	0
ROH12	CNS	ROH	0	-	0
NU5436	*S. epidermidis*	HH	0	-	2
NCIMB40948	*S. epidermidis*	QEH	3	+	0
CAPD1	*S. epidermidis*	QEH	1	-	1
CAPD15	*S. epidermidis*	QEH	2	-	1
023	*S. epidermidis*	QEH	0	-	2
CAPD7	*S. epidermidis*	QEH	2	-	0
CAPD9	*S. epidermidis*	QEH	1	-	0
NU27	*S. epidermidis*	HH	2	-	1
NU257	*S. epidermidis*	HH	0	-	1
EN1	CNS	QEH	0	-	0
[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
CVC1	*S. epidermidis*	QEH	1	-	0
CVC12	*S. epidermidis*	QEH	2	-	0
NU11	*S. epidermidis*	HH	0	-	2
EN32	*S. epidermidis*	QEH	1	+	0
NU104	*S. epidermidis*	HH	3	+	2
NU779	*S. epidermidis*	HH	0	-	0
CAPD2	*S. epidermidis*	QEH	1	-	2
ROH11	*S. epidermidis*	ROH	2	-	2
CVC19	*S. epidermidis*	QEH	0	+	2
CVC20	*S. epidermidis*	QEH	0	-	1
BONE2	*S. epidermidis*	ROH	0	-	2
EN23	*S. epidermidis*	QEH	1	+	2
CAPD14	*S. epidermidis*	QEH	0	-	2
CAPD10	*S. epidermidis*	QEH	0	-	2
CAPD11b	*S. epidermidis*	QEH	0	-	2
CVC7	*S. epidermidis*	QEH	2	+	2
NU3	*S. epidermidis*	HH	0	-	0
ROH13	*S. epidermidis*	ROH	2	+	0
CVC9	*S. epidermidis*	QEH	3	+	0
9665	*S. epidermidis*	QEH	1	+	0
EN13	*S. epidermidis*	QEH	2	-	0
NU1	*S. epidermidis*	HH	2	+	1
NU168	*S. epidermidis*	HH	1	+	1
EN5	CNS	QEH	3	+	0
EN8	CNS	QEH	1	+	0
CVC17	*S. epidermidis*	QEH	2	+	0
EN10	*S. epidermidis*	QEH	1	+	0
CAPD25	*S. epidermidis*	QEH	1	+	0
ROH3	*S. epidermidis*	ROH	0	-	0
CAPD4	*S. epidermidis*	QEH	0	-	1
CAPD13	*S. epidermidis*	QEH	1	+	1
NU33	CNS	HH	2	+	2
BONE8	*S. epidermidis*	ROH	1	+	0
CAPD6	*S. epidermidis*	QEH	3	+	1
CAPD21	*S. epidermidis*	QEH	2	+	1
EN27	*S. epidermidis*	QEH	1	-	2
NU5436	*S. epidermidis*	HH	0	-	0
NCIMB40945	*S. epidermidis*	QEH	2	-	0
CAPD3	*S. epidermidis*	QEH	0	-	1
CAPD23	*S. epidermidis*	QEH	1	-	1


7
8
9
10
11
12
13
CVC10 S. lugdunensis - - - 0 0
CAPD12 S. lugdunensis - - - 0 0
ROH15 S. hominis - - - 4 0
BONE10 S. xyl. or S. lug. - - - 0 0
EN4 S. simulans + + + 0 0
NU142 S. hominis - - - 4 2
CAPD11a S. capitis + - - 1 0
CAPD17 S. sciuri - - - 4 0
ROH9 S. simulans - - - 0 0
CAPD24 S. simulans - +/- - 0 0
NU296 S. simulans - - - 0 0
EN7 S. simulans + + + 0 0
BONE5 S. hominis - - - 4 1
EN34 S. hominis + + + 1 0
EN9 S. haemolyticus + + + 0 0
ROH7 S. caprae - - + 16 4
BONE1 S. capitis - - - 0 0
CVC16 S. epidermidis + - +/- 0 0
EN19 S. epidermidis + + + 0 0
NU7 S. epidermidis - - - 0 0
NU24 S. epidermidis + - - 0 0
EN33 S. epidermidis + + + 0 0
ROH8 S. epidermidis - - - 0 0
ROH1 S. epidermidis + + +/- 0 0
ROH4 S. saprophyticus - - - 0 0
ROH6 S. epidermidis - - + 1 0
ROH10 S. epidermidis - - - 1 0
NCIMB40946 S. epi. or S. hom. + - - 0 0
NCIMB40947 S. epidermidis + - - 0 0
BONE3 S. epidermidis - - - 0 0
CAPD5 S. epidermidis + + + 0 0
CAPD18 S. epidermidis + - - 0 0
CAPD19 S. epidermidis + +/- + 0 0
ROH5 S. epidermidis + - + 0 0
CAPD20 S. epidermidis + + + 0 0
NCIMB40896 S. epidermidis + +/- + 0 0
CVC11 S. epidermidis + + + 0 0
CAPD8 S. epidermidis + + + 0 0
NU160 S. epidermidis + + + 0 0
ROH14 S. epidermidis + - + 0 0
CAPD16 S. epi. or S. hom. + +/- - 0 0
NU159 S. epidermidis - +/- + 0 0
ROH2 S. epidermidis + +/- + 1 0
ROH12 CNS - +/- - 0 0
NU5436 S. epidermidis + + + 0 0
NCIMB40948 S. epidermidis + + + 0 0
CAPD1 S. epidermidis + - + 0 0
CAPD15 S. epidermidis + +/- + 1 0
023 S. epidermidis + + + 0 0
CAPD7 S. epidermidis + +/- - 0 0
CAPD9 S. epidermidis + - + 0 0
NU27 S. epidermidis + +/- + 0 0
NU257 S. epidermidis + + + 0 0
EN1 CNS + + + 4 0
[redacted] [redacted] [redacted] [redacted] [redacted] [redacted] [redacted]
CVC1 S. epidermidis + + + 0 0
CVC12 S. epidermidis - - - 0 0
NU11 S. epidermidis - - - 0 0
EN32 S. epidermidis + + + 0 0
NU104 S. epidermidis - - - 0 0
NU779 S. epidermidis - - +/- 0 0
CAPD2 S. epidermidis - - - 0 0
ROH11 S. epidermidis + +/- + 0 0
CVC19 S. epidermidis + + + 0 0
CVC20 S. epidermidis + - - 0 0
BONE2 S. epidermidis - - - 0 0
EN23 S. epidermidis + + + 0 0
CAPD14 S. epidermidis + +/- + 0 0
CAPD10 S. epidermidis + - + 0 0
CAPD11b S. epidermidis + - + 0 0
CVC7 S. epidermidis + +/- + 0 0
NU3 S. epidermidis + +/- + 1 0
ROH13 S. epidermidis + - + 0 0
CVC9 S. epidermidis + +/- - 0 0
9865 S. epidermidis + + - 1 0
EN13 S. epidermidis + + + 0 0
NU1 S. epidermidis + + + 0 0
NU168 S. epidermidis - - - 0 0
EN5 CNS + + + 0 0
EN8 CNS + + + 0 0
CVC17 S. epidermidis + - - 0 0
EN10 S. epidermidis + + + 0 0
CAPD25 S. epidermidis + +/- + 0 0
ROH3 S. epidermidis + + - 0 0
CAPD4 S. epidermidis + + +/- 0 0
CAPD13 S. epidermidis + +/- - 1 0
NU33 CNS + + + 0 0
BONE6 S. epidermidis + + + 0 0
CAPD6 S. epidermidis + - - 0 0
CAPD21 S. epidermidis + +/- - 0 0
EN27 S. epidermidis + + + 0 0
NU5438 S. epidermidis - - - 0 0
NCIMB40945 S. epidermidis - - - 0 0
CAPD3 S. epidermidis + +/- - 0 0
CAPD23 S. epidermidis - +/- - 0 0
0.23
0.4 0.5 0.6 0.7 0.8 0.9



	14	15	16	17	18	19	20	21
CVC10	*S. lugdunensis*	+	+	+/-	0	2	3
CAPD12	*S. lugdunensis*	+	+	-	0	1	0
ROH15	*S. hominis*	+	+	+	0	0	1
BONE10	*S. xyl. or S. lug.*	+	+	+	0	3	3
EN4	*S. simulans*	+	+	+	1	3	2
NU142	*S. hominis*	+	+	+	0	0	0
CAPD11a	*S. capitis*	-	+	-	1	0	3
CAPD17	*S. sciuri*	+	+	+	1	0	0
ROH9	*S. simulans*	+	-	+	0	1	2
CAPD24	*S. simulans*	+	-	+	0	0	0
NU296	*S. simulans*	+	-	+	0	1	0
EN7	*S. simulans*	+	-	+	0	1	0
BONE5	*S. hominis*	+	+	+	0	0	2
EN34	*S. hominis*	-	-	-	1	0	1
EN9	*S. haemolyticus*	+	+	+	0	0	1
ROH7	*S. caprae*	+	+	+	3	0	1
BONE1	*S. capitis*	+	+	-	0	0	1
CVC16	*S. epidermidis*	+	+	+	0	3	5
EN19	*S. epidermidis*	+	+	+	0	2	0
NU7	*S. epidermidis*	+	+	+	0	0	3
NU24	*S. epidermidis*	+	+	+	0	1	3
EN33	*S. epidermidis*	+	+	+	0	1	3
ROH8	*S. epidermidis*	+	+	+	0	2	4
ROH1	*S. epidermidis*	+	+	+	0	0	3
ROH4	*S. saprophyticus*	+	+	-	0	0	3
ROH6	*S. epidermidis*	+	+	+	0	0	1
ROH10	*S. epidermidis*	+	+	+	0	1	1
NCIMB40946	*S. epi. or S. hom.*	+	+	+	0	1	5
NCIMB40947	*S. epidermidis*	+	+	+	0	2	6
BONE3	*S. epidermidis*	+	+	+	0	1	3
CAPD5	*S. epidermidis*	+	+	+	0	2	5
CAPD18	*S. epidermidis*	+	+	+	0	0	4
CAPD19	*S. epidermidis*	+	+	+	0	1	3
ROH5	*S. epidermidis*	+	+	+	0	2	2
CAPD20	*S. epidermidis*	+	+	+	0	1	4
NCIMB40896	*S. epidermidis*	+	+	+	0	2	4
CVC11	*S. epidermidis*	+	+	+	0	2	2
CAPD8	*S. epidermidis*	+	+	+	0	3	4
NU160	*S. epidermidis*	+	+	+	0	2	2
ROH14	*S. epidermidis*	+	+	+	0	1	5
CAPD16	*S. epi. or S. hom.*	+	+	+	0	1	3
NU159	*S. epidermidis*	+	+	+/-	0	2	0
ROH2	*S. epidermidis*	+	+	+	0	0	1
ROH12	CNS	+	+	+	0	0	0
NU5436	*S. epidermidis*	+	+	+	0	0	3
NCIMB40948	*S. epidermidis*	+	+	+	0	2	2
CAPD1	*S. epidermidis*	+	+	+	0	1	3
CAPD15	*S. epidermidis*	+	+	+	0	0	3
023	*S. epidermidis*	+	+	+	0	0	4
CAPD7	*S. epidermidis*	+	+	+	0	1	2
CAPD9	*S. epidermidis*	+	+	+	0	1	2
NU27	*S. epidermidis*	+	+	+	0	2	0
NU257	*S. epidermidis*	+	+	+	0	0	0
EN1	CNS	+	+	+	0	0	0
[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
CVC1	*S. epidermidis*	-	+	+	0	2	1
CVC12	*S. epidermidis*	+	+	-	0	1	3
NU11	*S. epidermidis*	+	+	+/-	0	1	3
EN32	*S. epidermidis*	-	+	-	0	0	3
NU104	*S. epidermidis*	+	+	+	0	2	3
NU779	*S. epidermidis*	+	+	+	0	3	4
CAPD2	*S. epidermidis*	+	+	+	0	0	6
ROH11	*S. epidermidis*	+	+	+	0	1	5
CVC19	*S. epidermidis*	+	+	+	0	2	5
CVC20	*S. epidermidis*	+	+	+/-	0	1	5
BONE2	*S. epidermidis*	+	+	+	0	2	2
EN23	*S. epidermidis*	+	+	+	0	1	5
CAPD14	*S. epidermidis*	+	+	+	0	3	5
CAPD10	*S. epidermidis*	+	+	+	0	1	6
CAPD11b	*S. epidermidis*	+	+	+	0	1	6
CVC7	*S. epidermidis*	+	+	+	0	1	6
NU3	*S. epidermidis*	+	+	+	0	0	3
ROH13	*S. epidermidis*	+	+	+	0	3	5
CVC9	*S. epidermidis*	+	+	+	0	1	5
9885	*S. epidermidis*	+	+	+	0	1	1
EN13	*S. epidermidis*	+	+	+	0	0	1
NU1	*S. epidermidis*	+	+	+	0	2	1
NU166	*S. epidermidis*	+	+	+	0	0	3
EN5	CNS	+	+	+	0	3	4
EN8	CNS	+	+	+	0	2	2
CVC17	*S. epidermidis*	+	+	+	0	1	5
EN10	*S. epidermidis*	+	+	+	0	1	5
CAPD25	*S. epidermidis*	+	+	+	0	1	6
ROH3	*S. epidermidis*	-	+	+	0	0	0
CAPD4	*S. epidermidis*	+	+	+	0	3	2
CAPD13	*S. epidermidis*	+	+	+	0	1	3
NU33	CNS	+	+	+	0	3	3
BONE6	*S. epidermidis*	+	+	+	0	2	3
CAPD6	*S. epidermidis*	+	+	+	0	1	1
CAPD21	*S. epidermidis*	+	+	+	0	3	1
EN27	*S. epidermidis*	+	+	+	0	0	0
NU5438	*S. epidermidis*	+	-	+	0	1	2
NCIMB40945	*S. epidermidis*	-	-	-	0	2	0
CAPD3	*S. epidermidis*	+	+	+	0	2	0
CAPD23	*S. epidermidis*	+	+	+	0	3	2

Figure 4.10. The expression of potential virulence determinants was transposed onto the dendrogram generated for the *Sma*I macrorestricted fragments of each isolate. a) column 1, strain; 2, species; 3, patient location; 4, biofilm formation; 5, polysaccharide slime (Congo-red method); 6, polysaccharide slime (concanavalin A method); b) 7, strain; 8, species; 9, non-specific proteinase; 10, proteinase – elastase (elastin agar); 11, proteinase – elastase (elastin-Congo-red method); 12, cytolytic toxin (horse RBC); 13, cytolytic toxin (sheep RBC); c) 14, strain; 15, species; 16, non-specific lipase/esterase; 17, lipase; 18, esterase; 19, DNase; 20, urease; 21, number of antimicrobials against which the strain was resistant (of seven tested). CVC-associated strains are illustrated in green, CAPD-related strains are in blue, prosthetic hip-associated isolates are shown in red, internally fixed bone fractures are illustrated in black, IE strains are shown in black with yellow highlight, and the type strain *S. epidermidis* NCTC 11047 is highlighted in green. For the majority of assays the result is indicated as a positive or negative result, where a number is shown the reader is referred to the appropriate section of the results.

4.4 DISCUSSION

4.4.1 Proteinase activity

In this study the majority of strains associated with catheter-related sepsis secreted a proteinase enzyme (CVC-sepsis $p = 0.0324$, CAPD-associated peritonitis $p = 0.0596$), whereas substantially fewer isolates recovered from the other infections exhibited enzymic activity. It is not known if proteinase activity is a virulence factor associated with catheter related infections but it may be postulated that cleavage of the serum proteins, which coat a catheter surface on insertion, by proteinase activity may aid bacterial attachment. Therefore, for this infection type proteinase activity may facilitate the establishment of infection. Alternatively, such enzymes can destroy immunoglobulin thus reducing opsonization and allowing evasion of the immune response (Travis, Potempa and Meada, 1995). For example, *S. aureus* secretes proteinases shown to cleave IgG (Projan and Novick, 1996). Although a number of staphylococcal products have proteinase activity, few have been fully investigated (Zhang and Maddox, 2000). A *S. aureus* proteinase capable of activating the kallikrein-kinin system, is one that has been characterised (Molla *et al.*, 1989).

A greater proportion of the CNS strains investigated were elastase positive by the elastin-Congo-red assay than the elastin-agar method, indeed only two strains expressed enzymic activity by the plate method alone. This suggests that the former method was the most sensitive assay. Elastolytic activity was confined to isolates of the species *S. epidermidis,* except for weak activity observed in *S. caprae* ROH7. This confirmed the findings of a previously published study (Janda, 1986). However, in contrast to the study by Janda (1986), not all the *S. epidermidis* strains were able to degrade the macroprotein. Less than half of the CNS strains screened in each infection group, regardless of the technique employed, expressed a functional enzyme and no statistical difference was found between infections. This implies that elastase producing CNS strains did not have a predilection to cause a specific infection over any other type of sepsis included in this study and that secretion of this enzyme was not a prerequisite for pathogenicity.

Though elastase activity has been correlated with the pathogenesis observed during *S. epidermidis* perifollicular macular atrophy (Varadi and Saqueton, 1968), no

evidence was found to suggest that the enzyme contributed to CNS-related bovine intramammary infections (Watts, Naidu and Wadström, 1990). Whether an elastolytic enzyme contributes to connective tissue degradation during staphylococcal IE remains to be determined.

4.4.2 Lipase and esterase expression

Staphylococcal lipase is expressed *in vivo* during infection (Rollof, Hedström and Nilsson-Ehle, 1987; Christensson, Fehrenbach and Hedström, 1985). Christensson *et al.* (1985) demonstrated an antibody response to the enzyme during serious *S. aureus* infections (IE and complicated septicaemia) whereas antibody titres were not significantly elevated during non-invasive infections (such as furunculosis). In support of this evidence, higher levels of lipase production were observed in staphylococcal isolates recovered from deep-seated and subcutaneous infections (for example septicaemia) than strains related to superficial infections (impetigo) (Rollof, Hedström and Nilsson-Ehle, 1987). This indicated that the enzyme might facilitate tissue invasion.

In this study, at least three-quarters of the strains in each infection group secreted a detectable level of both of the enzymes investigated. There was no statistical difference between elaboration and the infection type. Since these isolates were recovered from significant invasive infections the ability of the majority of strains to secrete lipase was not unexpected in view of the work published by Christensson *et al.* (1985) and Rollof *et al.*, (1987). It is unclear whether expression in these strains was purely a metabolic function or whether it was a virulence factor commonly expressed during significant CNS infections.

A lipase/esterase virulence function has been identified in staphylococcal abscesses. The host imports bactericidal lipids into the abscess. In response to this the staphylococci secrete a fatty acid modifying enzyme (FAME), which converts the bactericidal fatty acids into non-bactericidal fatty acid esters (Mortensen, Shryock and Kapral, 1992). However, the host also imports FAME inhibiting glycerides. A staphylococcal lipase removes the inhibitory glycerides allowing the FAME to resume activity and the microorganism to circumvent the host's immune response

(Long *et al.*, 1992). In addition to facilitating virulence within an abscess, staphylococcal lipase has been shown to interfere with the structure and function of human granulocytes (Rollof *et al.*, 1988). Since granulocytic function is essential to the host's immunity against these microorganisms, staphylococcal lipase appears to be involved in evasion of host defences and tissue penetration.

Interestingly, experimental evidence suggests that lipase expression is controlled by *agr*, a locus strongly associated with the expression of virulence factors (Vuong, Götz and Otto, 2000). Furthermore, the *S. epidermidis* lipase gene *geh* is located next to the *ica* operon which encodes genes for the biosynthesis of PIA (section 1.3.6.3) (Götz, Verheij and Rosenstein, 1998). This extracellular substance is involved in biofilm formation and is implicated in the pathogenicity of device-related infections (Rupp *et al.*, 1999a; Rupp *et al.*, 1999b).

4.4.3 Extracellular polysaccharide slime layer

During the course of this investigation production of a polysaccharide slime layer was assessed by two methods: concanavalin A agglutination and colony phenotype on Congo-red agar. The interaction of the mannose-specific lectin, concanavalin A, with the sugar residues identified in high concentrations in slime has been reported to be a useful test for distinguishing slime producing from slime non-producing strains of CNS (Ludwicka *et al.*, 1984). However, the composition of the slime substance is not yet fully understood and it has been suggested that the lectin might be interacting with terminal glucose molecules in wall teichoic acid (Hussain, Hastings and White, 1991). The Congo-red agar method, originally described for distinguishing virulent from avirulent Gram-negative bacteria (Kay *et al.*, 1985; Ishiguro *et al.*, 1985), was modified by Freeman *et al.*, (1989) to separate CNS into strains that secreted an extracellular polysaccharide layer and those that did not. Heilmann and Götz (1998) further modified the Freeman Congo-red agar. During this study, the modified medium was retrospectively compared to the original Freeman formula but was not found to be a significant improvement. Recently it has been reported that strains expressing PIA correlate to those producing the black colony phenotype (slime-positive) on Congo-red agar (Ziebuhr *et al.*, 1997). The Christensen tube method of slime detection was assessed (Christensen *et al.*, 1982), but the results proved not to

be reproducible, a finding that has been reported by other groups (Deighton and Balkau, 1990). Furthermore, this technique probably evaluates attachment and biofilm formation rather than extracellular polysaccharide production.

The concanavalin A method generated a positive result in 33% to 64% of strains in each infection group, whereas Congo-red agar identified 19% to 53% assay positive strains. Although the frequencies of positive strains in each group as detected by the two methods were similar there was little correlation between individual strains (appendix 3). Clearly, at least one of these methods was not detecting the polysaccharide substance specifically. Whilst there is a growing body of evidence supporting the Congo-red agar method (Ziebuhr *et al.*, 1997), there is some doubt as to the validity of the lectin-based method (Hussain, Hastings and White, 1991; Deighton and Balkau, 1990). There was no significant association found between strains positive for either test and infection type.

Ziebuhr *et al.* (1997) screened CNS strains recovered from episodes of septicaemia and from normal skin for biofilm formation, possession of the *ica* operon and PIA expression using the Congo-red agar method. Whilst all strains possessed the *ica* operon, the clinical isolates generally formed a biofilm and produced PIA (87% of strains positive for both assays) whereas the skin isolates did not (11% and 6% of strains positive, respectively) (Ziebuhr *et al.*, 1997). In contrast between 19% and 53% of the clinical strains screened in this study were positive by the Congo-red agar method. Since PIA expression is subject to phenotypic variation (section 1.3.6.3), differences in culture methods may account for the different results obtained in the two investigations (Ziebuhr *et al.*, 1999; Ziebuhr *et al.*, 1997).

The role of slime production and biofilm formation in CNS infections and prosthetic device-related infections in particular has been extensively investigated and reviewed (Ing, Baddour and Bayer, 1996; Christensen, Baldassarri and Simpson, 1994; Kloos and Bannerman, 1994; Goldmann and Pier, 1993). Shiro *et al.* (1994, 1995) have investigated the role of PS/A in *S. epidermidis* IE specifically (section 1.3.6.3). Initially the group demonstrated that *S. epidermidis* strains deficient in the slime component PS/A were virtually avirulent in an IE model when the origin of infection was bacteraemia (none of the animals infected with a PS/A$^-$ strain developed IE,

whereas all of the animals infected with a PS/A$^+$ strain had infected vegetations) (Shiro *et al.*, 1994). It was demonstrated that PS/A prevented complement C3 deposition on the bacterial cell surface thus preventing complement mediated killing (Shiro *et al.*, 1994). During earlier investigations Baddour *et al.* (1984) noted that *S. epidermidis* strains that did not secrete slime were more susceptible to phagocytic killing, supporting the observations of Shiro *et al.* (1994). Conversely, production of the polysaccharide was shown not to be essential to the establishment of intra-operative-acquired *S. epidermidis* PVE (Shiro *et al.*, 1995). These studies indicate that PS/A has an essential virulence role when IE develops by an haematogenous route, but is of less importance when infection is acquired by direct contamination of an implanted device.

4.4.4 Adherence to a polymer surface

When discussing the attachment of bacterial cells to a surface the terms slime, polysaccharide substance, glycocalyx and biofilm are frequently used interchangeably. In this section of the study the overall ability of bacterial strains to adhere to and form a biofilm on a native polystyrene surface was investigated and not the specific bacterial components that are potentially involved.

Attachment of staphylococci onto the polymer surface is the essential first stage of a prosthetic device-related infection (figure 1.7) (Hogt, Dankert and Feijen, 1987). Indeed, blocking this initial interaction can significantly reduce the ability of a microorganism to cause infection (Takeda *et al.*, 1991). In this investigation, in which all strains were considered to be clinically significant, a wide range of abilities to adhere to the plastic surface was exhibited. There were isolates associated with each particular infection that were found to be non-adherent (28% to 65% of strains per infection). Similarly, there was a distribution of weakly adherent, adherent and strongly adherent strains associated with the various infections. These results, in contradiction to those of Deighton and Balkau (1990), suggest that adherence to polystyrene cannot be used as a virulence marker. Furthermore, there was no statistical difference between infection type and the ability of an isolate to adhere to polystyrene, except the bone fracture-related strains, which displayed a marked inability to adhere.

Since bacterial attachment is fundamental to the initiation of infection, it might be expected that all clinical strains of CNS recovered from prosthesis-related infections would exhibit a pronounced ability to form a biofilm. However, attachment is complex and involves both physical properties and surface structures, for example MSCRAMMs which interact with host proteins present in the conditioning layer that coats the prosthetic-device (Chapter 5) (Heilmann *et al.*, 1997; Patti *et al.*, 1994a; Timmerman *et al.*, 1991). The assay utilised in this section of the study screened CNS for the ability to interact directly with unconditioned polystyrene. It is possible that the adhesion negative strains may be capable of interacting with host proteins absorbed onto the prosthesis surface immediately following implantation. This interaction is investigated in Chapter 5.

4.4.5 Urease activity

Urease activity has previously been demonstrated in isolates of *S. aureus*, *S. epidermidis*, *S. xylosus* and *S. saprophyticus* (Mobley, Island and Hausinger, 1995; Schäfer and Kaltwasser, 1994). In this study activity was also observed in strains of *S. lugdunensis* and *S. simulans*. The majority of CNS strains screened produced a urease enzyme (52% to 94% of isolates in each infection group). Indeed, the high percentage of CVC sepsis-related strains exhibiting enzymic activity in comparison to strains recovered from other infections was considered significant ($p = 0.0180$). Urease activity has not been shown to act as a virulence factor during episodes of catheter-related sepsis but it has been suggested that the high pH generated at a localised point, particularly if the microorganisms elaborate a diffusion limiting slime layer, might promote physical damage to the biomaterial surface (Jansen *et al.*, 1991). Franson *et al.* (1984) showed by scanning electron microscopy that CNS adhere to flaws in the catheter surface.

No enzymically active protein band, detected by native PAGE, was larger than the 272kDa sub-unit marker of the Jack Bean urease. However, the number of bands detected varied from one to three per strain. This multiple band pattern, which was also observed following native-PAGE of the *S. saprophyticus* urease, was thought to represent the holoenzyme and catalytically active degradation products (Schäfer and Kaltwasser, 1994). Two independent groups have reported the urease enzyme of

S. saprophyticus to be a heteropolymeric protein of approximately 420kDa (Gatermann, John and Marre, 1989b; Schäfer and Kaltwasser, 1994).

4.4.6 DNase activity

Only a small minority of the strains investigated (five out of ninty-four) produced an observable level of DNase activity. All were species other than *S. epidermidis*: *S. simulans*, *S. capitis*, *S. sciuri*, *S. hominis*, and *S. caprae*. Although a positive DNase reaction is generally considered a characteristic of *S. aureus*, a minority of CNS are capable of hydrolysing DNA (Duguid, 1989). The failure of the majority of strains to hydrolyse DNA suggests that the activity of this enzyme had little if any pathogenic function in the infections investigated.

4.4.7 Cytolytic toxins

The capacity of a microorganism to produce cytolytic toxins can be readily evaluated using erythrocytes, since lysis of RBC can be visually assessed and is generally applicable to other eukaryotic cell types (Bernheimer, 1988). The production of α, β and δ-toxins has been demonstrated in various species of CNS (Molnàr *et al.*, 1994; Rozgonyi *et al.*, 1994; Hébert, 1990; Lambe *et al.*, 1990; Gemmell, 1987; Gemmell and Thelestam, 1981).

Initially agar plates and tubes containing horse or sheep RBC that had been washed with PBS, washed with PBS/$CaCl_2$, or had not been treated were used to assess haemolysis by the CNS (Chart *et al.*, 1998). As a further modification the various agar preparations were supplemented with 10mM $CaCl_2$. Although Chart *et al.*, (1998) demonstrated that $CaCl_2$ treatment was essential for expression of the *E. coli* α-toxin (magnesium or calcium ions are required by some haemolytic toxins for maximal activity (Bernheimer, 1988)), calcium ions have been shown to protect against *S. aureus* α-haemolysin activity (Harshman and Sugg, 1985). None of these agar methods was shown to be sufficiently sensitive for the assessment of haemolytic toxins. The second technique employed measured erythrocyte lysis by a microtitre plate method. Horse or sheep RBC were washed in PBS, washed in PBS

supplemented with 1mM $MgSO_4$ (Jordens, Duckworth and Williams, 1989), or used without treatment. Although washing the erythrocytes prior to the assay was shown to improve the detection of haemolytic toxins, the addition of $MgSO_4$ to the wash was not found to benefit the assay. Since the addition of $MgSO_4$ to the wash solution conveyed no detectable advantage and in view of the inhibitory effect of divalent cations on certain haemolysins, the preparation of RBC for the assay involved washing in PBS alone (Harshman and Sugg, 1985). Measuring the absorbance at 570nm was shown to be appropriate for detecting haemolysis. Incubation at 37°C was sufficient for haemolysis to occur, however β-toxin required "cold-shock" after incubation at 37°C to produce lysis (Jordens, Duckworth and Williams, 1989; Bernheimer, 1988).

Staphylococcal α and β-toxins are active against sheep but not horse RBC (Hébert and Hancock, 1985). The δ-toxin is active against both sheep and horse RBC, however small amounts of the lysin may not be detected with sheep erythrocytes (Hébert and Hancock, 1985). In this study twelve strains were active against horse but not sheep RBC, whilst three isolates lysed both species of erythrocytes. No additional lysis was observed in any of the CNS strains after incubation at 4°C. These results suggested that all the haemolytic strains secreted a δ-toxin. This is consistent with the findings of a previous study (Hébert and Hancock, 1985). No significant association was found between the observed frequencies of haemolysis and the infection type. In contrast, Gemmell (1987) reported that CAPD-related strains were more likely to produce haemolysins than isolates recovered from CVC-sepsis or IE patients. Similarly, CNS associated with urinary tract infections were more frequently haemolytic than strains recovered from skin infections (Gemmell, 1987). The discrepancies observed between these two studies might reflect the different culture conditions and variation in the assay methodologies.

Although cytolytic toxins produced by *S. aureus* are reported to be involved in the pathogenesis of IE (Bayer *et al.*, 1997), there is no evidence to suggest that CNS haemolysins enhance infection of heart valves. Indeed there are few reports to indicate that these CNS toxins are virulence factors associated with any type of infection (Scheifele and Bjornson, 1988). This study found no evidence to suggest

that expression of a cytolytic toxin either enhanced virulence or was essential to any of the infections studied.

4.4.8 Antibiotic resistance

Generally the catheter-related sepsis strains were sensitive to fewer of the antibiotics than the osteomyelitis or IE–associated strains. This probably reflects the origin of the pathogens; nosocomial-acquired microorganisms tend to be more resistant to antimicrobials than community-acquired infections. There were no distinct antibiograms associated with a particular infection type.

The hypothesis that a specific CNS phenotype is associated with IE will be further investigated in Chapter 5. A second group of potential virulence factors, which mediate attachment of the bacterial cell to the extracellular matrix proteins of the host will be investigated.

CHAPTER 5- MSCRAMM-MEDIATED ADHERENCE OF COAGULASE-NEGATIVE STAPHYLOCOCCI TO HOST TISSUES

5.1 INTRODUCTION

Microbial surface components recognising adhesive matrix molecules (MSCRAMMs), which mediate bacterial colonisation and possible tissue invasion, can be considered as virulence factors (Patti *et al.*, 1994a). The aim of this section of the study was to investigate MSCRAMM expression in CNS isolates recovered from two types of infection (IE and CAPD-associated peritonitis) and evaluate any possible contribution to tissue tropism.

5.2 MATERIALS AND METHODS

5.2.1 Preparation of the extracellular matrix protein

5.2.1.1 Fibrinogen

A fresh solution of bovine plasma fibrinogen (10μg/ml) was prepared with PBS as required.

5.2.1.2 Collagen type I

Bovine collagen type I (Becton Dickinson) was diluted to a working concentration of 10μg/ml in 0.01M HCl, dispensed into 10ml aliquots and stored at 4°C until required.

5.2.1.3 Fibronectin

Human fibronectin (Becton Dickinson), supplied as a lyophilized powder, was allowed to equilibrate to room temperature before being reconstituted in sterile distilled water to 1mg/ml. The fibronectin suspension was dispensed in 0.1ml aliquots and stored at -20°C until required. For use the protein suspension was

diluted with PBS to a working concentration of 20μg/ml (Valentin-Weigand, Timmis and Chhatwal, 1993; Schou, Bøg-Hansen and Fiehn, 1999).

5.2.1.4 Laminin

Engel-Holm-Swarm mouse tumour laminin (Becton Dickinson) solution was allowed to thaw at 4°C. The suspension was diluted with PBS to a stock solution concentration of 1mg/ml, dispensed in 0.1ml aliquots, and stored at -70°C. For use an aliquot of laminin was defrosted on ice and diluted to 20μg/ml with PBS (Schou, Bøg-Hansen and Fiehn, 1999).

5.2.1.5 Heparin

Heparin, sodium salt grade II prepared from porcine intestinal mucosa, was reconstituted in PBS to a stock solution concentration of 1mg/ml and stored at 4°C until required. For use, the stock solution was diluted with PBS to a working concentration of 50μg/ml (Schou, Bøg-Hansen and Fiehn, 1999).

5.2.2 Coating microtitre plates with matrix protein

A 0.2ml aliquot of the protein suspension (fibrinogen, fibronectin, collagen type I, laminin or heparin) prepared to a working concentration was dispensed into the first well of each row of a microtitre plate (Immulon 2) and 0.1ml of the appropriate diluent into wells 2-10. The final two wells of each row remained as empty control wells. From well 1, 0.1ml of the protein suspension was transferred to well 2, mixed thoroughly with the diluent and 0.1ml transferred to the next well. This method of two-fold serial dilution was performed between the adjacent wells up to and including well 8 from which the excess 0.1ml was discarded. Wells 9 and 10 contained only diluent and served as further control wells. The plate was covered with aluminium foil and incubated at 4°C for 18h. The protein suspension was removed from the wells by inversion, the plate tapped dry onto an absorbent material and finally submerged into blocking solution [0.01M Tris-HCl (pH 7.4), 0.9% (w/v) NaCl, 0.3% (v/v) Tween 20]. The Tris-buffered saline-Tween 20 was immediately

removed and the blocking procedure repeated a further three times. The blocking solution of the final submersion was retained in the wells and the plate incubated at 20°C for 3h to block protein free sites of the microtitre plate. Finally, the Tris-buffered saline-Tween 20 was removed by inversion, the wells tapped dry on absorbent material and the plate either used immediately or sealed with a microtitre tray sealing strip and stored at -20°C (fibrinogen) or 4°C (laminin, fibronectin, collagen type I and heparin) until required.

5.2.3 Microtitre plate inoculation

The bacterial strains (IE n = 14, CAPD n = 25) were cultivated in 20ml TSB at 37°C for 18h with aeration at 200rpm. The cells were harvested from 1ml of culture (6,700*g* × 5min), washed once in PBS and resuspended in PBS to an optical density (OD) at 600nm of 1.0. Each well in one row of the protein coated microtitre tray was inoculated with 0.1ml of the bacterial suspension and incubated at 37°C for 2h. The bacterial suspension was removed from the wells by the rapid inversion of the plate and unbound bacteria removed by immersing the plate in blocking solution. The plate was emptied and the washing procedure repeated a further two times. Finally, the OD_{405} was measured to assess bacterial adherence to the protein matrix (Nilsson *et al.*, 1998).

5.2.4 Assay evaluation

5.2.4.1 Reproducibility

A panel of control organisms were used to assess the reproducibility of the technique for each of the ECM proteins; *S. epidermidis* NCTC 11047, *S. aureus* NCTC 6571 (Oxford strain), *S. aureus* ATCC 12598 (Cowan I strain), and a clinical strain *S. aureus* (HL). Four protein coated plates were simultaneously inoculated and assessed for bacterial binding. Additionally, binding assays performed on different days, plates prepared in different batches, freshly prepared and stored plates were compared for stability and reproducibility.

5.2.4.2 Effect of growth phase

To assess the effect of growth phase on MSCRAMM expression, stationary and exponential phase cells of the control strains were evaluated for ECM protein binding. The control bacterial strains were cultivated in 20ml TSB at 37°C for 16h with aeration at 200rpm. Fresh 20ml TSB flasks were inoculated with 0.4ml of the 16h cultures and all flasks incubated for a further 2h. The bacterial cells from all eight flasks were prepared as outlined in section 5.2.3 and assessed for MSCRAMM-mediated ECM protein binding.

5.2.4.3 Inoculum effect

For each ECM protein the effect of varying the cell concentration of the control strain inoculum was assessed. Four aliquots of the prepared cells (section 5.2.3) were adjusted to an OD_{600} of 0.7, 0.8, 0.9 and 1.0 and the binding assay performed as outlined in section 5.2.3.

5.2.4.4 Specificity - inhibition by extracellular matrix protein

To assess the specificity of bacterial attachment to ECM proteins, strains that exhibited binding were evaluated for inhibition by that particular protein. A suspension of prepared cells (section 5.2.3), adjusted to an OD_{600} of 1.0, was divided into two aliquots. One aliquot was maintained at 4°C until required. The second suspension was combined with 100µg/ml of the appropriate protein and incubated on a roller at 4°C for 2h. Finally the two samples were dispensed into the wells of a protein coated microtitre plate and evaluated for bacterial cell-protein binding (section 5.2.3) (Ljungh and Wadström, 1995; Maxe *et al.*, 1986).

5.2.5 MSCRAMM-mediated adherence assay

5.2.5.1 Screening coagulase-negative staphylococci

The positive control strains selected for use in the screening assay were *S. aureus* NCTC 6571 and *S. aureus* HL and the negative control isolate was *S. epidermidis* NCTC 11047. Fourteen IE-associated CNS, twenty-five CAPD-related peritonitis

CNS, together with the appropriate controls, were screened for MSCRAMM-mediated host protein binding (section 5.2.3). The assay was repeated for any strains that exhibited attachment. Those strains that repeatedly bound to the protein were subjected to an inhibition assay with the appropriate ECM protein to assess specificity (section 5.2.4.4).

5.2.5.2 Effect of pre-treating cells with heparin and plasma

A panel of ten CNS strains (type strain *S. epidermidis* NCTC 11047, the two strains shown to bind to one of the proteins (EN4 and EN5), and seven negative strains which included both IE and CAPD-related strains) and the two *S. aureus* control strains were pre-treated with heparin and human plasma. A 0.5ml aliquot of prepared bacterial cells (section 5.2.3), adjusted to an OD_{600} of 1.3, was combined with 0.5ml human plasma (blood bank, QEH) and incubated at 4°C on a roller for 2h. A second 1ml aliquot of prepared bacterial cells, adjusted to an OD_{600} of 1.0, was combined with 5µl of heparin solution [1mg/ml heparin in distilled water]. This suspension was incubated at 4°C on a roller for 2h (Ljungh and Wadström, 1995). The untreated remainder of the cell suspension (OD_{600} of 1.0) was maintained at 4°C until required. Following incubation both the plasma and heparin treated cells were recovered from the suspension (6,700*g* × 5min), washed once with PBS and resuspended in PBS to an OD_{600} of 1.0. The treated and untreated control cells were assessed for their ability to attach to microtitre tray wells (two wells each) coated with 10µg/ml fibrinogen, fibronectin or collagen type I.

5.3 RESULTS

5.3.1 Standardisation of the assay

5.3.1.1 Protein coating of wells and measurement of protein binding

Firstly, the protocol for protein coating microtitre tray wells was standardised. Collagen type I, fibronectin or laminin adsorption onto the well surface of microtitre plates was initially conducted over a 1h period at room temperature, as indicated in the manufacturer's coating protocol. Limited adsorption was achieved using this method. The procedure was, therefore, modified to 18h incubation at 4°C, as used for fibrinogen coating wells. During the initial assessment of fibrinogen coating the microtitre plates were blocked either with BSA [2% (w/v) BSA in PBS] or with Tris-buffered saline-Tween 20. Since neither was found to be superior the Tween based blocking solution was used for all assays. Three washes with Tris-buffered saline-Tween 20 were demonstrated to be sufficient to remove loosely attached and non-adherent bacterial cells. Bacterial binding to immobilised protein was assessed at a range of different optical densities (340, 405, 450, 492, 550, 570, 620nm). An OD_{405}, which has been used in previous studies (Nilsson *et al.*, 1998), was shown to be the most discriminating wavelength.

5.3.1.2 Inoculum preparation

All inoculum cell concentrations assessed, OD_{600} of 0.7-1.0, were sufficient to saturate the solid phase bound protein, for example see figure 5.1.

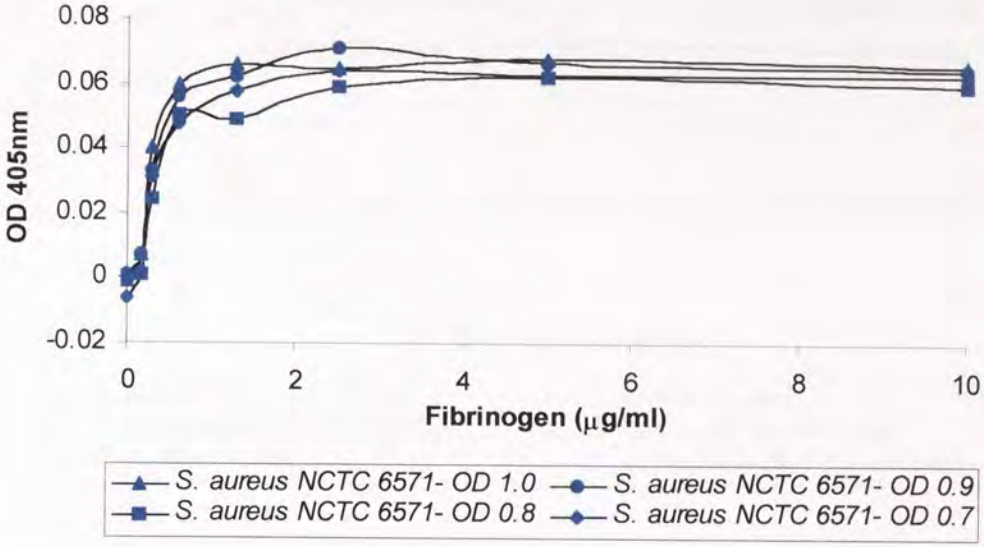


Figure 5.1. Standardisation of the inoculum. A range of *S. aureus* NCTC 6571 cell concentrations (OD_{600} 0.7-1.0) were incubated with solid phase bound fibrinogen for 2h at 37°C. After washing to remove non-adherent cells, bacterial attachment was assessed by measuring the optical density at 405nm. There were a sufficient number of bacterial cells in all four cell concentrations assessed to achieve saturation of the protein binding sites.

The effect of varying the inoculum size for the fibronectin, collagen type I and laminin assays was essentially the same as for fibrinogen (figure 5.1) and all cell concentrations were sufficient to achieve saturation. The assays were standardised with an inoculum OD_{600} of 1.0.

The level of MSCRAMM expression during different bacterial growth phases (exponential and stationary phases) was assessed. Stationary phase cells of the positive controls (*S. aureus* NCTC 6571 and/or *S. aureus* HL) exhibited a greater level of attachment to all the ECM proteins tested than exponential stage cells. The phase of growth had no effect on the negative control. Figure 5.2 illustrates the

differences in binding to fibrinogen by exponential and stationary stage cells. The fibronectin, collagen type I and laminin assays were essentially the same as for fibrinogen, therefore, stationary phase cells were used for the assay.

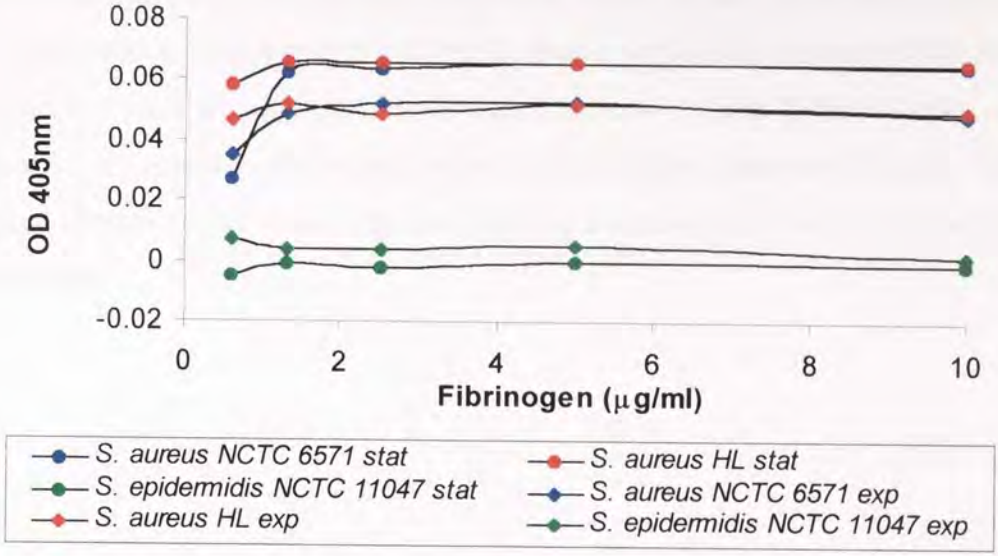


Figure 5.2. Fibrinogen binding by exponential and stationary phase cells. Differences in the level of MSCRAMM expression by exponential (*exp*) and stationary (*stat*) phase cells of the control strains were assessed. Stationary phase cells of *S. aureus* NCTC 6571 and *S. aureus* HL exhibited a greater level of binding to fibrinogen than exponential stage cells. Neither exponential nor stationary phase cells of *S. epidermidis* NCTC 11047 exhibited any capacity to bind to immobilised fibrinogen.

5.3.1.3 Positive and negative controls

The inclusion in each assay of four uncoated wells (two treated with diluent and blocked, two blocked only) per strain functioned as both negative controls and blocking controls. The average OD_{405} of these control wells was calculated to determine background levels of binding. To assess bacterial binding the background reading was subtracted from the OD_{405} of each protein coated well. The OD_{405} of bacterial binding (Y-axis) was plotted against the protein concentration (X-axis) and the ability of the organism to bind to the specific ECM protein evaluated by comparing to the positive and negative control organisms.

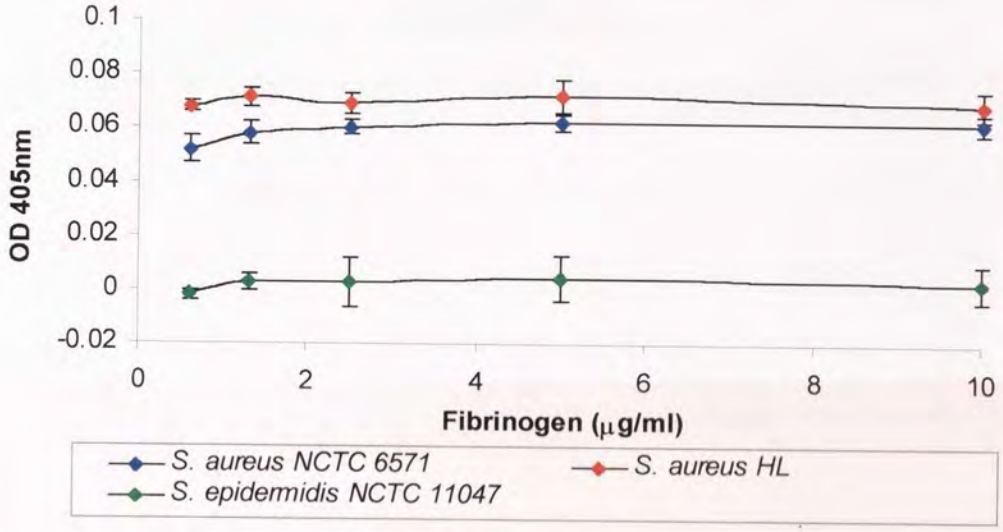
The positive control strains demonstrated that there was no batch to batch variation and confirmed the stability of protein coated plates stored prior to use. The strain *S. aureus* ATCC 12598 (which has been used in many reported studies as a positive control strain (Schou, Bøg-Hansen and Fiehn, 1999; Maxe *et al.,* 1986; Speziale *et al.,* 1986)) was included in the initial trial assays performed for each ECM protein. This strain bound to each protein screened. However, these bacterial cells, which expressed a clumping phenotype during broth culture, generated highly variable results. Therefore, the strain was not used as a positive control for the screening experiments.

5.3.2 Evaluation of the assay

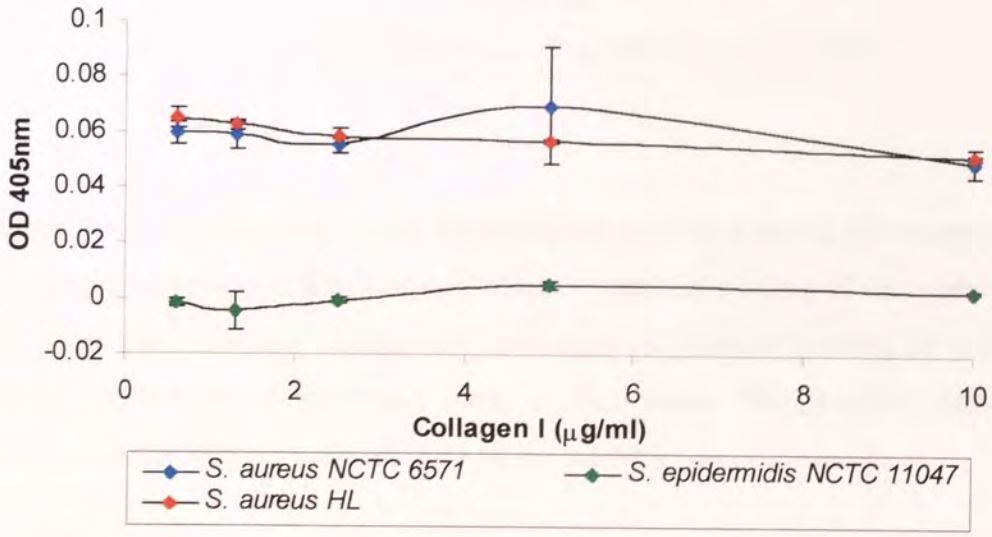
5.3.2.1 Reproducibility

Assays to evaluate the reproducibility of binding to each of the four ECM proteins (fibrinogen, fibronectin, collagen type I and laminin) were replicated three or four times and the standard deviation assessed (figure 5.3). No strains, including the positive control panel of microorganisms, bound to immobilised heparin.

a



b



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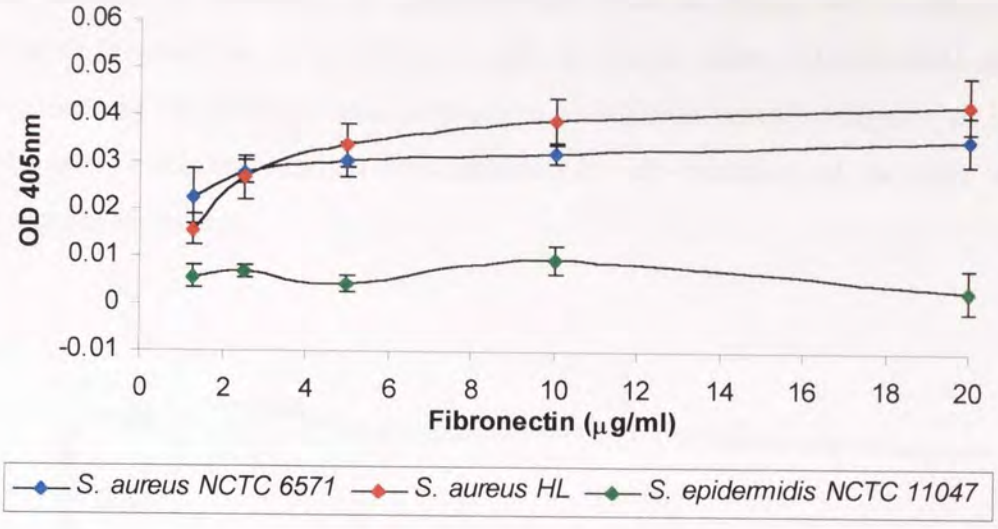


d

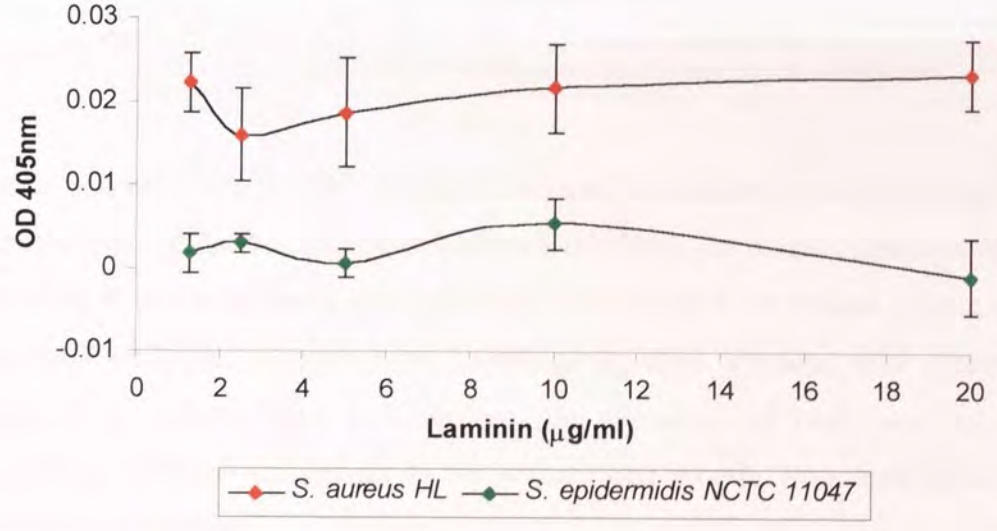


Figure 5.3. Reproducibility of the MSCRAMM binding assay. a, fibrinogen binding; b, collagen I binding; c, fibronectin binding; d, laminin binding of the control strains. The error bars illustrate the standard deviation of bacterial binding to each of the protein concentrations replicated three or four times. The standard deviation of adhesion by the control stains to each of the proteins was comparable.

5.3.2.2 Specificity

The specificity of the binding assay was demonstrated by inhibiting adhesion with free protein; for example, the prepared cells were incubated with collagen type I prior to inoculation of a collagen type I coated plate. MSCRAMM-mediated adherence by the positive control strains to solid phase bound collagen type I (figure 5.4), fibronectin and laminin was inhibited by pre-treatment of the cells with the appropriated protein.

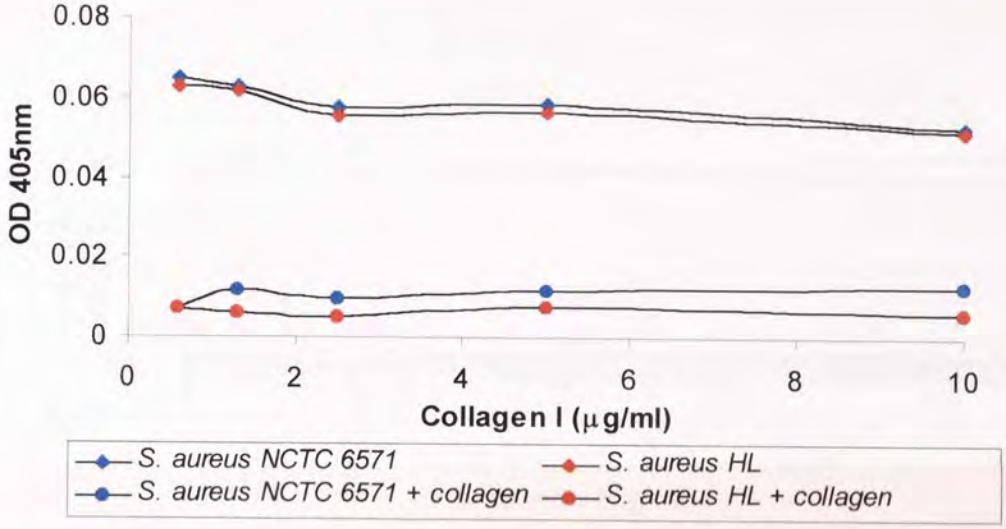


Figure 5.4. Inhibition of MSCRAMM-mediated attachment to collagen type I. The bacterial cells of each *S. aureus* strain were prepared and divided into two aliquots. One sample of the prepared cells remained untreated and the second aliquot of cells was incubated with collagen type I (100µg/ml). Both aliquots were assessed for adhesion to collagen type I. Collagen type I coating of both bacterial strains completely inhibited attachment to the immobilised protein, thus demonstrating the specificity of binding.

Fibronectin and laminin attachment were similarly inhibited, whilst fibrinogen binding was either unaffected (*S. aureus* NCTC 6571) or increased (*S. aureus* HL).

5.3.3 Screening endocarditis and continuous ambulatory peritoneal dialysis-associated coagulase-negative staphylococcal strains for MSCRAMM-mediated adherence.

Of the CNS screened (IE-associated CNS n = 14, CAPD-related peritonitis CNS n = 25) none bound to fibrinogen or laminin, one IE-related CNS strain attached to fibronectin (EN5) and one IE-associated *S. simulans* strain exhibited limited binding to collagen type I (EN4). An example of ten IE-associated strains tested for binding to fibrinogen is shown in figure 5.5.

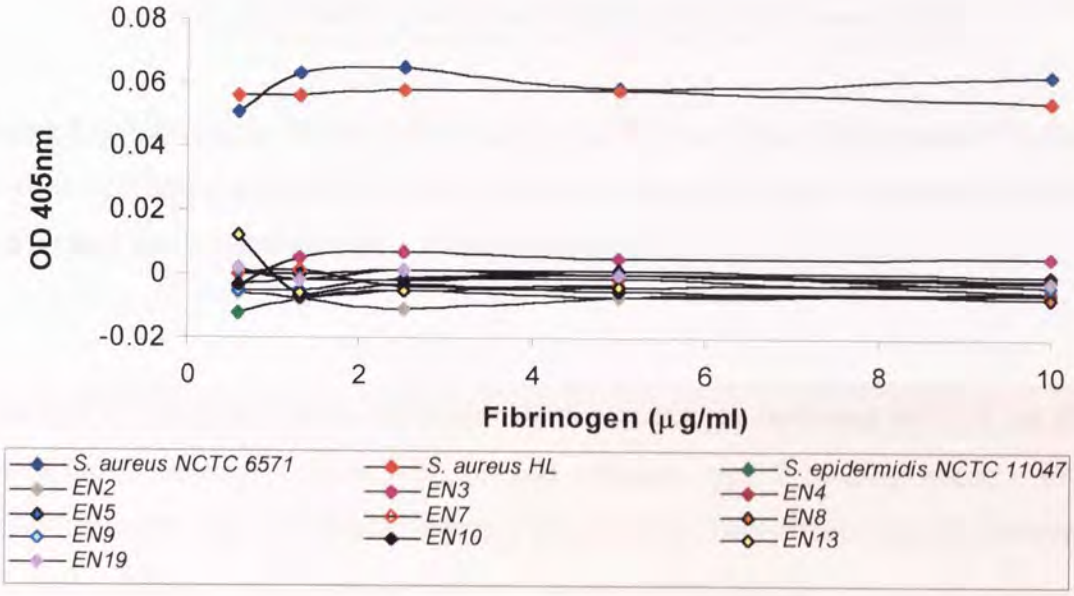


Figure 5.5. MSCRAMM-mediated fibrinogen binding by IE-associated CNS strains. Whilst the positive control strains attached to the immobilised protein, none of the ten IE-related strains screened or the negative control bound to the fibrinogen.

Fibronectin binding was exhibited by the IE-associated CNS strain EN5 (figure 5.6) and the specificity of attachment was demonstrated by the complete inhibition of binding following pre-treatment of the cells with soluble fibronectin (data not shown).

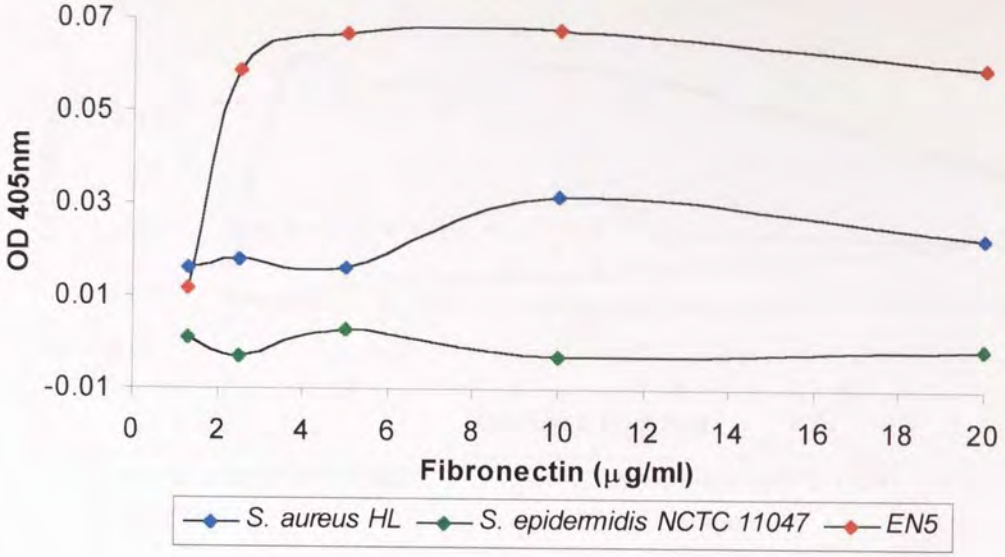


Figure 5.6. Fibronectin binding exhibited by the IE-associated CNS strain EN5. The level of attachment exhibited by EN5 (the only isolate to exhibit a capacity to attach to fibronectin) was greater than achieved with the positive control strain.

Coating EN5 with fibronectin inhibited adherence, but pre-treatment of EN4 (an IE-associated *S. simulans* strain with limited collagen type I binding ability) with soluble collagen type I enhanced binding (figure 5.7). This effect was not observed following collagen type I coating of the *S. aureus* control strains.

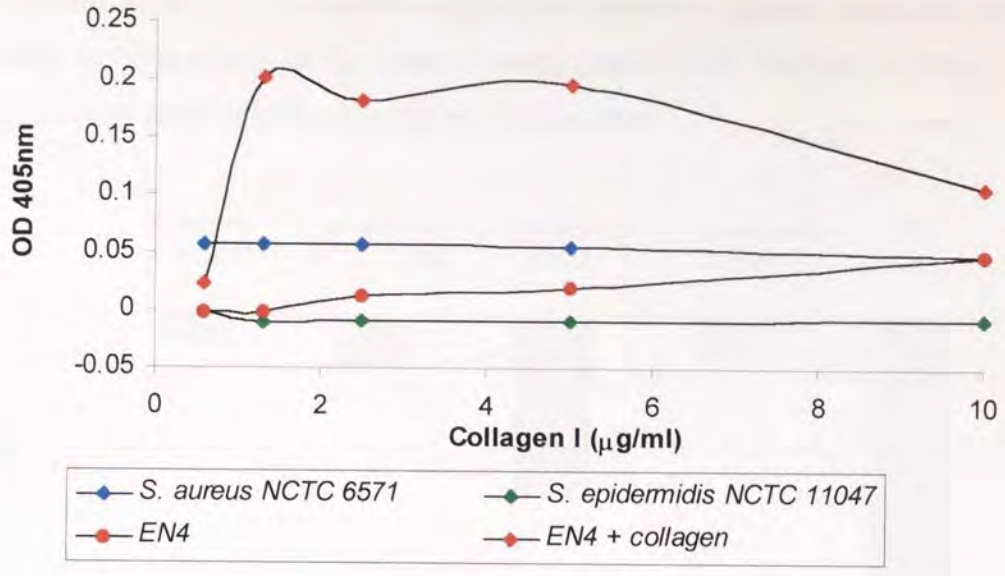


Figure 5.7. Enhancement of binding to collagen type I by the IE-related *S. simulans* strain EN4 pre-treated with soluble collagen type I. Cells of EN4 exhibited minimal binding to the collagen type I layer, however, pre-coating the cells with the protein enhanced binding to the immobilised collagen type I.

All strains were screened for MSCRAMM-mediated binding to heparin coated microtitre plate wells. No strains, including the positive control panel, bound during the assay.

5.3.4 Pre-treatment of cells with heparin or plasma

5.3.4.1 Heparin

A number of ECM proteins (Paulsson *et al.*, 1994) and bacteria have been shown to interact with heparin (Rostand and Esko, 1997; Winters, Ramasubbu and Stinson, 1993; Liang *et al.*, 1992; Choi and Stinson, 1989). The aim of this section of the study was to investigate heparin-mediated ECM protein (fibrinogen, fibronectin and collagen type I) adhesion. Pre-treatment with heparin did not induce binding to fibrinogen, collagen type I or fibronectin by MSCRAMM negative CNS strains. Neither did the treatment alter the capacity of the positive strains to bind protein, except binding of CNS EN5 to fibronectin was inhibited (figure 5.8). Interestingly, untreated control cells of *S. epidermidis* CAPD2 and *S. epidermidis* EN23 bound to

fibronectin whilst heparin treated cells of both strains exhibited decreased levels of binding in comparison to the control wells (figure 5.8). Neither of these strains exhibited any level of binding in the previous assays.

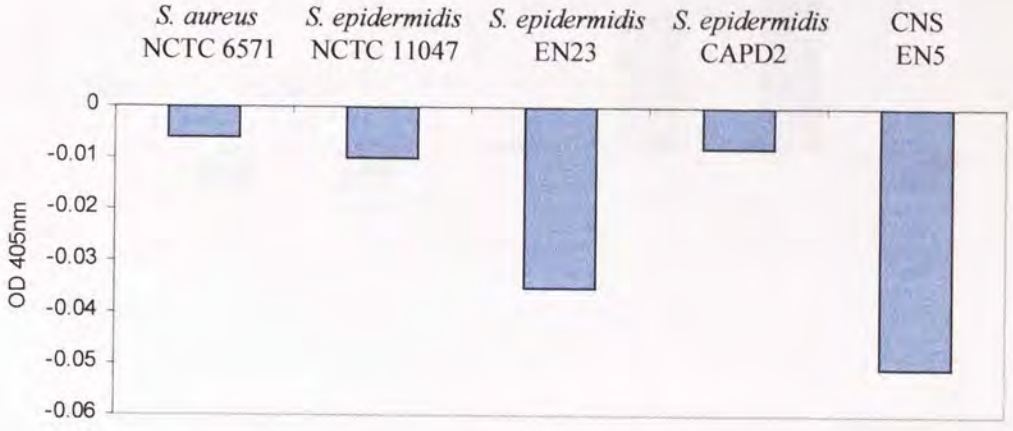


Figure 5.8. Effect of pre-treatment of cells with heparin on binding of CNS and *S. aureus* cells to fibronectin. The negative values were calculated by reference to the level of binding achieved with untreated cells. Heparin coating *S. epidermidis* EN23 and CNS EN5 cells inhibited attachment to fibronectin. The binding of the *S. aureus* control strain was unaffected.

5.3.4.2 Plasma

Plasma treatment of the bacterial cells did not induce MSCRAMM negative strains to bind to fibrinogen. Two IE-associated strains (*S. epidermidis* EN19 and *S. epidermidis* EN23) were induced to adhere to collagen type I in increased levels following plasma coating and *S. epidermidis* EN19 also exhibited a weak level of adherence to fibronectin (figure 5.9b). Adherence of the *S. aureus* positive control strains to both collagen type I and fibronectin was reduced. Similarly, the binding of the previously positive CNS strain EN5 to fibronectin was reduced whilst attachment of *S. simulans* EN4 to collagen type I was also slightly decreased (figure 5.9a). As observed with the heparin assay, untreated cells of *S. epidermidis* CAPD2 and *S. epidermidis* EN23 bound to fibronectin (neither strain exhibited any level of binding in the previous assessments). Plasma treated cells of both these strains exhibited decreased levels of binding in comparison to the control wells (figure 5.9, section 5.3.4.1).

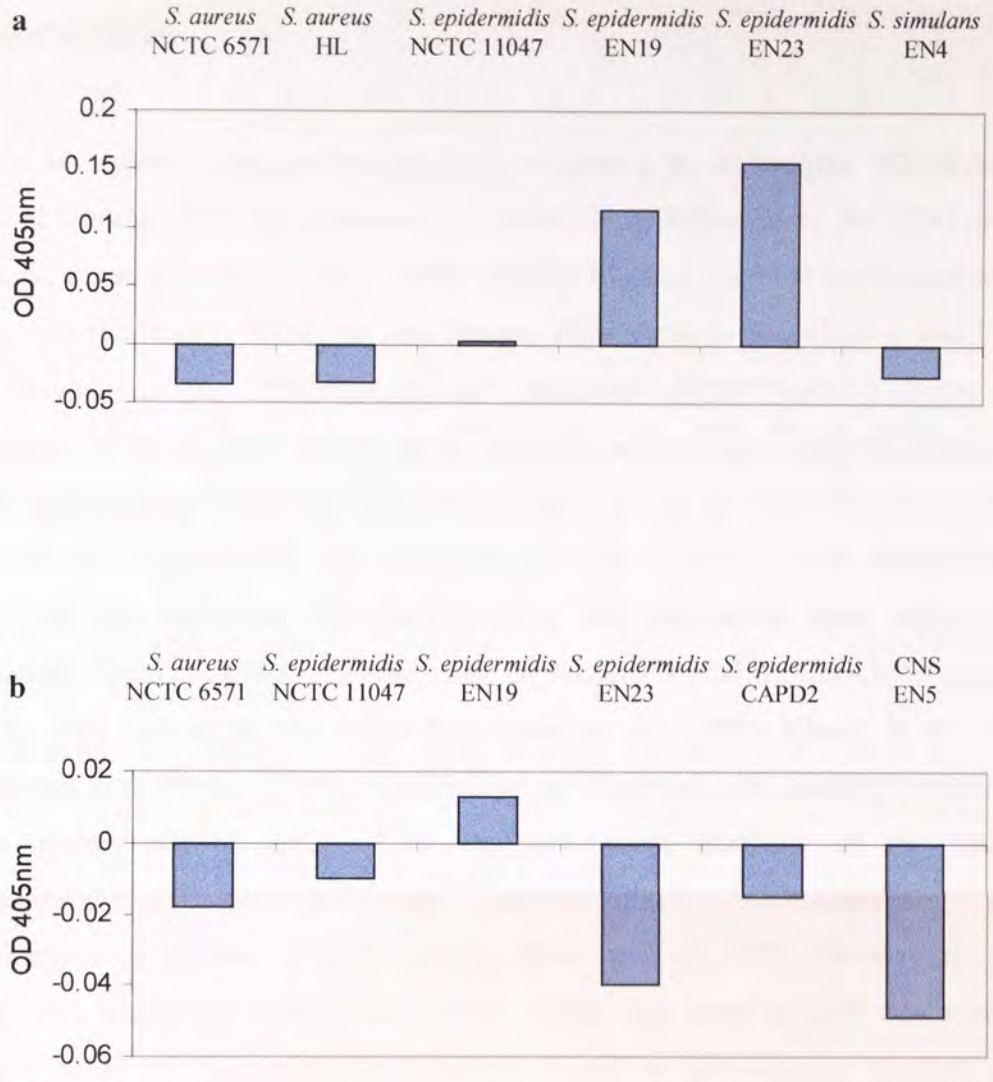


Figure 5.9. The effect on adherence of cells pre-treated with plasma. a, cells pre-treated with plasma and challenged to bind to immobilised collagen type I; b, cells coated with plasma and assessed for attachment to solid phase bound fibronectin. All changes in adherence were calculated by reference to the level of binding achieved with untreated cells. Plasma coating the two *S. epidermidis* strains EN19 and EN23 induced attachment to collagen type I but slightly inhibited binding by the type I collagen MSCRAMM-positive *S. simulans* strain EN4. Conversely, plasma pre-treatment of *S. epidermidis* EN23, *S. epidermidis* CAPD2 and the fibronectin MSCRAMM-positive CNS EN5 inhibited fibronectin binding but induced a low level of attachment in the strain *S. epidermidis* EN19.

5.4 DISCUSSION

Various techniques have previously been employed to investigate MSCRAMM-mediated binding including radiometric methods, immobilisation of the ECM protein to a solid phase (microtitre plate wells), affinity blotting, particle agglutination and cell culture (Duensing, Wing and van Putten, 1999; Schou, Bøg-Hansen and Fiehn, 1999; Gillaspy *et al.,* 1998; Ljungh and Wadström, 1995; Paulsson, Ljungh and Wadström, 1992). Initially, in this study a particle agglutination method using ECM protein coated latex beads was attempted (Paulsson, Ljungh and Wadström, 1992). However, auto-agglutination was demonstrated with a number of the protein-coated beads and the technique abandoned. Since the microtitre plate method had successfully been used in the investigation of staphylococcal MSCRAMM-mediated binding, this technique was utilised (Nilsson *et al.,* 1998; Flock *et al.,* 1996; Gatermann and Meyer, 1994). Quantitation of bacterial cell binding to the solid phase protein can be achieved by the fluorescent labelling of the bacteria, measurement of bioluminescence, enzyme-linked immunosorbent assay, measurement of OD and bacterial staining (Nilsson *et al.,* 1998; Flock *et al.,* 1996; Ljungh and Wadström, 1995; Maxe *et al.,* 1986). The latter method was evaluated but the level of discrimination was not found to be superior to the direct measurement of the OD_{405} of adherent whole bacterial cells.

In previous studies CNS strains have been reported to bind to fibrinogen, collagen, fibronectin and laminin (Nilsson *et al.,* 1998; Paulsson *et al.,* 1993; Paulsson and Wadström, 1990; Switalski *et al.,* 1983). Therefore, it was anticipated that a number of the isolates screened in this study would also exhibit a level of adhesion to these ECM proteins. The lower than expected number of CNS strains exhibiting MSCRAMM-mediated binding to ECM proteins might be explained by the following:

- Many of the ECM proteins were obtained from animal sources and, therefore, may have had structural differences in the binding region of the protein (Ljungh and Wadström, 1995). Where possible extracellular matrix proteins derived from a human source were used, for example fibronectin, although the majority

utilised were derived from an animal source; collagen type I, laminin, heparin and fibrinogen.

- The method of purification used in the commercial preparation of the ECM protein may have induced denaturation or conformational changes in the binding region (Ljungh and Wadström, 1995).
- Slime production by the CNS may have masked the bacterial receptor and prevented the MSCRAMM-ligand interaction (Gillaspy *et al.*, 1998; Baldassarri *et al.*, 1997).
- CNS may not have expressed MSCRAMMs during the stationary phase of growth. This form of regulation is observed with the *S. aureus* ClfB receptor that is only produced to the early exponential phase of growth (Ní Eidhin *et al.*, 1998). A number of previous studies have found that most bacteria express ECM protein receptors during the exponential phase of growth as opposed to the stationary phase (Gillaspy *et al.*, 1998; Ljungh and Wadström, 1995). However, during the initial assessment of the assay the stationary phase positive control strains clearly adhered to a greater level than exponential stage cells.
- The growth medium and incubation temperature used for cultivation can have pronounced effects on MSCRAMM expression (Ljungh and Wadström, 1995). In this study the bacteria were cultured in conditions identical to those used in previous investigations (Switalski *et al.*, 1983; Paulsson and Wadström, 1990).
- The exposed binding domains of a protein vary between the soluble and absorbed forms and even between the different polymer types onto which the protein is coated (Paulsson *et al.*, 1993; Proctor, 1987). In this study only the ability to bind to protein sites exposed by immobilisation to polystyrene were investigated.

5.4.1 Fibrinogen

S. aureus produces a number of cell wall associated fibrinogen binding proteins, ClfA (clumping factor), ClfB and FbpA, in addition to at least three secreted binding proteins; an 87kDa fibrinogen binding coagulase, a 60kDa fibrinogen-prothrombin coagulase and a 19kDa fibrinogen binding protein (Fib) (Foster and Höök, 1998; McDevitt *et al.*, 1997; Palma *et al.*, 1996; Cheung *et al.*, 1995). The *S. epidermidis* gene *fbe*, which shares a level of homology with *clfA*, has been implicated in

fibrinogen binding (Nilsson *et al.*, 1998). Although Fbe has homologous regions with ClfA it is clear the two proteins interact with fibrinogen differently and in this respect Fbe more closely resembles ClfB (Pei *et al.*, 1999). PCR analysis has shown that although the majority of *S. epidermidis* clinical isolates investigated carried the *fbe* genes (forty out of forty-three), a number of those strains failed to bind to solid phase bound protein (Pei *et al.*, 1999; Nilsson *et al.*, 1998). In this study none of the CNS strains investigated were found to adhere to immobilised fibrinogen despite using the same protocol as Nilsson *et al.* (1998).

S. aureus clumping factor mediates adherence to fibrinogen and fibrin, both of which are present in large amounts in the endocardial vegetation (Ing, Baddour and Bayer, 1996; McFarland, 1992). It is also involved in platelet aggregation which, is another important component of the valvular vegetation (McFarland, 1992). In a rat IE model, a fibrinogen-binding ($ClfA^-$) *S. aureus* mutant was significantly less virulent than the wild type parental strain (Moreillon *et al.*, 1995). It is interesting to speculate that, if CNS strains do possess a functional fibrinogen binding protein, the receptor-ligand interaction may aid bacterial colonisation of a sterile vegetation present on a damaged heart valve. Furthermore, since this ECM protein is one of the plasma components that rapidly coats the surface of prostheses after implantation it may also be involved in the pathogenesis of PVE (Vaudaux *et al.*, 1995; Herrmann *et al.*, 1988).

5.4.2 Collagen type I

Although an affinity for collagen by *S. aureus* was reported in 1983 (van de Water, Destree and Hynes, 1983), studies investigating binding by CNS strains are contradictory; from no bacterial adhesion to binding by the majority of isolates investigated (Paulsson, Ljungh and Wadström, 1992; Paulsson and Wadström, 1990; Holderbaum *et al.*, 1987). Since a number of studies have shown that *S. aureus* collagen binding is not collagen-type specific, CNS binding in this study was assessed only against type I collagen (one of at least fifteen species of collagen) (Foster and Höök, 1998; Gillaspy *et al.*, 1998; Ljungh and Wadström, 1995; Switalski, Speziale and Höök, 1989; Holderbaum *et al.*, 1987; Speziale *et al.*, 1986). However, one investigation has indicated that there are differences between adhesion

to fibre forming collagens (types I, II and III) and those associated with basement membranes (for example type IV) (Naidu, Ekstrand and Wadström, 1989). Of the CNS strains screened during this investigation only one IE-associated *S. simulans* strain (EN4) exhibited a low level of collagen type I binding. Although pre-coating *S. aureus* cells with collagen type I reduced or inhibited attachment to the immobilised protein, pre-treatment of *S. simulans* EN4 with soluble collagen I enhanced binding. Considering there is no circulating form of this protein in plasma the relevance of this finding is unclear (Holderbaum *et al.*, 1987).

Since bone, cartilage, valvular and aortic tissues are amongst those containing collagen a number of groups have investigated the role of collagen binding in *S. aureus* associated IE, osteomyelitis and arthritis (Montanaro *et al.*, 1999; Hienz *et al.*, 1996; Patti *et al.*, 1994b; Switalski *et al.*, 1993). IE and arthritis animal models infected with *S. aureus* strains expressing the collagen receptor Cna were more likely to be associated with clinical signs of infection than mice infected with Cna$^-$ strains (Hienz *et al.*, 1996; Patti *et al.*, 1994b). Furthermore, Holderbaum *et al.* (1987) and Switalski *et al.* (1993) both reported that *S. aureus* strains associated with complicated bacteraemia (septic arthritis, osteomyelitis and IE) were more likely to bind to collagen than strains related to uncomplicated bacteraemia. However, Thomas *et al.* (1999) concluded that there was no significant difference between strains isolated from different infection types and their ability to bind to this ECM protein. Despite the contradictory reports, there is good evidence to suggest that collagen binding by *S. aureus* strains is a virulence factor associated with a number of different infections (Rhem *et al.*, 2000; Hienz *et al.*, 1996; Patti *et al.*, 1994b). If CNS strains possess a functional *cna* homologue this would enhance their ability to adhere to basement membrane collagen exposed by damage to the endothelium.

5.4.3 Fibronectin

The ability of *S. aureus* to bind fibronectin was first reported in 1978 (Kuusela, 1978), but since then no single staphylococcal species has been found to consistently bind this ECM protein (Switalski *et al.*, 1983). Most *S. aureus* strains express two fibronectin-binding proteins; FnBPA and FnBPB (Foster and Höök, 1998; Greene *et al.*, 1995; Jönsson *et al.*, 1991). A number of studies investigating the ability of CNS

to bind fibronectin have reported conflicting results (Valentin-Weigand, Timmis and Chhatwal, 1993; Paulsson, Ljungh and Wadström, 1992; Herrmann *et al.,* 1988; Switalski *et al.,* 1983; Myhre and Kuusela, 1983; Doran and Raynor, 1981; Verbrugh *et al.,* 1981). In this investigation only one out of forty CNS strains screened exhibited any level of fibronectin binding. The ability to inhibit this attachment by pre-treatment of EN5 (the one IE-related CNS strain that adhered to fibronectin) demonstrated the specificity of binding (Maxe *et al.,* 1986).

Valentin-Weigand *et al.* (1993) showed that the adherence of *S. epidermidis* to fibrin thrombi and plastic was increased through interactions with immobilised fibronectin, whilst soluble fibronectin had no effect. Conversely, soluble fibronectin did increase the adherence of *S. aureus* to fibrin thrombi and endothelial cells (Valentin-Weigand, Timmis and Chhatwal, 1993; Vann *et al.,* 1989). Therefore, it is apparent that *S. aureus* and CNS have different mechanisms of adherence. *S. aureus* adhere to a matrix via soluble fibronectin (as present in wound exudate) whilst CNS interact directly with the immobilised protein (for example, coating prosthetic devices after insertion). The different affinities might reflect the conformational differences in soluble (globular protein) and immobilised fibronectin (two arms of the dimer unfold completely) (Proctor, 1987). The assay used in this experiment assessed only the ability to bind to immobilised fibronectin.

The capacity of a bacterium to bind to fibronectin is potentially an important virulence factor associated with IE and has been extensively reviewed (Hamill, 1987). In addition to soluble fibronectin being incorporated into and covering the surface of an endocardial vegetation the immobilised form of the protein is also present in the subendothelial layer of heart valves and, thus, exposed following tissue damage (Campbell and Johnson, 1990; Hamill, 1987). Furthermore, this ECM polypeptide is one of the major plasma proteins that form a "conditioning layer" on the surface of prosthetic heart valves after implantation (Valentin-Weigand, Timmis and Chhatwal, 1993). A number of studies have investigated the role of fibronectin binding and IE (Flock *et al.,* 1996; Kuypers and Proctor, 1989; Vann *et al.,* 1989; Hamill, Vann and Proctor, 1986). A *S. aureus* fibronectin-binding parent strain was found to attach to rat heart valves, at sites of catheter induced valve trauma, in numbers significantly higher than those achieved by a fibronectin non-binding

mutant strain. The difference was shown not to be due to differing growth rates, collagen, fibrinogen or laminin binding and the number of organisms recovered from blood was identical (Kuypers and Proctor, 1989). Furthermore, a fibronectin binding wild-type strain of *Streptococcus sanguis* has been demonstrated to be more virulent than a non-binding mutant (Lowrance, Baddour and Simpson, 1990). Conversely, Flock *et al.* (1996) using a similar rat IE model to Kuypers and Proctor (1989), found no attachment differences between the fibronectin non-binding mutant strain of *S. aureus* and the parental wild-type. Current interest is centred on the possibility that the bacterial cells directly invade heart tissues in a process initiated by a MSCRAMM-mediated interaction. A number of studies have presented evidence for fibronectin-mediated internalisation of *S. aureus* by non-professional phagocytes including epithelial cells (Dziewanowska *et al.*, 1999; Lammers, Nuijten and Smith, 1999; Hamill, Vann and Proctor, 1986).

Currently, there are no published studies investigating the virulence role of fibronectin binding by CNS in IE, although one group has demonstrated that the majority of IE-associated *S. epidermidis* strains carried the *fbnA* gene or a homologue (Minhas *et al.*, 1995). Based on this evidence and *S. aureus* related studies, a fibronectin MSCRAMM may aid CNS colonisation of damaged valve tissue during the initial stages of endocarditis. The results in this thesis indicate that fibronectin-binding by IE-associated CNS isolates may contribute to pathogenesis in a minority of strains but receptor expression is not essential to the infection.

5.4.4 Laminin

Laminin receptors have been identified in *S. aureus* (Lopes, dos Reis and Brentani, 1985). As the most abundant glycoprotein in the basement membranes of cardiac tissues the ability of bacterial cells to bind to this ECM protein would be a virulence factor of IE (Martin and Timpl, 1987). Switalski *et al.* (1987) found that only 25% of strains of oral viridans streptococci expressed the laminin MSCRAMM, whereas >80% of strains recovered from IE bound this ECM protein. In accordance with the work of Herrmann *et al.* (1988) and Lopes *et al.* (1985) none of the CNS strains screened in this investigation were shown to adhere to immobilised laminin. Though these studies indicate the inability of CNS to bind laminin, Paulsson *et al.* (1992)

demonstrated expression of a laminin MSCRAMM in strains recovered from a variety of infections including IE. The conflicting results probably reflect the different methodologies used; for example, fibronectin is known to expose dissimilar binding domains when absorbed onto different polymer surfaces (Paulsson *et al.*, 1993)

5.4.5 Heparin

S. aureus, Bordetella pertussis, Haemophilus influenzae, Streptococcus mutans, Streptococcus pyogenes and *Chlamydia trachomatis* have the ability to adhere to glycosaminoglycans (Rostand and Esko, 1997; Winters, Ramasubbu and Stinson, 1993; Liang *et al.*, 1992; Choi and Stinson, 1989). Glycosaminoglycans such as heparin and heparan sulphate, which are present on the surface of cardiac endothelium (Yu *et al.*, 1994), may be ECM components that pathogenic microorganisms bind to facilitate colonisation of host tissues. *S. pyogenes* and *S. mutans*, frequent aetiological agents of IE, express a glycosaminoglycan-binding protein that selectively binds to the basement membrane of cardiac tissue (Winters, Ramasubbu and Stinson, 1993; Choi and Stinson, 1989). There are currently no reported studies investigating glycosaminoglycan-mediated bacterial attachment and the development of staphylococcal IE.

In addition to a direct microorganism-glycosaminoglycan–host tissue interaction heparin may mediate adhesion via another ECM protein. Heparin interacts with many ECM proteins (including fibronectin and laminin) because of its strongly anionic nature (sulphate and carboxyl groups) (Cardin *et al.*, 1991) and the relatively low specificity of the binding consensus sites within the proteins (Westerlund and Korhonen, 1993; Cardin *et al.*, 1991; Martin and Timpl, 1987). *Neisseria gonorrhoeae* does not have specific receptors for proteins such as fibronectin. However, coating this organism with heparan sulphate allowed binding to both serum vitronectin and fibronectin (Duensing, Wing and van Putten, 1999). The enrolment of host proteins via a heparan sulphate bridge was also shown to enhance the invasive potential of the bacterium into epithelial cells and interfere with the formation of a cytokine-induced chemotactic gradient (Duensing, Wing and van Putten, 1999). Dextran sulphate (a heparin related sulphated polysaccharide) coating

of staphylococci was similarly shown to facilitate attachment to ECM proteins, including vitronectin (Duensing, Wing and van Putten, 1999).

In this thesis the binding of CNS to immobilised heparin was assessed by a microtitre plate method. The failure of all the control strains and the CNS test isolates to adhere suggested that either none of the strains expressed a glycosaminoglycan-binding protein or the technique was not be capable of detecting adhesion. Heparin and other glycosaminoglycans are highly charged, hydrophilic molecules and, therefore, do not easily interact with hydrophobic surfaces such as microtitre trays (Paulsson *et al.*, 1994). Although this method was used by Schou *et al.* (1999), they also failed to detect binding by streptococci. It is therefore probable that the heparin coating procedure used was unsuitable for attaching the heparin to polystyrene microtitre tray wells.

The potential ability of CNS to bind to heparin has further implications. The surface of some prosthetic devices such as CVC are heparinized to create a thromboresistant surface (Paulsson *et al.*, 1994; Yu *et al.*, 1994). This might, however, promote bacterial attachment and thus aid the establishment of a prosthetic device-related infection. Heparinization of a polyethylene film decreased CNS binding but the adherence of cells to the serum-coated heparinized surface was of the same magnitude or greater than that observed for the non-heparinized serum-coated film (Paulsson *et al.*, 1994). Treatment of the two surfaces with a single protein species, such as fibronectin, increased the binding of CNS to the heparinized over the non-heparinized polyethylene (Paulsson *et al.*, 1994). This again illustrates the complexity of *in vivo* ECM protein interactions.

5.4.6 Summary of coagulase-negative staphylococcal pathogenicity and MSCRAMMs

There is a vast amount of published literature, of which much is contradictory, investigating the role of MSCRAMMs and infection. Understanding the relevance of bacterial attachment to ECM protein in a complex and dynamic *in vivo* system is complicated further by the enormous variety of methods and protein combinations used in various published studies. Certain combinations of ECM proteins can have an

enhanced effect on bacterial attachment (for example collagen type I and vitronectin with CNS) whilst others can be inhibitory (as observed with heparin and fibronectin with *S. aureus*) (Paulsson and Wadström, 1990; van de Water, Destree and Hynes, 1983). Due to these difficulties, a genetic approach might generate more useful information than investigating MSCRAMMs at the phenotypic level. Many *S. aureus* MSCRAMM genes, for example fibrinogen (*fib*, *clfA* and *fbpA*), collagen (*cna*) and fibronectin (*fnbA* and *fnbB*), have been identified and could be used to screen CNS by PCR for homologous genes. This approach has already proved successful in the investigation of a CNS fibronectin-binding gene (Minhas *et al.*, 1995). This would indicate the potential of the organisms to bind to ECM proteins prior to investigating *in vivo* gene expression.

In this study no CNS strains investigated attached to either fibrinogen or laminin and the majority exhibited no collagen or fibronectin binding ability. Unexpectedly, heparin coating the bacterial cells did not enhance or induce binding to the ECM proteins but inhibited fibronectin binding by two CNS strains. The involvement of plasma proteins with bacterial adhesion is not clear. As noted in previous studies, incubation of bacterial cells with plasma either inhibited attachment (Galliani *et al.*, 1994; Muller *et al.*, 1991) or induced binding (Vaudaux *et al.*, 1989) - further illustrating the complexity of MSCRAMM-ligand interactions.

The importance of CNS MSCRAMMs and their potential role as virulence factors is not clearly understood. Indeed, few CNS-ligand interactions have been fully investigated. Binding soluble ECM proteins, such as fibrinogen and fibronectin (Patti *et al.*, 1994a), may provide the bacterial cell with a protein coat that prevents recognition by the immune system. Conversely, a coat of fibronectin might act as an opsonin. Furthermore, these soluble proteins rapidly coat the surface of implanted devices. MSCRAMM-mediated attachment to a device would aid the establishment of prosthesis-related infection. Binding to a ligand found exclusively in the ECM, such as collagen and laminin (Patti *et al.*, 1994a), is thought to mediate direct bacterial attachment to host tissues and possibly initiate internalisation. An organism possessing a specific MSCRAMM is more likely to attach to and invade host tissues containing that ECM protein than tissues without the protein. This hypothesis of tissue tropism suggests that there might be subsets of staphylococci with a

predilection to certain types of infection, such as IE (Patti *et al.*, 1994a). In this and two previously published studies (Paulsson, Ljungh and Wadström, 1992; Paulsson and Wadström, 1990), there were no identified subsets of strains. Indeed, in this investigation no CNS strains related to IE or CAPD-peritonitis exhibited any ability to bind to fibrinogen or laminin and only one strain bound to either collagen or fibronectin. Since not all staphylococcal strains associated with a particular infection, either in this study or the published literature, expressed a specific receptor, for example the collagen adhesin Cna, other virulence factors must be involved. In Chapter 6 the immune response of an IE patient will be used to screen *in vivo* expressed bacterial products for other potential IE-associated virulence factors.

CHAPTER 6: A STAPHYLOCOCCAL GENOMIC DNA LIBRARY

6.1 INTRODUCTION

6.1.1 Lambda Zap Express

Lambda Zap Express (Stratagene) is an insertion vector with multiple cloning sites within plasmid sequences and a cloning capacity of 0-12kb (Short *et al.*, 1988). Expression is driven by the *lac*Z promoter, which is repressed in the presence of the LacI protein and induced in the presence of isopropyl-1-thio-β-D-galactopyranoside (IPTG). The pBK polylinker is placed downstream from this promoter in the amino terminus of the α-complementing portion of the β-galactosidase gene allowing blue/white colour screening of recombinant clones. The inserted gene product, expressed as a fusion protein, is screened with antibody probes. DNA fragments are excised *in vivo* from the vector to generate kanamycin-resistant subclones in pBK-CMV phagemid vector (Stratagene).

6.1.2 Aim

The aim of this section of the study was to generate and screen a genomic DNA library of *S. epidermidis* NCTC 11047 with serum collected from a patient with active *S. epidermidis* IE. This approach screened for cellular and secreted staphylococcal antigens that were expressed *in vivo* and induced an immune response within the patient. These *in vivo* expressed bacterial products may be virulence factors.

6.2 MATERIALS AND METHODS

6.2.1 Isolation of Genomic DNA

6.2.1.1 Preparation of genomic DNA

Genomic DNA was prepared essentially by the Ausubel *et al.* method (1995). A *S. epidermidis* NCTC 11047 starter culture, cultivated in 20ml TSB at 37°C on an orbital shaker at 200rpm for 4h, was used to inoculate 5ml of Luria broth [1% (w/v)

pancreatic digest of casein, 0.5% (w/v) NaCl, 0.1% (w/v) glucose, 0.5% (w/v) yeast extract]. This second culture was incubated in identical conditions for 18h. Cells were harvested by centrifugation (11,400*g* × 5min), re-suspended in 0.25ml TE [10mM Tris-HCl (pH 7.4), 1mM Na_2EDTA (pH 7.4)] supplemented with 80μg/ml lysostaphin and 1mg/ml lysozyme, and incubated at 37°C for 90min. Two μl of proteinase K [25mg/ml in TE (pH 7.4)] was added to the cell wall digest and mixed followed by 27μl of 10% (w/v) sodium dodecyl sulphate (SDS) in water. The solution was inverted to mix and incubated at 37°C for 20min. Following incubation 97μl of 5M NaCl was added and vortex mixed followed by 81μl of cetyltrimethylammonium bromide (cetrimide) [10% (w/v) cetrimide in 0.7% (w/v) NaCl] pre-heated to 65°C. The suspension was vortex mixed and incubated at 65°C for 30min with occasional inversion to mix. An equal volume of chloroform:iso-amyl alcohol (24:1) was added, vortex mixed and subjected to centrifugation (11,400*g* × 5min). The upper layer was retained, an equal volume of isopropanol added and gently mixed. Pelleted DNA was recovered from solution by centrifugation (11,400*g* × 4min), washed once in 70% (v/v) ethanol and re-pelleted as previously. Ethanol was removed from the DNA pellet by heating to 45°C for 15min prior to re-hydration of the nucleic acid at 65°C with 20μl of double distilled water for a minimum of 30min. The DNA suspension was stored at -20°C.

6.2.1.2 Quantitation and purity of the DNA preparation - Absorption method

The purity and concentration of nucleic acid in the preparation was assessed by spectrophotometric measurement of absorption at wavelengths 260nm and 280nm (Cecil CE 3041, Cambridge, UK) (Sambrook, Fritsch and Maniatis, 1989).

6.2.2 Partial restriction endonuclease digestion of genomic DNA

6.2.2.1 Selection of restriction endonuclease for partial digestion of genomic DNA

Five restriction endonucleases: *Eco*RI (Boehringer Mannheim), *Bam*HI (Boehringer Mannheim), *Bgl*II (Boehringer Mannheim), *Mbo*I (MBI Fermentas) and *Sau*3AI (Sigma), were assessed for their ability to cleave *S. epidermidis* NCTC 11047 DNA.

Each 10µl restriction endonuclease digest contained 1µg DNA and 10 units restriction endonuclease in restriction endonuclease buffer prepared with sterile double distilled water. The digest for each restriction endonuclease was prepared in duplicate, one eppendorf incubated for 2h at 37°C, the second for 18h at 37°C. Restriction digestion of the DNA was assessed by agarose gel electrophoresis.

6.2.2.2 Electrophoresis of DNA

A 0.8% (w/v) agarose gel (molecular biology grade agarose, BioRad) was prepared with TBE [1mM Na_2EDTA (pH 8.0), 0.45mM Tris-borate]. On cooling to 60°C 0.5µg/ml ethidium bromide was added and the gel cast (10cm × 6.5cm × 1cm). The hardened gel was transferred to a mini sub™ DNA cell electrophoresis tank (BioRad) containing TBE electrode buffer. To each 10µl restriction digest 2µl of 6 × loading buffer [0.25% (w/v) bromophenol blue, 40% (w/v) sucrose] was added, mixed, and the sample loaded into the agarose gel (Sambrook, Fritsch and Maniatis, 1989). An undigested control sample of DNA was included. To assess the size of the fragments, 0.5µl of *Eco*RI/*Hin*dIII digested λ DNA was mixed with an equal volume of 6 × loading buffer and included in the gel as a DNA sizing ladder (size range 125-21,226bp). The gel was electrophoresed at 5.7V/cm for 40min and the DNA visualised with an UV light scanner.

6.2.2.3 Optimising partial restriction endonuclease digestion of genomic DNA

S. epidermidis NCTC 11047 DNA was partially restriction endonuclease digested with serial diluted concentrations of the restriction endonuclease *Eco*RI. *Eco*RI was diluted with restriction endonuclease buffer and combined with 1µg DNA to give final concentrations of 10, 8, 6, 4, 2 and 1 units *Eco*RI/µg DNA in a total volume of 50µl. The six prepared restriction digests were incubated for 2h at 37°C. After 2h incubation 25µl of the digest was mixed with 5µl of 6 × loading buffer and loaded into a 0.8% (w/v) agarose gel. Samples of control undigested DNA and *Eco*RI/*Hin*dIII digested λ phage DNA sizing ladder were included in the gel. Electrophoresis was conducted at 5.7V/cm for 40min and the DNA visualised using an UV light scanner. The remaining 25µl of the digest was returned to 37°C and

incubated for a further 16h. After a total of 18h incubation the remainder of the restriction digests was electrophoresed as previously outlined. The optimum concentration of *Eco*RI and incubation time was determined by the comparison of the two gels.

6.2.2.4 Extraction of partially digested genomic DNA fragments from agarose gel

Genomic DNA fragments were recovered from the agarose gel using an Agarose gel DNA extraction kit (Boehringer Mannheim), based on the technique developed by Vogelstein and Gillespie (1979). DNA fragments (~800bp to ~10kb), produced by the partial restriction digestion of 20µg genomic DNA with 80 units of the restriction endonuclease *Eco*RI (4 units *Eco*RI/µg DNA) for 2h at 37°C, were cut from an agarose gel visualised on a transilluminator (UVP Products). The agarose gel block containing the DNA fragments was dissolved with agarose solubilisation buffer (Boehringer Mannheim) (0.3ml per 100mg of agarose gel) and mixed with re-suspended silica resin (Boehringer Mannheim) (4µl per µg of DNA). The mixture was incubated at 60°C and vortex mixed every 2min for 10min. The suspension was centrifuged (11,440g × 30s), the supernatant discarded and the pelleted resin-DNA matrix re-suspended with 0.5ml nucleic acid binding buffer (Boehringer Mannheim). The recovered resin-DNA matrix pellet (11,400*g* × 30s) was washed with 0.5ml washing buffer (Boehringer Mannheim), centrifuged, and the supernatant discarded as previously. The washing step was repeated once. The eppendorf tube was inverted onto absorbent tissue to remove all the supernatant and heated to 45°C for 15min, using a heating block, to ensure the complete removal of ethanol from the resin-DNA matrix. To elute the DNA from the silica resin 25µl of freshly boiled double distilled water, cooled to 60°C, was mixed with the pellet and incubated at 60°C for 20min with frequent vortex mixing. The suspension was centrifuged (11,400*g* × 30s) and the supernatant containing the DNA recovered. The DNA concentration was determined by the ethidium bromide fluorescence method and the sample stored at 4°C.

6.2.2.5 Quantitation and purity of the DNA preparation - Ethidium bromide fluorescence method

A 1µl aliquot of the DNA sample was mixed, on a strip of Parafilm, with an equal volume of TE (pH 7.6) containing 2µg/ml ethidium bromide. The fluorescence of the sample under UV excitation was visually compared to that of similarly prepared standards of bacteriophage λ DNA (3µg/ml to 50µg/ml) and the amount of DNA in the sample estimated (Sambrook, Fritsch and Maniatis, 1989).

6.2.3 Genomic DNA Library

6.2.3.1 Ligation of DNA inserts to vector arms

The Zap Express® predigested vector (*Eco*RI/CIAP-treated) (Stratagene) bacteriophage λ cloning kit was used to generate a *S. epidermidis* NCTC 11047 genomic DNA library according to the manufacturer's protocol.

An equal molar ratio of vector: sample DNA (1:1) was used in the ligation;

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

$$\frac{1000 \times 5}{38.9} \times \frac{1}{1} = 128\text{ng insert}$$

(Promega, 1996)

The *S. epidermidis* NCTC 11047 sample ligation consisted of 1µg vector arms and ~128ng of *Eco*RI partially restriction digested genomic DNA with 0.5µl 10mM rATP (pH 7.5), 2 units T4 DNA ligase [50mM Tris-HCl (pH 7.5), 7mM $MgCl_2$, 1mM dithiothreitol (DTT)] and ligase buffer to a final volume of 5µl. A test ligation to insert pRheo into the ZAP Express vector consisted of 1.0µg Zap Express vector and 0.4µg pRheo test insert, 0.5µl 10mM rATP (pH 7.5), 2 units T4 DNA ligase and ligase buffer to a total volume of 5µl. Both ligations were incubated at 4°C for 44h.

6.2.3.2 Packaging of ligated DNA

Three samples required packaging into phage: a) the ligated sample of *S. epidermidis* NCTC 11047 DNA; b) the pRheo test ligation; and c) λ*c*I857 *Sam*7 wild-type λ control DNA to test packaging efficiency. A single packaging extract (Gigapack III Gold Packaging Extract, Stratagene) was removed from -80°C storage, defrosted at room temperature until the contents were half thawed, and 4µl of *S. epidermidis* ligated DNA-vector sample added immediately. The mixture was gently stirred with a pipette tip, pulse spun for 3s and incubated at room temperature for 90min. The packaging procedure was repeated for the remaining two samples as outlined except 3µl of the pRheo test ligation and 1µl (0.2µg) of the λ*c*I857 *Sam*7 wild-type λ control were packaged. Following incubation, 0.5ml of SM buffer [0.1M NaCl, 8mM $MgSO_4.7H_2O$, 0.05M Tris-HCl (pH 7.5), 0.01% (w/v) gelatin] was added to the packaged *S. epidermidis* and pRheo tubes (1.0ml to the λ*c*I857 *Sam*7 wild-type λ control DNA tube). Finally, 20µl of chloroform was added to each tube, the contents gently mixed, debris removed by centrifugation (11,400*g* × 30s) and the supernatant containing the phage stored at 4°C.

6.2.3.3 Titration of recombinant phage

The host strain *E. coli* XL1-Blue MRF' (Stratagene) was grown in Luria-Bertani (LB) broth [1% (w/v) NaCl, 1% (w/v) tryptone, 0.5% (w/v) yeast extract, pH 7.0] supplemented with 0.2% (w/v) maltose and 10mM $MgSO_4$, for 18h at 30°C with aeration at 200rpm. The cells were harvested (1,200*g* × 5min) and resuspended in 10mM $MgSO_4$ to an OD_{600} of 0.5. Immediately, 0.2ml of the prepared host strain was combined with 1µl of the *S. epidermidis* NCTC 11047 packaged phage. A further 0.2ml of host strain cells were combined with 1µl of the packaged phage diluted 10^{-1} with 10mM $MgSO_4$. Both suspensions were incubated at 37°C for 15min to allow phage attachment. NZY top agar [0.5% (w/v) NaCl, 0.2% (w/v) $MgSO_4.7H_20$, 0.5% (w/v) yeast extract, 1% (w/v) NZ amine (casein hydrolysate), 0.7% (w/v) agarose, pH 7.5] was supplemented with 5µl/ml IPTG [stock solution 0.5M in distilled water, filter sterilised] (Stratagene) and 16.6µl/ml 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside [250mg/ml in N,N-dimethylformamide (DMF)] (X-gal, Stratagene). The phage infected cells were combined with 3ml of the

prepared NZY top agar cooled to ~48°C, and poured immediately onto dry, pre-warmed NZY agar plates (NZY top agar with the agarose replaced by 1.5% (w/v) agar no. 2). The plates were maintained at room temperature for 10min before transfer to 37°C for 18h. Recombinant phage containing pRheo ligated product was also titered as outlined. Following incubation the blue and white plaques were counted and the recombinant phage titre calculated.

6.2.3.4 Packaging efficiency

E. coli VCS257 (Stratagene), cultivated in LB broth supplemented with 0.2% (w/v) maltose and 10mM $MgSO_4$ for 18h at 30°C, on an orbital shaker at 200rpm, was harvested (1,200*g* × 5min) and the cells resuspended in 10mM $MgSO_4$ to an OD_{600} of 0.5. To assess packaging efficiency two consecutive 10^{-2} dilutions of the λ*c*I857 *Sam*7 wild-type λ control DNA packaging reaction in SM buffer were prepared. A 10µl aliquot of the packaged phage, 10^{-4} dilution, was mixed with 0.2ml of the prepared *E. coli* VCS257 host strain and incubated for 15min at 37°C. The phage-VCS257 mixture was combined with 3ml of LB top agar [LB broth supplemented with 0.7% (w/v) agarose (pure agarose-electrophoresis grade, GibcoBRL, UK)] cooled to ~48°C, poured onto dry, pre-warmed LB agar plates [LB broth supplemented with 2% (w/v) agar no. 2] and incubated at 37°C for 18h. The plaques were counted and the efficiency of packaging assessed.

6.2.3.5 Amplification and storage of the genomic library

E. coli XL1-Blue MRF' cells were cultivated and prepared to an OD_{600} of 0.5 (section 6.2.3.3). An aliquot of the DNA genomic library containing ~5 × 10^4pfu of bacteriophage was combined with 0.6ml of the *E. coli* suspension in a polypropylene tube (Falcon® 2059, Becton Dickinson, USA) and incubated at 37°C for 15min. A sufficient number of aliquots were prepared (twenty in total) to represent the entire library containing 1 × 10^6pfu (section 6.3.3.1). Each aliquot of infected bacteria was mixed with 6.5ml of NZY top agar cooled to ~48°C and poured evenly onto a freshly prepared, pre-warmed NZY agar plate (150mm diameter plate, Sarstedt, Germany). The plates were maintained at room temperature for 10min and then transferred to

37°C until plaques developed (~7h). The plates were overlaid with 10ml of SM buffer, sealed with Parafilm and maintained at 4°C for 18h. The buffer, containing the diffused phage, was harvested from all twenty plates by aspiration. Each plate was rinsed with an additional 2ml of SM buffer. The phage, pooled in a polypropylene centrifuge tube, was thoroughly mixed with chloroform added to a final concentration of 5% (v/v) and incubated at room temperature for 15min. Cell debris was removed by centrifugation (500*g* × 10min). The supernatant was transferred to a second polypropylene centrifuge tube and remaining cellular debris removed by chloroform treatment as previously. The amplified phage library was combined with 0.3% (v/v) chloroform and 7% (v/v) dimethyl sulphoxide (DMSO), dispensed into 1.5ml aliquots and stored at -80°C.

6.2.3.6 Titering the amplified genomic DNA library

An aliquot of the amplified library was defrosted on ice. The phage suspension was serially diluted with SM buffer (10^{-2}, 10^{-4}, 10^{-6}) and 10µl of each dilution combined with 0.2ml of freshly prepared *E. coli* XL1-Blue MRF' (section 6.2.3.3). The cell-phage suspension was incubated at 37°C for 15min, mixed with 3ml of NZY top agar cooled to ~48°C, and spread evenly over the surface of a pre-warmed NZY agar plate. The plates were solidified at room temperature and transferred to 37°C for 18h. The plaques on each plate were counted and the titre of the amplified library calculated.

6.2.4 Immunoscreening of the genomic library

6.2.4.1 Dot blot analysis of serum PRT

Dot blot of PRT

Six nitrocellulose membrane strips (6cm × 1cm) (0.2µm pore size, BioRad) were marked with a 6 × 1 grid. One strip was labelled AB and five EC. Six two-fold dilutions (1:100-1:3,200) of serum PRT were prepared with antibody diluent [20mM Tris-HCl (pH 7.5), 150mM NaCl, 1% (w/v) BSA] and 1µl of each dilution spotted onto AB. Four serial-dilutions of *E. coli*/phage lysate (Stratagene) (neat-1:1,000) were prepared with blocking solution [20mM Tris-HCl (pH 7.5), 150mM NaCl, 1%

(w/v) BSA] and 1µl of each dilution spotted onto rows EC. The strips were air-dried for 5min, transferred to blocking solution for 1h at room temperature with constant agitation, and finally washed 5 × 5min in Tris-buffered saline/Tween 20 (TBST) [20mM Tris-HCl (pH 7.5), 150mM NaCl, 0.05% (v/v) Tween 20]. Four two-fold dilutions of PRT (1:800-1:6,400) were prepared with antibody diluent and one EC strip incubated in each dilution for 90min at room temperature with agitation. These four strips were washed 5 × 5min with TBST and transferred, together with the remaining two strips (AB, EC), to conjugate solution [0.125µg/ml protein A-alkaline phosphatase in antibody diluent] and incubated at room temperature for 1h with agitation. The strips were washed 4 × 5min in TBST, 1 × 5min in TBS [20mM Tris-HCl (pH 7.5), 150mM NaCl] (final wash) and blotted with Whatman® 3MM paper (Whatman, Maidstone, UK) to removed excess moisture.

Colour development reaction

Colour development solution was freshly prepared and protected from light [100mM Tris-HCl (pH 9.5), 100mM NaCl, 5mM $MgCl_2$, 0.3mg/ml nitroblue tetrazolium (NBT) [stock solution 10mg/ml in deionized water], 0.15mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) [stock solution 50mg/ml in DMF]. All the nitrocellulose dot blot strips were immersed in colour development solution and the colour reaction allowed to proceed in the dark until purple-blue positive reactions were clearly visible (~3min). The strips were rinsed once in TBS for 10s and immersed in stop solution [20mM Tris-HCl (pH 2.9), 1mM Na_2EDTA (pH 7.0)] for 10s to terminate the reaction. Once air-dried, the membranes were stored protected from light.

6.2.4.2 Adsorption by *E. coli*/phage lysate

Antibodies cross-reactive with *E. coli*/phage antigens were removed using the picoBlue™ Immunoscreening kit protocol (Stratagene). Four strips of nitrocellulose (5cm × 10cm) were immersed in *E. coli*/phage lysate, diluted 1:10 with TBST, at room temperature with constant agitation for 30min. The strips were placed on Whatman 3MM paper and air-dried before washing 5 × 5min in TBST. Unbound sites on the nitrocellulose were blocked by incubation in blocking solution for 90min

with agitation at room temperature. The strips were rinsed 3 × 1min in TBST and air-dried. One strip was placed into a pre-blocked plastic universal (i.e. washed with TBST for 5min and drained) containing 10ml of PRT diluted 1:10 with TBST. The universal was rolled continuously for 10min at 37°C. The strip was removed from the universal, discarded, replaced with a fresh strip and incubated as previously. This was repeated until all the strips had been used. The lysate treated serum was dispensed into 1ml aliquots and stored at -20°C.

6.2.4.3 Dot blot assessment of *E. coli*/phage lysate treatment of PRT

Five nitrocellulose strips (1cm × 6cm) were prepared with a 1 × 6 grid. Each strip was marked P, PEC, EC-P, EC-PEC, or AP.

- Strip P was spotted with 1µl of PRT diluted 1:100-1:3,200 with antibody diluent.
- Strip PEC was spot inoculated with 1µl of *E. coli*/phage lysate treated-PRT diluted 1:100-1:3,200 with antibody diluent.
- The remaining three strips (EC-P, EC-PEC, AP) were spot inoculated with *E. coli*/phage lysate (neat-1:1,000 diluted with blocking solution).

All strips were air-dried for 5min, blocked with blocking solution at room temperature for 1h with agitation, and rinsed 3 × 1min with TBST. EC-P was placed in PRT (diluted 1:400) and EC-PEC was placed in *E. coli*/phage lysate treated-PRT (diluted 1:400) and incubated at room temperature for 1h with agitation. These two strips were washed in TBST (5 × 5min) and then submerged, together with the remaining three strips (P, PEC, AP), in the protein A-alkaline phosphatase conjugate solution for 1h at room temperature with agitation. Finally, the strips were washed and developed using the colour development solution as previously (section 6.2.4.1).

6.2.4.4 Plating Phage

E. coli XL1-Blue MRF' cells were cultivated and prepared to an OD_{600} of 0.5 (section 6.2.3.3). An aliquot of the genomic DNA library containing ~5 × 10^4pfu of bacteriophage was combined with 0.6ml of the *E. coli* suspension in a polypropylene tube and incubated at 37°C for 15min. NZY agar plates (150mm diameter plate) were prepared a minimum of 24h before use and thoroughly dried at 37°C for 1h

prior to phage plating to prevent the top agar from lifting with the nitrocellulose membrane on removal. The infected cells were mixed with 7.5ml of NZY top agar cooled to ~48°C and spread evenly over the surface of a NZY agar plate. The top agar was allowed to completely solidify at room temperature before the plates were inverted and incubated at 42°C until plaques became visible (~4h). Nitrocellulose membrane discs (137mm diameter, Stratagene) were soaked in 10mM IPTG for 2min and air-dried on Whatman 3MM paper. Each membrane was labelled with a reference number and placed onto the agar surface of the appropriate plate. A syringe needle was used to mark three alignment points by piercing the membrane and the agar around the periphery of the plate. The plates were transferred to 37°C and incubated for a further 3.5h. During the final 20min of incubation the lids of the agar plates were lifted at one edge to allow drying before the plates were placed at -20°C for 5min to aid membrane removal. Finally, the membrane was slowly lifted from the plate and placed in TBST. Agar adhering to the nitrocellulose filter surface was removed and the membrane washed 5 × 15min with TBST. The nitrocellulose discs were blocked overnight in blocking solution at room temperature with agitation and the agar plates sealed with Parafilm and stored at 4°C.

Antibody and protein A-alkaline phosphatase incubations

The nitrocellulose membranes were transferred from blocking solution to the primary antibody (*E. coli*/phage lysate treated-PRT diluted with antibody diluent to a final concentration of 1:400) and incubated for 1h at room temperature with agitation. Following incubation the primary antibody was recovered for further use and the membranes washed to remove any residual unbound antibody (5 × 5min in TBST). Finally, the membranes were immersed in conjugate solution for 1h at room temperature with agitation, washed as previously to remove non-specifically bound or unbound conjugate and then submerged in colour development solution (section 6.2.4.1).

Identification of immunopositive plaques and phage recovery

Immunopositive plaques were identified on the membrane and located on the original agar plate using an acetate template. Once identified the plaques were removed, as plaque-agar plugs, with the wide aperture end of a sterile glass Pasteur pipette. Twenty-four plaque-agar plugs were placed together in 10ml SM buffer supplemented with 5% (v/v) chloroform and incubated at 4°C for 18h. The buffer containing the diffused phage was recovered, supplemented with a further 0.3% (v/v) chloroform and 7% (v/v) DMSO and stored at -70°C. The titre of the phage suspension was calculated (section 6.2.3.6) and the phage plated onto a lawn of *E. coli* XL1-Blue MRF' at a concentration of 5×10^3 plaques per 150mm plate as above. Plaques were lifted from the plate on a disc of nitrocellulose and screened for reaction with antibody as described above. Ten immunopositive plaques were identified, recovered in agar plugs and eluted individually in 1ml SM buffer supplemented with chloroform as previously. The titre of each individual phage elute was determined and the phage plated onto a lawn of *E. coli* XL1-Blue MRF' at a concentration of 100 plaques per 150mm plate. An agar plug containing a single plaque was selected from each plate, the phage eluted, plated on an *E. coli* XL1-Blue MRF' lawn and immunoscreened to ensure the homogeneity of the bacteriophage. The phage elutes, confirmed homogeneous, were amplified (section 6.2.3.5) and the titre of the amplified suspension calculated (section 6.2.3.6). A second batch of twelve phage were purified from the original genomic DNA library by the process of plating and screening as outlined, except the initial pooling of the plaque-agar plugs was not performed, until a homogeneous population of recombinant bacteriophage was achieved.

6.2.5 Clone analysis

6.2.5.1 Single clone excision

Using a Falcon polypropylene tube, 0.2ml of *E. coli* XL1-Blue MRF' prepared to an OD_{600} of 1.0 (section 6.2.3.3) was combined with 0.25ml of the single phage stock containing 1×10^6pfu in SM buffer and 1µl of ExAssist helper phage (Stratagene) containing $>1 \times 10^6$pfu/ml. The mixture was incubated at 37°C for 15min, 3ml of NZY broth added and incubation continued for a further 3h at 37°C on an orbital

shaker at 200rpm. On completion of excision the phagemid suspension was heated to 65°C for 20min to lyse the bacterial cells and bacteriophage λ leaving the heat resistant phagemid intact. The pBK-CMV phagemid vector, packaged as filamentous phage particles, was collected in the supernatant by centrifugation (1,000*g* × 15min). *E. coli* XLOLR cells, cultivated in NZY broth for 18h at 30°C with aeration at 200rpm, were harvested (1,200*g* × 5min) and resuspended in 10mM $MgSO_4$ to an OD_{600} of 1.0. Two aliquots of the excised phagemid, 10µl and 0.1ml, were combined with 0.2ml of the freshly prepared XLOLR cells and incubated at 37°C for 15min. On addition of 0.3ml NZY broth the tubes were incubated for a further 45min at 37°C. Colonies containing the phagemid were selected by plating 0.2ml of the *E. coli* XLOLR-phagemid suspension onto LB-kanamycin plates (50µg/ml) and incubating in air at 37°C for 18h. A TSB-glycerol stock of each transformed bacterium (clone) was prepared and stored at -80°C (section 2.1.4).

6.2.5.2 SDS-PAGE analysis

Preparation of bacterial cells

SDS-PAGE was based on the gel method of Lugtenberg *et al.* (1975). Bacterial clone cells were cultivated in 10ml of NZY broth supplemented with 50µg/ml kanamycin. *E. coli* XLOLR was cultivated in NZY broth without kanamycin and *S. epidermidis* NCTC 11047 was grown in LB broth. All cultures were incubated at 37°C for 18h on an orbital shaker at 200rpm. Following incubation 50% of each culture was replaced with fresh broth and the bacterial clone and *E. coli* XLOLR cultures were supplemented with 1mM IPTG. All flasks were incubated for a further 3h at 37°C with aeration at 200rpm. The bacterial cells were harvested from 1ml of broth (4,300 × 10min) and re-suspended in PBS to an OD_{600} of 1.0. Equal volumes of the bacterial suspension and SDS-PAGE sample buffer (table 6.1) were combined and heated to 100°C for 10min to denature the bacterial proteins.

SDS-PAGE

An 11% (w/v) separating gel (80 x 70 x 1mm) (table 6.1) was cast using the PAGE gel casting apparatus (section 4.2.6).

Table 6.1. Composition of the SDS-PAGE separating and stacking gels, sample buffer and electrode buffer used to detect urease activity in culture supernatant

	Separating gel 11% (w/v)	**Stacking gel 5% (w/v)**	**Sample buffer**	**Electrode buffer (pH 8.0)**
Acrylamide stock I	5ml	-	-	-
Acrylamide stock II	-	2.5ml	-	-
10% (w/v) SDS	0.5ml	0.15ml	5ml	10ml
1.5M Tris-HCl (pH 8.8)	6ml	-	-	-
0.5M Tris-HCl (pH 6.8)	-	3.75ml	2.5ml	-
Distilled water	8ml	8ml	5ml	1L
TEMED (N,N,N^1N^1-tetramethyl-ethylenediamine)	50µl	40µl	-	-
10% (w/v) ammonium persulphate - freshly prepared	70µl	50µl	-	-
Glycerol	-	-	2.5ml	-
2-mercaptoethanol	-	-	0.25ml	-
5% (w/v) bromophenol blue	-	-	0.2ml	-
Tris	-	-	-	3g
Glycine	-	-	-	14.4g

Acrylamide stock I - 44% (w/v) acrylamide and 0.8% (w/v) Bis(N,N^1,- methylene-bis-acrylamide) (Severn Biotech Ltd, UK).
Acrylamide stock II - 30% (w/v) acrylamide and 0.8% (w/v) Bis(N,N^1,-methylene-bis-acrylamide).

For each bacterial strain 20µl of the denatured sample was loaded into the gel. A pre-stained protein marker (range 6.5kDa-175kDa) (BioLabs, New England) was included. The gel was electrophoresed at 200V for 45min. Duplicate gels were prepared to allow both Coomassie Blue staining and western blotting.

Coomassie Blue staining

On completion of electrophoresis, the gel was placed in Coomassie Blue stain [20% (v/v) methanol, 10% (v/v) acetic acid, 0.1% (w/v) Coomassie Blue] for 1h at room temperature with gentle agitation. After staining the Coomassie Blue solution was replaced with destaining solution [20% (v/v) methanol, 10% (v/v) acetic acid] and the gel again gently agitated at room temperature. The solution was replaced as required until all the background stain had been removed.

6.2.5.3 Western blot analysis

Bacterial proteins resolved by SDS-PAGE were transferred from the gel to a nitrocellulose membrane using a western blot method (Towbin, Staehelin and Gordon, 1979). The gel was placed onto a nitrocellulose membrane, slightly larger than the gel, pre-soaked in transblot buffer [25mM Tris, 192mM glycine, 20% (v/v) methanol, pH 8.3]. Both the gel and the membrane were sandwiched between two sheets of Whatman 3MM paper pre-soaked in transblot buffer. The gel-membrane assembly was placed between foam pads and positioned within the electrophoretic blotting apparatus (BioRad). Transblot buffer within the tank was cooled with an internal ice block. Additional ice packed externally around the tank maintained a 4°C running temperature. The proteins were electrophoretically transferred to the membrane at 100V for 42min. On completion of transfer the membrane was blocked with blocking solution [PBS supplemented with 5% (w/v) skimmed milk powder (Marvel, Sainsbury Plc.) and 0.05% (v/v) Tween 20] for 1h with agitation at room temperature. The membrane was rinsed with PBS supplemented with 0.05% (v/v) Tween 20, blotted to remove excess moisture and stored at -20°C, or probed directly.

6.3 RESULTS

6.3.1 Isolation of genomic DNA

Genomic DNA prepared by the modified cetrimide technique was quantitated by UV absorption:

S. epidermidis NCTC 11047 genomic DNA preparation (diluted 1:100):

OD_{260} = 1.918

OD_{280} = 0.969

An OD_{260} of 1.0 corresponds to approximately 50μg/ml dsDNA (Sambrook, Fritsch and Maniatis, 1989). Therefore the sample contained 9.59mg nucleic acid/ml.

Pure nucleic acid has an OD_{260}/OD_{280} ratio value of 1.8 (Sambrook, Fritsch and Maniatis, 1989) and must be above 1.75 for successful ligation (Promega, 1996). The sample ratio 1.918/0.969 = 1.979 indicated acceptable purity.

6.3.2 Partial digestion of genomic DNA

Five restriction endonucleases were assessed for their ability to digest genomic DNA prepared from *S. epidermidis* NCTC 11047 over 2h and 18h incubation periods (figure 6.1). *Bam*HI, *Bgl*II and *Sau*3AI achieved very limited digestion of the nucleic acid. Both *Eco*RI and *Mbo*I attained good partial digestion, however, *Eco*RI generated DNA fragments which were of a size appropriate to the λ phage cloning vector. An incubation time of 2h was adequate for partial digestion with *Eco*RI.

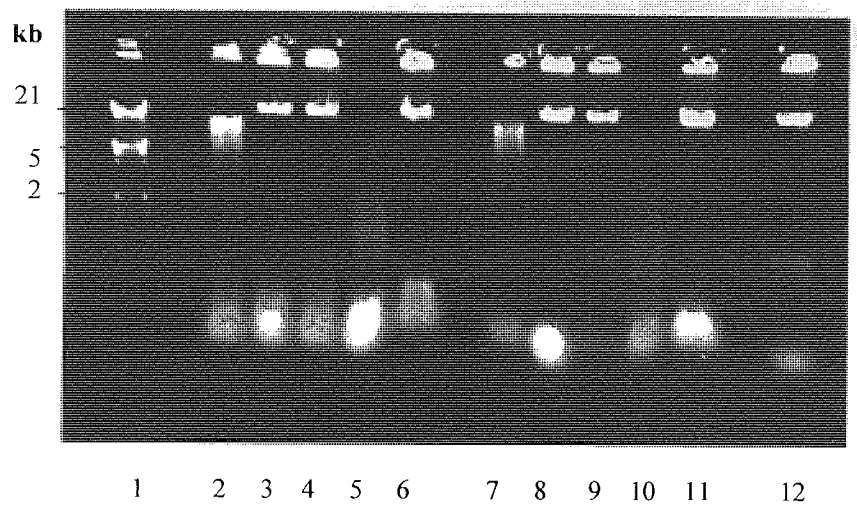


Figure 6.1. Restriction endonuclease partial digest fragments of *S. epidermidis* NCTC 11047 genomic DNA, incubated at 37°C for 2h or 18h, resolved by electrophoresis through a 0.8% agarose gel. Lane 1, *Eco*RI/*Hin*dIII digested phage λ DNA sizing ladder; 2, *Eco*RI × 2h; 3, *Bam*HI × 2h; 4, *Bgl*II × 2h; 5, *Mbo*I × 2h; 6, *Sau*3AI × 2h; 7, *Eco*RI × 18h; 8, *Bam*HI × 18h; 9, *Bgl*II × 18h; 10, *Mbo*I × 18h; 11, *Sau*3AI × 18h; 12, undigested DNA.

To assess the optimum restriction endonuclease concentration to attain good partial digestion the genomic DNA preparation was digested with serially diluted restriction endonuclease (figure 6.2). Restriction digestion of genomic DNA with 4 units *Eco*RI/μg DNA incubated at 37°C for 2h generated fragments suitable of ligation.

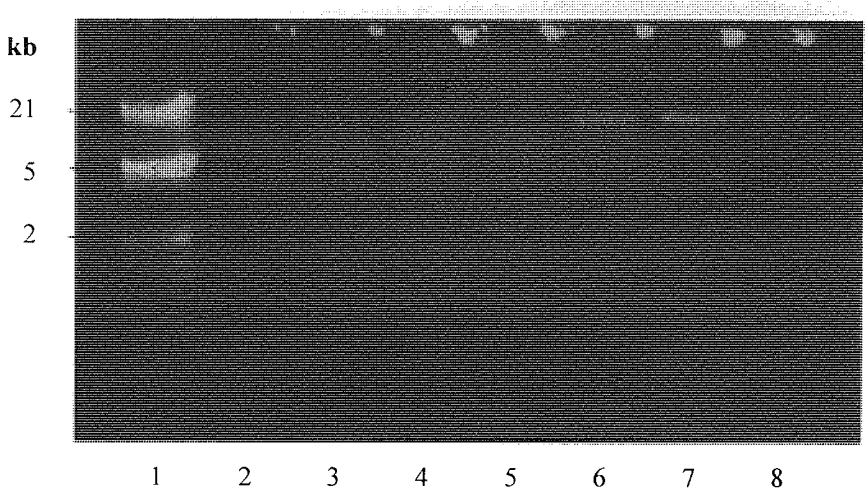


Figure 6.2. Genomic DNA restriction endonuclease digest fragments, prepared with various concentrations of *Eco*RI, were resolved by electrophoresis through a 0.8% agarose gel. All digests were incubated at 37°C for 2h. Lane 1, *Eco*RI/*Hin*dIII digested phage λ DNA sizing ladder; 2, 10 units *Eco*RI; 3, 8 units *Eco*RI; 4, 6 units *Eco*RI; 5, 4 units *Eco*RI; 6, 2 units *Eco*RI; 7, 1 unit *Eco*RI; 8, undigested DNA.

6.3.2.1 Quantitation of the partially digested DNA - Ethidium bromide fluorescence method

Samples that contained small amounts of DNA (<500μg/ml) were quantitated using the ethidium bromide fluorescence method. Approximately 25ng/μl-50ng/μl (25μg/ml-50μg/ml) DNA (fragment size ~800bp-10kbp) was extracted from the restriction endonuclease partial digest of 20μg genomic DNA. This concentration of recovered DNA was sufficient for ligation.

6.3.3 Packaging ligated DNA

6.3.3.1 Titration of recombinant phage

Recombinant plaques appeared as colourless or white plaques, whereas background plaques were blue (table 6.2).

Table 6.2. The number of recombinant and background plaques identified

	Recombinant plaques (white)	**Background plaques (blue)**
***S. epidermidis* NCTC 11047 (neat)**	~2,160	98
***S. epidermidis* NCTC 11047 (10^{-1} dilution)**	203	11
pRheo test insert (neat)	semi-confluent	3
pRheo test insert (10^{-1} dilution)	~1,800	0

Ligation/packaging efficiency was calculated by:

$$\frac{\text{number of plaques} \times \text{dilution factor} \times \text{total packaged volume}}{\text{total number of micrograms of vector arms packaged}} = \text{plaques/}\mu\text{g arms}$$

Test ligation:

Pre-digested arms, when ligated to a test insert (pRheo), should yield ~5.0×10^6 recombinant pfu/μg arms (Stratagene):

$$\frac{1{,}800 \times 10 \times 500}{1} = 9.0 \times 10^6 \text{ recombinant pfu/}\mu\text{g of arms}$$

Recombinant plaques should be 10-100-fold above the background (Stratagene):

$$\frac{3 \times 1 \times 500}{1} = 1.5 \times 10^3 \text{ background pfu/}\mu\text{g of arms}$$

The insert pRheo test ligation indicated high efficiency ligation and packaging.

Sample ligation:

If an insert is free from contaminants and contains a high percentage of ligatable ends 1.0×10^6 to 1.5×10^7 recombinant phage should be generated (Stratagene):

$$\frac{203 \times 10 \times 500}{1} = 1.0 \times 10^6 \text{ recombinant pfu/}\mu\text{g of arms}$$

Background plaques should be less than 1×10^5 pfu/μg of arms and recombinant plaques should be 10 to 100-fold above the background (Stratagene).

$$\frac{11 \times 10 \times 500}{1} = 5.5 \times 10^4 \text{ background pfu/}\mu\text{g of arms}$$

Therefore, ligation of *S. epidermidis* NCTC 11047 genomic DNA was efficiently achieved.

6.3.3.2 Packaging efficiency

A total of 248 plaques of packaged λ*c*I857 *Sam*7 were identified on the lawn of *E. coli* VC5257. The efficiency of Gigapack III Gold packaging extract was calculated using the following equation:

$$\frac{\text{number of plaques} \times \text{dilution factor} \times \text{total packaging volume}}{\text{total number of micrograms packaged} \times \text{number of microliters plated}}$$

$$\frac{248 \times 10^4 \times 1{,}000}{1 \times 10} = 2.5 \times 10^8 \text{ pfu/μg vector}$$

A total of 400 pfu/plate or 4×10^8 pfu/μg vector was expected (Stratagene).

6.3.4 Is the library representative of the genome?

Clarke and Carbon (1976) derived a formula, which related the probability (P) of including any DNA sequence in a random library of independent recombinants (N):

$$N = \frac{ln\,(1 - P)}{ln\,(1 - {}^1/_n)}$$

where n = the number of DNA sequences of cloned fragment size in the entire genome (Old and Primrose, 1994; Clarke and Carbon, 1976).

The *S. epidermidis* genome was taken to be 2,800kb (based on *S. aureus* genome size of 2,700kb-2,800kb (Iandolo, Bannantine and Stewart, 1996). The average fragment size was estimated as 5kb (range 800bp-10,000kb), therefore, n = 2,800/5 = 560.

At P = 95%

$$N = \frac{ln\,(1 - 0.95)}{ln\,(1 - {}^1/_{560})}$$

$$= 1.7 \times 10^3$$

To achieve a 95% probability of including any particular sequence of ~5kb size in a random *S. epidermidis* NCTC 11047 genomic DNA library 1.7×10^3 recombinants are required.

To account for the inserted gene being incorporated into the vector in two possible directions and in three possible reading frames it is necessary to multiply N by a factor of six:

$$1.7 \times 10^3 \times 6$$
$$= 1.02 \times 10^4$$

Therefore, to achieve a 95% probability of detecting a functional gene on a ~5kb DNA fragment a random library of 1.02×10^4 recombinants was required. A *S. epidermidis* NCTC 11047 genomic DNA library of 1×10^6 recombinants was generated.

6.3.5 Titering the amplified library

After amplification of the library ~10^9 to 10^{10} pfu/ml would be expected (Stratagene). An amplified library of 10^9 pfu/ml was generated (table 6.3).

Table 6.3. Titering the amplified library

Dilution of amplified library	Plaques counted	Titre of amplified library (pfu/ml)
10^{-2}	semi-confluent	-
10^{-4}	~1,300	-
10^{-6}	10	1.0×10^9

6.3.6 Immunoscreening of the genomic library

6.3.6.1 Dot blot analysis of serum PRT

Dot blot analysis assessed the level of cross-reactive antibodies to *E. coli* and phage antigens present in serum PRT. The 1:100-1:400 dilution spots on strip AB, which was dot inoculated with PRT and then immersed in conjugate, were rapidly immunopositive. The 1:800-1:3,200 spots were visible within 1min. This

demonstrated both the presence of IgG antibody in the serum and the avidity of the conjugate (0.125µg/ml) for the antibody.

Four EC strips, which were inoculated with *E. coli*/phage antigen and then immersed in PRT (dilutions of 1:800-1:6,400), demonstrated the presence of cross-reactive antibodies in the serum. At a PRT dilution of 1:800 all four spots of *E. coli*/phage lysate (neat-1:1,000) were intensely coloured. For the 1:1,600 and 1:3,200 PRT dilutions the lysate neat-1:100 dilution spots were clearly visible. At a 1:6,400 dilution only the neat and 1:10 lysate spots were strongly immunopositive. The immunopositive reaction of the 1:100 lysate with cross-reacting antibody in serum diluted up to 1:3,200 indicated the presence of contaminating antibodies that required removal. The level of cross-reacting antibody in the serum at a working dilution (1:400) would be sufficient to create false positives during screening.

No immunopositive spots were detected on the single EC strip, which was dot inoculated with *E. coli*/phage lysate and then submerged in conjugate. The failure to detect spots indicated that the blocking procedure was sufficient to block non-specific protein binding sites.

6.3.6.2 Dot blot analysis to assess *E. coli*/phage lysate treatment of PRT

A series of dot blots were used to assess the removal of cross-reactive *E. coli*/phage antibodies from serum PRT following lysate treatment:

- P: Serially diluted PRT incubated with conjugate.

  All spots were visible within 30s indicating reaction with antibody (IgG) in the serum and indicating the avidity of the conjugate for the antibody (figure 6.3).

- PEC: *E. coli*/phage lysate treated-PRT serially diluted and incubated with conjugate.

  The spots 1:100 and 1:200 dilution were visible within 30s, 1:400 and 1:800 were visible within 1min. The dilutions 1:1,600 and 1:3,200 were weakly visible in

comparison to the lower dilutions (figure 6.3). Thus demonstrating that lysate treatment has not reduced the avidity of the conjugate for the antibody.

- EC-P: *E. coli*/phage lysate serially diluted and incubated with PRT followed by conjugate.

  Neat and 1:10 dilution spots were visible within 30s followed by the 1:100 spot. The 1:1,000 dilution was not detected (figure 6.3).

- EC-PEC: *E. coli*/phage lysate serially diluted and incubated with PRT-*E. coli*/phage lysate treated followed by conjugate.

  After 1min incubation the neat and 1:10 spots were visible and the 1:100 spot weakly discernible. The 1:1,000 spot was not detected (figure 6.3). By comparison to strip EC-P, lysate treatment had reduced the level of cross-reactive antibodies.

- AP: *E. coli*/phage lysate serially diluted and incubated with conjugate.

  After 1min incubation no spots were perceived demonstrating successful blocking (figure 6.3).

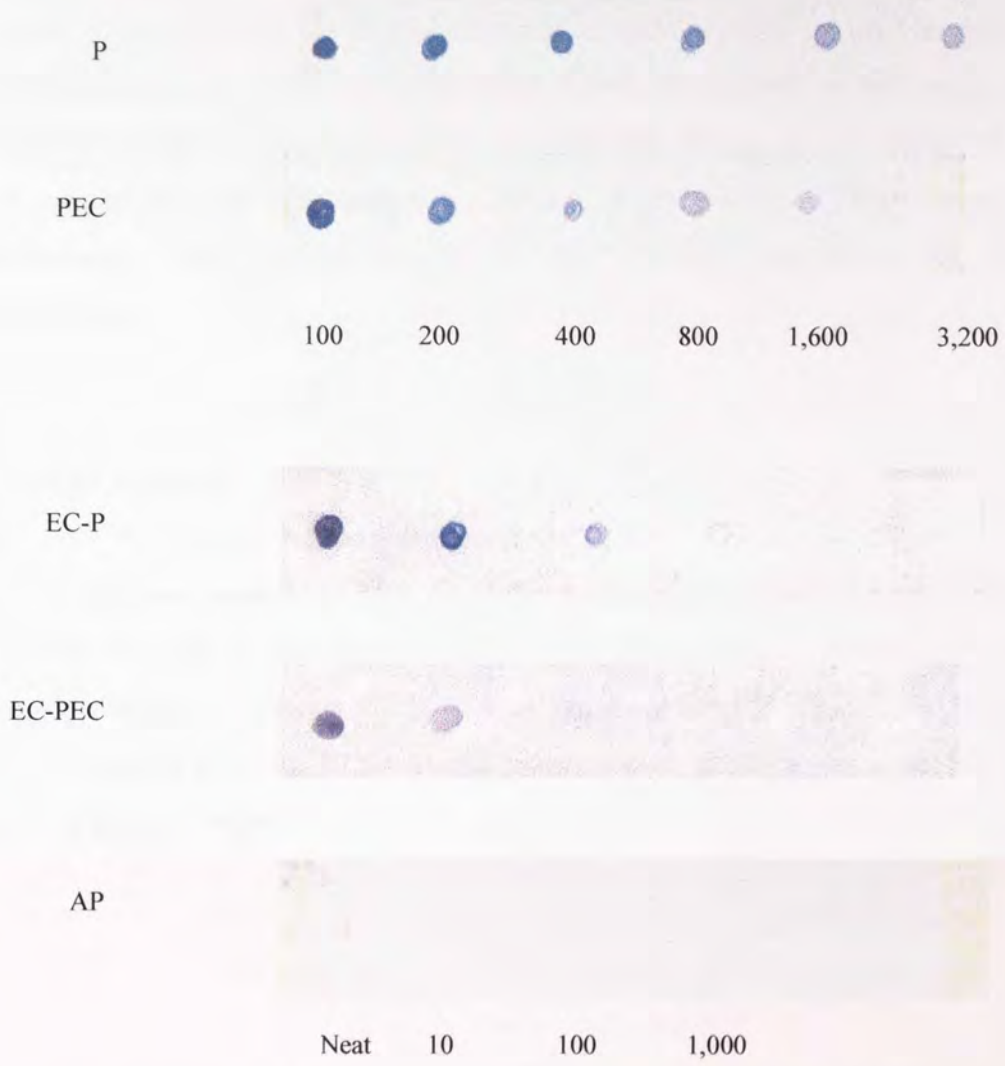


Figure 6.3. Dot blot analysis to assess *E. coli*/phage lysate treatment of serum PRT. P, serially diluted PRT incubated with conjugate; PEC, *E. coli*/phage lysate treated-PRT serially diluted and incubated with conjugate; EC-P, *E. coli*/phage lysate serially diluted and incubated with PRT followed by conjugate; EC-PEC, *E. coli*/phage lysate serially diluted and incubated with PRT-*E. coli*/phage lysate treated followed by conjugate; AP, *E. coli*/phage lysate serially diluted and incubated with conjugate.

The *E. coli*/phage lysate treatment, therefore, reduced the level of *E. coli* and phage antibodies in PRT by one two-fold dilution; an acceptable background level.

6.3.6.3 Immunoscreening plaques

The method used to select the recombinant phage initially relied on the identification of antigenic products, produced by the DNA insert, by antibody in the serum PRT. Twenty-two phage were selected from the genomic library on the basis of their strong colour reaction, indicating the presence of specific IgG. These twenty-two bacteriophages were excised into the *E. coli* XLOLR host strain for further investigation.

6.3.7 Clone analysis

6.3.7.1 SDS-PAGE and western blot analysis

Of the twenty-two immunopositive clones investigated, six encoded a proteinaceous product as detected by SDS-PAGE (figure 6.4). Five of these six clones produced identical proteins of ~66kDa (E2). The sixth, designated *E. coli* XLOLR/pSsaA, encoded a protein of ~30kDa (SsaA). No proteinaceous product was identified in the remaining sixteen clones.

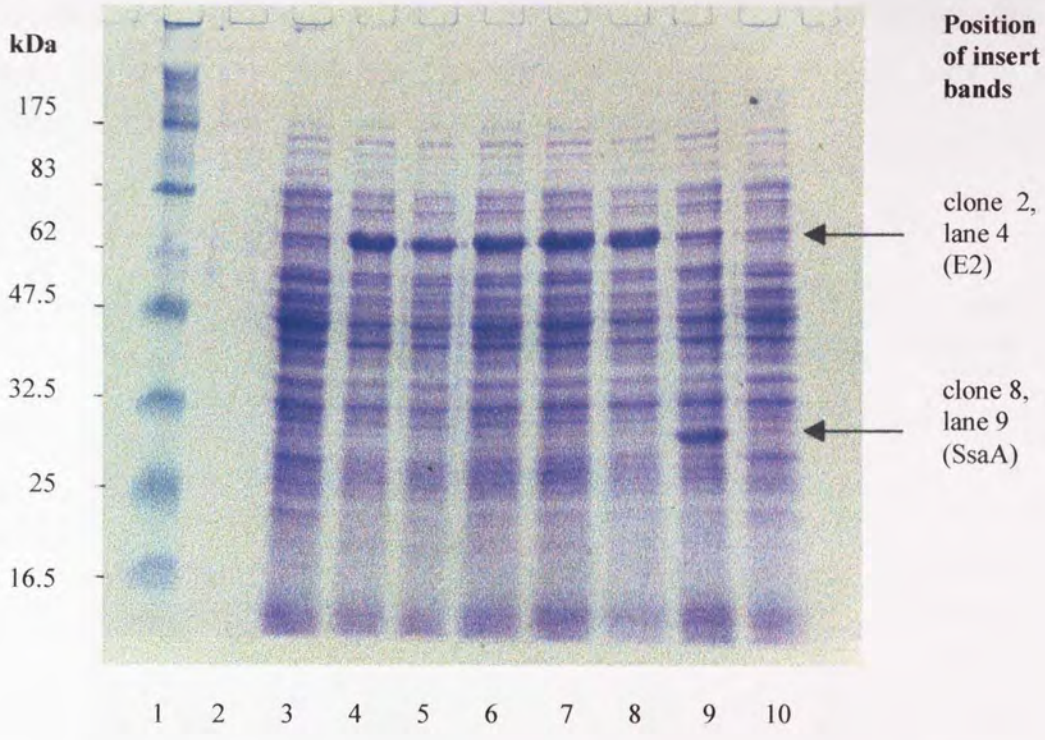


Figure 6.4. The cellular proteins of *E. coli* XLOLR (IPTG induced), six clones of *E. coli* XLOLR with inserts of *S. epidermidis* NCTC 11047 genomic DNA (IPTG induced) and *S. epidermidis* NCTC 11047 separated by SDS-PAGE and stained with Coomassie Blue. Lane 1, protein marker sizing ladder; 2, *S. epidermidis* NCTC 11047; 3 and 10, *E. coli* XLOLR; 4, clone 2 (*E. coli* XLOLR/pE2); 5, clone 3; 6, clone 4; 7, clone 6; 8, clone 7; 9, clone 8 (*E. coli* XLOLR/pSsaA). The proteins of interest encoded in clone 2 (E2) and clone 8 (SsaA) are indicated.

The western blot of the six clones encoding a proteinaceous product indicated that the 30kDa protein was highly antigenic but the 66kDa protein was apparent as a "ghost" band (figure 6.5). Though the remaining sixteen clones were immunopositive, no antigen was identified by western blot analysis.

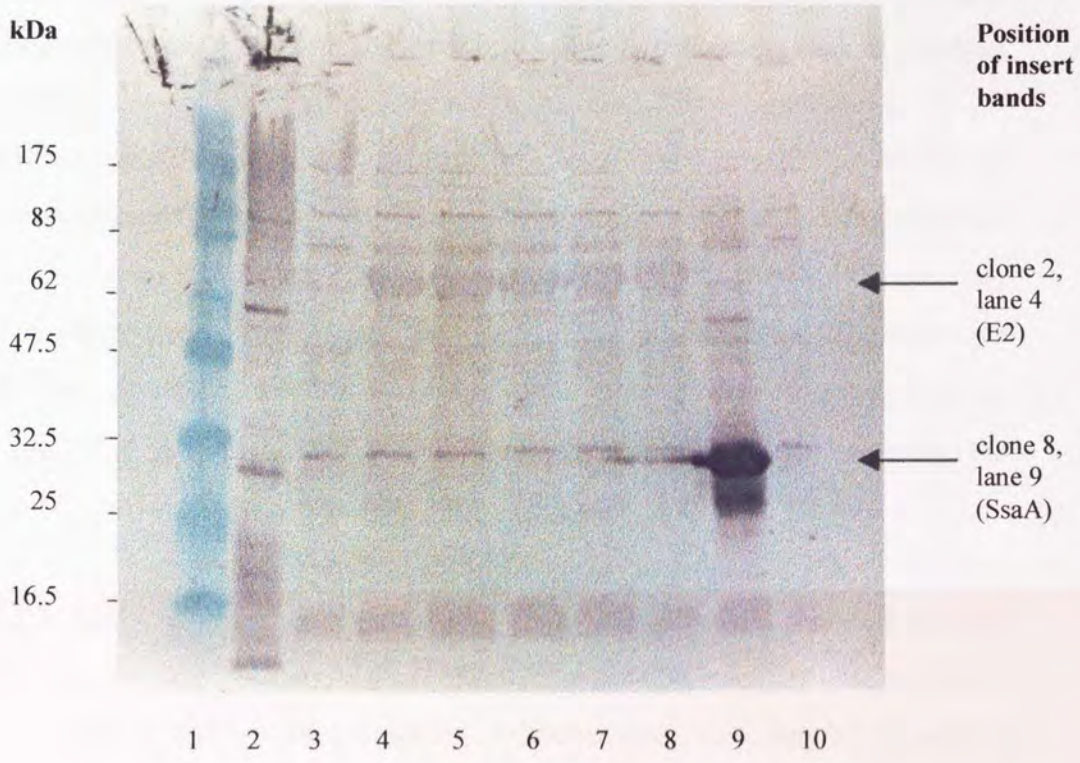


Figure 6.5. The cellular proteins of *S. epidermidis* NCTC 11047, *E. coli* XLOLR (IPTG induced) and six clones of *E. coli* XLOLR with inserts of *S. epidermidis* NCTC 11047 genomic DNA (IPTG induced) separated by SDS-PAGE, western blotted and immunodetected with serum PRT (diluted 1:400). Lane 1, protein marker sizing ladder; 2, *S. epidermidis* NCTC 11047; 3 and 10, *E. coli* XLOLR; 4, clone 2 (*E. coli* XLOLR/pE2); 5, clone 3; 6, clone 4; 7, clone 6; 8, clone 7; 9, clone 8 (*E. coli* XLOLR/pSsaA).

6.4 DISCUSSION

A genomic library is a collection of random recombinants, which together contain all the DNA sequences of an entire genome. The size of the library i.e. the number of recombinants that have to be screened to have a reasonable chance of isolating a particular DNA sequence is dependent on the genome size and average fragment size. The larger the ligated fragments the fewer the number of recombinants required to ensure that the library is representative of the genome. The *S. epidermidis* NCTC 11047 genomic DNA library (average fragment size ~5kb) required a library of 1.7×10^3 recombinants to ensure that all sequences had a 95% probability of being represented. However to account for two possible insertion orientations (where only one would produce gene product) and insertion in three possible reading frames (of which only one would be functional) 1.02×10^4 recombinants were necessary. A library of 1×10^6 recombinants was generated and therefore was representative of the genome.

Short DNA fragments for ligation can be generated by mechanical shearing or more commonly by the use of restriction endonucleases. Restriction endonuclease digestion has the advantage of generating cohesive ends that can be ligated to similarly produced ends of the vector arms. A restriction endonuclease reaction allowed to continue to completion would destroy any genes with internal restriction sites. Therefore, by allowing only partial digestion the bulk of genetic material remains as large, almost random fragments, which are convenient for the production of a library. *Eco*RI generated cohesive ends compatible with the chosen vector, produced a good partial digest of the DNA (indicated by the smear of nucleic acid as opposed to the distinct banding pattern of complete digestion), and generated the bulk of partially digested pieces in a size range ideal for ligation.

The library was amplified once to produce a large, stable quantity of high titre phage stock. Further amplification was avoided since this can lead to a significant under-representation of weakly growing recombinant phage (Promega, 1996). The recovery of a 66kDa protein from five out of ten (50%) clones demonstrated the dominance of a phage species over slower growing bacteriophage during purification. The pooling

of plaque-agar plugs during the first batch of phage purification, which led to the over-representation of a single species, was retrospectively discontinued for subsequent rounds of purification.

The immune response of a patient with a severe *S. epidermidis* infection (serum PRT) was exploited to identify antigenic staphylococcal proteins that were synthesised *in vivo* during sepsis. Of twenty-two immunopositive plaques selected, excised as phagemid and immunoblotted, six expressed an antigenic protein; *E. coli* XLOLR/pSsaA encoded a highly antigenic 30kDa protein and five clones expressed a 66kDa protein (one of these five clones was selected for investigation and designated *E. coli* XLOLR/pE2). The western blot "ghost" band of the 66kDa protein suggested that although antigenic (only immunopositive plaques were selected) the expressed protein was unable to either resume its epitope reactivity on transfer to the nitrocellulose or the high concentration of protein in the band effectively blocked itself from binding specific antibody efficiently. It is possible that the bacteriophage was selected as a false positive due the background antibodies to *E. coli*/phage antigens, however, since the serum was lysate treated and only strongly positive plaques were selected, this is unlikely. Though the remaining sixteen clones were immunopositive, no antigen was identified by western blot analysis. These clones could encode proteins obscured by *E. coli* XLOLR proteins, although this is improbable since IPTG induction leads to massive over expression of the insert that would be apparent on a Coomassie Blue stained gel. Alternatively the DNA insert could code for a non-proteinaceous antigen that would not be detected by either Coomassie Blue staining or the western blot.

The two clones *E. coli* XLOLR/pSsaA and *E. coli* XLOLR/pE2 expressing a 30kDa and 66kDa protein, respectively, were selected for further investigation in Chapter 7.

CHAPTER 7: IDENTIFICATION AND CHARACTERISATION OF A STAPHYLOCOCCAL SECRETORY PROTEIN ANTIGEN (SsaA) AND A PYRUVATE DEHYDROGENASE COMPLEX SUBUNIT (E2)

7.1 INTRODUCTION

The aim of this section of the study was to investigate and characterise the two staphylococcal antigens, encoded in the clones *E. coli* XLOLR/pSsaA and *E. coli* XLOLR/pE2, selected from the *S. epidermidis* NCTC 11047 genomic DNA library by screening with serum collected from a patient with active *S. epidermidis* IE. This approach, which identified antigens that were expressed *in vivo* and induced an immune response in the patient, might identify new virulence determinants leading to an improved understanding of CNS-associated pathogenesis and may form the basis of a serodiagnostic test.

7.2 MATERIALS AND METHODS

7.2.1 DNA insert recovery

7.2.1.1 Purification of phagemid

Phagemid was purified using the Wizard™ *Plus* Minipreps DNA Purification System (Promega, USA). The transformed bacterial (*E. coli* XLOLR/pSsaA and *E. coli* XLOLR/pE2) cells were cultivated in 20ml of NZY broth supplemented with kanamycin (50μg/ml) at 37°C for 18h with aeration at 200rpm. The cells from 3ml of the culture were harvested by centrifugation (10,000*g* × 2min) and re-suspended in 0.2ml of cell re-suspension solution [50mM Tris-HCl (pH 7.5), 10mM EDTA, 100μg/ml RNase A] (Promega). To the cell suspension 0.2ml of cell lysis solution [0.2M NaOH, 1% (w/v) SDS] (Promega) was added and mixed followed by 0.2ml of neutralization solution [1.32M potassium acetate, pH 4.8] (Promega). The lysate was mixed by inverting four times and then centrifuged (10,000*g* × 5min). A minicolumn was attached to a syringe barrel and then to a vacuum system. A 1ml aliquot of thoroughly re-suspended resin (Promega) followed by the recovered lysate was dispensed into the minicolumn/syringe assembly and the vacuum applied until the

sample had passed through the column. Two ml of the column wash solution [80mM potassium acetate, 8.3mM Tris-HCl (pH 7.5), 40μM EDTA, 55% (v/v) ethanol] (Promega) was dispensed into the barrel and the vacuum re-applied. The vacuum was allowed to draw for a further 30s after the sample had passed through the column to dry the resin. The minicolumn was detached from the barrel, placed into an eppendorf tube, and centrifuged (10,000*g* × 2min) to remove residual wash solution. To elute the DNA, 50μl of sterile double distilled water pre-warmed to 70°C was applied to the column, placed in a fresh eppendorf tube, and allowed to stand for 2min. Finally, the DNA was recovered by centrifugation (10,000*g* × 30s), quantitated by the ethidium bromide fluorescence method (section 6.2.2.5) and stored at 4°C.

7.2.1.2 Determination of insert size

An aliquot of the phagemid suspension (pSsaA and pE2) containing 30ng of DNA was restriction endonuclease digested with 10 units of *Eco*RI for 1h at 37°C. The DNA fragments, and a sample of undigested phagemid, were resolved by electrophoresis through a 0.8% (w/v) agarose gel at 5.7V/cm for 40min (section 6.2.2.2). The inclusion of a DNA sizing ladder (*Eco*RI/*Hin*dIII digested λ phage DNA) allowed the size of the DNA insert to be estimated.

7.2.2 DNA and amino acid sequence analysis

7.2.2.1 DNA insert sequencing

Both strands of the two inserts were independently sequenced with an Applied Biosystems 377 automated DNA sequencer using the T3 and T7 primer regions of the phagemid. Sequencing was performed by Alta Bioscience, Birmingham, UK.

7.2.2.2 DNA and amino acid sequence analysis

The EMBL nucleic acid sequence database was searched, using the FASTA software through the GCG10 package (http://www.hgmp.mrc.ac.uk) for deposited sequences with homology to the two DNA inserts. Similarly, the GenBank database was searched using the BLAST software (http://www.ncbi.nlm.nih.gov/PubMed).

The insert nucleotide sequence of both strands was translated into peptide sequence for each of the three possible open reading frames (ORFs). The peptide sequence databases SWISSPROTNEW, SWISSPROT, SPTREMBL, REMTREMBL and TREMBLNEW were searched, using the FASTA software, for peptide sequences homologous to the translated ORFs.

Deposited nucleic acid and amino acid sequences with homology to the cloned sequence were aligned with the insert sequence using the Clustal multiple sequence alignment software available through the GCG10 package.

7.2.2.3 Analysis of a cloning artefact

To assess a possible cloning artefact in the insert sequence of pSsaA (deletion of 4bp), detected by Clustal alignment with homologous DNA sequences, the *E. coli* XLOLR/pSsaA nucleotide sequence was compared to the corresponding region of the *S. epidermidis* NCTC 11047 genome amplified by the polymerase chain reaction (PCR).

PCR primers

The DNA of *S. epidermidis* NCTC 11047 was amplified by PCR using primers flanking the possible deletion (primers A and B, Alta Bioscience) (table 7.1).

Table 7.1. Primer A and B data

	Upstream primer-Primer A	**Downstream primer-Primer B**
Sequence	$^{5'}$CGC AAG CTA TTC AAC ATC$^{3'}$	$^{5'}$ GCA TTA AAC AAC ATC ATA CAA TG $^{3'}$
Length	18bp	23bp
Melting temperature	49.2°C	53.4°C
G/C concentration	44%	30%
n/t number*	360-377	820-842

* n/t number refers to the nucleotide number of the primer sequence in the *ssaA* deposited sequence, AF162275 (deposited from the studies in this thesis by S. Lang, June 2000).

Ethanol precipitation of primers

Ethanol precipitation of the oligonucleotides (originally prepared by Alta Bioscience for sequencing purposes) was used to desalt the primers prior to use. One volume of the primer, resuspended in deionized water, was combined with 0.1 volumes of 3M sodium acetate (pH 5.2) and 2 volumes of absolute ethanol (-20°C). The solution was vortex mixed, incubated at -20°C for 20min, and centrifuged (11,400*g* × 15min). The supernatant was removed and the pelleted DNA washed in 0.5 volumes of 70% (v/v) ethanol. The DNA was recovered by centrifugation as previously and dried by heating to 45°C for 15min. Finally, the oligonucleotide pellet was resuspended in 100µl of deionized water and rehydrated by heating to 65°C for 30min. The oligonucleotide sequence concentration was evaluated by the ethidium bromide fluorescence method (section 6.2.2.5) and adjusted to 20pmol/µl by addition of deionized water.

Polymerase chain reaction

PCR was performed as outlined in section 7.2.7, except the annealing temperature was 50°C. The PCR product was resolved by agarose gel electrophoresis (section 6.2.2.2), recovered from the gel using an agarose gel DNA extraction kit (section 6.2.2.4) and quantitated by the ethidium bromide fluorescence method (section 6.2.2.5). The PCR product was sequenced using a 377 automated DNA sequencer (section 7.2.2.1) and compared to the nucleotide sequence of the cloned insert pSsaA.

7.2.3 Analysis and characterisation of E2 and SsaA

7.2.3.1 Protein data

The relative molecular mass (M_r) and isoelectric point (pI) of SsaA and E2 was determined by the Peptidesort software available through the GCG10 package (section 7.2.2.2).

7.2.3.2 Prediction of secondary protein structure

Secondary protein structure (Chou-Fasman) and hydrophobicity/hydrophilicity (Kyte-Doolittle) of SsaA were predicted using the Protylze structure predictor software (Scientific and Educational Software, Durham, USA).

7.2.4 Detection of SsaA by SDS-PAGE and western blotting of bacterial protein

Duplicate western blots of *E. coli* XLOLR/pSsaA and cellular and supernatant proteins recovered from *S. epidermidis* NCTC 11047 were probed with serum PRT or affinity purified anti-SsaA antibody.

7.2.4.1 Bacterial protein preparation

Expression of SsaA and phase of growth

The cellular and secreted expression of SsaA by *S. epidermidis* NCTC 11047 cultures in different phases of growth (cellular proteins harvested from 4, 6 and 18h cultures and ethanol precipitated culture supernatant proteins from 6 and 18h cultures) were compared by western blot analysis.

Cell-associated protein

S. epidermidis NCTC 11047 was cultivated in 20ml of LB broth at 37°C for 18h with aeration at 200rpm. Transformed *E. coli* XLOLR/pSsaA was grown in NZY broth supplemented with kanamycin (50μg/ml) and induced with 1mM IPTG (section 6.2.5.2). The bacterial cells from 1ml of broth were harvested (4,300*g* × 10min) and resuspended in PBS to an OD_{600} of 1.0. The *S. epidermidis* cells were maintained at 4°C for a minimum of 24h prior to use to allow autolytic enzymes to release cellular proteins. This method of cell lysis was as efficient as lysostaphin treatment.

Ethanol precipitation of the culture supernatant protein

Extracellular bacterial proteins of *S. epidermidis* NCTC 11047 were concentrated by ethanol precipitation of the culture supernatant (Heilmann and Götz, 1998). A

staphylococcal starter culture was cultivated in 10ml of LB broth for 8h at 37°C with aeration at 200rpm. A 50ml LB broth was inoculated with 0.5ml of the starter culture and incubated for 18h at 37°C with aeration at 200rpm. The bacterial cells were removed by centrifugation (15,000*g* × 10min) and the culture supernatant retained. An equal volume of absolute ethanol (-20°C) was added to the supernatant and incubated at 4°C for 5h. The precipitated protein was harvested by centrifugation (22,000*g* × 30min), resuspended in 0.2ml of distilled water (exocellular protein concentrated × 250) and stored at -20°C.

7.2.4.2 Affinity purification of anti-SsaA antibody

Whole cells of IPTG induced *E. coli* XLOLR/pSsaA were resuspended in PBS to an OD_{600} of 1.0 (section 6.2.5.2). Equal volumes of the bacterial suspension and SDS-PAGE sample buffer were combined and heated to 100°C for 10min. The cellular proteins of 0.5ml of denatured cell suspension were separated by SDS-PAGE using an 8cm wide sample slot and western blotted onto nitrocellulose. After blocking, incubation with 20ml of diluted serum PRT (1:400) and washing, bound antibody was eluted from the membrane with 5ml of glycine-saline buffer [150mM NaCl, 200mM glycine, pH 2.8] at room temperature with agitation for 30min. The eluted antibody was neutralised immediately with 0.04g Tris and stored at -20°C (Brooks and Burnie, 1994).

7.2.4.3 Investigation of an 85kDa exocellular protein band of *S. epidermidis* detected with anti-SsaA antibody

Affinity purified anti-SsaA antibody bound to two *S. epidermidis* NCTC 11047 proteins, the 32kDa SsaA and an 85kDa protein. To investigate this 85kDa protein further five aliquots of *S. epidermidis* NCTC 11047 ethanol precipitated culture supernatant protein (concentrated × 250) were mixed with an equal volume of SDS-PAGE sample buffer. The samples were denatured at 100°C for 15, 30, 60, 90 or 120min. An *E. coli* XLOLR/pSsaA cell suspension (section 6.2.5.2) was combined with an equal volume of sample buffer and denatured at 100°C for 10min. The

polypeptides of each sample were separated by SDS-PAGE, western blotted and probed with affinity purified anti-SsaA antibody.

7.2.4.4 Protein micro-sequencing of the SsaA band

To further confirm the identity of the SsaA band detected by SDS-PAGE of *S. epidermidis* NCTC 11047 the protein band was subjected to N-terminal sequencing based on the technique of Edman and Begg (1967). A 0.5ml aliquot of ethanol precipitated culture supernatant protein (concentrated × 50) was separated by SDS-PAGE, using an 8cm wide sample slot, and electroblotted onto a polyvinyldifluoridine (PVDF) membrane (Stratagene) using a modified nitrocellulose-electroblotting method. The PVDF membrane was immersed in 100% methanol for 5s, rinsed in distilled water for a further 5s, and finally submerged in CAPS buffer [0.01M 3[cyclohexylamino]-propane sulphonic acid, 10% (v/v) methanol, pH 11.0] for 15min. The SDS-PAGE gel of resolved *S. epidermidis* NCTC 11047 culture supernatant protein was placed in CAPS buffer for 15min to allow both the removal of Tris and glycine and to equilibrate the gel in electroblotting buffer. The gel and membrane were assembled in the mini trans-blot transfer cell as for western blotting with CAPS as the electrode buffer. Electroblotting was conducted at 69V (0.3A) for 30min with constant cooling to 4°C. On completion the membrane was rinsed (3 × 5min) with distilled water, stained with concentrated Coomassie Blue [0.1% (w/v) Coomassie Brilliant Blue, 50% (v/v) methanol] for 5min and destained with destain [50% (v/v) methanol, 10% (v/v) glacial acetic acid] for 15min. The membrane was air dried and stored at -20°C. N-terminal sequencing of the band suspected to be SsaA was performed by Alta Bioscience using an Applied Biosystems 473A automated protein sequencer. The 85kDa protein was not sequenced due to insufficient protein on the PVDF blot.

7.2.5 Expression of SsaA under iron restriction conditions

A range of TSB and LB broths with the following supplements were prepared;

0.01mM deferoxamine mesylate (desferrioxamine mesylate, desferal) (an iron-chelator)

0.01mM deferoxamine mesylate and 0.1mM $FeCl_3$

0.05mM deferoxamine mesylate

0.05mM deferoxamine mesylate and 0.1mM $FeCl_3$

A starter culture of *S. epidermidis* NCTC 11047, cultivated in 20ml of TSB broth for 18h at 37°C with aeration at 200rpm was used to inoculate each of the prepared LB and TSB broths (inoculum dilution 1:200). All cultures were incubated at 37°C for 18h with aeration at 200rpm. The OD_{600} of each flask was recorded to assess any effect of iron restriction on growth. The bacterial cells were removed by centrifugation (10,000*g* × 10min) and the culture supernatant retained. The cells were resuspended in PBS to an OD_{600} of 1.0 and maintained at 4°C for 24h. Microbial products present in the culture supernatants were ethanol precipitated and concentrated eighty-fold (section 7.2.4.1). Western blots of the cell-associated and culture supernatant proteins were probed with serum PRT (diluted 1:400).

7.2.6 Detection of a SsaA-like protein in Gram-positive bacteria

Cell-associated and ethanol precipitated culture supernatant proteins were harvested from various staphylococcal, streptococcal, enterococcal species and *Propionibacterium acnes* strains as outlined previously (section 7.2.4.1), except streptococci and enterococci were cultured initially in BHI broth. *P. acnes* strains were grown in 20ml of LB broth in static universal containers at 37°C for 3 days. The bacterial proteins were separated by SDS-PAGE, transferred to nitrocellulose membrane and immunodetected with serum PRT (diluted 1:400).

7.2.7 PCR to screen for *ssaA*-like genes

PCR was utilised to screen for the presence of *ssaA*-like genes in various Gram-positive and Gram-negative bacteria: *S. epidermidis* (n = 69); *S. simulans* (n = 5); *S. hominis* (n = 4); *S. capitis* (n = 2); *S. lugdunensis* (n = 2); *S. haemolyticus* (n = 1); *S. caprae* (n = 1); *S. sciuri* (n = 1); *S. saprophyticus* (n = 1); non-typable CNS (n = 8); *S. epidermidis* NCTC 11047; *S. carnosus* TM300; *S. aureus* ATCC 12598; *S. aureus* NCTC 6571; strains of *S. aureus* isolated from patients with associated

infections (n = 5); *Enterococcus faecium* NCTC 7171; *E. faecalis* var. *zymogenes* NCTC 5957; *Streptococcus bovis* NCTC 11436; *S. milleri* NCTC 10708; *S. mutans* NCTC 10449; *S. salivarius* NCTC 8618; *S. sanguis* NCTC 7863; single clinical strains of *S. gordonii*, *S. mitis*, β-haemolytic streptococcus groups A and G and *S. pneumoniae*; *Bacillus megaterium* (n = 1); *Micrococcus luteus* (n = 1); *Kocuria rosea* (n = 1); *Escherichia coli* (n = 7); *Citrobacter diversus* (n = 1); *Enterobacter cloacae* (n = 1); *Klebsiella pneumoniae* (n = 1); and *Serratia* species (n = 1).

7.2.7.1 Primer preparation

PCR primers (table 7.2, PE-Applied Biosystems, UK) were designed for optimal detection of *ssaA* using the following criteria (Promega, 1996):

- 15bp to 30bp in length.
- No polypurine or polypyrimide sequences, which could result in secondary structures such as hair pin loops.
- No complementarity between the two primers which could result in primer-dimer formation.
- No run of three or more G or C bases at the 3' end of the primer, which may result in non-specific binding to the target DNA,
- Between 40% and 60% G/C content.
- Ideally the primers should anneal at the same temperature.
- The primers should be separated on the DNA strand by approximately 250bp-400bp.

Table 7.2. Primer 1 and 2 data

	Upstream primer-Primer 1	**Downstream primer-Primer 2**
Sequence	$^{5'}$TCC AAA CGA CCC TTA TTC$^{3'}$	$^{5'}$GTA CAT TGA CCT GAA GTG$^{3'}$
Length	18bp	18bp
Melting temperature	45.6°C	45.6°C
G/C content	44%	44%
n/t number*	528-545	891-908

* n/t number refers to the nucleotide number of the primer sequence in the *ssaA* deposited sequence, AF162275 (deposited from the studies in this thesis by S. Lang, June 2000).

7.2.7.2 PCR of whole bacterial cells

The PCR assay components (except template) were combined as a single volume master mix and dispensed as 25µl final volume aliquots. Each 25µl reaction consisted of reaction buffer (with added 1.5mM $MgCl_2$) (Perkin-Elmer, USA), 100µM individual dNTPs (Amersham Pharmacia, UK), 10pmol of the upstream and downstream primers, 1 unit *Taq* DNA polymerase (BioLine, UK) (added last to the master mix), and deionized water (to a final volume of 25µl). The bacterial template, two to three bacterial colonies taken from horse blood agar plates, were placed in a 0.5ml eppendorf tube and overlaid with one drop (~50µl) of sterile mineral oil. A 25µl aliquot of the master mix was combined with the template, briefly vortex mixed, and pulse microcentrifuged. The PCR assay was composed of a 3min hot start at 95°C followed by a thermal cycle of 95°C for 30s (to denature the DNA), 40°C for 30s (annealing of the primers to the DNA) and 72°C for 1min (to allow DNA elongation) repeated 30 times. On completion the PCR products were analysed by agarose gel electrophoresis using a 2% (w/v) agarose gel prepared with TAE buffer [40mM Tris, 1mM Na_2EDTA (pH 8.0), 0.1% (v/v) glacial acetic acid] and pre-stained with 0.5µg/ml ethidium bromide. Inclusion of a DNA sizing ladder (Hyperladder IV, Bioline) in the gel allowed sizing of the PCR product. Electrophoresis was conducted at 80V for 45 minutes in TAE buffer.

7.3 RESULTS

7.3.1 Phagemid DNA analysis

7.3.1.1 Determination of the DNA insert size

The *S. epidermidis* NCTC 11047 DNA insert carried by phagemid pSsaA was estimated to be ~1,600bp and the insert of phagemid pE2 ~1,700bp (figure 7.1).

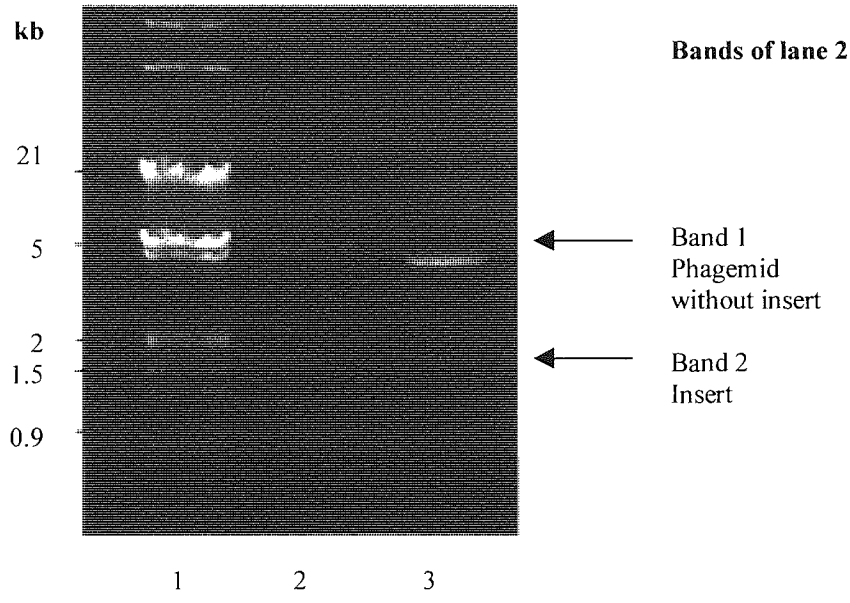


Figure 7.1. Restriction endonuclease digested phagemid pE2 and undigested phagemid pE2 resolved by agarose gel electrophoresis. Lane 1, *Eco*RI/*Hin*dIII digested λ phage DNA sizing ladder; 2, *Eco*RI digested pE2. Band 1 is pE2 with the insert removed by *Eco*RI digestion. Band 2 is the insert cut from pE2; 3, undigested pE2. Superhelical, nicked circular or linear DNA of the undigested phagemid is resolved into three separate bands. The third band of lane 3 is not visible in the printed figure because of the low concentration of DNA present.

7.3.2 pE2

The two strands of the DNA insert pE2 were independently sequenced and translated into peptide sequence for each of the possible six ORFs. Comparison of the pE2 DNA insert sequence with the EMBL and GenBank databases identified homology with a number of prokaryotic and eukaryotic sequences (including human and

mouse) encoding pyruvate dehydrogenase complex (PDH) subunits (table 7.3). Alignment of the translated amino acid sequence identified homology with a specific subunit of the pyruvate dehydrogenase complex; subunit E2-dihydrolipoamide acetyltransferase (table 7.3). The genes carried by the pE2 insert are referred to using the *S. aureus* PDH gene complex nomenclature; *pdhB* (subunit E1β), *pdhC* (subunit E2), and *pdhD* (subunit E3).

Table 7.3. A selection of prokaryotic DNA and amino acid sequences with high homology to the pE2 insert identified by alignment with database deposited sequences

Search	**EMBL/ SwissProt* hit**			
	Accession number	**Name**	**Organism**	**Homology**
DNA	X58434	*pdhB*, *pdhC*, *pdhD* genes for pyruvate decarboxylase, dihydrolipoamide acetyltransferase and dihydrolipoamide dehydrogenase (Hemilä, 1991)	*S. aureus*	82.5% over 1416 na
	M57435	pyruvate dehydrogenase complex genes (Hemilä *et al.*, 1990)	*Bacillus subtilis*	72.5% over 1019 na
	X53560	*pdhA*, *pdhB*, *pdhC*, *pdhD* genes for pyruvate dehydrogenase multi-enzyme complex (Borges *et al.*, 1990)	*Bacillus stearotherm-ophilus*	68.2% over 1011 na
Translation – ORF1 of forward strand	Q59821	Dihydrolipoamide acetyltransferase - subunit E2	*S. aureus*	86.2% over 435 aa
	P11961	Dihydrolipoamide acetyltransferase - subunit E2 (Borges *et al.*, 1990)	*Bacillus stearotherm-ophilus*	68.3% over 435 aa
	P21883	Dihydrolipoamide acetyltransferase - subunit E2 (Hemilä *et al.*, 1990)	*Bacillus subtilis*	67.7% over 444 aa
	P35489	Dihydrolipoamide acetyltransferase - subunit E2 (Wallbrandt *et al.*, 1992)	*Acholeplasma laidlawii*	40.0% over 433 aa
	AAF09841	Dihydrolipoamide acetyltransferase - subunit E2	*Deinococcus radiodurans*	37.8% over 439 aa
	Q49110	Dihydrolipoamide acetyltransferase - subunit E2	*Mycoplasma capricolum*	35.6% over 438 aa
	Q59638	Dihydrolipoamide acetyltransferase - subunit E2	*Pseudomonas aeruginosa*	35.8% over 439 aa
	P06959	Dihydrolipoamide acetyltransferase - subunit E2 (Stephens, Darlison and Guest, 1983)	*Escherichia coli*	35.0% over 432 aa
	Q9Y817	Dihydrolipoamide acetyltransferase - subunit E2	*Haloferax volcanii*	40.0% over 502 aa

Nucleic acid, (na); amino acid, (aa). *The peptide databases searched included SWISSPROTNEW, SWISSPROT, SPTREMBL, REMTREMBL and TREMBLNEW.

Within the 1,716bp insert of pE2 the partial coding region *pdhB* (E1β subunit-pyruvate dehydrogenase), the complete sequence of *pdhC* (E2 subunit-dihydrolipoamide acetyltransferase) and the partial coding sequence of *pdhD* (E3 subunit-dihydrolipoamide dehydrogenase) were identified (figure 7.2). A start codon (gtg), stop codon (taa), potential promoter region and Shine-Dalgarno sequence (located nine bases upstream of the gtg start codon) were identified in the coding sequence of *pdhC* (1,302bp) (figure 7.2). The gtg codon, which is an occasional start codon (Dale, 1998), was conserved in *S. aureus, B. stearothermophilus*, *B. subtilis* (deposited sequences X58434, X53560 and M57435) and other E2 homologues. A lipoamide binding lysine residue was conserved at amino acid position 43 (Wallbrandt *et al.,* 1992; Hemilä *et al.,* 1990; Stephens, Darlison and Guest, 1983) and a putative acetyltransferase active site histidine was conserved at amino acid position 404 (Wallbrandt *et al.,* 1992; Borges *et al.,* 1990). No termination sequence was identified within the DNA insert. DNA alignment of the insert with accession number X58434 (*S. aureus* PDH complex) revealed the partial coding sequence of *pdhB* (E1β), including the stop codon (taa), and the partial coding sequence of *pdhD* (E3), including a possible Shine-Dalgarno and start codon (atg). However, since the *pdhB* and *pdhD* sequences were incomplete they were not investigated further. The insert pE2 sequence was deposited with GenBank, accession number AF261757, from the studies in this thesis by S. Lang, May 2000.

```
-138                                                  5'aattctaattattaaaaa
                                                -35
-120 agcaaacgtaaaaatatcaatcaaataaagtttacatttagttgtaaggttttccttacc
                     -10
-60  ttacaacttttgtataaattttcaataatttaaatacgtaaatcttaggagggacaagaac
     E2
1    gtggcatttgaatttagattacccgatatcggggaaggtatccacgaaggtgaaattgtt
1    M  A  F  E  F  R  L  P  D  I  G  E  G  I  H  E  G  E  I  V
61   aaatggtttattaaagccggcgatacaattgaagaagatgatgtattagcagaagttcaa
21   K  W  F  I  K  A  G  D  T  I  E  E  D  D  V  L  A  E  V  Q
121  aatgataaatctgtagtagaaattccttctccagtaagtggtactgttgaagaagtgtta
41   N  D  K  S  V  V  E  I  P  S  P  V  S  G  T  V  E  E  V  L
181  gtagatgaaggaacagtggcagtagtaggagatgtcatcgttaaaattgatgcacctgat
61   V  D  E  G  T  V  A  V  V  G  D  V  I  V  K  I  D  A  P  D
241  gcagaagaaatgcaatttaaaggtcatggcgatgatgaggattctaagaaagaagaaaaa
81   A  E  E  M  Q  F  K  G  H  G  D  D  E  D  S  K  K  E  E  K
301  gaacaagaatcaccagtgcaagaagaagcttcatcaactcaatcacaagaaaagacagaa
101  E  Q  E  S  P  V  Q  E  E  A  S  S  T  Q  S  Q  E  K  T  E
361  gtagatgaaagtaaaactgttaaagcgatgccgtcagtgcgtaagtatgcacgtgaaaat
121  V  D  E  S  K  T  V  K  A  M  P  S  V  R  K  Y  A  R  E  N
421  ggtgtcaatattaaagctgtaaatggttctggtaaaaatggacgaatcacaaaagaagac
141  G  V  N  I  K  A  V  N  G  S  G  K  N  G  R  I  T  K  E  D
481  atcgatgcatacttaaatggtggtagttccgaagaaggttcaaacactagcgcagcatct
161  I  D  A  Y  L  N  G  G  S  S  E  E  G  S  N  T  S  A  A  S
541  gaatcaacttctagtgatgtcgttaatgcttctgcaacacaagcattaccagaaggcgac
181  E  S  T  S  S  D  V  V  N  A  S  A  T  Q  A  L  P  E  G  D
601  ttccctgaaactacagaaaaaatacctgcaatgcgcaaagcaattgctaaagcaatggtt
201  F  P  E  T  T  E  K  I  P  A  M  R  K  A  I  A  K  A  M  V
661  aattctaaacacactgcacctcatgttacattaatggatgaaattgatgtgcaagaatta
221  N  S  K  H  T  A  P  H  V  T  L  M  D  E  I  D  V  Q  E  L
721  tgggatcaccgtaagaaatttaaagaaattgctgctgaacaaggtacaaaacttactttc
241  W  D  H  R  K  K  F  K  E  I  A  A  E  Q  G  T  K  L  T  F
781  ttaccatatgttgttaaagcattagtttctgcacttaaaaaatatccagcacttaatact
261  L  P  Y  V  V  K  A  L  V  S  A  L  K  K  Y  P  A  L  N  T
841  tctttcaatgaagaagctggagaggttgtacacaaacattactggaatattggtattgct
281  S  F  N  E  E  A  G  E  V  V  H  K  H  Y  W  N  I  G  I  A
901  gcagatacggataaaggattattagtaccagtagttaaacatgccgatcgtaaatcaata
301  A  D  T  D  K  G  L  L  V  P  V  V  K  H  A  D  R  K  S  I
961  ttcgaaatttctgatgaaattaatgaactagctgtaaaagcacgtgatggtaaattaact
```

```
321  F  E  I  S  D  E  I  N  E  L  A  V  K  A  R  D  G  K  L  T
1021 tcagaagaaatgaaaggtgcaacatgcacaattagtaatatcggttccgctggtggacaa
341  S  E  E  M  K  G  A  T  C  T  I  S  N  I  G  S  A  G  G  Q
1081 tggttcactccagttatcaatcacccagaagtagctatcttaggaattggccgtatcgct
361  W  F  T  P  V  I  N  H  P  E  V  A  I  L  G  I  G  R  I  A
1141 caaaaacctatcgttaaagatggagaaattgtagctgcaccagtgttagctttatcatta
381  Q  K  P  I  V  K  D  G  E  I  V  A  A  P  V  L  A  L  S  L
1201 agctttgaccatagacaaatcgatggtgctactggacaaaatgctatgaatcacattaaa
401  S  F  D  H  R  Q  I  D  G  A  T  G  Q  N  A  M  N  H  I  K
1261 cgcttattaaataatccagaattattattaatggaggggtaaaaaacatggtagttggaga
421  R  L  L  N  N  P  E  L  L  L  M  E  G
1321 tttcccaattgaaacagatactattgtaataggagcaggtccaggtggatatgtcgcagc

1381 cattcgcgcggctcaattaggacaaaaggtaacaatcgttgagaaaggtaatttaggtgg

1441 tgtatgcttaaacgttggttgtataccttcaaaagcattactacatgcttctcatcgctt

1501 tgttgaagcgcaaaattcagaaaacttaggggtaattgctgaaagcgtttcgttaaacta

1561 tcaaaaagttcaagaatt3'
```

Figure 7.2. The nucleotide sequence of the pE2 insert (partial coding sequence of *pdhB* (E1β), coding sequence of *pdhC* (E2) and the partial coding sequence of *pdhD* (E3)) and the translated amino acid sequence of E2. (□), start and stop codons; tttaca/ tataaa, potential promoter region; (_), potential Shine-Dalgarno; K, lipoamide binding lysine residue; H, putative active site histidine.

The translated E2 amino acid sequence (ORF1) was aligned with the three deposited prokaryotic sequences identified with the highest level of homology (table 7.3); Q59821 (*S. aureus*), P11961 (*B. stearothermophilus*) and P21883 (*B. subtilis*) (figure 7.3).

```
Q59821  MAFEFRLPDIGEGIHEGEIVKWFVKAGDTIEEDDVLAEVQNDKSVVEIPSPVSGTVEEVM
E2      MAFEFRLPDIGEGIHEGEIVKWFIKAGDTIEEDDVLAEVQNDKSVVEIPSPVSGTVEEVL
P11961  MAFEFKLPDIGEGIHEGEIVKWFVKPGDEVNEDDVLCEVQNDKAVVEIPSPVKGKVLEIL
P21883  MAFEFKLPDIGEGIHEGEIVKWFVKPNDEVDEDDVLAEVQNDKAVVEIPSPVKGKVLELK
         ****.***************** *..*...*****.******.********.*.*.*..

Q59821  VEEGTVAVVGDVIVKIDAPDAEDMQFKGHDDDSSSKEEPAKEE----------APAEQA
E2      VDEGTVAVVGDVIVKIDAPDAEEMQFKGHGDDEDSKKEEKEQE----------SPVQEE
P11961  VPEGTVATVGQTLITLDAPGYENMTFKGQ-EQEEAKKEEKTET-------VSKEEKVDAV
P21883  VEEGTVATVGQTIITFDAPGYEDLQFKGSDESDDAKTEAQVQSTAEAGQDVAKEEQAQEP
        * *****.**......***..* ..***. .....*.*.. ..             ..

Q59821  PVATQTEE-----VDENRTVKAMPSVRKYAREKGVNIKAVSGSGKNGRITKEDVDAYLNG
E2      ASSTQSQEK--TEVDESKTVKAMPSVRKYARENGVNIKAVNGSGKNGRITKEDIDAYLNG
P11961  APNAPAA---EAEAGPNRRVIAMPSVRKYAREKGVDIRLVQGTGKNGRVLKEDIDAFLAG
P21883  AKATGAGQQDQAEVDPNKRVIAMPSVRKYAREKGVDIRKVTGSGNNGRVVKEDIDSFVNG
        .  ..  .    .... ..*.*********** **.*..* *.*.***..***.*....*

Q59821  G-APTASNESADS--ATNEEVAETPAAPAAVSLEGDFPETTEKIPAMRRAIAKAMVNSKH
E2      GSSEEGSNTSAASE-STSSDVVNASATQALP--EGDFPETTEKIPAMRKAIAKAMVNSKH
P11961  G-----AKPAP----AAAEEKAAPAAAKPATT-EGEFPETREKMSGIRRAIAKAMVHSKH
P21883  G-----AQEAAPQETAAPQETAAKPAAAPAP--EGEFPETREKMSGIRKAIAKAMVNSKH
        *     .. .. ..  .   .    *  . .  **.****.**....*.*******.***

Q59821  TAPHVTLMDEIDVQALWDHRKKFKEIAAEQGTKLTFLPYVVKALVSALKKYPALNTSFNE
E2      TAPHVTLMDEIDVQELWDHRKKFKEIAAEQGTKLTFLPYVVKALVSALKKYPALNTSFNE
P11961  TAPHVTLMDEADVTKLVAHRKKFKAIAAEKGIKLTFLPYVVKALVSALREYPVLNTSIDD
P21883  TAPHVTLMDEVDVTNLVAHRKQFKQVAADQGIKLTYLPYVVKALTSALKKFPVLNTSIDD
        **********.**. *..***.**..**..*.***.********.***...*.****...
```

```
Q59821  EAGEIVHKHYWNIGIAADTDRGLLVPVVKHADRKSIFQISDEINELAVKARDGKLTADEM
E2      EAGEVVHKHYWNIGIAADTDKGLLVPVVKHADRKSIFEISDEINELAVKARDGKLTSEEM
P11961  ETEEIIQKHYYNIGIAADTDRGLLVPVIKHADRKPIFALAQEINELAEKARDGKLTPGEM
P21883  KTDEVIQKHYFNIGIAADTEKGLLVPVVKNADRKSVFEISDEINGLATKAREGKLAPAEM
        ...*...***.********..******.*.****..*....***.**.***.***.  **

Q59821  KGATCTISNIGSAGGQWFTPVINHPEVAILGIGRIAQKPIVKDGEIVAAPVLALSLSFDH
E2      KGATCTISNIGSAGGQWFTPVINHPEVAILGIGRIAQKPIVKDGEIVAAPVLALSLSFDH
P11961  KGASCTITNIGSAGGQWFTPVINHPEVAILGIGRIAEKPIVRDGEIVAAPMLALSLSFDH
P21883  KGASCTITNIGSAGGQWFTPVINHPEVAILGIGRIAEKAIVRDGEIVAAPVLALSLSFDH
        ***.***.****************************.*.**.********.*********

Q59821  RQIDGATGQNAMNHIKRLLNNPELLLMEG
E2      RQIDGATGQNAMNHIKRLLNNPELLLMEG
P11961  RMIDGATAQKALNHIKRLLSDPELLLMEA
P21883  RMIDGATAQNALNHIKRLLNDPQLILMEA
        *.*****.*.*.*******..*.*.***.
```

Figure 7.3. Clustal alignment of E2, from *S. epidermidis* NCTC 11047, with deposited sequences Q59821 (*S. aureus*), P11961 (*B. stearothermophilus*) and P21883 (*B. subtilis*). (*), conserved amino acids between the four proteins; (.), conserved amino acids between E2 and at least one other protein; K, lipoamide binding lysine residue; H, putative active site histidine.

7.3.3 Analysis and characterisation of E2

7.3.3.1 Protein data

The E2 subunit had a predicted M_r of 46,930 and a pI of 4.67.

Due to the limited binding of antibody to the E2 polypeptide, as indicated by the western blot "ghost" band (section 6.3.7.1, figure 6.5), the protein would not be useful as a marker of infection and, therefore, was not investigated further.

7.3.4 pSsaA

The two strands of the DNA insert were independently sequenced and translated into peptide sequence for each of the possible six ORFs. Comparison of the pSsaA DNA

insert sequence with the EMBL and GenBank databases identified homology with two deposited sequences: a staphyloxanthin biosynthesis protein of *S. aureus* (accession number X97985) and SceB, a protein of unknown function from *S. carnosus* (accession number U96107) (Wagner, Doskar and Götz, 1998) (table 7.4).

Table 7.4. DNA and amino acid sequences homologous to the pSsaA insert identified by alignment with database deposited sequences

Search	**EMBL/ SwissProt* hit**			
	Accession number	**Name**	**Organism**	**Homology**
DNA	X97985	SA1234 - Staphyloxanthin biosynthesis	*S. aureus*	67.2% over 1211 na
	U96107	SceB - exoprotein (Wagner, Doskar and Götz, 1998)	*S. carnosus*	70.1% over 588 na
Translation – ORF1 of forward strand	Q53587	SA1234- Staphyloxanthin biosynthesis	*S. aureus*	72.9% over 221 aa
	054487	SceB - exoprotein (Wagner, Doskar and Götz, 1998)	*S. carnosus*	65.2% over 227 aa
Translation – ORF3 of forward strand	Q53587	SA1234- Staphyloxanthin biosynthesis	*S. aureus*	88.9% over 36 aa
	054487	SceB -exoprotein (Wagner, Doskar and Götz, 1998)	*S. carnosus*	94.3% over 35 aa

Nucleic acid, (na); amino acid, (aa). *The peptide databases searched included SWISSPROTNEW, SWISSPROT, SPTREMBL, REMTREMBL and TREMBLNEW.

Within the 1,630bp insert of pSsaA a coding region of 690bp was identified with start (atg) and stop (tga) codons, peptide leader sequence, signal peptidase cleavage sequence (AQA↓A), a region of dyad symmetry characteristic of a termination sequence (rho-independent), potential Shine-Dalgarno sequence located 11bp upstream of the atg start codon and the position of a mutation event or possible cloning artefact resulting in the deletion of 4bp and loss of the correct reading frame (figure 7.4). The product coded by pSsaA was termed staphylococcal secretory antigen (SsaA).

```
-120  5'ataaagtaatgcttgtcgttaaaaaagaacgaaaccaaggataacaaagtaaatatttta

 -60  gtcacatgactattcattatatgaaattaattatttaaatttttaggaggatatttaaca

   1  atgaaaaaaatcgctacagctacaattgcaactgcaggaatcgctactttcgcatttgca
   1  M  K  K  I  A  T  A  T  I  A  T  A  G  I  A  T  F  A  F  A
  61  caccatgacgcacaagcagcagaacaaaataatgatgggtacaatccaaacgacccttat
  21  H  H  D  A  Q  A  A  E  Q  N  N  D  G  Y  N  P  N  D  P  Y
 121  tcatatagctacacttacacaatcgatgctgaaggtaactaccactacacttggaaaggt
  41  S  Y  S  Y  T  Y  T  I  D  A  E  G  N  Y  H  Y  T  W  K  G
 181  aactggagtccagatcgtgtaaatacttcatataactataataattataataactacaac
  61  N  W  S  P  D  R  V  N  T  S  Y  N  Y  N  N  Y  N  N  Y  N
 241  tactatggttacaataactatagcaactacaataactacagtaattacaacaattacaac
  81  Y  Y  G  Y  N  N  Y  S  N  Y  N  N  Y  S  N  Y  N  N  Y  N
 301  aactatcaatcaaacaacacgcaatcacaaagaacaactcaaccgactggtggtttaggc
 101  N  Y  Q  S  N  N  T  Q  S  Q  R  T  T  Q  P  T  G  G  L  G
 361  gcaagctattcaacatcaagtagtaatgttcacgttacaacaacttctgcgccatcatca
 121  A  S  Y  S  T  S  S  S  N  V  H  V  T  T  T  S  A  P  S  S
 421  aacggtgtatctttatcaaacgctcgctcagcatctggtaacttatacacttcaggtcaa
 141  N  G  V  S  L  S  N  A  R  S  A  S  G  N  L  Y  T  S  G  Q
 481  tgtacatattatgtatttgacagagtaggtggcaaaatcggttcaacgtggggtaacgca
 161  C  T  Y  Y  V  F  D  R  V  G  G  K  I  G  S  T  W  G  N  A
 541  aacaactgggcaaacgctgcagcacgttctggttacacagtaaacaattcgcctgctaaa
 181  N  N  W  A  N  A  A  A  R  S  G  Y  T  V  N  N  S  P  A  K
 601  ggtgcaatcttacaaacgtcacaaggtgcatacggacacgtagcatacgttgaaggtgta★
 201  G  A  I  L  Q  T  S  Q  G  A  Y  G  H  V  A  Y  V  E  G  V
 661  gcaatggttcaatcagagtttcagaaatgaactacggtcacggtgcaggtgttgtcactt
 221  A  M  V  Q  S  E  F  Q  K
 721  cacgtacaatctctgcgagccaagctgcttcatataactatattcactaatcatatgtga

 781  ttaataatatactgatatttaaagcattaaactaacattgtatgatgttgtttaatgctt

 841  ttttt3'
```

Figure 7.4. The nucleotide sequence of *ssaA* and the translated amino acid sequence (ORF1) of SsaA. aggagg, potential Shine-Dalgarno; (□), start and stop codons; AQA, signal peptidase cleavage sequence, (_), rho-independent termination sequence; (★), the position of a mutation event or cloning artefact, as indicated in Clustal analysis of homologous DNA sequences.

The translated SsaA amino acid sequence (ORF1) was aligned with the amino acid sequences of the deposited proteins 054487 (*S. carnosus*) and Q53587 (*S. aureus*) using Clustal (figure 7.5). Strong homology existed between the three proteins up to amino acid 219 of SsaA after which point homology was transferred to ORF3 (figure 7.6).

```
SsaA     MKKIATATIATAG--IATFAFAHHDAQAAEQNNDGYNPNDPYSYSYTYTIDAEGNYHYTW
054487   MNKIATTTIATVGTGIAALTLSHHDADAAE-NNNGYNPNDPYSYSYSYTIDQQGNYHYTW
Q53587   MKKIVTATIATAG--LATIAFAGHDAQAAEQNNNGYNSNDAQSYSYTYTIDAQGNYHYTW
         *.**.*.****.*  .*. ....***.***.** ***.**..****.****. *******

SsaA     KGNWSPD-RVNTSY-NYNNYNN--YNYYGYNNYSNYNNYSNYN-NYNNYQSNNTQSQRTT
054487   EGNWNKDNRFNNNYSTYAQYNNGYSNNTSSNNYSSYSNYNTANNNYSNYSSYNNNNAGST
Q53587   TGNWNPS-QLTQNN-TYY-YNN--YNTYSYNNAS-YNNYYNHSYQYNNY-TNNSQTATNN
          *** .. . .  .  *  ***  .* . .**.* *.** . . .*.** ..* .    .

SsaA     QPTGGLGASYSTSSSNVHVTTTSAPSSNGVSLSNARSASGNLYTSGQCTYYVFDRVGGKI
054487   PRTGGMGATYSTSDNNVKVSTTSAPSTSSNTMSSRTSSGANYYTAGQCTYYAFDRAGGKV
Q53587   YYTGGSGASYSTTSNNVHVTTTAAPSSNGRSISNGYASGSNLYTSGQCTYYVFDRVGGKI
           *** **.***.. **.*.**.***... . *.  .   *.**.******.***.***.

SsaA     GSTWGNANNWANAAARSGYTVNNSPAKGAILQTSQGAYGHVAYVEGV----AMVQSEFQK
054487   GSTWGNANNWASAAAAAGYTVNNSPAAGAIMQTSQGAYGHVAYVESVNSNGSIRVSEMNY
Q53587   GSTWGNASNWANAAASSGYTVNNTPKVGAIMQTTQGYYGHVAYVEGVNSNGSVRVSEMNY
         *******.***.***  .******.*. *** **.**.********.*★        **

SsaA     □
054487   GHGPGVVTSRTISASQAAGYNYIR
Q53587   GHGAGVVTSRTISANQAGSYNFIH
```

Figure 7.5. Clustal alignment of SsaA (ORF1) with 054487 (*S. carnosus*) and Q53587 (*S. aureus*). (*), conserved amino acids between the three proteins; (.), conserved amino acids between SsaA and one other protein; (★), the position of a mutation event or cloning artefact, as indicated in Clustal analysis of homologous DNA sequences; (□), stop codon.

```
                                        *
SsaA     KQFACRCNLTNVTRCIRTRSIRRCSNGSIRVSEMNYGHGAGVVTSRTISASQAASYNYIH
054487   VAYVESVN----------------SNGSIRVSEMNYGHGPGVVTSRTISASQAAGYNYIR
Q53587   VAYVRGVN----------------SNGSVRVSEMNYGHGAGVVTSRTISANQAGSYNFIH
                *                ****.**********.**********.**..**.*.
```

Figure 7.6. Clustal alignment of SsaA (last 60 amino acids of ORF3) with 054487 (*S. carnosus*) and Q53587 (*S. aureus*). (*), conserved amino acids between the three proteins; (.), conserved amino acids between SsaA and one other protein; (*), the position of a mutation event or cloning artefact, as indicated in Clustal analysis of homologous DNA sequences.

Alignment of the DNA sequences identified a 4bp $^{5'}$taac$^{3'}$ deletion at the position of amino acid 219 of SsaA (figure 7.7) producing a frameshift mutation and thus the formation of a truncated protein (nine incorrect and a further twenty-eight amino acid residues missing from the carboxy terminus).

```
SsaA      5'ggtgt----agcaat3'
U96107    5'agtgttaacagcaat3'
X97985    5'ggcgttaacagcaac3'
```

Figure 7.7. Clustal alignment of the DNA sequences of *ssaA*, U96107 (*S. carnosus*) and X97985 (*S. aureus*) illustrating the deletion of 4bp from *ssaA* and the loss of the correct ORF.

Sequencing both strands of the PCR product amplified from the *S. epidermidis* NCTC 11047 genome confirmed the presence of 4 nucleotides $^{5'}$aaac$^{3'}$ in the position of the 4bp deletion identified in the cloned *ssaA* gene. During cloning a 4bp deletion was introduced into the insert pSsaA - a cloning artefact. Data base sequences were re-screened with the modified DNA sequence and the translated amino acid sequences (table 7.5).

Table 7.5. DNA and amino acid sequences homologous to SsaA, from *S. epidermidis* NCTC 11047 identified by alignment with deposited database sequences

Search	**EMBL/ SwissProt* hit**			
	Accession number	**Name**	**Organism**	**Homology**
DNA	X97985	SA1234- Staphyloxanthin biosynthesis	*S. aureus*	67.5% over 1211 na
	U96107	SceB-exoprotein (Wagner, Doskar and Götz, 1998)	*S. carnosus*	70.6% over 588 na
Translation – ORF1 of forward strand	Q53587	SA1234- Staphyloxanthin biosynthesis	*S. aureus*	75.2% over 258 aa
	054487	SceB-exoprotein (Wagner, Doskar and Götz, 1998)	*S. carnosus*	69.2% over 263 aa

Nucleic acid, (na); amino acid, (aa). *The peptide databases searched included SWISSPROTNEW, SWISSPROT, SPTREMBL, REMTREMBL and TREMBLNEW.

The native *ssaA* sequence shown was essentially the same as the cloned *ssaA* however the correct stop codon (taa) was at the position nucleic acids 772-774 and the coding region was extended from 690bp to 774bp (figure 7.8). The *ssa*A sequence was deposited with GenBank, accession number AF162275, from the studies in this thesis by S. Lang, June 2000.

```
-120  5'ataaagtaatgcttgtcgttaaaaaagaacgaaaccaaggataacaaagtaaatatttta

-60   gtcacatgactattcattatatgaaattaattatttaaatttttaggaggatatttaaca

 1    atgaaaaaaatcgctacagctacaattgcaactgcaggaatcgctactttcgcatttgca
 1    M  K  K  I  A  T  A  T  I  A  T  A  G  I  A  T  F  A  F  A
 61   caccatgacgcacaagcagcagaacaaaataatgatgggtacaatccaaacgacccttat
 21   H  H  D  A  Q  A  A  E  Q  N  N  D  G  Y  N  P  N  D  P  Y
 121  tcatatagctacacttacacaatcgatgctgaaggtaactaccactacacttggaaaggt
 41   S  Y  S  Y  T  Y  T  I  D  A  E  G  N  Y  H  Y  T  W  K  G
 181  aactggagtccagatcgtgtaaatacttcatataactataataattataataactacaac
 61   N  W  S  P  D  R  V  N  T  S  Y  N  Y  N  N  Y  N  N  Y  N
 241  tactatggttacaataactatagcaactacaataactacagtaattacaacaattacaac
 81   Y  Y  G  Y  N  N  Y  S  N  Y  N  N  Y  S  N  Y  N  N  Y  N
 301  aactatcaatcaaacaacacgcaatcacaaagaacaactcaaccgactggtggtttaggc
 101  N  Y  Q  S  N  N  T  Q  S  Q  R  T  T  Q  P  T  G  G  L  G
 361  gcaagctattcaacatcaagtagtaatgttcacgttacaacaacttctgcgccatcatca
 121  A  S  Y  S  T  S  S  S  N  V  H  V  T  T  T  S  A  P  S  S
 421  aacggtgtatctttatcaaacgctcgctcagcatctggtaacttatacacttcaggtcaa
 141  N  G  V  S  L  S  N  A  R  S  A  S  G  N  L  Y  T  S  G  Q
 481  tgtacatattatgtatttgacagagtaggtggcaaaatcggttcaacgtggggtaacgca
 161  C  T  Y  Y  V  F  D  R  V  G  G  K  I  G  S  T  W  G  N  A
 541  aacaactgggcaaacgctgcagcacgttctggttacacagtaaacaattcgcctgctaaa
 181  N  N  W  A  N  A  A  A  R  S  G  Y  T  V  N  N  S  P  A  K
 601  ggtgcaatcttacaaacgtcacaaggtgcatacggacacgtagcatacgttgaaggtgta
 201  G  A  I  L  Q  T  S  Q  G  A  Y  G  H  V  A  Y  V  E  G  V
 661  aacagcaatggttcaatcagagtttcagaaatgaactacggtcacggtgcaggtgttgtc
 221  N  S  N  G  S  I  R  V  S  E  M  N  Y  G  H  G  A  G  V  V
 721  acttcacgtacaatctctgcgagccaagctgcttcatataactatattcactaatcatat
 241  T  S  R  T  I  S  A  S  Q  A  A  S  Y  N  Y  I  H
 781  gtgattaataatatactgatatttaaagcattaaactaacattgtatgatgttgtttaat

 841  gctttttttt3'
```

Figure 7.8. The nucleotide sequence of *ssaA* and the translated amino acid sequence of SsaA. aggagg, potential Shine-Dalgarno; (□), start and stop codons; AQA, signal peptidase cleavage sequence; (_), rho-independent termination sequence.

Similarly, the peptide sequence of native SsaA was essentially the same as the cloned SsaA, however the amino acid chain was extended by 37 amino acids (figure 7.9).

```
SsaA     MKKIATATIATAG--IATFAFAHHDAQAAEQNNDGYNPNDPYSYSYTYTIDAEGNYHYTW
054487   MNKIATTTIATVGTGIAALTLSHHDADAAE-NNNGYNPNDPYSYSYSYTIDQQGNYHYTW
Q53587   MKKIVTATIATAG--LATIAFAGHDAQAAEQNNNGYNSNDAQSYSYTYTIDAQGNYHYTW
         *.**.*.****.*  .*. ....***.***.** ***.**..****.****. *******

SsaA     KGNWSPD-RVNTSY-NYNNYNN--YNYYGYNNYSNYNNYSNYN-NYNNYQSNNTQSQRTT
054487   EGNWNKDNRFNNNYSTYAQYNNGYSNNTSSNNYSSYSNYNTANNNYSNYSSYNNNNAGST
Q53587   TGNWNPS-QLTQNN-TYY-YNN--YNTYSYNNAS-YNNYYNHSYQYNNY-TNNSQTATNN
          *** .. . .  .  *  ***  .* . .**.* *.** . . .*.** ..* .    .

SsaA     QPTGGLGASYSTSSSNVHVTTTSAPSSNGVSLSNARSASGNLYTSGQCTYYVFDRVGGKI
054487   PRTGGMGATYSTSDNNVKVSTTSAPSTSSNTMSSRTSSGANYYTAGQCTYYAFDRAGGKV
Q53587   YYTGGSGASYSTTSNNVHVTTTAAPSSNGRSISNGYASGSNLYTSGQCTYYVFDRVGGKI
           *** **.***.. **.*.**.***... . *.  .   *.**.******.***.***.

SsaA     GSTWGNANNWANAAARSGYTVNNSPAKGAILQTSQGAYGHVAYVEGVNSNGSIRVSEMNY
054487   GSTWGNANNWASAAAAAGYTVNNSPAAGAIMQTSQGAYGHVAYVESVNSNGSIRVSEMNY
Q53587   GSTWGNASNWANAAASSGYTVNNTPKVGAIMQTTQGYYGHVAYVEGVNSNGSVRVSEMNY
         *******.***.*** .******.*. *** **.**.********.******.*******

SsaA     GHGAGVVTSRTISASQAASYNYIH
054487   GHGPGVVTSRTISASQAAGYNYIR
Q53587   GHGAGVVTSRTISANQAGSYNFIH
         ***.**********.**..**.*.
```

Figure 7.9. Clustal alignment of SsaA, from *S. epidermidis* NCTC 11047, with accession numbers 054487 (*S. carnosus*) and Q53587 (*S. aureus*). (*), conserved amino acids between the three proteins; (.), conserved amino acids between SsaA and one other protein.

7.3.5 Analysis and characterisation of SsaA

7.3.5.1 Protein data

The precursor polypeptide (before signal peptidase cleavage) had a predicted M_r of 27,911 and the pI was 9.02. The mature exocellular protein (after cleavage of the signal peptide) had a M_r of 25,271 and a pI of 8.40.

7.3.5.2 Prediction of secondary protein structure

The secondary protein structure (α-helix, β-sheet or β-turns) was predicted using the rules proposed by Chou and Fasman (Chou and Fasman, 1978) (figure 7.10).

Hydrophilicity/hydrophobicity, predicted using Kyte-Doolittle (Kyte and Doolittle, 1982), revealed a hydrophobic domain corresponding to a peptide leader sequence followed by a hydrophilic region consistent with a surface exposed epitope within a region of 100 amino acids residues (amino acids 30-130) (figure 7.11).

Figure 7.10. Chou-Fasman secondary protein structure prediction for SsaA. The values of the vertical axis are the relative probabilities of amino acid residue occurrence in different globular protein secondary structures (Chou and Fasman, 1978).

Figure 7.11. Kyte-Doolittle hydrophilicity profiles for SsaA, Q53587 (*S. aureus*) and 054487 (*S. carnosus*). On the vertical axis positive relative values indicate hydrophobicity and negative relative values indicate hydrophilicity (Kyte and Doolittle, 1982).

The SsaA amino terminus was characteristic of a signal peptide:

- One to three positively charged amino acids at the amino terminus (SsaA had two lysine residues).
- A hydrophobic sequence of fourteen to twenty amino acids (the Kyte-Doolittle prediction of SsaA revealed a hydrophobic domain at the amino terminus).
- One or two proline or glycine residues within the hydrophobic domain (SsaA had a single glycine residue).
- Presence of a serine or threonine residue within the hydrophobic region (SsaA had four threonine residues within the hydrophobic region of the peptide).
- Alanine or glycine residue at the carboxy terminus of the signal peptide cleavage site (SsaA had an alanine residue) (Duffaud, Lehnhardt and March, 1985; Perlman and Halvorson, 1983).

The $^{-3}$A-$^{-2}$X-$^{-1}$A↓ motif (where ↓ indicates the cleavage site) at amino acid position 24-26 was identified as a signal peptidase cleavage sequence (Perlman and Halvorson, 1983). No homologous sequences were identified within the databases searched of the amino acid motif Y-N-N-Y, which is repeated several times within the hydrophilic region of the sequence (amino acid residues 71-102). The function of this unusual amino acid motif remains unknown. The hydrophilicity profile of SsaA showed remarkable similarity to those of both SceB and the staphyloxanthin biosynthesis protein (figure 7.11).

7.3.6 Detection of SsaA in *S. epidermidis* NCTC 11047

7.3.6.1 Screening with affinity purified anti-SsaA antibody

Affinity purified antibody, eluted from a western blot of IPTG induced *E. coli* XLOLR/pSsaA, bound to *S. epidermidis* NCTC 11047 culture supernatant protein bands of approximately 32kDa and 85kDa (figure 7.12).

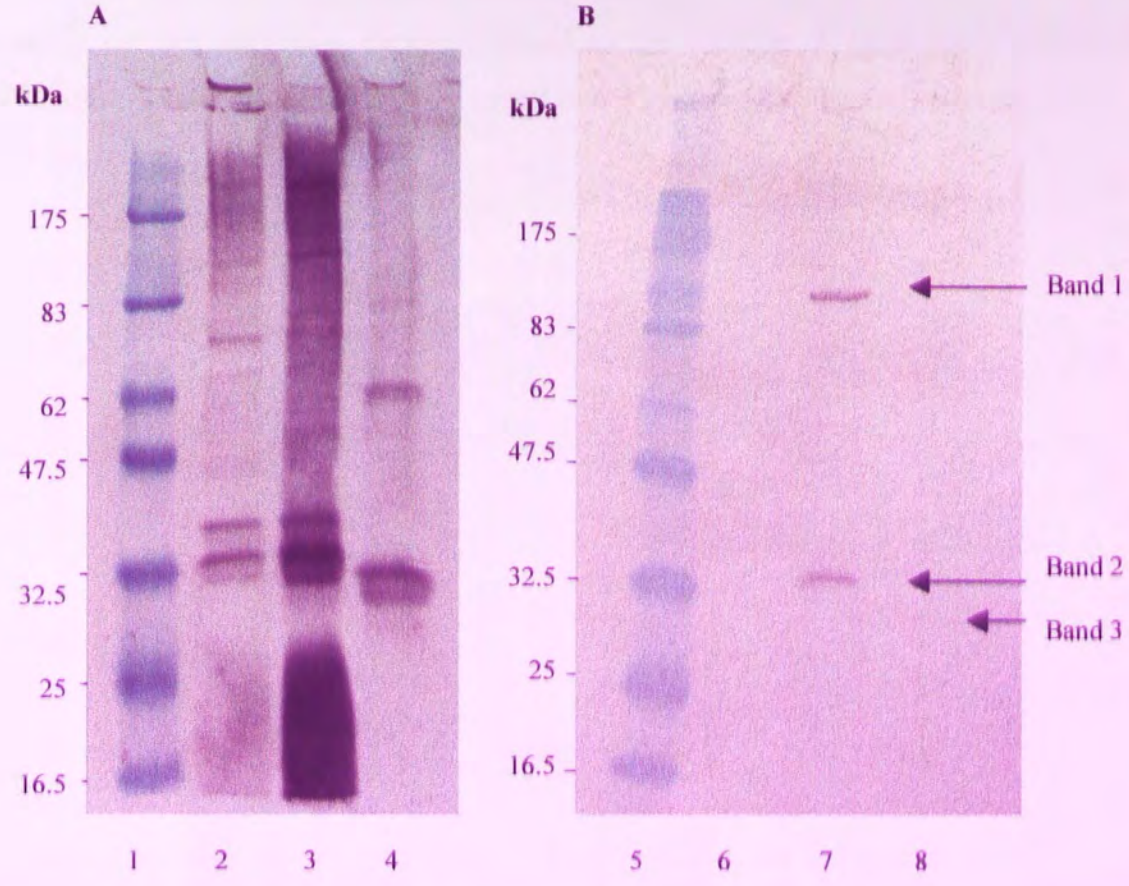


Figure 7.12. Western blots of cellular protein and ethanol precipitated culture supernatant protein of *S. epidermidis* NCTC 11047 and *E. coli* XLOLR/pSsaA probed with PRT or affinity purified anti-SsaA antibody. Lane 1 and 5, sizing ladder; 2 and 6, *S. epidermidis* NCTC 11047 cellular protein; 3 and 7, *S. epidermidis* NCTC 11047 culture supernatant protein; 4 and 8, *E. coli* XLOLR/pSsaA. Blot A was probed with PRT (diluted 1:400); blot B was probed with affinity purified anti-SsaA antibody. Band 1 is ~85kDa; band 2 is SsaA, ~32kDa; band 3 (only faintly visible on the blot) is the 30kDa fusion protein.

7.3.6.2 Investigation of the 85kDa protein band

Affinity purified anti-SsaA antibody bound to the ~85kDa *S. epidermidis* NCTC 11047 protein despite denaturing the samples at high temperatures in the presence of SDS for up to 2h (figure 7.13).

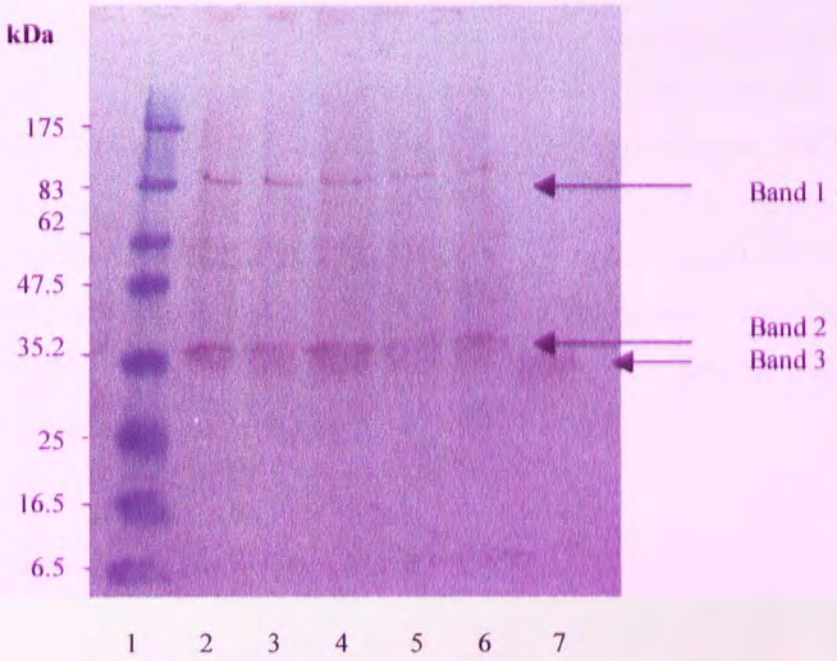


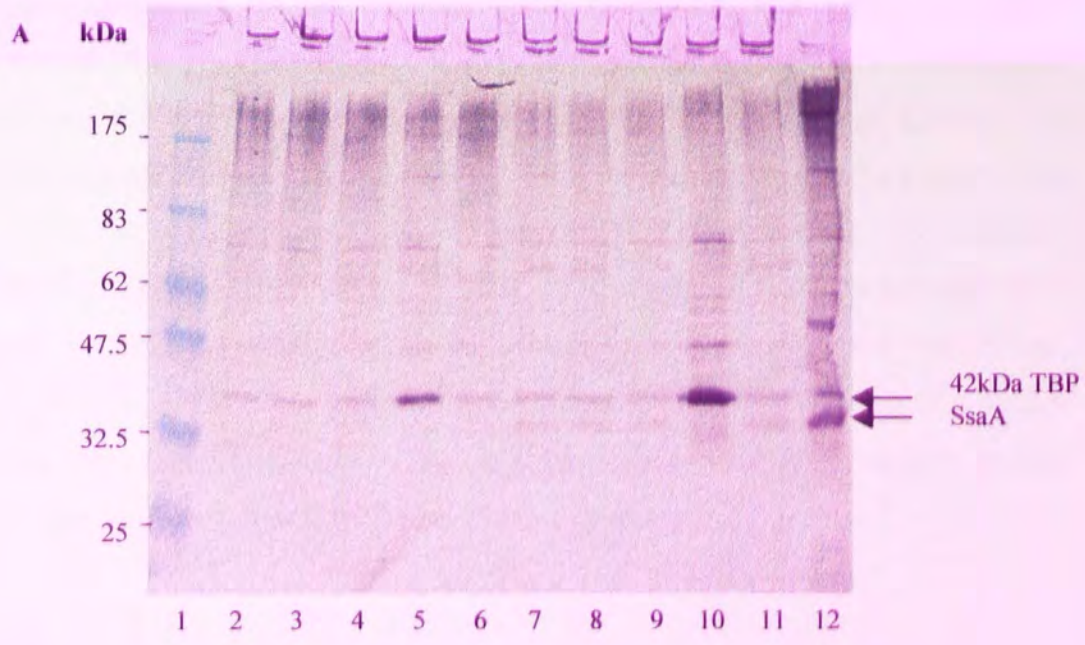
Figure 7.13. Western blot of *S. epidermidis* NCTC 11047 ethanol precipitated culture supernatant protein, denatured with SDS at 100°C for varying time duration, probed with affinity purified anti-SsaA antibody. Lane 1, sizing ladder; 2, *S. epidermidis* NCTC 11047 ethanol precipitated culture supernatant protein denatured with SDS at 100°C for 15min; 3, 30min; 4, 60 min; 5, 90min; 6, 120min; 7, *E. coli* XLOLR/pSsaA denatured with SDS at 100°C for 10min. Band 1 is ~85kDa; band 2 is SsaA, ~32kDa; band 3 is the 30kDa fusion protein of *E. coli* XLOLR/pSsaA.

7.3.6.3 Protein micro-sequencing

N-terminal sequencing of five amino acids of the protein band suspected to be SsaA generated the sequence ETTNV. This amino acid sequence was not present in either the precursor polypeptide or the mature secreted peptide SsaA. Database analysis identified only one deposited sequence with homology; VP4 fragment of foot and mouth disease virus (100% homology over 5 amino acids).

7.3.7 Detection of SsaA expression in iron restricted conditions

TSB and LB broths were found to give similar levels of SsaA expression in iron restricted growth conditions. The reduced OD_{600} of the 0.05mM deferoxamine mesylate supplemented flasks indicated that growth had been limited, but not inhibited, by the addition of the iron-chelator. This reduced growth was not caused by deferoxamine mesylate toxicity since a high level of growth was achieved in the broths supplemented with 0.05mM deferoxamine mesylate and 0.1mM $FeCl_3$. Additionally, a protein of approximately 42kDa was expressed in greater concentration in the two flasks with the highest concentration of the iron-chelator (figure 7.14). This protein was predicted to be the transferrin-binding protein expressed by staphylococci under iron limited conditions created by the addition of transferrin to growth media (Modun, Kendall and Williams, 1994). There was no detectable difference in the expression of cell-associated or secreted SsaA under iron restricted growth conditions (figure 7.14).



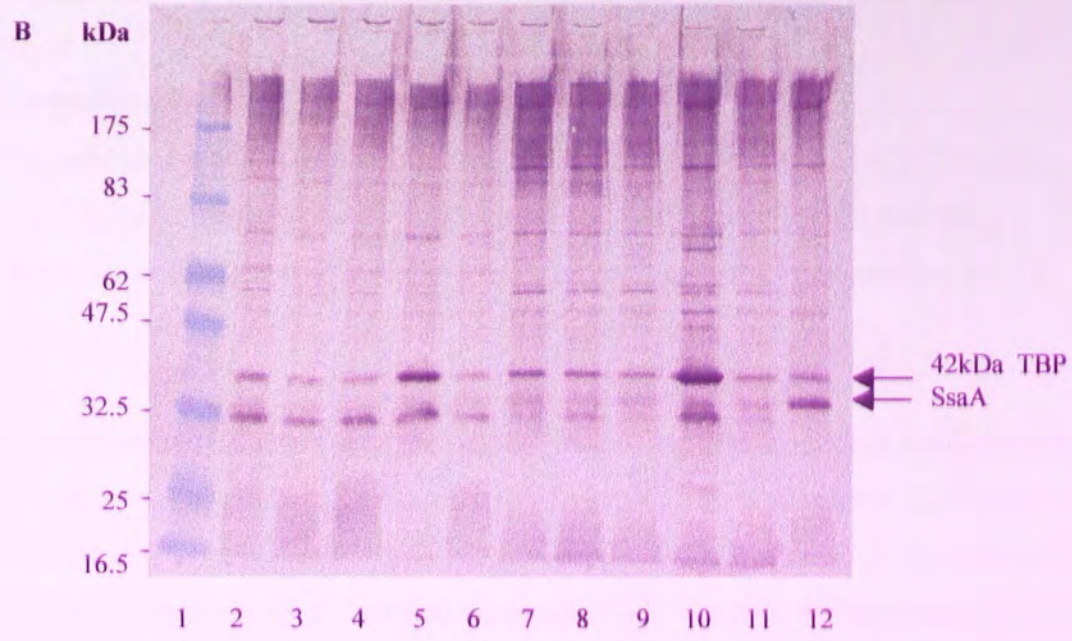


Figure 7.14. Expression of SsaA by *S. epidermidis* NCTC 11047 grown under iron restricted conditions in two different broth types (LB and TSB). a, cellular preparation of *S. epidermidis*; b, ethanol precipitated culture supernatant (concentrated × 10) preparation of *S. epidermidis*. Lane 1, sizing ladder; 2, LB; 3, LB + 0.01mM deferoxamine mesylate (DF); 4, LB + 0.01mM DF + 0.1mM $FeCl_3$; 5, LB + 0.05mM DF; 6, LB + 0.05mM DF + 0.1mM $FeCl_3$, 7, TSB; 8, TSB + 0.01mM DF; 9, TSB + 0.01 DF + 0.1mM $FeCl_3$; 10, TSB + 0.05mM DF; 11, TSB + 0.05mM DF + 0.1mM $FeCl_3$; 12, original stock of ethanol precipitated culture supernatant (*S. epidermidis* NCTC 11047 in LB, concentrated × 12.5). The position of a 42kDa iron regulated protein, predicted to be the staphylococcal transferrin-binding protein (TBP) (Modun, Kendall and Williams, 1994), is indicated.

7.3.8 Detection of proteins in other bacteria cross-reacting with anti-SsaA antibody

7.3.8.1 Staphylococci

Cell-associated and secreted SsaA was expressed by *S. epidermidis* NCTC 11047 in all of the different growth phases investigated (cellular proteins harvested from 4, 6 and 18h cultures and ethanol precipitated culture supernatant proteins from 6 and 18h cultures).

Cell-associated and supernatant precipitated protein profiles of eleven CNS strains (six *S. epidermidis*, and one strain each of *S. capitis*, *S. lugdunensis*, *S. hominis*, *S. haemolyticus*, *S. simulans*) were compared. Briefly, all of the *S. epidermidis* strains, the *S. lugdunensis*, *S. haemolyticus* and the *S. hominis* strains gave positive western blots whilst the *S. capitis* and *S. simulans* were negative for cell-associated SsaA. The *S. epidermidis* strains were the only isolates positive for secreted SsaA (figures 7.15, 7.16).

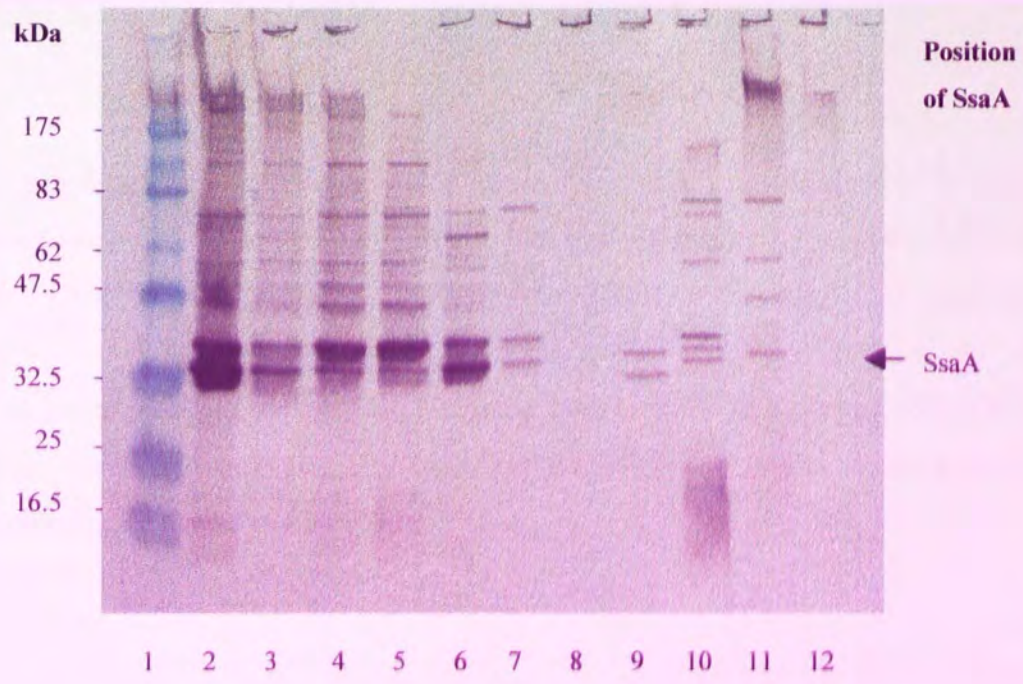


Figure 7.15. Cell-associated proteins of various CNS strains immunodetected with serum PRT (diluted 1:400). Lane 1, sizing ladder; 2, *S. epidermidis* NCTC 11047; 3-7 *S. epidermidis* strains; 8, *S. capitis*; 9, *S. lugdunensis*; 10, *S. hominis*; 11, *S. haemolyticus*; 12, *S. simulans*.

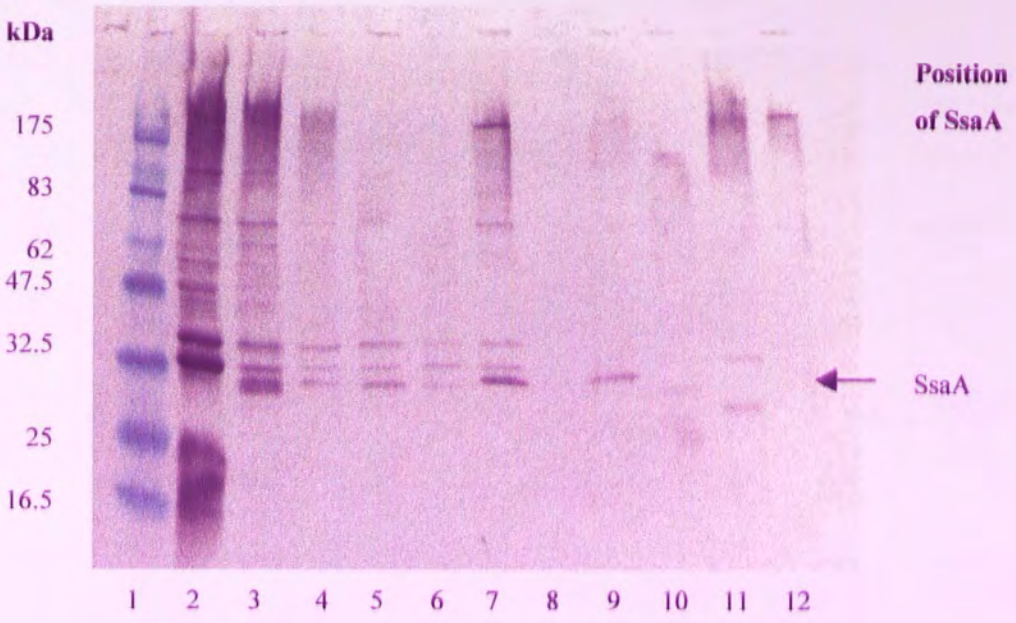


Figure 7.16. Ethanol precipitated culture supernatant proteins of various CNS strains immunodetected with serum PRT (diluted 1:400). Lane 1, sizing ladder; 2, *S. epidermidis* NCTC 11047; 3-7 *S. epidermidis* strains; 8, *S. capitis*; 9, *S. lugdunensis*; 10, *S. hominis*; 11, *S. haemolyticus*; 12, *S. simulans*.

SsaA was detected in both cellular and culture supernatant preparations of *S. aureus* strains (seven out of seven) by western blot probed with PRT (figure 7.17). To confirm that *S. aureus* NCTC 6571 protein was cross-reacting with anti-SsaA antibody and not *S. aureus* IgG in the serum, a western blot of both cellular and culture supernatant preparations of *S. aureus* NCTC 6571 was probed with affinity purified anti-SsaA antibody. A SsaA homologue was detected in both protein preparations (data not shown).

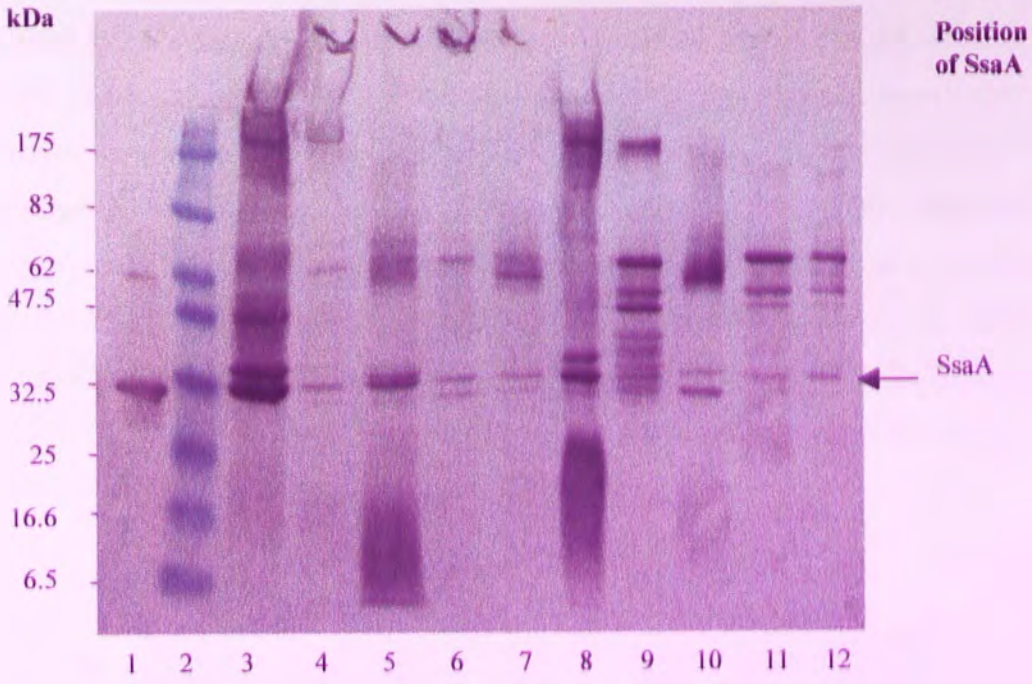


Figure 7.17. Cell-associated and ethanol precipitated culture supernatant proteins of four *S. aureus* strains immunodetected with serum PRT (diluted 1:400). Lane 1, *E. coli* XLOLR/pSsaA; 2, sizing ladder; 3, *S. epidermidis* NCTC 11047 cellular protein; 4, *S. aureus* NCTC 6571 cellular protein; 5, *S. aureus* ATCC 12598 cellular protein; 6-7, *S. aureus* clinical strains cellular protein; 8, *S. epidermidis* NCTC 11047 supernatant protein; 9, *S. aureus* NCTC 6571 supernatant protein; 10, *S. aureus* ATCC 12598 supernatant protein; 11-12, *S. aureus* clinical strains supernatant protein.

7.3.8.2 Other Gram-positive organisms

A number of streptococci, enterococci and *P. acnes* strains were examined for a *ssaA*-like gene. Western blot analysis of both cellular and exocellular material probed with serum PRT (diluted 1:400) identified no homologue from the *P. acnes* strains (n = 6), *Streptococcus* (n = 4, *S. mutans* NCTC 10449, *S. bovis* NCTC 11436, *S. sanguis* NCTC 7863, group G streptococcus), and *Enterococcus* (n = 2, *E. faecium* NCTC 7171, *E. faecalis* var. *zymogenes* NCTC 5957) species investigated.

7.3.9 PCR

PCR was employed to increase the number of bacterial genomes screened for the presence of *ssaA*-like genes. The sequence was detected within various CNS strains (86 out of 88, 98%) (figure 7.18) but no PCR product was obtained from either the *S. aureus* strains (n = 7) or any of the non-*Staphylococcus* species (n = 20) investigated, except *E. coli* strains (six out of seven) (Table 7.6). Agarose gel analysis of the PCR product, generated using *E. coli* PCR product as a template a second round of PCR, revealed a high intensity product band. However, the band was smaller (~300bp) than the expected product (381bp). All PCR-negative staphylococcal strains (seven *S. aureus* and two CNS) were shown to be positive for a SsaA homologue by western blot analysis.

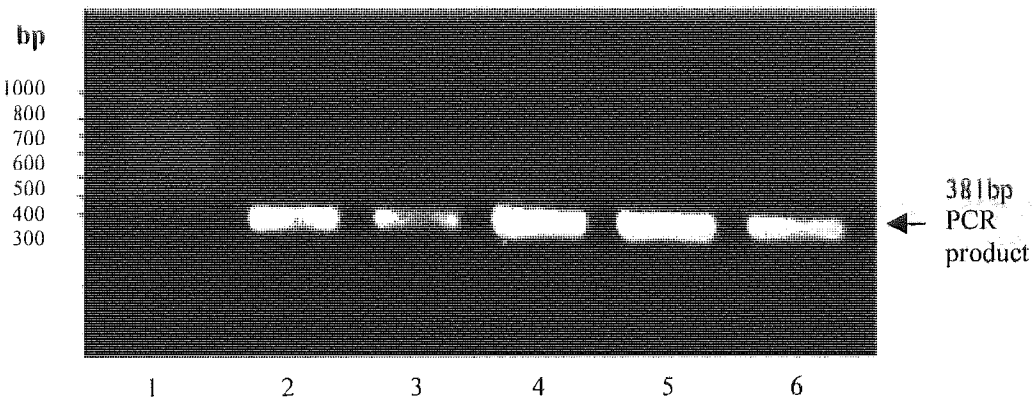


Figure 7.18. Visualisation of PCR products, generated using *ssaA* specific primers, by agarose gel electrophoresis and ethidium bromide staining. Lane 1, sizing ladder; 2, *S. epidermidis* NCTC 11047 positive control; 3-6, *S. epidermidis* clinical strains.

Table 7.6. Bacterial strains screened by PCR for the presence of *ssaA* or a homologue

Bacterial species	**Number positive/number tested**
S. aureus	0/7
S. capitis	2/2
S. caprae	1/1
S. carnosus TM300	1/1
S. epidermidis	70/70
S. haemolyticus	1/1
S. hominis	3/4
S. lugdunensis	1/2
S. saprophyticus	1/1
S. sciuri	1/1
S. simulans	5/5
Staphylococcus species	8/8
Bacillus megaterium	0/1
Citrobacter diversus	0/1
Enterobacter cloacae	0/2
Enterococcus species	0/2
Escherichia coli	6/7
Klebsiella pneumoniae	0/1
Kocuria species	0/1
Micrococcus species	0/1
Serratia species	0/1
Streptococcus species	0/10
Total	**100/130**

7.4 DISCUSSION

The immune response of a patient (serum PRT) with a severe *S. epidermidis* infection was exploited to identify antigenic cellular or secreted staphylococcal antigens that were synthesised *in vivo* during sepsis. The study identified two novel antigens not previously reported in *S. epidermidis*: staphylococcal secretory antigen (SsaA) and the E2 subunit protein of a PDH complex.

7.4.1 E2 subunit protein

The range of prokaryotes with highly conserved PDH genes (Gram-positive bacteria, *Bacillus* and *Staphylococcus* species; Gram-negative bacteria, *E. coli* and *P. aeruginosa*; halophilic Archaea, *Haloferax volcanii*; mycoplasmas, *Acholeplasma laidlawii*) reflects the central importance of the metabolic enzymes comprising the PDH complex (Wallbrandt *et al.*, 1992; Vettakkorumakankav and Stevenson, 1992). The high level of gene conservation extended to eukaryotic cells; the polypeptide sequence of human and mouse dihydrolipoamide acetyltransferase shared 34.9% over 473 amino acids and 35.5% over 459 amino acids, respectively (deposited sequences P11182 and P53395).

The cloned antigenic insert pE2 carried the complete E2 sequence and the partial E1β and E3 subunit sequences (figure 7.19).

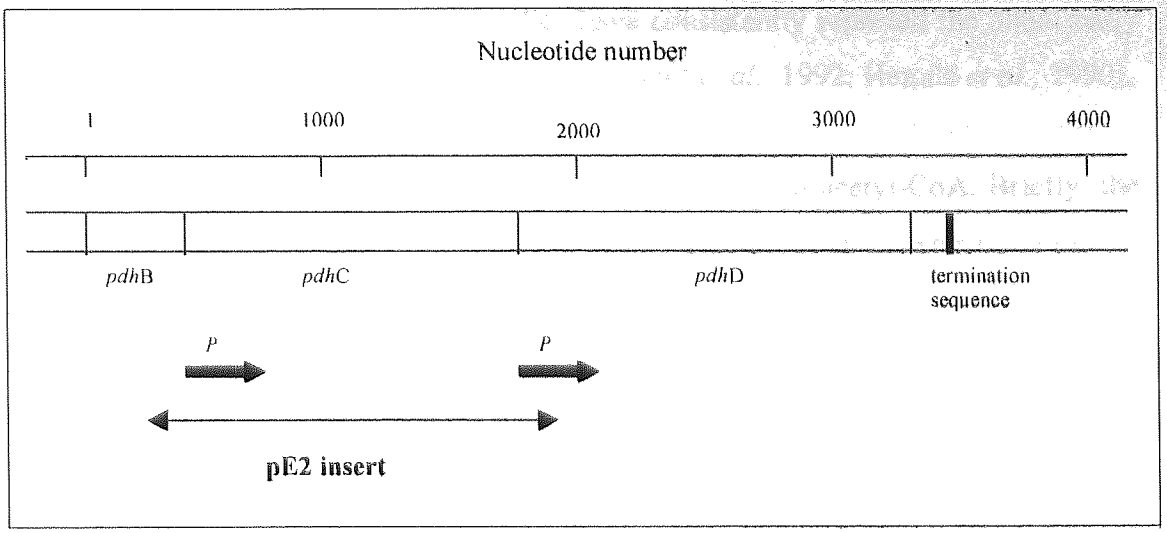


Figure 7.19. Schematic diagram of the PDH complex genes, based on the deposited sequence X58434 of *S. aureus*, and the region of the complex coded within the pE2 DNA insert. *P*, putative promoter region. Adapted from Hemilä, 1991.

An E2 promoter was identified in both *S. aureus* (figure 7.19) and *B. subtilis* (Hemilä, 1991; Hemilä *et al.*, 1990). A potential promoter region was identified within the pE2 DNA sequence; TATAAa (-10) and TTtACA (-35) separated by 36 nucleotides and located 42 nucleotides upstream of the start codon. The distance between the –10 and –35 boxes and the promoter region and the start codon were both greater than expected. The absence of a termination sequence indicated that the *pdhC* and *pdhD*, or possibly the entire gene complex, are transcribed together with termination downstream of *pdhD* (Hemilä, 1991). The reading frame for the genes in the pE2 insert was discontinuous. However, this is not unusual and occurs in the PDH complex genes of *A. laidlawii, B. subtilis, B. stearothermophilus* and *E. coli* (Wallbrandt *et al.*, 1992; Hemilä *et al.*, 1990; Borges *et al.*, 1990; Stephens, Darlison and Guest, 1983).

The E2 subunit expressed by the pE2 insert had a theoretical M_w of 47kDa but an experimental M_w of ~66kDa. The E2 subunit of various bacterial species has been reported to have an estimated M_w of 57kDa to 75kDa and a predicted M_w of 46kDa to 65kDa (Wallbrandt *et al.*, 1992; Hemilä *et al.*, 1990; Borges *et al.*, 1990; Stephens, Darlison and Guest, 1983). The size differences were largely due to the method used

for estimation, however, previous studies have consistently reported the abnormally low mobility of E2 during SDS-PAGE (Wallbrandt *et al.*, 1992; Hemilä *et al.*, 1990).

The E2 subunit is involved in the conversion of pyruvate to acetyl-CoA. Briefly, the E1 subunit accepts a two-carbon aldehyde group. This group is transferred to the E2 subunit (lipoamide arm) and simultaneously oxidized. The resultant acetyl group is transferred onto a second arm (the location of a second arm, if present was not identified), which positions it for transfer to the next step of the cycle (Mathews and van Holde, 1990) (appendix 5). The lipoamide arm is linked to the E2 protein via a lysine residue, located in *S. epidermidis* NCTC 11047 at amino acid position 43. The number of identified lipoamide binding lysine residues varies with different species; *E. coli* has three and *A. laidlawii* one (Wallbrandt *et al.*, 1992; Stephens, Darlison and Guest, 1983). Interestingly, alignment of the amino acid sequences of pE2 and the nine organisms identified with homologous sequences (table 7.3) revealed conservation of this single lipoamide binding lysine residue and a preceding aspartic acid residue (appendix 6). By comparison to the deposited sequences of *A. laidlawii and B. stearothermophilus* a putative acetyltransferase active site histidine residue was identified, which was also highly conserved in all the organisms investigated (appendix 6).

The clone was selected from the genomic DNA library based on an induced immune response of a patient with *S. epidermidis* IE. However, western blot analysis of the clone product revealed limited antibody binding to the fusion protein shown by the generation of a "ghost" band. The E2 subunit is antigenic, selection of plaques was made by an immunopositive reaction and anti-human E2 antibody has been reported in association with primary biliary cirrhosis (Fussey *et al.*, 1989). Additionally, antibody to the *Neisseria meningitidis* E2 subunit was detected in patients infected with meningococci (AlaAldeen *et al.*, 1996). It is possible that the fusion protein was unable to regenerate its full epitope reactivity on transfer to the nitrocellulose or that the high concentration of the induced protein within the band effectively blocked itself from binding antibody. Since E2 exhibited limited antibody binding and was unlikely to function as a virulence determinant it was not investigated further.

7.4.2 SsaA

Affinity purified anti-SsaA antibody reacted with a 32kDa protein of *S. epidermidis* NCTC 11047 present in both the cellular and secreted protein preparations. The band size difference of the *S. epidermidis* protein (32kDa) and the *E. coli* XLOLR/pSsaA expressed fusion protein (30kDa) reflected the 28 amino acid truncation of the polypeptide due to the deletion that arose during cloning. The slight variation in the predicted M_w (precursor peptide M_w 28kDa and mature peptide M_w 25kDa) and the observed M_w of SsaA (32kDa) probably resulted from either incomplete denaturing of the protein, abnormal migration during SDS-PAGE (as observed with E2), or inaccuracy of the sizing ladder (covalent coupling of the dye to the proteins affects their electrophoretic behaviour) (BioLabs).

A second protein band of approximately 85kDa, which also reacted with antibody eluted from the 30kDa band, indicated either the formation of SDS-stable oligomers, synthesis of a complex containing SsaA with another protein, or the presence of a staphylococcal protein with a shared epitope. The corresponding bands were present in both the cellular and exocellular protein preparations. Failure of N-terminal sequencing to detect either SsaA or the 85kDa protein band reflected the high number of closely positioned proteins on the gel and the low concentration of each polypeptide transferred to the PVDF membrane.

The presence of a signal peptide is characteristic of proteins that are exported from bacterial cells, including the LamB receptor and OmpA of *E. coli*, histidine-binding-protein of *Salmonella typhimurium,* and diphtheria toxin of *Corynebacterium diphtheriae* (Duffaud, Lehnhardt and March, 1985; Silhavy, Benson and Emr, 1983; Michaelis and Beckwith, 1982). The $^{-3}$A-$^{-2}$X-$^{-1}$A motif ($^{-3}$A^{-2}Q-$^{-1}$A-$^{+1}$A^{+2}-E in SsaA) is a sequence frequently associated with signal peptidase activity (Perlman and Halvorson, 1983; Michaelis and Beckwith, 1982; Higgins and Ames, 1981) and is commonly the site of cleavage for secreted staphylococcal polypeptides including staphylococcal lipases, *S. aureus* protein A, the *icaB* component of the PIA and the *atlE* autolysin of *S. epidermidis* (Heilmann *et al.*, 1997; Heilmann *et al.*, 1996; Farrell, Foster and Holland, 1993; Duffaud, Lehnhardt and March, 1985). The amino acid at position –1 (last residue of the leader sequence) is most frequently an amino acid with a small side chain, frequently alanine, sometimes glycine or occasionally

serine. The -2 amino acid is generally an amino acid with a large or charged side chain, such as histidine, phenylalanine, tyrosine, glutamine, asparagine. The +1 residue often has a small side chain (i.e. alanine, cysteine, glycine) and the amino acid occupying the +2 position is frequently acidic. Based on these criteria and the characteristics outlined in section 7.3.5.2, the SsaA leader sequence is a classic example of a signal peptide. Identification of a leader sequence, a signal peptidase cleavage site and detection of SsaA in culture supernatant confirmed secretion of the protein. However, why the bacterial cell secretes SsaA remains unknown.

Western blotting of CNS species other than *S. epidermidis* showed limited detection of an homologous protein antigen. However, PCR indicated that 98% of the CNS species screened possessed a *ssaA*-like gene. This indicated a degree of diversity in the gene present in the CNS species. A different pattern of results was obtained when *S. aureus* strains were investigated. Using primers designed for optimal detection of *ssaA*, the *S. aureus* isolates did not yield a positive PCR product but all were shown to produce a protein of the appropriate size which reacted with PRT and, furthermore, at least one strain reacted with affinity purified anti-SsaA antibody. Failure of PCR to detect the *ssaA* homologue, X97985 shown by sequence analysis to be present in *S. aureus* strains, could be explained by the six out of eighteen (nucleotide number 641-658 in deposited sequence X97985 for *S. aureus*) and two out of eighteen (nucleotide number 998-1015) base pair mismatch of primers 1 and 2, respectively. The *E. coli* strains (six out of seven) generated slightly smaller than expected PCR product bands of weak intensity in comparison to both the positive control and the product generated by other positive organisms suggesting low affinity binding of the primer to template sequence. This together with the failure of western blotting to detect a SsaA-like protein suggested that *E. coli* strains did not express a SsaA homologue. Therefore, neither molecular nor immunological techniques detected a SsaA-like protein or *ssaA* homologue in any of the non-staphylococcal bacterial species screened demonstrating that SsaA and homologues were unique to staphylococcal species.

Many bacterial virulence genes are regulated by environmental factors, such as extracellular iron concentration (section 1.3.5.1). As a potential virulence determinant, SsaA expression was investigated during iron restricted growth.

Although *S. aureus* has been shown to be capable of utilising desferrioxamine bound iron (Brock and Ng, 1983), the availability of the metal ion was limited in the culture medium supplemented with 0.05mM deferoxamine mesylate. This was demonstrated by reduced bacterial growth and the enhanced production of a 42kDa protein suspected to be the *S. epidermidis* transferrin-binding protein (Modun *et al.*, 1998). However, SsaA expression was unaffected by iron restriction. Although this demonstrates that expression of *ssaA* was not iron-regulated, this does not remove the possibility that this protein is involved in pathogenesis.

Despite the identification of homologous gene sequences in *S. epidermidis* NCTC 11047 (accession number AF162275), *S. carnosus* TM300 (accession number U96107) and *S. aureus* Newman strain (accession number X97985), no clear functional role for SsaA, SceB or the staphyloxanthin biosynthesis protein has yet been established in these organisms. The antibody response to SsaA during staphylococcal infection indicated that the protein antigen was expressed during infection (Chapter 8). The reported association of Q53587 with staphyloxanthin biosynthesis in *S. aureus* (GenBank deposition Q53587) presented the interesting possibility of a protective role against oxidative host defences. Staphyloxanthin (α-D-glucopyranosyl-1-O-(4,4'-diaponeurosporene-4-oate)6-O-(12-methyltetradecan -oate) is a major triterpenoid carotenoid pigment of *S. aureus* that gives the distinctive yellow-orange colony coloration (Marshall and Wilmoth, 1981a; Marshall and Wilmoth, 1981b; Wieland *et al.*, 1994). Although *S. epidermidis* are not normally pigmented, a carotenoid glucoside similar to staphyloxanthin has been identified in *S. epidermidis* (Ogo, 1985; O'Leary and Wilkinson, 1988). Carotenoid pigments protect against desiccation, provide resistance against linolenic acid, and have the ability to convert toxic singlet oxygen to a non-toxic form and convey resistance to phagocytic killing as observed in *S. aureus* (Clements and Foster, 1999; Brock and Madigan, 1991; Grinsted and Lacey, 1973). Indeed, *S. aureus* mutants with reduced staphyloxanthin synthesis have also increased sensitivity to hydrogen peroxide (Kullik, Giachino and Fuchs, 1998). It is also interesting to note that the *S. aureus* staphyloxanthin operon is a direct target of the sigma factor σ^B which has also been implicated in the expression of the global regulator Sar and a number of potential virulence factors (Kullik, Giachino and Fuchs, 1998). It is possible that, based on current knowledge, SsaA and related genes may be involved in the

biosynthetic pathway of a staphylococcal carotenoid. The elaboration of pigments such as staphyloxanthin provides a protective role against singlet oxygen encountered in the environment, as for the non-pathogenic *S. carnosus*, and against the host defence mechanisms encountered by pathogenic strains of staphylococci, such as *S. epidermidis* and *S. aureus*. However, the possible involvement of SsaA in carotenoid synthesis does not explain why SsaA is secreted from the bacterial cell. The serodiagnostic potential of the SsaA antigen is discussed in Chapter 8.

CHAPTER 8: THE IMMUNE RESPONSE TO THE COAGULASE-NEGATIVE STAPHYLOCOCCAL PROTEIN - SsaA

8.1 INTRODUCTION

8.1.1 Enzyme-linked immunosorbent assay

The enzyme-linked immunosorbent assay (ELISA) technique uses an enzyme-labelled conjugate to quantitate a specific antigen-antibody reaction (Wood and Wreghitt, 1990). A heterogeneous indirect ELISA is a simple method for the quantitation of immunoglobulin specific to a particular antigen. The various reagents (sample containing antibody of interest, enzyme-labelled conjugate, chromogenic substrate) are added sequentially to solid phase bound antigen with an incubation and washing step between each stage. The coloured product generated by the enzyme on addition of substrate, and quantitated using a spectrophotometer, is directly related to the amount of antibody in the sample. Clinical microbiology laboratories routinely employ this technique in the diagnosis of many infectious diseases including *Helicobacter pylori*, *Chlamydia* species, hepatitis B and HIV.

8.1.2 Aim

In Chapters 6 and 7 the antigenicity of the secreted staphylococcal protein SsaA was demonstrated. The aim of this chapter was to evaluate the serodiagnostic potential of the SsaA antigen as a marker of CNS infection. The immune response to SsaA by patients with various infections caused by both Gram-positive and Gram-negative organisms and control patients with no evidence of infection was assessed by a blotting technique and by ELISA. The diagnostic potential of the SsaA ELISA was evaluated in comparison to the results of the lipid S ELISA, a test for Gram-positive sepsis (section 1.3.8.1). Finally, the antibody titres of sequential sera were investigated as a means of following therapy and evaluating outcome. This work may contribute to the development of a serological test for staphylococcal or more specifically *S. epidermidis* infection.

8.2 MATERIALS AND METHOD

8.2.1 Screening multiple sera for anti-SsaA antibody

E. coli XLOLR/pSsaA was grown in NZY broth supplemented with kanamycin (50μg/ml) and induced with 1mM IPTG and *E. coli* XLOLR was cultivated in NZY broth (section 6.2.5.2). The bacterial cells were recovered by centrifugation (4,300*g* × 10min) and resuspended in PBS to an OD_{600} of 1.0. The cellular proteins of 0.5ml of denatured *E. coli* XLOLR/pSsaA and *E. coli* XLOLR cells were separated by SDS-PAGE using an 8cm wide sample slot (section 7.2.4.2). A pre-stained size marker was included in each gel. The resolved proteins were transferred to nitrocellulose membrane by western blotting (section 6.2.5.3). After blocking, each membrane was cut into vertical strips 0.5cm wide and labelled on the reverse side. Thirty-five serum samples were collected from patients with various staphylococcal infections including IE, CVC-associated sepsis, CAPD-related peritonitis and prosthetic hip joint-associated osteomyelitis (section 2.2). Serum PRT was included as a positive control and sera from patients with bone fractures but no evidence of infection were included as negative controls. One strip from each membrane (*E. coli* XLOLR/pSsaA and *E. coli* XLOLR) was placed into a bijou bottle containing 3ml of serum diluted 1:400 with TBS-BSA. The bijoux bottles were maintained at 4°C on a roller for 18h. After washing the strips in TBST, bound antibody was detected using protein A-alkaline phosphatase and NBT/BCIP colour development solution (section 6.2.4.1).

8.2.2 Purification of SsaA by gel permeation chromatography

8.2.2.1 Supernatant preparation

The supernatants of two 18h cultures of *S. epidermidis* NCTC 11047, cultivated in 50ml LB broth at 37°C with aeration at 200rpm, were harvested by centrifugation (15,000*g* × 10min). The supernatant of one culture was concentrated ten-fold by ethanol precipitation (section 7.2.4.1) and the second concentrated ten-fold by freeze-drying. The recovered supernatant was transferred to a freeze-drying flask, frozen by placing in liquid nitrogen, freeze-dried, and re-dissolved in distilled water to achieve a ten-fold concentration. The concentrated supernatant of both samples was stored in

1.5ml aliquots at -20°C. Control samples of freeze-dried and ethanol precipitated LB broth concentrated ten-fold was similarly prepared.

8.2.2.2 Gel permeation chromatography by fast protein liquid chromatography

Samples of freeze-dried and ethanol precipitated *S. epidermidis* NCTC 11047 culture supernatant and the two control samples were subjected to gel permeation chromatography by fast protein liquid chromatography (FPLC). Insoluble material was removed from 1.5ml of the concentrated culture supernatant (11,400*g* × 2min). A 1ml aliquot of the supernatant was loaded onto a Superose 12 prepacked HR 10/30 FPLC column (Pharmacia) equilibrated with double distilled water. Fifty fractions of 0.8ml were eluted with double distilled water at 0.4ml/min. The fractions were stored at -20°C.

8.2.2.3 Antibody detection of SsaA by dot blot

A strip of nitrocellulose membrane (9cm × 5cm) was divided into a grid of fifty numbered squares. Each square was dot inoculated with 1µl of the corresponding fraction recovered by FPLC. This was repeated with the fractions from both the *S. epidermidis* culture supernatant preparations and the two control samples. The four nitrocellulose strips were air-dried, submerged in blocking solution, rinsed and probed directly with serum PRT (diluted 1:400). After washing, bound antibody was detected with protein A - alkaline phosphatase (section 6.2.4.1). A second dot blot of ethanol precipitated culture supernatant FPLC fractions was probed with affinity purified anti-SsaA antibody to confirm the presence of SsaA (section 7.2.4.2).

8.2.2.4 Antibody detection of SsaA by western blot

Each fraction that gave a strong colour reaction on dot blotting was subjected to western blotting. The bacterial proteins within each fraction were separated by SDS-PAGE and transferred to nitrocellulose membrane. The membrane was blocked, immunodetected with serum PRT (diluted 1:400) and bound antibody detected with protein A - alkaline phosphatase (section 6.2.4.1).

8.2.3 Purification of SsaA by elution from polyacrylamide

The bacterial cells of IPTG induced *E. coli* XLOLR/pSsaA and *E. coli* XLOLR, cultivated in LB broth for 18h at 37°C with constant agitation at 200rpm, were harvested (4,300*g* × 10min) and resuspended in PBS to an OD_{600} of 1.0 (section 6.2.5.2). The cell-associated proteins of 0.5ml of denatured *E. coli* XLOLR/pSsaA and *E. coli* XLOLR cells were separated by SDS-PAGE using an 8cm wide sample slot (section 7.2.4.2).

Electro-elution was performed using a Model 422 Electro-Eluter (BioRad) according to the manufacturer's guidelines (Harrington, 1990). A silicone adaptor fitted with a dialysis membrane cap was filled with elution buffer (25mM Tris, 192mM glycine, 0.1% (w/v) SDS, pH 8.0) and attached to the lower end of a glass tube containing a frit. The tube was filled with elution buffer and located into the elution module. On completion of SDS-PAGE the gels were rinsed with distilled water, stained for 5min in 0.3M cupric chloride and destained for 2 to 3min with water. The polyacrylamide band containing SsaA, located as a non-stained area on a blue background, was cut from the *E. coli* XLOLR/pSsaA gel in 1cm lengths and placed into a single glass tube of the elution module. The corresponding region of the polyacrylamide gel was excised from the *E. coli* XLOLR gel and placed in a second glass tube. The upper and lower chambers of the module and buffer chamber were filled with ~100ml and ~600ml of buffer, respectively. A magnetic stirrer placed in the buffer chamber prevented bubble formation on the dialysis membrane. Elution was performed at a constant 10mA/glass tube (Model 300Xi computer controlled electrophoresis power supply, BioRad) for 4h. After the elution was completed the glass tube was removed from the module, the buffer above the frit discarded, the tube detached from the silicone adapter and the buffer containing the eluted protein recovered and stored until required at -20°C. The success of elution was determined by western blot analysis of the eluate and immunodetection with serum PRT (diluted 1:400) and protein A - alkaline phosphatase.

8.2.4 SsaA ELISA

8.2.4.1 Optimisation of protein coating microtitre plate wells

Protein coating of wells

The eluted SsaA fraction was coated onto the wells of two rows of a microtitre plate (Immulon 2) by serial two-fold dilution (neat to 1:2,024) to a final volume of 0.1ml with sodium carbonate/bicarbonate buffer (0.5M, pH 9.6) as the diluent. A further two rows were coated with sample eluted from the *E. coli* XLOLR gel (referred to as the control elute). The microtitre plate was covered with aluminium foil and maintained at 4°C for 18h. Unbound protein was removed from the wells by plate inversion and gentle tapping onto absorbent material. Protein free sites were blocked by submerging the plate into TBS-Tween blocking solution [0.01M Tris-HCl pH 7.4, 0.9% (w/v) NaCl, 0.3% (v/v) Tween 20] and immediately emptying. This washing/blocking process was repeated three times before the plate was filled for a fourth time, covered with aluminium foil, and maintained at 4°C for 3h. The TBS-Tween solution was removed, the plate sealed with a microtitre tray sealing strip and stored at -20°C until required.

Detection of SsaA IgG antibody in the control serum

Sera PRT (the positive control) and LLD (the negative control), diluted 1:400, were each applied to one row of wells coated with SsaA and one row coated with the control elute to a final volume of 0.1ml. The plate was covered with aluminium foil and maintained at 4°C for 18h. Serum was removed from the microtitre plate by rapid inversion, the plate immersed in TBS-Tween, ensuring complete filling of all the wells, and immediately emptied by rapid inversion. This was repeated a further three times to ensure that all unbound antibody was removed and the plate gently tapped dry on an absorbent material. Protein A-peroxidase conjugate was diluted to 0.25µg/ml in TBS-Tween and 0.1ml dispensed into each well. The plate was covered with aluminium foil and maintained at 4°C for 2h. Conjugate was removed from the plate by rapid inversion and unbound protein A - horseradish peroxidase removed by washing as described above. A chromogenic peroxidase substrate solution was prepared by combining 5mg of 3,3',5,5'-tetramethylbenzidine dissolved in 0.5ml of DMSO with 50ml of 0.1M sodium acetate/citrate buffer (pH 6.0) containing 5µl of

6% (w/v) hydrogen peroxide. Into each well 0.1ml of chromogenic substrate was dispensed and the plate maintained at room temperature for exactly 10min. The reaction was stopped by the addition of 0.1ml 1M sulphuric acid. The absorbance at 450nm of the yellow coloured product was read immediately using a plate reader.

8.2.4.2 Screening patient serum for IgG antibody to SsaA

Coating microtitre plates with eluted SsaA

Eluted SsaA was diluted 1:100 with 0.5M sodium carbonate/bicarbonate buffer (pH 6.0) and 0.1ml was dispensed into all the wells of a microtitre tray. The plate was covered with aluminium foil and maintained at 4°C for 18h. For each microtitre tray coated with SsaA a corresponding plate was coated with control elute. All microtitre plates were blocked with TBS-Tween (section 8.2.4.1).

Assay of serum IgG by ELISA

Positive (PRT) and negative (LLD) control sera, included in every assay batch, were diluted 1:400 in TBS-Tween. Test sera were similarly prepared. Into the first well of a row, 0.2ml of the diluted serum was dispensed and 0.1ml of TBS-Tween into the next five wells of the row. From the first well 0.1ml of the sample was transferred to the second well, thoroughly mixed with the diluent TBS-Tween, and 0.1ml transferred to the next well. This method of two-fold serial dilution was performed between the adjacent wells up to and including well five from which the excess 0.1ml was discarded. Well six remained as a control well. Each sample was assayed in parallel for anti-SsaA IgG and antibody to the control elute. Inoculated plates were covered and maintained at 4°C for 18h. The plates were washed and bound antibody detected using protein A-peroxidase conjugate and chromogenic substrate (section 8.2.4.1). The absorbance obtained for the control elute plate was subtracted from the corresponding value obtained for the SsaA coated plate and the resulting absorbance (Y-axis) plotted against the serum dilution (X-axis, exponential scale). The absorbance (x) at which the positive control, PRT, had a titre of 1,000 was recorded from the plot and the dilution of each serum sample that reduced the absorbance to x was estimated.

8.2.4.3 Sequential sera collected from a patient with *S. epidermidis* endocarditis

Nine sequential serum samples were collected from a patient with *S. epidermidis* IE (serum PRT). These samples were collected over a period of 1 month from the time of diagnosis. All samples were screened for anti-SsaA (section 8.2.4.2) and anti-lipid S IgG (section 8.2.5.2) using the ELISAs.

8.2.5 Serum IgG antibody to Lipid S

8.2.5.1 Preparation of lipid S coated microtitre plates

The method for preparation of lipid S preparation was based on that described by Elliott *et al.*, (2000). The culture supernatants from seven strains of CNS cultivated in BHI broth at 37°C for 18h with aeration at 200rpm were harvested (10,000*g* × 10min), pooled, freeze-dried and re-dissolved in distilled water to achieve a ten-fold concentration. The concentrated supernatant was subjected to gel permeation chromatography by FPLC (section 8.2.2.2). The antigenic material was collected in fractions 10-15, eluted immediately following the void volume of the column. The pool of these fractions was diluted with 100 volumes of sodium carbonate/bicarbonate buffer (0.05M, pH 9.6) and 0.1ml dispensed into each well of a 96-well microtitre tray. The plate was covered with aluminium foil and maintained at 4°C for 18h. The antigen was removed and unbound sites blocked with TBS-Tween (section 8.2.4.1). Prepared plates were stored at -20°C until required.

8.2.5.2 Screening patient sera for anti-lipid S IgG antibody

All sera to be assayed, including a positive and a negative control, were diluted 1:400 in TBS-Tween. Into the first well of each row 0.2ml of diluted serum was dispensed and 0.1ml of TBS-Tween into the remaining wells of each row (wells 2-12). Each serum sample was serially diluted two-fold between each well (wells 1-11) with thorough mixing in each well before transfer. From well 11 the excess 0.1ml was discarded. Well 12 remained as a control well. The plate was covered with aluminium foil and maintained at 4°C for 18h. The serum was removed from the wells, the plate washed, protein A-horseradish peroxidase conjugate added to each well and the plated developed using the chromogenic substrate as outlined in section

8.2.4.1, except the reaction was stopped after exactly 5min. The absorbance at 450nm (Y-axis) was plotted against the serum dilution (X-axis, exponential scale). The absorbance (x) at which the positive control had a titre of 100,000 was recorded from the plot and the dilution of each test sample that reduced the absorbance to x was estimated. A number of samples included in this study were assayed for anti-lipid S IgG by S.E. Tebbs and T. Worthington at QEH using the same method (Elliott *et al.*, 2000).

8.3 RESULTS

8.3.1 Screening multiple sera for anti-SsaA antibody

Western blots of *E. coli* XLOLR/pSsaA and *E. coli* XLOLR were probed with sera (diluted 1:400) recovered from patients with staphylococcal IE, CVC-sepsis, CAPD-related peritonitis, prosthetic hip-associated osteomyelitis and control patients with bone fractures but no evidence of infection. The colour intensity of the SsaA band on the *E. coli* XLOLR/pSsaA strip was scored (-, +, ++, or +++) by comparison to the PRT blot (figure 8.1). The PRT strip also served as a positive control and the corresponding *E. coli* XLOLR strip as a negative control.

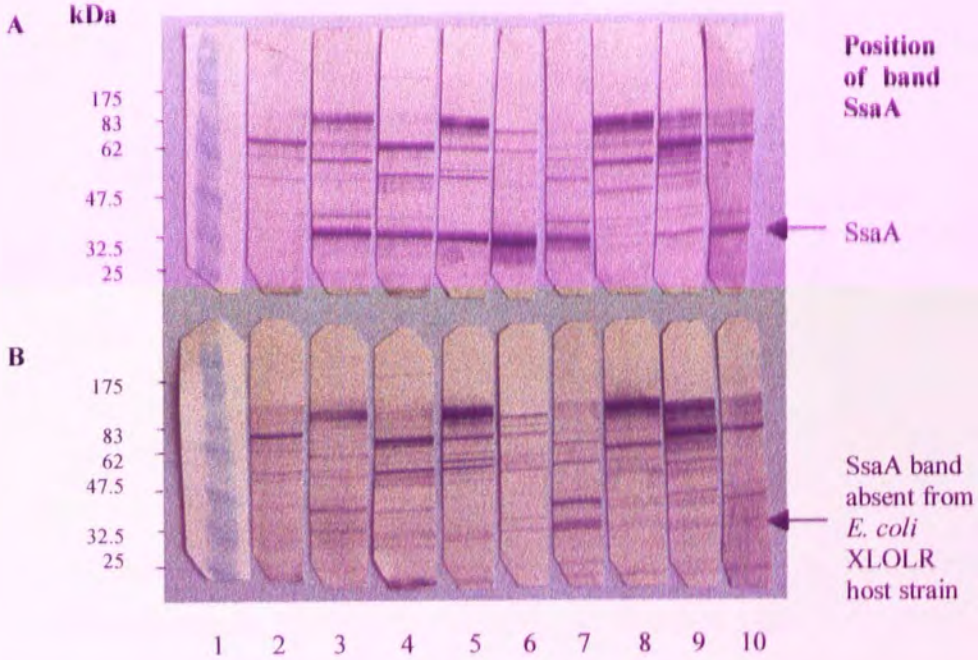


Figure 8.1. Screening multiple sera from IE patients for anti-SsaA IgG antibody. A, western blot of *E. coli* XLOLR/pSsaA; B, western blot of *E. coli* XLOLR. Lane 1, sizing ladder; 2, 9, 10, probed with serum recovered from a patient with a CNS infection; 3, 4, 6, probed with serum recovered from a patient with a *S. epidermidis* infection; 5, probed with serum recovered from a patient with a *S. hominis* infection; 7, probed with serum recovered from a patient with a *S. warneri* infection; 8, probed with serum recovered from a patient with a *S. aureus* infection (rejected by Duke criteria and investigated no further, section 1.2.5). All sera were diluted 1:400 with TBS-BSA. Lane 6 contained the positive control, PRT.

All IE samples in figure 8.1 were strongly positive for SsaA except for the samples in lanes 2 (CNS) and 8 (*S. aureus*) (figure 8.1, table 8.1). However, all the control sera recovered from patients with no evidence of infection also generated strong intensity bands at the position of SsaA (figure 8.2).

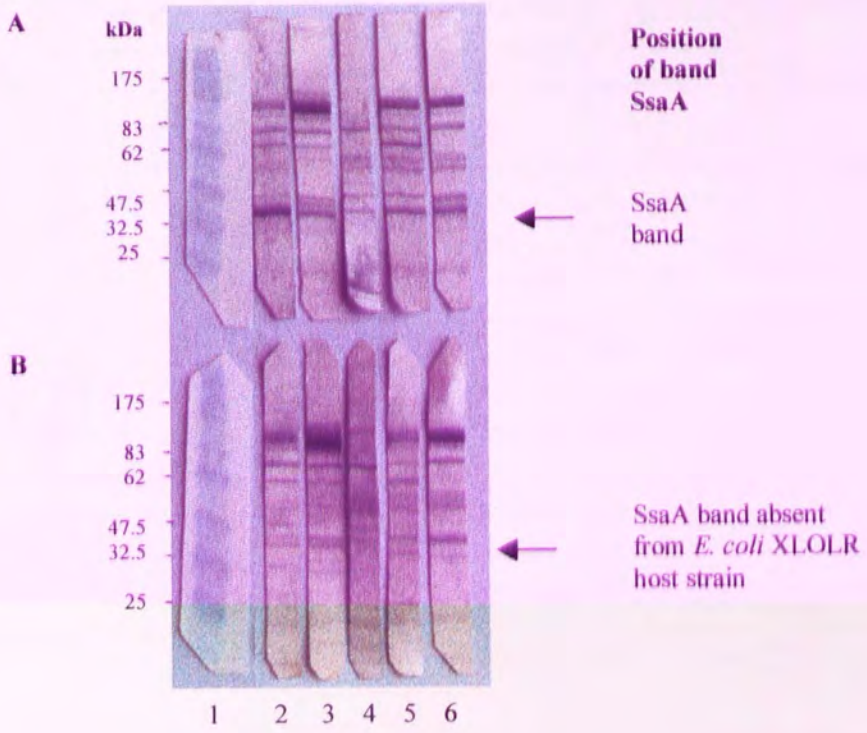


Figure 8.2. Screening multiple control sera for anti-SsaA antibody. A, western blot of *E. coli* XLOLR/pSsaA; B, western blot of *E. coli* XLOLR. Lane 1, sizing ladder; 2-6, probed with serum (diluted 1:400 with TSB-BSA) recovered from patients with bone fractures but no evidence of infection.

Additional IE and control patient sera and sera from CVC, CAPD catheter, and prosthetic hip joint-associated infections (41 serum samples in total) were screened for anti-SsaA antibody (table 8.1).

Table 8.1. Thirty-five serum samples from patients with various staphylococcal infections including IE, CVC-associated sepsis, CAPD-related peritonitis and prosthetic hip joint-associated osteomyelitis and six negative control sera from patients with bone fractures but no evidence of infection were screened for anti-SsaA antibody by western blot

Infection Type	**Organism**	**Anti-SsaA antibody**
IE	*S. aureus*	+++
IE	*S. aureus*	+++
IE	*S. aureus*	+++
IE	*S. aureus*	+++
IE *(lane 6 fig 8.1)	*S. epidermidis*	+++
IE (lane 3 fig 8.1)	*S. epidermidis*	+++
IE (lane 4 fig 8.1)	*S. epidermidis*	+++
IE (lane 5 fig 8.1)	*S. hominis*	+++
IE	*S. simulans*	+
IE (lane 7 fig 8.1)	*S. warneri*	+++
IE (lane 2 fig 8.1)	CNS	+
IE	CNS	++
IE	CNS	+
IE (lane 10 fig 8.1)	CNS	+++
IE (lane 9 fig 8.1)	Possible CNS	++
IE	Polymicrobial with CNS	+++
IE	Polymicrobial with *S. epidermidis* and *S. haemolyticus*	+++
CVC	*S. epidermidis*	+++
CVC	*S. epidermidis*	+
CVC	*S. epidermidis*	-
CVC	*S. epidermidis*	-
CVC	*S. epidermidis*	-
CVC	*S. epidermidis* and *S. lugdunensis*	+++
CAPD	*S. epidermidis*	+++
CAPD	*S. epidermidis*	+++
CAPD	*S. epidermidis*	++
CAPD	*S. epidermidis*	+
CAPD	*S. haemolyticus*	+
CAPD	CNS	+
Hip	*S. aureus*	+++
Hip	*S. aureus*	+
Hip	CNS	+++
Hip	CNS	+++
Hip	CNS	+++
Hip	CNS	+
Control (lane 2 fig 8.2)	-	+++
Control (lane 3 fig 8.2)	-	+++

Control (lane 5 fig 8.2)	-	+++
Control (lane 6 fig 8.2)	-	+++
Control	-	+++
Control (lane 4 fig 8.2)	-	+

IE*, positive control serum. For clarity those strains shown on the western blots (figure 8.1 and 8.2) are indicated.

8.3.2 Purification of SsaA

8.3.2.1 Gel permeation chromatography by fast protein liquid chromatography

Dot blot analysis of fractions 1-50, collected by FPLC of ethanol precipitated culture supernatant, probed with PRT (diluted 1:400) and protein A - alkaline phosphatase identified antibody binding in fractions 8-24 (figure 8.3). Dot blot analysis of the freeze-dried culture supernatant was identical to the ethanol precipitated culture supernatant dot blot. No bound antibody was detected on either of the two control blots.

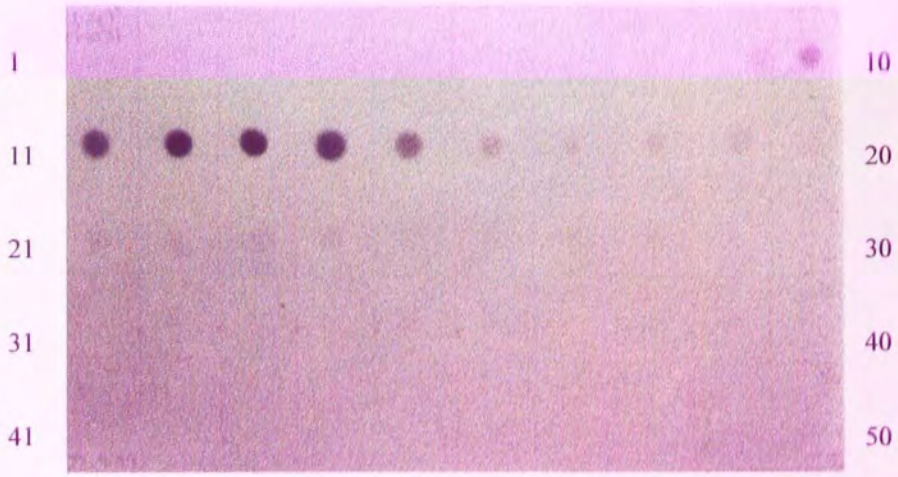


Figure 8.3. Dot blot analysis of fractions 1-50, collected by FPLC of *S. epidermidis* NCTC 11047 ethanol precipitated culture supernatant. Antibody (serum PRT diluted 1:400) reacted with fractions 8-24, although some of the spots are only faintly visible.

Immunodetection of a second dot blot of ethanol precipitated culture supernatant probed with affinity purified anti-SsaA antibody confirmed the presence of SsaA in fractions 11-14 (data not shown).

The proteins of fractions 8-24 were resolved by SDS-PAGE and transferred to nitrocellulose membrane by western blotting. SsaA was identified in fractions 11-14 (figure 8.4) as predicted by the dot blot probed with affinity purified anti-SsaA. Calibration of the column, by the manufacturer, predicted that a 160kDa polypeptide (IgG) would elute in fraction 15 and a 35kDa protein (β-lactoglobulin) would elute in fraction 18 (Pharmacia calibration of Superose 12 column). The collection of SsaA in fractions immediately after the void volume (fractions 11-14) indicated a molecular weight of >160kDa. The perceived co-purification of lipid S with SsaA in the fractions might explain this observation (figure 8.4). The western blot of the freeze-dried culture supernatant fractions was identical to the ethanol precipitated culture supernatant blot.

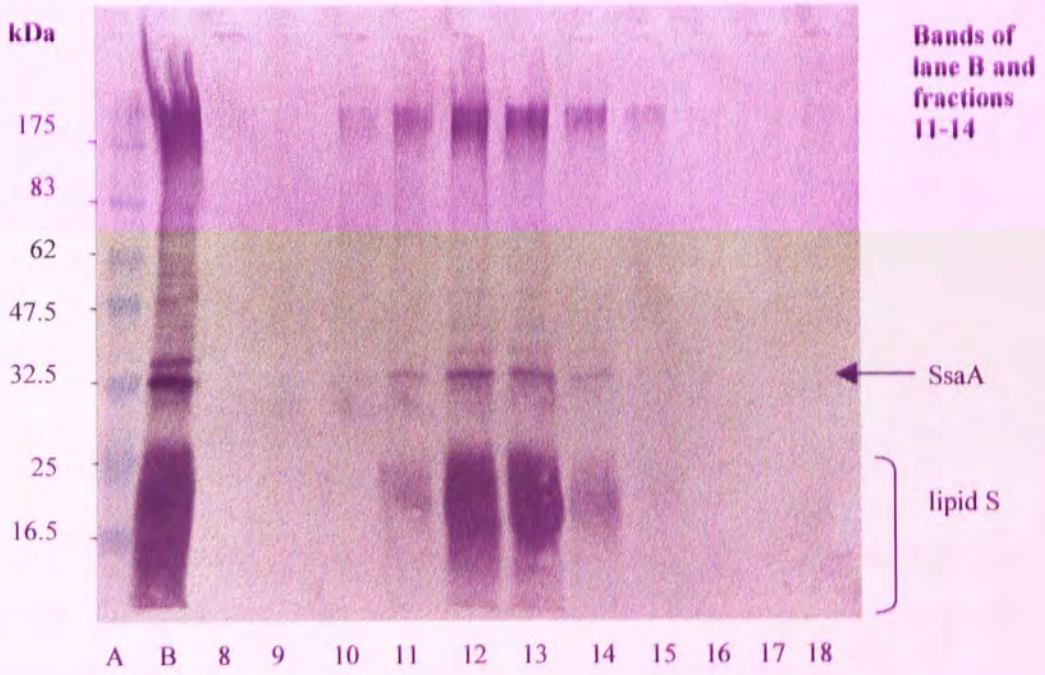


Figure 8.4. Western blot of fractions 8-18, collected by FPLC of *S. epidermidis* NCTC 11047 ethanol precipitated culture supernatant, probed with serum PRT (diluted 1:400). Lane A, sizing ladder; B, *S. epidermidis* NCTC 11047 cell-associated protein. Fractions 11-14 revealed the co-purification of SsaA with lipid S.

8.3.2.2 Electro-elution

Since gel permeation chromatography proved to be an unsatisfactory method of SsaA purification the protein was electro-eluted from a polyacrylamide gel. The success of electro-elution and fraction purity was evaluated by western blot analysis of the eluted samples immunodetected with serum PRT diluted 1:400 (figure 8.5). The *E. coli* XLOLR/pSsaA fraction contained a single antigenic band of the appropriate size. The *E. coli* XLOLR fraction contained no antigenic material recognised by serum PRT.

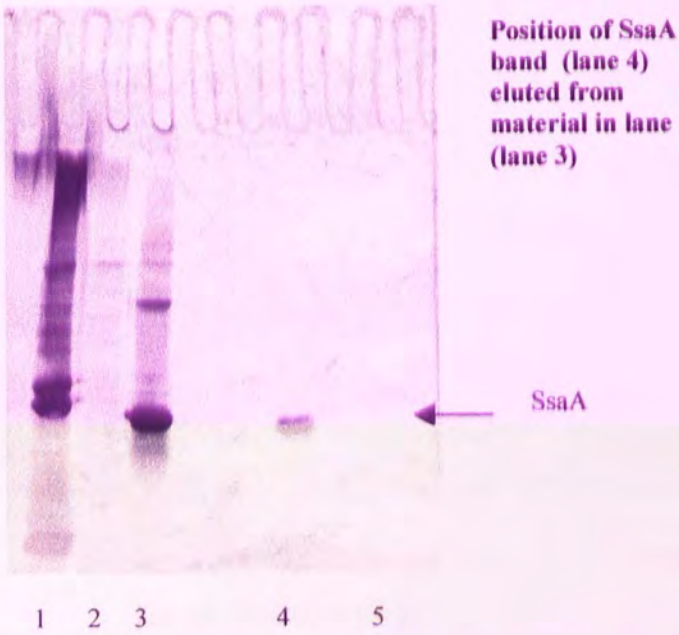


Figure 8.5. Purity of the electro-eluted fractions evaluated by western blot and probed with serum PRT (diluted 1:400). Lane 1, *S. epidermidis* NCTC 11047 cell-associated protein; 2, *E. coli* XLOLR cell-associated protein; 3, *E. coli* XLOLR/pSsaA cell-associated protein; 4, fraction eluted from *E. coli* XLOLR/pSsaA; 5, fraction eluted from *E. coli* XLOLR.

8.3.3 Assay of anti-SsaA serum IgG by ELISA

8.3.3.1 Optimisation of protein coating microtitre plate wells

To estimate the optimum concentration of purified SsaA for use in an ELISA assay the eluted protein was coated onto the wells of a microtitre plate by serial two-fold dilution (neat to 1:2,048) and detected with 1:400 diluted positive (PRT) and negative (LLD) serum. A hundred-fold dilution of the eluted protein was selected as the concentration which gave both good discrimination between sera positive and negative for anti-SsaA IgG and the highest possible dilution without the loss of sensitivity, so as to increase the number of samples which could be screened (figure 8.6).

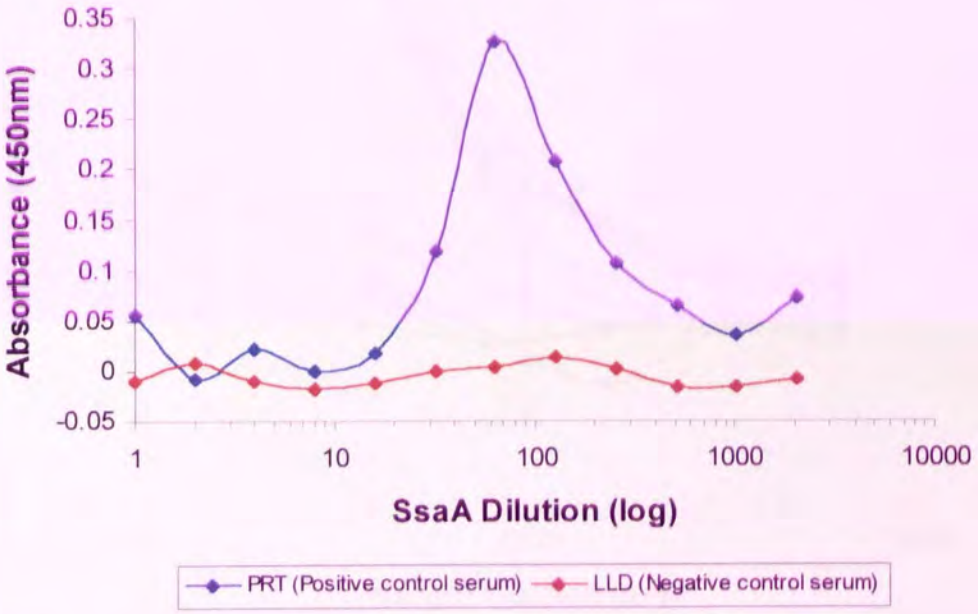


Figure 8.6. Optimisation of protein coating ELISA plates. Serial two-fold dilutions of purified SsaA and the control fraction were reacted with anti-SsaA antibody positive and negative sera. The absorbance 450nm (control elute absorbance subtracted from the SsaA absorbance) was plotted against protein dilution (exponential scale).

Although the efficiency of electro-elution is estimated to be 80-100% (BioRad) a six-fold dilution of the eluted protein occurred during the purification stage. Therefore, the concentration of SsaA used to coat the microtitre plate for ELISA assay was equivalent to the SsaA concentration of 250µl of IPTG induced-*E. coli* XLOLR/pSsaA resuspended in PBS to OD_{600} 1.0 diluted six hundred-fold.

8.3.3.2 SsaA ELISA

A total of seventy-one serum samples recovered from various infection types and aetiologies were screened for anti-SsaA antibody using the optimised ELISA. The serum IgG titre of antibody reacting with SsaA varied from <400 (considered negative) to >6,400, relative to the positive control titre of 1,000 (>400 was considered positive) (figure 8.7, table 8.2).

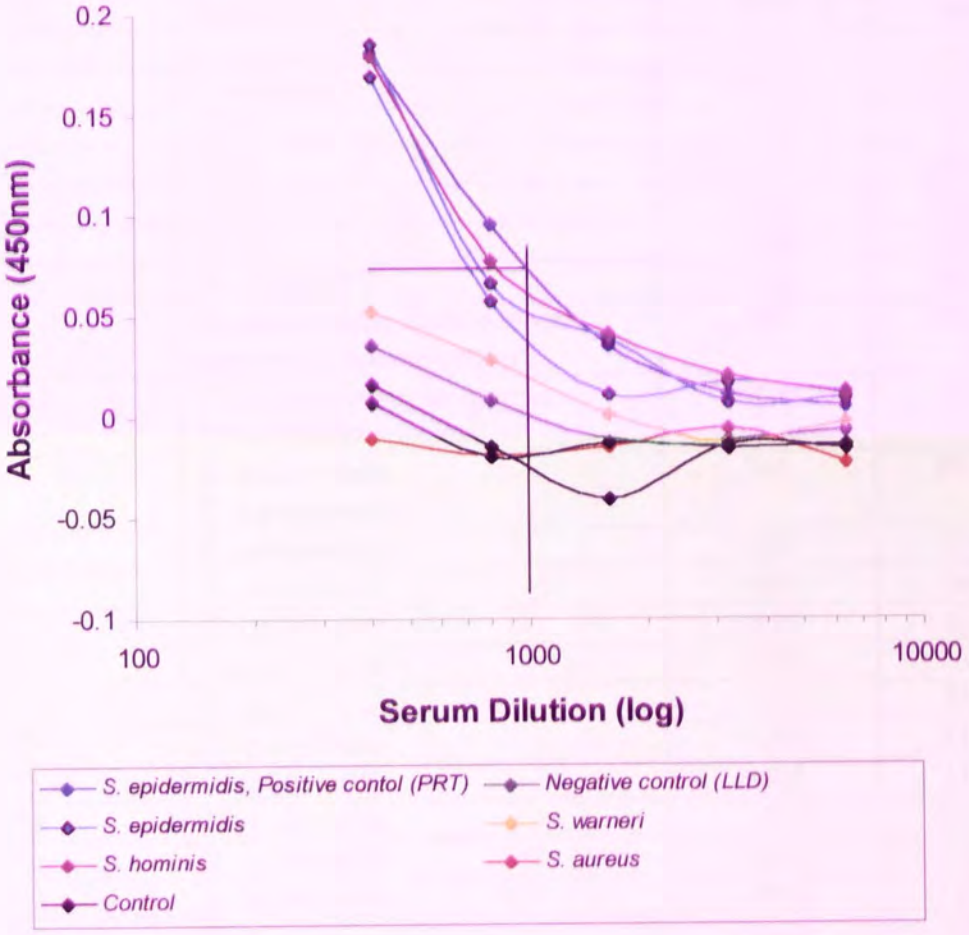


Figure 8.7. An ELISA to detect anti-SsaA antibody. The absorbance 450nm (control elute absorbance subtracted from the SsaA absorbance) plotted against the serum dilution (exponential scale). The dilution of each sample that reduced the absorbance to *x* (the absorbance at which the positive control, PRT, had a titre of 1,000) was estimated.

Table 8.2. The anti-SsaA IgG antibody titre and anti-lipid S IgG titre of seventy-one sera screened by ELISA.

Infection Type	Organism	SsaA titre-IgG	Lipid S titre-IgG
IE	*S. aureus*	<400	38,000
IE	*S. aureus*	400	100,000
IE	*S. aureus*	<400	130,000
IE	*S. aureus*	<400	38,000
IE	***S. epidermidis***	**700**	**205,000**
IE	***S. epidermidis***	**800**	**149,000**
IE*	***S. epidermidis***	**1,000**	**400,000**
IE	***S. hominis***	**700**	**70,000**
IE	*S. simulans*	<400	30,000
IE	*S. warneri*	<400	50,000
IE	CNS	<400	38,000
IE	CNS	<400	40,000
IE	CNS	<400	270,000
IE	CNS	<400	50,000
IE	Possible CNS	<400	40,000
IE	***S. epidermidis, Enterococcus* species, *S. haemolyticus***	**500**	**51,000**
IE	CNS, *Enterococcus* species *Enterobacter* species	<400	13,000
CVC	***S. epidermidis, S. lugdunensis***	**700**	**29,000**
CVC	***S. epidermidis***	**700**	**13,000**
CVC	*S. epidermidis*	<400	16,000
CVC	*S. epidermidis*	<400	6,000
CVC	*S. epidermidis*	<400	26,000
CVC	*S. epidermidis*	<400	38,000
CAPD	*S. epidermidis*	<400	31,000
CAPD	*S. epidermidis*	<400	41,000
CAPD	*S. epidermidis*	<400	102,000
CAPD	*S. epidermidis*	<400	26,000
CAPD	*S. haemolyticus*	<400	119,000
CAPD	CNS	<400	58,000
Hip	*S. aureus*	<400	77,000
Hip	*S. aureus*	<400	84,000
Hip	CNS	<400	31,000
Hip	CNS	<400	91,000
Hip	CNS	<400	17,000
Hip	CNS	<400	35,000
Osteomyelitis	*S. aureus*	<400	ND
Osteomyelitis	*Staphylococcus* species	<400	ND
IE	*Enterococcus faecalis*	<400	20,000
IE	*Enterococcus faecalis*	<400	25,000
IE	*Enterococcus faecalis*	<400	25,000

IE	*Enterococcus faecalis*	<800	11,000
IE	*Enterococcus faecalis*	<400	22,000
IE	*Enterococcus faecalis*	<400	31,000
IE	*Enterococcus faecalis*	<400	4,000
IE	***Streptococcus mitior***	**900**	**90,000**
IE	Nutrient variant streptococcus	<400	70,000
IE	**Group G streptococcus**	**>6,400**	**>400,000**
N/K	*Streptococcus sanguis*	<400	2,000
Osteomyelitis	***Enterococcus faecalis***	**2,000**	**200,000**
CAPD	***Enterococcus faecalis***	**3,000**	**ND**
Nephrostomy fluid	***Enterococcus faecalis***	**3,000**	**ND**
GNS1	**Gram-negative organism**	**600**	**12,000**
GNS2	Gram-negative organism	<400	11,000
GNS3	*Proteus mirabilis*	<400	11,400
GNS4	***Klebsiella pneumoniae* sepsis/ *S. aureus* and *Enterococcus* species wound infection**	**1,000**	**17,000**
GNS5	Gram-negative organism	<400	10,000
GNS6	*Enterobacter* species	<400	200,000
GNS7	*Acinetobacter* species and *Candida* species	<400	10,000
GNS8	***Pseudomonas aeruginosa***	**3,000**	**30,000**
GNS9	*Enterobacter cloacae, Citrobacter* species, *Candida* species, *S. aureus* carrier	<400	11,000
GNS10	*Pseudomonas aeruginosa*	<400	33,000
Control	-	<400	6,000
Control	-	<400	10,000
Control	-	<400	11,000
Control	-	<400	8,000
Control	-	<400	10,000
Control	-	<400	11,000
Control	-	<400	9,000
Control	-	<400	5,000
Control	-	<400	10,500
Control	-	<400	2,500
Lipid S positive control		1,000	100,000
SsaA negative control (LLD)		<400	19,000
Lipid S negative control		<400	6,000

Sera positive for anti-SsaA IgG antibody are indicated by bold text. IE*, SsaA positive control PRT; ND, not determined.

The lipid S titre, as expected distinguished between patients with Gram-positive infections (titre >20,000) and those without (titre <20,000). SsaA IgG antibody was not detected in any of the control sera but was identified in samples collected from IE and other infections (table 8.3). This indicated that SsaA recognition by the immune system was not unique to IE.

Table 8.3. Association of infection type with anti-SsaA IgG antibody

Infection type	**Number of sera positive for SsaA**	**Percentage positive**
IE n=27	7	26
Other n=34	8	24
Control sera n=10	0	0

Neither was the detection of antibody found to be specific to an infection caused by any specific group of organisms (table 8.4).

Table 8.4. Association of infection type and related microorganism with anti-SsaA IgG antibody

Infection type by associated organism	**Number of sera positive for SsaA**	**Percentage positive**
Staphylococcal IE n = 17	5	29
Staphylococcal other n = 20	2	10
Streptococcal/enterococcal IE n = 10	2	20
Streptococcal/enterococcal other n = 4	3	75
Gram-negative sepsis n = 10	3	30
Control n = 10	0	0

Since infection type did not appear to be related to the production of anti-SsaA antibody the association by organism alone was investigated (table 8.5). Although there did not appear to be a correlation with a single streptococcal/enterococcal species, *S. epidermidis* and *S. hominis* were the only staphylococcal species associated with antibody production to the SsaA protein

Table 8.5. Association of specific bacterial species with anti-SsaA IgG antibody

Species	Number of sera positive for SsaA	Percentage positive
Staphylococci n = 37	7	**19**
S. aureus n = 7	0	0
S. epidermidis n = 12	4	33
S. haemolyticus n = 1	0	0
S. hominis n = 1	1	100
S. simulans n = 1	0	0
S. warneri n = 1	0	0
Staphylococcus species n = 1	0	0
CNS n = 10	0	0
Polymicrobial (with a *Staphylococcus* species) n = 3	2	66
Streptococci/enterococci n = 14	**5**	**36**
E. faecalis n = 10	3	30
S. mitior n = 1	1	100
S. sanguis n = 1	0	0
Group G streptococcus n = 1	1	100
Nutrient variant streptococcus n = 1	0	0

The total number of staphylococci, streptococci and enterococci is indicated in bold text. The breakdown into *Staphylococcus*, *Streptococcus* and *Enterococcus* species is indicated in plain text.

S. epidermidis strains, which were associated with the production of SsaA antibody, were investigated by infection type (table 8.6). These results, although the numbers were small, suggested that *S. epidermidis* IE infections were more likely to induce antibody than *S. epidermidis* infections at other sites, such as CAPD-peritonitis.

Table 8.6. Association of *S. epidermidis* by infection type with anti-SsaA IgG antibody

Infection type - *S. epidermidis* associated	Number of sera positive for SsaA	Percentage positive
IE n = 4	4	100
CVC sepsis n = 5	1	20
CAPD peritonitis n = 4	0	0

8.3.3.3 Sequential Sera from a patient with *S. epidermidis* endocarditis

The anti-SsaA IgG titre remained constant at 1,000 for the period of one month following the diagnosis of IE. Similarly, anti-lipid S antibody remained elevated at 200,000 - 400,000 over the period.

Table 8.7. Anti-SsaA and anti-lipid S IgG titres of sequential sera from a patient with *S. epidermidis* IE

Serum sample	Sample date	SsaA titre-IgG	Lipid S titre-IgG
PRT 1	23.02.98	1,000	400,000
PRT 2	25.02.98	1,000	200,000
PRT 3	26.02.98	1,000	200,000
PRT 4	02.03.98	1,000	300,000
PRT 5	04.03.98	1,000	300,000
PRT 6	09.03.98	1,000	200,000
PRT 7	15.03.98	1,000	200,000
PRT 8	16.03.98	1,000	300,000
PRT 9	19.03.98	1,000	300,000

8.3.4 Anti-lipid S IgG titres

The anti-lipid S IgG titres were evaluated for all the seventy-one sera screened for SsaA antibody (table 8.2, figure 8.8). A titre of <20,000 was considered to be negative and >20,000 was considered positive.

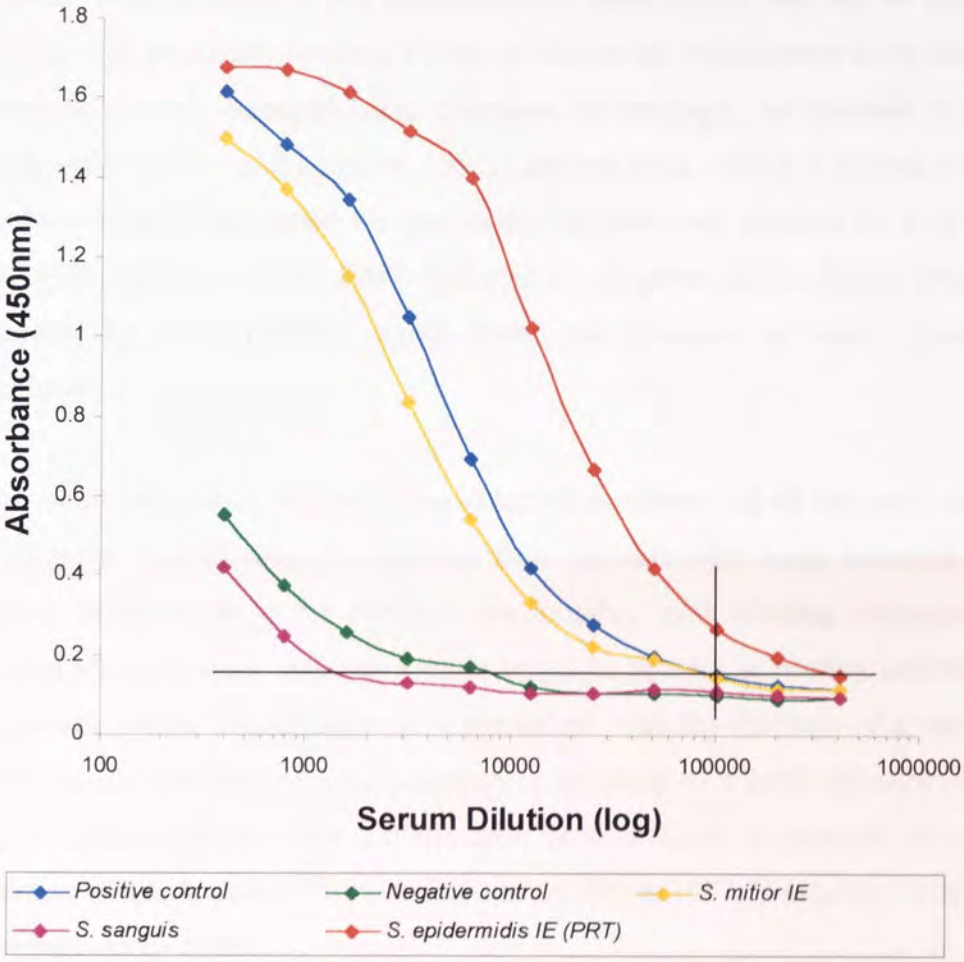


Figure 8.8. An ELISA to detect anti-lipid S antibody. The absorbance at 450nm plotted against the serum dilution (exponential scale). The titre of each sample that reduced the absorbance to *x* (the absorbance at which the positive control had a titre of 100,000) was estimated.

8.4 DISCUSSION

Previous studies, which have investigated the possibility of a serodiagnostic test for staphylococcal infection, have concluded that the high level of pre-existing anti-staphylococcal antibodies in the general population would prevent this development (Plaunt and Patrick, 1991; Wergeland *et al.,* 1989; Bell, Pennington and Petrie, 1987; Wergeland *et al.,* 1984). Indeed, the few assays which have been commercially developed, (ENDO-STAPH and staphylolysin), have limited use due to their poor sensitivity and specificity (section 1.3.8). A number of investigators have attempted to diagnose specific staphylococcal infections by serology, for example *S. aureus* osteomyelitis (Krikler and Lambert, 1992; Lambert *et al.,* 1992). The lipid S ELISA is the latest attempt to develop a sensitive and specific assay (section 1.3.8.1) (Elliott *et al.,* 2000; Lambert *et al.*, 2000; Rafiq *et al.,* in press 2000). Whilst this test is diagnostic for Gram-positive sepsis there still remains no assay specific to staphylococci.

In this study anti-SsaA antibody was detected in almost all of the sera screened, including the control samples collected from patients with bone fractures but no evidence of infection in the previous six months, by a blotting technique. This indicated that anti-SsaA immunoglobulin might be present in healthy individuals at background levels. This hypothesis is consistent with the findings of a number of studies, which have reported the presence of antibody to a range of staphylococcal antigens (peptidoglycan, wall teichoic acid, protein A) in the absence of infection (Plaunt and Patrick, 1991; Bell, Pennington and Petrie, 1987; Wergeland *et al.,* 1989; Wergeland *et al.,* 1984).

To investigate the possibility of background levels of anti-SsaA antibody in the general population and elevated titres during infection an ELISA to quantitate anti-SsaA IgG was devised. Initially, the SsaA required to coat wells of microtitre plates was to be purified by gel permeation chromatography of concentrated culture supernatant recovered from *S. epidermidis* NCTC 11047. However, FPLC of the prepared supernatant, concentrated either by freeze-drying or ethanol precipitation, purified SsaA together with lipid S. SsaA co-purified with the highly antigenic lipid S antigen was unsuitable for use in an ELISA. Interestingly, this implied that the SsaA protein might be associated with lipid S.

The recovery of SsaA from *E. coli* XLOLR/pSsaA culture supernatant by the same method was also shown to be unsatisfactory. Although the protein was detected in a small-scale trial preparation of *E. coli* XLOLR/pSsaA culture supernatant the protein could not be identified in any of the FPLC fractions of ethanol precipitated culture supernatant. It was possible that *E. coli* cellular components interfered with the SsaA protein during either the supernatant preparation or the purification stage.

Elcctro-clution of SsaA from *E. coli* XLOLR/pSsaA cells overcame the problem of lipid S contamination and yielded a protein concentration sufficiently high to coat a number of microtitre plates. Non-specific polypeptide detection methods, such as Coomassie Blue staining, did not have sufficient sensitivity to detect the low concentration of eluted protein. Therefore, the eluate was assessed for purity by western blotting with serum PRT. Although sensitive, this technique would only detect antigens recognised by antibody in the serum PRT.

All the staphylococcal infections associated with positive anti-SsaA IgG levels (anti-SsaA antibody titre >400) were caused by *S. epidermidis*, except for one *S. hominis* sepsis. *S. hominis* is closely related to *S. epidermidis* and it is not surprising that there was cross reactivity between SsaA and *S. hominis* antibody (Kloos, 1996). A number of sera recovered from patients with infectious aetiologies other than staphylococci were shown to have anti-SsaA antibody titres >400: three were *Enterococcus faecalis*, one *Streptococcus mitior*, one group G streptococcus, three Gram-negative organisms (an unspecified Gram-negative bacillus, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*). Of these, the sera recovered from patients with Gram-positive infections (*E. faecalis*, *S. mitior* and a Group G streptococcus) contained high levels of anti-lipid S IgG antibody (titres of 90,000 to 200,000) and the majority also had anti-SsaA IgG titres that were substantially higher than the positive control (titres of 2,000, 3,000, 3,000, 6,400). Since it was demonstrated in the previous section of this study that *Streptococcus* and *Enterococcus* species did not have the gene *ssa*A or a homologue, these results suggest that the high level of anti-lipid S IgG might either cross-react with the SsaA protein or bind non-specifically with SsaA. However, high anti-lipid S titres were not always associated with high anti-SsaA IgG levels. For example, six sera (associated with various infections and

aetiologies) had anti-lipid S IgG levels of >100,000 but were negative for SsaA (a titre of <400).

Three of the ten GNS patients had anti-SsaA antibody titres between 600 and 3,000 and anti-lipid S IgG titres of 12,000 to 30,000. The anti-lipid S levels indicated the absence of a Gram-positive bacterial infection (one of these patients GNS8 was borderline positive/negative). In contrast the anti-SsaA IgG titres implied, based on the results obtained in this study, a *S. epidermidis* or *S. hominis* infection. There are three possible explanations for this observation:

- Contamination of the SsaA eluate with *E. coli* antigens during electro-elution. The clinical records of patient GNS1 (anti-SsaA IgG titre 600) were unavailable and, therefore, the species of the Gram-negative organism is not known. If the patient had an *E. coli* infection specific antibody might react with the contaminating antigen to generate a false positive result. Alternatively, the contaminating protein might cross-react with antibody generated to a range of Gram-negative microorganisms.
- These patients would have very high antibody titres to Gram-negative organisms and, therefore, it is possible that non-specific reactions gave rise to false positives as perceived with the streptococcal/enterococcal infections.
- Although diagnosed with Gram-negative sepsis it is conceivable that these hospitalised patients would have CVC *in situ* and, therefore, possibly an undiagnosed staphylococcal infection - particularly *S. epidermidis* associated. Patient GNS8 did have a catheter *in situ* and GNS4 was diagnosed with multiple focus cancer and consequently would be catheterised. Notably, patient GNS8 had a borderline positive/negative anti-lipid S titre of 30,000.

The low titres of anti-SsaA IgG associated with *S. epidermidis* CVC-related sepsis and CAPD-associated peritonitis compared to IE suggests that the anti-SsaA immune response is associated with chronic and not acute infections. However, none of the patients with chronically infected prosthetic hip joints expressed high anti-SsaA IgG titres. Although none of the joint associated isolates were identified beyond CNS at the time of recovery it is probable that a number were *S. epidermidis*. The evidence suggests that an anti-SsaA IgG titre >400 is indicative of *S. epidermidis* IE infection.

Further testing of an increased number of samples is required to confirm or disprove this hypothesis.

The aim of this part of the study was to evaluate the serodiagnostic potential of the SsaA antigen as a marker of CNS infection. An assay for staphylococci or specifically *S. epidermidis* would be of benefit to the clinical diagnostic services. For example, serological tests are of particular importance in investigating patients that have been given antibiotics prior to samples being taken. Also as CNS are frequent specimen contaminants a test would allow the confirmation of diagnosis where only a single *S. epidermidis* isolate had been recovered. Testing of sequential samples has shown that anti-SsaA IgG titres remain elevated for at least 4 weeks following onset of IE. Further investigation to monitor IgG levels during the course of infection is required to establish when titres fall or whether they remain high for a prolonged period. If titres were to fall following resolution of infection, monitoring a patient's anti-SsaA antibody level might allow assessment of treatment success. Improvement of the SsaA ELISA requires further investigation of false positives (anti-SsaA IgG levels of >400 associated with streptococcal and enterococcal infections) and refinement of the assay to remove non-specific reactivity and improve the level of discrimination between positive and negative samples. This study suggests that the test might be specific to *S. epidermidis* IE.

Chapter 9- Final Discussion

Although IE was first described in the 16th century (Weinstein and Brusch, 1996), recognition of the role of CNS as major aetiological agents of heart valve infections is a relatively recent phenomenon. These bacteria were and continue to be extremely rare causes of NVE, accounting for between 1% and 4% of episodes, but are more frequently associated with PVE (33% of early PVE cases). This observed increase in the frequency of this microorganism and infection can be explained by the formation of an "at risk" population that did not previously exist; patients with prosthetic devices including intravascular catheterization, artificial heart valves and vascular grafts. Why this group of normally avirulent commensal microorganisms causes infection in association with prosthetic devices and cardiac implants in particular is currently unclear. Many of the virulence factors that are elaborated by *S. aureus* are absent in CNS, however, these bacteria do produce other potential virulence factors. The most extensively investigated of these possible pathogenic determinants is the polysaccharide slime layer. It is believed that this slime substance is responsible for the attachment of the bacteria to biomaterials, the formation of a physical barrier between the bacteria and antimicrobial agents, and the inhibition of certain components of the host's immune response. Whilst there is a growing body of evidence supporting the role of the polysaccharide layer during infection, the production of this substance alone is not sufficient to cause the aggressive pathogenic phenotype that has been described for cases of CNS-related IE.

The aims of this study were to:

- Ascertain whether there are specific CNS genotypes with a predilection to cause IE.
- To identify any virulence characteristics that are primarily expressed by IE-associated strains.
- To assess any identified virulence factors as markers of infection.

PFGE of macrorestricted chromosomal DNA is ideally suited to the genotyping of staphylococci. Unlike phenotypic methods, analysis of the genome is not affected by

environmental conditions; the profile does not vary with different culture conditions. A further advantage of the method is that it samples the entire genome and not a subsection, for example plasmid typing assesses only plasmid DNA. Despite the stability and versatility of PFGE, the technique has been applied primarily to epidemiological investigations of epidemic strains of CNS during outbreak situations. In this study the technique was applied for the identification of a genotype or genotypes common to CNS strains associated with IE. The failure to identify recurring PFGE profiles within the group of IE-associated isolates, and indeed within strains related to other infections, indicates that various CNS genotypes are capable of causing IE and that no specific genotype is primarily associated with the infection. This suggests that different strains are equally capable of causing different forms of sepsis and that the site of bacterial entry may influence the type of resulting infection caused by the opportunistic pathogen. For example, impaction of bacteria onto a catheter tip during skin insertion or contamination of a hip-joint at the time of operation predispose the patient to CVC-associated sepsis or prosthetic hip-associated osteomyelitis. Given the ubiquitous nature of CNS and frequency at which transient bacteraemia occurs within a given individual (simple tooth brushing is sufficient to cause bacteraemia (Livornese and Korzeniowski, 1992)), it might be expected that more patients with a predisposing risk factor should develop associated infection. This introduces the interesting possibility that certain patients have further immunological deficiencies, which predispose them to infection by this group of microorganisms. For example, it is already recognised that the elderly and low birth-weight neonates have a predisposition to catheter-associated sepsis caused by CNS (Michalopoulos and Geroulanos, 1996).

A number of groups have investigated the potential virulence factors of CNS but few have attempted to correlate the resulting phenotype to particular types of infection. In this study the expression of putative virulence determinates was compared with both the associated disease and the genotype. Unfortunately no characteristics common to IE specifically were identified, indeed no virulence factors were found to be specific to any of the infection types investigated. The failure to detect a specific IE-related phenotype introduces a number of possibilities. Firstly, the CNS-associated with IE may express a virulence factor essential to infection of heart valves which has not been previously identified. Secondly, it is possible that all the strains assessed were

equally capable of causing any of the infections studied. Since a number of potential pathogenic determinants were common to the majority of the strains investigated these factors may be essential to pathogenicity in generally whilst the site of infection is irrelevant. In the absence of a control group of "non-pathogenic" CNS (which may not actually exist) it is impossible by this method to identify virulence factors common to pathogenic strains. Finally, it is recognised, although not fully understood, that expression of exoproteins and cell surface receptors (MSCRAMMs), which include potential virulence factors of CNS is strictly controlled by extracellular conditions. These regulating stimuli include iron availability and cell density. Therefore, it is possible that the CNS strains investigated in the course of this study express virulence factors *in vivo* that were not detected *in vitro*.

Since it is highly probably that the CNS strains elaborate virulence factors that were not specifically screened for during this study an alternative method of investigating isolate pathogenicity was utilised. This involved screening a *S. epidermidis* genomic DNA library with patient's serum for staphylococcal products that had stimulated an immune response in the patient during an episode of *S. epidermidis*-associated IE. The advantages of this approach included screening for products that were expressed *in vivo* and the potential for identification of previously unknown factors that induced an immune response. Furthermore, this approach circumvented the problem of product regulation.

Two antigenic staphylococcal products that were expressed by a strain of *S. epidermidis* during IE were identified; a pyruvate dehydrogenase complex E2 subunit and SsaA. The E2 subunit was identified as a metabolic component, but SsaA proved to be a novel *S. epidermidis* protein with homology to two other staphylococcal proteins of unknown function. Characterisation of this previously unrecognised product identified a secreted 32kDa antigenic protein. Although no virulence function was identified, homology to a *S. aureus* protein involved in biosynthesis of the pigment staphyloxanthin suggested a protective role against oxidative damage.

The potential of SsaA as a marker of endocardial infection was investigated. The problems associated with the diagnosis of CNS-related IE are widely acknowledged

and the availability of a simple, rapid and inexpensive assay for CNS would be of particular value. Although areas requiring modification were identified, for example to improve the specificity of the assay, the SsaA ELISA demonstrated the potential to diagnose *S. epidermidis*-related IE. However, a test that specifically detects a single staphylococcal species may be of limited value unless incorporated into a multi-antigen assay (Kjerulf, Espersen and Tvede, 1994).

The natural progression of the work presented in this thesis would include the investigation of the virulence factors assessed in this study and other non-specified determinants in conditions that resemble those encountered *in vivo*. Representational difference analysis of cDNA is a newly described technique that subtracts *in vitro* generated mRNA and rRNA from an amplified library of mRNA transcribed under conditions of interest, for example an iron restricted medium or *in vivo* (Bowler, Hubank and Spratt, 1999). The mRNA that remains after subtraction represents genes switched on in the test conditions and not *in vitro*, i.e. only genes transcribed *in vivo*. Further molecular analysis and database searches would allow the gene to be investigated. This is just one of a growing number of techniques designed to investigate expression of genes *in vivo* and thus associated with pathogenicity (Handfield and Levesque, 1999).

The genotyping section of this study introduced the possibility that certain individuals might be immunologically susceptible to the development of IE. Infection induces the inflammatory response; a cascade of events aimed at restricting tissue damage. This response is mediated by cytokines (for example, TNF-α, IL-1 and IL-6) and acute-phase proteins and results in the increased expression of cell-adhesion molecules (such as ICAM-1 and ELAM-1) (Kuby, 1994). Comparison of factors associated with the inflammatory response in the serum of IE patients to those in a control group might allow the identification immunological deficiencies in the IE patients which predisposed them to CNS infections.

SsaA was identified as a potential virulence factor. Transposon mutagenesis with Tn917 could be used to generate mutants defective in SsaA expression (Foster, 1998). This method has previously been used successfully with *S. epidermidis* to

study the role of the intercellular adhesin in biofilm formation (Heilmann *et al.*, 1997). Considering the homology of SsaA to the *S. aureus* protein, which is potentially associated with resistance to phagocytosis, the phenotype of the *ssaA*⁻ mutants could be examined in terms of sensitivity to phagocytosis and oxidative stress. Alternatively the wild type strain and the mutant could be compared using an animal model to assess differences in virulence.

A greater understanding of the virulence factors that allow commensal CNS strains to present as aggressive pathogens would provide useful information on the mechanisms of infection and the host response. This work could promote the development of a diagnostic test and ultimately contribute to prevention strategies.

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Appendix 1

Table I. Duke criteria for the diagnosis of infective endocarditis

Definite infective endocarditis

Pathologic criteria

Microorganisms: demonstrated by culture or histology in a vegetation, *or* in a vegetation that has embolized, *or* in an intracardiac abscess, *or*

Pathologic lesions: vegetation or intracardiac abscess present, confirmed by histology showing active endocarditis

Clinical criteria, using specific definitions listed in table II

2 major criteria, *or*

1 major and 3 minor criteria, *or*

5 minor criteria

Possible infective endocarditis

Findings consistent with infective endocarditis that fall short of the "definite", but not "rejected"

Rejected

Firm alternate diagnosis for manifestations of endocarditis, *or*

Resolution of manifestations of endocarditis, with antibiotic therapy for 4 days or less, *or*

No pathologic evidence of infective endocarditis at surgery or autopsy, after antibiotic therapy for 4 days or less

From Durack *et al.*, 1994

Appendix 1 continued

Table II. Definitions of terminology used in the Duke criteria

Major criteria

Positive blood culture for infective endocarditis

Typical microorganism for infective endocarditis from two separate blood cultures:

(i) Viridans streptococci,* *Streptococcus bovis*, HACEK group, *or*

(ii) Community-acquired *Staphylococcus aureus* or enterococci, in the absence of a primary focus, *or*

Persistently positive blood cultures, defined as recovery of a microorganism consistent with infective endocarditis from:

(i) Blood cultures drawn more than 12 hours apart, *or*

(ii) All of three or a majority of four or more separate blood cultures, with first and last drawn at least 1 hour apart

Evidence of endocardial involvement

Positive echocardiogram for infective endocarditis:

(i) Oscillating intracardiac mass, on valve or supporting structures, or in the path of regurgitant jets, or on implanted material, in the absence of an alternative anatomic explanation, *or*

(ii) Abscess, *or*

(iii) New partial dehiscence of prosthetic valve, *or*

New valvular regurgitation (increase or change in pre-existing murmur not sufficient)

Minor criteria

Predisposition: predisposing heart condition *or* intravenous drug use

Fever: $\geq$ 38.0°C (100.4°F)

Vascular phenomena: major arterial emboli, septic pulmonary infarcts, mycotic aneurysm, intracranial haemorrhage, conjunctival haemorrhages, Janeway lesions

Appendix 1 continued

Immunologic phenomena: glomerulonephritis, Osler's nodes, Roth spots, rheumatoid factor

Microbiologic evidence: positive blood culture but not meeting major criterion as noted previously† *or* serologic evidence of active infection with organism consistent with active endocarditis

Echocardiogram: consistent with infective endocarditis but not meeting major criterion as noted previously

HACEK = *Haemophilus* species, *Actinobacillus actinomycetemcomitans*, *Cardiobacterium hominis*, *Eikenella* species, and *Kingella kingae*.

*including nutritional variant strains.

†excluding single positive cultures for coagulase-negative staphylococci and organisms that do not cause endocarditis.

From Durack *et al.*, 1994

Appendix 2

Clinical details of the endocarditis patients recruited into the study

Patient	Patient details- M/F, age	Organism	Dukes	Predisposing factor identified	Clinical presentation	Surgery	Type of surgery	Survival
EN 1	M, 24	CNS	Possible	Yes- pacemaker	2cm mass in right atrium on pacing lead. Native tricuspid valve vegetation.	NK	-	NK
EN 4	F, 70	*S. simulans*	Definite	NK	Hypertrophic cardiomyopathy with aortic regurgitation, perforated left coronary cusp. Vegetation on native mitral valve.	No	-	Died
EN 5	M, 72	CNS	Definite	No	Aortic stenosis with moderate aortic regurgitation. Large vegetation on aortic valve.	No	-	Survived
EN 7	F, 49	*S. simulans*	Possible	Yes- congenital heart defect	Large vegetation on native posterior mitral valve leaflet.	NK	-	NK
EN 8	M, 78	CNS	Definite	Yes- prosthetic valve	Dehiscence of prosthetic aortic valve with septal abscess	Yes	Homograft aortic valve and debridement	Survived
EN 9/ 10	F, 61	*S. haemolyticus* (EN9), *S. epidermidis* (EN10), *Enterococcus* spp. *Serratia* spp.	Definite	Yes	Vegetation on right coronary cusp, aortic regurgitation.	No	-	Survived

EN 13	M, 64	*S. epidermidis*	Definite	NK	Posterior mitral valve leaflet prolapse with chordal rupture. Severe mitral valve regurgitation	No	-	Survived
EN 19	M, 74	*S. epidermidis*	Definite	Yes-pacemaker	Mass on atrial lead. Moderate aortic regurgitation	Yes	Explant of dual chamber pacing system	Survived
EN 23	F, 61	*S. epidermidis*	Definite	Yes-prosthetic valve	Mass on prosthetic mitral valve (late PVE) with partial dehiscence	Yes	Prosthetic mitral valve replaced	Survived
EN 27	M, 66	*S. epidermidis*	Definite	NK	Vegetation on mitral valve, fronds in left atrium.	Yes	Mitral valve replacement and aortic valve debridement	Survived
EN 32	F, 82	*S. epidermidis*	Definite	No	Aortic and mitral valve regurgitation. Vegetation on aortic valve.	No	-	Survived
EN 33	M, NK	*S. epidermidis*	Definite	No	Aortic valve vegetation with severe aortic regurgitation.	Yes	Homograft aortic valve replacement	Survived
EN 34	F, 71	*S. hominis*	Definite	NK	Vegetation on posterior mitral valve leaflet.	Yes	Posterior mitral valve repair	Survived

NK, not known; M, male; F, female.

Appendix 3

Potential virulence determinant results of the CNS strains investigated

Specimen	Species	Non-specific proteinase	Proteinase -elastase (elastin agar)	Proteinase -elastase congo red-elastin	Non-specific lipase/ esterase	Lipase	Esterase	Adherence to polystyrene	Slime-congo red agar	Slime-concana -valin A	DNase	Haemolysis (Horse red blood cells)	Haemolysis (Sheep red blood cells)	Urease
CVC1	*S. epidermidis*	+	+ 5	0.417 +	-	+	3 +	0.133 1	-	0	0	0	0	2
CVC7	*S. epidermidis*	+	+/-	0.120 +	+	+	5 +	0.223 2	+	2	0	0	0	1
CVC9	*S. epidermidis*	+	+/-	0.068 -	+	+	2 +	0.253 3	+	0	0	0	0	1
CVC10	*S. lugdunensis*	-	-	0.063 -	+	+	+/-	0.153 2	-	0	0	0	0	2
CVC11	*S. epidermidis*	+	+ 11	0.186 +	+	+	5 +	0.115 1	-	2	0	0	0	2
CVC12	*S. epidermidis*	-	-	0.060 -	+	+	-	0.177 2	-	0	0	0	0	1
CVC16	*S. epidermidis*	+	-	0.083 +/-	+	+	3 +	0.175 2	+	0	0	0	0	3
CVC17	*S. epidermidis*	+	-	0.066 -	+	+	3 +	0.172 2	+	0	0	0	0	1
CVC19	*S. epidermidis*	+	+ 8	0.127 +	+	+	3 +	0.109 0	+	2	0	0	0	2
CVC20	*S. epidermidis*	+	-	0.071 -	+	+	+/-	0.107 0	-	1	0	0	0	1
PAL1	*S. epidermidis*	+	+/-	0.780 +	+	+	7 +	0.267 3	+	0	0	0	0	2
PAL2	*S. epidermidis*	+	-	0.071 -	+	+	7 +	0.105 0	+	2	0	0	0	1
PAL3	*S. epidermidis*	-	-	0.063 -	-	-	-	0.152 2	-	0	0	0	0	2
PAL4	*S. epidermidis*	+	+ 21	0.601 +	+	+	5 +	0.268 3	+	0	0	0	0	2
PAL5	*S. epidermidis*	+	-	0.066 -	+	+	5 +	0.099 0	-	2	0	0	0	2
023	*S. epidermidis*	+	+ 11	0.159 +	+	+	3 +	0.102 0	-	2	0	0	0	0
9865	*S. epidermidis*	+	+ 21	0.078 -	+	+	3 +	0.115 1	+	0	0	1	0	1
CAPD1	*S. epidermidis*	+	-	0.087 +	+	+	2 +	0.120 1	-	1	0	0	0	1
CAPD2	*S. epidermidis*	-	-	0.072 -	+	+	11 +	0.120 1	-	2	0	0	0	0
CAPD3	*S. epidermidis*	+	+/-	0.065 -	+	+	2 +	0.102 0	-	1	0	0	0	2
CAPD4	*S. epidermidis*	+	+ 11	0.083 +/-	+	+	2 +	0.100 0	-	1	0	0	0	3
CAPD5	*S. epidermidis*	+	+ 21	0.142 +	+	+	7 +	0.099 0	+	0	0	0	0	2
CAPD6	*S. epidermidis*	+	-	0.062 -	+	+	2 +	0.420 3	+	1	0	0	0	1
CAPD7	*S. epidermidis*	+	+/-	0.075 -	+	+	1 +	0.151 2	-	0	0	0	0	1
CAPD8	*S. epidermidis*	+	+ 3	0.092 +	+	+	2 +	0.125 1	-	2	0	0	0	3
CAPD9	*S. epidermidis*	+	-	0.096 +	+	+	2 +	0.112 1	-	0	0	0	0	1

Appendix 3

Potential virulence determinant results of the CNS strains investigated

Specimen	Species	Non-specific proteinase	Proteinase -elastase (elastin agar)	Proteinase -elastase congo red-elastin	Non-specific lipase/ esterase	Lipase	Esterase	Adherence to polystyrene	Slime-congo red agar	Slime-concana -valin A	DNase	Haemolysis (Horse red blood cells)	Haemolysis (Sheep red blood cells)	Urease
CVC1	*S. epidermidis*	+	+ 5	0.417 +	-	+	3 +	0.133 1	-	0	0	0	0	2
CVC7	*S. epidermidis*	+	+/-	0.120 +	+	+	5 +	0.223 2	+	2	0	0	0	1
CVC9	*S. epidermidis*	+	+/-	0.068 -	+	+	2 +	0.253 3	+	0	0	0	0	1
CVC10	*S. lugdunensis*	-	-	0.063 -	+	+	+/-	0.153 2	-	0	0	0	0	2
CVC11	*S. epidermidis*	+	+ 11	0.186 +	+	+	5 +	0.115 1	-	2	0	0	0	2
CVC12	*S. epidermidis*	-	-	0.060 -	+	+	-	0.177 2	-	0	0	0	0	1
CVC16	*S. epidermidis*	+	-	0.083 +/-	+	+	3 +	0.175 2	+	0	0	0	0	3
CVC17	*S. epidermidis*	+	-	0.066 -	+	+	3 +	0.172 2	+	0	0	0	0	1
CVC19	*S. epidermidis*	+	+ 8	0.127 +	+	+	3 +	0.109 0	+	2	0	0	0	2
CVC20	*S. epidermidis*	+	-	0.071 -	+	+	+/-	0.107 0	-	1	0	0	0	1
PAL1	*S. epidermidis*	+	+/-	0.780 +	+	+	7 +	0.267 3	+	0	0	0	0	2
PAL2	*S. epidermidis*	+	-	0.071 -	+	+	7 +	0.105 0	+	2	0	0	0	1
PAL3	*S. epidermidis*	-	-	0.063 -	-	-	-	0.152 2	-	0	0	0	0	2
PAL4	*S. epidermidis*	+	+ 21	0.601 +	+	+	5 +	0.268 3	+	0	0	0	0	2
PAL5	*S. epidermidis*	+	-	0.066 -	+	+	5 +	0.099 0	-	2	0	0	0	2
023	*S. epidermidis*	+	+ 11	0.159 +	+	+	3 +	0.102 0	-	2	0	0	0	0
9865	*S. epidermidis*	+	+ 21	0.078 -	+	+	3 +	0.115 1	+	0	0	1	0	1
CAPD1	*S. epidermidis*	+	-	0.087 +	+	+	2 +	0.120 1	-	1	0	0	0	1
CAPD2	*S. epidermidis*	-	-	0.072 -	+	+	11 +	0.120 1	-	2	0	0	0	0
CAPD3	*S. epidermidis*	+	+/-	0.065 -	+	+	2 +	0.102 0	-	1	0	0	0	2
CAPD4	*S. epidermidis*	+	+ 11	0.083 +/-	+	+	2 +	0.100 0	-	1	0	0	0	3
CAPD5	*S. epidermidis*	+	+ 21	0.142 +	+	+	7 +	0.099 0	+	0	0	0	0	2
CAPD6	*S. epidermidis*	+	-	0.062 -	+	+	2 +	0.420 3	+	1	0	0	0	1
CAPD7	*S. epidermidis*	+	+/-	0.075 -	+	+	1 +	0.151 2	-	0	0	0	0	1
CAPD8	*S. epidermidis*	+	+ 3	0.092 +	+	+	2 +	0.125 1	-	2	0	0	0	3
CAPD9	*S. epidermidis*	+	-	0.096 +	+	+	2 +	0.112 1	-	0	0	0	0	1

Specimen	Species	Non-specific proteinase	Proteinase -elastase (elastin agar)	Proteinase -elastase congo red-elastin	Non-specific lipase/ esterase	Lipase	Esterase	Adherence to polystyrene	Slime-congo red agar	Slime-concana -valin A	DNase	Haemolysis (Horse red blood cells)	Haemolysis (Sheep red blood cells)	Urease
CAPD10	*S. epidermidis*	+	-	0.100 +	+	+	5 +	0.093 0	-	2	0	0	0	1
CAPD11a	*S. capitis*	+	-	0.058 -	-	+	-	0.121 1	-	0	1	1	0	0
CAPD11b	*S. epidermidis*	+	-	0.301 +	+	+	5 +	0.107 0	-	2	0	0	0	1
CAPD12	*S. lugdunensis*	-	-	0.060 -	+	+	-	0.228 2	-	2	0	0	0	1
CAPD13	*S. epidermidis*	+	+/-	0.071 -	+	+	1 +	0.115 1	+	1	0	1	0	1
CAPD14	*S. epidermidis*	+	+/-	0.088 +	+	+	5 +	0.107 0	-	2	0	0	0	3
CAPD15	*S. epidermidis*	+	+/-	0.158 +	+	+	3 +	0.233 2	-	1	0	1	0	0
CAPD16	CNS	+	+/-	0.069 -	+	+	5 +	1.632 3	+	2	0	0	0	1
CAPD17	*S. sciuri*	-	-	0.064 -	+	+	5 +	0.155 2	-	2	1	4	0	0
CAPD18	*S. epidermidis*	+	-	0.076 -	+	+	3 +	0.149 1	-	0	0	0	0	0
CAPD19	*S. epidermidis*	+	+/-	0.379 +	+	+	5 +	0.127 1	+	0	0	0	0	1
CAPD20	*S. epidermidis*	+	+ 5	0.335 +	+	+	5 +	0.163 2	+	0	0	0	0	1
CAPD21	*S. epidermidis*	+	+/-	0.059 -	+	+	5 +	0.180 2	+	1	0	0	0	3
CAPD23	*S. epidermidis*	-	+/-	0.077 -	+	+	3 +	0.112 1	-	1	0	0	0	3
CAPD24	*S. simulans*	-	+/-	0.057 -	+	-	1 +	0.096 0	-	0	0	0	0	0
CAPD25	*S. epidermidis*	+	+/-	0.090 +	+	+	3 +	0.138 1	+	0	0	0	0	1
NU1	*S. epidermidis*	+	+ 7	0.150 +	+	+	3 +	0.166 2	+	1	0	0	0	2
NU3	*S. epidermidis*	+	+/-	0.161 +	+	+	5 +	0.107 0	-	0	0	1	0	0
NU7	*S. epidermidis*	-	-	0.055 -	+	+	3 +	0.102 0	-	1	0	0	0	0
NU11	*S. epidermidis*	-	-	0.055 -	+	+	+/-	0.094 0	-	2	0	0	0	1
NU24	*S. epidermidis*	+	-	0.060 -	+	+	11 +	0.098 0	-	2	0	0	0	1
NU27	*S. epidermidis*	+	+/-	0.182 +	+	+	11 +	0.172 2	-	1	0	0	0	2
NU33	CNS	+	+ 11	0.126 +	+	+	11 +	0.167 2	+	2	0	0	0	3
NU104	*S. epidermidis*	-	-	0.055 -	+	+	3 +	0.661 3	+	2	0	0	0	2
NU142	CNS	-	-	0.048 -	+	+	1 +	0.176 2	-	1	0	4	2	0
NU159	*S. epidermidis*	-	+/-	0.084 +	+	+	+/-	0.106 0	+/-	0	0	0	0	2
NU160	*S. epidermidis*	+	+ 21	0.451 +	+	+	7 +	0.100 0	+/-	2	0	0	0	2
NU168	*S. epidermidis*	-	-	0.045 -	+	+	7 +	0.150 1	+	1	0	0	0	0
NU257	*S. epidermidis*	+	+ 21	0.198 +	+	+	5 +	0.099 0	-	1	0	0	0	0
NU296	CNS	-	-	0.057 -	+	-	1 +	0.083 0	-	0	0	0	0	1

Specimen	Species	Non-specific proteinase	Proteinase -elastase (elastin agar)	Proteinase -elastase congo red-elastin	Non-specific lipase/ esterase	Lipase	Esterase	Adherence to polystyrene	Slime-congo red agar	Slime-concana -valin A	DNase	Haemolysis (Horse red blood cells)	Haemolysis (Sheep red blood cells)	Urease
NU779	CNS	-	-	0.083 +/-	+	+	11 +	0.109 0	-	0	0	0	0	3
NU5436	*S. epidermidis*	+	+ 7	0.140 +	+	+	5 +	0.085 0	-	2	0	0	0	0
NU5438	*S. epidermidis*	-	-	0.069 -	+	-	7 +	0.109 0	-	0	0	0	0	1
BONE1	*S. capitis*	-	-	0.049 -	+	+	-	0.160 2	-	0	0	0	0	0
BONE2	CNS	-	-	0.063 -	+	+	3 +	0.102 0	-	2	0	0	0	2
BONE3	CNS	-	-	0.056 -	+	+	3 +	0.110 0	-	2	0	0	0	1
BONE5	CNS	-	-	0.053 -	+	+	2 +	0.107 0	-	2	0	4	1	0
BONE6	*S. epidermidis*	+	+ 5	0.382 +	+	+	2 +	0.129 1	+	0	0	0	0	2
BONE10	CNS	-	-	0.079 -	+	+	7 +	0.188 2	+	0	0	0	0	3
ROH1	*S. epidermidis*	+	+ 14	0.083 +/-	+	+	3 +	0.117 1	-	0	0	0	0	0
ROH2	*S. epidermidis*	+	+/-	0.121 +	+	+	3 +	0.112 1	-	0	0	1	0	0
ROH3	*S. epidermidis*	+	+ 14	0.064 -	-	+	3 +	0.093 0	-	0	0	0	0	0
ROH4	*S. saprophyticus*	-	-	0.059 -	+	+	-	0.107 0	-	0	0	0	0	0
ROH5	*S. epidermidis*	+	-	0.168 +	+	+	5 +	0.138 1	+	1	0	0	0	2
ROH6	*S. epidermidis*	-	-	0.109 +	+	+	3 +	0.189 2	-	0	0	1	0	0
ROH7	*S. caprae*	-	-	0.093 +	+	+	11 +	0.209 2	-	-	3	16	4	0
ROH8	*S. epidermidis*	-	-	0.064 -	+	+	11 +	0.176 2	-	1	0	0	0	2
ROH9	*S. simulans*	-	-	0.066 -	+	-	2 +	0.096 0	-	0	0	0	0	1
ROH10	*S. epidermidis*	-	-	0.056 -	+	+	3 +	0.124 1	-	0	0	1	0	1
ROH11	*S. epidermidis*	+	+/-	0.104 +	+	+	5 +	0.168 2	-	2	0	0	0	1
ROH12	CNS	-	+/-	0.057 -	+	+	5 +	0.092 0	-	0	0	0	0	0
ROH13	*S. epidermidis*	+	-	0.094 +	+	+	2 +	0.157 2	+	0	0	0	0	3
ROH14	*S. epidermidis*	+	-	0.089 +	+	+	5 +	0.118 1	-	1	0	0	0	1
ROH15	CNS	-	-	0.063 -	+	+	2 +	0.101 0	-	0	0	4	0	0
EN1	CNS	-	-	0.082 +/-	+	-	11 +	0.082 0	-	0	0	0	0	0
EN4	*S. simulans*	-	-	0.062 -	+	+	3 +	0.099 0	-	0	1	0	0	3
EN5	CNS	+	+ 21	0.098 +	+	+	5 +	0.692 3	+	0	0	0	0	3
EN7	*S. simulans*	+	-	0.058 -	+	-	3 +	0.091 0	-	0	0	0	0	1
EN8	CNS	+	-	0.137 +	+	+	3 +	0.135 1	+	0	0	0	0	2
EN9	CNS	-	-	0.059 -	+	+	7 +	0.196 2	-	2	0	0	0	0

Specimen	Species	Non-specific proteinase	Proteinase -elastase (elastin agar)	Proteinase -elastase congo red-elastin	Non-specific lipase/ esterase	Lipase	Esterase	Adherence to polystyrene	Slime-congo red agar	Slime-concana -valin A	DNase	Haemolysis (Horse red blood cells)	Haemolysis (Sheep red blood cells)	Urease
EN10	*S. epidermidis*	+	-	0.084 +	+	+	2 +	0.137 1	+	0	0	0	0	1
EN13	*S. epidermidis*	+	-	0.135 +	+	+	5 +	0.161 2	-	0	0	0	0	0
EN19	*S. epidermidis*	+	-	0.059 -	+	+	7 +	0.087 0	-	2	0	0	0	2
EN23	*S. epidermidis*	+	-	0.086 +	+	+	7 +	0.118 1	+	2	0	0	0	1
EN27	*S. epidermidis*	+	-	+	+	+	11 +	0.119 1	-	2	0	0	0	0
EN32	*S. epidermidis*	-	-	-	-	+	-	0.111 1	+	0	0	0	0	0
EN33	*S. epidermidis*	-	-	-	+	+	3 +	0.131 1	+	2	0	0	0	1
EN34	*S. hominis*	-	-	-	-	-	-	0.410 3	-	2	1	1	0	0
NCTC 11047	*S. epidermidis*	-	-	0.060 -	+	+	3 +	0.629 3	-	0	0	0	0	0

The specimen is colour coded to indicate the infection from which the isolate was recovered;
CVC-related sepsis
CAPD-associated peritonitis
Internally fixed bone fracture-associated osteomyelitis
Prosthetic hip-related osteomyelitis
Endocarditis
NCTC type strain

Explanation of the table;
The "proteinase-elastase (elastin agar)" and the "esterase" results indicate the minimum incubation time required (days) for the strain to produce a positive result, whilst a +/- symbol indicates that both negative and positive results were obtained for successive assays.
The "proteinase- elastase (congo red-elastin)" result shows the OD_{492} and interpretation of that value: positive; negative; intermediate (+/-).
The "adherence to polystyrene" result is presented as the OD_{595} and interpretation of the value: non-adherent (0); weakly adherent (1); adherent (2); strongly adherent (3).
The "concanavalin A" result is recorded as negative (0), weak agglutination (1), or strong agglutination (2).
The "DNase" result is recorded as negative (0), reduced zone of nuclease activity (1), comparable zone size (2), and increased zone size (3) in comparison to the *S. aureus* Oxford NCTC 6571 control strain.
"Haemolysis" of both horse and sheep erythrocytes is recorded as no haemolysis (0), or the reciprocal of the dilution at which haemolysis was observed.
"Urease" activity is presented as no activity observed (0), weak band (1), clear band (2), or strong band observed (3).

Appendix 4

The antibiograms of the coagulase-negative staphylococcal strains

CNS	Meth	Pen	Ery	Cip	Gent	Fus	Van
CVC1	S	R	S	S	S	S	S
CVC7	R	R	R	R	R	R	S
CVC9	R	R	R	R	R	S	S
CVC10	R	R	S	S	S	R	S
CVC11	S	R	R	S	S	S	S
CVC12	R	R	S	S	R	S	S
CVC16	R	R	R	R	R	S	S
CVC17	S	R	R	R	R	R	S
CVC19	S	R	R	R	R	R	S
CVC20	R	R	R	R	R	S	S
NCIMB40896	R	R	R	R	S	S	S
NCIMB40946	R	R	R	R	R	S	S
NCIMB40945	S	S	S	S	S	S	S
NCIMB40948	R	R	S	S	S	R	S
NCIMB40947	R	R	R	R	R	R	S
023	R	R	R	S	S	R	S
9865	S	R	S	S	S	S	S
CAPD1	R	R	R	S	S	S	S
CAPD2	R	R	R	R	R	R	S
CAPD3	S	S	S	S	S	S	S
CAPD4	R	R	S	S	S	S	S
CAPD5	R	R	R	R	R	S	S
CAPD6	S	R	S	S	S	S	S
CAPD7	S	R	S	S	S	R	S
CAPD8	R	R	R	S	R	S	S
CAPD9	S	R	S	S	S	R	S
CAPD10	R	R	R	R	R	R	S
CAPD11a	R	R	S	S	S	R	S
CAPD11b	R	R	R	R	R	R	S
CAPD12	S	S	S	S	S	S	S
CAPD13	R	R	R	S	S	S	S
CAPD14	R	R	R	R	R	S	S
CAPD15	R	R	S	S	S	R	S
CAPD16	R	R	S	S	R	S	S
CAPD17	S	S	S	S	S	S	S
CAPD18	R	R	R	R	S	S	S
CAPD19	S	R	R	R	S	S	S
CAPD20	S	R	R	R	R	S	S
CAPD21	S	R	S	S	S	S	S
CAPD23	R	R	S	S	S	S	S
CAPD24	S	S	S	S	S	S	S
CAPD25	R	R	R	R	R	R	S
ROH1	R	R	S	S	R	S	S
ROH2	S	R	S	S	S	S	S
ROH3	S	S	S	S	S	S	S
ROH4	S	R	R	S	S	R	S
ROH5	S	R	S	R	S	S	S
ROH6	S	R	S	S	S	S	S
ROH7	S	R	S	S	S	S	S
ROH8	R	R	R	S	S	R	S
ROH9	S	R	R	S	S	S	S
ROH10	S	R	S	S	S	S	S

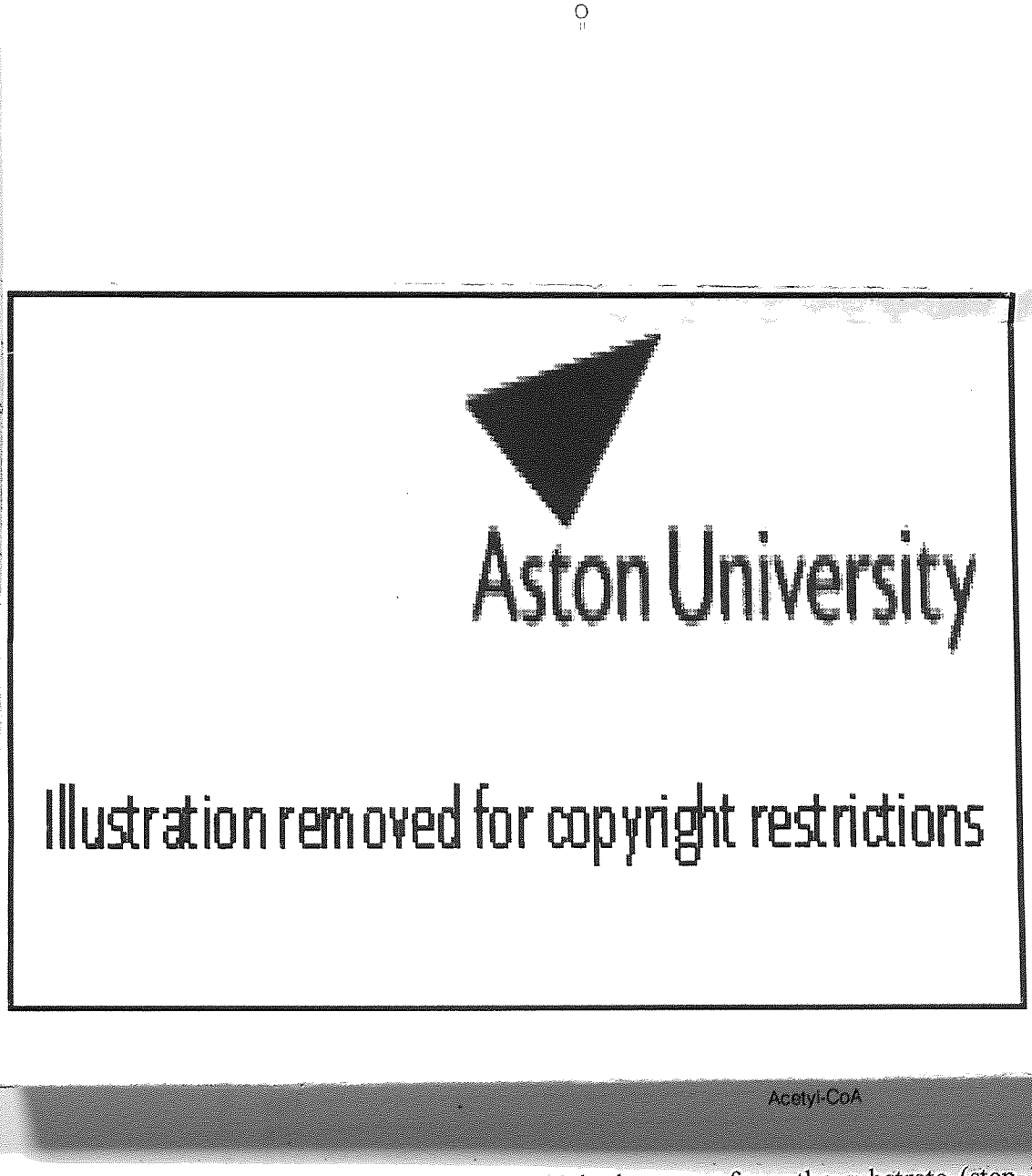
CNS	Meth	Pen	Ery	Cip	Gent	Fus	Van
ROH11	R	R	R	R	R	S	S
ROH12	S	S	S	S	S	S	S
ROH13	R	R	R	R	R	S	S
ROH14	R	R	R	R	R	S	S
ROH15	S	R	S	S	S	S	S
BONE1	S	S	S	S	S	S	S
BONE2	S	R	S	S	R	S	S
BONE3	S	R	R	S	R	S	S
BONE5	S	R	R	S	S	S	S
BONE6	R	R	R	S	S	S	S
BONE10	R	R	S	S	S	R	S
NU1	R	S	S	S	S	S	S
NU3	R	R	R	S	S	S	S
NU7	R	R	R	S	S	S	S
NU11	R	R	S	S	R	S	S
NU24	R	R	R	S	S	S	S
NU27	S	S	S	S	S	S	S
NU33	R	R	R	S	S	S	S
NU104	R	R	R	S	S	S	S
NU142	S	S	S	S	S	S	S
NU159	S	S	S	S	S	S	S
NU160	S	R	R	S	S	S	S
NU168	R	R	R	S	S	S	S
NU257	S	S	S	S	S	S	S
NU296	S	S	S	S	S	S	S
NU779	R	R	S	S	R	R	S
NU5436	R	R	R	S	S	S	S
NU5438	R	R	S	S	S	S	S
EN1	S	S	S	S	S	S	S
EN4	S	R	R	S	S	S	S
EN5	R	R	S	R	R	S	S
EN7	S	S	S	S	S	S	S
EN8	R	R	S	S	S	S	S
EN9	S	S	R	S	S	S	S
EN10	R	R	S	R	R	R	S
EN13	S	R	S	S	S	S	S
EN19	S	S	S	S	S	S	S
EN23	S	R	R	R	R	R	S
EN27	S	S	S	S	S	S	S
EN32	R	R	S	S	R	S	S
EN33	R	R	R	S	S	S	S
EN34	S	R	S	S	S	S	S
NCTC11047	S	R	S	S	S	S	S

meth, methicillin; pen, penicillin; ery, erythromycin; cip, ciprofloxacin; gent, gentamicin; fus, fusidic acid; van, vancomycin.

The CNS are colour coded to indicate the infection type from which the isolates were recovered;
CVC-related sepsis
CAPD-associated peritonitis
Internally fixed bone fracture-associated osteomyelitis
Prosthetic hip-related osteomyelitis
Endocarditis
NCTC type strain

Appendix 5

The conversion of pyruvate to acetyl-CoA by the pyruvate dehydrogenase complex



E1 accepts a two-carbon aldehyde group from the substrate (step 1); this group is transferred to the first arm on E2 and simultaneously oxidized (step 2). The resultant acetyl group is then transferred to the second arm (step 3), which positions it for transfer to CoA-SH (step 4), Finally, E3 oxidizes the reduced lipoamide arm (step 5), with oxidation of the reduced flavin by NAD^+ (step 6) (From Mathews and van Holde, 1990).

Appendix 6

Clustal alignment of the E2 subunit (dihydrolipoamide acetyltransferase) amino acid sequence of ten bacterial species

```
P. aer ----------------------MSE-LIRVPDIGN--GEGEVIELLVKPGDKVEADQSL
E. col ----------------------AI-EIKVPDIGA--DEVEITEILVKVGDKVEAEQSL
S. epi ----------------------MAF-EFRLPDIGEGIHEGEIVKWFIKAGDTIEEDDVL
S. aur ----------------------MAF-EFRLPDIGEGIHEGEIVKWFVKAGDTIEEDDVL
B. ste ----------------------MAF-EFKLPDIGEGIHEGEIVKWFVKPGDEVNEDDVL
B. sub ----------------------MAF-EFKLPDIGEGIHEGEIVKWFVKPNDEVDEDDVL
H. vol ----------------------MALKEFKLPDVGEGVAEGELVTWHVAPGDEVTEDQVL
D. rad MAAMVSHSSPVDGAPVSRPRSPVMAT-ELKLPDVGDNIEKGTVVTVLVNPGDSVTEGQPI
A. lai ------------------------MYEFKFADIGEGIHEGTVLQWNFKVGDKVKEGETL
M. cap ------------------------MFKVKFADIGEGLTEGTVAEVLVKVGDVVKEGQSL
                                      * *                 *

P. aer LTLESDKASMEIPSPKAGVVKSIKAKVGDTLKEGDEILELEVEGGEQ-----PAEAKAEA
E. col ITVEGDKASMEVPSPQAGIVKEIKVSVGDKTQTGALIMIFDSADGAADAAPAQAEEKKEA
S. epi AEVQNDKSVVEIPSPVSGTVEEVLVDEGTVAVVGDVIVKI-------------------D
S. aur AEVQNDKSVVEIPSPVSGTVEEVMVEEGTVAVVGDVIVKI-------------------D
B. ste CEVQNDKAVVEIPSPVKGKVLEILVPEGTVATVGQTLITL-------------------D
B. sub AEVQNDKAVVEIPSPVKGKVLELKVEEGTVATVGQTIITF-------------------D
H. vol AEVETDKALVDVPSPFDGTVKELLAEEGEVVPVGDVIITIQEDGDDEE----AAEAADAD
D. rad IEIETDKAVVEVPASAAGTIEAVNVKVGDTIPVGGVIATLGGG----------AASASDS
A. lai VIVETDKVNAELPSPVDGTIVSLGAKEGEEIHVGQIIVTIDDG---------------TG
M. cap YFVETDKVNSEIPAPVAGKIAVINIKAGQEIKVGDVVMEIEDG---------------SD
            **    *   *        *    *

P. aer APAQP-----------------------------------------EAPKA--EAPAP--
E. col APAAAPAAAAAKDVNVPDIGSDEVEVTEILVKVGDKVEAEQSLITVEGDKASMEVPAPFA
S. epi APDAEE-------------------------------------MQFKGHG----------
S. aur APDAED-------------------------------------MQFKGHD----------
B. ste APGYEN-------------------------------------MTFKGQ-----------
B. sub APGYED-------------------------------------LQFKGSD----------
H. vol AEAAGS-------------------------------------ESAEGDDG---------
D. rad APSASAP-----------------SP---------------ANGGGDQAANVESTPAT
A. lai TPAAAPAP-----------------------------------AQVSAPTP---------
M. cap TSATSE-------------------------------------PKAETKS----------

P. aer ------------------------------APSESKPAAPAAA-----SVQDIKVPDI
E. col GTVKEIKVNVGDKVSTGSLIMVFEVAGEAGAAAPAAKQEAAPAAAPAPAAGVKEVNVPDI
S. epi ------------------------------------------------------------
S. aur ------------------------------------------------------------
B. ste ------------------------------------------------------------
B. sub ------------------------------------------------------------
H. vol ------------------------------A----SDDESGSGGR-----VFAPPSVRRL
D. rad DSG------TAQRVAQAQQDAQKEQAGQPTSGGTADANQAQATPSEGQSSGGQQVTLPDV
A. lai ------------------------------APAAAPQVAAPAASG---DIYDFKFADI
M. cap ------------------------------------------------------------
```

Appendix 6 continued

```
P. aer GSAGKA-NVIEVMVKAGDTVEADQSLITLESDKASMEIPSPASGVVESVSIKVGDEVGTG
E. col GGD-EV-EVTEVMVKVGDKVAAEQSLITVEGDKASMEVPAPFAGVVKELKVNVGDKVKTG
S. epi ----------------------------DDEDSKKEEKEQE----------------
S. aur ----------------------------DDSSSKEEPAKEE----------------
B. ste ----------------------------EQEEAKKEEKTET----------------
B. sub ----------------------------ESDDAKTEAQVQST----------------
H. vol ARELGV-DLDAVDGSGPSGRVTEGDVRAAADDDGDEDDEPSGP----------------
D. rad GDNIEKGTVVTILVNVGDTVSEGQPVIELETDKAVVEVPANASGTVQSVAVKIGDSIPVG
A. lai GEGIHEGTILQWNFKVGDKVKEGETLVVVETDKVNAELPSPVDGTILKLGKAEGEVIHVG
M. cap ----------------------------EAKVEVVEENASVVG---------------


P. aer DLILKLK-VEGAAPAAEEQPAAAPAQAAAPAAEQKPAAAV-----PAPAKADTPAPVGAP
E. col SLIMIFE-VEGAAPAAA--PAKQEAAAPAPAAKAEAPAAA-----PA-AKAEGKSEF--A
S. epi ------------------SPVQEEASSTQSQEK-----------T-----------E
S. aur ------------------APAEQAPVATQTEE--------------------------
B. ste -------------VSKE--EKVDAVAPNAPAA-------------EA-----------E
B. sub ------A-EAGQDVAKE--EQAQEPAKATGAGQQ-----D-----QA-----------E
H. vol ------R-TVQTNGKSA--TAKRDEGTSASASSS-----A-----PT-----------E
D. rad GTILTLSGAASTQPTAPAPESAQPASQSQQSTQPEPARPAGAPQAQAAAPQQSGTQNPQT
A. lai ------ETVVLIGQNGATLEQAQAPKAEAPVSEPKKGAGVVG-EIEVSDDIIGGSEEVHV
M. cap --------------------ATPVSNDVIVRKQTT----------T-------------


P. aer SRDGAKVHAGPAVRMLAREFGVELSEVKASGP-KG--RILKEDVQVFVKEQ-----LQRA
E. col END-AYVHATPLIRRLAREFGVNLAKVKGTGR-KG--RILREDVQAYVKEA-----IKRA
S. epi VDESKTVKAMPSVRKYARENGVNIKAVNGSGK-NG--RITKEDIDAYLNGGSSEEGSNTS
S. aur VDENRTVKAMPSVRKYAREKGVNIKAVSGSGK-NG--RITKEDVDAYLNGG-APTASNES
B. ste AGPNRRVIAMPSVRKYAREKGVDIRLVQGTGK-NG--RVLKEDIDAFLAGG-----AKPA
B. sub VDPNKRVIAMPSVRKYAREKGVDIRKVTGSGN-NG--RVVKEDIDSFVNGG-----AQEA
H. vol SADREQTLAAPATRALAKEEGVDIDAVPATEMRDGEAFVSPEAVQEYAQAQREAQAADAE
D. rad FDGRPVVPAAPSVRRLAREIGIDIHAVHGTGI-AG--RISEEDVRRTAGTP----SVQAQ
A. lai VATTGKVLASPVARKLASDLGVDIATIKGSGE-QG--RVMKDDVQNSKAPA----EAQAP
M. cap VNKSSTIKATPLARKVAADLNIDLSLVTPTGP-NQ--RILVADIKNHQASS-----TQLA
                 * *  *  *


P. aer KS------AVPAPRGAGIP---PIPEV-DFSKFGEVEEVAMTRLIEVGAANLHRSWLNVP
E. col E-------AAPAATGGGIPGMLPWPKV-DFSKFGEIEEVELGRIQKISGANLSRNWVMIP
S. epi AA----SE-STSSDVVNASATQALP---EGDFPETTEKIPAMR--KAIAKAMVNSKHTAP
S. aur AD----S--ATNEEVAETPAAPAAVSL-EGDFPETTEKIPAMR--RAIAKAMVNSKHTAP
B. ste P--------AAAEEKAAPAAAKPATT--EGEFPETREKMSGIR--RAIAKAMVHSKHTAP
B. sub AP----QETAAPQETAAKPAAAPAP---EGEFPETREKMSGIR--KAIAKAMVNSKHTAP
H. vol AVSAEADAGTATAEATVDAASEPAPPE-AGPGAGERVPYKGVR--KAIGDQMQRSKYTAP
D. rad A--------SAASAAAPAPMAAPLPNF-EKWGTVRREDMSGIR--KATVRSMTTAWTTIP
A. lai VQ-------QTQAPAQAAASVAPSFAA-AGKPQGDVEVVKITRLRKAVSNAMTRSKSIIP
M. cap SQP--ISQPAPTPSPSAHQTIAPTIKVVEPSAPLSWDEVPMNGVRKATVKAMTKSHTEIA


P. aer HVTQFDQSDITDMEAFRVAQ--KAAAEKAGVKLTVLPILLKACAHLLKELPDFNSSLAPS
E. col HVTHFDKTDITELEAFRKQQNEEAAKRKLDVKITPVVFIMKAVAAALEQMPRFNSSLSED
S. epi HVTLMDEIDVQELWDHRKKF--KEIAAEQGTKLTFLPYVVKALVSALKKYPALNTSFNEE
S. aur HVTLMDEIDVQALWDHRKKF--KEIAAEQGTKLTFLPYVVKALVSALKKYPALNTSFNEE
B. ste HVTLMDEADVTKLVAHRKKF--KAIAAEKGIKLTFLPYVVKALVSALREYPVLNTSIDDE
B. sub HVTLMDEVDVTNLVAHRKQF--KQVAADQGIKLTYLPYVVKALTSALKKFPVLNTSIDDK
H. vol HVTHHDEVDVTELVELREQL--KPVAEERGSRLTYMPFVMKAVVAALKGFPYLNSQLDEE
D. rad MVTHFDKADVTAMEETRKRFG--ARVEKEGGKLTMTHILMKVVANALRKFPKFNASLDLG
A. lai ETVLMDEINVDALVNFRNEA--KGLAESKGIKLTYMAFIAKAVLIALKEFPMFNASFNHD
M. cap AFTGMKNTDITETHKMRTEL--KDHAAASGIKLTYLAFIIKAVAKSLRDMPNINVRGDFA
                         *                 *     *     *   *  *
```

Appendix 6 continued

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P. aer GKALIRKKYVHIGFAVDTPDGLLVPVIRDVDRKSLLQLAAEAADLADKARNKKLSADAMQ
E. col GQRLTLKKYINIGVAVDTPNGLVVPVFKDVNKKGIIELSRELMTISKKARDGKLTAGEMQ
S. epi AGEVVHKHYWNIGIAADTDKGLLVPVVKHADRKSIFEISDEINELAVKARDGKLTSEEMK
S. aur AGEIVHKHYWNIGIAADTDRGLLVPVVKHADRKSIFQISDEINELAVKARDGKLTADEMK
B. ste TEEIIQKHYYNIGIAADTDRGLLVPVIKHADRKPIFALAQEINELAEKARDGKLTPGEMK
B. sub TDEVIQKHYFNIGIAADTEKGLLVPVVKNADRKSVFEISDEINGLATKAREGKLAPAEMK
H. vol NEEIVLRDEYNIGVAAATDAGLLVPVVHDADRKGMLELADEMNEKVEKARNRKIAPEEMR
D. rad AEQVIYKEFVNIGVAVDTPVGLLVPVVKDADRKGITELVLDLSELAGRARERKLKPDEMQ
A. lai TDEVYIKKFINLGMAVDTPDGLIVPNIKNADRLSVFELASQVRSLADDTIARKISMDQQT
M. cap NNKIQFMHNINIGIAVDTPNGLMVPVIKGADHLSVFEIAIKISELANKAKDGKLTRAEMT
                  * *  *  ** **                                  *

P. aer GACFTISSLGHIGGTGFTPIVNAPEVAILGVSKATMQP-VWDGK--AFQPRLMLPLSLSY
E. col GGCFTISSIGGLGTTHFAPIVNAPEVAILGVSKSAMEP-VWNGK--EFVPRLMLPISLSF
S. epi GATCTISNIGSAGGQWFTPVINHPEVAILGIGRIAQKPIVKDG---EIVAAPVLALSLSF
S. aur GATCTISNIGSAGGQWFTPVINHPEVAILGIGRIAQKPIVKDG---EIVAAPVLALSLSF
B. ste GASCTITNIGSAGGQWFTPVINHPEVAILGIGRIAEKPIVRDG---EIVAAPMLALSLSF
B. sub GASCTITNIGSAGGQWFTPVINHPEVAILGIGRIAEKAIVRDG---EIVAAPVLALSLSF
H. vol GGTFTITNVGGIGGEYATPIINYPEVAILALGAIKEKPRVVDG---EVVPRNVLTLSLSF
D. rad GATFTISNLGGIGGNAFTPIVNSPEVAILGVSRGGFEP-VWNKEKGEFEPRNMLPLSLTY
A. lai GGTFTITNFGSAGIAFGTPVINYPELAILGIGKIDRKPWVVGN---EIKIAHTLPLSLAV
M. cap EATFTVSNFGSVGLDYATPIINSPESAILGVGTMSQTPLYING---ELQKRFIMPLSMTC
          *    *  *     *  * ** ***                              *

P. aer DHRVINGAAAARFTKRLGELLADIRTLLL--
E. col DHRVIDGADGARFITIINNTLSDIRRLVM--
S. epi DHRQIDGATGQNAMNHIKRLLNNPELLLMEG
S. aur DHRQIDGATGQNAMNHIKRLLNNPELLLMEG
B. ste DHRMIDGATAQKALNHIKRLLSDPELLLMEA
B. sub DHRMIDGATAQNALNHIKRLLNDPQLILMEA
H. vol DHRVV-------------------------
D. rad DHRLIDGADAARFLRYICESLEDPFLISL--
A. lai DHRIIDGADGGRFLMRVKELLTNPTLLLLS-
M. cap DHRIIDGADAGRFLIKVQDYLSKPVLLFM--
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P. aer, *Pseudomonas aeruginosa*; E. col, *Escherichia coli*; S. epi, *Staphylococcus epidermidis*; S. aur, *Staphylococcus aureus*; B. ste, *Bacillus stearothermophilus*; B. sub, *Bacillus subtilis*; H. vol, *Haloferax volcanii*; D. rad, *Deinococcus radiodurans*; A. lai, *Acholeplamsa laidlawii*; M. cap, *Mycoplasma capricolum*. K, conserved lipoamide binding lysine residue; H, conserved putative active site histidine.

CONFERENCES/ WORKSHOPS ATTENDED

British Society for Antimicrobial Chemotherapy (BSAC): Bacterial Endocarditis. Derby, 28th October 1998.

The International Society of Cardiovascular Infectious Diseases: 5th International Symposium on Modern Concepts in Endocarditis and Cardiovascular Infections (ISCVID). Amsterdam, The Netherlands, 1st–3rd July 1999.

The Royal Society: The Activities of Bacterial Pathogens *in vivo*. London, 20th-21st October 1999.

Society for General Microbiology: Millennium Meeting. University of Warwick, 10-14th April 2000.

British Pharmaceutical Conference: Medicines – The Future Horizon. Birmingham, 11th September 2000.

Federation of Infection Societies: 7th Conference. Manchester, 29th November – 1st December 2000.

PUBLICATIONS SUBMITTED IN THE COURSE OF THIS RESEARCH

Publications as lead author

Lang, S., M. A. Livesley, P. A. Lambert, J. Elliott, and T. S. J. Elliott. 1999. The genomic diversity of coagulase-negative staphylococci associated with nosocomial infections. *Journal of Hospital Infection* **43**:187-193.

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Lang, S., M. A. Livesley, P. A. Lambert, T. S. J. Elliott, M. Connaughton, W. A. Littler. The genotypic and phenotypic characterisation of coagulase-negative staphylococci associated with endocarditis. Poster P-12, 5th International Symposium on Modern Concepts in Endocarditis and Cardiovascular Infections, Amsterdam, The Netherlands, 1st– 3rd July 1999.

Lang, S., P. A. Lambert, T. Worthington, M. Connaughton, W. A. Littler, T. S. J. Elliott. Serodiagnostic potential of a novel *Staphylococcus epidermidis* antigen. Poster P14, 7th Conference of the Federation of Infection Societies. Manchester, 29th November – 1st December 2000.

Publications as associated author

Connaughton, M., S. Lang, S. E. Tebbs, W. A. Littler, P. A. Lambert, T. S. J. Elliott. Rapid serodiagnosis of Gram-positive bacterial endocarditis: refining the Duke criteria. Submitted to *The Journal of Infection*.

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Connaughton, M., S. Lang, S. E. Tebbs, M. A. Livesley, P. A. Lambert, T. S. J. Elliott, W. A. Littler. Prediction of infecting organism and clinical outcome in endocarditis is aided by quantitative serodiagnosis. Abstract 2-05, 5th International Symposium on Modern Concepts in Endocarditis and Cardiovascular Infections, Amsterdam, The Netherlands, 1st– 3rd July 1999.