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Nottingham

Characterization of Staphylococcal Small Colony Variants and their Pathogenic Role in Biomaterial–Related Infections with Special Reference to *Staphylococcus epidermidis*

By

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Thesis Submitted to the University of Nottingham for the Degree of Doctor of Philosophy

November 2004

Dedications

This thesis is dedicated with deepest love to my parents. Their support has sustained me in the most challenging of circumstances.

Acknowledgments

I would like to express my gratitude to the Division of Orthopaedic and Accident for their financial support for the last six months of my PhD.

I am grateful to my examiners, Dr. Alan Cockayne and Dr. Primrose Freestone, for their generous help and advice in the preparation of this thesis.

My special thanks to Prof. D.A.A. Ala'Aldeen for his assistance towards using his lab facilities. I appreciate his support and advice in some difficult moments through my PhD.

My thanks also go to Dr. Adrian Robins for his assistance in the Flow Cytometry analysis.

Last but not least, my special thanks will go to my parents, brothers and friends for their tremendous moral support throughout my studies in England.

Abstract

There are many surgical implanted devices in current use and all are prone to biomaterial-related infections (BRI) associated with staphylococcal biofilm formation. BRI are usually associated with *S. epidermidis* or *S. aureus* and are characterized by treatment failure and chronicity resulting in re-operation, removal of the implant, and loss of function or death. Staphylococcal small colony variants (SCVs) may be generated by exposure to sublethal concentrations of antibiotics or nutrient limitation which may occur in biofilms. Although the characteristics of *S. aureus* SCVs have been well studied, little information on SCVs of *S. epidermidis* and their potential role in BRI is currently available. This study was designed to investigate the biochemical and phenotypic characteristics of *S. epidermidis* SCVs to further identify characteristics which may contribute to their ability to cause these increasingly important infections.

Exposure to two to four times the gentamicin MIC led to the emergence of stable *S. epidermidis* SCVs, and the ability to produce SCVs was strain dependent. These variants were isogenic by PFGE and less immunogenic by western blotting, and SDS-PAGE analysis of whole cell preparations and cell wall fractions showed altered protein profiles when compared to wild type strains.

S. epidermidis SCVs were resistant to aminoglycosides such as amikacin and/or netilmicin and they were thiamine and/or menadione auxotrophs. Chemiluminescence assays showed a decreased ATP content reflecting the deficiency in electron transport systems which results in a growth rate – all characteristics similar to those of *S. aureus* SCVs.

Analysis of virulence factor production indicated that *S. epidermidis* SCVs showed increased lipolytic and proteolytic activity when compared to those of *S. aureus*. Some *S. epidermidis* SCVs showed phase variation in exopolysaccharide production which enabled them to be more adherent to uncoated plastic - a property that may also be important for the later stages of development of biofilms.

Invasion assays demonstrated that some *S. epidermidis* and *S. aureus* SCVs were internalised by HUVECs by a receptor-mediated mechanism which differed from that of the wild type strains. Interaction of staphylococci with HUVECs induced cytokine production but SCVs stimulated production of IL1, IL-6 and IL-8 at lower concentrations than their related wild type parents in the first 6 hours of co-incubation. SCVs were also less damaging to the HUVEC cell line after 24 hours when compared to wild type strains.

This study supports the suggestion that a switch to the *S. epidermidis* SCV phenotype could be a mechanism exploited by the wild type strains to facilitate their survival inside the host. The chronicity and increased antibiotic resistance associated with BRI could in part, be explained by the characteristics of SCVs identified in this study. In particular the ability to survive intracellularly combined with reduced immunogenicity and resulting decreased cytokine production, may contribute to persistence of infection. Although SCVs are resistant to some antibiotics, surviving intracellularly may further protect staphylococci from other drugs which are unable to enter mammalian cells. Resistance may be further enhanced for some strains in biofilms where enhanced polysaccharide production may also limit antibiotic access.

Table of Contents

List of Tables

List of Figures

Chapter One

General Introduction

1:1 Staphylococcus

Members of the *Staphylococcus* genus are gram-positive cocci about 1μm in diameter and are most often seen as grape-like clusters of cells in gram-stained specimens. They have a unique cell wall peptidoglycan characterized by multiple glycine residues in an inter-peptide bridge, which renders them susceptible to lysostaphin.

Staphylococci are usually facultative anaerobes, capable of generating energy by respiratory or fermentative pathways. They are catalase-positive and usually oxidase-negative.

Staphylococci evolved as free-living organisms. They have typically been regarded as non-invasive extracellular pathogens that damage host cells after adhering to the extracellular matrix. Sir Alexander Ogston, a late nineteenth century Scottish ophthalmic surgeon, who was the first to bring *Staphylococcus aureus* to prominence by demonstrating the important role of the organism (which he termed *Staphylococcus Pyogenes aureus*) in suppurative post-operative infections. He appreciated immediately that the investigation of the defence and immunology of the host was as important as the biology of staphylococci for understanding of staphylococcal diseases.

A variety of phenotypic characteristics are used to identify a bacterial species, including colony morphology, cell wall composition, fatty acid composition, resistance to certain antibiotics and various biochemical activities (Kloos, 1990). The ability of *S. aureus* to clot blood plasma, with its connotations of virulence, has been used to divide staphylococci into two major groups: the **Coagulase-Positive** and **Coagulase-Negative Staphylococci (CoNS).**

Staphylococci have been, and continue to be, a major cause of human disease, especially in the hospital setting. They are responsible for a significant number of serious infections in the normal population, such as endocarditis, osteomyelitis, and septic shock. *S. aureus* appears to differ considerably from the CoNS with respect to its pathogenicity, in that it elaborates a large number of extracellular virulence factors, while the CoNS, which are rather indolent pathogens, generally

produce few if any of these factors. CoNS rely much more on their ability to colonise and infect catheters and other indwelling medical devices from which they may spread systemically.

Again members of the genus Staphylococcus exhibit a characteristic phenotypic variation. Staphylococci can acquire a very slow metabolic rate, in a phenotypic alteration termed **Small Colony Variants (SCVs).** Slow growing bacteria have been known to be resistant to antibiotics since 1942, it being well recognised that active cell wall synthesis is necessary for antibiotics such as penicillin to be bactericidal (Schnitzer *et al.*, 1943; Czens *et al.*, 1986).

S. aureus SCVs were described for the first time in 1932 by Hoffstadt and Youmans as pinpoint bacterial colonies (less than 1 mm) that grew very slowly and often required magnification to be seen (Hoffstadt and Youmans, 1932). Documenting the emergence of colonial variants in cultures of CoNS and *S. aureus* is important, because the presence of atypical colonial forms in a diagnostic culture is usually interpreted as evidence of contamination and is disregarded. SCVs have been recovered from patients with persistent infections, particularly those patients who have experienced long disease-free intervals. SCVs have also been isolated from patients who are chronically exposed to aminoglycosides, suggesting that these clinical situations are those in which SCVs should be suspected and that clinical laboratories should carefully search for these forms of bacteria (Proctor and Peters, 1998).

The use of synthetic materials for temporary or permanent implantation has been accompanied by the emergence of a new challenging entity, namely, biomaterials-related infections **(BRI)**. The pathogenesis of BRI is a complex process with various contributing factors, such as bacterial virulence, physicochemical properties of the biomaterial, and alterations in host defence (Boelens *et al.*, 2000 (a)).

The pathogenesis of these biomaterials-related infections, which are often due to staphylococci, is related to bacterial adherence to host tissue and artificial surfaces within the host (Hogt *et al.*, 1986). The infecting organisms are either introduced during implantation of the prosthesis or are carried to the biomaterial surface by a

temporary bacteremia, where they adhere and grow to form a biofilm (Neut *et al.*, 2001).

A series of studies have reported that some isolates of *S. epidermidis* which produced a mucoid growth *in vitro* that adhered to the walls of culture tubes, were more commonly associated with sepsis, including intravenous catheter-related bacteremia and other prosthetic device-associated infections (Ishak *et al.*, 1985; Kotilainen, 1990; Peters *et al.*, 1982; Rogers, 1979; deSilva *et al.*, 2002). Implant infections are usually persistent until removal of the prosthesis (Zimmerli *et al.*, 1994; Zimmerli and Ochsner, 2003), and are often associated with a high mortality especially in patients with cardiovascular prostheses (Lyte *et al.*, 1996; Zimmerli and Widmer, 1991), and BRIs therefore represent a major problem in modern medicine.

1:1:1 Coagulase-Positive Staphylococcus in Human Disease

S. aureus is coagulase-positive, and the best known and by far most studied species of disease-causing bacteria. The pathogenic capacity of *S. aureus* is the combined effect of extracellular factors and toxins together with the invasive properties of the strain. In general, *S. aureus* is not considered a significant intracellular pathogen when compared with genera such as *Listeria* and *Shigella*. However, there is growing evidence that *S. aureus* has the ability to invade and persist within eukaryotic cells (Hamill *et al.*, 1986; Sinha *et al.*, 1999). From a clinical standpoint, this observation could yield insights into mechanisms relevant to persistent and recurrent staphylococcal infections and to better understanding of at least a subset of instances where staphylococcal infections are refractory to antimicrobial therapy.

S. aureus infections result from direct contamination of a wound, e.g. postoperative staphylococcal wound infections or infection following trauma. If *S. aureus* disseminates and bacteraemia ensues, acute haematogenous osteomyelitis or potentially fatal endocarditis, meningitis, or pulmonary infections can result.

Methicillin – resistant *Staphylococcus aureus* (MRSA) was first observed in the 1960's soon after the introduction of cloxacillin into clinical use (Cookson and Phillips 1990), and is known to be highly transmissible leading to highly publicised hospital outbreaks. Often this occurs when a patient or health care worker is colonized with an MRSA strain and, through contact with others, spreads the strain to others who are susceptible to infection. Interestingly, by the 1980s, a high number of methicillin-resistant S*taphylococcus epidermidis* (MRSE) strains were also being isolated from nosocomial infections (Jones *et al.,* 1989; Karchmer *et al.,* 1983).

1:1:2 Coagulase-Negative Staphylococci in Human Disease

Over the last three decades other staphylococci associated with human disease have been recognized and well documented. Several members of CoNS other than *S. epidermidis* have been associated with BRI such as *Staphylococcus haemolyticus and Staphylococcus warneri* (Kloos and Bannerman, 1994). They have also been isolated in native valve endocarditis (Kloos and Bannerman, 1995). *Staphylococcus saprophyticus* is an important opportunistic pathogen infecting the urinary tract and is second to *Escherichia coli* as the most common cause of urinary tract infection (UTI) in the community (Hovelius and Mårdh, 1984).

S. epidermidis, as the name implies, is one of the most common bacteria of the skin flora. However, it requires a predisposed host in order to change from a normal inhabitant of the human skin to an infective agent, and therefore clearly has to be described as opportunistic. In contrast to *S. aureus*, *S. epidermidis* does not usually cause pyogenic infections in non-compromised patients, with the only exception being native valve endocarditis (Caputo *et al.,* 1987). This is due to its distinctly reduced arsenal of toxins compared to *S. aureus*. Because of the lack of severely tissue-damaging toxins, *S. epidermidis* infections are usually sub-acute or chronic. Lambe *et al* (1990) studied CoNS virulence factors and correlated their

production with disease progression (abscess formation) in mice. He and his colleagues found that while *S. epidermidis* possessed a variety of virulence factors such as α-haemolysin and δ-haemolysin, lipase and esterase, the production of these exoenzymes did not always correlate with the formation of abscesses in mice (Lambe *at al.*, 1990).

S. epidermidis very often becomes the major infective agent in compromised patients, such as intravenous drug abusers and immuno-compromised patients (patients under immuno-suppressive therapy, AIDS patients, and premature new-borns). The port of entry into the human body in all of these infections is usually an intravascular catheter (Domingo *et al.*, 2001) or a dirty injection.

1:1:2:1 Staphylococcal Biomaterial-Related Infections and Biofilms

The majority of BRI are caused by the relatively non-pathogenic CoNS, especially *S. epidermidis*. The pathogenesis of *S. epidermidis* BRI is characterised by the ability of this species to colonise the implant surface by the formation of a thick, multilayered biofilm. Biofilm formation is often the cause of the difficulty in eradicating the biomaterials related infection, due to an impaired penetration of antibiotics, resistance of inherent biofilm organisms to antibiotics, and the effect of the biofilm on the host immune response. The biofilm present on infected devices plays an important role in the pathogenicity of the infecting organism. Biofilms protect the embedded staphylococci and reduce the efficacy of antimicrobial killing and host defences (von Eiff *et al.*, 1999), often necessitating removal of the implanted device (Hodgson *et al.*, 1995; Hoyle and Costerton, 1991; Zimmerli *et al.*, 1994).

The extracellular substance surrounding the multilayered cell clusters, often referred to as 'slime' in the past, is composed of bacterial products of different chemical composition. In recent years, the components have in part been identified, and biochemical and genetic investigations have produced a much more defined idea of what a staphylococcal biofilm consists of and how it is produced.
In the course of BRIs, two different stages have been distinguished.

(1) The primary attachment which may either proceed as direct attachment of the bacteria to the biomaterial surface or via binding to host-derived matrix proteins which have previously coated the biomaterial (Fletcher, 1976; Heilmann *et al.*, 1996(b); Hussain *et al.*, 1997). (2) The formation of multi-layered cell clusters with cell-cell adherence depending on the production of a 'slimy' extracellular substance (Heilmann and Götz, 1998).

The attachment of *S. epidermidis* to uncoated biomaterial is dependent on the physico-chemical properties of the biomaterial, the suspending medium and the bacterial surface. The main parameter determining bacterial adhesion is the hydrophobicity of the bacterial surface (Vacheethasanee *et al.*, 1998). In fact, detergent-like substances of artificial and bacterial origin interfering with the hydrophobic interaction have been shown to lower the degree of adhesion (Vacheethasanee and Marchant, 2000). Adherence is obviously a crucial step in the initiation of BRI. After implantation of the medical device, the polymer material rapidly becomes coated with plasma and extracellular matrix proteins such as fibronectin, fibrinogen, vitronectin, thrombospondin, and von Willebrand factor (Cottonaro *et al.*, 1981; Dickinson and Bisno, 1989; Kochwa *et al.*, 1977) forming what is called conditioning film.

For *S. aureus* proteins have also been identified that are involved in biofilm formation, such as the accumulation-associated protein (AAP), the clumping factor A (ClfA), the staphylococcal surface protein (SSP1), the biofilm-associated protein (Bap), MSCRAMMS (Götz, 2002), extracellular adhesion protein (Eap) (Hussain *et al.*, 2001) and extracellular matrix protein-binding protein (Emp) (Hussain *et al.*, 2001), all of which mediate the attachment to extracellular matrix substrates and eukaryotic cells.

The formation of multicellular cell clusters on top of the monolayer of cells attached to biomaterial or host cells constitutes the second stage of biofilm formation by *S. epidermidis*. Clearly, the mechanisms underlying the adhesion between staphylococcal cells must be different from those enabling the bacteria to adhere to a hydrophobic surface or to specific receptors on host cells. Several molecules have been proposed to be responsible for cell-cell adhesion, mostly

polymeric carbohydrates and proteins. Most genetic and biochemical evidence has been obtained for the involvement of an extracellular polysaccharide in intercellular adhesion, named **PIA** (polysaccharide intercellular adhesin).

PIA is composed of a major polysaccharide, termed I, and a minor polysaccharide II. Polysaccharide I is a linear β-1,6-linked 2 deoxy-2-amino-D-glucopyranosyl homopolymer which shows N-acetylation at about 80-85% of the polysaccharide I monomers. Polysaccharide II is very similar in structure, but harbours a limited omission of phosphate and ester-linked succinate (Mack *et al.*, 1996). PIA also mediates haemagglutination of erythrocytes by *S. epidermidis* strains and has been found to be an important virulence factor in the pathogenesis of *S. epidermidis* BRI (Fey *et al.*, 1999; Rupp *et al.*, 1995).

Capsular polysaccharide-adhesin (PS/A) is another component of the extracellular biofilm layer that mediates cell adhesion to biomaterials (McKenney *et al.*, 1998; Muller *et al.*, 1993; Shiro *et al.*, 1995) and protects bacterial cells from opsonophagocytosis (Kojima *et al.*, 1990; Tojo *et al.*, 1988). PS/A is a virulence factor for *S. epidermidis* infections in animal models of endocarditis (Shiro *et al.*, 1994). Chemical studies proposed that PS/A was a high-molecular-weight polymer of β(1,6)-linked glucosaminyl residues (Mckenney *et al.*, 1998; McKenney *et al.*, 1999). PIA and PS/A are closely related chemically and immunologically and both are synthesized by the protein products of the *ica*ADBC locus (Gerke *et al.*, 1998; McKenney *et al.*, 1998). PIA had been proposed to be functionally distinct from PS/A, with PS/A mediating initial adherence to solid surfaces and PIA mediating accumulation of cells into biofilm (Mack *et al.*, 1996; Mack *et al.*, 1994), although data evaluating PS/A's function indicate that it can mediate both processes (McKenney *et al.*, 1998).

1:1:2:2: Mechanisms of Bacterial Resistance in Biomaterial-Related Infections (BRI)

The incidence of antibiotic resistance in pathogenic bacteria is rising. Bacterial infections respond to antibiotics alone, but implant infections do not

because biofilm-associated bacteria are generally less susceptible to antibiotics. Since implants are not vascularized, antibiotics, immune cells and substances active against infection can arrive only by diffusion from adjacent tissue. Antibiotic resistance can be achieved via three distinct routes: inactivation of the drug, modification of the target of action, and reduction in the concentration of drug that reaches the target. It has long been recognized that specific antibiotic resistance mechanisms can be acquired through mutation of the bacterial genome or by gaining additional genes through horizontal gene transfer.

Bacteria have developed mechanisms to mitigate the growth disadvantages conferred by antibiotic resistance while still ensuring survival in the presence of antibiotics. It is well established that within biofilms there will exist huge physio-chemical gradients such as pH, Oxygen and nutrients. These will cause bacteria to express different physiologies (Gilbert *et al.*, 1993). This will lead to some cells sited in biofilms becoming auxotrophic for substances like haemin, menadione, thiamine and CO_2 (Proctor and von Humboldt, 1998). These organisms have been shown to be very slow growing upon plating on nutrient agar. These phenotypic variants have been reported to be SCVs. SCVs are considered to be responsible for the two major clinical characteristics of BRI: resistance to antibiotic treatment and chronicity. Since SCVs reproduce at a very slow rate, the antibiotics will have little effect on the bacterial cell. Aminoglycosides, which are commonly used in the treatment of BRI, are dependent on membrane proton motive force generated by the bacterial electron transport system (Mates *et al.*, 1982) and are not able to enter SCVs because they show a defective electron transport system. For the reason stated above, the production of SCVs could be considered another staphylococcal mechanism for selecting SCVs during antibiotic therapy in BRI resistance.

1:1:3 Pathogenicity and Virulence of Staphylococci

Staphylococci can produce disease both through their ability to multiply and spread widely in tissues and through their production of many extracellular

substances. Some of these substances are enzymes; others are considered to be toxins, though they may function as enzymes. In contrast to *S. aureus*, little is known about mechanisms of pathogenesis of *S. epidermidis* infections. Some of the major staphylococcal virulence factors identified are discussed below.

Catalase is an enzyme with biochemical properties that enhance staphylococcal survival in phagocytes. Catalase converts hydrogen peroxide, normally toxic, into water and oxygen. All staphylococci strains are catalase positive. This test has been used to distinguished staphylococci from streptococci which are catalase negative.

S. aureus carries several surface adhesins **MSCRAMMs,** (microbial surface components recognizing adhesive matrix molecules) (Foster and Höök, 1998) that interact with various human or animal tissues, plasma proteins, and polypeptides of the extracellular matrix (Patti *et al.,* 1994; Vaudaux *et al.,* 1995). The fibronectin-binding protein A and B, the collagen-binding protein and the fibtinogen-binding proteins, clumping factor A and B, belong to this family. In general, *S. epidermidis* has fewer MSCRAMMs than *S. aureus* that mediates adherence to components in the extracellular matrix.

Coagulase is one of the extracellular proteins which binds to prothrombin in the host to form a complex called staphylothrombin. The protease activity characteristic of thrombin is activated in the complex, resulting in the conversion of fibrinogen to fibrin (Phonimdaeng *et al.,* 1990). Coagulase is a traditional marker for identifying *S. aureus* in the clinical microbiology laboratory. Coagulase may deposit fibrin on the surface of staphylococci, perhaps altering their ingestion by phagocytic cells and their destruction within such cells. Coagulase production is considered synonymous with invasive pathogenic potential (van der Vijver *et al.,* 1975).

Fibrinogen-Binding Protein (Fbp), is the so-called 'bound coagulase' widespread among strains of *S. aureus* (Cheung *et al.,* 1995; Duthie, 1954). Fbp promotes virulence by attachment.

Clumping Factor (ClfA and ClfB), is considered to be another form of fibrinogen-binding protein in *S. aureus.* The binding of clumping factors to fibrinogen promotes bacterial adhesion to thrombi on the surfaces of heart valves (Moreillon *et al.,* 1995). ClfA-related fibrinogen-binding protein was identified in *S. epidermidis* but has not yet been analysed (Nilsson *et al.*, 1998).

Fibronectin-Binding Protein (Fnb A and Fnb B) are believed to be important in the attachment of *S. aureus* to foreign objects (Vaudaux *et al.,* 1995; Flock *et al.,* 1987; Jonsson *et al.,* 1991). Fibronectin in plasma binds to *S. aureus* first suggested a possible role for Fn adherence as a virulence factor (Kuusela, 1978).

Vitronectin-Binding Protein has been found in both *S. epidermidis* and *S. aureus.* This protein recognises vitronectin found in extracellular matrix sites which might contribute in bacterial colonization of host tissues (Liang *et al.,* 1993).

AtlE (accompanied transposon insertion) is a major autolysin found in both *S. epidermidis* (Heilmann *et al.*, 1997) and *S. aureus* (Oshida *et al.*, 1995). AtlE binds strongly to vitronectin (Heilmann *et al.*, 1997), a protein which is found in the extracellular matrix and in serum.

Proteins other than AtlE contribute to biofilm formation in staphylococci such as the accumulation-associated protein **(AAP)** and the biofilm-associated protein **(Bap)** (Hussain *et al.,* 1997). All staphylococcal isolates harbouring Bap are highly adherent and strong biofilm producers. In a mouse infection model, Bap is involved in pathogenesis, causing a persistent infection (Cucarella *et al.,* 2001).

Polysaccharide Intercellular Adhesin (PIA): consists of linear β-1,6-linked glucosaminoglycan which is found in *S. epidermidis* and *S. aureus* (Mack *et al.*, 1996). However, PIA is considered to be the major virulence factor produced by the CoNS. It has been shown to promote the attachment of cells of *S. epidermidis* to each other producing the biofilm (Heilmann *et al.*, 1996; Heilmann and Götz, 1998; Mack *et al.*, 1996).

Bacterial survival during an infection is dependent on the ability of the staphylococci to circumvent the host's defences. Virulence factors involved in evasion of host defences include *S. aureus* **Protein A,** which binds to the Fc portion of IgG antibodies (Forsgren and Sjöquist, 1966). The binding of protein A to IgG antibodies is involved in evading the immune response, perhaps in the prevention of opsonization (Verhoef *et al.*, 1979). Bacteria will bind IgG molecules in an inappropriate orientation on their surface which disrupts opsonization and phagocytosis.

In response to an infection, the host can produce a variety of fatty acids and other lipid molecules that act as surfactants to disrupt the bacterial membrane, especially when an abscess is formed. Virtually all strains of staphylococci of human origin are lipolytic. **Lipase** and a **Fatty Acid Modifying Enzyme (FAME)** provide nutrients for the bacteria. However, The FAME enzyme is important in abscesses, where it could modify anti-bacterial lipids and prolong bacterial survival. FAME has been proposed to function to detoxify bactericidal fatty acids at the site of an infection. Consistent with a role in abscess formation, both lipase and FAME have been reported to be expressed extracellularly during postexponential phase in staphylococcal strains (Götz *et al.*, 1985).

Lipases (Lee and Landolo, 1986) have been proposed to have a negative effect on immune function. It has been suggested that lipases may be important for the colonization and persistence of resident organisms on the skin by the release of free fatty acids which may promote adherence (Farrell *et al.*, 1993). Lipases, like proteases (Carmona and Gray, 1987), may well represent scavenging enzymes,

one of whose roles is to harvest nutrients from the environment. Lipase and protease production have been found to be related to the pathogenicity of *S. epidermidis* in mice (Molnar *et al.,* 1994), and *S. aureus* in the induction and progression of septic arthritis in mice (Abdelnour *et al.,* 1993).

Proteases have been shown to have the ability to cleave and degrade a number of important host proteins, including the heavy chains of all human immunoglobulin classes, plasma proteinase inhibitors, and elastin (Dubin, 2002; Karlsson and Arvidson, 2002).

Iron uptake system, iron is the common prerequisite for all bacterial pathogens to establish an infection and to proliferate within the mammalian host. As do all bacteria, staphylococci need iron for their growth; however, the free iron concentration (10^{-18} M) in the extracellular body fluids, owing to the presence of high-affinity iron-binding glycoproteins such as transferrin or lactotransferrin, is much too low to support staphylococcal growth. There are two main mechanisms for iron acquisition. One mechanism involves the synthesis and secretion of low-molecular-mass iron chelators (siderophores), which remove iron from transferrin. The siderophores (staphyloferrin A and B) have been seen to be produced by *S. epidermidis* under iron-restricted conditions (Lindsay and Riley, 1994). The second mechanism by which bacteria assimilate iron depends on direct contact between the host transferrin and a bacterial surface receptor. Both *S. epidermidis* and *S. aureus* isolated from infected individuals or from experimentally infected animals express several iron-repressible cell-wall-associated and cytoplasmic-membrane-associated proteins which may have a role in transferrin binding (Modun *et al.,* 1998).

Haemolysins are virulence factors involved in invasion/tissue penetration. α,β and δ-haemolysins are cytotoxic agents that promote invasion of tissue. **α-Toxin** (α-haemolysin) causes the formation of pores in cell membranes, which explains its ability to cause lysis of certain red blood cells. After binding the

toxin, a complex series of secondary reactions ensues, causing release of cytokines that trigger production of inflammatory mediators (Menzies and Kourteva, 2000). Although α-haemolysin is well known to be produced by *S. aureus* strains, CoNS do produce a variety of exoproteins such as α- and δ haemolysins (Stout *et al.*, 1992).

β-Haemolysin (phospholipase C) has been shown to act as a sphingomyelinase which damages membranes rich in sphingomyelin (Wadström and Möllby, 1972; Katerov *et al.*, 1994). The classical test for β-toxin is lysis of sheep erythrocytes. This toxin was found to play an important role in profound cell damage in *S. aureus*-associated eye infection (O' Callaghan *et al.*, 1997).

δ-Haemolysin has been observed to potentiate the action of β-haemolysin *in vitro*, enhancing its haemolytic properties (Ruzickova, 1994). Staphylococcal δ-Haemolysin has surfactant-like or channel-forming properties, based on its ability to lyse bacteria lacking cell walls (Bernheimer and Rudy, 1986).

To date, several toxins secreted by *S. aureus* have been identified such as **enterotoxin A and B** (Irwin and Gascoigne, 1993). They induce vomiting and diarrhea when ingested at submicrograms levels as well as toxic shock, a systemic response to infectious agents (Zumla, 1992). Another toxin produced by some isolates of *S. aureus*, toxic shock syndrome toxin-1 (**TSST-1**), also causes systemic toxic shock (Irwin and Gascoigne, 1993). These proteins are also referred to as **superantigens** because of their ability to stimulate the proliferation of large numbers of T-lymphocytes (Irwin and Gascoigne, 1993) and this activation is associated with the release of cytokines such as IL-6 and IL-8.

1:1:4 Staphylococcal Virulence Regulators

For the majority of staphylococcal diseases, pathogenesis is multifactorial, so it is difficult to determine precisely the role of any given factor. However, there is correlation between strains isolated from particular diseases and expression of particular virulence determinants, which suggests their role in a particular disease.

The application of molecular biology has led to advances in unraveling the pathogenesis of staphylococcal diseases. Genes encoding potential virulence factors have been cloned and sequenced, and many protein toxins have been purified.

Virulence factors are expressed in different environmental situations. Tompkins *et al* (1992) suggested that *S. aureus* surface components whose expression is growth-dependent can adhere to human endothelial cells *in vitro*. Table 1.1 shows examples of virulence factors whose expression in related to growth phase.

Table 1-1: Effect of Growth Phase on Staphylococcal Virulence Factor Production

Early Log Phase	Late Log Phase	Stationary Phase
Coagulase	α-Toxin	FAME
Protein A	β-Haemolysin	PIA
Clumping Factor	δ-Haemolysin	
MSCRAMMs	Protease	
Fibronectin-Binding Proteins	Lipase	
Fibrinogen-Binding Proteins	Capsular Polysaccharide	

Several studies have been published describing different loci which regulate expression of multiple exoproteins and/or virulence factors. The ***agr*** *(accessory gene regulator)* (Recsei *et al.,* 1986) and the ***sar*** *(staphylococcal accessory regulator)* (Cheung *et al.,* 1992) are two of these loci which have been cloned, sequenced, and studied in detail.

Agr is a locus which is affected by a quorum-sensing system and therefore, the expression of *agr*-regulated targets is cell density-dependent. Generally, when cell density and *agr* activity are low, surface proteins are expressed to allow for tissue colonization. With increasing cell density, *agr* becomes active, the production of

surface protein stops, and extracellular degrading exoenzymes and toxins are produced (Novick and Muir, 1999).

Mutations in *agr* showed that the production of most of the extracellular virulence factors was depressed, while the cell wall-associated proteins were over expressed (Ji *et al.*, 1995; Manna *et al.*, 1998). Table 1-2 shows how the phenotypic expression of specific virulence factors is affected by mutations in *agr*.

Table 1-2: Effect of Agr on Virulence Factor Expression

Decreased Expression in *agr* Mutants	Increased Expression in *agr* Mutants
α-Toxin	Protein A
β-Haemolysin	Fibronectin-Binding Proteins
δ-Haemolysin	Clumping Factor
Lipase	Vitronectin Binding Protein
Protease	PIA
Capsular Polysaccharide	
FAME	

Sar, the second regulatory locus was identified in much the same manner as the *agr* locus; a Tn917TV1 transposon insertion gave rise to a mutant displaying multiple changes in the phenotypic expression of several virulence factors (Cheung *et al.*, 1992). The phenotype of the original *sar* mutant appears to be the inverse of the *agr* mutant strain (increased production of α-toxin and decreased production of cell wall-associated proteins) (Cheung *et al.*, 1992).

Further regulatory loci, such as the secondary sigma factor *sigB*, also influence the expression of virulence factors. Although *agr, sar,* and *sigB* have all meanwhile been found in *S. epidermidis*, it has remained obscure for a long time whether or not these loci fulfil the same tasks as in *S. aureus*, i.e. regulating the expression of virulence factors. This has mainly been because of the reduced

extracellular and surface-attached virulence factors in *S. epidermidis* compared to *S. aureus*.

The global regulatory loci of *S. epidermidis* and *S. aureus* also influence biofilm formation. *Agr* mutants of *S. aureus* show increased attachment to polystyrene (Vuong *et al.*, 2000).

The *sigB* locus has been reported in *S. aureus* to influence biofilm formation. A transposon mutant in *sigB* shows decreased production of PIA and decreased biofilm formation in *S. aureus* (Knobloch *et al.*, 2001). Kullik and Giachino (1997) showed that *sigB* is induced during stationary phase and speculated that it is directly or indirectly involved in the regulation of virulence genes (Kullik *et al.*, 1998).

Virtually nothing is known about the regulation of potential virulence determinants in *S. epidermidis.* Until recently, *agr*-regulated targets of *S. epidermidis,* which are likely to include virulence determinants, have not been identified. Li *et al* (2004) had investigated *agr* locus and its genetic polymorphism in different Chinese *S. epidermidis* isolates. They found that genetic polymorphisim of *agr* in *S. epidermidis* was linked to its pathogenecity (Li *et al.*, 2004).

Intercellular adhesin locus regulator (*icaR)* has been found to be involved in phenotypic variation in biofilm formation in *S. epidermidis* isolates (Handke *et al.*, 2004). *IcaR* gene product was found to be a transcriptional repressor which plays an adaptive role in *S. epidermidis* biofilms formation by modulating the regulation of *ica* expression in response to specific environmental conditions (Conlon *et al.*, 2002).

It is obvious that there is a concerted action of the virulence determinants and their global regulators which lead to staphylococcal disease. *In-vitro* and *in-vivo* results suggest that attachment and growth are precursors to the production of most exotoxins (Sioderquist *et al.*, 1998). The fact that many attachment proteins require ongoing cell wall assembly to become anchored to the cell wall is good reason for cells to produce these only during cell division, and not post exponentially (Cucarella *et al.*, 2002; Heyer *et al.*, 2002). Likewise, to have an

optimum effect in the immediate environment of the bacterium, extracellular proteins would be best produced when the cells are localized and in sufficient quantity to produce enough material to have a beneficial effect for the bacterium. This is true for those exoproteins that are enzymes such as metalloproteases or pore-forming invasins such as haemolysins. This suggests that sepsis due to staphylococcal infection is not the result of a generally disseminated contamination of the bloodstream. Rather, it is the result of a focal infection in which growing bacteria reach a cell density sufficient to lower the local pH, deplete available nutrients, and then produce degrading enzymes, the pore-forming invasins and immunomodulators, which produce the symptoms associated with the disease.

1:1:5 Clinical Significance and Laboratory Detection of Staphylococcal Isolates

While *S. aureus* causes infections ranging from cutaneous infections to toxin-induced food poisoning, CoNS are predominantly members of the normal skin flora and are generally considered to be non-virulent. In the last three decades, however, it has become clear that CoNS are important nosocomial pathogens with a propensity to colonize foreign material, such as indwelling catheters, graft material and orthopaedic prostheses (Patrick, 1990; Hall, 1991). CoNS are most likely to be significant causes of infection in the following situations: presence of implants, neonates requiring intensive care (usually having intravenous catheters), immunocompromised patients, some specific clinical disease settings such as post operative sternotomy wound infections (closed with wire sutures), urinary tract infection caused by *S. saprophyticus*, and native valve endocarditis.

It is often difficult to decide whether recovery of CoNS from blood or other normally sterile body fluids represents contamination by skin flora or a true infection requiring antibiotic therapy and/or removal of biomaterial for resolution. Therefore, several actions should be considered to facilitate the correct

interpretation of culture results. It is essential that good aseptic technique be followed during sample collection. Similarly, care should be taken not to contaminate the collection bottles during sample introduction. In situations where the interpretation of the culture result is unclear, repeat culture(s) with strict attention to collection technique should usually be done. Repeated recovery of the same strain is indicative of infection rather than contamination.

In the hospital microbiology laboratory, identification of staphylococci is often limited to a rapid screening test for *S. aureus*, while *non-S. aureus* isolates are simply reported as CoNS. Because of their increasing importance, clinically significant CoNS should be identified to the species level by a reliable, simple, rapid, and preferably, inexpensive method (Bannerman *et al.*, 1993; Kleeman *et al.*, 1993).

Molecular techniques have greatly enhanced the ability to better characterize and more accurately identify *Staphylococcus spp.* and their mechanisms of antimicrobial resistance. However, the adoption of molecular diagnostic tests has not been possible in most laboratories because of the increased cost and expertise required.

Staphylococcus is first differentiated from other gram-positive cocci by the catalase test. All *Staphylococcus* species are catalase positive. The coagulase test further differentiates *Staphylococcus* species. For practical purposes, a combination of a slide and a thermonuclease (DNAse) test should recognize the great majority of *S. aureus* strains. If there is disagreement between results of these tests, or suggestive colonial features, a commercially available biochemical testing kit should be used. The testing kits such as API STAPH (BioMérieux, Basingstoke, UK) are based on the biochemical characteristics to differentiate the staphylococcal species.

Beginning with the use of penicillin in the 1940s, drug resistance developed in the staphylococci within a short time of introduction of an antibiotic into clinical use. Some strains are now resistant to most conventional antibiotics, and there is concern that new antibiotics have not been forthcoming.

Typing of *S. aureus* is often required for the investigation of an outbreak or as part of ongoing surveillance, whereas typing of CoNS is often used to determine whether two or more strains from a patient represent contamination or infection. A typing system must be able to characterize readily all or most strains tested, be reproducible, and be able to discriminate between unrelated isolates. These tests can be broadly divided into phenotypic, such as antibiograms and biotypes, or genotypic, such as plasmid analysis and pulsed-field gel electrophoresis (PFGE) (Bergeron and Ouellette, 1998).

Expression analysis of *S. epidermidis* virulence factors by microarrays will answer questions such as which virulence factors are expressed in different environmental situations, especially in a biofilm setting, and how regulatory systems work and interact.

1:2 Phase Variations: Mechanisms for Bacterial Survival and Growth under Changing Environmental Conditions

A potentially important property for staphylococcal survival is their ability to make phenotypic changes. The staphylococcus is an extremely adaptable micro-organism capable of changing rapidly its phenotypic and genotypic characteristics (Gemmell, 1995). Phase variation may represent a mechanism for bacterial survival and growth under changing environmental conditions (Chan and Foster, 1988; Ernst *et al*, 1999). Such information mostly comes from experiments *in vitro*, but there is a growing body of information about what virulence factors are produced at different periods of infection and how *in vivo* conditions select bacterial populations that are able to cause disease (Smith 1998; Wesson *et al.*, 1998).

It would seem that bacterial populations have the capacity to respond to different growth opportunities by the selection of suitable variants (Kurland, 1992). Specifically, staphylococcal species have a high frequency of phase variation. Phase variation is a generic term for a group of genetic mechanisms in which the expression of a gene varies in a reversible manner from generation to generation at a relatively rapid rate. These phase variants differ in a variety of characteristics including biofilm production, antibiotic resistance and virulence (Christensen *et al.*, 1990). Elek pointed out the major problem confronting microbiologists due to the emergence of colonial variants which could be mistaken for a mixed culture, or a mixed culture could be mistaken for the emergence of colonial variants (Elek, 1959).

Antibiotic resistant staphylococcal variants have been recovered following treatment with penicillin and related antibiotics (Annear and Grub, 1973; Devriese, 1973; Wise and Spink, 1954) and aminoglycosides (Kaplan and Dye, 1976; Lacey, 1969; Li *et al.*, 1995; Miller *et al.*, 1980; Yegian *et al.*, 1959). Variants showing differing degrees of phenotypic variation from the parent strain have also been isolated from clinical samples (Acar *et al.*, 1978; Lacey and Mitchell, 1969) and

the experimental induction of variants in animals has also been reported (Miller *et al.,* 1978, Musher *et al*, 1977).

Previous studies have reported phenotypic variation in *S. aureus* in association with changes in colonial morphology (Lee and Bergdoll, 1985; Parisi, 1966). Production of toxins, haemolysins, extracellular enzymes, protein A, and bacteriophage susceptibility may also vary. This phenomenon is not limited to *S. aureus,* as *S. epidermidis* also demonstrates phase variation in biofilm production, antibiotic resistance and the expression of virulence-related functions in the pathogenesis of *S. epidermidis* foreign body infections. Ziebuhr *et al* (1997) have detected an intercellular adhesion gene cluster (*ica*) indicating that *S. epidermidis* strains from clinical material differ from saprophytic strains in terms of the presence of the *ica* gene cluster as well as in the capacities for phase variation, polymer adherence, autoaggregation, and in colony morphology on Congo red agar. Results from this study indicate that adherence, slime production, and resistance to antibiotics can be induced or acquired and are influenced by global regulators. Conlon *et al* (2002) found the *icaR* plays a role in biofilm formation by regulating *ica* operon expression and biofilm formation in a biofilm-forming clinical isolate of *S. epidermidis*.

Staphylococci, as mentioned before, are the most important pathogens in BRI (Emori and Gaynes, 1993; Kawamura *et al.,* 1994; Kloos and Bannermann, 1994). Phenotypic methods have been used as a discriminatory tool correlating these variants with their pathogenicity, infectivity and virulence. Although staphylococci are easy to identify by classical microbiological techniques, in some cases the correct species diagnosis is difficult. This might be due to the fact that at least some clinical staphylococcal isolates can differ in a wide range of phenotypic properties, leading to misinterpretations of biochemical tests (Deighton *et al.,* 1992(a); Deighton *et al.,* 1992(b); Lewis *et al.,* 1990; Proctor *et al.,* 1995) therefore, genotypic identification is emerging as an alternative or complement to established phenotypic methods.

Genotypic and phenotypic methods have provided new insight into the understanding of pathogenesis associated with staphylococcal infections due to

phase variations of *S. aureus* small colony variants as a persistent and intracellular pathogen. Changes in local environmental factors, both *in vitro* and *in vivo*, seem to affect the rates of phase change. The discovery that antibiotics are capable of modifying both the structural appearance and physiology of pathogenic bacteria when they are incorporated in the culture medium at sub-growth inhibitory concentrations, has led to numerous examples in which the expression of one or more virulence factors (both structural and soluble) is inhibited or potentiated (Gemmell, 1991). Accordingly, the finding that SCVs have been recovered from patients with unusually persistent infections, particularly those patients with long disease-free intervals, and from patients who are chronically exposed to aminoglycosides and trimethoprim sulfamethoxazole (TMP-SMZ), suggest that these clinical situations are those in which SCVs should be suspected and the clinical laboratory should carefully search for them (Proctor and Peters, 1998). These observations support the suggestion that SCVs are phenotypic variants which could be induced by antibiotic effects on the wild type leading to the appearance of phenotypic variations including slow growth, atypical colonial morphology and unusual biochemical profile (Proctor *et al.*, 1998).

Generally, variations could result from plasmid loss or gain, insertion or deletion of phage DNA, transposition of DNA sequences to a new expression site, or promoter inversion (Borst and Greaves, 1987). For some organisms, programmed gene rearrangements may control the expression of surface antigens and virulence determinants and thus regulate the interaction of the microorganism with its environment (Borst and Greaves, 1987). The purpose of these programmed gene rearrangements appears to be to prepare a small fraction of cells for environmental changes (Borst and Greaves, 1987).

1:2:1 Staphylococcal Small Colony Variants (SCVs): Variants Associated with Persistent and Recurrent Infections

Although *S. aureus* SCVs were recognized more than 80 years ago, these phenotypes has only been linked to persistent, resistant, and recurrent infections by

many researchers in the last ten years or so (Mitsujama *et al.*, 1997; Proctor *et al.*, 1995; von Eiff *et al.*, 1997). A wide variety of other species are known to form SCVs, e.g. *Pseudomonas aeruginosa, Salmonella typhimurium, Shigella species, Brucella abortus, E. coli, Lactobacillus acidophilus, Serratia marcescens,* and *Neisseria gonorrhoeae* and infection with these organisms may also cause persistent infections (von Eiff *et al.*, 2000).

Even in patients whose acute infection initially seems to respond to antimicrobial treatment, chronic relapsing disease characterised by long periods of quiescence between episodes of acute illness may occur (Korovessis *et al.*, 1991; Waldvogel, 1995). Specific microbiological factors associated with such antibiotic failure have not been fully defined, but small colony variants (SCVs) of *S. aureus* have been isolated from patients with chronic, relapsing and antibiotic-resistant infections (Proctor *et al.*, 1994; Proctor *et al.*, 1995). In the past, the frequency of *S. aureus* SCVs among clinical isolates has not been established by prospective studies, except for one blood culture study where stable SCVs were isolated from 1% of patients (Proctor *et al.*, 1995). Since then, clinical specimens from cystic fibrosis (CF) patients and from patients with chronic osteomyelitis, both groups of patients who frequently have staphylococcal infections and receive large quantities of antibiotics, were prospectively evaluated for the presence of *S. aureus* SCVs (Acar *et al.*, 1978; Proctor *et al.*, 1995)

The longest disease free interval was 53 years, in a patient who had had osteomyelitis of the left femur at age 16 and whose symptoms did not recur until age 69 years (Proctor *et al.*, 1995). The potential for *S. aureus* SCVs to cause resistant, persistent, and recurrent infections may arise from their ability to reside within host cells without causing lysis of the host cell (Balwit *et al.*, 1994; Vann and Proctor, 1988). SCVs are ingested by non-professional phagocytes as efficiently as their parental strains, but they produce only small amounts of α-toxin and are therefore able to persist within the host cells for a prolonged time (Vesga *et al.*, 1996; Proctor *et al.*, 1994). As SCVs are often relatively unstable, they can revert to the highly virulent, rapidly growing form and lyse the host cell, once the host immune response has decreased or antibiotic therapy is completed.

Compared to SCVs of *S. aureus,* relatively little is known regarding infections caused by SCVs of coagulase-negative staphylococci (CoNS). There are only two well-described cases of infection due to phenotypic variants of CoNS (Baddour *et al.,* 1990). Most recently, another two cases of pacemaker electrode infections due to SCVs of *S. epidermidis* and *S. capitis*, respectively, were described (Von Eiff *et al.,* 1999). In these patients, isolates from blood cultures were obtained over at least a two-week interval and visible growth of SCVs on subculture plates was seen only after 48-72 h incubation at 37°C yielding very small colonies. The organisms were colonially pleomorphic and polymicrobial infection was suspected. For most isolates biochemical testing with API Staph strips and conventional macrotube testing was not possible, except for the finding of catalase positivity, because their limited growth over 48 hours was not sufficient to provide positive test results in the reaction vials. Therefore, two approaches were used to identify the positive blood cultures. First, analysis of a portion of the 16S rDNA sequence analysis confirmed the identity of the staphylococcal species as *S. capitis* and *S. epidermidis*. Second, *SmaI* digests of the whole bacterial genome of all isolates analyzed by PFGE showed that they were clonal, even though the colony morphology was very different. Thus, the isolates in both cases were proved to be from the same species and the subpopulations were also from the same clone. These cases show that SCVs of CoNS, as with those of *S. aureus*, must be actively sought, because they grow very slowly and can easily be missed and indicate that molecular techniques may be required for identification of SCVs.

1:2:2 Emergence of Variant Forms after Exposure to Aminoglycosides: Mechanism for Development of SCVs

In the past, most studies have addressed the problem of antibiotic-resistant infectious diseases from the standpoint of classic forms of antibiotic resistance mainly founded on the possession of resistance genes. However, bacteria such as *S. aureus* may have additional mechanisms for resisting therapy that extend beyond these classic mechanisms. In patients whose acute infection initially

responded to antimicrobial treatment, where infection recurred after long disease-free intervals or where infection persisted despite appropriate antibiotic treatment, SCVs of *S. aureus* were recovered. For many antibiotics, SCVs mechanism of resistance has not yet been identified, but it might involve some new mechanism related to their small colony morphology.

S. aureus SCVs are a subpopulation, which are identified in the microbiological laboratory as nonpigmented, non-haemolytic, and slow-growing pinpoint colonies. However, they can be induced *in vitro* by exposure of *S. aureus* strains to lithium and barium chloride, gentian violet, acridines, and antibiotics including aminglycosides and penicillin (Quie, 1969; Swingle, 1934; Youmans, 1937). Variants were initially distinguished as (a) those strains that maintain the minute colony phenotype only in the presence of the selective agent (Schnitzer *et al.*, 1943) and (b) strains which were stable in the absence of the selective agent. Although many variants spontaneously revert on continued subculture, some strains are stable on multiple passages (Schliefer and Kloos, 1975). A number of variants have reverted to normal *S. aureus* characteristics when metabolic defects were compensated by supplementation of the medium with amino acids, menadione, thiamine, haemin, pantothenate, or incubation in a carbon dioxide containing atmosphere (Kloos and Schliefer, 1975; Kloos and Bannerman, 1994). This leads to the definition of auxotrophy, as the inability of an organism to synthesize a particular organic compound required for its growth. This organism cannot grow on a minimal medium and therefore, requires nutrient supplements such as amino acids added to the minimal medium. When a bacterial culture is incubated in a medium lacking specific nutrient, auxotrophic bacteria present in the culture that require this nutrient will not be able to grow.

Many clinical and laboratory SCVs isolated as minute colonies on solid agar show auxotrophy for thymidine, menadione, or haemin (Acar *et al.*, 1978; Kahl *et al.*, 1998; Proctor *et al.*, 1997; Proctor *et al.*, 1995; von Eiff *et al.*, 1997). When the medium is supplemented with these compounds, SCVs grow as rapidly as the parent strains. Menadione and haemin are required for biosynthesis of electron transport chain components (Proctor *et al.*, 1997), while the mechanism

for thymidine auxotrophy formation is not likely to be related to that for the electron transport type SCVs (Gilligan *et al.*, 1987). However, thymidine auxotrophy could arise from inhibition of the synthesis of tetrahydrofolate, a cofactor for the synthesis of thymidine (Jones *et al.*, 1989) or by any agent interfering with DNA gyrase and topoisomerase IV, which are two essential ATP-dependent enzymes that function in double strand DNA breakage and rejoining to promote DNA unwinding during DNA replication and chromosome segregation at cell division (Drlica and Zhao, 1997; Wang, 1996). SCVs which are thymidine auxotrophs emerge due to long term treatment with trimethoprim-sulfamethoxazole (SXT). In addition, the often relatively unstable SCVs, demonstrate a number of other characteristics that are atypical for *S. aureus* making the correct identification difficult, including: reduced α-toxin production, delayed or absent coagulase activity, lack of haemolysins and pigment production (Balwit *et al.*, 1994), failure to ferment mannitol and increased resistance to aminoglycosides and cell-wall active antibiotics.

Davis and Lederberg many years ago established a way to select for auxotrophic bacteria. They noticed that upon exposure to penicillin, these non-growing auxotrophs would survive the antibiotic treatment while the majority of prototrophic cells will not (Davis, 1949; Lederberg, 1956). Afterwards, this technique was used for the selection of auxotrophic variants from many bacterial species

Aminoglycosides antibiotics, such as gentamicin, kanamycin, streptomycin, and neomycin, inhibit protein synthesis by binding to the 30S ribosomal subunits. SCVs are more resistant to aminoglycosides and can be recovered *in vitro* from cultures of normal *S. aureus* strains exposed to gentamicin (Balwit *et al.*, 1994; Musher *et al.*, 1977; Lewis *et al.*, 1990).

The major mechanism of aminoglycoside resistance observed in the staphylococci is drug inactivation by transferases and other enzymes. However, SCVs have additional mechanisms for resisting antibiotic therapy, apart from these classical ones. The biochemical basis for the SCV phenotype has been linked to deficient electron transport- impaired respiratory activity (Freman and White,

1967; Lewis *et al.,* 1990; Swingle, 1934), and is discussed in terms of bacterial auxotrophy (Goudie and Goudie, 1955; Pelletier *et al.,* 1979; Weinberg, 1950; Youmans, 1937). An interruption in electron transport reduces the electrochemical gradient across the bacterial membrane, resulting in decreased uptake of antimicrobial agents such as aminoglycosides (Acar *et al.,* 1978; Koo *et al.,* 1996) which depend on this to enter the cell and reach their target site.

Mitsujama *et al* (1997) had described the emergence of staphylococcal SCVs after exposure of wild-type parent strains to a MIC concentration of the fluoroquinolone pazufloxacin. Emergent SCVs were half as susceptible to pazufloxacin and ciprofloxacin as wild-type *S. aureus.* Reduced susceptibilities of SCVs to these compounds were not a result of mutations but rather were mediated by decreased fluoroquinolone uptake, perhaps as a result of permeability changes in the cell wall (Proctor *et al.,* 1994).

In comparison with SCVs of *S. aureus,* relatively little is known regarding factors that generate or properties of CoNS SCVs. Characteristics of SCVs form of *S. epidermidis* have been well described and include the following: (1) colonies that are translucent or sometimes opaque, (2) colonies that vary in size and are usually <1 mm in diameter. In the literature, SCVs isolated from cases of infection were not characterized for electron-transport deficiency or for their auxotrophism (Baddour *et al.,* 1987; Baddour *et al.,* 1990) until 1999, when von Eiff *et al* (1999) reported bloodstream infections caused by *S. epidermidis* and *S. capitis* following pacemaker implantation. These SCVs were shown to be haemin auxotrophs.

1:2:3 Pathogenicity and Virulence of Staphylococcus SCVs

The ability to form a variant subpopulation (SCVs) affords *S. aureus* a number of survival advantages that extend beyond simply increased resistance to antibiotics: (1) *S. aureus* with normal phenotype are phagocytosed by non-professional phagocytes and produce large amounts of α-toxin that quickly lyses

mammalian cells. In contrast, while it has been demonstrated that SCVs can be ingested by host cells, they produce only small amounts of α-toxin and are able to persist within the non-professional phagocytes (von Eiff *et al.*, 2000 (a) & (b)).

(2) As SCVs are often relatively unstable, they can hide within the host cell, then revert to the highly virulent rapidly growing form and lyse the host cell, once the host immune response has abated and antibiotic therapy is completed. Thus, the intracellular position shields SCVs from host defences and decreases exposure to antibiotics (Kahl *et al.*, 1998; Balwit *et al.*, 1994; von Eiff *et al.*, 1997; Proctor *et al.*, 1994; Vesga *et al.*, 1996).

Bayston and Wood (1997) postulated that SCVs might represent the true biofilm mode phenotype since some characteristics (slow growth rate, altered metabolism, resistance to antibiotics and auxotrophy) have been found in common.

1:3 Bacterial Invasion of Host Cells

Invasion of host cells by bacteria is a characteristic feature of a number of infectious diseases caused by S*higella spp, Salmonella spp, Listeria monocytogenes* and many others. Microorganisms capable of surviving within phagocytes are rare; but represent very successful pathogens, such as *Mycobacterium tuberculosis* (Ernst *et al.*, 1999). Other pathogens, such as *S. aureus* have developed strategies to evade phagocytosis. It has been suggested that invasion enables bacteria to evade host defences and seek out new supplies of nutrients or find new tissues to colonize. Bacteria have evolved a variety of invasion mechanisms; some common elements in these processes are evident as most involve the manipulation of normal host cell cytoskeletal components such as actin and tubulin, resulting in the invagination of host cell membrane to enclose the bacterium within a vacuole. This often occurs by interference with the cellular signalling pathways either by stimulation of inhibition of signal transduction, or both (Miller, 1995). During the past decade, the majority of investigations of bacterial invasion have employed cell culture models, where enormous amounts of information have been derived using epithelial, endothelial and macrophage cell lines to elucidate some aspects of the pathogenesis of bacterial disease.

The first stage in an invasion process is adhesion of the invading organism to a host cell, which ultimately leads to invasion. Obviously after an organism has invaded a host cell, there are consequences for the host cell and for the bacterium (Finlay and Cossart, 1997).

Invasion of a host cell by a bacterium can stimulate cytokine release, prostaglandin release, and expression of adhesion molecules; eventually it could cause cell death. Once a bacterium has invaded a host cell, it has a limited number of options regarding where it subsequently survives and grows. It may continue to live within the vacuole enclosing it, it may exit from the vacuole and live in the cytoplasm or it may exit the cell and maintain an extracellular existence (Garcia-del Portillo and Finlay, 1995). While the microbiocidal mechanisms of

phagocytes are extremely efficient and capable of killing most microorganisms, pathogenic microorganisms have developed mechanisms to resist phagocytes.

In general, *S. aureus* and S. *epidermidis* are not considered a significant intracellular pathogen when compared with genera such as *Listeria* and *Shigella.* Staphylococci have typically been regarded as non-invasive extracellular pathogens that damage host cells after adhering to the extracellular matrix; however, there is growing evidence that *S. aureus* has the ability to invade and persist within eukaryotic cells. From a clinical standpoint, this observation could yield insights into mechanisms relevant to persistent and recurrent staphylococcal infections, and to at least a subset of instances where staphylococcal infections are refractory to antimicrobial therapy (Alexander and Hudson, 2001). Indeed, when *S. aureus* is incubated with endothelial cells, fibroblasts and epithelial cells, a massive internalization of the bacteria by the cells is observed (Hamill *et al.,* 1986; Sinha *et al.,* 1999). Hussain *et al* (2002) have reported that Eap contributed to the pathogenecity of *S. aureus* by promoting adhesion of whole staphylococcal cells to complex eukaryotic substrate. *S. aureus* has been reported to be capable of escaping killing by professional phagocytes through hiding in the intracellular space of other cell types (Krause, 2000).

Vesga *et al* demonstrated that the intracellular milieu of endothelial cells selects for staphylococcal small colony variants (Vesga *et al.,* 1996). These menadione or haemin auxotrophs are alpha-toxin deficient and appear capable of prolonged intracellular survival.

How microorganisms are phagocytosed and killed, and why certain pathogens resist these mechanisms, are crucial questions for an understanding of the pathogenesis of infectious diseases and the development of innovative treatment approaches.

1:3:1 Staphylococcal Internalization by Mammalian Host Cells

Phagocytes have been divided into: (1) professional phagocytes (granulocytes and macrophages) which are capable of engulfing relatively large microorganisms and killing them with a combination of various microbiocidal systems, (2) non-professional phagocytes like fibroblasts, osteoblasts, epithelial and endothelial cells (Krause, 2000). While the microbiocidal mechanisms of phagocytes in general, and in particular the professional phagocytes which are extremely efficient and capable of killing most microorganisms, pathogenic microorganisms have developed mechanisms to resist phagocytes.

Staphylococci such as *S. aureus* and *S. epidermidis* are classically considered as extracellular pathogens (Lowy, 1998; Lowy, 2000). However, certain features of Staphylococcal diseases suggest that Staphylococci have the capacity to function as an intracellular pathogen. In this regard, recent experiments assessing invasion and intracellular survival of *S. aureus* in endothelial cells (Vesga *et al.,* 1996), epithelial cells (Almeida *et al.,* 1996; Bayles *et al.,* 1998), and osteoblasts (Ahmed *et al.,* 2001; Ellington *et al.,* 1999; Hudson *et al.,* 1995; Reilly *et al.,* 2000) have suggested that intracellular survival could contribute to the persistence of the pathogen in *S. aureus*-induced endocarditis, bovine mastitis, and osteomyelitis causing approximately 80-90% of all cases of human osteomyelitis (Nair *et al.,* 2000). *S. aureus* has been recognised as an important pathogen for a long time and is responsible for significant human morbidity and mortality, causing infections of soft tissues, joints, bones, and the cardiovascular system (Vann and Proctor; 1987). Studies have shown that *S. aureus* adheres to cultured endothelial cells (Ogawa *et al.,* 1985), and various reports indicate that 31 to 70% of *S. aureus* endocarditis occurs in patients having no known pre-existing heart disease (Thompson, 1982; Watanakunakorn, 1973). The discovery that cultured endothelial cells can phagocytize *S. aureus* (Hamill *et al.,* 1986; Menzies *et al.,* 1998; Ogawa *et al.,* 1985) suggests that *S. aureus* could be phagocytized by the endothelium *in vivo* as well, with the ingested *S. aureus* possibly playing a role in

the initiation of *S. aureus* infectious endocarditis and the persistence of *S. aureus* septicaemia.

The possibility that phagocytes, particularly neutrophils (granulocytes), could facilitate *S. aureus* infections has also been proposed by other investigators (Craven and Anderson, 1979; Rogers and Melly, 1965). *In vitro* studies from the 1950s and 1960s demonstrated that pathogenic strains of *S. aureus* could survive for long periods of time inside both neutrophils and monocytes isolated from different animals and humans (Melly *et al.*, 1960). Neutrophils have long been regarded as essential for host defence against *S. aureus* infection. Gresham *et al* suggested that the ability of *S. aureus* to exploit the inflammatory response of the host by surviving inside neutrophils is a virulence mechanism for this pathogen, and that modulation of the inflammatory response is sufficient to significantly alter morbidity and mortality induced by *S. aureus* infection (Gresham *et al.*, 2000).

There is little experimental data demonstrating the pathogenic role for intracellular *S. epidermidis*, simply because *S. epidermidis* has been considered for a long time to be apathogenic or commensal, due to its distinctly reduced arsenal of toxins compared to *S. aureus* (Vuong and Otto, 2002). *S. epidermidis* has received considerable interest in recent years because it has become the most important cause of nosocomial infections. *S. epidermidis* is one of the most often isolated bacterial species in hospitals in general and is recognized as the most important pathogen involved in nosocomial bloodstream infections and cardiovascular infections (Vuong and Otto, 2002).

S. epidermidis was found to be internalized by bovine mammary epithelial cells *in vitro* emphasizing their importance as a cause of mastitis (Almeida and Oliver, 2001). Merkel and Scofield on the other hand studied *S. epidermidis* interaction with endothelial cells. Electron microscopy demonstrated that *S. epidermidis* was internalized and appeared to exist free in the cytoplasm (Merkel and Scofield, 2001). This could be a contribution towards explaining the relapse in native endocarditis (Veltrop *et al.*, 2001) and the need for re-operation in prosthetic valve endocarditis (Hagl *et al.*, 2002).

Survival of *S. epidermidis* inside macrophages has been recognized as an important process in the pathogenesis of BRI. Local host defense is compromised because of the presence of the implanted biomaterials, resulting in macrophage deactivation and subsequent deficient intracellular killing (Boelens *et al.*, 2000(b)). Moreover, *S. epidermidis* isolated from infected hip prostheses was shown to have an enhanced capacity to resist phagocytosis and elicited a reduced inflammatory response, measured as the production of extracellular oxidative metabolites from the neutrophils (Augustinsson *et al.*, 2001). Moran *et al* (1998) suggested that *S. epidermidis* which are able to produce slime possess additional properties which make them resistant to the action of neutrophils which could contribute to their intracellular survival.

1:3:2 Mechanisms of Staphylococcal Invasion of Cultured Cells

The ability of bacteria to interact with host cell receptors and trigger their own internalization has been clearly demonstrated for members of several bacterial genera including *Salmonella* and *Yersinia* (Finlay and Cossart, 1997; Pace *et al.*, 1993). *S. aureus* is hypothesized to invade tissue via interaction with host cells in one of four ways: (1) by binding directly to epithelial cells; (2) by invading sites of cellular damage where matrix proteins are exposed; (3) by migrating between microvascular capillary endothelial cells; or (4) by binding to host cells via plasma or cellular proteins that are adherent to both cell and bacteria (i.e. bridging ligands) (Lowy, 2000). So for *S. aureus* to be internalized, bacterial adherence to the host cell is a prerequisite for invasion. *S. aureus* MSCRAMMs include fibrinogen-binding proteins (Boden and Flock, 1994; Cheung *et al.*, 1995), fibronectin-binding proteins (FnBPs) (Hynes, 1990; Jonsson *et al.*, 1991), elastin-binding adhesin (Park *et al.*, 1999), collagen-binding adhesin (Patti *et al.*, 1992), and a broad-specificity adhesin (Map) that facilitates low-affinity binding of *S. aureus* to several proteins, including osteopontin, collagen, bone sialoprotein, fibronectin, fibrinogen, and vitronectin (McGavin *et al.*, 1993). FnBPs are likely the most-important MSCRAMMs in *S. aureus* invasion of eukaryotic cells.

Eap, an analogue of Map, was found to be important in promoting adhesion of *S. aureus* to eukaryotic substrates which might contribute to *S. aureus* pathogenicity (Hussain *et al.*, 2001, Hussain *et al.*, 2002).

Studies comparing adhesion of FnBP mutants and their isogenic parental strains have revealed that FnBPs are required for *S. aureus* adherence to bovine mammary epithelial cells (Dziewanowska, 1999; Lammers *et al.*, 1999), human endothelial cells (Peacock *et al.*, 1999), and human osteoblasts (Ahmed *et al.*, 2001). After bacterial adhesion to host tissues, some signaling and biochemical pathways are activated for the next step: the internalization process. Studies have shown that *S. aureus* utilizes the cytoskeleton (actin microfilaments, microtubules) and receptor-mediated endocytosis for the uptake of *S. aureus* into host cells (Alexander and Hudson, 2001).

Receptor-mediated endocytosis (RME) involves the formation and uptake of clathrin-coated pits via rearrangement of the cytoskeleton. Experiments on *S. aureus* implicate actin microfilaments, microtubules, and RME in the staphylococcal internalization (Jevon *et al.*, 1999). Results indicate inhibition of actin microfilament polymerization with cytochalasin D (cytochalasin D binds to the rapidly growing F-actin and induces depolymerization) decreases numbers of intracellular *S. aureus* cells by 95% in a human osteoblast cell line (Jevon *et al.*, 1999) compared with controls. The use of colchicine (colchicine, which binds to tubulin dimers and inhibits polymerisation of microtubules) reduced intracellular *S. aureus* numbers by 40% in a human osteoblast cell line (Jevon *et al.*, 1999) compared with controls. However, a decrease in internalization was not observed with bovine mammary epithelial cells (Almeida *et al.*, 1996); though the use of monodansylcadaverine (MDC) (transaminase inhibitor, which inhibits clathrin-dependent RME) decreased intracellular *S. aureus* numbers by 95% in a human osteoblast cell line (Jevon *et al.*, 1999) compared with controls. These studies suggest that activation of signal transduction cascades plays an important role in internalization of *S. aureus* by non-professional phagocytes. Menzies and Kourteva studied the internalization of *S. aureus* by endothelial cells. Their work showed that cytochalasin D alone is

enough to inhibit *S. aureus* internalization by endothelial cells (Menzies and Kourteva, 1998). In an experiment to examine neutrophil concentrations that would be lethal to a clinical isolate of *S. epidermidis*, Li *et al* (2002) found that killing of *S. epidermidis* at all neutrophil concentrations was blocked by cytochalasin D confirming that killing depends on phagocytosis and not on microbiocidal substances secreted into the medium by neutrophils (Barkalow and Hartwig, 1995).

Almeida and Oliver (2001) have studied the interaction of *S. epidermidis* with bovine mammary epithelial cells, and investigated the involvement of host cell signal transduction and host cell cytoskeleton rearrangement on bacterial internalization. The inhibitory effect on internalization caused by cytochalasin B and D, but not colchicine, suggests the involvement of host cytoskeleton elements, particularly F-actin microfilaments. Also, it appeared that *S. epidermidis* generated a self uptake signal that was transduced into the host cell using the host cell's own signal transduction pathways. In addition, Mamo *et al* (1988) reported that *S. epidermidis* bound fibronectin and collagen and that could contribute to adherence to mammalian cells.

1:3:3 *Staphylococcus aureus* Small Colony Variants are Induced by the Endothelial Cell Intracellular Milieu: A Possible Link to Persistent Infection and Antimicrobial Resistance

To the best of my knowledge, Vesga *et al* (1996) were the first to show that SCVs are induced by the endothelial cell intracellular milieu. They suggested that this could be a possible mechanism for the intriguing patho-physiology of tissue persistence of staphylococci (Vesga *et al.*, 1996). Their experiments were designed to comprehend the process by which SCVs of *S. aureus* appear in subjects who have not received antibiotic treatment. Bovine endothelial cells were incubated with a wild type *S. aureus* strain for 72 h. and results showed that intracellular bacteria developed the SCV phenotype at a greater rate than control

bacteria not exposed to endothelial cells. The intracellular induction rate was approximately 10^{-3} versus a spontaneous rate of $<10^{-7}$ (Vesga *et al.*, 1996).

On the other hand, von Eiff *et al* (2001) described the first known case of persistent and antibiotic resistant skin infection with different phenotypes and genotypes of *S. aureus.* This is also the first demonstration of intracellular persistence within keratinocytes.

Recently, it has been reported that fibroblast cells are capable of restricting *Salmonella enterica serovar typhimurium* intracellular growth. Cano *et al* have shown that prolonged residence of bacteria in the intracellular environment of fibroblasts results in the appearance of genetically stable SCVs (Cano *et al.*, 2003). Their data demonstrate that *S. enterica* SCVs display enhanced intracellular persistence in eukaryotic cells. In addition, they also suggest that *S. enterica* SCVs may be favoured *in vivo.*

However, up to this date there is no publication describing CoNS SCVs induced by eukaryotic cells. The only data covering CoNS (*S. epidermidis*) SCVs, was their involvement in bloodstream infections following pacemaker implantation (von Eiff *et al.*, 1999).

1:3:4 Consequences of Internalization in Human Infections

The ability of a bacterium to adhere, invade, evade host defences and cause tissue damage is largely due to its ability to produce a variety of virulence factors. Additionally, internalization may provide a means of protection against host defences and certain classes of antibiotics. The ability of *S. aureus* to stay alive in the eukaryotic intracellular environment could explain several aspects of chronic staphylococcal diseases and long standing colonization. Many staphylococcal infections that tend to become chronic (e.g., osteomyelitis and mastitis) are associated with multiple recurrences and do not resolve even in the presence of an adequate humoral immune response.

Exploring bacterial invasion further, two aspects can be considered: (1) effects on host cells and (2) effect on bacteria. The two most important effects on host cells

are (1) cytokine release and (2) cell death. However invasion of a host cell also limits the options of the bacterium: (1) it may continue to live within the vacuole enclosing it, (2) it may live in the cytoplasm, (3) it may exit the vacuole and maintain an extracellular existence; (4) in staphylococcal internalization the production of SCVs could be another outcome (Proctor *et al.*, 1995).

Cytokines are inflammatory mediators which may serve to activate host defence systems or, because of their production, may have adverse consequences for the host. It has become clear over the past few years that commensal and pathogenic bacteria contain and release a wide range of proteins, carbohydrates and lipids which can induce cytokine synthesis (Henderson *et al.*, 1996(a)).

Lipopolysaccharide (LPS), which is a major cell wall component of Gram-negative organisms (Rietschel and Brade, 1992), triggers the inflammatory responses associated with sepsis including cell adhesion molecule expression and release of IL-8 in endothelial cells (Blease *et al.*, 1998; Brown *et al.*, 1994). However, currently most of the papers describing non-LPS cytokine inducing molecules from bacteria, have reported that they induce the formation of pro-inflammatory cytokines such as IL-1, TNFα, IL-6 or IL -8 (Henderson *et al.*, 1996(b)). In Gram-positive bacteria, the cell walls are made up of layer of peptidoglycan embedded with teichoic acids, including lipoteichoic acid (Bone, 1994). These components were shown to stimulate cell adhesion molecule expression and IL-8 release in HUVECs (Kawamura *et al.*, 1995). Endothelial cells are capable of producing and releasing IL-6 and IL-8 in response to exposure to *S. aureus* strains or staphylococcal exotoxins such as α-toxin, enterotoxin A and TSST-1 (Sioderquist *et al.*, 1998).

Monocytes and macrophages as well as neutrophils produce most of the cytokines involved in host defense but most of the cytokines can also be produced by other cells, including endothelial cells, fibroblasts, and lymphocytes. The early cytokines, TNFα and IL-1, induce an increase of adhesion molecules on endothelial cells and induce inflammatory cells to promote migration of neutrophils into the tissues invaded by bacteria. At the same time or somewhat later, cytokines IL-8, IL-10, and IL-12 are produced by the mononuclear cells and

neutrophils (Scott, 1993). IL-8 induces neutrophils to migrate through the endothelial cell layer into the tissue and activates the neutrophil respiratory burst so that the cell is better equipped to kill bacteria. However, IL-10 down regulates IL-8 and may play an important role in the resolution of an inflammatory response (Scott, 1993; Rajarathnam *et al.*, 1994; Wang *et al.*, 1994).

IL-12 is gaining more attention because it upregulates the production of interferon γ (IFN-γ) by natural killer cells (NK) cells. It is worth mentioning that IL-12 ↔ IFN-γ axis is of primary importance for adequate activation of the macrophage (Scott, 1993). One important activator of the process of chemotaxis is the cytokine IL-8. Other neutrophil and monocyte triggers include the platelet activating factor, a phospholipid that is induced by endothelial cells after stimulation with leukotriene, TNF-α, and IL-4. IL-8 not only induces chemotaxis of neutrophils but triggers their adhesion as well (Rajarathnam *et al.*, 1994). Many of the triggers needed for the adhesion of neutrophils to endothelial cells, and for subsequent chemotaxis, are produced by the endothelial cells themselves while others are from the monocytes and bacteria.

Endothelial cells were initially thought to have a passive role in vascular physiology, functioning mainly to modulate coagulation (Mantovani *et al.*, 1992). Recent studies, however, have demonstrated a potent inflammatory role for endothelial cells, either by secretion of inflammatory mediators or by modulation of adhesion of leukocytes to their surfaces (Wang *et al.*, 1996; Cannan *et al.*, 1998). Endothelial cells are influenced by cytokine producers and are themselves capable of producing a number of endogenous cytokines.

Human endothelium is capable of expressing interleukins IL-1, IL-5, IL-6, IL-8, monocytes chemotactic protein-1 (MCP-1), growth-related oncogene-α (GRO-α) protein, and colony stimulating factors, granulocytes-macrophage CSF (GM-CSF), and platelet-derived growth factor (PDGF) (Krishnaswamy *et al.*, 1999). Endothelial cells also have been shown to express IL-11 and IL-15 (Mohamadzadeh *et al.*, 1996; Suen *et al.*, 1994).

Subsequent studies revealed that various *S. aureus* strains induce EC activation that resulted in production of the chemokines IL-8 and monocyte chemotactic protein-1, surface expression of intercellular adhesion molecule-1 (ICAM-1; CD54) and vascular cell adhesion molecule-1 (VCAM-1; CD106), as well as enhanced monocyte adhesion (Veltrop *et al*, 1999). In addition, internalization of *S. aureus* by human umbilical vein endothelial cells (HUVECs), results in the synthesis of mRNA for both IL-6 and IL-1β, enhancing endothelial secretion of the pro-inflammatory cytokines IL-1 and IL-6 (Yao *et al.,* 1995; Yao *et al.,* 1996), and eliciting procoagulant activity in these cells (Drake and Pang, 1988). Treatment of the HUVEC cells with cytochalasin D inhibited cytokine gene expression, demonstrating that cytokine induction was a consequence of bacterial invasion rather than adhesion (Yao *et al.,* 1995).

Neutrophil-activating protein, or IL-8, is a potent neutrophil chemoattractant and is directly involved in neutrophil recruitment (Smith *et al.,* 1991(b)). Circulating levels of IL-8 are correlated with the severity of tissue damage after *S. aureus* infection (Mulligan *et al.,* 1993). The pro-inflammatory cytokines tumor necrosis factor alpha (TNF) and IL-1β have been shown to induce IL-8 gene expression in a variety of cell types, including monocytes, neutrophils, and endothelial cells (Yao *et al.,* 1996). *S. aureus* invasion of osteoblasts stimulates the secretion of IL-6, IL-12, and colony-stimulating factors (Bost *et al.,* 1999; Bost *et al.,* 2000; Yao *et al.,* 1995). These cytokines can potentially aggravate bone destruction by activation of osteoclasts.

Cytokine release induced by *S. epidermidis* has rarely been studied. It received attention in patients who were treated with continuous ambulatory peritoneal dialysis (CAPD) and who might suffer from recurrent peritonitis episodes caused by *S. epidermidis*. Betjes *et al* (1993) have shown that human peritoneal mesothelial cells produced high levels of IL-8 in response to IL-1β and TNF-α but not *S. epidermidis*. However, co-culture of peritoneal macrophages with S. *epidermidis* induced high levels of IL-1α, IL-1β, and TNF-α. Taken together, these data indicate that mesothelial cells are important for the recruitment of granulocytes into the peritoneal cavity (Betjes *et al.,* 1993).

Intracellular growth and replication within host cells often leads to the death of the colonized cell as a result of nutrient depletion, degradation of important intercellular constituents and the build-up of toxic end products of bacterial metabolism – this is often termed necrotic cell death. However, bacterial invasion of the host cells can sometimes induce a second form of cell death known as apoptosis. Apoptosis, otherwise known as programmed cell death, is a natural process by which the host controls cell numbers and it is important in embryogenesis and the response to genetic damage. Apoptosis is a highly regulated process which involves the active participation of the cell and is controlled by complex signal transduction pathways. The apoptotic cells exhibit a characteristic appearance: membrane blebbing, typical chromatin condensation and fragmentation and the presence of apoptotic bodies (partition of cytoplasmic and nucleus into membrane bound-vesicles) which contain ribosomes, morphologically intact mitochondria and nuclear material (Cohen, 1993).

Staphylococcal infections are typically associated with death of tissue, and evidence suggests that intracellular bacteria are capable of inducing apoptosis. *S. aureus* provoking apoptosis has been reported in epithelial cells (Bayles *et al.*, 1998; Kahl *et al.*, 2000; Wesson *et al.*, 1998), endothelial cells (Menzies and Kourteva, 1998; Wesson *et al.*, 1998), osteoblasts (Tucker *et al.*, 2000) and keratinocytes (Nuzzo *et al.*, 2000).

The induction of apoptosis upon infection results from a complex interaction of bacterial proteins with cellular proteins finally mediating apoptosis. Thus, bacteria are able to activate several pro-apoptotic proteins, e.g. caspases.

Monitoring bacterial internalization provides useful information regarding studies on virulence and immunity of intercellular pathogens. Transmission electron micrographs of bovine aortic endothelial cell monolayers infected with *S. aureus* have demonstrated phagocytosis following a specific sequence of events:

(i) adherence of *S. aureus* to the endothelial cells, (ii) formation of cuplike processes on the endothelial cell surface underlying the adherent bacteria, and (iii) elongation of the cup and engulfment of the bacteria within phagosomes (Lowy *et al.*, 1988). Despite lysosomal fusion, the endothelial cell is unable to kill the

ingested *S. aureus*. Viable staphylococci can be isolated after treatment of infected endothelial cell monolayers with lysostaphin, which lyses extracellular staphylococci only. Uptake of *S. aureus* by endothelial cells offers a unique explanation for the frequency and persistence of invasive staphylococcal infections involving endothelialized surfaces such as heart valves, prosthetic vascular grafts, and catheterised vessels. Moreover, *S. aureus* invades a variety of mammalian cells and escapes from the endosome to multiply in the cytoplasm (Shompole *et al.*, 2003). More information on staphylococcal intracellular pathogenesis is required to give us new strategies for prevention and treatment of staphylococcal diseases. The production of *S. aureus* SCVs by the endothelial milieu could be another mechanism used by staphylococci to evade host defences and cause chronic infections-a major observed concern in infections caused by these organisms. SCVs intracellular survival also suggests a possible way for *S. aureus* to resist antibiotics.

1:4 Research Aims

1:4:1 Introduction

Altered phenotypes or small colony variants (SCVs) of *S. epidermidis* and *S. aureus* have been most frequently isolated from infected implants. Biomaterial-related infections (BRI) associated with SCV are characterized by treatment failure and chronicity resulting in re-operation, removal of the implant, and loss of function or even death. Therefore, BRI represents a major problem in modern medicine (Bayston, 2000).

The ability to produce SCVs could be considered as a virulence factor possessed by some wild type strains to avoid host defenses. Resistance of SCVs to antibiotics and their ability to avoid host defenses due to their intracellular survival could be behind the difficulty in curing chronic BRI. However, the present knowledge of SCVs is incomplete; much more fundamental work has to be directed towards better understanding of the pathogenic role of *S. epidermidis* SCV in BRI. Knowing the exact pathogenesis of BRI due to staphylococcal SCVs might allow the development of new strategies for the prevention of foreign body infections.

1:4:2 Aims

The overall aim of this project is therefore to investigate the potential role of staphylococcal SCVs as causative organisms in chronic, recurrent and antibiotic-resistant BRI. Particular reference will be made to *S. epidermidis* SCVs, as these staphylococci are most frequently implicated in BRI. The specific experimental objectives are as follows;

1. Determining the ability of wild type parent strains of staphylococci to produce stable SCVs following exposure to gentamicin *in vitro*.

2. Screening for metabolic or physiological characteristics of SCVs that might be connected with the true biofilm phenotype and contribute to pathogenesis of BRI.
3. Evaluating the potential role of SCVs to initiate BRI via distinct mechanisms of adhesion to polymer materials or conditioning film.
4. Investigating intercellular persistence of SCVs in infected HUVEC cells *in vitro* and their mechanisms of cell entry.
5. Seeking to determine whether the production of cytokines by HUVECs infected with SCVs differs from these infected with wild type parent staphylococci, a factor that could contribute to persistence of SCVs in BRI infections.

Chapter Two

Diagnostic Methods for Identification of Staphylococcal Clinical Isolates and their Corresponding Phenotypic Small Colony Variants (SCVs)

2:1 Introduction

The identification of a bacterial strain is a key element in the effective management and control of bacterial infections. Commercial kits are now available to ease the testing and to decrease the completion time required to get the identification information to the physicians on time.

Special consideration should be taken for the identification of the organism because the presence of atypical colonial forms in a culture was usually interpreted as evidence of contamination and has previously been disregarded by laboratory technicians. Probably due to this reason, until fairly recently *S. epidermidis* was often not regarded as a pathogen but as a culture contaminant, though it is now increasingly being recognized as a cause of infection in implants (Fleer and Verhoef, 1984).

Studies on SCVs of *S. aureus* have shown that they are often nonpigmented, non-haemolytic and coagulase-negative (Bayston and Wood, 1997). As they give the appearance of mixed cultures, they have frequently led to delay in diagnosis (Proctor *et al.*, 1995). SCVs are infrequently reported by clinical laboratories because they form very small colonies that are frequently not visible until 48-72 hours after inoculation (Proctor *et al.*, 1995). Therefore, clinicians and clinical lab personnel should consider making special efforts to search for SCVs.

Phenotypic variation (SCVs) within staphylococcal populations appearing in the presence of gentamicin could be a mechanism developed by the bacterium to survive in the presence of antibiotics. Entry into the SCV form is reversible upon growth in antibiotic-free medium. Cycling between the resistant SCV and the antibiotic sensitive wild colony form can occur multiple times, indicating that SCVs are not formed by mutation but through a regulatory mechanism or phase variation (Hogan and Kolter, 2002).

Chapter two describes the identification and the characterisation of members of the genus *Staphylococcus* which exhibit a characteristic phenotypic variation. The identification included morphological, growth, metabolic and biochemical properties. The genetic identification is included in Chapter three in this thesis.

2:2 Materials and Methods

2:2:1 Bacterial Strains and Growth Conditions

Clinical isolates of *Staphylococcus aureus*, *S. epidermidis* and *S. haemolyticus* from BRI were taken from laboratory -20°C freezer stocks which had been frozen in 10% vol/vol glycerol/TSB (Tryptone Soya Broth) in the Division of Orthopaedic and Accident Surgery at Nottingham City Hospital, UK. These organisms are registered by F (freezer) numbers, and for simplicity they are referred to in this thesis by numbers (1-28) as indicated in Table 2.1.

Whenever experimental work had to be undertaken, 5 μL of the frozen stock was plated on Sheep Blood agar ready poured plates (Oxoid, Basingstoke, UK) (BA) and on Tryptone soya agar plates (Oxoid, Basingstoke, UK) (TSA), incubated for 24 hours at 37°C. After incubation, a portion of one colony was plated out again on BA and TSA and incubated for 24 hours at 37°C.

Table 2.1: The origin of staphylococcal isolates used in this study

Bacterial Identification Number	Origin
1- F1585	*S. epidermidis,* Arterial graft infection
2- F1537*	*S. epidermidis,* Ventricular peritoneal shunt
3- F1483	*S. epidermidis,* Clinical isolate, laboratory modified PIA (+)*
4- F1594	*S. epidermidis,* No data about the origin
5- F1578	*S. epidermidis*, Pus in hip replacement
6- F1480	*S. epidermidis,* Clinical isolate, laboratory modified PIA(-)**
7- F1481	*S. epidermidis,* Clinical isolate, laboratory modified PIA(+)
8- F1484	*S. epidermidis,* Clinical isolate, laboratory modified PIA(-)
9- F1576	*S. epidermidis*, Tissue-hip replacement
10- F1577	*S. epidermidis*, Septic arthritis of prosthetic knee
11- F1490	*S. aureus,* Canadian strain, wound abscess
12- F1482	*S. epidermidis,* Clinical isolate laboratory modified as PIA(-)
13- F1513	*S. epidermidis,* Hip replacement
14- F1365	*S. epidermidis,* Catheter infection
15- F1538	*S. epidermidis*, Shunt valve infection
16- F1589	*S. haemolyticus*, Ventrical peritoneal Shunt infection
17- F1479	*S. epidermidis,* Hamburg Germany PIA (+)
18- F1342	*S. epidermidis*, Shunt infection
19- F1485**	*S. aureus,* MRSA***, wound abscess
20- F1596	*S. aureus*, Osteomyelitis
21- F1593	*S. epidermidis*, Ventrical peritoneal. Shunt infection
22- F1536*	*S. epidermidis*, 4th Ventrical peritoneal tip
23- F1588	*S. epidermidis,* CSF
24- F1575	*S. epidermidis*, Pus from hip replacement
25- F1592	*S. epidermidis*, shunt infection
26- F1512	*S. epidermidis,* Hip replacement
27- F1547**	*S. aureus*, MRSA wound abscess
28- F22	*S. epidermidis*, Shunt infection
Ox Staph	Oxford *S. aureus* used as control in antibiograms

*** 2 and 22 are from the same patient but different site. ** 19 and 27 are from the same patient but different sites. PIA(+)* = polysaccharide intercellular adhesin, PIA(-)**=the mutant.**
MRSA = methicillin resistant *S. aureus*, CSF = cerebrospinal fluid.

Table 2.1 summarises the origins of staphylococcal origin isolates from chronic infections which were used in this study. The isolates were *S. epidermidis* (n=23), *S. haemolyticus* (n=1) and *S. aureus* (n=4).

S. epidermidis strains (3, 6, 7,8,12 and 17) are PIA (polysaccharide intercellular adhesin) laboratory modified strains but they were originally isolated from chronic infections. These PIA mutant strains were provided by Professor Dietrich Mack, University of Krankenhaus Eppendorf, Hamburg, Germany.

2:2:2 Determination of Gentamicin MIC by E-Test

E-test- Epsilometer for antimicrobial susceptibility testing of bacteria (AB BIODISK, Sweden) - is a quantitative technology used to determine the Minimum Inhibitory Concentration (MIC in mg/mL) of individual antibiotics on agar medium. The Etest strip for gentamicin contains a gentamicin gradient that covers a concentration range of 0.016 to 256mg/mL and is applied to the surface of pre-seeded agar plates. After 24 hours of incubation, bacterial growth becomes visible with an inhibition ellipse which intersects with the strip indicating the MIC. Gentamicin MIC determinations of staphylococcal strains using E-test strips were performed according to the following protocol.

1. One well-defined isolated colony of a wild type strain was emulsified in phosphate buffered saline (PBS) (Oxoid, Basingstoke, UK) to achieve a suspension having a turbidity equivalent to a 0.5 McFarland standard (BioMérieux, Basingstoke, UK)
2. A sterile swab was dipped into the suspension and the swab was streaked over the entire half of an iso-sensitest agar plate (Oxoid Ltd, Basingstoke, UK) The other half of the plate was swabbed with a control Oxford *S. aureus* strain which is susceptible to all tested antibiotics (Ox-Staph)
3. The strip was then placed on the few millimetre gap left between the two areas
4. Agar plates were incubated at 37°C for 18 hours
5. After the required period of incubation, during which growth became distinctly visible, the MIC value was determined at the point of intersection between the zone edge and the E-test strip

6. In the ellipse, if small colonies were seen they were recorded as SCVs as indicated in Table 2.2

2:2:3 Gentamicin Microbroth Dilution Assay for SCVs Production

Microbroth dilution assay is a special susceptibility test used in the clinical setting and in research and was used to determine MICs for gentamicin in the present study. Gentamicin was tested at twofold serial dilutions, and the lowest concentration that inhibited visible growth of an organism was recorded as the MIC. Regrowth of the organisms after a certain time at concentrations equal or greater than the MIC triggers these heteroresistant subpopulations of gentamicin resistant staphylococci to emerge under antibiotic selection pressure. Such resistant clones may then change in the presence of subinhibitory concentrations of antibiotic to higher levels of gentamicin resistance. Microbroth dilution assay was used to generate SCVs over E-test because it showed reproducible results in generating stable SCVs.

The methodology used to determine the MIC is based on similar protocols used by other workers (Balows, 1991; Sören and Nilsson, 1984; Taylor *et al.,* 1982) and it is described below.

1. Autoclaved and cooled Mueller-Hinton broth (MHB) (Oxoid Ltd, Basingstoke, UK) was supplemented 50 mg/L filter-sterilized $CaCl_2$ (Sigma, Poole, UK) and 25mg/L $MgCl_2$ (Sigma, Poole, UK) solutions
2. A gentamicin solution was made up in MHB in a sufficient amount to achieve the desired starting concentration. Gentamicin was added to the first well of a flat-bottomed microtitre tray and was serially diluted along the plate to produce final concentrations in the range 256 to 0.06 μg/mL
3. Standard density turbidity (McFarland 0.5) was used to adjust the inoculum to ~10^8 CFU/mL. The inoculum was added to the wells to a final inoculum concentration of $5x10^4$ CFU per well
4. Standard strain of *Staphylococcus aureus* (Ox-Staph) was used in the same manner with each batch of susceptibility tests to serve as control (MIC 0.16mg/L for gentamicin)
5. Following inoculation, the trays were sealed with tape to prevent evaporation and incubated for 20 hours at 35°C

6. Trays were read by a micro ELISA reader (SpectraCount, Packard Bioscience, Bangbourne, UK) at 490nm and were further incubated for another day to check for any regrowth in the wells where no growth was seen after the first incubation for 20 hours

7. Five microlitres was taken out of the wells after two days incubation and plated out on TSA at 35 ^{0}C and incubated overnight to check for the presence of SCVs

8. The MIC was determined as the concentration of antibiotic in the well before the one displaying a sudden increase in absorbency (Table 2.3). The concentration of gentamicin in the wells containing the re-growth of the bacteria was subsequently used for SCVs production.

Based on data in Table 2.3, a protocol for producing pure and stable SCV was established as the following:

A. 1 mL of bacterial suspension (0.5 McFarland) was added to 9 mL MHB supplemented with $CaCl_2$ (50mg/L) and $MgCl_2$ (25mg/L) and gentamicin indicated by MHB^0 in Table 2.4. Broths were incubated overnight at 35°C statically

B. Next day, 1 mL of the bacterial suspension MHB^0 was added to another 9mL MHB with gentamicin, as indicated by MHB^1 in Table 2.4. Broths were incubated overnight at 35°C

C. On the third day, 5 µL from MHB^1 was plated out on TSA with 3 different concentrations of gentamicin in order to generate the most stable SCVs and least revertants. The three different concentrations of gentamicin used were: Half the concentration in MHB^1, gentamicin concentration same as in MHB^1, and twice the concentrations of MHB^1. Plates were incubated at 35°C for another day

D. Next day all three plates were examined for SCVs. The plate with the highest purity of SCVs was used for further consecutive subculturing. Table 2.4 summarises all the consecutive subcultures with different gentamicin concentrations for each strain. Figure 2.1 shows *S. epidermidis* wild strain (26) plated on BA before exposure to gentamicin. Figure 2.2 shows the SCVs generated in the same *S. epidermidis* strain after exposure to successive subculturing on plates supplemented with gentamicin.

2:2:4 API Staph

The API Staph (BioMérieux, Basingstoke, UK) is used as an identification system to identify staphylococci according to their biochemical profile. The strip of substrates is inoculated with a bacterial suspension, incubated and the results are read and interpreted with reference to the information contained in the kit. The identification is facilitated by the use of the API STAPH Analytical Profile Index or the identification software.

Wild colonies from TSA or SCV colonies from TSA with appropriate concentration of gentamicin were suspended in the medium provided in the kit and suspensions adjusted to a density equivalent to McFarland 0.6. Each cupule in the test strip was filled with the bacterial suspension. The arginine and urea cupules were sealed with mineral oil. The strip was then placed in a tray (with added sterile water) provided in the kit, covered and placed in a plastic box and incubated overnight at 37°C. Appropriate reagents were then added and the assays left for 10 minutes before the API reference number was read. The final reference number was a 7-digit Figure produced by adding the triplicate scores of the individual results. The data with the substrates tested and the readings of a positive and a negative result are illustrated in the Appendix 8:2:1 for the identification of the strains through their numerical profile. Any difference in the 7-digit Figure was typed in bold and interpreted accordingly as shown in Table 2.5.

2:2:5 Antibiotic Susceptibility Test (Antibiogram)

The antibiogram is another digital based reference test for bacterial strain identification (Bayston *et al.*, 1983). The antibiogram uses various antibiotic discs to determine the sensitivity of bacteria when compared to a fully sensitive control strain Oxford *Staphylococcus aureus* (OX-Staph). The antibiotics used and their concentrations are detailed in the Appendix 8:2:3.

Two diagnostic sensitivity test agar (D.S.T.A.) with plates containing 7% defibrinated horse blood (Oxoid, Basingstoke, UK) were prepared for each strain (Figure 2.3). A rotary inoculator was used to plate an OX-Staph suspension of McFarland 0.6 density in the inner circle and the test organism

around the outside leaving a boundary of 3mm between the two. Eight antibiotic discs were placed on the boundary of each plate by a dispenser and the plates incubated overnight at 37°C. The degree of susceptibility was ascertained by comparing the zone of inhibition of the test organism to that of the OX-Staph. Strains were classed as resistant if their zone of inhibition were more than 3mm smaller than the zone produced by the OX-Staph (Bayston, 1978). The resulting 5-digit figure, which defines the antibiogram produced for a particular strain was generated by combining together the triplicate scores. Teicoplanin and ciprofloxacin were included in the test; they were reported separately on separate plates. The sensitivity to methicillin was tested separately by spotting the test and control organisms (McFarland 0.6 density) onto two plates of TSA agar supplemented with 4% polyvinylpyrrolidone (PVP) with and without methicillin (12mg/L), followed by incubation overnight at 30°C (Bayston, 1978). The results for these tests are shown in Table 2.6.

2:2:6 Api Zym

API ZYM (BioMérieux, Basingstoke, UK) is a semi-quantitative and rapid micromethod designed for the detection of 19 enzymatic activities. The system consists of a strip with 20 microwells, the base of which contains the enzyme substrate and its buffer. The reactions are read according to the reading Table in the Appendix 8:2:2. The Api Zym profiles were generated as shown below.

1. A bacterial suspension with a turbidity of 5-6 McFarland units was prepared in sterile distilled water
2. Using a pipette, 65µL of suspension was dispensed into each microwell
3. After inoculation, the strip was placed in its box and incubated for 4 hours at 37°C
4. After the incubation, one drop of ZYM A (BioMérieux, Basingstoke, UK) reagent and one drop of ZYM B (BioMérieux, Basingstoke, UK) was added to each microwell
5. The colour was allowed to develop for at least 5 minutes

6. The reactions were read and recorded according to the reading table placed in the Appendix (8:2:2).
7. Each enzyme activity was assigned with a number. Any differences among the strains in their enzymatic activity were reported in bold font. The test was repeated twice for each strain. The results for these tests are presented in Table 2.7.

2:2:7 Screening for Catalase, Coagulase, Haemolysis and DNase

Catalase, coagulase and haemolysis are standard tests used in the diagnostic laboratory for the identification of *S. aureus* strains.
Staphylococci produce the enzyme catalase which decomposes hydrogen peroxide (H_2O_2) into oxygen (O_2) and water (H_2O). The catalase test has been used in laboratories to differentiate between staphylococci and streptococci which are catalase negative.
Coagulase causes blood plasma to clot. It is present only in *S. aureus* but not in CoNS and its property is the basis for the coagulase test. Two different coagulase tests can be performed: a tube test for free coagulase and a slide test for bound coagulase, or clumping factor (Seguin *et al.*, 1999). While the tube test is definitive, the slide test is used as a rapid screening technique to identify *S. aureus*.
Haemolysis is a simple test to detect haemolysin activities causing lysis of red blood cells. Haemolysis is recorded as α-haemolysis, β-haemolysis, no haemolysis or double haemolysis (α-, β-haemolysis) on sheep blood agar plates (Laevens *et al.,* 1996).
The DNase test is used to screen for *S. aureus* strains producing DNase which hydrolyse the deoxyribonucleic acid supplemented in the agar. After the incubation period, the addition of diluted hydrochloric acid (1N HCl) causes the DNA to precipitate out (turbidity) and clear zones appear around DNase-positive colonies.

Screening for Catalase: A portion of a colony growing on TSA was emulsified in one drop of water on the top of a slide. One drop of (3% v/v H_2O_2 in water) was added to the emulsified colony. Oxygen bubbles released

indicated a positive reaction. Results for SCVs and their wild type parents are shown in Table 2.7.

Coagulase Slide Test: A small portion of a colony was emulsified in one drop of citrated human plasma on a slide and left for 5 minutes incubated at 37°C in a sealed box. Any coagulation was reported as a positive test. Results for SCVs and their wild type parents are shown in Table 2.7.

Coagulase Tube Test: Approximately 0.1 mL of bacterial suspension was added to 0.3 mL of citrated human plasma. Tubes were placed in a water bath set at 37°C and read at 1,2,3,4 hours and after standing at room temperature for an additional 20 hours. The tube was tilted on its side gently to take the reading. Clotting of the plasma at the bottom of the tube was recorded as a positive test. The tube was tilted on its side gently to take the reading. Data for these analyses are presented in Table 2.8.

Haemolysis on Sheep Blood Agar: the presence of haemolysis activity was determined by the plating of a portion of a colony on a sheep blood agar plate and incubating the plate at 37°C overnight. Plates with SCVs profile were incubated for another day to check if they would maintain the same picture of haemolysis. The results were reported as α-haemolysis if the haemolysis was greenish in colour, while β-haemolysis was recorded if a clear zone surrounded the colonies and a double haemolysis (α-, β-haemolysis) when a clear zone of haemolysis was framed by a greenish colour. Results are displayed in Table 2.8 and Table 2.9.

DNase Agar Test: With a sterile cotton swab, 2-3 colonies were taken from TSA plate and placed (dotted) on DNase agar (Oxoid, Basingstoke, UK) plates. The plates were incubated at 37°C overnight. Next day the plates were flooded with 1N HCl and left for 5 minutes at room temperature. A clear zone around the growth was recorded as positive DNase test. Results from the DNase tests are shown in Table 2.8.

2:2:8 Lysostaphin Sensitivity

Lysostaphin is 25-kDa glycyl-glycine endopeptidase from *Staphylococcus simulans*. The enzyme's staphylolytic activity restricts its antibacterial activity to staphylococcal species (Schindler and Schuhardt, 1964). The test is considered positive if the tube contents (staphylococcal strain and lysostaphin) turn clear after 2 hours. All the 28 strains (the wild and their corresponding SCVs) were studied individually. The procedure was carried out as described below.

1. One isolated colony from a TSA agar plate was transferred with an inoculating loop to 0.2 mL PBS, and emulsified
2. Half of suspended cells were transferred to another tube and mixed with 0.1 mL PBS as control
3. 0.1mL of a lysostaphin solution (Sigma, Poole, UK) dissolved in PBS was added to the original tube to produce a final lysostaphin concentration of 25μg/mL
4. Control and test tubes were then incubated at 37°C for 2 hours; at half-hour intervals, the turbidity at 550nm was measured using a spectrophotometer (Pharmacia Biotech, Ultraspec 2000, UK). PBS was used as a blank
5. If turbidity decreased in the tube containing lysostaphin, the test was considered positive. If reduction in turbidity had not occurred in 2 hours, the test was considered to be negative
6. The test was repeated twice for each strain. Each point represents the mean of all the strains added together ± SD as indicated in Figure 2.4.

2:2:9 Auxotrophism Assay

Gentamicin-induced staphylococcal auxotrophs show a conditional drug-dependent auxotrophy. Studies on SCVs of *S. aureus* revealed a deficiency in the electron transport system that lead to the appearance of menadione, and thiamine auxotrophy (Jonsson *et al.*, 2003). Examination of auxotrophy in this study required the constant presence of gentamicin in the agar plates to sustain the stability of SCVs. Supplying the cultures with the auxotrophic substance should transform SCVs to their wild type appearance

(revertants) by compensating for the metabolic defect found in SCVs. The method for determination in the current study is described below.

1. Two sets of TSA plates were prepared for auxotrophism assays. Both sets were supplemented with the auxotrophy test substance, and one set was additionally supplemented with gentamicin at the concentrations shown in Table 2-4 (end point gentamicin concentration)
2. Auxotrophic substances were prepared as separate stock solutions and added individually, and along with gentamicin when appropriate to the melted TSA at 45°C. Auxotrophic substances used in this study included menadione sodium bisulfite (Sigma, Poole, UK) (10 μg/mL), thymidine 5′ -diphosphate sodium salt (100 μg/mL) (Sigma, Poole, UK), thiamine hydrochloride (100 μg/mL) (Sigma, Poole, UK) and proline methyl ester hydrochloride (100 μg/mL) (Sigma, Poole, UK). Plates supplied with menadione and thiamine were kept covered because they are sensitive to light exposure
3. One colony of a SCV culture was taken and emulsified in 1 mL of deionised water and subjected to five serial dilutions (10^{-1}-10^{-5}). A sterile swab was immersed in the 10^{-5} dilution and used to inoculate the surface of the agar plate
4. Plates were incubated overnight at 37°C. Next day, plates were screened for SCVs. Plates were incubated for an extra day at 37°C if they failed to show any bacterial growth after the first overnight incubation. Each plate was screened for revertants and reversion recorded as a percentage:

Revertant %= [Revertant count/Total Count] x 100%

5. The assay was repeated twice. The results are presented in Table 2.10

2:2:10 Lipolytic and Proteolytic Activities

Many staphylococci are able to produce the exoproteins, lipase and protease. These enzymes are known to be virulence factors in *S. aureus* (Goguen *et al.*, 1995; Götz *et al.*, 1998) and may contribute to the pathogencity of *S. epidermidis* (Vuong *et al.*, 2000).

Lipase is synthesised as a preproenzyme forms and after secretion into the medium, proteolytic processing results in mature, enzymatically active forms (Rollof and Normark, 1992). Staphylococcal lipases have been found to have differences in pH optimum, calcium affinity, and substrate specificity

(Nikoleit *et al.*, 1995; Simons *et al.*, 1996). Tween 20 and tributyrin are two lipids often used in agar plate screens for staphylococcal lipolytic activity. Lipolysis of tributyrin is reported positive when a clear zone is observed around the bacterial growth; in contrast, lipolysis of Tween 20 is reported as positive when a turbid zone is observed around the bacterial growth.

Production of extracellular proteases by staphylococci may represent a bacterial defence system which could inactivate IgG antibodies and protect against antimicrobial peptides such as the neutrophil defensins (Selsted *et al.*, 1996) and the platelet microbicidal proteins (PMPs) (Yeaman *et al.*, 1994). The detection of protease activity using an agar plate-based assay usually involves the addition of skimmed milk. If staphylococcal strains exhibit a proteolytic activity, a clear zone around the staphylococcal growth will appear after one-day incubation.

Lipase and protease activities were determined by an agar plate assay with slight modifications to the method of Vuong *et al* (2000).

Lipase Assay

1. Tributyrin agar base (Sigma, Poole, UK) was supplied with $CaCl_2$ (25mg/L). Autoclaved Tween 20 at 1% vol/vol was added to TSA (Sigma, Poole, UK) or tributyrin solution at 1% vol/vol was added to tributyrin base agar (Sigma, Poole, UK) at 45°C. Plates were left to solidify at room temperature
2. Bacterial suspensions were prepared in PBS at a cell density of 6 McFarland unit standards
3. Bacterial suspension (25µL) was placed on the plates and left to dry. Dried plates were incubated for 2 days at 37°C
4. A clear zone appearing on tributyrin plates and a turbid zone on Tween 20 plates were considered as a positive indication of the presence of lipase
5. The test was repeated twice for each strain. Results for SCVs and their parents are presented in Table 2.11

Protease Assay

1. Sterile skimmed milk (Sainsbury, Nottingham, UK) was added at a final concentration of 1% wt/vol to autoclaved 1% wt/vol tryptone (Sigma, Poole, UK), 0.5% wt/vol yeast extract (Sigma, Poole, UK), 0.5% wt/vol NaCl (Sigma, Poole, UK), and 1.5% wt/vol agar (Sigma, Poole, UK) at 45°C. The contents were mixed well and poured into plates and left to solidify and dry
2. Bacterial strains were prepared exactly as described for the lipase assay as in section 2:2:10
3. The result was considered to be positive for the presence of protease if a clear zone was observed around the growth after 24 hours incubation at 37°C.
4. The test was repeated twice for each strain. Results for these tests are presented in Table 2.11

2:2:11 Comparative Growth Curves for Staphylococcal Wild Type Strains and SCVs

Several studies have showed that clinical *S. aureus* isolates as well as CoNS have the capacity to change their phenotypic features (Baddour and Christensen, 1987; Deighton *et al.*, 1992(a,b); Baselga *et al.,* 1993; Proctor *et al.*, 1995). There are specific properties which correspond with these features such as colony morphology, growth rate and antibiotic susceptibility (Ziebuhr *et al.*, 1999).

The Vialight ™ MDA Kit (BioWHITTAKER, CAMBREX, Nottingham, UK) is based upon the bioluminescent measurement of ATP that is present in metabolically active cells and allows an indirect measurement of bacterial growth to be determined. In the present study growth was monitored for the wild strains and SCVs in MHB with and without gentamicin supplementation. ATP contents per cell were determined for the wild type strains and their SCVs as described below. Plating of aliquots of the cultures on MHA plates was also carried out to enable the detection of any reversion to wild type.

1. A bacterial suspension was made in MHB with standard density turbidity (McFarland 0.5) for the wild strains or their corresponding SCV
2. One millilitre from the suspension was mixed with 9 mL of MHB to produce 10^{-1} dilution, and then 2mL was taken from this and transferred to 18 mL of MHB to produce a 10^{-2} dilution was obtained
3. The 20 mL 10^{-2} diluted suspension was divided into two equal volumes. One was used as control and the other supplemented with gentamicin. Gentamicin concentrations for wild types were the MIC. However, gentamicin concentrations for SCVs were the concentrations which resulted in most stable maintenance of SCVs as indicated in Table 2.4
4. The suspensions were shaken at 150 rpm at 37°C
5. Triplicate aliquots of 125μL were taken at zero, 1,3,6, and 12 hour time points and transferred to a 96 well plate (white walled, clear-bottomed chemiluminescence plates) (BioWHITTAKER, CAMBREX, Nottingham, UK)
6. With a sterile loop, a small volume was also taken from the suspensions and plated out on MHA with and without gentamicin to screen for revertants. Data from these screens are presented in Table 2.12
7. 125μL of Bactolyse (a solution used to extract ATP) was added to the wells containing the staphylococcal cultures and extraction was allowed to occur for at least 5 minutes at room temperature
8. The plate was then transferred to a luminometer with a dispensing system, where 25μL of ATP Monitoring Reagent (formulated luminescence cocktail) was automatically added to each well
9. The light emission was measured and the results expressed as relative light units are Figure 2.5 and 2.6
10. For calculating the amount of ATP per cell, aliquots at zero time were diluted and plated out for CFU (cell forming unit) count. The count was then adjusted for 125μL. Since RLUs obtained were proportional to the ATP content of the bacterial cells, a relative measure of ATP content was calculated using RLU data according to the following equation:

RLU per cell = Relative Light Units (RLU)/Count of bacteria in 125μL

11. The assay was repeated twice for each strain. The readings shown in Figure 2.5 and 2.6 are means ±SD.

2:3 Results

Table 2.2: Gentamicin E-test results for the staphylococcal isolates used in this study

Strain No.	Gentamicin MIC readings (mg/L)	Gentamicin concentrations at which SCVs emerged (mg/L)
1(SE)*	4	none
2(SE)	0.125	none
3 (SE)	0.064	0.75-1.0
4(SE)	16	> 96
5(SE)	32	512
6(SE)	0.064	none
7(SE)	0.25	1-5
8(SE)	0.047	1-4
9(SE)	0.38	none
10(SE)	16	64 and 1024
11(SA)**	1.0	none
12(SE)	19	none
13(SE)	0.06	0.5-1.0
14(SE)	16	>32
15(SE)	0.19	none
16(SH)***	0.064	none
17(SE)	0.125	0.25-0.75
18(SE)	0.064	none
19 (SA)	0.032	none
20(SA)	0.50	none
21(SE)	64	512
22(SE)	0.125	none
23(SE)	0.25	none
24(SE)	12	none
25(SE)	24	256
26(SE)	32	none
27(SA)	32	none
28 (SE)	0.06	0.5-1
Oxford *S .aureus*	0.16	none

*SE= *S. epidermidis.* ** SA = *S. aureus*, ***SH = *S. haemolyticus*, none= no SCVs detected

Data in Table 2.3 summarises gentamicin MIC (mg/L) obtained by E-test for staphylococcal isolates. Gentamicin concentration in the ellipse at which SCVs emerged was two fold the MIC or more. Only twelve out of twenty eight staphylococci tested produced SCVs.

Table 2.3: Gentamicin MIC determined by microbroth dilution

Strain No.	Gentamicin MIC readings (mg/L)	Gentamicin concentrations at which SCVs emerged (mg/L)
1(SE)*	4	none
2(SE)	0.25	none
3 (SE)	0.25	0.5
4(SE)	8	64
5(SE)	16	none
6(SE)	0.064	none
7(SE)	0.25	0.125
8(SE)	0.125	0.25
9(SE)	64	none
10(SE)	128	none
11(SA)**	2	1
12(SE)	0.125	none
13(SE)	0.125	0.25
14(SE)	16	none
15(SE)	0.125	none
16(SH)***	0.125	0.25
17(SE)	0.125	none
18(SE)	0.125	0.25
19 (SA)	0.25	none
20(SA)	1	none
21(SE)	32	128
22(SE)	0.125	none
23(SE)	0.125	0.5
24(SE)	32	none
25(SE)	16	32
26(SE)	32	64
27(SA)	32	128
28 (SE)	0.06	0.125
Oxford *S.aureus*	0.16	none

*SE = *S. epidermidis*, ** SA =*S. aureus*, ***SH = *S. haemolyticus*

The data in Table 2.3 summarises the gentamicin MIC (mg/L) obtained by microbroth dilution. SCVs from 14 strains were isolated from wells with re-growth on day two.

Table 2.4: Consecutive subcultures in TSA supplemented with gentamicin for the production of stable SCVs

Strain No.	Starting point (MHB with gentamicin mg/mL)		Serial plating out (TSA plates with gentamicin mg/L)	Collecting point (TSA plates with gentamicin mg/L)	Ending point (TSA plates with gentamicin mg/L)
	MHB^0	MHB^1			
3(SE)	0.5 →	1 →	T1→T1→T5 →	T5 →	5
4(SE)	64 →	128 →	T512→T512→T1024→	T1024 →	2048
7(SA)	0.125 →	1 →	T0.5→T1→T2→T5 →T10→T23→	T64→	526
8(SE)	0.25 →	1 →	T1→T1→T5 →	T10→	23
11(SA)	1 →	2 →	T1→T2→T5→T5 →	T10 →	23
13(SE)	0.25 →	1 →	T0.5→T0.5 →	T1→	2
16(SH)	0.06 →	1 →	T5→T10 →	T32 →	526
18(SE)	0.125 →	1 →	T1→T5→T10 →	T32 →	64
21(SE)	128 →	256 →	T512→T1024 →	T2048 →	4096
23(SE)	0.5 →	1 →	T0.5→T1→T5→T5 →	T5 →	256
25(SE)	32 →	1024 →	T256 →	T1024→	2048
26(SE)	32 →	512 →	T512 →	T1024→	1024
27(SA)	128 →	1024 →	T1024 →	T2048→	2048
28(SE)	0.125 →	1 →	T1→T5 →	T5 →	23

→ = consecutive plating. SE = *S. epidermidis*, SA = *S. aureus*, SH = *S. haemolyticus*

Table 2.4 summarises the consecutive subcultures at different gentamicin concentrations to produce pure and stable SCVs. This procedure was repeated whenever SCVs strains were needed for further experimental work.

Each arrow illustrates one subculture on that particular day. After 5-7 subcultures, reversion was unlikely to occur because of the continuous and corresponding increase in gentamicin concentration.

The procedure shown in Table 2.4 consisted of 4 steps. **The starting point** included growing wild type in MHB with gentamicin MICs at which SCVs emerged (MHB^0) as shown in Table 2.3 then subculturing into MHB with gentamicin at a concentration of 2MIC (MHB^1). **Serial plating** consisted of 2-5 consecutive subcultures onto TSA with gentamicin >2 fold the MIC. Serial plating ended with a **collecting point** where SCVs were transferred to plates or broths supplemented with gentamicin at concentration indicated in the **ending point** in Table 2.4.

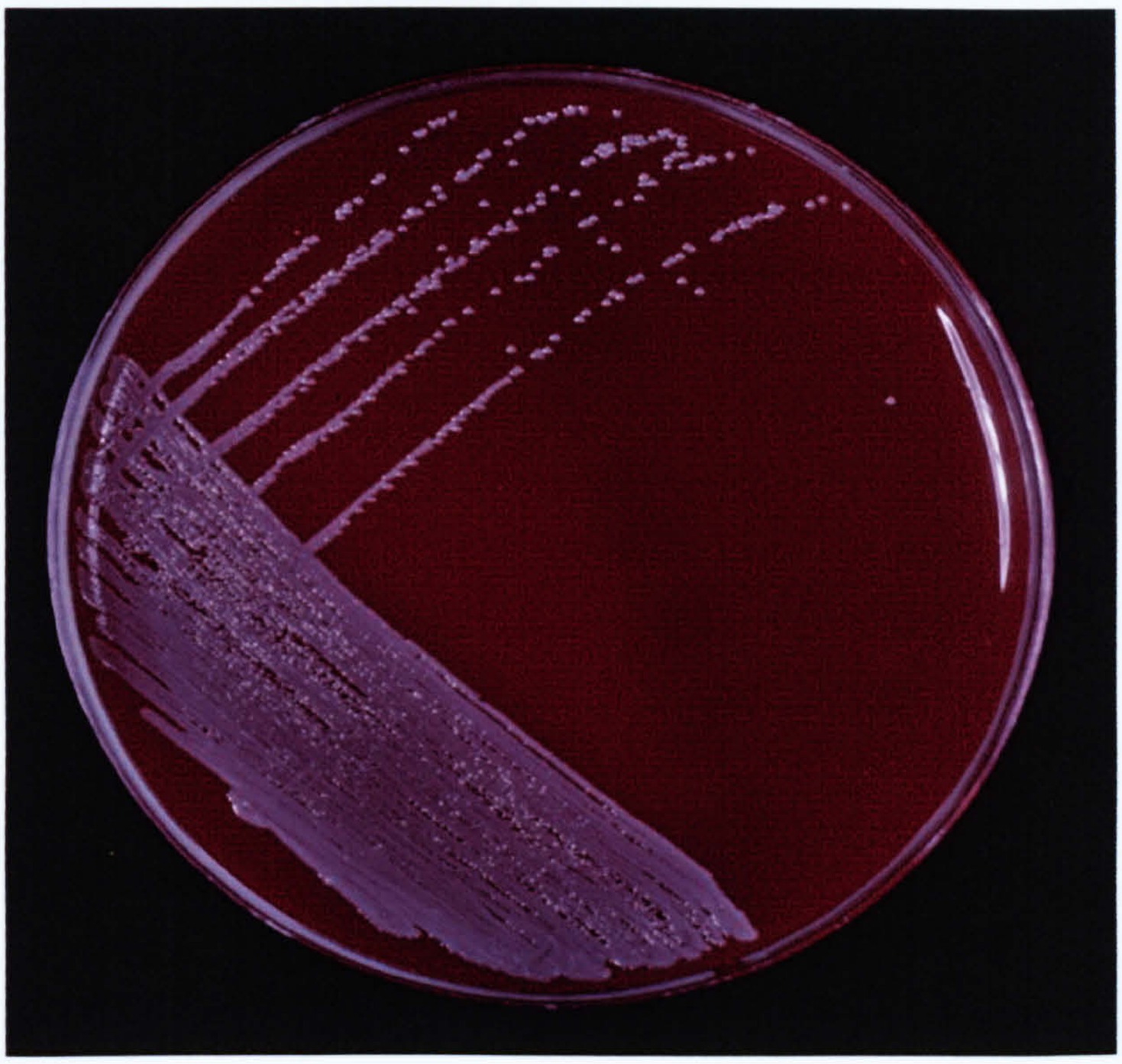

FIG 2.1: *S. epidermidis* strain 26 wild type before exposing to gentamicin

Figure 2.1 shows the morphology *S. epidermidis* wild strain colonies when plated on BA. The colonies are ≈ 1mm in diameter.

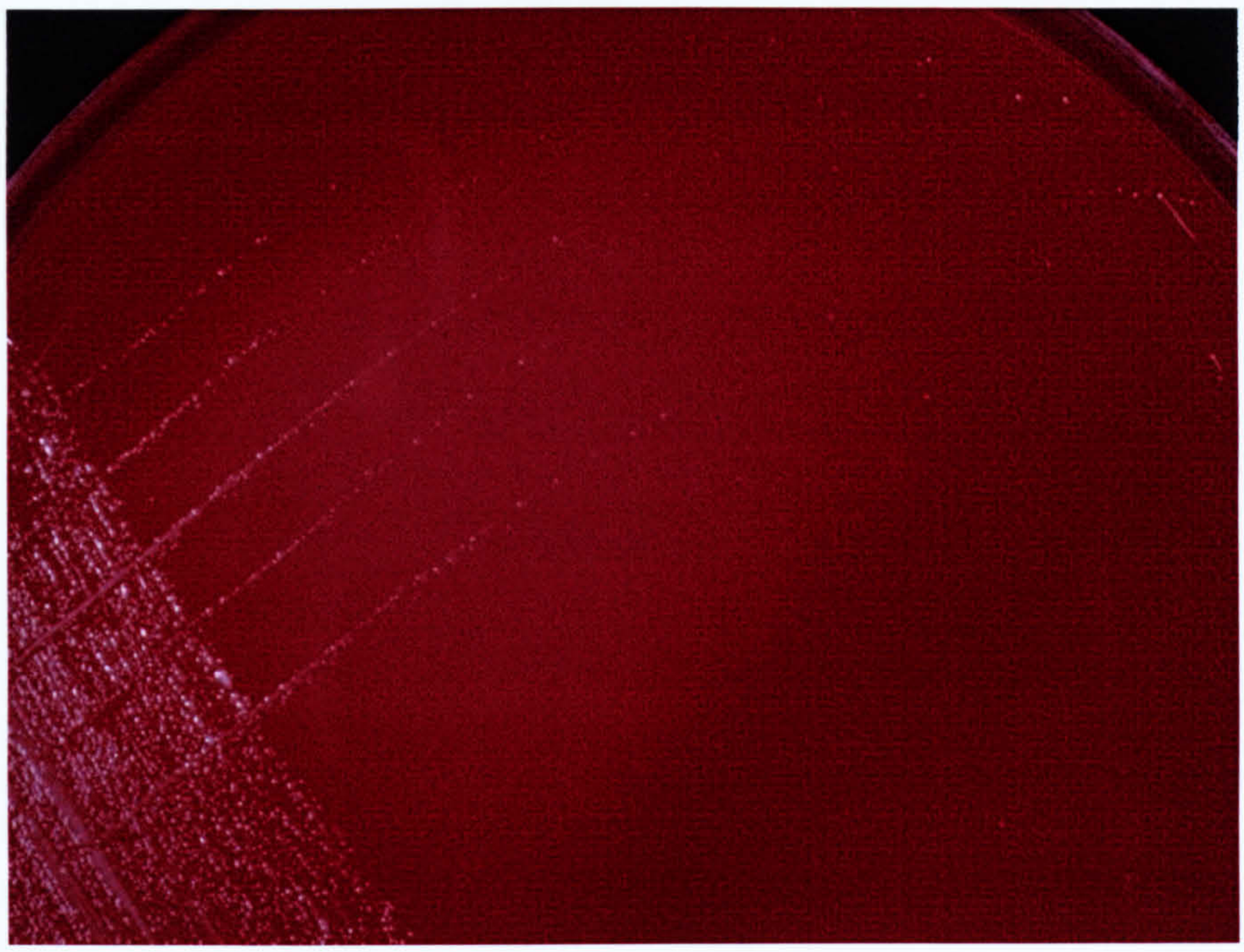

FIG 2.2: *S. epidermidis* strain 26 SCVs after exposure of wild type to successive concentrations of gentamicin

Figure 2.2 shows the morphology of *S. epidermidis* after experiencing successive subculturing on gentamicin supplemented agar plates. These are *S. epidermidis* SCVs at their collecting point plated on BA. SCVs colonies appeared to be < 0.5 mm in diameter.

Table 2-5: Api Staph scores and their interpretation for staphylococcal strains and their corresponding SCVs

Strain No.	Api Staph Score	Interpretation
3 (SE) W	6706113	SCVs showed negative reaction for NIT and PAL
3 (SE) S	6704113	
4 (SE) W	6706113	SCVs showed negative reaction for ADH
4 (SE) S	6706112	
7 (SE) W	6706112	SCVs showed negative reaction for ADH and URE
7 (SE) S	6706110	
8 (SE) W	6706113	No Differences but weaker reactions in case of SCVs
8 (SE) S	6706113	
11 (SA) W	6736153	SCVs showed negative reaction for URE
11 (SA)S	6736151	
13 (SE) W	6606013	SCVs showed positive reaction for VP but negative for URE
13 (SE) S	6706111	
16 (SH)W	6616151	SCVs showed positive reaction for MNE and URE but negative for NAG
16 (SH)S	6716113	
18 (SE) W	6706043	SCVs showed positive reaction for VP and SAC but negative for NAG
18 (SE) S	6706113	
21(SE) W	6606113	SCVs showed positive reaction for MNE
21 (SE) S	6706113	
23 (SE) W	6606113	SCVs showed positive reaction for MNE and negative for NAG
23 (SE) S	6706153	
25 (SE) W	6606111	SCVs showed positive reaction for MNE
25 (SE) S	6706111	
26(SE) W	6306112	SCVs showed positive reaction for MAN and negative for ADH
26 (SE) S	6326113	
27 (SA) W	6306113	SCVs showed positive reaction for LAC but negative for URE
27 (SA) S	6706111	
28 (SE) W	6606111	SCVs showed positive reaction for MNE and URE but negative reaction for NIT
28 (SE) S	6704112	

(SE) W = *S. epidermidis* wild strain, (SE) S = *S. epidermidis* SCV, SA = *S. aureus*
SH =*S. haemolyticus*

Table 2.5 summarises the Api Staph results (bold font) which showed the differences in metabolic profile between the wild strains and their corresponding SCVs. The interpretation of the observed differences is shown in the third column. The abbreviations for the substances are shown in the Appendix 8:2:2. Based on the results, SCVs differed from wild strains in their metabolic activity in one or more tests. However, for *S. epidermidis* strain 8,

SCVs carried the same Api Staph profile as their wild type parent but showed weaker reactions with the substrates compared to the wild strain.

Table 2.6: Antibiogram Staph Score for staphylococcal wild type strains and their corresponding SCVs

No Strain.	Antibiogram Staph score	Interpretation
3 (SE) W	51000 Cip(R), Tec (S)	SCVs showed resistance to gentamicin, amikacin, teicoplanin, and sensitivity to penicillin.
3 (SE) S	00240 Cip (R), Tec (R)	
4 (SE) W	40354 Cip(R), Tec (S)	SCVs showed resistance to methicillin, trimethoprim, netilmicin, and sensitivity to vancomycin
4 (SE) S	43370 Cip (R), Tec (S)	
7 (SE) W	60000 Cip (S), Tec (S)	SCVs showed resistance to gentamicin and amikacin
7 (SE) S	60240 Cip (S), Tec (S)	
8 (SE) W	45402 Tec (S), Cip (R)	SCVs showed resistance to gentamicin, amikacin and vancomycin and ciprofloxacin
8 (SE) S	45246 Tec (R), Cip (R)	
11 (SA) W	06000 Tec (R), Cip (R)	SCVs showed resistance to clindamycin, gentamicin, amikacin and netilmicin
11 (SA)S	06660 Tec (R), Cip (R)	
13 (SE) W	00000 Tec (S), Cip (S)	SCVs showed resistance to gentamicin and teicoplanin
13 (SE) S	00240 Tec (R), Cip (S)	
16 (SH)W	44000 Tec (S), Cip (S)	SCVs showed resistance to gentamicin, amikacin, netilmicin, cefuroxime
16 (SH)S	44261 Tec (S), Cip (S)	
18 (SE) W	00000 Tec (S), Cip (S)	SCVs showed resistance to clindamycin, amikacin, gentamicin, vancomycin and rifampicin
18 (SE) S	00744 Tec (S), Cip (S)	
21(SE) W	43210 Tec (S), Cip (S)	SCVs showed resistance to amikacin and netilmicin
21 (SE) S	43270 Tec (S), Cip (S)	
23 (SE) W	40001 Tec (S), Cip (S)	SCVs showed resistance to gentamicin, amikacin.
23 (SE) S	40241 Tec (S), Cip (S)	
25 (SE) W	44670 Tec (S), Cip (R)	SCVs is resistant to methicillin
25 (SE) S	46670 Tec (S), Cip (R)	
26(SE) W	40200 Tec (S), Cip (S)	SCVs showed resistance to amikacin, and sensitivity to penicillin
26 (SE) S	00240 Tec (S), Cip (S)	
27 (SA) W	40200 Tec (S), Cip (S)	SCVs showed resistance to amikacin, sensitivity to penicillin
27 (SA) S	40240 Tec (S), Cip (S)	
28 (SE) W	00000 Tec (S), Cip (S)	SCVs showed resistance to gentamicin
28 (SE) S	00200 Tec (S), Cip (S)	

(SE) W = *S. epidermidis* wild strain, (SE) S= *S. epidermidis* SCV. SA = *S. aureus*. SH = *S. haemolyticus*.

R = resistant to antibiotic. S = susceptible to antibiotics

Table 2.6 shows the antibiograms profiles in 5 digits. Some SCVs were more resistant to some aminoglycosides like gentamicin, amikacin, netilmicin but were sensitive to penicillin and vancomycin compared to their wild type parents.

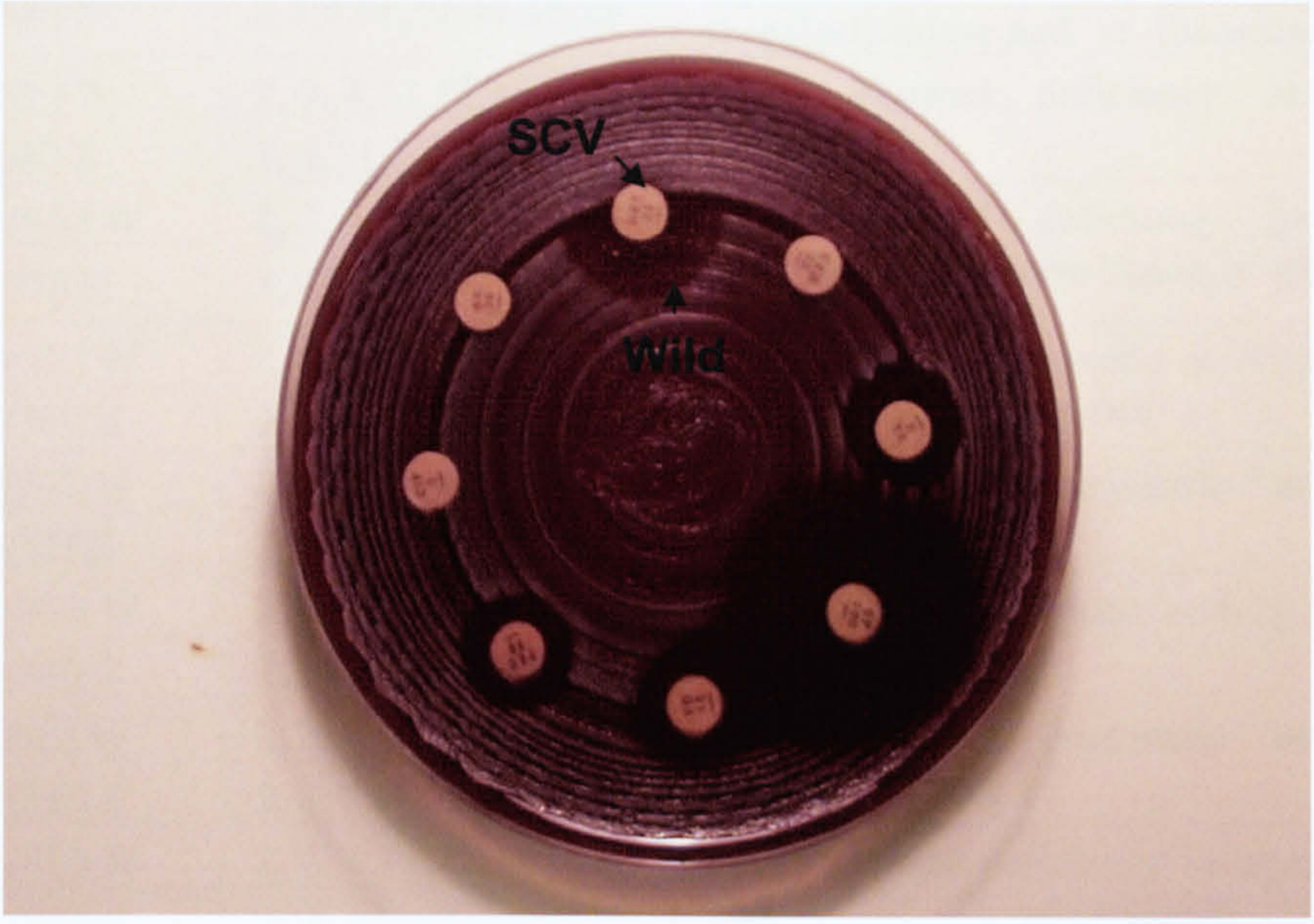

FIG 2.3: Antibiotic susceptibility test for *S. epidermidis* strain 23 and its SCVs showing antibiotic resistant SCVs when compared to the wild type

Figure 2.3 shows the SCVs and their wild strains susceptibility to gentamicin (indicated with arrow). SCV was resistant to gentamicin resulting in bacterial growth around the disc; however, its corresponding wild strain was inhibited by gentamicin (clear zone).

Table 2.7: The enzymatic activities for staphylococcal strains and their SCVs as determined by Api Zym

Strain No.	Positive Enzyme No	Interpretation
3 (SE) W*	**2, 3, 4,11,12,16**	SCVs showed deficiency in alkaline phosphatase, esterase, α-glucosidase
3 (SE) S**	4, 11, 12	
4 (SE) W	**2, 3, 4,11,12,16**	SCVs showed deficiency in esterase and α-glucosidase
4 (SE) S	2, 4, 11, 12	
7 (SE) W	2,3,4,**6**,11,12,16	SCVs showed deficiency in leucine arylamidase, but enzymatic activity in α -galactosidase and α -fucosidase
7 (SE) S	2,3,4,11,12,**13**,16,**20**	
8 (SE) W	**2**, 3, 4,11,12,16	SCVs showed deficiency in alkaline phosphatase
8 (SE) S	3, 4,11,12,16	
11 (SA) W	**2**, 3, 4,11,12,16	SCVs showed deficiency in alkaline phosphatase, esterase lipase, α -glucosidase
11 (SA)S	3, 11, 12	
13 (SE) W	2, 3, 4,11,12,16	SCVs showed enzymatic activity in lipase and cysteine arylamidase
13 (SE) S	2, 3,4,5,8,11,12,16	
16 (SH)W	2, 3, 4,11,12,16	SCVs showed enzymatic activity in α -chymotrypsin
16 (SH)S	2, 3,4,11,12,10,16	
18 (SE) W	2, 3, 4,11,12,16	SCVs showed enzymatic activity in lipase
18 (SE) S	2,3,4,**5**,11,12,16	
21(SE) W	2, 3, 4,11,12,16	SCVs showed enzymatic activity in α -galactosidase
21 (SE) S	2, 3,4,11,12,14,16	
23 (SE) W	2, 3, 4,11,12,16	SCVs showed deficiency in esterase lipase and α -glucosidase
23 (SE) S	2, 3, 11, 12	
25 (SE) W	2,3,4,11,12	SCVs showed enzymatic activity in α-galactosidase and α -glucosidase
25 (SE) S	2, 3,4,11,12,14,16	
26(SE) W	2, 3, 4,11,12,16	SCVs showed enzymatic activity in lipase
26 (SE) S	2,3,4,**5**,11,12,16	
27 (SA) W	3, 4,11,12,16	SCVs showed deficiency in acid phosphatase
27 (SA) S	3, 4, 12, 16	
28 (SE) W	2, 3, 4,11,12,16	SCVs showed deficiency in α -glucosidase
28 (SE) S	2,3,4,11,12	

(SE) W = *S. epidermidis* wild strain, (SE) S= *S. epidermidis* SCV. SA = *S. aureus*. SH = *S. haemolyticus*.

The differences in enzymatic activities have been indicated in bold font in Table 2.7. The results indicated that SCVs differed in their enzymatic activities when compared to the wild strains. SCVs were shown to be deficient in one or more of the enzymes screened including lipases, esterases and glucosidases and phosphatases. However, other SCVs (16, 18, 21 and 26) showed additional activities (lipase, galactosidase and α-chymotrypsin) which were not present in their wild type parent.

Table 2.8: Catalase, coagulase, DNAse and haemolysis activities on sheep blood agar plates for the differentiation of *S. aureus* wild and SCVs strains

Strain No.	Catalase	Coagulase		DNase	Haemolysis on sheep BA
		Slide	Tube		
11 (SA)W	+	+	+ at 2 hours	+	β-haemolysis
11 (SA) S	+	-	+ overnight	+	- haemolysis
27 (SA)W	+	+	+ at 4 hours	+	β-haemolysis
27 (SA)S	+	-	+ overnight	+	- haemolysis

(SA) W = *S. aureus* wild strains. (SA) S = *S. aureus* SCVs

Further biochemical tests on *S. aureus* wild and SCVs including assays for catalase, coagulase, DNAse and haemolysis are summarised in Table 2.8. All Staphylococcal strains tested showed positive results in catalase and DNase test. SCVs were non-haemolytic and coagulase negative (slide test).

Coagulase and haemolysin production has been considered to be important in staphylococcal pathogenesis. Vesga *et al* (1995) suggested that *S. aureus* SCVs could survive intracellularly because they did not produce sufficient coagulase and haemolysins, as compared to their wild parent. This is thought to enable SCVs to survive longer in host cells without lysing them.

Table 2.9: CoNS haemolysis on sheep blood agar

Strain No.	Haemolysis on Sheep BA
3 (SE) W	-Haemolysis
3 (SE) S	*- Haemolysis
4(SE) W	α-Haemolysis
4(SE) S	α -Haemolysis
7(SE) W	- Haemolysis
7(SE) S	- Haemolysis
8(SE) W	- Haemolysis
8 (SE) S	- Haemolysis
13 (SE) W	α -Haemolysis
13(SE) S	α -Haemolysis
16(SH) W	β-Haemolysis
16(SH) S	α, β -Haemolysis
18 (SE) W	α -Haemolysis
18 (SE) S	α -Haemolysis
21(SE) W	α, β -Haemolysis
21 (SE) S	α, β -Haemolysis
23(SE) W	α -Haemolysis
23 (SE) S	- Haemolysis
25(SE) W	- Haemolysis
25(SE) S	- Haemolysis
26(SE) W	- Haemolysis
26 (SE) S	- Haemolysis
28 (SE) W	- Haemolysis
28 (SE) S	- Haemolysis

***- = negative haemolysis; SE= *S. epidermidis*; SH= *S. haemolyticus*; W= wild strains; S= SCVs**

The results displayed in Table 2.9 indicated that *S. epidermidis* SCVs haemolysin production did not differ from wild strains.

However, *S. haemolyticus* SCVs showed an increase in haemolytic activity (around double) when compared to their wild strain.

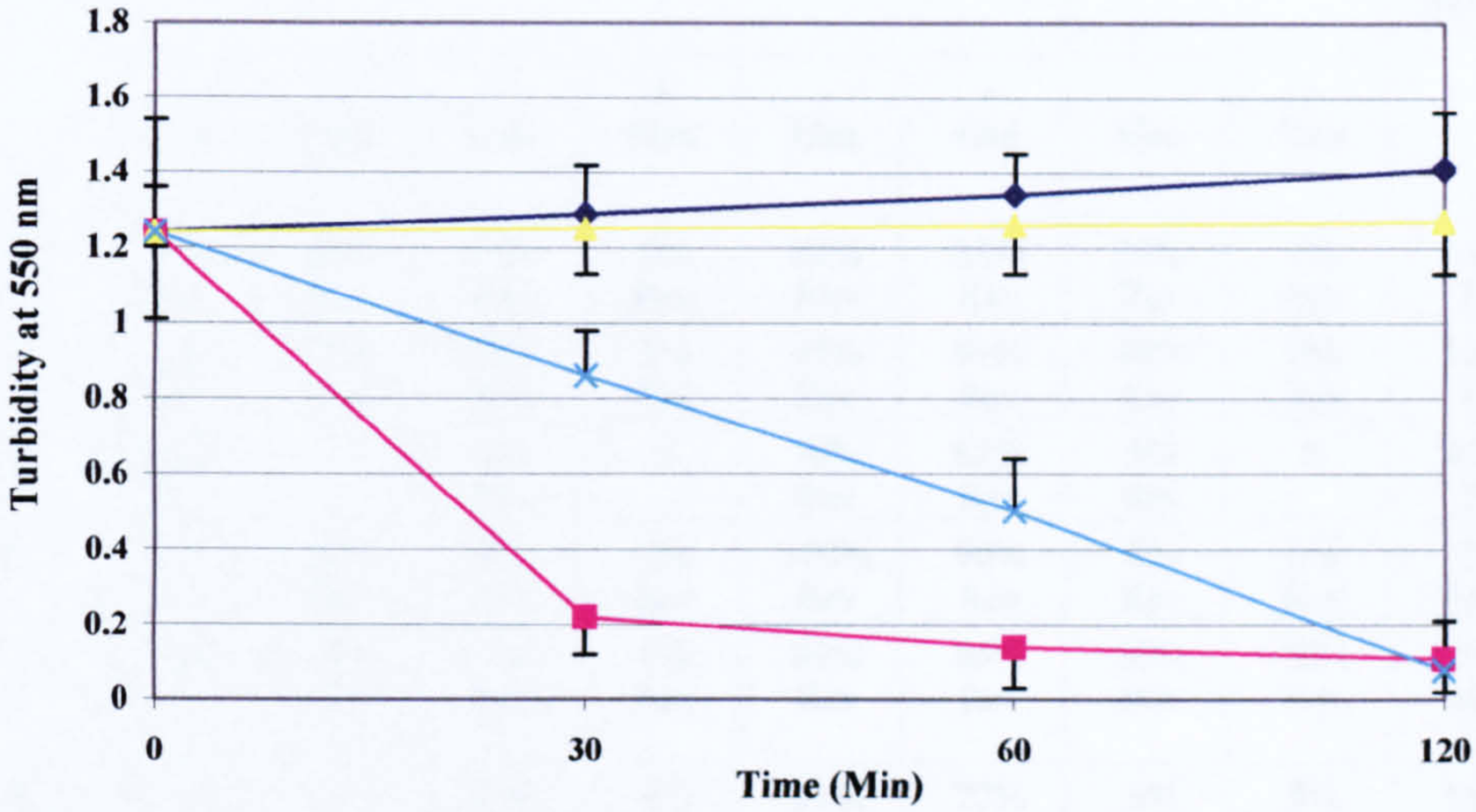

FIG 2-4: Influence of lysostaphin on the lysis of staphylococcal strains

Lysostaphin had different effects on staphylococcal wild and SCVs strains. Each point in Figure 2.4 represents the mean of the lysostaphin sensitivity assays of the fourteen staphylococcal strains shown in Table 2.4 tested individually. Figure 2.4 indicated that SCVs are more resistant to lysostaphin lytic action, since lysis occurred more slowly than wild type strains within the 2 hr incubation period. This resistance could be explained by change in cell wall composition which reduces lysostaphin activity or delays it.

Table 2.10: Screening for auxotrophy in SCV strains on TSA plates with and without gentamicin

SCVs Strain No.	Menadione 10 mg/L		Thymidine 100 mg/L		Thiamine 100 mg/L		Proline 100 mg/L		Auxotrophic Substance
	- Gen	+ Gen	- Gen	+ Gen	- Gen	+ Gen	- Gen	+ Gen	
3(SE)	85% Rev	80% Rev	15% Rev	1% Rev	89% Rev	82% Rev	15% Rev	1% Rev	Menadione Thiamine
4(SE)	91% Rev	87% Rev	18% Rev	3% Rev	95% Rev	91% Rev	18% Rev	3% Rev	Menadione Thiamine
7(SE)	80% Rev	-*	5% Rev	S	70% Rev	62% Rev	5% Rev	S	Menadione Thiamine
8(SE)	97% Rev	92% Rev	8% Rev	5% Rev	100% Rev	90% Rev	8% Rev	6% Rev	Thiamine Menadione
11(SA)	76% Rev	71% Rev	3% Rev	1% Rev	89% Rev	85% Rev	80% Rev	76% Rev	Thiamine Menadione Proline
13(SE)	95% Rev	-	6% Rev	4% Rev	81% Rev	77% Rev	6% Rev	5% Rev	Menadione Thiamine
16(SH)	92% Rev	-	3% Rev	2% Rev	3% Rev	2% Rev	3% Rev	1% Rev	Menadione
18(SE)	98% Rev	92% Rev	10% Rev	2% Rev	85% Rev	-	10% Rev	2% Rev	Menadione Thiamine
21(SA)	75% Rev	60% Rev	10% Rev	2% Rev	100% Rev	-	10% Rev	3% Rev	Menadione
23(SE)	76% Rev	69% Rev	10% Rev	3% Rev	65% Rev	60% Rev	10% Rev	2% Rev	Menadione Thiamine
25(SE)	96% Rev	86% Rev	15% Rev	2% Rev	15% Rev	2% Rev	15% Rev	2% Rev	Menadione
26(SE)	12% Rev	1% Rev	12% Rev	2% Rev	60% Rev	50% Rev	12% Rev	1% Rev	Thiamine
27(SA)	98% Rev	95% Rev	8% Rev	1% Rev	80% Rev	30% Rev	8% Rev	1% Rev	Menadione Thiamine
28(SE)	90% Rev	-	10% Rev	2% Rev	80% Rev	70% Rev	10% Rev	2% Rev	Menadione Thiamine

***(-) = no growth at all. Gen+= plates with gentamicin, Gen- = plates without gentamicin. Where no growth was detected in the initial screen, the auxotrophy assay was repeated on TSA plates containing half the original concentration of gentamicin. No growth was observed under these conditions. All SCVs showed variable levels of reversion in the presence of medium supplementation with the auxotrophic substances.**

The results in Table 2.10 showed that staphylococcal SCVs are menadione and/or thiamine auxotrophs. Auxotrophic SCVs were reported if two sets of plates (Gen + and Gen -) showed 50-100% revertants or no growth at all. However, the appearance of 100% SCVs or less than 50% revertants was recorded as no auxotrophy to the screened substance.

It is believed that the auxotrophic substance compensates for the defect found in SCVs' metabolic pathway(s) (Proctor *et al.*, 1997). The repaired pathway will cause reversion of SCVs to the wild strains rendering them susceptible to gentamicin. This would explain the lack of growth of some strains indicated in the auxotrophy assays in the presence of high concentrations (>MIC) of gentamicin (Table 2.10) e.g. SE 13 and SH 16 in the presence of menadione.

Table 2.11: Protease and lipase activities of staphylococcal wild type strains and SCVs

Strain No.	Protease Activity	Lipase Activity		Notes
		Tween 20	Tributyrin	
3 (SE) W*	-	-	-	No difference in lipolytic and proteolytic activity between SCVs and wild type
3 (SE) S**	-	-	-	
4 (SE) W	+	-	-	No difference in lipolytic and proteolytic activity between SCVs and wild type
4 (SE) S	+	-	-	
7 (SE) W	+	-	-	No difference in lipolytic and proteolytic activity between SCVs and wild type
7 (SE) S	+	-	-	
8 (SE) W	+	-	+	No difference in lipolytic and proteolytic activity between SCVs and wild type
8 (SE) S	+	-	+	
11 (SA) W	-	+	-	No difference in lipolytic and proteolytic activity between SCVs and wild type
11 (SA) S	-	+	-	
13 (SE) W	-	-	-	No difference in lipolytic and proteolytic activity between SCVs and wild type
13 (SE) S	-	-	-	
16 (SH) W	-	-	-	**SCV showed lipolytic activity against tributyrin compared to their corresponding wild strain**
16 (SH) S	-	-	+	
18 (SE) W	-	-	-	**SCV showed protease activity and lipase activity against tributyrin when compared to their corresponding wild strain**
18 (SE) S	+	-	+	
21(SE) W	+	-	-	No difference in lipolytic and proteolytic activity between SCVs and wild type
21 (SE) S	+	-	-	
23 (SE) W	-	-	-	No difference in lipolytic and proteolytic activity between SCVs and wild type
23 (SE) S	-	-	+	
25 (SE) W	+	-	-	**SCV showed lipase activity against tributyrin when compared to their corresponding wild strain**
25 (SE) S	+	-	+	
26(SE) W	+	-	-	No difference in lipolytic and proteolytic activity between SCVs and wild type
26 (SE) S	+	-	-	
27 (SA) W	-	-	+	No difference in lipolytic and proteolytic activity between SCVs and wild type
27 (SA) S	-	-	+	
28 (SE) W	+	-	+	No difference in lipolytic and proteolytic activity between SCVs and wild type
28 (SE) S	+	-	+	

SE (W) =*S. epidermidis* wild type, SE(S) = *S. epidermidis* SCVs, SA = *S. aureus*, SH = *S. haemolyticus

Table 2.11 summarises the results of the analysis of the lipolytic and proteolytic activity levels of the fourteen-staphylococcal strains with their corresponding SCVs. This study showed that only 2 staphylococcal CoNS SCVs (strains 16, 25) exhibited differences in lipolytic activity compared with their wild type parents (against tributyrin). Further, only one *S. epidermidis* SCV (strain 18) showed a difference in lipolytic and proteolytic activities when compared to their wild types.

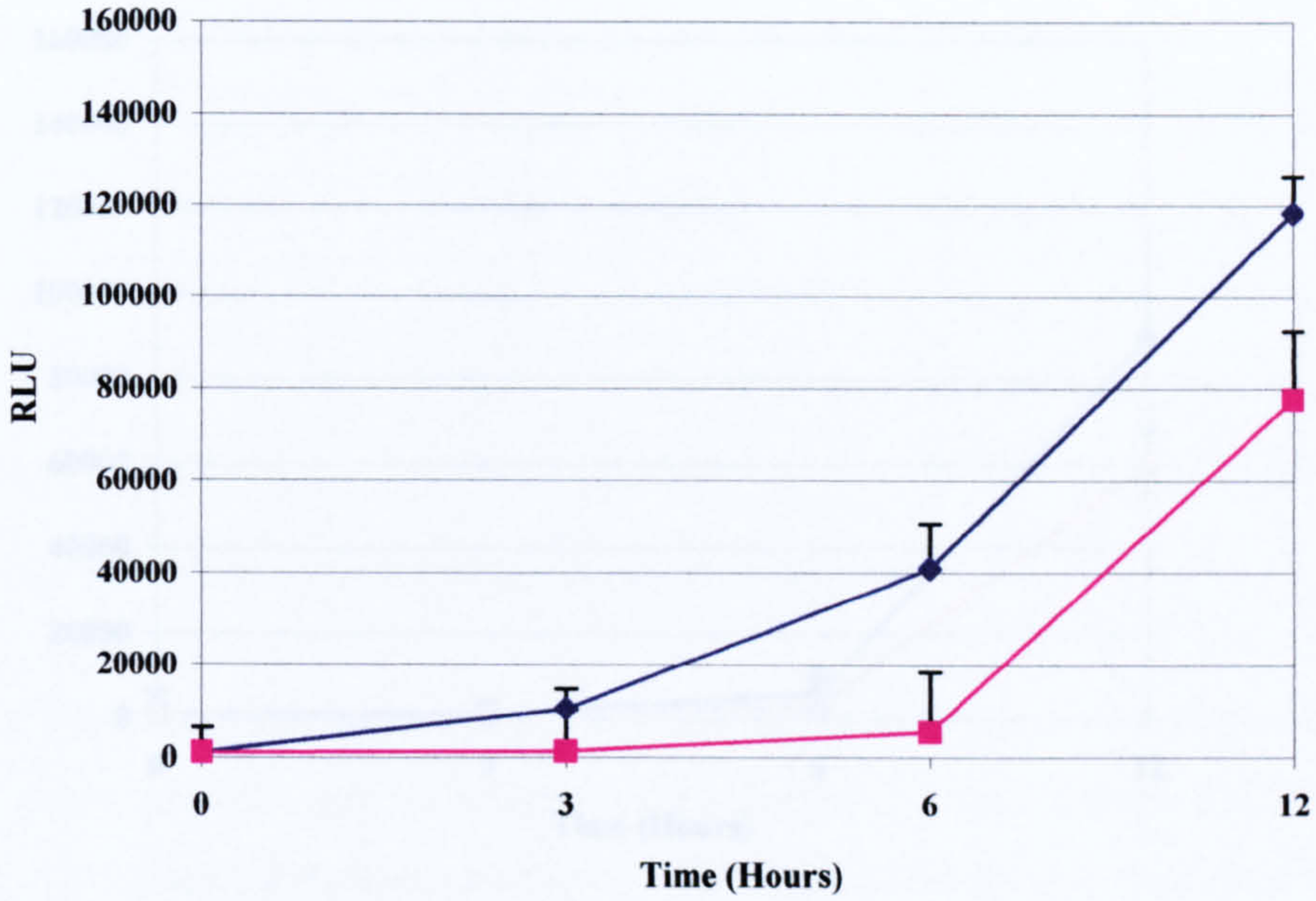

FIG 2-5 Growth curves for wild type strains in MHB supplemented with and without gentamicin.

The growth of the staphylococcal wild types was monitored through 4 time points. Each point in Figure 2.5 represents the mean value in RLU (Relative Light Units) for all the strains measured individually. Wild strains in MHB without gentamicin were used as controls. In the first three hours it was noticed that gentamicin resulted in a decrease in RLU when compared to controls. Aliquots that were taken at 3 hours and plated on MHA showed a decrease in CFU/mL (data not shown). This decrease was due to the bactericidal effects of gentamicin on the wild type cells. However, after 3 hours the RLU started to increase, and aliquots at 6 hours showed increased CFU/mL. It is believed that the emergence of gentamicin resistant staphylococcal strains (SCVs) were responsible for the increase based on data presented in Table 2.12. After 6 hours, the growth rates were similar to each other but the outcome at 12 hours showed that the CFU/mL (data not shown) was less in the presence of gentamicin when compared to controls, probably due to the gentamicin bactericidal action which took place at the start of the culture (data not shown). Staphylococcal wild type ATP contents were calculated from the curve and found to range from $4x10^{-2} - 3.7x10^{-2}$ RLU/Cell.

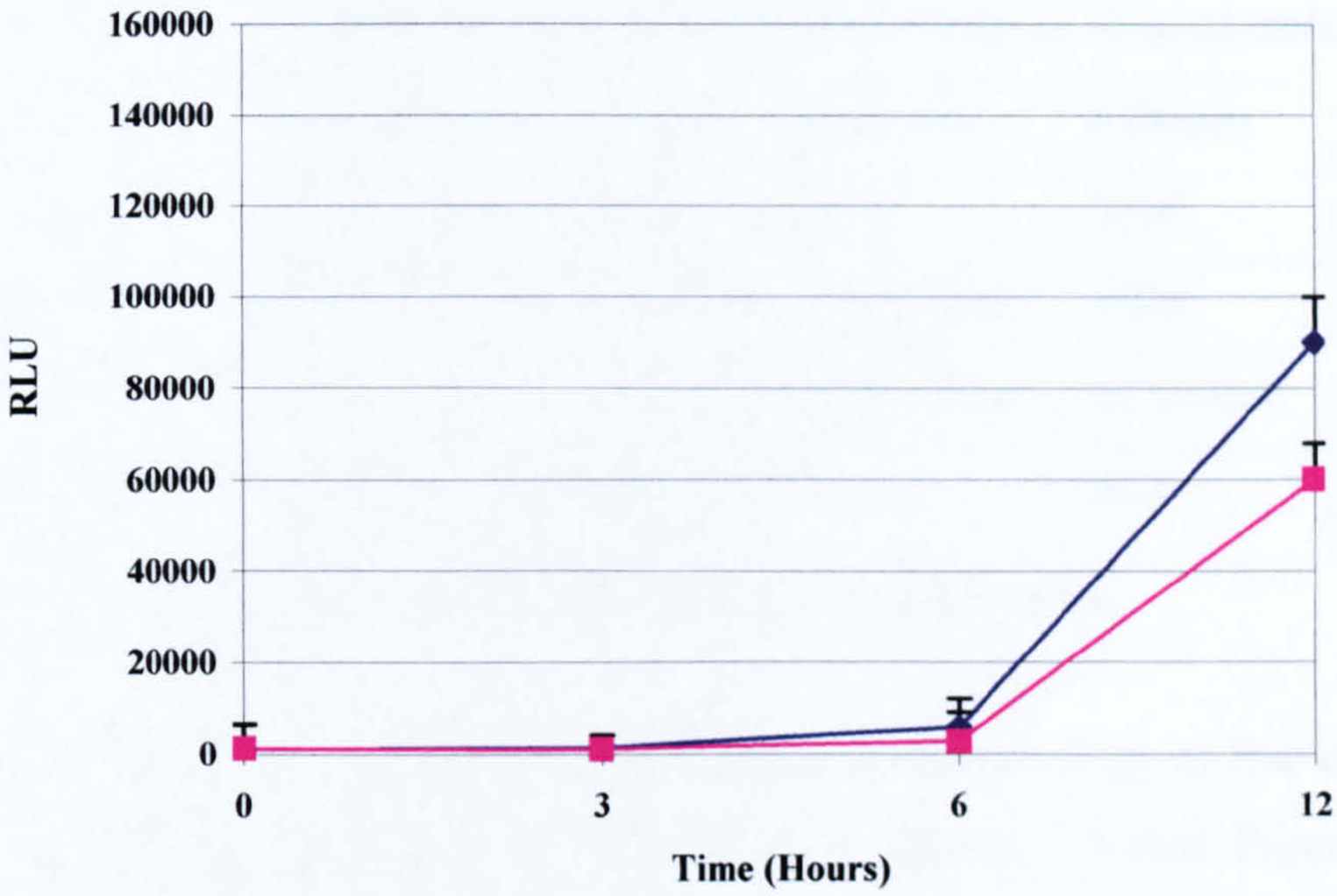

FIG 2-6 Growth curves for the SCVs in MHB with and without gentamicin through four different time points.

Figure 2.6 represents the growth of staphylococcal SCVs strains through 4 time points. Each point on the curve is the mean value of all the fourteen staphylococcal SCVs RLU tested individually. There was not any significant difference in RLU in the first 6 hours between staphylococcal SCVs strains grown in the presence of gentamicin or in antibiotic free MHB. The second six hours showed an increase in RLU in both curves with higher staphylococcal CFU/mL in the absence of gentamicin (data not shown). It is believed that reversion to the wild type had occurred in the second six hours and it is responsible for the increase in RLU in controls. Data in Table 2.12 showed that revertants had appeared at that time point, supporting the observed increase in RLU. The ATP contents of the SCVs were calculated from the curve and found to be in range of $3.9x10^{-5} - 4x\ 10^{-5}$ RLU/Cell.

Table 2.12: Monitoring staphylococcal morphology at five time points after growing in MHB in the presence and absence of gentamicin

Strains	Zero Time	1 Hour	3 Hours	6 Hours	12 Hours
Wild	Wild	Wild	Wild	Wild	Wild
Wild/Gen	Wild	Wild	Wild+SCV	Wild	Wild
SCV	SCV	SCV	SCV+*Rev	SCV+Rev	Rev
SCV/Gen	SCV	SCV	SCV	SCV	SCV+Rev

***REV= revertants (SCVs revert to wild type status). Gen=gentamicin**

Data in Table 2.12 shows the staphylococcal morphology at five different time points when growth was monitored as in Figure 2.5 and Figure 2.6. It is obvious that after three hours incubation staphylococcal morphology had been affected by the presence or the absence of gentamicin. SCVs appeared after 3 hours incubation of wild strains in the presence of gentamicin, and revertants appeared in cultures where SCVs growth was monitored without a stabilising concentration of gentamicin. However, after 6 hours incubation of wild types with gentamicin, SCVs were no longer detected. The reasons could be that the fast growth of wild strains could mask the appearance of SCVs, or that growth-associated decreases in the pH of the MHB could affect gentamicin ability to select for SCVs (Massey *et al*, 2001).

SCVs produced by microbroth dilution maintained stability through the first 6 hours when gentamicin was supplemented, however, after 12 hours incubation in the absence of antibiotic revertants appeared.

2:4 Discussion and Conclusions

This Chapter describes a systematic study in the following aspects of SCV physiology:

(a) Generating stable staphylococcal SCVs by treating the wild staphylococcal strains with increasing concentrations of gentamicin above the MIC.

(b) The use of diagnostic laboratory tests to screen for any virulence factors expressed by staphylococcal strains and to identify variation in expression of these factors between wild type and SCV staphylococci.

(c) The study of selected features of the metabolism of SCVs, that might correlate with their phenotypic variations.

Strains of interest were isolated from chronic infections mainly BRI (Table 2.1). SCVs are known to be associated with chronic infections, particularly those that are persistent and recurrent. SCVs have been isolated from sites of infection and following both *in vitro* and *in vivo* exposure to aminoglycosides (Looney, 2000; Proctor *et al.*, 1995, Wise and Spink, 1954).

In the current study SCVs were produced by exposing the wild type staphylococcal strains to serial passages in gentamicin above the MIC. MICs for gentamicin were determined by microbroth dilution for the wild type strains. This method was favoured over the E-test (Table 2.2) since the E-test failed to indicate the optimal concentration at which SCVs could be produced, and subsequently maintained in pure culture for several passages (Table 2.3). At the end of the selection assay, fourteen-staphylococcal strains out of twenty-eight produced stable SCVs which were then characterized (Table 2.4). Two of the strains were *S. aureus*, one was *S. haemolyticus* and eleven were *S. epidermidis*. The reason why some wild strains failed to produce SCVs is still unknown and this observation has not been considered in detailed in the current literature on staphylococcal SCVs. However, it is possible the gentamicin selection for SCVs could be species and strain dependent.

In order to begin to investigate the possible role of SCVs in BRI, virulence factor production by wild strains and their corresponding SCVs was investigated. The majority of virulence factors were screened using standard laboratory tests. Some virulence factors were selected for study based on previously published data from the literature. Metabolic profiles, auxotrophy

and growth rates of SCVs and their wild type strains were also compared to look for any pattern unique to the SCV phenotype.

Lipases and proteases are exoenzymes which are known to be staphylococcal virulence factors. These two classes of enzymes are important in allowing dissemination by tissue destruction (Goguen *et al.*, 1995). In this current study, *S. haemolyticus* (16) SCVs showed lipolytic against tributyrin and protease activities which were not shown by their wild type parental strain. However, while *S. epidermidis* SCVs (18 and 25) had differences in lipase activity, *S. aureus* SCVs had no difference in their lipolytic or proteolytic activity when compared to their corresponding wild type strains (Table 2.11).

Overnight incubation on sheep blood agar plates revealed that *S. aureus* SCVs were non-haemolytic, while the coagulase test showed delayed tube coagulation with human plasma. However, *S. aureus* SCVs sustained the ability to degrade DNA (Table 2.8). The haemolytic activity of coagulase-negative SCVs did not differ from wild type although *S. haemolyticus* SCVs strain 16 showed double haemolysis when compared to the wild strain parent (Table 2.9).

API Staph for staphylococcal identification revealed that the CoNS SCVs were unable to ferment NAG (N-acetyl-glucosamine) and to use arginine, but were able to ferment MNE (mannitol), while in contrast to their parental strains, *S. aureus* SCVs were urease negative (Table 2.5). Based on API Staph results, a conclusion can be drawn that staphylococcal SCVs are variants with altered metabolic pathways. These variants, of which there are different classes, have many atypical characteristics one of which is a defect in metabolic pathways which could be responsible for their slow growth and minute colony morphology. However, it is not possible to identify exactly which pathway is defective based solely on Api Staph results. The ApiZym profiles showed that the majority of SCVs have altered enzymatic activity in esterase, lipase, α-glucosidase and alkaline phosphatase irrespective of whether they were *S. aureus* or CoNS (Table 2.7).

Reduced activity of the electron transport system can account for most of the features typically seen in *S. aureus* SCVs. Based on the results presented in this study, CoNS SCVs features such as slow growth could be explained by reduced ATP cell content. Clinical and laboratory-generated *S. aureus* SCVs are frequently observed to be auxotrophic for menadione or thiamine, two

components required in the biosynthesis of menaquinone and cytochromes, components of the electron transport chain. Screening for auxotrophy in this study revealed that *S. aureus* and CoNS SCVs are menadione and/or thiamine auxotrophs. Only one *S. aureus* SCV was shown to be a proline auxotroph (Table 2.10). The decrease in electron transport activity in SCVs that was observed in the present study may provide a mechanism for their persistence within host tissues (Proctor *et al.*, 1995). Down regulating some aspects of their metabolic profiles, would enable staphylococcal SCVs to produce multiple changes that may contribute to their pathogenesis.

SCVs are reputed to be slow growing organisms (Goudie and Goudie, 1955; Proctor *et al.*, 1997) when compared to their wild type parents. Decreased availability of ATP would lead to slow growth, and hence small colonies. In this study, ATP activity (as represented by RLU per cell in the Vialight MDA assay) indicated that wild strains contained three logs more ATP than SCVs. The growth curves indicated that staphylococcal wild types entered log phase in about 3 hours, but that SCVs with a slower rate of growth entered log phase in about 6-7 hours (Figures 2.5 and 2.6).

Reversion on the other hand is important in the staphylococcal SCVs pathogenesis since reversal of the SCV phenotype might form the basis for recurrent infections. Reversion may represent a mechanism for bacterial survival and growth in response to environmental conditions. In this study, it was important to realise that entry to SCVs status is reversible upon growth in medium without antibiotics, showing it as not being mutation but a regulatory mechanism or phase variation (Table 2.12).

Lysostaphin, a peptidase has recently been studied as a potential therapeutic agent for use against *S. aureus* (Patron *et al.*, 1999). In this study, assessing lysis by lysostaphin indicated that *S. aureus* and CoNS SCVs were more resistant to lysostaphin than their corresponding wild type strains (Figure 2.4). Among *S. aureus* isolates the development of resistance to lysostaphin is known to be mediated by alterations in the crossbridge of the muropeptide of the cell wall (Climo *et al.*, 2001) and CoNS (Ehlert *et al.*, 2000). Based on these results, it could be concluded that SCVs may be more resistant to lysostaphin due to alterations in their cell wall composition (Figure 2.4).

Antibiograms showed that SCVs were resistant to gentamicin. Resistance to gentamicin was also accompanied by resistance to other aminoglycosides such as amikacin and /or netilmicin Table 2.6. Other researchers such as Miller et al (1980) also reported the same results when they used a *S. aureus* mutant with a SCV phenotype and found this mutant to be resistant to gentamicin, amikacin and netilmicin.

From the data presented in the literature, it is evident that there is no definitive means by which to either identify SCVs, or to predict the clinical usefulness of various regimes to identify their virulence factors. Therefore, the experiments described in this Chapter were intended to acquire and improve knowledge of aspects of SCVs' virulence (such as the production of secreted virulence factors) which might contribute to the pathogenesis of BRI. However, this is a pilot study of SCV virulence and it is recognised that it will be necessary to investigate other aspects of staphylococcal virulence in addition to the conventional ones described in this Chapter to provide a more complete profile of how the virulence of SCVs may differ from their wild type parents.

Having developed a method for generating SCVs and undertaken their preliminary characterization, these organisms were then used to further investigate specific aspect of the role of SCVs in the pathogenesis of BRI. These studies are described in Chapters 3-6.

Chapter Three

Genotypic and Further Phenotypic Characterisation of Staphylococcal Wild and Small Colony Variant Strains

3:1 Introduction

This Chapter investigates further the phenotypic and genotypic similarities and difference between staphylococcal SCVs and their wild-type strains. The typing systems used in the following comparisons have conventionally been classified as phenotypic techniques, those that detect characteristics expressed by the microorganisms, and genotypic techniques, those that involve direct DNA based analysis of chromosomal genetic elements.

One method of monitoring phenotypic variations is by the use of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), a widely used technique which is used for a variety of protein characterisation applications, both general and specific. Immuno-reactivity of Western blotted proteins, two-dimensional gel electrophoresis, and peptide mapping are related techniques used for investigation of the identity of specific proteins. Such techniques are also routinely used to study unknown proteins, to determine if they are similar to, or have any structure in common with, known proteins. SDS-PAGE has been used successfully as a taxonomic tool by several researchers. However, Clink and Pennington (1987) suggested that although SDS-PAGE can be used to identify staphylococcal species, this type of analysis will not provide the basis for a complete typing method since they showed the occurrence of minor differences in polypeptide pattern between strains of the same species, though each species did produce a protein pattern distinguishable from that of any other (Clink and Pennington, 1987).

Immuno-blotting is another way of typing proteins. The electrophoresed bacterial products are transferred from a gel to (blotted onto) a nitrocellulose membrane and then exposed either to antisera (antibodies) raised against a specific strain or to pooled human serum, which contains a broad spectrum of reactive antibodies. Antibody binding can be detected using enzyme-labelled anti-primary immunoglobulins. Depending on the detection system used to determine antibody reactivity (colorimetric or enhanced chemiluminescence based), as little as 1-5 ng of an average-sized protein can be detected.

Genotypic techniques to study microbial strain and species differences have been distributed and performed increasingly in clinical laboratories. The pulsed field gel electrophoresis (PFGE) technique is an adaptation of conventional agarose gel electrophoresis that allows extremely large DNA fragments from chromosomal digests to be resolved. PFGE is an extremely powerful tool commonly used for genotypic and epidemiology studies for pathogenic microorganisms. PFGE requires the preparation of restriction digests of DNA. A restriction endonuclease enzymatically cuts double-stranded DNA at a specific nucleotide recognition sequence. The fragments generated are then separated by size by agarose gel electrophoresis and visualised by staining the electrophoretic gel with ethidium bromide and viewing under ultraviolet (UV) light. Linhardt *et al* (1992) showed that PFGE is an important epidemiological tool with high discrimination power for *S. aureus* and coagulase-negative staphylococci. Typing of *S. aureus* is often required for the investigation of an outbreak or as part of ongoing surveillance, whereas typing of CoNS is often used to determine whether two or more strains from a patient represent contamination or infection.

Since PFGE has been reputed to be highly discriminatory and effective at identifying related isolates, PFGE was used in this study to investigate whether wild type strains and their corresponding SCVs were isogenic (clonal). Isogenic strains should have the same DNA profiles.

3:2 Materials and Methods

3:2:1 Characterising Staphylococcal Strains by Pulsed Field Gel Electrophoresis (PFGE)

PFGE is a DNA fingerprinting method for typing staphylococci and other bacteria. It is a variation of agarose gel electrophoresis in which the orientation of the electric field across the gel is changed periodically. This enables DNA fragments as large as megabases to be separated on the basis of size. The organisms under investigation are embedded in agarose plugs and the cell wall and the cellular proteins are digested enzymatically. The isolated genome is then digested *in situ* with selected restriction enzymes, such as *Sma*I. The staphylococcal chromosome is about 2,800 KB, and when digested with *Sma*I (recognition sequence CCCGGG), PFGE provides a profile of about 15-20 distinct and well-resolved fragments ranging from 10-800kb.

The fourteen staphylococcal wild strains which produced stable SCVs (Table 2.4) were investigated with PFGE. The protocol used in this Chapter is based on the methods of Bannerman *et al* (1995) and Leonard *et al* (1995). The procedure was spread out through several days of preparations to assure a good electrophoresis result as described. The recipes for preparing the solutions are found in the Appendix 8:1:3.

3:2:1 (A) Day One- Bacterial Culture Preparation

Fourteen-staphylococcal wild strains were plated on TSA whereas their corresponding SCVs were generated as described in (Table 2.4) by plating on TSA plates supplemented with gentamicin and incubation overnight at 37°C.

3:2:1 (B) Day Two- Preparation of Cell/Agarose Blocks and Cell Lysis

1. The colonies were resuspended in 5 mL Tris-EDTA buffer (TE) (pH 7.5) and the OD_{550} was adjusted to 1

2. Bacterial suspension (600µL) was transferred into a 1.5mL eppendorf and centrifuged at 13,000 rpm for 1 minute

3. The supernatant was discarded and the pellet was resuspended and washed 3 times in 3x500µL of TE buffer

4. Following centrifugation at 3000 rpm for 5 minutes, the pellet was resuspended into 250µL TE and vortexed. The eppendorf was then placed on ice

5. Low Melting Point (LMP) Agarose 2% wt/vol (Bio-Rad, Hemel Hempstead, UK) was dissolved in autoclaved de-ionised water in a 500mL conical flask

6. The flask with the agarose was placed in the microwave oven and heated until the agarose particles were completely dissolved

7. To prepare the plug moulds (Bio-Rad, Hemel Hempstead, UK), the bottoms were sealed with white tape

The next steps were done one tube at a time

8. Lysostaphin (1mg/mL) in distilled water (Sigma, Poole, UK) (10µL) was added to each eppendorf and very gently mixed (at this stage the eppendorfs were not vortexed to prevent the possible artefactual shear-damage of the DNA)

9. Immediately, 250µL LMP agarose solution was added into the eppendorf. The contents were mixed gently and briefly by pipeting

10. Immediately, the agarose/cell mixture was pipetted into the plug moulds (2 blocks were prepared per isolate)

11. The moulds were kept on ice for 10-15 minutes

12. For each isolate, 1 mL Lysis buffer was pre-warmed at 37°C and added to a fresh eppendorf

13. The plugs were dispensed into the Lysis buffer

14. The eppendorfs were incubated for 60 min at 37°C

15. After incubation, 100µL Proteinase K (Sigma, Poole, UK) (10mg/mL) in distilled water was added to the eppendorfs and these were then inverted several times

16. The eppendorfs were then incubated at 50°C overnight

17. Standard reference *S. aureus* NCTC 8325 was used as a control

3:2:1(C) Day Three- Digestion of DNA with Restriction Enzyme

1. The plugs were trimmed to produce 2mm x 3mm slices and placed in a 1.5ml eppendorf
2. PMSF (200µL) (0.5 mg/mL) in TE (pH 8.0) was added for 30 min at 50°C
3. The PMSF in TE was then aspirated carefully to ensure that the plugs were not damaged
4. The plugs were washed 3 times in 200µL of ice cold TE (pH 8.0) buffer
5. TE buffer was removed thoroughly from the eppendorfs
6 The restriction enzyme (RE) Master Mix (Promega, Southampton, UK) stock was then prepared. RE Master Mix (100µL) was added to each eppendorf (sterile distilled water (85µL), *Sma*I buffer x10 concentration (10 µL), BSA (Bovine serum Albumin) (1µL), *Sma*I (10 units/ µl) (4µL))
7. The eppendorfs were incubated at 30°C for 3 hours

3:2:1 (D) Day Four- Preparation of Agarose Gel for 30 Well Gels

1. The following components (distilled water, x10 Tris-Borate buffer (TBE) and PFGE grade agarose) were added to a 250mL conical flask

30 Well Gel	Distilled Water	X10 TBE	PFGE Grade Agarose
	142.5 mL	7.5mL	1.5g

2. Agarose was dissolved in a microwave oven at full power for 2 ½ minutes. The flask was removed and swirled carefully and placed again in the microwave at full power for another 1 minute. The mixture was then placed in a water bath to cool to 50°C
3. The agarose (50°C) was then poured into the gel mould and left to set for 30 minutes

3:2:1 (E) PFGE

1. Electrophoresis was performed using the CHEF-DRII Drive Module electrophoresis cell (Bio-Rad, Hemel Hempstead, UK). The device was set up according to the manufacturer's recommendations and instructions
2. 800mL of x 0.5 TBE buffer was poured in to the gel chamber. The Gel was placed onto the middle of the gel chamber and the digested plugs were treated at 52°C for 8 minutes before loading into wells. At this stage the wells were not covered with the buffer
3. TBE buffer (800mL) was poured very gently to cover the gel then the lid was closed
4. The apparatus was checked for correct assembly and the interlock cable was connected to the drive module to run the electrophoresis. The temperature was kept constant at 14°C by a cooler (Bio-Rad, Hemel Hempstead, UK) connected to the CHEF –DRII device

5. The running parameters were as follows:

	1st Setting	**2nd Setting**
Run Time	10 Hours	13 Hours
Initial Switch	5.0 Sec	15.0 Sec
Final Switch	15.0 Sec	60.0 Sec
Voltage	200 V	200 V

3:2:1 (F) Day Five- Gel Staining and Image Storing

1. On the fifth day, after the PFGE run had finished, the gel was transferred to a separate container and washed with distilled water at least once
2. The gel was carefully transferred to a staining box and stained with ethidium bromide (1mg/L in H_2O) for 30 minutes.
3. De-staining was performed in distilled water for 45 minutes
4. The gel was viewed under a UV transilluminater, photographed and processed by imaging software VisionWorks 3.1 from ultra violet Products

(UVP Ltd, Cambridge, UK). A separate PFGE profile was generated for each strain.

3:2:2 Staphylococcal Protein Profiles by Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot Analysis

SDS-PAGE is an analytical and/or preparative electrophoresis of proteins that ensures dissociation of the proteins into polypeptide subunits. SDS and heat is used to denature and solubilise the proteins before they are loaded on the gel where SDS-polypeptide complexes migrate through polyacrylamide gels in accordance with the size of the polypeptide. The gel is composed of a stacking gel into which the sample is loaded and the resolving gel where the polypeptides are separated.

In the current study, protein profiles were prepared as two sets: first, from whole cell preparations and second, from three cell fractions- cell wall, membrane and cytoplasmic. The two approaches were used to improve discrimination between the wild type strains and their corresponding SCVs.

In the immuno-blotting analyses electrophoresed polypeptides were transferred onto nitrocellulose blots which were then blocked to reduce non-specific antibody interactions, and probed for immuno-reactivity in two stages: an incubation with an unlabelled (polyclonal) primary antisera followed by washing, and incubation with a secondary anti-immunoglobulin coupled to a detector enzyme activity, in this case horseradish peroxidase (HRP). After further washing, the colour was developed by the addition of H_2O_2 and the blots then photographed. The appearance of purple coloured bands is indicative of antibody reactivity.

ECL (Amersham Biosciences, Buckinghamshire, UK) is a light emitting non-radioactive (luminescence) method for detection of immobilized antigens with (HRP) labelled antibodies. It is at least 10 times more sensitive than the colorimetric detection method, and is therefore useful in the detection of very lower quantities of proteins compared to colorimetric systems.

3:2:2 (A) Whole Cell Preparation

The method used for protein profile assessment was based on protocols used by other researchers (Cheung and Fischetti, 1988; Smith *et al.*, 1991; Modun *et al.*, 1994). SDS-PAGE was performed in 11% resolving polyacrylamide gels and 4% Stacking gels, as described previously (Sambrook, 1989). The compositions of the solutions used are found in the Appendix 8:1:1.

Bacterial suspension (10 mL) in PBS was prepared from colonies grown on TSA plates for wild type strains and TSA with gentamicin for SCVs (Table 2.4). The suspensions had a turbidity of 0.4-0.5 at 550 nm. This start point was repeated for whole cell preparation or for cell fractionating. The protocol used was as described below:

1. The 10-mL bacterial suspension was centrifuged at 13,000xg for 5 minutes
2. The pellet of bacteria was suspended in 1mL PBS and transferred to an eppendorf
3. The suspension was further centrifuged at 13,000x*g* for 5 minutes
4. The supernatant was then removed and 350 µL of (80µg/mL lysostaphin (Sigma, Poole, UK) and 1mg/mL benzamidine (Sigma, Poole, UK) in PBS was added to the pellet and tubes vortexed to resuspend the cells
5. The cell suspension was then incubated for 15-30 minutes at 37°C in a water bath
6. 350 µL of 2xSDS PAGE sample buffer was added to the suspension
7. The eppendorfs were boiled for 5 minutes
8. The gelatinous suspensions were sonicated for 15 sec with a fine sonicater probe
9. The eppendorfs were finally centrifuged at 13,000x*g* for 5 minutes to pellet any unlysed cells and cell debris
10. 15 µL of the supernatant was loaded per lane of an 11% SDS-PAGE mini gel
11. Molecular size standards-(prestained protein markers) (Bio-Rad, Hemel Hempstead, UK) were run concurrently in parallel wells

3:2:2 (B) Cell Wall, Membrane and Cytoplasmic Proteins Preparations

1. To dry cell pellets prepared as described above was added 500µL of the following cell wall digestion mixture (80µg/mL lysostaphin, 1mg/mL benzamidine and 0.5 mg/mL PMSF and 30% w/v raffinose in PBS. The raffinose was employed as a hypertonic medium to osmotically stabilize the protoplasts that are formed during lysostaphin digestion.

2. The eppendorfs were vortexed to resuspend the cells in the digestion buffer and incubated for 15 minutes at 37°C

3. The suspensions were centrifuged at 13,000xg for 5 minutes

4. The supernatants containing wall proteins were removed and the pellets containing protoplasts were used for further fractionation

5. To prepare membrane proteins, 500µL PBS was added to protoplast pellets and the eppendorfs were cooled on ice for 10 minutes

6. Protoplast suspensions were then sonicated for 15 second with a fine sonicater probe to lyse protoplasts. After sonication, the eppendorfs were centrifuged for 10 minutes at 13,000xg

7. The supernatants containing the cytoplasmic proteins were removed and retained. The pellets contained the membrane protein fraction

8. For SDS PAGE, 500µL of 2xSDS-sample buffer was added to wall or cytoplasmic protein preparations. Suspensions were boiled for 5 minutes and centrifuged at 13,000xg for 5 minutes

9. For the membrane protein preparations, 200µL of 1xSDS sample buffer was added to the membrane pellet which was boiled for 5 minutes and centrifuged at 13,000xg for 5 minutes

10. 15µL of each of the preparations was loaded per lane of an 11% SDS-PAGE mini gel

3:2:2 (C) Electrophoresis and protein visualisation

The BioRad Mini-Protein II electrophoresis gel system was used for electrophoresis of gels. The equipment was assembled, the gels cast and the chambers prepared as described in the BioRad instruction manual. The gels consisted of an 11% separating gel and 4% stacking gel. A current of 30 mA (per mini gel) was applied and the gel was run in a BioRad tank in SDS-PAGE electrophoresis buffer (Appendix 8:1:1) for about 60 minutes. Gels were prepared in duplicate; one was stained and used for visualisation of proteins and the other one was used for immunoblotting. Gels that were to be stained were washed after electrophoresis three times in PBS for 5 minutes and then transferred into a container for staining as described below.

3:2:2 (D) Staining and Destaining the Gels for SDS-PAGE

Simple Blue ™ Safe Stain (Invitrogen, Paisley, UK) was used to stain the gels. Gels were covered with the stain (20-mL) for one hour at room temperature and with gentle shaking. To destain, the gel was washed with water (100 mL) for a minimum of one hour. The gel was then photographed, and processed by imaging software VisionWorks 3.1 from ultra violet Products (UVP Ltd, Cambridge, UK). The data is presented in Table 3.1 and Figure 3.2.

3:2:2 (E) Transfer of Proteins from SDS-Polyacrylamide Gels to Nitrocellulose Filters for Western Blotting Analysis

1. When handling the gels, gloves were worn to avoid oil from the skin that could prevent the transfer of proteins from the gel to the filter and to avoid contamination of blots with skin proteins
2. Two pieces of blotting paper and one piece of nitrocellulose filter (Sigma, Poole, UK) were cut to the exact size of the SDS-polyacrylamide gel
3. The nitrocellulose filter and the blotting papers were allowed to wet from beneath by capillary action in the transfer buffer. Then, they were submerged in the buffer for at least 5 minutes to displace trapped air bubbles

4. The horizontal transfer blotting apparatus (BioRad Transblot system, Hemel Hempstead, UK) was set up as follows: the blotting paper was laid to the bottom electrode sheet, and the nitrocellulose placed on the blotting paper. The SDS-PAGE gel was then placed exactly on the top of the nitrocellulose filter. Trapped air bubbles were squeezed out gently with a gloved hand gently. The last sheet of blotting paper was placed on the top of the gel. Finally to get rid of any air bubbles which could be trapped between gel and membrane, a glass pipette was used as a roller over the gel-membrane sandwich

5. The upper electrode sheet was then placed on the top of the stack. The electrical leads were connected. The transfer was carried out at 14V for 20 minutes. After the electrophoresis the blots were briefly rinsed in PBS, and blocked prior to immuno probing as described below.

3:2:2 (F) Immuno Probing of Blotted Staphylococcal Proteins: Colorimetric-Secondary Antibody Detection Systyem

1. Nitrocellulose blots of staphylococcal proteins were blocked for 3 hours in blocking buffer, washed 3 times for 5 minutes in PBS, and incubated at room temperature with rabbit *S. epidermidis* antiserum (Eurogentec, Seraing, Belgium), diluted 1:100 in blocking buffer (0.1 mL of skimmed milk solution per cm^2 of filter)

2. After the primary antibody incubation the membranes were washed three times for 10 minutes with PBS and probed with horseradish peroxidase-conjugated goat anti-rabbit (HRP-GAR) diluted 1:2000 in blocking buffer and incubated at room temperature for three hours

3. To visualise immuno-reactive proteins, the membranes were washed with PBS three times for 10 minutes and transferred to a shallow tray to which was added the HRP substrate chromogen 4-chloro-1-naphthol (Sigma, Poole, UK). The substrate (3mg) was dissolved in 10 mL PBS. H_2O_2 (10 μL of 30% vol/vol in H_2O) was added and mixed and used immediately upon the membranes (0.1 mL of the substrate solution per square centimetre of filter)

4. The progress of the reaction was monitored visually. After 2-3 minutes the polypeptides had usually reached the desired intensity. To stop the reaction,

the membranes were washed briefly in water and then transferred to a container containing PBS

5. The developed membranes were then dried and scanned for a permanent record of the experiment. Data is presented in Table 2.2 and Figure 3.3.

3:2:2 (G) Enhanced Chemiluminescent (ECL) Antibody Detection System

ECL is as a method of visualising immuno-reactive proteins on membrane blots (Amersham International, Pharmacia Biotech, Buckinghamshire, UK) ideally suited to the detection of lower quantities of proteins that could not be detected by colorimetric methods.

The difference between the colorimetric and ECL detection system is in the way of detecting the secondary antibodies. In chemiluminescence the excitation is effected by a chemical reaction. HRP/Hydrogen Peroxide catalyse the oxidation of cyclic diacylhydrazides (luminol). ECL is achieved in the presence of chemical enhancers such as phenols. The light emission at wavelength of 428 nm is detected by a short exposure to blue-light sensitive film (Hyperfilm ECL, Amersham /Pharmacia Biotech).

1. An equal volume of detection solution (1) was mixed with detection solution (2). The final volume required is 0.125-mL/cm^2 membrane. The membrane and the detection solution were incubated for 1 minute at room temperature

2. Excess detection reagent was then drained off. The nitrocellulose membrane with protein side down was put between two transparency sheets (5 Star, UK)

3. This stage was carried out in a dark room using red safelights. A sheet of autoradiography film was placed on the top of the wrapped blots with protein side up

4. The wrapped blots with the film were placed in an x-ray film cassette and closed for 15 seconds

5. The film was removed from the box and immersed in the developer (Universal Developer) (Ilford Imaging UK Ltd, Cheshire, UK) tank for 3 minutes followed by a rinse in a water tank for 30 seconds and finally the film was transferred into a fixer (HYPAM) (Ilford Imaging UK Ltd, Cheshire, UK)

tank for about 3 minutes. The film was then given a wash in a water tank for about 30 seconds

6. The film was left to dry and subsequently scanned for a record. Data was presented in Table 2.3.

3:3 Results

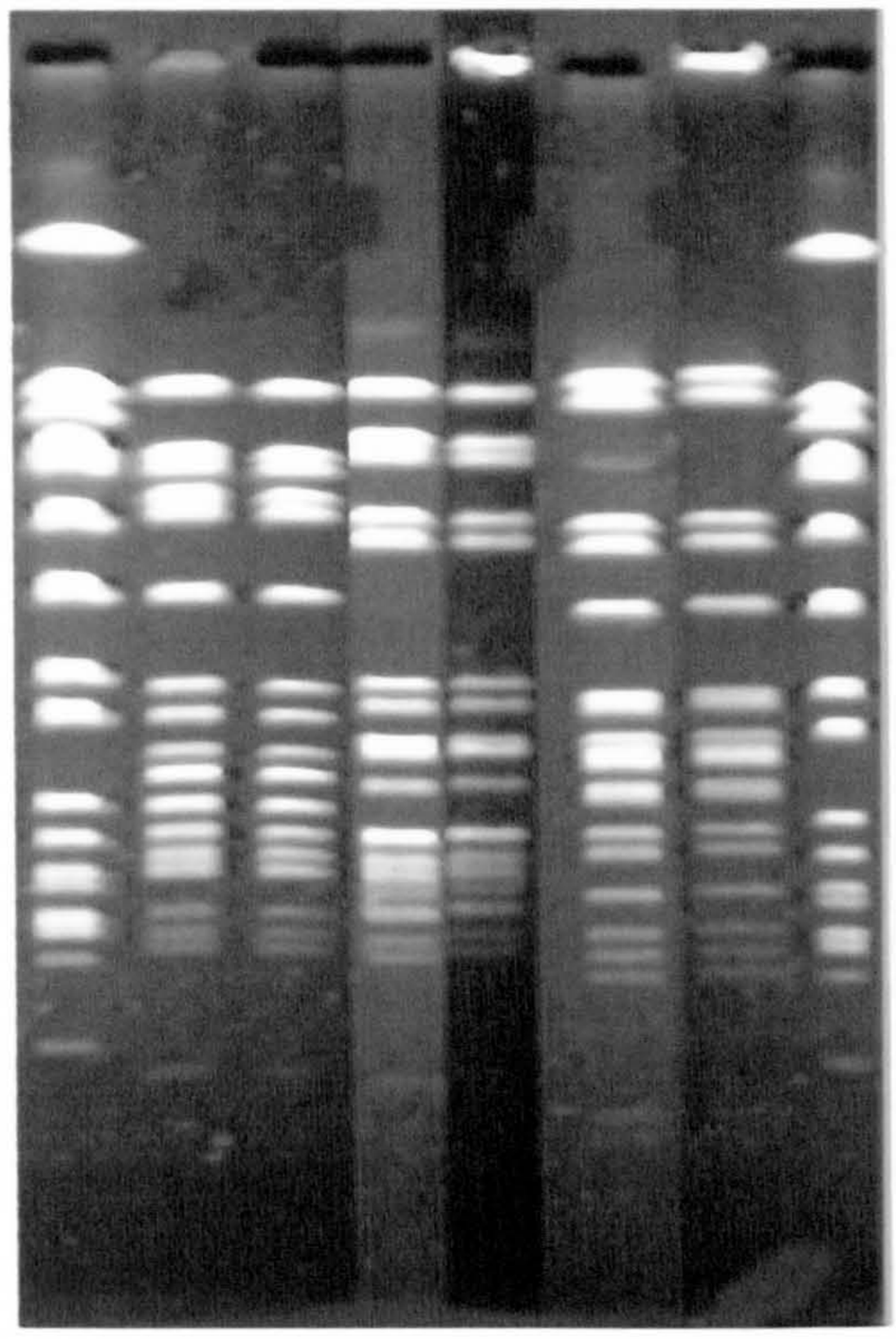

FIG 3-1: Pulsed field gel electrophoresis of *SmaI* cleaved genomic DNA of three different wild (W) staphylococcal strains and SCVs (S)

Representative PFGE profiles are demonstrated in Figure 3.1 for three different staphylococcal strains (*S. aureus* 11, *S. epidermidis* 25, *S. haemolyticus* 16) along with their corresponding SCVs. NCTC 8325 is a *S. aureus* control strain shown on the right and left side of the gel. Comparing the DNA profiles for the wild strains and SCV strains showed that there was essentially no difference within the same species, although different species showed different DNA profiles. The genomic profiles of cleaved *SmaI* DNA investigated by PFGE suggest that the wild strains and their SCVs are isogenic derived from the same origin and there is no obvious DNA alterations detected.

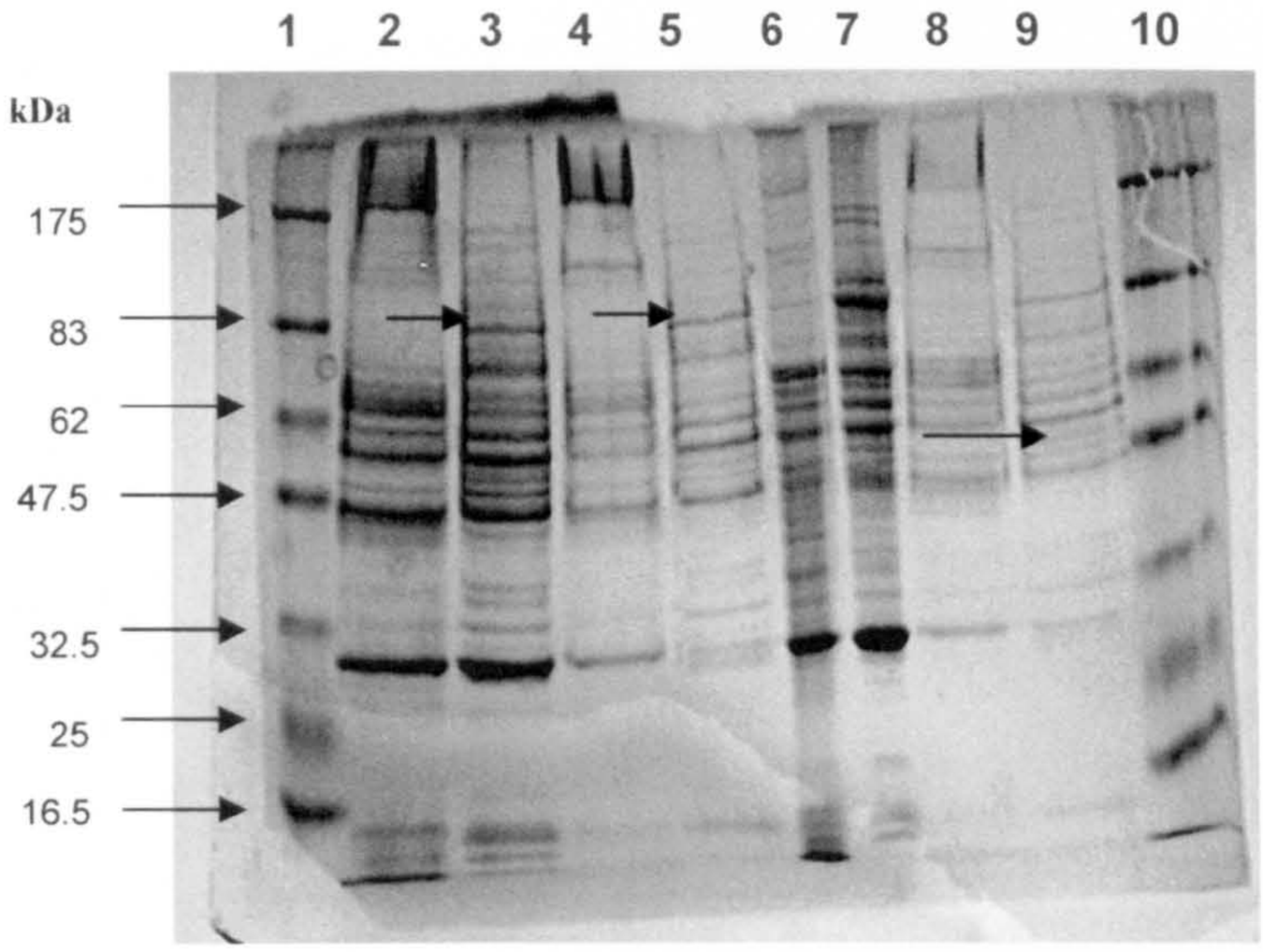

FIG 3-2: SDS-PAGE of wild type and SCVs whole cell preparation and cell fractions.

Pre-stained protein marker (1 and 10), wild whole cell prep (2), SCVs whole cell prep(3), wild cell wall fraction (4), SCVs cell wall fraction (5), wild membrane fraction(6), SCVs membrane fraction (7), wild cytoplasmic fraction (8), SCVs cytoplasmic fraction (9)

Figure 3.2 is a representative gel showing SDS-PAGE analysis of *S. epidermidis* 7 (wild type and SCV) whole cell and cellular protein fractions with at least two additional proteins of 83 kDa in SCVs in whole and cell wall protein factions and 34 kDa in the cytoplasmic fraction (indicated by arrows). SDS-PAGE profiles for all the staphylococcal SCV strains analysed usually revealed extra or missing polypeptides when compared with the equivalent protein fraction obtained from their wild type parent. The molecular weights of the most prominent extra or missing proteins in SCVs when compared to their wild parental strains are presented in Table 3.1 below.

Table 3-1: Differences in protein SDS-PAGE expression profiles between staphylococcal wild type and SCV strains

Strains	Whole Preparation	Cell Wall Fraction	Cell Membrane Fraction	Cytoplasm Fraction
SE 3	+83, -60	+83		-60, -45
SE 4	+77	+83, +60		
SE 7	+83	+83		+34
SE 8	+77	+77		
SA 11	+83	+83		
SE 13	+83, -67	+83		
SE 16	+83	+83		-85
SH 18	+83, +34	+83		+34
SA 21	-77			-77
SE 23	+60	+60		
SE 25	+83	+83		
SE 26	+60	+60		
SA 27	-45			-45
SE 28	+83	+83		

- =Polypeptide missing from SCV; + = Extra polypeptide present in SCV; SE=*S. epidermidis*, SA=*S. aureus*, SH=*S. haemolyticus*

Table 3.1 summarises the approximate molecular weights of polypeptides separated by SDS-PAGE as a comparison between SCVs and wild type strains. Proteins were separated from whole cell preparations, cell wall, cell membrane and cytoplasmic fractions to screen for any differences in protein expression which could be related to the SCV phenotype generated by exposure to gentamicin. A protein present in whole cell preps and cell wall fractions of around 83 kDa was detected in 9 SCV strains. Cell membrane protein profiles were similar in wild type and SCVs; however, proteins of 85, 77, 60 and 45 kDa were missing from cytoplasmic fractions in some SCV strains.

Although these are preliminary analyses, the data from the above experiments indicates that the protein profiles of SCVs generated by exposure to gentamicin differ in a number of proteins compared to wild strains. Further studies could be directed to separate and identify these proteins in order to study their possible relation to known virulence factors or components of metabolic pathways. This could contribute to a much more complete understanding the pathogenic role of SCVs in BRI.

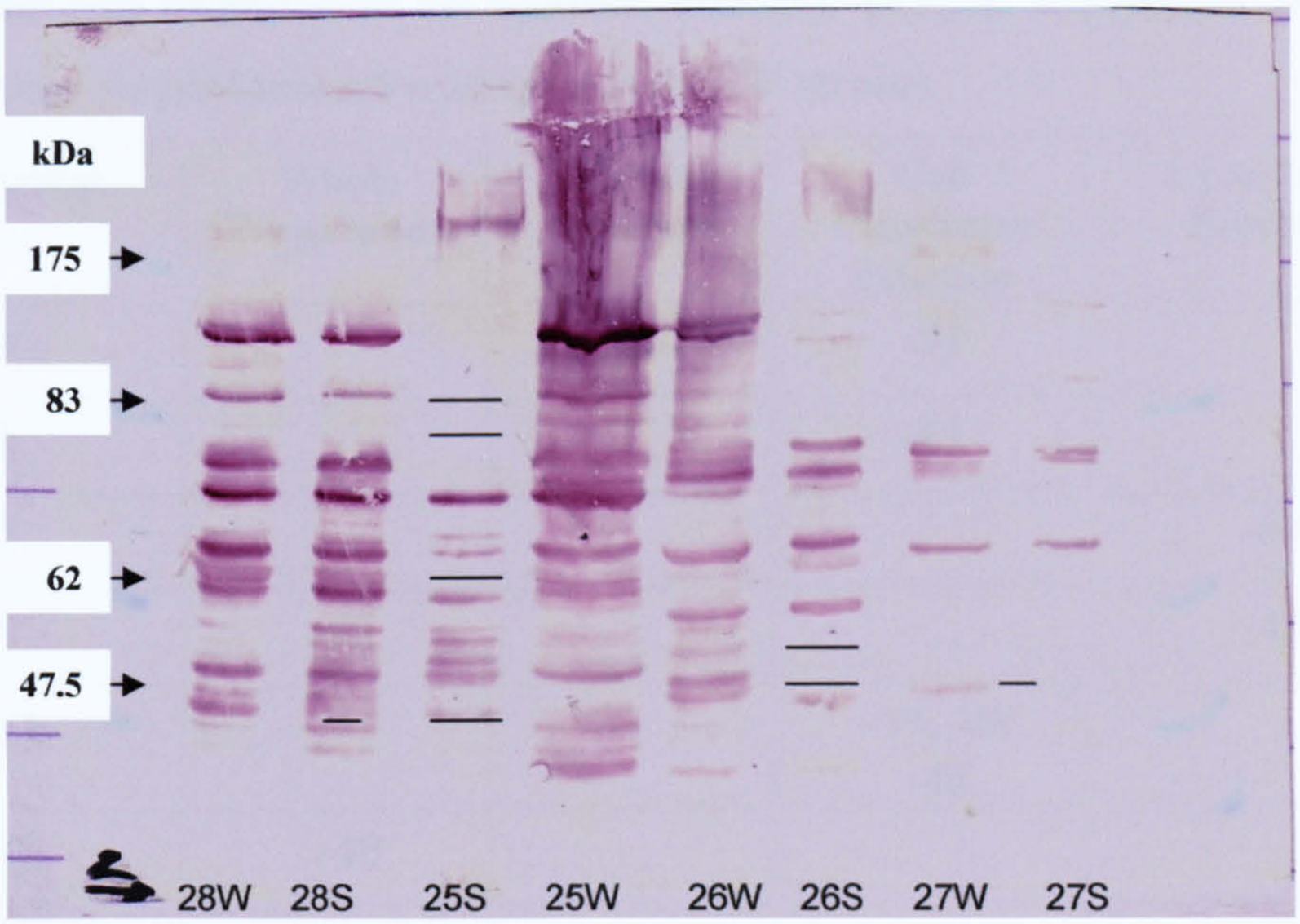

FIG 3-3: Antibody reactivity of Western blots of whole cell preparations for different SCV and wild type strains using a colorimetric detection method.
All strains above are *S. epidermidis*, except 27 which is *S. aureus*. Wild (W), SCVs (S)

Figure 3.3 is a representative western blot showing four different staphylococcal strains. The whole cell protein preparations for *S. epidermidis* strains 25, 26 and 28 and *S. aureus* 27 wild and SCVs are shown. The molecular weights of the immuno-reactive staphylococcal proteins were estimated by referring to pre-stained protein markers. In the figure protein bands which are missing from SCVs are indicated by lines. The molecular weights of missing or additional proteins in the fourteen SCVs generated by gentamicin exposure (Table 2.4) compared to their wild strains are presented in Table 3.2 below.

Table 3-2: Differences in immuno-reactive protein expression profiles between staphylococcal wild type and SCV strains

Strains	Whole Preparation	Cell Wall Fraction	Cell Membrane Fraction	Cytoplasm Fraction
SE 3		-36	-51	
SE 4			-51	
SE 7				-60
SE 8	-40			-40
SA 11			-77, -48 -40	
SE 13	-40			
SE 16		-60	-40	
SH 18	-32	-77		-32
SA 21				-60
SE 23				-60
SE 25	-40,-60,-80 -77		-60, -40	
SE 26	-51		-45	
SA 27	-51		-40, -54	
SE 28	-40		-48	

-=Polypeptide missing from SCV; +=Extra polypeptide present in SCV; SE=*S. epidermidis*, SA=*S. aureus*, SH=*S. haemolyticus*

Table 3.2 summarises the approximate molecular weights of differentially expressed SCV immuno-reactive proteins compared with their wild type parents. The data obtained above was obtained using two detection methods: colorimetric and ECL. Data obtained by the two detection methods were generally similar, though the ECL technique generally produced less background on the blots.

Proteins of different molecular weights were missing in all cell fractions from SCV strains and the whole cell preparations, though the precise molecular weight of the missing immuno-reactive proteins differed considerably between strains. For instance, proteins of 51 and 40 kDa were missing from

the whole cell preparations of SE strains 26 and 27, but not as far as could be determined from corresponding protein fractions of the other SCVs. Immuno-reactive proteins of 77, 60 and 36 kDa were found missing in SCVs cell wall fractions of SE 3 and 13 and SH 18. Membrane proteins of 77, 60, 54, 51, 48 and 40 kDa and the cytoplasimic fractions polypeptides of 60, 40 and 32 kDa were found missing from some SCVs, but not from all.

Considered collectively, the data in Table 3.2 suggests that SCVs may be less immunogenic when compared to their wild type parents. This may have implications for the prolonged survival of SCVs in the host and their contribution to BRI.

3:4 Discussion and Conclusions

The genetic profiles of the fourteen SCVs were compared by PFGE and all produced restriction patterns which were found to be similar to their wild parental strains (Figure 3.1). This indicates that SCVs are subpopulations and not contaminants even though differences in virulence factor expression between wild and SCV strains were detected in Chapter two. This supports the belief that SCVs are phenotypic but not genotypic variants.

PFGE was followed by SDS-PAGE to detect any phenotypic changes as identified by protein profiles. There were marked differences in protein expression profiles between SCVs and their wild type parents. Some SCVs were found to express additional polypeptides when compared with parental strains. For instance, proteins of approximately 83, 77, 60 and 34 KDa were present in SCV whole cell and cell wall preparations, which were not present in similar protein fractions from wild type strains, though as mentioned, increases in expression of these proteins were not seen in all SCVs. The protein whose expression showed a most consistent increase was the 83 kDa cell wall associated protein and whole cell preparations whose expression was induced/increased in 9/14 of the SCV strains tested (*S. epidermidis* [strains 3, 4, 7, 13, 18, 25, and 28], *S. aureus* [strain 11], and *S. haemolyticus* [strain 16]) (Table 3.1). However, polypeptides of 77, 67, 60 and 45 kDa were missing in the whole cell preparations as well as in the cytoplasmic fractions in some of the SCVs. The cell membrane fractions did not show any obvious differences between SCVs and their corresponding wild strains (Table 3.1).

One can often characterize the SDS-PAGE profile of a protein sample based on information already known about the biological system that is being studied. A survey of the recent literature investigating staphylococcal SCVs indicates that nothing has been published about phenotypic properties investigated by SDS-PAGE comparing wild and SCVs strains. This current study is therefore probably the first to recognise some of the differences which might help to not only identify SCVs, but also to provide some guidance as to future systematic and detailed study of this class of organisms. Due to time and resources restrictions it was impossible in the current project to perform any further

experimental work to identify the proteins whose expression is modulated in the SCV morphological state. However, this is a very promising area for future study. Those SCV polypeptides whose expression is up or down regulated could be purified and then sequenced. Once the protein is identified, there are many techniques which could be used to characterise the role of the gene concerned in SCV physiology, and BRI generally (e.g. mutagenesis, over expression, reporter-fusion etc).

A similar study to that described in this Chapter was performed on *P. aeruginosa* SCVs generated by gentamicin exposure (Langford *et al.*, 1989). Langford and his colleagues (1989) found that gentamicin induced the expression of an outer membrane protease of 34 kDa in SCVs with increased protease activity when compared to the wild type strain. This might tempt us to think that the staphylococcal SCV proteins identified in the current study as being increased in expression relative to wild type, could similarly be virulence factors.

The initial SDS-PAGE protein profile characterization provided a basis for western blotting and immuno-analyses using anti-staphylococcal antisera to be undertaken. Usually, western blotting and antibody reactivity is used to identify the expressed known proteins by challenging the SDS-PAGE profiles with sera raised against known antigens. However, that was not the case in this study in view of the fact that there was no information about the identity of any polypeptide separated. Polyclonal *S. epidermidis* anti-serum was used in this study for this reason. Western blotting results showed a high number of polypeptides in the SCV cell membrane preparations to be missing (77, 60, 54 and 40 kDa). Proteins of approximately 60, 40 and 32 kDa were also found to be missing in some SCVs in the cytoplasmic fractions. However, analysis of antibody reactivity of blots of whole cell preparations and cell wall fractions showed proteins of 77, 60, 40 and 32 kDa to be absent in several SCVs (Table 3.2).

Based on results shown in Table 3.2, it seems that SCVs protein preparations are considerably less immunogenic when compared to their wild parental strains, particularly with regard to cell surface associated antigens. It can easily be imagined that suppressing the immunogenecity of cell surface associated protein antigens could be in the SCVs favour by eliciting a less

aggressive reaction by the immune system. That could be an important factor in the BRI pathogenesis due to SCVs as it may contribute to their persistence by affecting the efficacy of clearance by phagocytic cells, thereby leading to the chronicity found in BRI.

Chapter Four

Staphylococcal Adhesion Assessment by Chemiluminescence Assay

4:1 Introduction

Bacterial biofilms have long been recognised as a common source of persistent and relapsing infections (Ziebuhr *et al.*, 2000). Biofilm formation appears to be a widespread attribute of bacteria and may allow increased survival ability under stressful conditions such as low nutrients or exposure to antimicrobials (Costerton *et al.*, 1995, Mah and O'Toole, 2001)

Biofilm formation plays an important role in the pathogenesis of staphylococcal infection. This is particularly true for *Staphylococcus epidermidis*. Indeed, there is an almost direct correlation between the emergence of *S. epidermidis* as a pathogen and its capacity to colonize indwelling medical devices (Voung and Otto, 2002). However, *S. aureus* is also capable of forming a biofilm, and this presumably contributes to its ability to cause at least some forms of infection such as those associated with cystic fibrosis.

The genetic and molecular basis of biofilm formation in staphylococci is multifaceted. Two properties at least are responsible for biofilm formation: the adherence of cells to a surface and accumulation to form multilayered cell clusters. No single mechanism has ever been identified as being solely responsible for the adhesion of microbial strains to surfaces (Busscher *et al.*, 1992; Van der Mei *et al.*, 1997).

S. aureus and *S. epidermidis* often elaborate adherent biofilms which contain the capsular polysaccharide-adhesin (PS/A) that mediates the initial cell adherence to biomaterials. PS/A has been identified to be important in staphylococcal colonization of catheters (Kojima *et al.*, 1990). Biofilm cells produce another antigen, termed polysaccharide intercellular adhesion (PIA). PIA has been found to act as an adhesin on glass and probably other hydrophilic surfaces (Götz, 2002). PIA and PS/A are closely related chemically and immunologically and both are synthesized by the *ica* locus (Gerke *et al.*, 1998; McKenney *et al.*, 1999). PIA had been proposed to be functionally distinct from PS/A, with PS/A mediating the initial adherence to solid surfaces and PIA mediating the accumulation of cells into

biofilms (Mack *et al.*, 1994; Mack *et al.*, 1996). These extracellular polysaccharides which mediate adherence to implants and bacterial colonization were collectively named slime by Arciola and her colleagues (Aricola *et al.*, 2003).

Proteins have been identified that are also involved in biofilm formation, such as the accumulation-associated protein (AAP), the clumping factor A (ClfA), the staphylococcal surface protein (SSP1) and the biofilm-associated protein (Bap) (Götz, 2002). AtlE is another protein which binds strongly to vitronectin and is involved in binding to plastics (Heilmann *et al.*, 1997). Teichoic acid is another substance produced by staphylococci which affects the charge of the cell surface which may contribute to the highly viscous appearance of the colonies and strong biofilm formation (Götz, 2002).

Whole plasma and some isolated plasma proteins were found to influence bacterial adherence to tested materials (Zdanowski *et al.*, 1993; Keogh and Eaton, 1994). Hussain *et al* (2001) have shown that when host plasma proteins are deposited on the implanted device, *S. epidermidis* bind to fibronectin, an observation which might play a role in *S. epidermidis* BRI pathogenicity. From a clinical point of view, bacterial binding to plasma-coated rather than non-coated materials may more adequately reflect the *in-vivo* colonization of various materials (Zdanowski et al., 1993).

Much attention has been paid towards evaluating the prevalence of polysaccharide production among bacterial strains in an attempt to assess the role of polysaccharide production in the pathogenesis of BRI. Colony appearance on Congo red agar plates represents a reference method most often used to phenotypically detect slime (exopolysaccharide) production for *S. epidermidis* (Arciola *et al.*, 2001; Heilmann and Götz, 1998). However, nowadays more reliable techniques have been introduced which are able to specifically identify the genes necessary for polysaccharide production, such as *ica*. In addition, anti-exopolysaccharide immunofluorescence is another way to detect polysaccharide production (Lyte *et al.*, 2003).

Quantitation of microbes adhering to a surface by measuring bacterial ATP (adenosine triphosphate) by chemiluminescence is commonly used in studies of microbial adhesion to different surfaces. The method is a sensitive tool to quantify adherent bacteria during experiments lasting for less than 6 hours and constitutes a valuable method to be used in conjunction with various microscopical techniques (Stollenwerk *et al.*, 1998). The number of attached bacterial cells is determined by measuring the light emission resulting from the reaction between firefly luciferase and ATP present in adhered staphylococcal cells. The chemiluminescence assay developed has been found to be a relatively rapid alternative method to enumerate bacterial cells (Sunderland *et al.*, 1995).

In this Chapter, the experiments were designed to compare the initial adhesion of staphylococcal wild strains and their related SCVs to untreated (plain) plates (microtitre 96 well plates) and to human plasma-coated plates. The assessment of adhesion by chemiluminescence could contribute to a more complete understanding of the initial steps of the pathogenesis of persistent and recurrent infections due to bacterial strains with abilities to form biofilms. The ability to form a biofilm on the surface of a prosthetic device is probably a significant determinant of virulence for these bacteria.

4:2 Materials and Methods

4:2:1 Using Congo Red to Study Phenotypic Variation of Staphylococcal Strains

S. aureus (n=2) and CoNS (*S. epidermidis* (n=11) and *S. haemolyticus* (n=1)) wild type strains along with SCVs were initially classified for *in-vitro* polysaccharide production by examining their colonial morphology on Congo red agar (CRA).

CRA was prepared by adding 0.8 g of Congo red (Sigma, Poole, UK) and 36 g of sucrose (Sigma, Poole, UK) per litre of MHA (Zeibuhr *et al.*, 1997). The mixture was poured in to sterile plates and left to solidify and dry at room temperature. A portion of a wild strain colony or SCV was plated out on CR plates and incubated for 24 hours at 37°C and subsequently overnight at room temperature.

In the present study, the original CRA test was optimised adopting a three-colour reference scale for a fine classification of colony colour (Arciola *et al.*, 2002). The three-colour tones of the scale were as follows: black which was considered as a positive result (Figure 4.2), and bordeaux or red interpreted as negative (Figure 4.1). Results are presented in Table 4.1.

4:2:2 Adhesion Assay Protocol of Staphylococcal Strains to Plasma Coated and Plain Plates

The chemiluminescence assay of ATP requires the use of analytical equipment which measures light emission in an automatic microplate Luminometer (Microlumat plus,LB 97V, version 2.0, Berthold technologies Ltd, Herfordshire, UK). WINGLOW MS Windows was used as a PC software package for analysing the data and automatic transfer and evaluation of measured data was processed in Microsoft Excel. The ViaLight ™ MDA (Microbial Detection Assay) (LumiTech Ltd, Nottingham, UK) Kit was used for these assays, which were carried out in opaque white 96-well microtitre trays with transparent bottoms (Berthold Technologies Ltd, Hertfordshire, UK).

The light emitted was measured by the luminometer and the results were expressed as relative light units (RLU). RLU readings derived from ATP content of cells can therefore be used as an indirect measure of the viable cell numbers present in the culture sample. The method used is described below.

1. One colony of wild type strains from TSA plates was inoculated into MHB and shaken for 4 hours at 37°C until early-mid log phase was reached. However, five colonies of SCVs from TSA/gentamicin plates were inoculated to MHB/gentamicin and shaken for 7 hours on a rocker at 37°C to reach a similar turbidity since SCVs are slow growing organisms. The suspensions were then centrifuged at 3000rpm for 5 minutes and pellets resuspended in $1/10^{th}$ strength MHB and adjusted to OD (0.7-0.8) at A_{550}
2. To study the effect of plasma (as a conditioning film) on the adherence of staphylococcal strains, 25μL of 50% human plasma (Blood Bank, City Hospital, Nottingham-UK) (diluted in water) was transferred into each well of the microtitre plate and incubated for one hour at 37°C. At the end of the incubation time, the plasma was pipetted out and wells were washed gently three times with distilled water to remove unbound plasma proteins. A second set of plates was left untreated as a control
3. Bacterial solution (125μL) was transferred into triplicate wells in the flat-bottomed, 96 well microtitre tray, and incubated for 5 minutes at 37°C
4. The wells were then washed gently with distilled water three times
5. To obtain a reading for ATP content of the total number of bacteria in the suspension, 125μL of each strain was also added to appropriate control wells
6. Distilled water (125μL) was placed into 2 wells of the plate as a reagent control
7. Bactolyse (125μL) was added to all wells and the plates incubated for 10 minutes at room temperature, and then transferred to the luminometer
8. ATP monitoring reagent, 25 μL was added automatically to each well
9. Data were saved in Excel where they were represented in clustered columns, which allowed the comparison between experimental categories
10. The data were represented as % adhesion as the following

(RLU of adhered/RLU of total bacterial count obtained in step 5) x100

11. For each strain the experiment was done on two occasions in triplicate

12. Statistical analysis of the data was performed using t-test where the confidence level is 95%. Differences were considered significant when t-test $P<0.05$. Each point presented means of groups ± SD. The data for these experiments are found in Figures 4.3- 4.5.

4:3 Results

4:3:1 Polysaccharide production assessed by Congo red agar

Examples of the phenotype of exopolysaccharide-positive and negative CoNS colonies as detected by growth on Congo red agar are shown below.

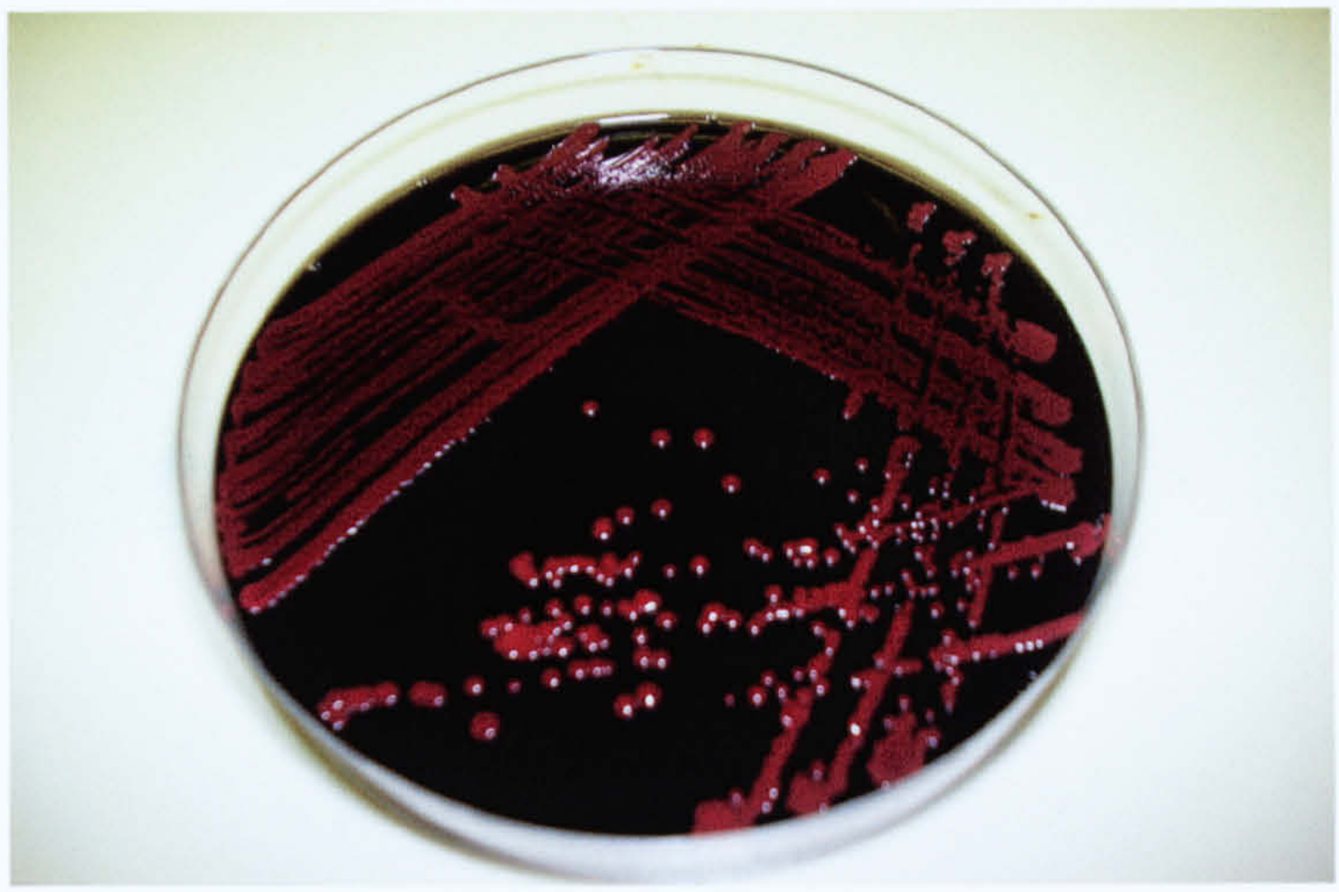

FIG 4-1: Congo red agar plate showing *S. epidermidis* (SE 28) wild type with red colonies indicating lack of exopolysaccharide production by the strain

Incubation of *S. epidermidis* (SE 28) wild type on Congo red agar plate for 24 hours at 37°C followed by 24 h at room temperature yielded red coloured colonies indicating lack of exopolysaccharide production by this strain (Figure 4.1).

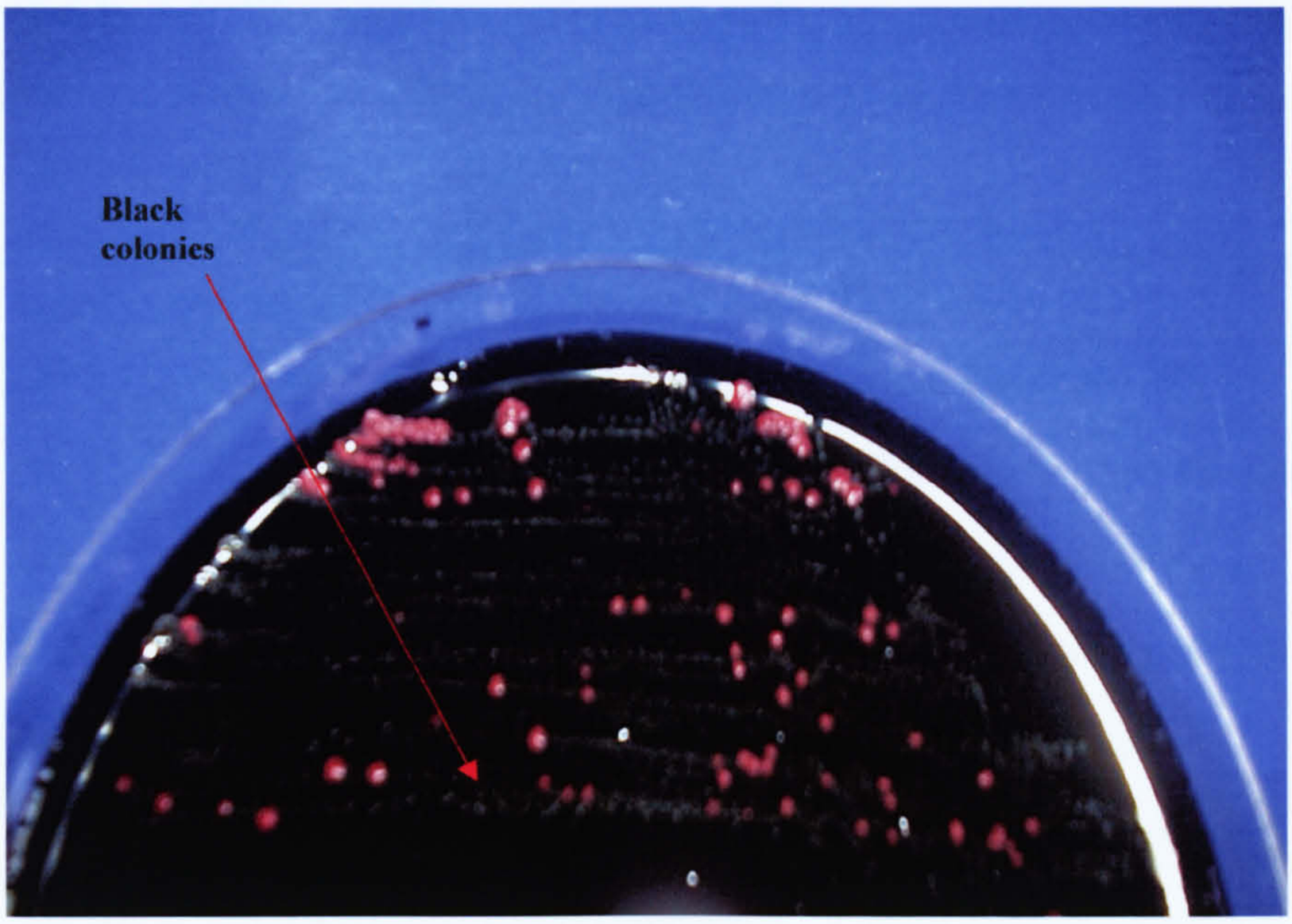

FIG 4-2: Congo red agar plate showing exopolysaccharide non-producer *S. epidermidis* (SE 28) wild type (red colonies) and exopolysaccharide producer SCVs (black colonies).

In contrast, incubation of *S. epidermidis* 28 SCVs on Congo red agar produced mainly black, exopolysaccharide producer colonies (Figure 4.2). Red colonies on these plates were non-exopolysaccharide producing revertants.

Table 4-1: Three-colour reference scale for classification of staphylococcal colony colour on CRA for identification of exopolysaccharide-producing strains

Strain No.	Wild Strain	SCVs	Interpretation
SE 3	(+) Black	(+) Black	Both are producers
SE 4	(-) Red	(-) Red	Both are non- producers
SE 7	(+) Black	(+) Black	Both are producers
SE 8	(-) Red	(-) Red	Both are non-producers
SA 11	(+) Black	(+) Black	Both are producers
SE 13	(-) Red	(-) Red	Both are non-producers
SE 16	(-) Red	(-) Bordeaux	Both are non-producers
SH 18	(+) Black	(+) Black	Both are producers
SE 21	(-) Bordeaux	(+) Black	**Wild type is non-producer while SCV is producer**
SE 23	(-) Red	(-) Bordeaux	Both are non-producers
SE 25	(-) Bordeaux	(+) Black	**Wild type is non-producer while SCV is producer**
SE 26	(-) Red	(-) Bordeaux	Both are non-producers
SA 27	(+)Black	(+) Black	Both are producers
SE 28	(-) Bordeaux	(+) Black	**Wild type is non-producer while SCV is producer**

SE= *S. epidermidis*, SA= *S. aureus*, SH= *S. haemolyticus*

Table 4.1 summarises the colony colour of different staphylococcal strains plated on Congo red agar plates. The appearance of black colonies was considered to indicate an exopolysaccharide-producer strain, however, production of bordeaux or red coloured colonies was considered to indicate exopolysaccharide non production. The results from analysis of three SCV strains and their wild type parents showed that exopolysaccharide production in SCVs is a phenotypic

variation, which corresponded with the appearance of SCVs. Three *S. epidermidis* strains 21, 25 and 28 showed bordeaux colony colour for wild type strains, which classified them as exopolysaccharide non-producers and black colony colour for their equivalent SCVs, classifying the latter as being positive for exopolysaccharide production. Other staphylococcal strains did not show any colour differences between wild type and SCV.

4:3:2 Adherence Assays Measured by ATP Chemiluminescence

Results of adherence assays are presented in Figure 4.3 which shows adherence of wild type strains and their related SCVs to plasma-treated and un-treated control (plain) plastic plates. Data are presented as the means for three groups of strains.

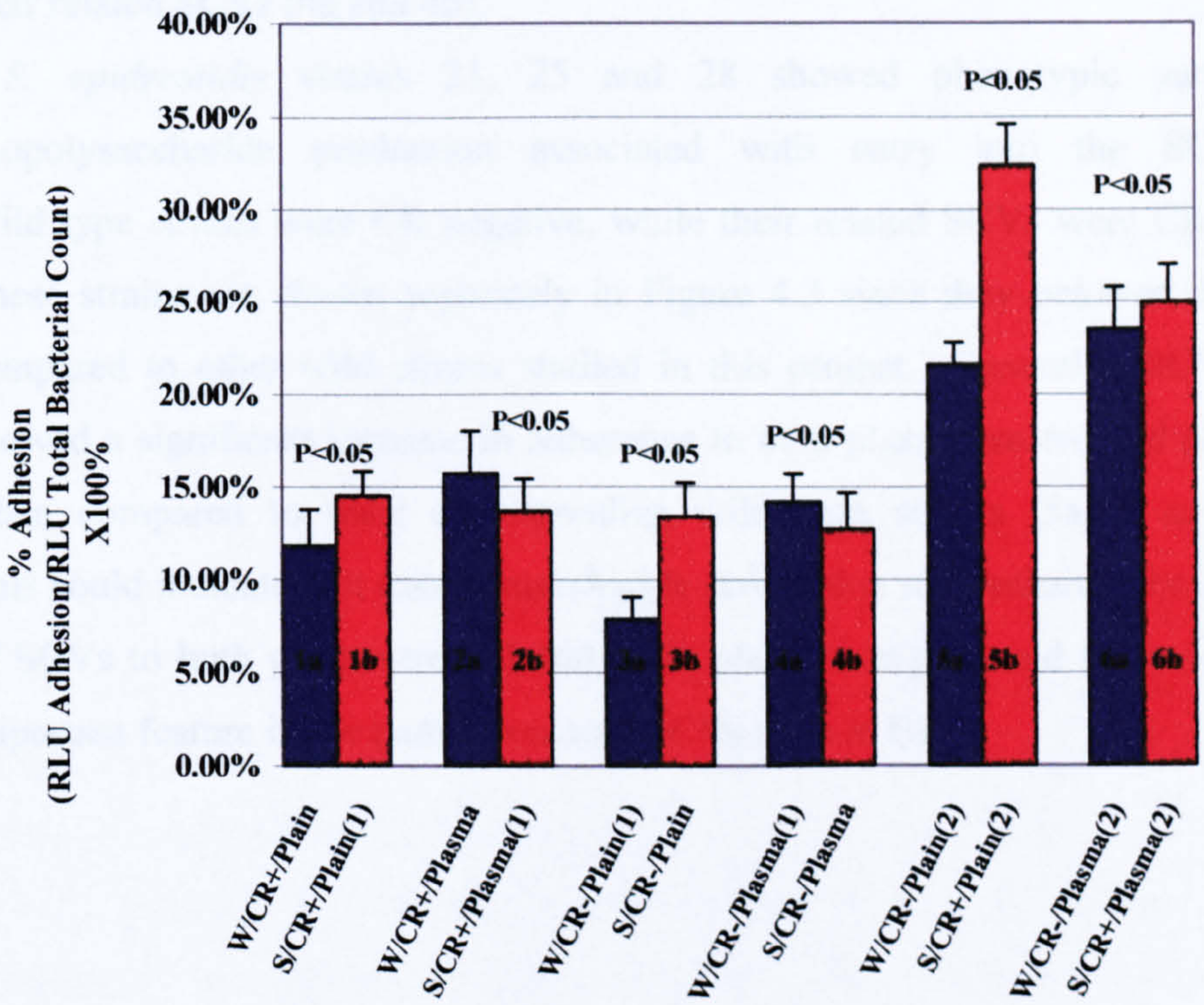

FIG 4-3: Adherence of Staphylococcal wild and SCVs to plasma coated and plain wells.

W/CR+and S/CR+(1) are strains (3,7, 11,18, 27)
W/CR-(1) and S/CR- are strains (4, 8, 13, 16, 23, 26)
W/CR-(2) and S/CR+(2) are strains (21,25,28)

Figure 4.3 summarizes the adherence of wild strains and corresponding SCVs to plasma-treated and plain wells. Congo red (CR) positive *S. epidermidis* SCVs 3 and 7, *S. haemolyticus* 18 and *S. aureus* 11 and 27 showed a significant increase

in adherence to plain wells when compared to their corresponding wild type parents (1a and 1b). However, wild type strains showed a significant increase in adherence to plasma-treated wells when compared to their corresponding SCVs (2a and 2b).

CR negative *S. epidermidis* 4, 8,13,16,23, and 26 SCV strains showed a significant increase in adherence to plain wells when compared to their corresponding wild strains (3a and 3b). However, the wild type strains showed a significant increase in the adherence to plasma coated wells when compared to their related SCVs (4a and 4b).

S. epidermidis strains 21, 25 and 28 showed phenotypic variation in exopolysaccharide production associated with entry into the SCV state. Wild type strains were CR negative, while their related SCVs were CR positive. These strains are shown separately in Figure 4.3 since they behaved differently compared to other wild strains studied in this project. Generally, SCVs strains showed a significant increase in adherence to both plasma-treated and plain wells when compared to their corresponding wild type strains (5a, b and 6a, b). This could indicate that exopolysaccharide production may enhance the adherence of SCVs to both plasma-treated and plain plastic surfaces, and that might be an important feature in the pathogenic role SCVs have in BRI.

4:4 Discussion and Conclusions

Surface components of staphylococcal cells are important in promoting adherence to plastic surfaces. Surface components involved in binding include exopolysaccharides, teichoic acids and surface attachment proteins (Götz, 2002). The data presented in this Chapter investigated the possible effect of variations in exopolysaccharide production on adherence of staphylococcal wild type and SCV strains to a plastic surface. The influence of the presence of a conditioning film (plasma products) on the adherence was also investigated.

Assessment of the presence of exopolysaccharide was achieved by the CRA method using the colorimetric scale according to Arciola *et al* (2001). Although there are more accurate methods to assess exopolysaccharide production, CRA was used for its simplicity and reliability. Although, there is currently no published explanation of the mechanism by which growth on Congo red agar permits detection of exopolysaccharide, one possibility is that adsorption of Congo red to some residue(s) in exopolysaccharide products could elicit the reaction which leads to a black colouration, or the reaction is solely pH dependent where certain substances in the exopolysaccharide could change the pH and subsequently change the colour indirectly, so differentiating the exopolysaccharide producers from non exopolysaccharide producers.

The appearance of either red or bordeaux colonies on Congo red agar plates was considered to indicate exopolysaccharide non producers (Figure 4.1). However, black colonies were considered as exopolysaccharide producers (Figure 4.2). Plating the fourteen wild strains along with their corresponding SCVs (Table 2.4) on Congo red agar plates resulted in differences in Congo red dye adherence to these colonies allowing discrimination between exopolysaccharide producer and non-producer strains (Table 4.1).

S. epidermidis 3 and 7, *S. haemolyticus* 18 and *S. aureus* 11, 27 were CR positive along with their related SCVs. However, *S. epidermidis* 4, 8, 13, 16, 23 and 26 were CR negative along with their corresponding SCVs (Table 4.1).

There was a third category which represented by *S. epidermidis* strains 21, 25 and 28 in which the wild type strains were CR negative but their corresponding SCVs were CR positive (Table 4.1), suggesting that for these SCV strains exopolysaccharide production was a phenotypic variation.

Adherence studies were carried out using the chemiluminescence assay for ATP levels manufactured by ViaLight ™ MDA (Microbial Detection Assay) (LumiTech Ltd, Nottingham, UK). The RLU results were used as an indirect measure of the viable cell numbers found within the culture sample. The percentage of adherence to plasma-treated and plain wells was compared for wild type strains against their related SCVs.

Adherence of wild type strains to plasma coated wells showed a significant increase when compared to plain wells (Figure 4.3). However, SCVs generally showed insignificant differences in adherence to plasma-treated or plain wells. The exception to this was those CR positive SCVs whose wild type parents were CR negative, which showed a significant increase in adherence to plain wells when compared to plasma coated wells (Figure 4.3).

Studies of adherence of wild strains and SCVs to plasma-treated and plain wells showed that wild type strains showed a significant increase in adherence to plasma coated wells when compared to SCVs (Figure 4.3). However, SCVs showed a significant increase in adherence to plain wells when compared to their corresponding wild parents (Figure 4.3). *S. epidermidis* CR positive SCV strains 21, 25 and 28 showed a significant increase in adherence to both plasma coated and plain wells when compared to their corresponding wild CR negative strains (Figure 4.3).

Based on the data presented in this Chapter, comparison of the adherence of SCVs to wild type strains showed that adherence to surfaces is multifactorial. Exopolysaccharide production leads to intercellular adhesion, which can also be mediated by proteins or non-proteinaceous cell wall structures. Extracellular binding proteins, human plasma proteins and cellular hydrophobicity all have important roles for the whole conclusive picture of adherence and biofilm production by the staphylococci.

Previous studies showed that initial bacterial adherence to surfaces is non-specific and reversible, which is then followed by a specific adhesion mediated by adhesin receptors, which strongly fixes the microorganisms to the surface of the biomaterial (Hermann *et al.,* 1988). Based on the Congo red results presented in (Table 4.1), along with data from Chapter three (Table 3.1 and 3.2) which showed the differences in cell -associated protein expression between SCVs and their wild type strains, it might be possible to explain the reason behind the adhesion differences found in SCV strains and their wild type parents.

Data in these studies showed that wild type strains, irrespective of whether or not they produced exopolysaccharide, were better in adhering to plasma coated wells than their related SCVs, which in turn were better than the wild type parents in adhering to plain wells. Adherence to plasma proteins is mediated by bacterial adhesins (e.g. MSCRAMMS) found on the surface of the staphylococcal cell. It is possible that such attachment factors might not be expressed by SCVs or might be altered in expression by gentamicin action during SCVs production. However, adherence of bacteria to plain wells is probably mediated by the cell surface hydrophobicity of the cells, with the presence of exopolysaccharide acting as an extra advantage to SCVs promoting adherence to biomaterials. Although proof that exopolysaccharide increase hydrophobicity is lacking, Götz *et al* (2002) believed that PIA itself mediates adherence to hydrophobic surfaces. Studies on *S. aureus* mutants with altered teichoic acid structure were shown to be severely affected in adherence to polystyrene or glass surfaces and less hydrophobic (Gross *et al.*, 2001).

SCVs that showed phenotypic variation in exopolysaccharide production behaved differently than other SCVs with respect to adhesion. Three *S. epidermidis* SCV strains that were producing exopolysaccharide showed a significant increase in the adhesion to both plasma coated and plain wells when compared to their exopolysaccharide non producer wild type parents. This suggests that exopolysaccharide production is still a crucial factor in adherence to materials coated with host plasma or other extracellular matrix proteins.

The induction of exopolysaccharide synthesis in the presence of environmental stresses such as antibiotics may be an important bacterial survival strategy. The presence of the antimicrobials would select for resistant SCVs to that particular antibiotic, resulting in increased adherence to the implant irrespective of the presence of a host-derived conditioning film. Therefore, it is very important to understand the various aspects of biofilm formation. Only when we have this knowledge will we be able to develop more specific ways to overcome staphylococcal biomaterials-related infections due to SCVs.

Chapter Five

Analyses of Staphylococcal-Host Cell Interactions:

(A) The Use of Flow Cytometry to Assess Staphylococcal Internalization by Cultured Human Umbilical Vein Endothelial Cells

(B) The Effect of Staphylococci on Host Production of Cytokines

Introduction

Staphylococci are considered to be primarily extracellular pathogens. Since they have been recognized to cause invasive diseases that are persistent and refractory to standard therapies, the necessity to understand the pathogenesis of such diseases has prompted further study of the possible role of bacterial invasion of host cells in staphylococcal disease. It is believed that bacteria sequestered from the host immune system inside the cell may provide a reservoir of bacteria for recurring infections, and may be more relevant to chronic disease.

The adhesion or the attachment of the bacterium to the host cells is of importance in the initiation of microbial invasion and in the progression of many bacterial infections. Staphylococcal species have evolved a range of surface molecules and structures to enable them to adhere specifically to a particular surface or a limited variety of host cell surfaces (Aly and Levit, 1987; Jonsson *et al.*, 1991; Kiers *et* al., 2001; McDevitt *et al.*, 1994; Ohshima *et al.*, 1990; Park *et al.*, 1996; Patti *et al.*, 1992; Soell *et al.*, 1995).

Invasion and Internalization of bacteria by host cells utilizes a variety of mechanisms including phagocytosis and clathrin-dependent receptor-mediated endocytosis (RME) (Mukherjee *et al.*, 1997). To study the mechanisms of invasion, several inhibitors specific to those internalization pathways could be used. Colchicin, cytochalasin D and monodansylcadaverine (MDC) were used by a number of researchers to study bacterial internalization by eukaryotic cells (Almeida *et al.*, 1996; Almeida *et al.*, 2001; Jevon *et al.*, 1999; Menzies and Kourteva, 1998).

Effects of bacterial adhesion to host cells can range from no apparent effect on the host cell, to altered morphology, induction of cytokine release, apoptosis and invasion. Although the outcome of the host-cell interaction is very much dependent on the type of host cell with which the bacterium has interacted, several reports have documented *S. aureus* stimulation of endothelial cytokine responses (Soell *et al.*, 1995; Wang *et al.*, 2000).

The production of proinflammatory cytokines such as interleukin-1β (IL-1β), and IL-6 which have been associated with inflammatory diseases and

septic shock, and the neutrophil chemoattractant IL-8 all contribute to the host defence mechanisms in response to bacterial colonization or invasion, and when secreted in excess can also induce various host immuno-pathological disorders (Gray *et al.*, 1996; Rabehi *et al.*, 2001; Yao *et al.*, 1996).

Flow cytometry is an analytical tool that allows the discrimination of different particles on the basis of size and colour. It is a technology that has also recently been used recently to study host-bacterium interaction due to its ability to detect the bacteria present on, or within single mammalian cells in suspension and provides rapid, quantitative, multiparameter analyses of bacterial-host cell interactions (Fattorossi *et al.*, 1989; Giaimis *et al.*, 1994). The bacteria of interest are targeted with a fluorochrome label and single cells are scanned and detected through a fluorescence detector. Data obtained may be plotted on a histogram representing the number of cells detected and values of one or more measurements made on individual cells. WinMDI version 2.8-windows 3.95/DOS 7.10 is used to plot the histogram and provides the multiple document interfaces for flow cytometry analysis.

Flow cytometry combined with cytometric bead array (CBA) is used to measure cytokines in a particle-based immunoassay. The advantages of the technique are that it requires small volume samples, and substantially less time compared to conventional ELISA. The bead populations are separated by their fluorescence excited by the red laser of the flow cytometer. The light emitted is filtered for phycoerythrin (575 nm) (PE) which measures the PE labelled anti-cytokine antibody binding to each bead population.

5:2 Materials and Methods

5:2:1 Flow Cytometric Analysis to Distinguish between Adherent and Internalised Staphylococci by Cultured HUVECs

In order to start using the flow cytometry technique, it was first important to label the bacteria with a dye that could be detected. BODIPY® Fluorescein (Molecular Probes Europe BV, Leiden, Netherlands) is an amine-reactive compound, which is pH-insensitive. It emits green fluorescence that is collected via a 525-nm bandpass filter by the flow cytometer.

The cryopreserved HUVECs (Clonetics, CC2519 Lot of 1742) were purchased from BioWhittaker Ltd (Berkshire, UK). The cell culture process for Clonetics HUVECs was according to the supplier's manual. For the maximum efficiency for growing Clonetics HUVECs, Clonetics medium (growth medium) EGM-2 BulletKit System, and subculture reagents were used (Clonetics, BioWhittaker Ltd, Berkshire, UK). This medium contains human epidermal growth factor (hEGF), hydrocortisone, GA-1000 (gentamicin and amphotericin B), fetal bovine serum (FBS), vascular endothelial growth factor (VEGF), human basic fibroblast growth factor (hFGF-B), long R^3-IGF-1 (human recombinant insulin-like growth factor), ascorbic acid, Heparin, amino acids and phenol red as pH indicator. HUVECs were seeded at 20,000 cells per well onto 24-well 0.2% gelatin (Sigma, Poole, UK) -coated tissue culture plates in 1 mL of Clonetics medium and cultured at 37°C in 5%CO_2 for 1-2 days until 80-90% confluent at (≈ 56,000 cells per well counted using haemocytometer). The viability of the HUVECs was confirmed by staining with 0.2% w/v trypan blue (Sigma, Poole, UK). The measurement of HUVEC viability was obtained by counting 100 cells and calculating the percentage of cells excluding the stain. The viability of the control cultured cells was maintained as 98%.

Bacteria attached to and ingested by HUVECs were quantified using a flow cytometer (Beckman Coulter EPICS Altra, Luton, UK) using a fluorescence-quenching assay that permits differentiation between attachment and ingestion of the bacterium by the cells. Trypan blue (0.2 mg/mL) in distilled water (VWR International Ltd, Leicestershire, UK) was found to be the best

quenching agent of extracellular fluorescence (Sahlin *et al.*, 1983, Wan *et al.*, 1993).

5:2:1:1 Staphylococcal Strain Cultivation and Labelling with BODIPY®FL

All fourteen wild type staphylococcal strains (*S. aureus* and *S. epidermidis* and *S. haemolyticus*) were included in this study along with their related SCV produced by exposure to gentamicin (Table 2.4). Each strain was labelled in duplicate.

1. Colonies from TSA plates incubated overnight at 37°C were resuspended in MHB supplemented with gentamicin for SCVs (Table 2.4) and adjusted to an A_{490} of 0.01. The suspensions were incubated at 37°C, 150 rpm for 4 hours for wild strains and 7 hours for SCVs strains
2. Bacterial suspensions were centrifuged and the pellet washed twice with PBS. The turbidity of the suspensions was adjusted to 0.6 at 550nm
3. BODIPY-FLwas dissolved at 10 mg/mL in dimethylsulfoxide (DMSO) (Sigma, Poole, UK)
5. One-mL of the bacterial suspension was transferred to an eppendorf. While vortexing the suspension, fifty microlitre of the reactive dye solution per each mL of bacterial suspension was slowly added
6. The mixture was incubated for 1 hour at room temperature with shaking
7. The bacterial labeling mixtures were centrifuged for 5 minutes. The bacterial pellets were washed three times with PBS and then resuspended in one-mL of HUVECs growth medium containing no antibiotics
8. One of the duplicate bacterial labeling suspensions was used to test the efficiency of the fluoresceinating procedure using fluorescence microscopy, while the other was used in HUVEC adhesion and invasion assays [5:2:1:3, 5:2:1:4 and 5:2:1:5].
9. Labeling of bacteria with BODIPY-FL dye was quantified in arbitrary units defined by flow cytometry which reflected the amount of protein found on the surface of the staphylococci. Data are presented in Table 5.1.

5:2:1:2 Flow Cytometric Analysis

Flow Cytometry was used in conjunction with the trypan blue fluorescence-quenching technique to investigate any differences in HUVEC uptake of wild type and SCV staphylococcal strains
Measurements were recorded at the following settings:

Forward scatter (FS) Lin and side scatter (SS) Lin. The x-axis represented the degree of forward light scatter and measures cell size. This is expressed only in relative terms, not as a unit of size, while the y-axis is a log-scale measurement of the fluorescence in each cell. A minimum of 5000 cells was measured in each sample.

An extracellular fluorescence-quenching dye (trypan blue) was used to differentiate between adherent (membrane-bound) and internalised staphylococci. Trypan blue quenches the fluorescence emitted by the bacteria adhered to the cell surface but cannot quench internalized bacteria because the HUVEC cell is not permeable to the dye. The fluorescence emitted from inside the cell by the labelled bacteria was assessed by the flow cytometry and was taken as representative of internalised staphylococci. All the data were analysed using (WinMDI 2.8) software.

5:2:1:3 Staphylococcal Adhesion to HUVECs as Assessed by Flow Cytometry (Adhesion Assay)

Staphylococcal adhesion to HUVECs was studied as described below.

1. HUVECs monolayers were washed twice with one mL of PBS. One mL of EGM-2 BulletKit medium without antibiotics was then added per well
2. Bacterial suspensions ($5x10^6$ cfu) (25 µL) which were labelled with BODIPY-FL as described in section 5:2:1:1 and were inoculated into the wells. This volume resulted in a multiplicity of infection (m.o.i.) of approximately 40 bacteria per endothelial cell. After inoculation of all wells, the 24-well plates were briefly and gently shaken on a rotating platform to distribute the bacterial inoculum throughout the HUVEC growth medium
3. The plates were incubated for 15 min at 37°C in 5% CO_2

4. To stop the internalisation, plates were placed on ice for 5 minutes
5. Non-adherent staphylococci were removed by washing the cells three times with ice-cold PBS
6. Trypsin 0.025% w/v EDTA 0.01% w/v (0.5 mL) (BioWhittaker) was added per well for 2 minutes to detach the HUVEC monolayer from the bottom of the plate. 0.5-mL of trypsin-neutralising reagent (BioWhittaker) was then added and plates incubated for a further 2 minutes. The cells were removed and transferred to clean tubes and centrifuged (200xg, 5min, 4°C)
7. The cell pellets were resuspended in one mL of cold HUVEC growth medium and then processed through the flow cytometry and the number of staphylococci per HUVEC was calculated. Results for wild type and SCV staphylococci are shown in Figures 5.1 and 5.2
8. This experiment was repeated three times for each strain

5:2:1:4 Staphylococcal Internalization by HUVECs as Assessed by Flow Cytometry (Invasion Assay)

The internalization of fourteen staphylococcal wild type strains with their corresponding SCVs (Table 2.4) were studied as described below.

1. Bacterial suspensions were co-cultured with HUVECs in exactly the same way as described for the adhesion assay (5:2:1:3) except that gentamicin was added to wells containing SCVs strains
2. The inoculated strains were incubated for 3 hours at 37°C in 5% CO_2.
3. After incubation, the monolayers were washed three times with PBS
4. Trypsin/EDTA (0.5-mL) was added per well for 2 minutes followed by 0.5-mL of trypsin-neutralizing reagent. The cells were centrifuged at 200xg for 5 minutes
5. The cell pellet was resuspended in one-mL of growth medium and analysed via the flow cytometer as described above. Readings obtained represent the total number of staphylococci found in the cell pellet aliquots (the extracellular and the internalised staphylococci)
6. Trypan blue was added to a second aliquot of the HUVEC suspension to a final concentration of 0.2 mg/mL and re-processed through the flow cytometer.

Readings obtained represent the internalised staphylococci in the HUVEC suspension

7. The experiment was repeated three times for each strain. The data was presented as median ±SD. Results for wild type and SCV staphylococci are shown in Figures 5.3 and 5.4.

5:2:1:5 Staphylococci Internalization in the Presence of Inhibitors

To study the mechanism of invasion, the assays were performed in the presence of 10 μM colchicine (Sigma, Poole, UK), 250 μM monodansylcadaverine (Sigma, Poole, UK) or 2 μM cytochalasin (Sigma, Poole, UK) in the growth medium. Preparation of these stock solutions is described in the Appendix (8:1:4). The concentrations used were selected according to similar studies investigating staphylococcal internalization of eukaryotic cells (Jevon *et al.*, 1999). *S. epidermidis* strains 4 and 25, *S. aureus* strains 11 and 27 along with their corresponding SCVs were studied. These strains were chosen because they were the strains that had been shown to be subject to internalization by HUVECs in preliminary studies. The method used is described below.

1. Ten μM colchicine, 250 μM monodansylcadaverine or 2 μM cytochalasin D were prepared in HUVECs growth medium containing no antibiotic and filter sterilized (0.20μm)
2. Monolayers were then washed three times with PBS. One mL of growth medium was placed into each well followed by 25μL of labelled bacteria. In the case of SCVs, the growth medium was also supplemented with gentamicin
3. Monolayers were incubated for 3 hours at 37°C
4. Steps (3-6) in 5:2:1:3 were repeated
5. The viability of the cells was determined to be ≈ 97% in both the control and the tested cells, as tested by 0.2% (w/v) trypan blue exclusion method
6. The number of bacteria internalized in an untreated monolayer was set as 100%

7. The experiment was repeated three times and the readings were represented as medians ±SD. Results for these assays are presented in Figures 5.3, 5.4 and Table 5.2.

5:2:2 Cytokine Production in HUVECs after Stimulation with Staphylococci

The BD™ Cytometric Bead Array (CBA) (BD Biosciences, Oxford, UK) employs a series of particles with discrete fluorescence intensities to simultaneously detect multiple cytokines. Each bead on a CBA provides a capture surface for a specific cytokine. The BD CBA system uses the sensitivity of amplified fluorescence detection by flow cytometry to measure soluble cytokines in a particle-based immunoassay.

The BD human inflammation CBA kit was used to quantitatively measure Interleukin-8 (IL-8), IL-1β, IL-.6, IL-10, IL-12p70 protein levels and TNF-α in a single HUVEC cell sample. Six bead populations with distinct fluorescence intensities have been coated with capture antibodies specific for IL-8, IL-1β, IL-6, IL-10, TNF-α and IL-12p70 proteins. The six bead populations were mixed together to form the CBA which was resolved in a flow cytometer.

The capture beads, phycoerythrin-conjugated detection antibodies, and recombinant standards or test samples are incubated together to form sandwich complexes. Following acquisition of sample data using the flow cytometer, the sample results are generated in graphical and tabular format using the BD CBA Analysis Software.

CBA has previously been used by several researchers (Chen *et al.*, 1999; Collins *et al.*, 1998) for simultaneous detection of several cytokines in single assay samples.

5:2:2:1 Analysis of Cytokine Levels in HUVECs Co-cultured with Staphylococci

Four staphylococcal strains were co-cultured with HUVECs for screening for cytokine release. The four strains were *S. epidermidis* strains 4 and 25 and *S. aureus* strains 11 and 27 which were shown to be internalized by HUVECs, as mentioned in 5:2:1:4 and 5:2:1:5. The experiment was performed as described below.

1. One colony from wild type staphylococcus or 5 colonies from staphylococcal SCVs were resuspended in 10 mL MHB or MHB with gentamicin, respectively. The bacterial suspensions were incubated in a shaking incubator set at 200rpm and at 37°C for 4-hours in the case of wild type strains, and 7-hours for SCVs. After incubation, the suspensions were pelleted and the turbidity was adjusted to 0.7 at 490 nm in fresh HUVEC growth medium.
2. Prior to incubation with bacteria, the HUVEC monolayers (grown in 24-well tissue culture plates) were washed with HEPES buffer (BioWhittaker) to remove any traces of antibiotics
3. Fifty five μL aliquot of a mid-log phase bacterial culture was suspended in 1 mL of antibiotic free HUVECs growth medium, and added directly to the wells containing the HUVEC cell line. Three wells were used for each bacterial strain. The 24-well plate was then incubated at 37°C in a 5% CO_2 humidified incubator for one hour.
4. One hundred microlitre of supernatant from infected HUVECs was removed after one hour incubation. Supernatants were then centrifuged for 5 minutes at 13,000 xg and stored at –20°C
5. The HUVEC cell lines were washed three times with HEPES, followed by addition to each well of 1.5-mL HUVEC growth medium supplemented with 80-mg/L lysostaphin and incubated for 30 minutes in order to kill any extracellular bacteria. The cell lines were washed three times with HEPES. One-mL fresh standard HUVEC medium with antibiotics was added to the cell lines, which were then incubated at 37°C in 5% CO_2 for up to 24 hours.

6. One hundred μL aliquots were collected after 3, 6 and 24 hours of incubation. They were centrifuged for 5 minutes at 13,000xg and the supernatants were stored at -20°C until required for cytokine measurement by BD CBA and flow cytometry.

7. At the 24 hour point, trypan blue exclusion was used to assess HUVEC viability

8. For screening for cytokine production, supernatant aliquots which had been stored at -20 were thawed to room temperature. The mixed cytokine capture beads were vortexed, and 50 μL added to the appropriate supernatant assay tubes.

9. Human inflammation PE detection reagent (50μL) (BD Biosciences, Oxford, UK) was then added the cytokine assay tubes

10. The human inflammation standard dilutions (50μL) (BD Biosciences, Oxford, UK) were added to the control assay tubes

11. Fifty μL of each test sample was added to the test assay tubes

12. The assay tubes were protected from direct exposure to light and incubated for 3 hours at room temperature.

13. Washing buffer (1mL) was then added to each assay tube, and the contents centrifuged at 200x *g* for 5 minutes

14. The supernatant was carefully aspirated and discarded from each assay tube

15. Wash buffer (300 μL) was added to each assay tube to resuspended the bead pellet

16. The tubes were vortexed for 3-5 seconds immediately before analysing on the flow cytometer

17. Data acquisition was performed using the BD FACSComp™ software. BD CellQuest™ Software was used for analyzing samples and formatting data for subsequent analysis using the BD™ CBA Software. Data for HUVECs co-cultured with *S. epidermidis* strains 4 and 25 and *S. aureus* strains 11 and 27 are presented in Figures 5.5 and 5.6.

5:3 Results

5:3:1 Preliminary Studies of Labelling of Staphylococcal Strains with BODIPY-FL

Table 5-1: BODIPY-FL labelling of staphylococcal strains assessed by flow cytometry

Staphylococcal Strains	Median ± SD (n=6)
CoNS (Wild)	139.49 ± 34.30
CoNS (SCVs)	81.7 ± 6.27
S. aureus (Wild)	199.23 ± 33.89
S. aureus (SCVs)	80.64 ±7.01

Data in Table 5.1 show results of preliminary experiments of labelling of staphylococcal strains with BODIPY-FL dye. Labelling is expressed in arbitrary units and reflects availability of proteins on the staphylococcal surface for binding to the fluorescent tag.

Fourteen wild type staphylococcal strains along with their related SCVs were labelled with BODIPY-FL. Labelling units were used indirectly as a measurement of surface proteins per cell. CoNS (*S. epidermidis* strains plus *S. haemolyticus*) and *S. aureus* SCVs median labelling units were compared with their related wild strains. A decrease of 48% in labelling was found in SCVs when compared to their wild strains in both CoNS and *S. aureus*. However, *S. aureus* wild strains showed a significantly higher ($P<0.05$) surface labelling (199.23± 34.30) when compared to CoNS wild strains (139.49 ± 34.30).

Binding was found to be reproducible for both *S. aureus* and CoNS and labelling with BODIPY-FL was considered to be an appropriate method for later use in studies of bacterial adherence and internalization.

5:3:2 Adherence of *S. aureus* Wild Strains and SCVs to HUVEC Cells

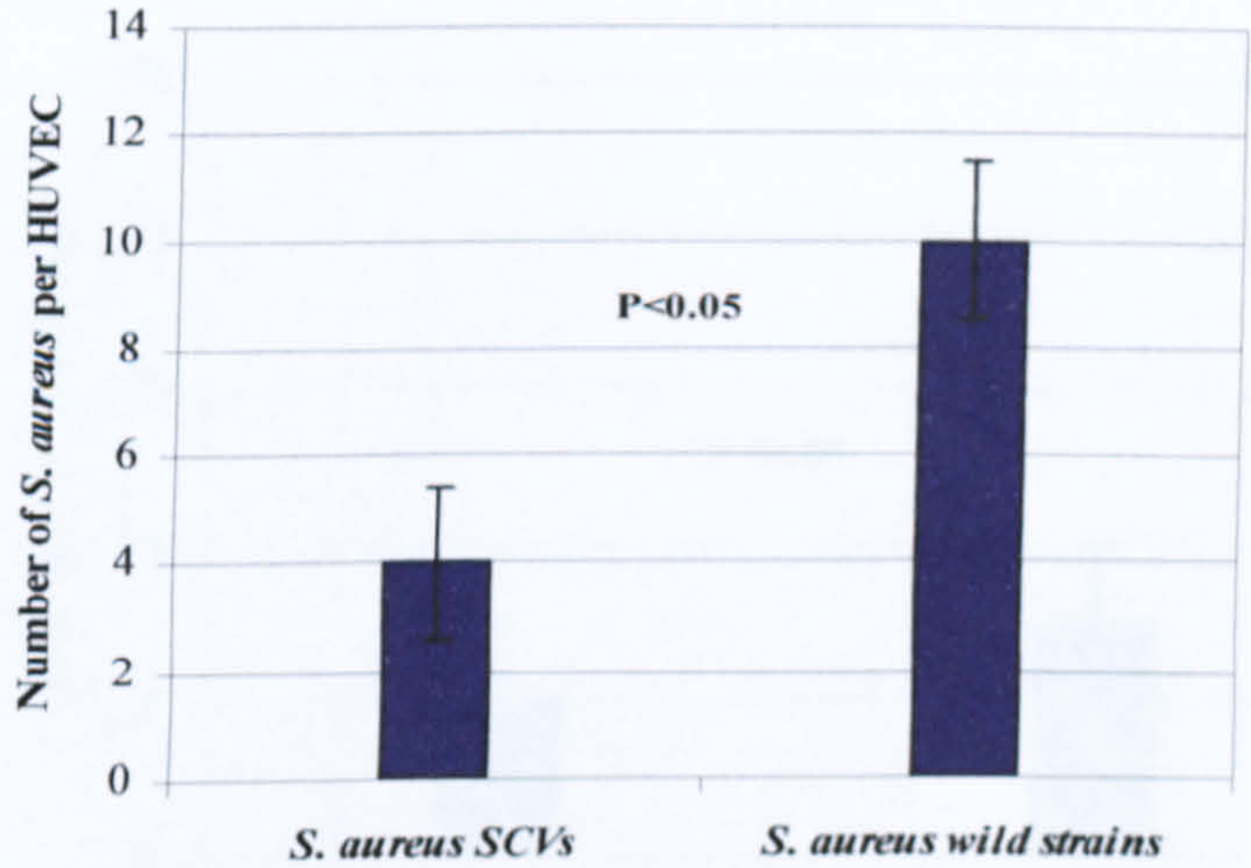

FIG 5-1: Comparison of adherence of *S. aureus* wild and SCVs per HUVEC cell.

The number of cells of *S. aureus* wild type strains 7, 11 and 27 adhered per HUVEC cell was compared with adherence of the related SCVs after incubating the monolayers with bacteria for 15 minutes. Figure 5.1 shows that there was a significantly higher adherence of wild type strains (10±1 per HUVEC) when compared with their related SCVs (4±1 per HUVEC) ($P<0.05$).

5:3:3 Adherence of CoNS Wild Strains and SCVs to HUVEC Cells

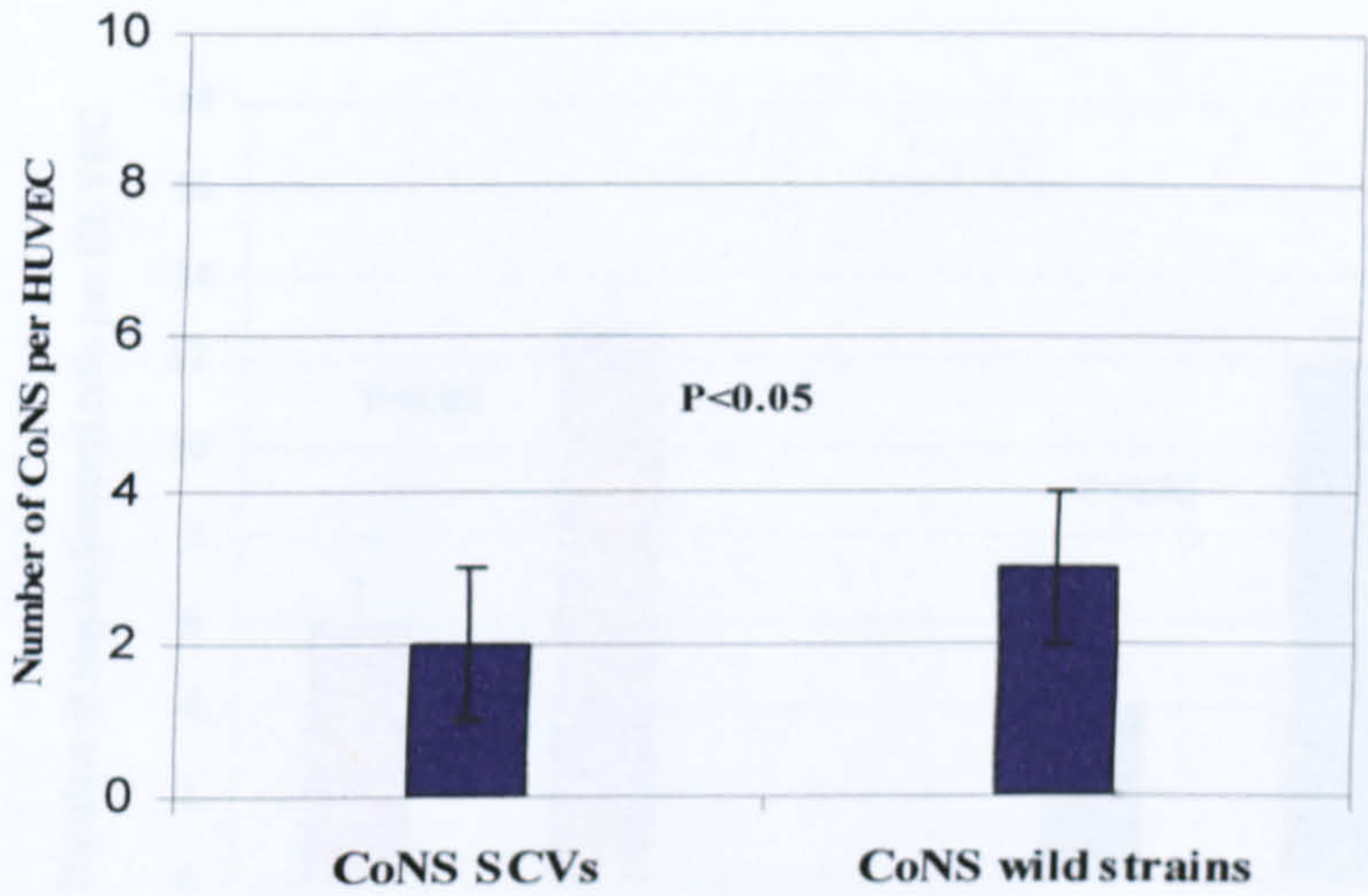

FIG 5-2: Comparison of adherence of CoNS wild and SCVs per HUVEC cell.

The number of cells of CoNS wild type strains adhered per HUVEC cell was compared with that of their related SCVs after 15 minute co-incubation. Figure 5.2 shows that there was not any significant difference between the adherence of wild type strains (3±1 per HUVEC) when compared with their related SCVs (2±1 per HUVEC). The data are pooled from all CoNS strains 3, 4, 8, 13, 16, 18, 21, 23, 25, 26 and 28.

In addition, when the number of cells of *S. aureus* wild and SCVs adhering to HUVECs were compared to the number of adherent cells of CoNS strains, it was found that the number of *S. aureus* cells adhering to host cells was significantly higher than that of the CoNS.

5:3:4 Adherence of Staphylococcal Wild Strains and SCVs to HUVEC Cells after 3 Hours Incubation

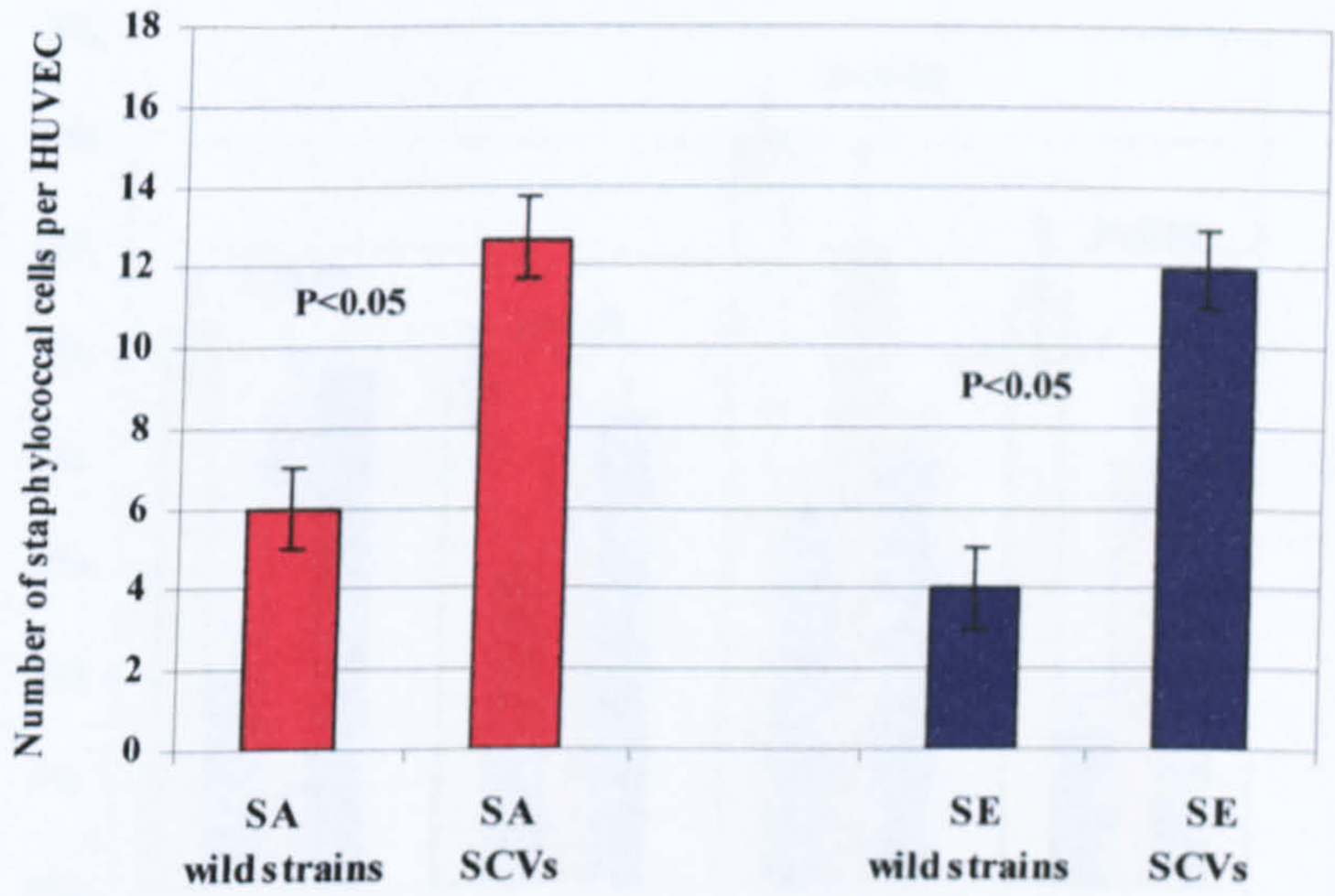

FIG 5-3: Staphylococcal adherence to HUVECs after 3 hours incubation.

SA= *S. aureus* strains 11 and 27; SE=*S. epidermidis* strains 4 and 25

The number of staphylococcal cells of wild type strains adhered per HUVEC cell was compared with that of their related SCVs. Adherence of four staphylococcal strains, *S. aureus* strains 11 and 27, and *S. epidermidis* strains 4 and 25 (which were internalized by HUVECs), was also assessed after three hour incubation. Figure 5.3 shows that after 3 hours contact with HUVECs adherence of *S. aureus* and *S. epidermidis* SCVs to HUVECs was significantly higher than their corresponding wild parents ($P<0.05$).

Longer incubations could result in changes in expression of HUVECs surface receptors or SCV adhesins which might be responsible for this noticeable increase in SCVs adhering to HUVECs after 3 hours incubation.

5:3:5 Internalization Assays of Staphylococcal Wild Type Strains in the Presence of Internalization Inhibitors

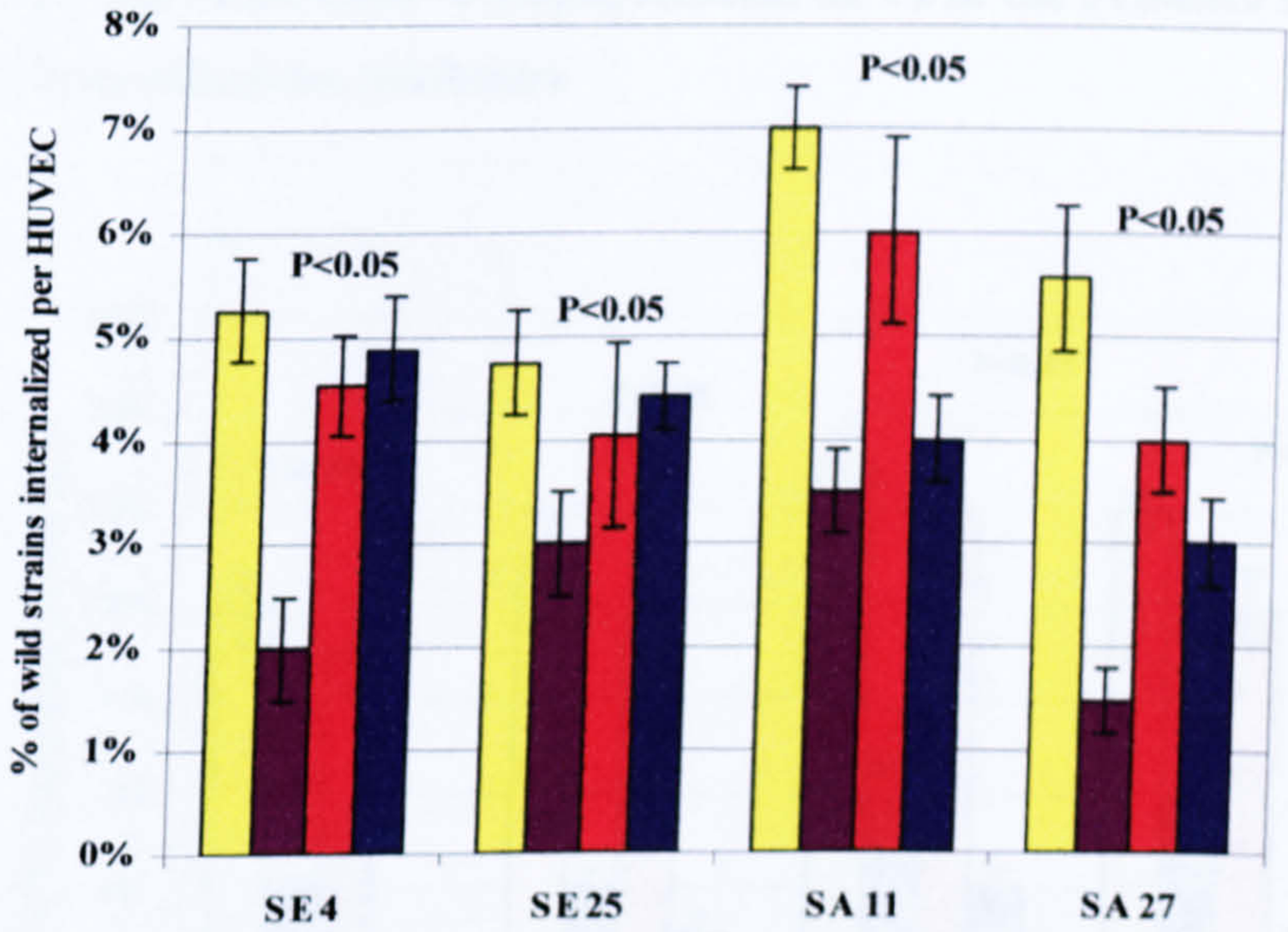

FIG 5-4: Percentage of wild strains internalized with and without internalization inhibitors.
P- values are related to internalization without inhibitors
SE=*S. epidermidis*; SA=*S. aureus*

Preliminary experiments (data not shown) showed that only four out of fourteen staphylococci strains co-cultured with HUVECs were internalized. These were (*S. epidermidis* strains 4, 25 and *S. aureus* strains 11, 27. These strains were studied in more detail, to investigate the mechanism of staphylococcal uptake.

Based on data presented in Figure 5.4, the percentage of *S. aureus* cells internalized by HUVECs was significantly higher than *S. epidermidis* strains internalized when incubated in the absence of internalization inhibitors.

However, setting the number of staphylococci internalized to 100%, in the presence of cytochalasin D, the numbers of bacteria internalized by the HUVECs decreased by 85% in *S. epidermidis* and by 80% in *S. aureus*. In addition, Colchicin also decreased staphylococcal internalization, by about 20% for both *S. epidermidis* and *S. aureus*.

However, differences were observed in the extent to which MDC inhibited internalization of staphylococci, reducing *S. epidermidis* internalized by around 10% and *S. aureus* by 40%.

5:3:6 Internalization of Staphylococcal SCVs in the Presence and Absence of Internalization Inhibitors

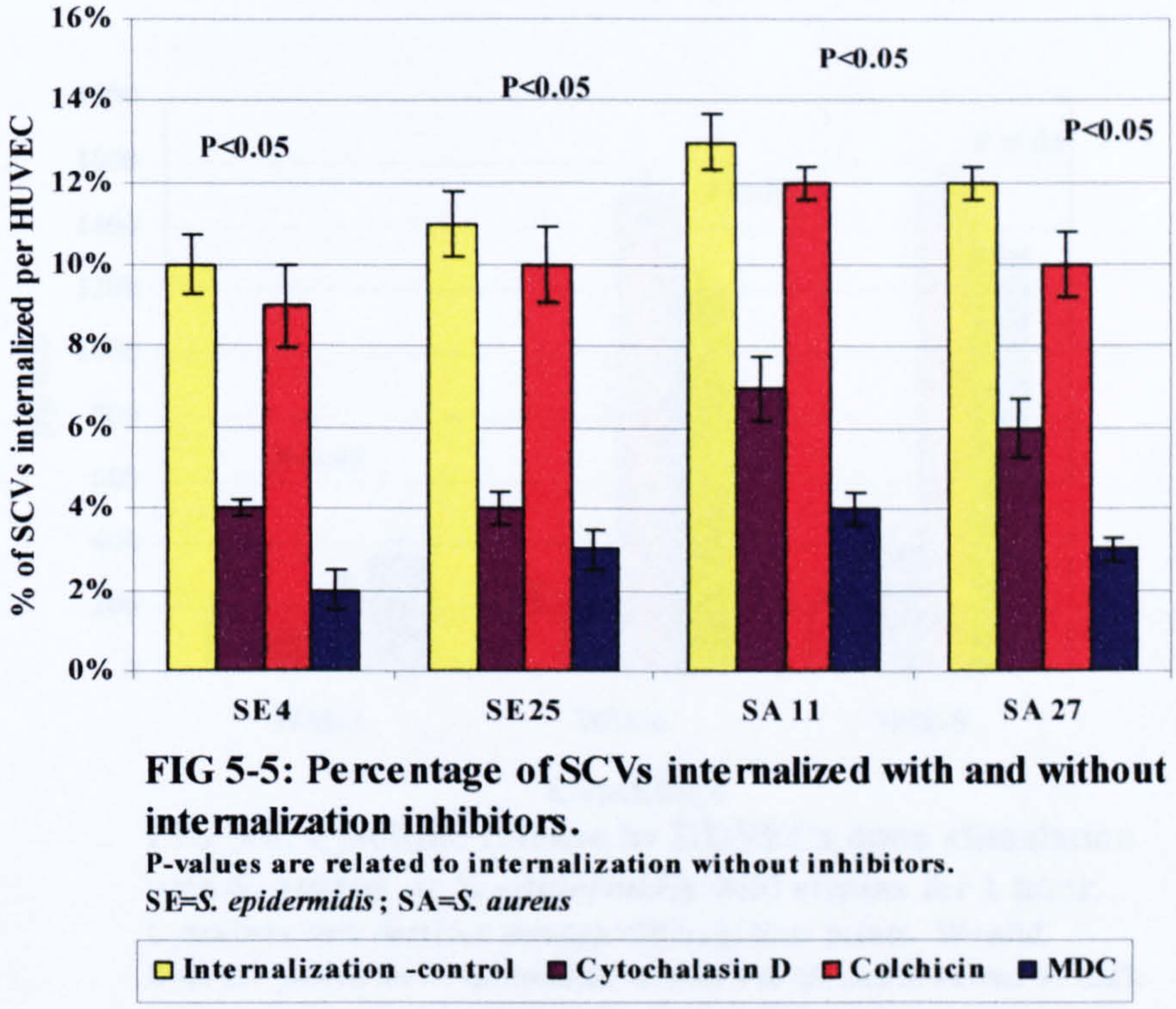

FIG 5-5: Percentage of SCVs internalized with and without internalization inhibitors.

P-values are related to internalization without inhibitors.

SE=*S. epidermidis*; SA=*S. aureus*

Figure 5.5 summarizes the experiments investigating the effect of internalization inhibitors on the numbers of staphylococci SCVs internalised by HUVECs. Data shown are for *S. epidermidis* SCV strains 4, 25 and *S. aureus* strains 11, 27 co-cultured with HUVECs for 3hours (gentamicin was present to stabilise the SCVs).

Comparison of the experiments summarised in Figures 5.4 and 5.5 shows that there were differences between wild type strains and their corresponding SCVs in the extent to which the internalization inhibitors affected uptake of bacteria by the HUVECs. Based on data presented in Figure 5.4, setting the number of SCVs internalized (control) to 100%, Cytochalasin D decreased

wild type staphylococcal internalization by 45% in case of *S. epidermidis* and 50% for *S. aureus*. However, Colchicin decreased SCV internalization of *S. epidermidis* and *S. aureus* SCVs by only 10%. In addition, MDC inhibited the number of internalized *S. epidermidis* and *S. aureus* SCVs by 95% and 90% respectively.

5:3:7 Cytokine Production by HUVECs Following Incubation with Staphylococcal Wild Strains

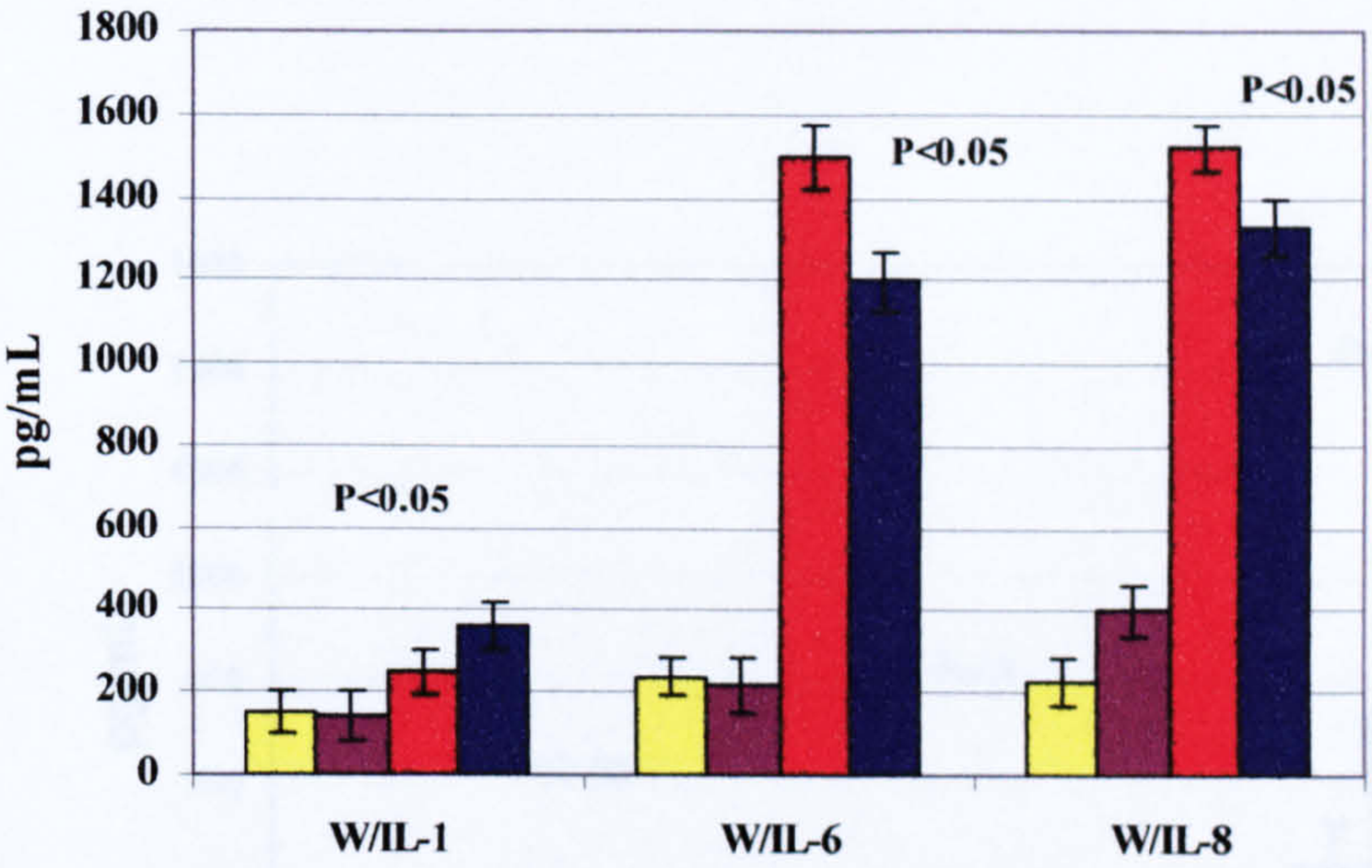

FIG 5-6: Cytokine release by HUVECs upon stimulation with *S. aureus* or *S. epidermidis* wild strains for 1 hour.
Cytokines were detected through different time points. W=wild.
Data are pooled for *S. epidermidis* strains 4 & 25, and *S. aureus* 11 &27.

The CBA cytokine assay kit was used to quantitatively measure Interleukin-8 (IL-8), IL-1β, IL-.6, IL-10, IL-12p70 levels and TNF-α in HUVEC supernatants collected after incubation for 1,3,6 and 24 hours with wild type *S. epidermidis* strains 4, 25 and *S. aureus* strains 11, 27.

Data in Figure 5.6 show cytokine levels for IL-1β, IL-6 and IL-8 which were released by HUVECs over time. The levels of IL-10, IL-12 and TNF-α detected were near the lower limit of assay detection and were not considered reliable, and so this data is not represented.

Data showed that levels of IL-1β, IL-6 and IL-8 increased significantly with time when compared to HUVEC only controls (data not shown). However, after 24 hours cytokine levels began to fall possibly due to decreasing HUVECs viability in the presence of wild type staphylococci (HUVECs incubated with the bacteria underwent a 45% decrease in viability when compared to HUVEC controls cultures (data not shown).

5:3:8 Cytokine Production by HUVECs Following Incubation with Staphylococcal SCVs

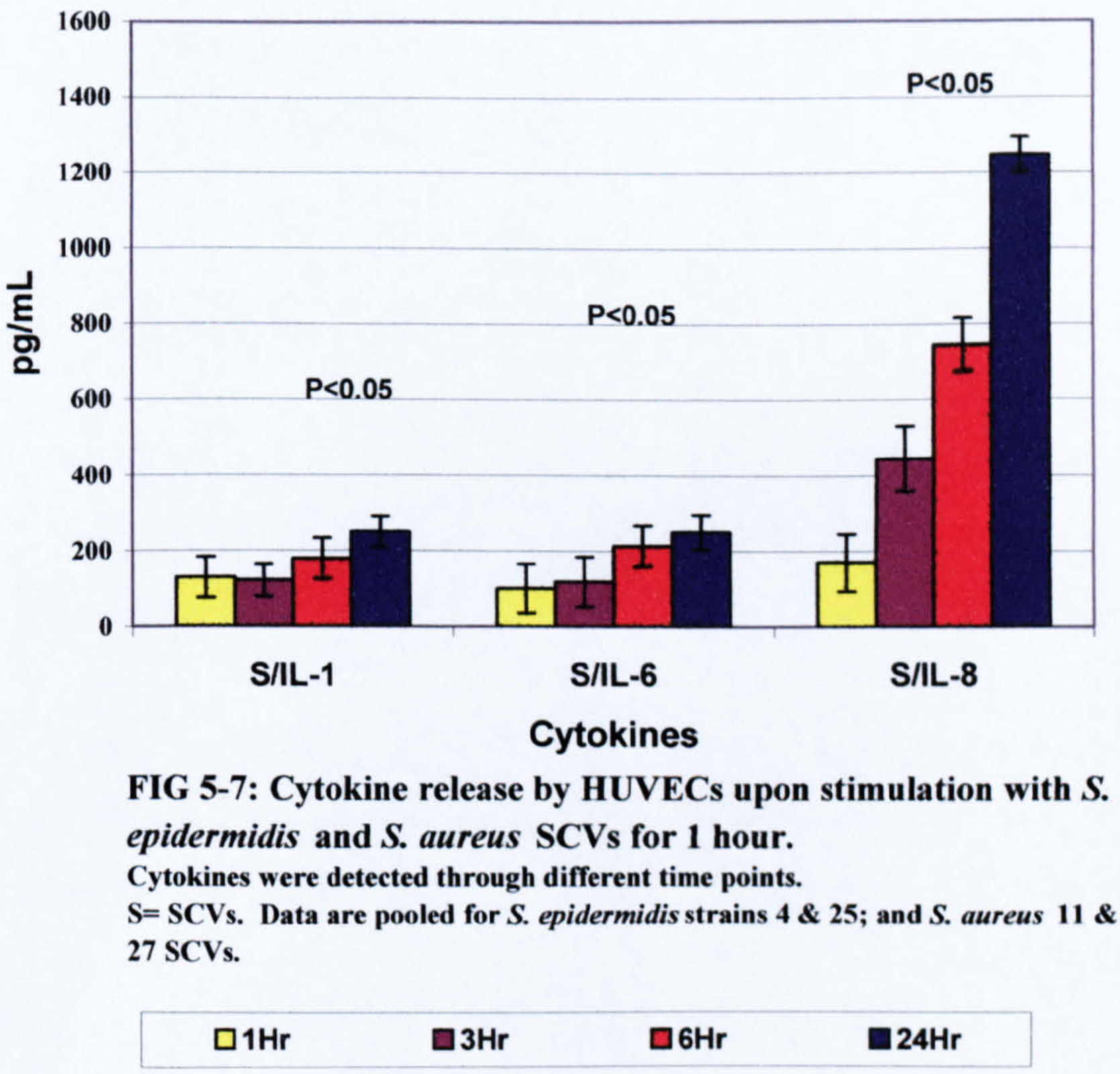

FIG 5-7: Cytokine release by HUVECs upon stimulation with *S. epidermidis* and *S. aureus* SCVs for 1 hour.
Cytokines were detected through different time points.
S= SCVs. Data are pooled for *S. epidermidis* strains 4 & 25; and *S. aureus* 11 & 27 SCVs.

Figure 5.7 summarizes cytokine levels released by HUVECs after being co-cultured for 1 to 24 hours with SCVs from *S. epidermidis* strains 4, 25 and *S. aureus* strains 11, 27. IL-1β, IL-6 and IL-8 were detectable after only 1h incubation, with levels of these cytokines increasing over 24 hours of the incubation in a manner similar to that seen with the wild type staphylococci.

It was notable that levels of IL-6 produced in the presence of the *S. epidermidis* and *S. aureus* SCVs were very much reduced compared to that produced when HUVECs were incubated with the equivalent wild type parental strains. While levels of IL-1 and IL-8 production by HUVECs were similar overall between wild type and SCVs, the SCVs were less cytotoxic, and HUVEC viability was reduced by only 15% by the end of the incubation compared to 45% for the wild type strains.

5:4 Discussion and Conclusions

The purpose of the work presented in this Chapter was to investigate and explore the possibility that SCVs that are persistant intracellularly can promote long-term staphylococcal colonization in the host and play a role in recurrent chronic infections such as in BRI.

Adhesion assays in which bacteria were in contact with host cells over a relatively short period (15 minutes) revealed that *S. aureus* wild strains adhered more extensively to HUVECs than SCVs; this difference was statistically significant ($P<0.05$) (Figure 5.1) . Such differences in adherence abilities of strains could affect the process of subsequent internalization and the ability of these strains to cause persistent infections. However, *S. epidermidis* SCVs did not show any significant difference in their adherence patterns to HUVECs when compared to their related wild type strains (Figure 5.2) after 15 minute incubation.

Adherence assays for four staphylococcal SCVs (*S. aureus* 11, 27 and *S. epidermidis* 4, 25) after 3 hours of incubation showed a significant increase in the numbers of adherent SCVs when compared to their related wild type strains (Figure 5.3). These same SCVs had a four fold increase in adherence after 3 hours incubation when compared with 15 minutes incubation (Figure 5.3).

Although staphylococcal adherence to HUVECs did not show any strain or species dependence, internalization was a strain-specific process. Out of 14 strains tested, only *S. aureus* strains 11, 27 and *S. epidermidis* 4, 25 and their corresponding SCVs were internalized after co-culture with HUVECs (Figure 5.4 and 5.5). This suggests that adherence was necessary but not sufficient to induce internalization by HUVECs, possibly because surface proteins and subsequent events including signal transduction must be specifically induced prior to internalization. In addition, the host cell internalization process is known to require activation and participation of the host cell cytoskeleton (Cossart, 1997). Therefore, the mechanism of internalization of the wild type staphylococci and their corresponding SCVs

was investigated by including different inhibitors of phagocytic pathways such as cytochalasin D, colchicines and MDC in assays of internalization..

Based on results summarized in Figures 5.4 and 5.5, pre-treatment of HUVECs with internalization inhibitors had variable effects upon subsequent staphylococcal uptake by HUVECs. Coated pit formation was inhibited by the addition of MDC, resulting in approximately 10% and 95% ($P<0.05$) reduction in HUVEC uptake of *S. epidermidis* wild type strains and their related SCVs respectively (Figure 5.3 and 5.4). MDC inhibited HUVEC uptake of *S. aureus* wild strains and SCVs by 40% and 90% respectively.

The microfilament depolymerization agent cytochalasin D, inhibited internalization of *S. aureus* wild type strains and their related SCVs by 80% and 50% respectively ($P<0.05$). Similar results were obtained with *S. epidermidis* wild types and SCV strains, with uptake showing an inhibition of 85% and 45% respectively ($P<0.05$) (Figure 5.4 and 5.5). Depolymerization of microtubules by colchicine reduced internalization *S. epidermidis* and *S. aureus* wild strains and corresponding SCV by a similar amount, some 20% and 10% respectively ($P<0.05$) (Figure 5.4 and 5.5).

These data suggest that internalization of SCVs by HUVECs occurred mainly via a receptor-mediated pathway, while their related wild strains were mainly internalized by microfilament-mediated pathways. This correlates with the identified virulence factors (e.g. surface proteins that promote adherence to host cell extracellular matrix proteins) produced by *S. aureus* when compared to CoNS.

To assess the role of surface proteins in adherence and internalization by HUVECs, labelling staphylococci wild type and SCVs strains with BODIPY®FL showed a reduction in dye binding to surface proteins by 58.57% in CoNS SCVs when compared to their wild type parental strains ($P<0.05$) (Table 5.1). Similarly, dye binding to surface proteins on *S. aureus* wild type strains showed a reduction of 40.79% when compared with the equivalent SCVs ($P<0.05$) (Table 5.1). This suggests that the synthesis of the outer cell surface proteins of SCVs had been affected by gentamicin. Since SCVs have been found to show decreased ATP production (Chapter three) which is essential for a lot of metabolic pathways to operate e.g. protein synthesis, it

might be that SCVs could have a defect in protein synthesis or protein secretion across the plasma membrane. This might be an explanation for the observed decrease in BODIPY®FL binding to SCVs compared to their wild strains (Table 5.1).

Since chronicity is linked with cytokine production, a comparison of cytokine production by HUVECs stimulated with staphylococcal wild and SCVs strains was undertaken in this study. Levels of the cytokines IL-1β, IL-6, IL-8, IL-10, IL-12p70 and TNF-α in the culture supernatants of HUVEC cells co-cultured with staphylococci wild type and SCV strains were determined by CBA and flow cytometry analysis. After infection with staphylococcal strains, HUVECs produced elevated levels of IL-1β, IL-6 and IL-8 compared to uninfected controls, which were detected after 1 hour incubation (Figure 5.5 and 5.6). The elicited release of cytokines by HUVECs infected with wild type staphylococci was found subsequently to increase the damage to the cell line, and after 24 hours cytokine levels deceased in cultures infected with wild strains. The decrease in cytokine production was associated with decreased HUVEC viability by 40%. However, cytokines levels continued to increase up to 24 hours in HUVECs infected with SCVs, though they were less than the levels found in infected HUVECs with wild type staphylococcal strains. HUVEC viability at 24 hours following infection with SCVs was 86% when compared with controls viability of 92% indicating that the SCV strains exhibited considerably less cytotoxicity than their wild type parents.

Little is known about the endothelial-activating properties of cell wall components of gram-positive bacteria. However, lipoteichoic acid (LTA) has been found to activate cell adhesion molecule expression (CAM) in HUVECs infected by *S. aureus* (Kawamura *et al.*, 1995). Infection of HUVEC with live *S. aureus* induces ICAM expression and IL-8 release (Beekhuizen *et al.*, 1997; Yao *et al.*, 1996). Soderquist *et al* (1998) found that endothelial cells produced IL-6 and IL-8 in response to exposure to *S. aureus* strains or staphylococcal exotoxins such as α-toxin, enterotoxin A and TSST-1. Capsular polysaccharide of *S. aureus* was also found to stimulate the endothelial production of IL-6 and IL-8 (Soell *et al.*, 1995).

Since infection of mammalian cells is evaluated by the level of cytokines released and subsequently the amount of destruction occurring to host tissues, the results from this study suggest that SCVs could be associated with chronicity found in staphylococcal BRI. That could be due to altered surface proteins (virulence factors), different internalization pathways and decreased destructive effect on tissues due to decreased cytokine released when compared to their wild type strains. Cytokine release has been known to be dependent on the strain and/or the virulence of the bacterium and cellular activation by different pathogens does not necessarily induce similar pathways of intracellular signalling (Rabehi *et al.*, 2001).

Chapter Six

General Discussion and Conclusions

General Discussion and Conclusions

One of the major complications associated with the use of implanted medical devices is biomaterials-related infections (BRI) due to staphylococcal species, in particular, *S. epidermidis* (Bayston, 1999; Kloos and Bannerman, 1994; Pfaller and Herwaldt, 1988). The staphylococcal BRI are distinguished by their chronic nature and resistance to antibiotic therapy, and by the characteristic formation of a bacterial biofilm (Mah and O'Toole, 2001).

Chronic infections such as osteomyelitis and hip replacement infections have been associated with the formation of biofilm involving *S. aureus* SCVs. This led Chuard *et al* (1997) to assume that there is a close association between SCVs and biofilm formation. The ability of the staphylococci to form biofilms is a major factor in their success as pathogens and is an example of their phenotypic flexibility (Brown and Williams, 1985) in their response to diverse environmental stimuli. A variety of environmental factors, including sub-lethal concentrations of antibiotics and intracellular antibacterial mechanisms, as well as nutrient deprivation occurring in biofilms are known to be capable of generating SCVs (Massey *et al.,* 2001; Proctor, 1994).

6:1 Metabolic Profiling of Staphylococcal SCVs

Certain physiological characteristics of SCVs including decreased metabolism, reduced growth rate, increased resistance to cell-wall-active antibiotics, and decreased uptake of aminoglycoside antibiotics, are relevant to the main features of BRIs; chronicity with persistence of infection and treatment failure or relapse. SCVs have therefore been suggested to represent the true biofilm mode phenotype (Bayston and Wood, 1997, Chuard *et al.*, 1997). Compared with *S. aureus* SCVs, very little is currently known regarding infections caused by SCVs of the CoNS.

One important mechanism by which invasive *S. aureus* infection tends to persist within patients, or induces relapse despite adequate antibiotic therapy is believed to occur through the emergence of antibiotic-resistant SCVs (Proctor *et al.*, 1995). Massey *et al* (2001) described how SCVs create an environment that protects wild type *S. aureus* from the lethal effect of aminoglycoside antibiotics. *S. aureus* SCVs were reported in patients with infective osteomyelitis who had undergone gentamicin bead administration (Balwit *et al.*, 1994; Von Eiff *et al.*, 1997) and in a pacemaker-related infection where a gentamicin-containing sponge was applied to the infection site (Seifert et al., 2003). Staphylococcal sub-populations with extremely high MICs to gentamicin were isolated from some cases of BRI in a study by Tshefu *et al* (1983) although these workers explained that these infections only occasionally result in emergence of resistant organisms even after prolonged antibiotic treatment, which could mean that there are additional unknown intrinsic factors that might be important in regulating the ability of staphylococcal strains to produce SCVs. Differences in SDS-PAGE and immunoblotting profiles between strains (such as wild type and SCVs) have been correlated with the antibiotic susceptibility/resistance differences observed for some bacteria. For instance, Langford *et al* (1989) related the changes in the outer membrane protein profiles which were found in gentamicin-induced *P. aeruginosa* SCVs, to their resistance to aminoglycosides.

In this current work, it is reported that *S. aureus* and CoNS SCVs emerged during serial passage in broth culture containing increasing gentamicin concentrations above the MIC. Results indicated that not all of the clinical isolates of staphylococci tested were capable of producing SCVs following gentamicin exposure. The majority of the wild type staphylococcal strains which produced SCVs were initially sensitive to gentamicin; however, some staphylococci which were originally resistant to gentamicin also produced resistant sub-populations (SCVs) with higher MICs than the parental wild type strains. Information on previous antibiotic therapy of patients from whom these particular strains were isolated was not available, but it is conceivable that these strains could be from patients who were previously treated with gentamicin where staphylococcal

resistance was generated by classical mechanisms. Exposure to higher concentrations of gentamicin in the laboratory may have resulted in expression of additional or alternative altered resistance mechanisms - in particular generation of SCV phenotype. Alternatively, culture conditions used to isolate the original isolate may have allowed reversion of strains exhibiting a SCV phenotype *in vivo*. Such strains would be gentamicin resistant, but it would be impossible to determine on the primary isolation plates, if the bacteria isolated were wild type or SCV revertants. In support of this possibility, are the results of one of the pilot investigations done in the present study which involved assessing the gentamicin MIC by Etest for three staphylococcal isogenic strains: the wild parental strain, revertants and SCVs. Some of the revertants showed an intermediate antibiotic resistance between the wild and SCVs.

It was important to constantly supplement both agar and broth cultures with gentamicin to keep the variants stable and to retain their auxotrophy for compounds such as thiamine, thymidine, menadione or proline. This suggests that gentamicin could have affected some metabolic pathways which contributed to the auxotrophy of these variants. Auxotrophy to menadione and thiamine could be due to a defect in electron transport as proposed by Proctor and his colleagues (1997 and 1998). Auxotrophy to proline could be the result of a defect in amino acid synthesis pathways, while auxotrophy to thymidine is connected to a defect in the synthesis of nucleotides.

Several reports support the observation that some of the metabolic characteristics of SCVs are similar to those of other organisms recovered from foreign-body infections (Brown *et al.*, 1990; Chuard *et al.*, 1993). These include a slow mode of growth and decreased *in vitro* susceptibility to bactericidal antibiotics. The altered growth phenotype arises from defects in menadione and haemin biosynthesis resulting in impaired electron transport and decreased cellular ATP concentrations, while the auxotrophic characteristics shown by SCVs suggest that gentamicin directly or indirectly may have affected several diverse metabolic pathways. McNamara and Proctor (2000) found that intracellular *S. aureus* had an altered metabolic state characterised by defective electron transport. Proctor *et al*

(1995) and Von Eiff *et al* (1997) suggested that the defect in electron transport resulted in auxotrophy for menadione or hemin and decreased α-toxin production, possibly allowing the bacteria to persist rather than lyse the host cell (Von Eiff *et al*, 1997). These features were found to be characteristics of SCVs which appear to be associated with persistent staphylococcal infections (Chuard *et al.*, 1997). In the present study staphylococcal SCVs were found to be menadione and/or thiamine auxotrophs, and to have decreased levels of ATP production when compared to their related wild type strains, suggesting a defective electron transport system. These findings confirm the results of earlier studies, and suggest that these alterations in metabolism could contribute to the ability of SCVs to cause persistent infections and chronic inflammatory changes. It is possible that an intra-endothelial reservoir of SCVs could trigger such longstanding inflammation. The mechanism of persistence of SCVs inside endothelial cells and the reason why SCVs have a less destructive effect on endothelial cells compared to the wild type strains are unclear. Decreased haemolysin production by SCVs, delayed coagulase production by *S. aureus* SCVs (Proctor *et al.*, 1995) and a slow growth rate all are likely to contribute to their reduced toxicity for endothelial cells.

This suggestion is also supported by the current results from the API Staph and Api Zym studies, which identified differences in the biochemical and enzymatic profiles displayed by SCVs and their parental strains. However, there was no common enzyme or biochemical defect detected for all pairs of wild and SCV strains tested. This suggests that multiple mechanisms may be affected in staphylococci resulting in generation of the SCV phenotype.

Analysis of the growth characteristics of SCVs confirmed that they have decreased concentration of ATP when measured by chemiluminescence, and that they are slow growing when compared to their related parental strains (Chapter Two). In his review, Smith (1998) showed the importance of bacterial growth rate on bacterial virulence. He indicated that slow growth led to less stimulation of the host immune response which may consequently lead to chronicity of infection (Smith, 1998). In addition to their decreased metabolic activity, chronicity could also be due to the ability of SCVs to survive intracellularly and to their decreased

susceptibility to antibiotics (Vesga *et al.*, 1995). In this present study, SCVs showed substantially decreased susceptibility to antimicrobial agents. Several factors may contribute to this effect. Firstly, slowly growing micro-organisms may be relatively resistant to cell-wall active antibiotics, which require active bacterial cell wall synthesis for maximal efficacy (Schnitzer *et al.*, 1943). Secondly, SCVs located intracellularly would be exposed to sub-therapeutic concentrations of many antimicrobial agents due to their failure to penetrate efficiently into eukaryotic cells (Mandell and Vest, 1972).

Resistance of SCVs to gentamicin has been associated with different susceptibility patterns for other antibiotics. Mickelsen *et al* (1985) stated that isolates of a single strain of *S. epidermidis* usually exhibit the same susceptibility pattern, though expression of antibiotic resistance to gentamicin, chloramphenicol, erythromycin and clindamycin in some strains of *S. epidermidis* may be unstable. This observation was supported by the work of O' Connor-Scarlet and Pierson (1983). Different susceptibility patterns to antibiotics displayed by SCVs could be explained by a decrease in the bacterial uptake of the antibiotic, and resistance to gentamicin would render the SCVs resistant to other aminoglycosides such as amikacin and netilmicin (Proctor and Peters, 1998).

6:2 Phase Variation in SCVs Exopolysaccharide Production

In this study, it has been demonstrated that some *S. epidermidis* strains (21, 25 and 28) showed phase variation in producing exopolymer that facilitates and enhances adherence and might consequently affect biofilm formation. The detection of staphylococcal strains that are able to increase exopolysaccharide production when in the SCV phase, demonstrates the elasticity of these organisms to switch from one state to another under certain environmental circumstances. Formation of biofilms is proposed to occur in two steps: a rapid initial attachment to a synthetic surface which is mediated by polysaccharide adhesin PS/A (Mack *et al.*, 1994) and surface proteins such as autolysin (Heilmann *et al.*, 1997) and a cellular accumulation process to form mature biofilm which is due to production

of polysaccharide intercellular adhesin (PIA) (Heilmann *et al.*, 1996). These two polysaccharide adhesins are encoded by the intercellular adhesin operon (*ica*) (Heilmann *et al.*, 1997) and are closely related chemically (McKenney *et al.*, 2000). Production of PIA has been found to be subject to phase variation (Ziebuhr *et al.*, 1999). Recent evidence has indicated that alternating insertion and excision of an insertion sequence element (IS*257*) is one mechanism responsible for *ica* operon switching in *S. epidermidis* (Ziebuhr *et al.*, 1999).

It is possible that strains which show phase variation due to antibiotic exposure might have advantages in establishing a BRI by enhancing the later stages of biofilm formation. However, results of the present study also indicate differences between wild type strains and SCVs in their initial interaction with plastic surfaces. Wild type strains adhered better to a plasma-coated plastic surface, while SCVs attached better to untreated plastic. These data suggest staphylococci are likely to be capable of adhering to biomaterials irrespective of whether they are coated by host proteins or not - a variable that may be dependent on the localised environment around any implanted material. The observation that staphylococci which did not produce exopolysaccharide also adhered in these *in* vitro assays, supports earlier evidence that initial adhesion to biomaterials depends on factors other than exopolymer production. The characteristics of SCVs of *S. epidermidis* isolates (21, 25 and 28) support the speculation that SCVs could represent the staphylococcal phenotype associated with biofilm formation. Although the wild type strains did not produce exopolymer, they still retained their capability to adhere to a plasma coated plastic surface. Consequently, the initial step in the formation of biofilm could be enhanced for wild type strains since biomaterials are covered by host proteins as soon as they are introduced into the body. Bacterial adherence to implants is determined by general physiochemical interactions between biomaterials and bacteria as well as more specific bridging through various proteins. So following adhesion to a surface, a wild type strain may undergo a phenotypic change that alters many of its surface molecules and de-represses exopolysaccharide synthesis (Davies *et al.*, 1993). In addition, due to antibiotic pressure and nutritional deprivation, these wild strains could undergo

phase variation resulting in the production of SCVs. This possibility was supported by pilot studies undertaken in this project, which demonstrated that SCVs were produced by nutritional limitation as well as by antibiotic stress.

Production of SCVs which are exopolymer producers could therefore strengthen the establishment of biofilm by production of intercellular adhesins such as PIA. Since these variants are not mutants, they could also revert to their parental state if the environmental circumstances change. However, for the invasion of endothelial and epithelial cells, sepsis and spreading, it might be advantageous for some staphylococcal cells to be able to escape the biofilm network, to become biofilm negative and to move on to other tissues. On their passage through the circulation, they might regain a biofilm-positive phenotype allowing them to settle down on heart valves, the endocardium, etc. Once a staphylococcal biofilm is formed the bacteria are very difficult to eradicate, as they are shielded from the immune system and resistant to antibiotic treatment. For these reasons, it is very important to understand the various aspects of staphylococcal biofilm formation, as this knowledge may lead to development of more specific ways of overcoming staphylococcal resistance in chronic infections.

6:3 Analysis of Staphylococcal-Host Cell Interaction

Presently, there is no rapid, simple, and reproducible method available for simultaneous measurement of association (attachment) and uptake (internalization) of staphylococci at the individual cell level. The procedures involving flow cytometry described in this study allowed investigations of various aspects of host-bacteria interactions. Making use of flow cytometry could be extended beyond studying attachment and internalization, and further studies could be possible to investigate the phagocytic process and intracellular killing.

6:3:1 Host Cell Attachment and Invasion

Initial adherence assays (Chapter Five) showed that staphylococci (*S. aureus* and CoNS) were both able to adhere to HUVECs, irrespective of whether or not they were of wild type or SCV status. However, the relative adherence of wild type and SCV strains was dependent on the incubation time with HUVEC cells. After 15 minutes incubation with host cells, *S. aureus* wild type strains adhered better than SCVs, although for this interaction period there was not any significant difference in adherence between *S. epidermidis* wild type and SCVs. However, after 3 hour incubation with HUVECs, *S. aureus* and *S. epidermidis* SCVs adhered better than their wild type strains. Only four wild type strains with their corresponding SCVs out of fourteen staphylococcal strains were internalized by HUVECs. Extended incubation with host cells could result in several changes in surface expression of HUVEC adhesion molecules resulting in increased SCV adherence and/or changes in bacterial expression of cell-associated proteins for host cell attachment, which could also increase aggregation of SCV cells to each other.

Although adhesion is the first step in host cell invasion, some studies indicate that not all bacteria that adhere are capable of invasion. Studies have shown that the potential of bacteria to invade varies with cytotoxicity (ability to kill cells) (Fleiszig *et al.*, 1996). Differences in adherence and internalization have been observed for veterinary mammary pathogens such as *S. aureus* and can be caused by differences in strain virulence (Wanasinghe, 1981). Moreover, streptococci in which particular genes encoding adhesin proteins had been inactivated did not completely lose their ability to invade HUVECs, indicating that other adhesion mechanisms can still mediate attachment, albeit at a diminished level (Stinson *et al.*, 2003). It has been demonstrated that endothelial cells are capable of phagocytosing *S. aureus* strains (Vann and Proctor, 1987). This ability indicates that endothelial cells play a role in local host defence mechanisms or, alternatively, that these cells can act as a hiding place for bacteria. Whether these cells are also

capable of killing the ingested bacteria is unknown; most studies have found no proof of any intracellular killing (Vann and Proctor, 1987; Visser *et al.*, 1996).

The ability of the bacteria to survive intracellularly for 24 hours after internalization has been considered as an indication of chronicity in *in vitro* assays (Döpfer *et al.*, 2001). Based on this definition, this study has proven that SCVs of both *S. aureus* and *S. epidermidis* are capable of surviving intracellularly for 24 hour after being co-cultured with HUVECs, with minimal effects on host cell viability when compared to controls.

Bacterial internalization by host cells is a multifactorial process which is frequently strain dependent and specific for host tissue from which any cell line used in *in vitro* models is derived. This could partially explain why some strains in this study were successfully internalized by HUVECs and others were not. Unfortunately, time limitations meant that it was not possible to include different host cell lines in the present study but other researchers have done extensive work studying *S. aureus* internalization by different cell lines (Veltrop *et al.*, 2001; Visser *et al.*, 1996). These studies showed that *S. aureus* strains were internalized by endothelial, epithelial and mesothelial cells, all of which were capable of digesting intracellular staphylococci. Usui *et a*l (1992) also showed that *in vitro*, different *S. aureus* strains varied in their internalization by murine fibroblasts. This finding was supported by Krut *et al* (2003) who reported that different *S. aureus* strains differed in their uptake into mammalian cells and showed different degrees of virulence and host cell cytotoxic activity.

Previous studies had shown that staphylococcal infections are both inoculum and time dependent (Vann and Proctor, 1987). In this study the incubation times used are comparable to the maximum incubation time used by other researchers (Vesga *et al.*, 1997; von Eiff *et al.*, 1997). However, the inoculum size may have differed in the present work from these published studies, since the intention of the current work was not to analyse the effect of inoculum size on internalization, but to compare the ability of wild type strains and their related SCVs to be internalized by HUVECs, an aim which the inoculum employed (40 bacteria per cell) fulfilled satisfactorily.

Attachment of staphylococci to eukaryotic cells is in part, dependent on surface molecules (mainly proteins) displayed by the bacteria. In this study, labelling the staphylococcal strains with BODIPY-FL provided some comparative evidence about expression of cell wall or surface associated proteins by SCVs and their wild type strains. These proteins could play an important role in adherence of bacteria to biomaterials (Götz, 2002), in host cell attachment and internalization (Kiers *et* al., 2001; McDevitt *et al.*, 1994; Ohshima *et al.*, 1990; Park *et al.*, 1996; Patti *et al.*, 1992; Soell *et al.*, 1995) and in cytokine production (Soell *et al.*, 1995; Wang *et al.*, 2000). All of these factors could affect the ability of a particular strain to cause infection. The results of SDS-PAGE and immunoblotting experiments presented in Chapters 2 and 3 showed that SCVs have reduced surface protein content compared to wild types. This could be due to a decrease in the amount of the same proteins found in wild strains or to altered patterns of protein expression.

The mechanism of invasion for staphylococcal SCVs of HUVECs is poorly understood, but investigation of the HUVEC interaction with SCVs showed that SCVs are internalized by a mechanism different from that of their related wild strains. The mode of internalization is very important and influences the intracellular events following internalization (Ernst *et al.*, 1999). This could promote intracellular survival by decreasing host cell cytokine production which may be a contributor to establishment of a chronic infection. Results from this study indicated that SCVs had been internalized by receptor-mediated endocytosis. This mechanism involves the direct interaction between bacterial ligands and cellular receptors, which leads to the engulfment of the bacteria by the membrane and the formation of a tightly adherent phagosome. However, although phagocytosis and receptor mediated endocytosis require actin polymerization, they are morphologically different and use different host signalling mechanisms (Ireton *et al.*, 1996). In contrast, wild type strains were mainly internalized by a classical phagocytosis mechanism which involves actin polymerization. This could be evidence that the mechanism of internalization is determined by the repertoire of bacterial products or proteinaceous molecules found on the cell wall of

staphylococci. In Chapter 3 SDS-PAGE SCVs profiles showed extra proteins present in cell wall fractions which may be important in influencing internalization mechanisms and subsequent cytokine production.

6:3:2 Alternations in SCV Virulence Factor Expression

The altered enzymatic activity detected in ApiZym profiles (Chapter 2-3) reflects altered metabolic activity between wild and SCV strains which may result in expression of different surface molecules. SDS-PAGE analysis of proteins from staphylococcal whole cell preparations and cell wall fractions, showed differences in protein expression profiles between wild type strains and their related SCVs. It is tempting to speculate that the observed differences in protein expression could account for some of the phenotypic differences seen between wild type and SCV strains, and could contribute to the differences in virulence observed. During the present study, it was not possible to identify the proteins whose expression differed between wild and SCV strains. However, a detailed proteomic comparison between SCVs and their wild type parents (using the staphylococcal genome database) should be regarded as a priority in any future study of staphylococcal SCVs. Proteomic approaches have previously been used to identify a number of staphylococcal virulence-associated proteins including some involved in attachment to host tissues, proteins involved in biofilm formation; host iron-binding proteins, virulence factor such as *S. aureus* protein A and TSST, and proteases (Cheung *et al.*, 1987; Cockayne *et al.*, 1998; Hussain *et al.*, 2001; Liang *et al.*, 1995; Takeuchi *et al.*, 1998; Sugai *et al.*, 1989; Voung *et al.*, 2000).

Pelletier *et al* (1979) compared *S. aureus* wild type parents with SCVs and showed that SCVs were considerably less haemolytic and, therefore, less virulent. Pelletier et al (1979), showed also that *S. aureus* SCVs were as infective as the parent *S. aureus* but were overall less virulent. *S. aureus* SCVs were also significantly more infective than *S. epidermidis* strains in an endocarditis model (Pelletier *et al.*, 1979). Additional studies have indicated that only *S. aureus* whose production of haemolysin is decreased are able to penetrate intracellularly

and persist in endothelial cells (Proctor *et al.*, 1995). However, in an *in vitro* model of septic arthritis Jonsson *et al* (2003) came to the conclusion that SCVs of *S. aureus* were more virulent than the isogenic parental strain due to production of a host cell degrading protease by the SCV. In this present study, lipolytic and proteolytic activities were increased in three of the CoNS SCVs whose wild type parents were isolated from shunt infections.

This work has shown that in addition to differences in expression of proteases and lipases, SCVs also posses a range of virulence factors which differ from their related wild type strains. It is difficult on the basis of the data obtained from the present study to conclude definitively if SCVs are more or less virulent than their wild type strains. However, this could be studied further by studying the relative virulence of the SCV and parental strains in an appropriate animal model. However, it is possible to speculate that the capacity to produce a resistant from such as a SCV is by itself a virulence factor, acting by promoting intracellular survival inside HUVECs. Since bacterial pathogens vary their phenotypic properties in response to environmental signals, this enables them to optimize their interactions with their hosts and thereby to establish productive infections.

6:3:3 SCV Induction of Host Cell Cytokine Expression

Previous *in vitro* studies have shown that *S. aureus* infection of endothelial cells can stimulate the release of pro-inflammatory cytokines, and can directly cause endothelial cell injury (Pober and Cotran, 1990). Pro-inflammatory cytokines such as IL-1 and IL-6 play an important role in induction of the host immune response (Fantuzzi and Dinarello, 1996), and also correlate with disease severity (Casey *et al.*, 1993). IL-12 and IL-10 are involved in the regulation of T-cell differentiation (Romagnani, 1991), while IL-8 is a chemoattractant which can facilitate transendothelial migration of neutrophils in an *in vitro* assay (Yao *et al.*, 1996). However, IL-8 can also be produced due simply to direct stimulation of endothelial cells with staphylococci (Yao *et al.*, 1996). Previous observations have revealed that IL-1β can actually enhance the growth of *S. aureus* (Kanangat

et al., 2001). Thus it is possible that besides shaping immune responses, cytokines may also modulate the expression of staphylococcal virulence factors and thereby influence the course of an infection from the bacterial perspective. Previous studies have shown that host cell cytokine production could be triggered by stimulation with bacterial products (van Langevelde *et al.*, 1999), bacterial adhesins (Soell *et al.*, 1995), and mineral particles (Shi *et al.*, 1998).

In this study, it was observed that SCVs adherence to HUVECs increased after 3 hours incubation (Chapter Five). It might be that cytokine production could have altered expression of some staphylococcal cell wall-associated proteins, teichoic acid and peptidoglycan, all of which could enhance and increase their adherence to HUVECs. However, in the present study cytokine production in staphylococcal infections was focused on strains which were internalized by endothelial cells (Vriesema *et al.*, 2000; Yao *et al.*, 1995; Yao *et al.*, 1996). Due to time limitations and financial constrains, it was not possible to include all the strains in this study, although the effect of all the strains on the viability of HUVECs was assessed. HUVEC viability was similar for all strains irrespective of whether they could be internalized by the HUVECs or not. It is therefore possible that cytokine production may also be similar for all the strains tested, but this suggestion would require additional work.

In this study, both SCVs and their related wild strains (*S. aureus* and *S. epidermidis*) evoked cytokine production by HUVECs. There was no significant difference in cytokine profiles detected following incubation with *S. aureus* or *S. epidermidis*. This would suggest that *S. aureus* and *S. epidermidis* produce similar intrinsic factors (mechanisms) stimulating cytokine release by HUVECs.

IL-1β, IL-6 and IL-8 were detected at all the time point sampled for both wild type and SCV strains, but SCVs showed decreased cytokine production when compared to wild type strains. This ability of staphylococcal SCVs to induce reduced levels of cytokine expression and to persist intracellularly without damaging the host cell could be a key factor contributing to the chronicity of SCV

infections, since SCVs once internalised would be protected from the host immune response allowing persistence in tissue.

Apoptotic mammalian cells infected by staphylococci (wild type or SCVs strains) could also be considered as a transport vehicle transferring the internalized staphylococci to other site of organs or implants where they could establish another focus of infection. Moreover, cells undergoing apoptosis could provide an additional niche for SCV generation. Although this study was not designed to screen the production of SCVs induced intracellularly, findings by Proctor and his colleagues support altered *S. aureus* physiology during intracellular residence within endothelial cells (Vesga *et al.*, 1996). They cite evidence to suggest that the intracellular milieu of endothelial cells which have phagocytized *S. aureus* may induce between 24 and 48 h the emergence of a subpopulation of metabolically hypoactive *S. aureus* SCVs (Vesga *et al.*, 1996). Another study by Menzies and her colleagues (Menzies *et al.*, 1998) showed that the presence of apoptosis was observed as early as 1 h after *S. aureus* infection of endothelial cells. They speculated that *S. aureus* SCVs as observed in other studies (Vesga *et al.*, 1996) might have arisen in response to stimuli from the environment of nutritionally depleted and dying host cells.

The ability of HUVECs to produce IL-10, IL-12 and TNF-α was confirmed in a pilot study using the intracellular pathogen *Listeria monocytogenes* (data not shown). Although HUVECs are clearly capable of producing IL-10, IL-12 and TNF-α these were not detectable in later studies with HUVECs infected with staphylococci. The difference in cytokine profiles generated by *L. monocytogenes* and staphylococci may reflect different mechanisms of intracellular growth and ability of the two bacteria to stimulate different pathways. This could be related to the fact that *Listeria* is a true intracellular pathogen; whereas, staphylococci are mainly extracellular pathogens which could be considered as having a less damaging effect when compared to the potential cellular damage done by *Listeria*.

Bacteria are now routinely examined for the presence of putative virulence determinants by SDS-PAGE and immunoblotting. In this study differences in polypeptide bands found in between SCVs and their related parental strains could

be surface modifications that promote resistance to host immune responses and facilitate the intracellular survival potentials. Blots of SCV proteins probed with anti-staphylococcal immune sera showed a lower level of antibody reactivity than the equivalent protein fractions prepared from wild type staphylococci. This finding may reflect a more generalised reduction in the quantity or variety of other types of surface antigens expressed by SCVs which may be an important factor contributing to reduced cytokine production and ability of SCVs to cause chronicity.

6:4 Overall Conclusions

Although the pathogenesis of staphylococci and in particular that due to staphylococcal SCVs is complex, this study has highlighted potentially important characteristics of SCVs that may contribute to their ability to cause BRIs. However, much further research remains to be done to fully understand the metabolic changes that comprise the SCV phenotypic shift, and its role in staphylococcal disease. As an overall conclusion, in this study the findings suggest the following:

(1) *S. epidermidis* SCVs could be produced as well as *S. aureus* SCVs by exposure to the antibiotic gentamicin.

(2) There are differences in virulence-associated factors produced by staphylococcal wild type strains and their related SCVs (such as exopolymer production, haemolysin, coagulase, proteases, and lipases) which could contribute to some aspects of the correlation between SCV formation and BRI. The clinical characteristics of persistent and refractory staphylococcal infection may in turn reflect the various phenotypic changes associated with SCVs.

(3) Despite the SCVs displaying phenotypic differences, their genetic make up has been conserved, as they are clonal as determined by PFGE.

(4) The advantages of SCV production may permit staphylococci to survive much longer intracellularly, to revert to the parental strain when the host environment changes, allowing the bacteria to disseminate and invade new tissues, thus perpetuating the infection and producing late relapses as has been reported by others (Proctor *et al.*, 1994; Proctor *et al.*, 1995). Although it is well known that delayed coagulase and haemolysin production are the two major reasons for *S. aureus* SCVs intracellular survival (von Eiff *et al.*, 1997), there is no clear reason for the ability of *S. epidermidis* SCVs to survive intracellularly when compared to their related wild type strains.

(5) Staphylococcal SCVs (*S. aureus* and CoNS) are less antigenic than their wild type parents, which could play a role in chronicity of BRI. This was demonstrated by analysing the cross-reactivity of immunoblots of wild type and SCV protein extracts against staphylococcal antiserum. Results showed, however, that there were similarities in the staphylococcal proteins cross reactivity with anti-staphylococcal antibodies between strains (*S. aureus* and CoNS).

(6) *S. epidermidis* SCVs showed similarity to *S. aureus* SCVs in displaying menadione and thiamine auxotrophy. This suggests common defects in the electron transport system of the staphylococcal SCVs, as well as defects in other metabolite pathway reflected in variability in the patterns of auxotrophy seen with different strains.

(7) The ability of some strains of staphylococci to produce detectable exopolysaccharide only in the SCV mode raises the possibility that this phase variation may contribute to the ability of these variants to produce larger quantities and more persistent biofilm than their wild type parental strains. The possibility that SCVs may represent the true biofilm phenotype for the staphylococci requires additional investigation. Biofilm formation could be studied by using an

established *in vitro* model of catheter colonization with staphylococcal strains (Raad *et al.*, 1995).

(8) Differences in the internalization mechanism by HUVECs between SCVs and their wild type strains (*S. aureus* and *S. epidermidis*) could play a role in chronicity and relapsing infection features characteristic of staphylococcal infections.

(9) This study showed that *S. epidermidis* SCVs shared a number of similarities with *S. aureus* SCVs (such as auxotrophism, regulation of exopolymer production, decreased immunogenicity, host cell internalization mechanisms, and induction of HUVEC apoptosis and cytokine production). This may reflect the fact that all of SCVs investigated in the present study were produced following a common stimulus- exposure to the antibiotic gentamicin.

(10) This study investigated a wide range of the physiological characteristics of *S. aureus* and CoNS wild strains and their related SCVs. However, this study is essentially a foundation work and it will be necessary to extend the analyses described in this report to fully investigate the differences which exist between *S. aureus* SCVs and CoNS SCVs and their wild type strains, and what role each phenotype plays in the pathogenesis of biomaterial related infections.

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8: Appendix

Appendix

8:1 Reagents

8:1:1 Electrophoresis Solutions

8:1:1:1 SDS-PAGE Running Buffer

25 mM Tris

190 mM Glycine

1% SDS in H_2O,

pH 8.8

8:1:1:2 12% Separating Gel Preparation (0.375M Tris, pH 8.8)

To make two gels-

3.35ml De-ionized water

2.5ml 1.5 Tris HCL pH 8.8

100µl 10% (w/v) SDS in H_2O

4ml Acrylamide/Bis (30% stock)

50µl 10% Ammonium Persulphate in H_2O

5µl TEMED

8:1:1:3 4% Stacking Gel Preparation (0.125M Tris, pH 6.8)

6.1ml De-Ionized Water

2.5ml 0.5M Tris-HCL, pH 6.8

100µl 10% (w/v) SDS in H_2O

1.33ml Acrylamide/Bis (30% stock)

50µl 10% Ammonium Persulphate in H_2O

10µl TEMED

8:1:1:4 SDS-PAGE Loading Buffer

1.62g Tris base

10ml 2-Mercaptoethanol

4g SDS

4mg Bromophenol

20g Sucrose

Make up to 100ml with distilled water, pH 6.8

8:1:2 Western Blotting Solutions

8:1:2:1 Western Transfer Buffer

Glycine	39 mM
Tris Base	48mM
SDS	0.037%
Methanol	20%

To prepare 1 litre of transfer buffer (pH 8.3), mix 2.9 g of glycine, 5.8g of Tris base, 0.37g of SDS, and 200ml of methanol.

8:1:2:2 Blocking Solution

1g dried Skimmed Milk in 10ml distilled water

8:1:2:3 Colorimetric Substrate for Development of Western Blots

0.03g 4-Chloro-1-Naphthol

60ml Transfer Buffer

60µl H_2O_2

8:1:3 PFGE Solutions

8:1:3:1 Reagents for Plug Preparation

Final Concentration	Stock Concentration	Volume
TE (pH 7.5)		
10mM Tris-HCl (pH 7.5)	1M	5ml
50mM EDTA (pH 7.5)	0.5M	50ml
Autoclaved de-ionised water		445 up to 500ml
TE (pH 8.0)		
10mM Tris-HCl (pH 8.0)	1M	5ml
1mM EDTA (pH 8.0)	0.5M	1ml
Autoclaved de-ionised water		494ml up to 500ml
Lysis Buffer		
0.5M EDTA	0.5M	10ml
1% Sarkosyl	25%	400µl ≅10ml
***Lysostaphin Solution**		
Lysostaphin	5mg/vial	5mg
Autoclaved de-ionised water		5ml up to 5ml

Final Concentration	Stoke Concentration	Volume
***PMSF Solution	-	
0.1M PMSF		175mg
Isopropanol		10ml
		up to 10ml
PMSF in TE (pH 8.0)		
1mM PMSF	0.1M	100µl
1mM TE (pH 8.0)	1mM	10ml
		≅10ml

*** Lysostaphin should be dispensed into 0.5 ml microtube and stored at –20°C**

****Proteinase K should be dispensed into 1.5ml microtube and stored at -20°C**

***** PMSF: phenylmethylsulphonylfluoride, PMSF; should be dispensed into 1.5 microtube and stored at –20°C**

8:1:3:2 Running Buffer

X10 TBE stock Solution	
Tris	108g
Boric Acid	55g
0.5M EDTA	40ml
	up to 1000ml with autoclaved distilled water

8:1:4 Internalization Assay

8:1:4:1 Solvents for Substances used to Study the Mechanisms of HUVEC Internalization

10 μM colchicine (sigma) in ethanol at 0.4% final concentration, 250 μM monodansylcadaverine (sigma) 2 μM cytochalasin D (sigma) in DMSO at 1% final concentration were prepared in growth medium without antibiotic and filtered. Each was added one hour prior to the addition of bacteria and incubated at 37°C

8:2 Charts

8:2:1 Api Staph Chart

Tests		Substrate	Reactions /enzymes	Result	
				Negative	Positive
0	1	No Substrate	Negative control	Red	-
GLU	**2**	D-Glucose	(Positive control)	Red*	Yellow
FRU	**4**	D-Fructose			
MNE	**1**	D-Mannose			
MAL	2	Maltose			
LAC	4	Lactose	Acidification due to carbohydrate utilization		
TRE	1	D-Trehalose			
MAN	2	D-Mannitol			
XLT	4	Xylitol			
MEL	1	D-Melibiose			
NIT	2	Potassium nitrate	Reduction of nitrate to nitrite	NIT1 + NIT2 /10 min	
				Clorless-light pink	Red
PAL	4	β-naphthyl-acid phosphate	Alkaline phosphatase	ZYM A + ZYM B /10 min	
				Yellow	Violet
VP	1	Sodium Pyruvate	Acetyl-methyl-carbinol production	VP1 + VP2 /10 min	
				Clorless-light pink	Violet-pink
RAF	2	Raffinose	Acidification due to carbohydrate utilization	Red	Yellow
XYL	4	Xylose			
SAC	1	Sucrose			
MDG	2	α-methyl-D-glucoside			
NAG	4	N-acetyl-glucosamine			
ADH	1	Arginine	Arginine dihydrolase	Yellow	Orange-red
URE	2	Urea	Urease	Yellow	Red-violet

The acidification tests should be compared to the negative (0) and positive (GLU) controls.
*** When MNE and XLT are preceded or followed by positive tests, then an orange test should be considered negative.**
ADH and URE under anaerobic conditions.

8:2:2 Api Zym Chart

The API ZYM strip is composed of 20 cupules, especially designed for *the study of enzymatic* reactions. The enzymatic tests are inoculated with a dense suspension of organisms, *which is used to rehydrate the enzymatic* substrates. The metabolic end products produced during the incubation period (6 hours) are predicted through colored reactions revealed by the addition of ZYM A (Tris-hydroxymethyl-aminomethane, hydrochloric acid, sodium lauryl sulfate) and ZYM B (Fast Blue BB, 2-methoxyethanol)

No.	Enzyme Assayed For	Result	
		Positive	Negative
1	Control	Colourless	
2	Alkaline phosphatase	Violet	
3	Esterase	Violet	
4	Esterase Lipase	Violet	
5	Lipase	Violet	
6	Leucine arylamidase	Orange	
7	Valine arylamidase	Orange	
8	Cystine arylamidase	Orange	
9	Trypsin	Orange	
10	α-chymotrypsin	Orange	
11	Acid phosphatase	Violet	
12	Naphthol-AS-BI-phosphohydrolase	Blue	
13	α-galactosidase	Violet	
14	β-galactosidase	Violet	
15	β-glucuronidase	Blue	
16	α-glucosidase	Violet	
17	β-glucosidase	Violet	
18	N-acetyl-β-glucosaminidase	Brown	
19	α-mannosidase	Violet	
20	α-fucosidase	Violet	

Colourless

8:2:3 Antibiogram Chart

Antibiotic	Resistance Value
Penicillin (P)	4
Tetracycline (TE)	2
Chloramphenicol (C)	1
Erythromycin (E)	4
Methicillin (Meth)	2
Trimethoprim (W)	1
Clindamycin (Da)	4
Gentamicin (Cn)	2
Rifampicin (Rd)	1
Amikacin (AK)	4
Netilmicin (NET)	2
Cefuroxime (Cxm)	1
Vancomycin (Va)	4
Spiramycin (SP)	2
Fusidic acid (FD)	1
Teicoplanin (Tec)	Recorded as *S or **R
Ciprofloxacin (Cip)	Recorded as S or R

*** S= susceptible , R= resistant.**

8:3 Chemicals

8:3:1 BODIPY®FL, STP Ester, Sodium Salt

(Molecular Probes Cat. B-10006, Netherlands) Stored at ≤-20°C. Protect material from long term exposure to light. The dye has an amine-reactive 4 sulfo-2,3,5,6-tetrafluorophenyl (STP) ester group.