The *In Vitro* Development and Characterisation of two Clinically Important Biofilms

Thesis submitted in fulfilment of the requirements of the degree of Doctor of Philosophy, Cardiff University.

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LIST OF UNITS AND ABBREVIATIONS

Aap	accumulation associated protein
AFM	atomic force microscope
Agr	accessory gene regulator
AHL	acylated homoserine lactones
AIP	autoinducing peptide
ALS	agglutinin like sequence
°C	degree Centigrade
CF	cystic fibrosis
μl	microlitre
BHI	brain heart infusion
BEC	buccal epithelial cells
bp	base pairs
BSA	bovine serum albumin
cfu	colony forming units
CAC	chronic atrophic candidosis
CFTR	cystic fibrosis transmembrane conductance regulator
CGH	comparative genome hybridisation
CHC	chronic hyperplasitc candidosis
CLSI	clinical laboratory standards institute
CLSM	confocal laser scanning microscopy
cm	centimetre
CDFF	constant depth film fermenter
ConA	concanavalin A
Crc	catabolite repression control
Cup	chaperon usher
CVLU	chronic venous leg ulcers
ddNTPs	dideoxynucleoside triphosphate
DEPC	diethyl-pyrocarbonate
DGGE	denaturing gradient gel electrophoresis
DMSO	dimethyl sulphoxide
DNA	deoxynucleosidetriphosphates
dNTPS	deoxynucleosidetriphosphates
EPS	extracellular polysaccharide
FimH	fimbrial component H
FISH	fluorescence in situ hybridisation
FITC	fluorescein isothiocyanate
h	hours
HCl	hydrochloric acid
H and E	haematoxylin and eosin
HIV	human immunodeficiency virus
HSL	homoserine lactone
K_2HPO4	dipotassium hydrogen orthophosphate
KCL	potassium chloride
l	litre
LPS	lipopolysaccharide
M	molar
m	minutes
MDR	multidrug
ml	millilitre
mg	milligram
MgSO4	magnesium sulphate
MIC	minimum inhibitory concentration

mm	millimetre
MRSA	methicillin resistant *staphylococcus aureus*
MSCRAMMs	microbial surface components recognising adhesive matrix molecules
n	number
NBTE	non-bacterial thrombotic endocarditis
NCCLS	national committee for clinical laboratory standards
NVE	native valve endocarditis
OM	otitis media
PAS	periodic acid – Schiff
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEI	polyethyleneimine
Pel	pellicle formation
PIA	polysaccharide intercellular adhesion
PLs	phsopholipases
Pls	polysaccharide synthesis locus
PMC	pseudomembranous candidosis
PMLs	polymorphonuclear leukocytes
PMNs	polymorphonuclear neutrophils
PNA	peptide nucleic acid
PS/A	polysaccharide/adhesin
pta	phosphate acetyltransferase
PTFE	polytetrafluoroethylene
PVP1	polyvinylpyrrolidone iodine
QS	quorum sensing
RHE	reconstituted human epithelium
RNA	ribonucleic acid
rpoS	stationary sigma factor
s	seconds
SAP	secreted aspartyl proteinases
Sar	staphylococcal accessory gene regulator
SCC	squamous cell carcinoma
SD	standard deviation
SDA	sabourad's dextrose agar
SDS	sodium dodecyl sulphate
SEM	scanning electron microscopy
SPSS	statistical Package for the Social Sciences
TCA	trichloracetic acid
TE	Tris EDTA
VAP	ventilator associated pneumonia
v/v	volume per unit volume
w/v	weight per unit volume
YCB	yeast carbon base
YEPD	Yeast extract peptone dextrose
XTT	tetrazolium salt 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide

PREFACE

The work presented in this thesis was undertaken at the School of Dentistry, Cardiff University, United Kingdom.

Chapter 2 and Chapter 3 were partly published in:

S. Malic, K. E. Hill, J. R. Ralphs, A. Hayes, D. W. Thomas, A. J. Potts, D. W. Williams. 2007. Characterization of *Candida albicans* infection of an *in vitro* oral epithelial model using confocal laser scanning microscopy. *Oral Microbiol Immunology:* 22: 188-194

Abstract

Environmental biofilms are abundant and represent the most prevalent growth mode of microorganisms. In humans, biofilms are frequently encountered and responsible for numerous infections which can be difficult to treat since biofilms tend to be more resistant to antimicrobial agents and host immune defences.

The focus of this study was to characterise two biofilms associated with specific human infections, namely oral candidosis and chronic wound infection. *In vitro* biofilms using microorganisms from these infections were generated using microtitre plates, a constant depth film fermenter (CDFF) and reconstituted human epithelial tissue (RHE).

Confocal laser scanning microscopy of *Candida* biofilms on RHE showed strain-dependent tissue invasion, with differences also evident in surface colonisation and *Candida* morphology. Hyphal elements invaded epithelial cells and exhibited budding within the tissues. A relationship between high candidal tissue invasion and consistent expression of secreted aspartyl proteinase (SAP) genes 4-6 was found. There was no correlation between level of invasion and expression of phospholipase and agglutinin-like sequence (ALS) genes. These results demonstrate strain variation by *Candida* which relate to pathogenic potential.

Biofilms of the chronic wound bacteria, *Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus oralis* and *Micrococcus luteus*, showed species variation occurred in the extent of biofilm formation with antagonism evident in mixed species biofilms. Importantly, the pathogenic species of *P. aeruginosa* and *S. aureus* often inhibited biofilm formation by the commensal species (*M. luteus* and *S. oralis*). Susceptibility of wound bacterial biofilms to povidone iodine was reduced compared with planktonic cells. Up-regulation of biofilm genes by *P. aeruginosa* (alginate genes) and *S. aureus* (*ica* genes) was evident in the biofilm models. Extrapolation of results to chronic wound environments does require additional research on wound tissue, but the ability of the pathogenic species to antagonise commensal species could explain biofilm succession within a wound, possibly promoting a chronic wound phenotype.

Overview of the thesis structure

The focus of the present study was to characterise *in vitro*, two clinically important biofilms associated with human infections, namely oral candidosis and chronic wound infection.

In the first instance, confocal laser scanning microscopy was used to examine infection of an oral epithelium by *Candida albicans* strains from different oral conditions (Chapter 2) and to determine whether inherent strain differences existed that could relate to infection type. Reverse transcription polymerase chain reaction (RT-PCR) was used to detect products from recognised virulence gene families (Chapter 3).

Having established appropriate methodologies, the second part of the thesis focused on chronic wound biofilms. Biofilm formation and bacterial interaction was characterised in different models, including a microtitre plate assay and a constant depth film fermenter (CDFF) (Chapter 4). The CDFF was also used to determine susceptibility of biofilms to povidone iodine (PVP1). Spatial distribution and biofilm architecture within established biofilms from the CDFF and on a reconstituted human epidermis were studied by fluorescent *in situ* hybridisation (Chapter 5). The spatial detection/association of microorganisms on actual wound biopsies was also assessed.

In the final part of the study, expression of specific genes associated with biofilms was assessed for planktonic and biofilm growth phases. This approach involved RT-PCR and microarray analysis (undertaken at the Health Protection Agency in Colindale) (Chapter 6).

Chapter 1
Literature Review

1.1 INTRODUCTION

1.1.1 The concept of biofilm growth

Microbiology based research has in the past primarily focused on the *in vitro* study of monotypic organisms grown in liquid media. The study of such 'free growing' or planktonic organisms has undoubtedly been of value in expanding our knowledge on the physiology and biochemistry of microorganisms. However, in recent years, the concept of microbial growth occurring as a biofilm rather than a planktonic growth phase has been recognised and become the focus of much research (Percival and Bowler, 2004; Wilson, 2001).

The classic definition of a biofilm is that of a "community of microorganisms often adhered to a surface and encased in an extracellular polysaccharide matrix (EPS) or "glycocalyx" (Kumamoto and Marcelo, 2005; O'Toole et al., 2000a) (Fig.1.1). Whilst much is still to be learnt about the differences between biofilm and planktonic (free form) growth phases, what is apparent is that the two forms differ markedly in terms of behaviour, structure and physiology (Table 1.1).

1.1.2 Prevalence of biofilms in humans

Biofilms are ubiquitous, and in humans a wide range of surfaces are available for microbial attachment and subsequent biofilm formation. Typical examples of biofilms include: bacterial plaque on both the enamel of teeth (Costerton et al., 1999) and on the acrylic of denture prostheses (Douglas, 2003; Ramage et al., 2006); candidal biofilms on the oral and vaginal mucosa and on prosthetic hip joints (Garcia-Sanchez et al., 2004; Kumamoto and Marcelo, 2005; Wilson, 2001), and the microflora located in chronic wounds (James et al., 2007; Percival and Bowler, 2004; Percival and Rogers, 2005; Rhoads et al., 2007).
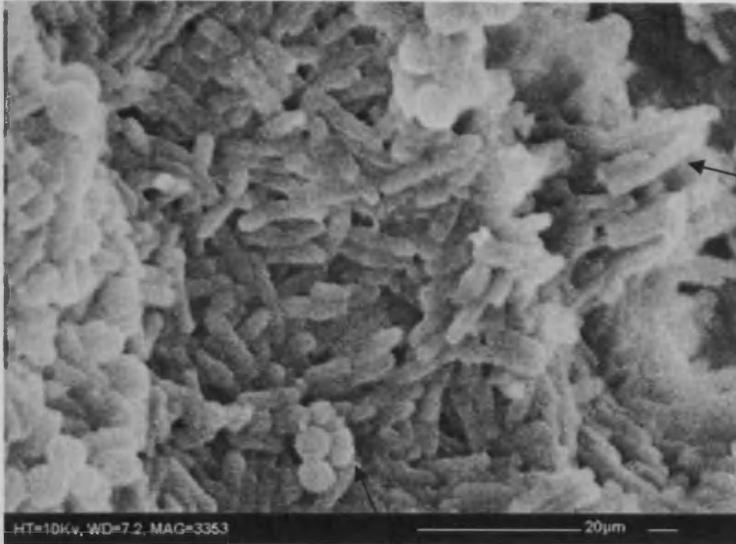
Bacillus;
Pseudomonas aeruginosa

Coccus;
Staphyloccus aureus

Fig. 1.1 Image of a mixed biofim generated in the CDFF, day 3. Scanning electron microscopy image (SEM).

Table 1.1 Comparison of biofilm and planktonic lifestyles

Characteristic	Lifestyle	References
Physiological		
Free growing often in aqueous environment	Planktonic	(Wilson, 2001)
Metabolic products continously removed from the milieu, Stresses of physical, chemical and biological nature	Planktonic	(Anwar et al., 1990; Costerton et al., 1987; Van Loosdrecht et al., 1990)
Slow growth	Biofilm	(Brown et al., 1988)
Found ubiquitously	Biofilm	(Costerton et al., 1995; O'Toole and Kolter, 1998; Potera, 1996)
Generally higher resistance to antibiotics, hydrogen peroxide, phagocytosis	Biofilm	(Ashby et al., 1994; Cochran et al., 2000; Gander, 1996; Hassett et al., 1999; Hoyle and Costerton, 1991; Jensen et al., 1990; Schierholz et al., 1999)
Stress conditions, lower metabolic activity (e.g. decreased nutrient accessibility, limitation of oxygen, increased osmotic pressure, pH variation, problems with accumulation of waste metabolites)	Biofilm	(Anwar et al., 1990; Beloin and Ghigo, 2005; Costerton et al., 1987; Van Loosdrecht et al., 1990)
Bacteria develop stress responses within biofilms	Biofilm	(Beloin and Ghigo, 2005)
Source of many infections	Biofilm	(Lewis, 2001)
Display unique gene expression patterns	Biofilm	(Davies, 2003)
Attachment can influence metabolic activities	Biofilm	(Breznak, 1984, Costerton et al., 1987, Van Loosdrecht et al., 1990)
Secreted virulence factors	Biofilm & Planktonic	(Davies et al., 1998)
Structural		
Attached to a solid substrate	Biofilm	(Costerton et al., 1987)
Many cells are dormant, likely to be smaller, not actively engaged in cell division	Biofilm	(Anwar et al., 1992; Rhoads et al., 2007)
Flagella function in transport and initiation of cell-to-surface interactions and detachment	Biofilm	(Sauer et al., 2002)

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Importantly, all these biofilms can act as reservoirs of infection and lead to human disease. Indeed, biofilm related infections are extremely prevalent and it has been estimated that over 65% of all hospital infections have a biofilm origin (Costerton et al., 1999; Donlan, 2001; Donlan and Costerton, 2002; Douglas, 2003; Ramage et al., 2006). Table 1.2 outlines some clinically significant examples of biofilms and these are discussed in more detail later in this Literature Review (section 1.6). Of additional significance, is that once established, biofilm originating infections can be very difficult to combat due to the resilience of this growth phase against removal by host defence mechanisms and antimicrobial agents. A greater understanding of the biofilm lifestyle could enhance our knowledge of pathogenic processes and possibly lead to novel and effective control strategies designed to improve the management of patients with biofilm infections (Donlan, 2002).

1.2 CHARACTERISTICS OF BIOFILMS

1.2.1. Biofilm formation

The formation of a mature biofilm is a multistage process and dependent on a number of variables, including the type of bacterial species present, the composition of the surface to which attachment occurs, environmental factors and the expression of biofilm essential genes (Carpentier and Cerf, 1993; Dunne, 2002). Figure 1.2 shows a schematic diagram of the various stages of biofilm formation consisting of attachment, colonisation and growth phases.

1.2.1.1 Initial adhesion

The mechanism of initial adhesion of a microorganism to a surface is dependant on both physical and chemical interactions. Primary adhesion between bacteria and abiotic surfaces is generally mediated by non-specific (*e.g.* hydrophobic) interactions, whereas adhesion to living tissue tends to be accomplished through specific molecular (lectin, ligand, or adhesion) mechanisms (Carpentier and Cerf, 1993; Dunne, 2002).

Table 1.2. Clinically significant examples of biofilm infections.

Infection or disease	Common bacterial species involved	Reference
Dental caries Periodontitis	*Streptococcus* spp., *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, *Bacteroides forsythus*, *Prevotella intermedia*	(Kenney and Ash, 1969; Mira et al., 2004; Socransky et al., 1998)
Otitis media	*Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis*, *S. aureus, S. epidermidis, P. aeruginosa*	(Ehrlich et al., 2002; Fergie et al., 2004; Ferguson et al., 1986)
Cystic fibrosis	*P. aeruginosa, Burkholderia cepacia*	(Bagge et al., 2004a; Hentzer et al., 2001)
Chronic wounds	Staphylococci, streptococci, enterococci, facultative gram-negative bacilli, anaerobic bacteria such as *Fusobacterium* spp. and peptostreptoocci	(Bjarnsholt et al., 2008; Davies et al., 2001; Hansson et al., 1995; Hill et al., 2003; James et al., 2007; Percival and Bowler, 2004; Serralta et al., 2001)

Infection associated with foreign body material

Urinary catheters	*Proteus mirabilis, Morganella morganii, P. aeruginosa, Klebsiella pneumoniae, Proteus vulgaris*	(Stickler, 2005; Stickler, 2002; Stickler et al., 1999; Trautner and Darouiche, 2004; Tunney et al., 1999)
Native valve endocarditis (NVE)	Streptococci, staphylococci, Gram negative bacteria, fungi	(Braunwald, 1997; Donlan and Costerton, 2002)
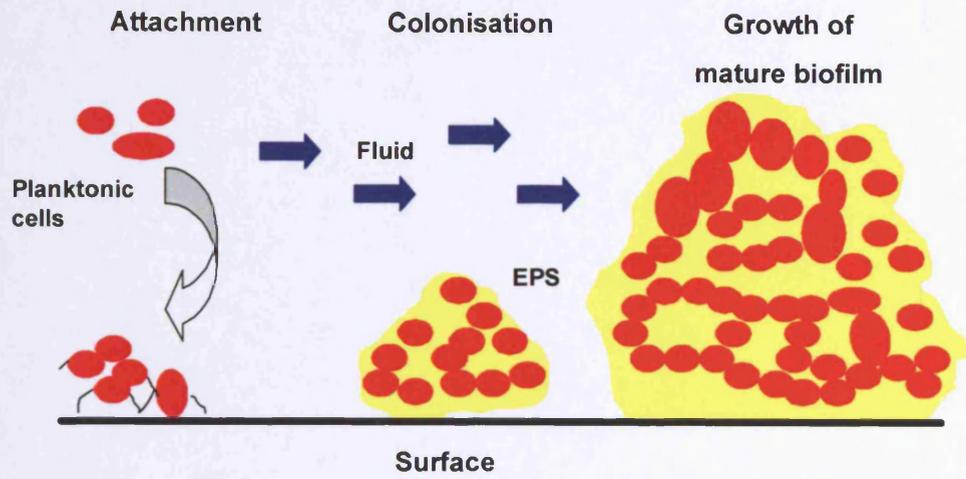
Fig. 1.2 Schematic diagram showing the various stages of biofilm formation consisting of attachment, colonisation and growth phases. Bacteria are depicted in red and the extracellular polysaccharide matrix of the biofilm is depicted in yellow.

Primary adhesion is reversible and in biological systems occurs between a conditioned surface and a planktonic microorganism. In the case of a biomaterial, conditioning represents the modification of the native surface through the adsorption of water, proteins, lipids, extracellular matrix molecules, complement, fibronectin and inorganic salts. This conditioning film may change the affinity of an organism to attach to the surface (Dunne, 2002).

In the initial stages of the adherence process, the organism needs to be in close proximity to the surface, either through random transportation in a stream of fluid or in a direct way via chemotaxis and motility (Dunne, 2002). In the case of *P. aeruginosa* biofilms, flagella motility is required to promote this primary adhesion (Dunne, 2002; O'Toole and Kolter, 1998a). Once in the vicinity of the host surface, bacterial attachment is mediated by multiple adherence factors (known collectively as adhesins), which can overcome any net repulsion between the two surfaces that may be present due to electrical charges (Boland et al., 2000; Carpentier and Cerf, 1993). The final determination of the initial adhesion depends on electrostatic and hydrophobic interactions, van der Waals forces, temperature and the hydrodynamic forces between the two surfaces (An et al., 2000; Carpentier and Cerf, 1993; Dunne, 2002).

Attachment is a complex progress regulated by diverse variables. Cell surface structures such as fimbriae, other proteins, lipopolysaccharide (LPS), EPS and flagella all play an important role. Attachment to hydrophobic substrata seems to be associated with cell surface polymer structures such as fimbriae, other proteins and cell wall components of certain Gram-negative bacteria (mycolic acids), while EPS and LPS are more important in attachment to hydrophilic materials (Donlan, 2002).

1.2.1.2 Secondary bacterial adhesion

Secondary bacterial adhesion involves covalent interaction between specific adhesins and receptors on cell surfaces (An et al., 2000). Bacteria and host surfaces generally express multiple adhesins and receptors respectively

(Table 1.3). The levels and activities of these components are modulated in response to different environmental and external conditions, nutrient sources available, static conditions or fluid flow (Dunne, 2002; Jenkinson et al., 2003). Certain adhesins are regulated at the transcriptional level, permitting organisms to switch from sessile to planktonic forms under different environmental influences (An et al., 2000; Ziebuhr et al., 1999). *Staphylococcus epidermidis* and *S. aureus* produce a polysaccharide intercellular adhesion (PIA) which is associated with cell-to-cell adhesion and subsequent biofilm formation (Cramton et al., 1999; Heilmann et al., 1996; Mack et al., 1996; Mack et al., 1994). *Pseudomonas aeruginosa* exhibits a surface-associated motion known as "twitching motility", where monolayer cells "walk" via twitching motility to form cellular aggregates on surfaces that eventually differentiate into microcolonies (Dunne, 2002). It has been shown that mutants deficient in type IV pili form monolayers but not cellular aggregates (Dunne, 2002).

1.2.2 Biofilm structure

As already stated, bacteria that form biofilms exist in two forms, planktonic or sessile. Those bacteria that are sessile attach to a surface, aggregate and form a matrix to produce a biofilm (Costerton et al., 1999). Attached bacteria multiply and encase their colonies with the generated 'slimy' matrix. The matrix surrounding the biofilm primarily consists of exopolysaccharides often collectively referred to as the glycocalyx.

Biofilms are heterogeneous environments and the consensus is that they comprise of aggregates of microbial cells within the glycocalyx, with interstitial voids and channels separating the microcolonies (Fig. 1.3a) (Sutherland, 2001). The planktonic population exists in the liquid state surrounding the biofilm. Importantly, sessile and planktonic bacteria of the same species have been shown to have different phenotypes.

Table 1.3 Examples of adhesins known to be involved in biofilm formation.

Microorganism	Adhesin	Reference
Escherichia coli	Fimbrial component H (FimH)	(Schembri and Klemm, 2001)
Streptococcus mutans	Ag I /II; receptor Salivary pellicle	(Hajishengallis et al., 1992; Hamada et al., 2004; Prakobphol et al., 2000)
Pseudomonas aeruginosa	Twitching motility: Type IV pili; Flagella motility, Fimbriae	(O'Toole and Kolter, 1998a; Vallet et al., 2004; Vallet et al., 2001)
Staphylococcus aureus	Polysaccharide intercellular adhesin (PIA)	(Arciola et al., 2001; Cramton et al., 1999; Knobloch et al., 2002; Martin-Lopez et al., 2002; Yazdani et al., 2006)
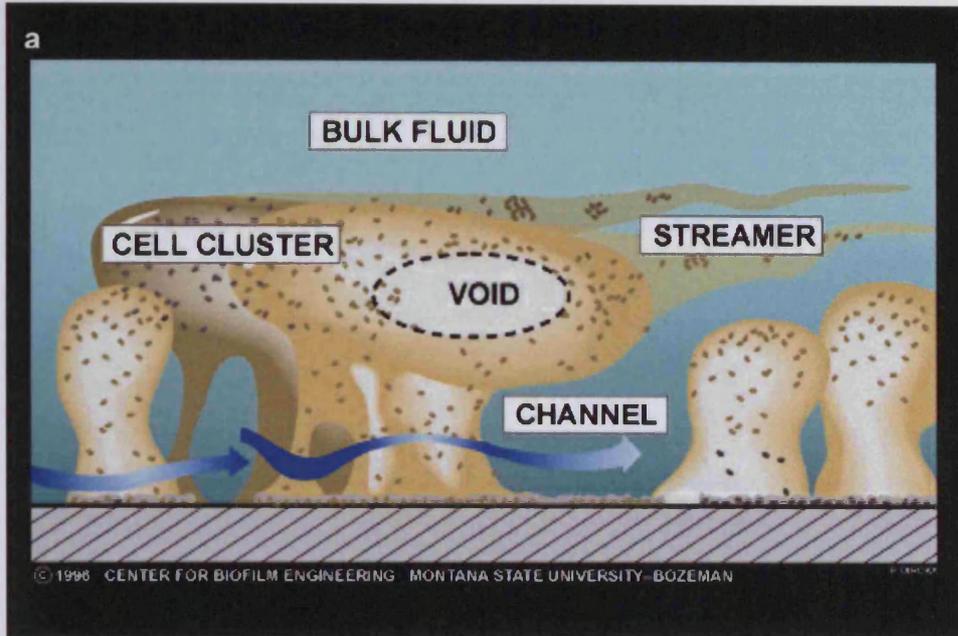
Fig. 1.3a A 3-dimensional structure of a biofilm including formation of distinct water channels. (Adapted from the Centre for Biofilm Engineering, Montana University, Bozeman; www.erc.montana.edu).
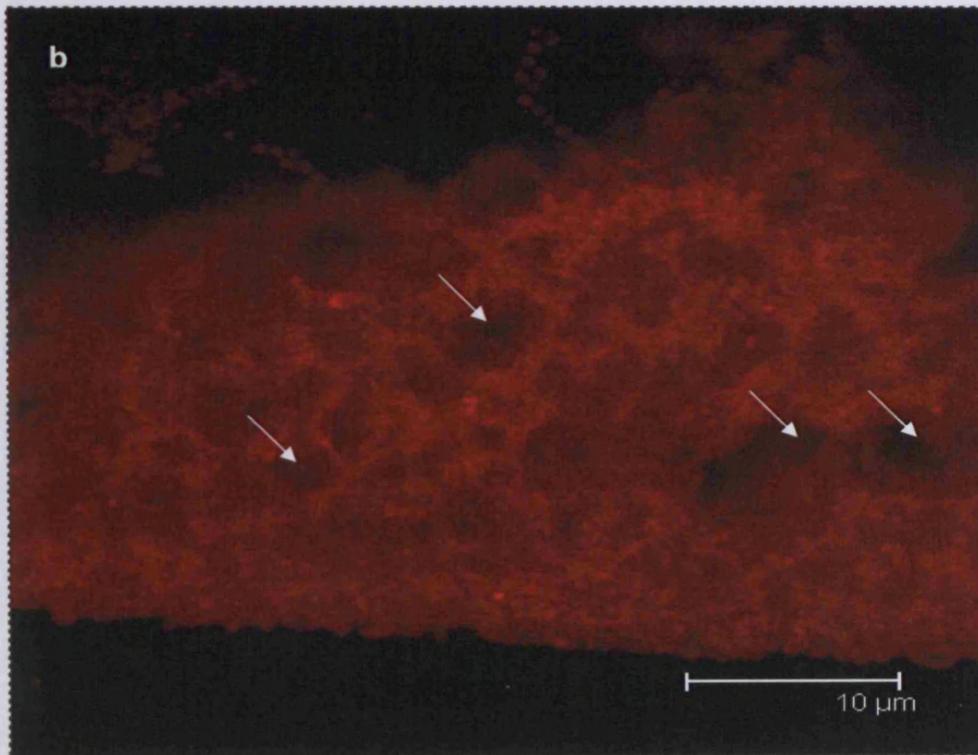
Fig. 1.3b Mixed biofilm generated in the Constant Depth Film Fermenter, stained with universal bacterial peptide nucleic acid probe (PNA, Bac-Uni1CY3-red). Arrows indicate water channels. (This study).

Figure 1.3b shows a mixed biofilm consisting of *P. aeruginosa*, *S. aureus*, *Micrococcus luteus* and *Streptococcus oralis* generated in the laboratory using the constant depth film fermenter (Chapter 3) over a five day incubation period. This image clearly depicts the water channels (spaces devoid of bacteria) which allow diffusion of nutrients and gases. This biofilm structure is typical of other descriptions found in the literature (Davey and O'Toole, 2000; Donlan, 2002).

1.2.3 Physiological properties of biofilms

The physiology of biofilm cells is extremely complex and profoundly different from those organisms grown planktonically (Costerton et al., 1987). The physiological status of biofilm cells is heterogeneous and determined by the location of each individual cell within the multiple layers of cells that form the biofilm (Anwar et al., 1992a). Surface biofilm cells are metabolically active and tend to be large in size since these have easy access to nutrients and oxygen, and have fewer problems with the discharge of metabolic waste products (Anwar et al., 1992a). The surface–associated biofilm cells are most likely to have properties similar to planktonically cultured cells. Sternberg *et al* (1999) has shown that cells at the base of the biofilm have reduced metabolic rates compared with cells at or near the surface, and highlights the importance of nutrient availability as a crucial factor influencing metabolic activity (Davies, 2003).

1.3 MICROBIAL INTERACTIONS WITHIN BIOFILMS

1.3.1 Microbial cell coaggregation

Biofilms can be comprised of single or mixed species populations. During the adhesion process, planktonic bacteria can form aggregates, thereby 'sticking' to neighbouring cells in a process known as coaggregation (Dunne, 2002).

Coaggregation is defined as the specific cell-to-cell recognition that occurs between both intra and inter species (Kolenbrander et al., 2006). This

mechanism of adhesion is highly specific and considered to have a role in the development of multi-species biofilms in many different environments. Coaggregation has been shown to be highly specific in dental plaque bacteria and mediated by growth phase-dependent adhesion-receptor interactions (Rickard et al., 2003a; Rickard et al., 2002; Rickard et al., 2003b).

Away from the oral cavity, coaggregation has also recently been reported for bacterial members of the urogenital flora (Reid et al., 1988), lactobacilli from the crops of chickens (Vandevoorde et al., 1992) and in aquatic biofilm-forming bacteria (Buswell et al., 1997; Rickard et al., 2000). The phenomenon has been attributed with enabling the survival of anaerobic species in an oxygenated environment by ensuring close association with certain aerobic species (Bradshaw et al., 1998).

Coaggregation may provide additional benefits to the interacting species beyond promoting adherence and facilitating bacterial succession (Bradshaw et al., 1998). It seems to promote a nutritionally beneficial, communal relationship, where metabolic cooperation results in the liberation of additional nutrients, which may help maintain the characteristic diversity of many biofilm communities (Rickard et al., 2003a).

1.3.2 Competitive interactions

Microorganisms in a biofilm are in close proximity to one another and therefore interact as a consequence. These interactions can be beneficial to one or more of the interacting populations, whilst others can be antagonistic (James et al., 1995; Stewart et al., 1997).

Donlan (2002) suggested that *P. aeruginosa* is competitive in mixed biofilms despite its slower growth rate compared with other bacteria because it is able to rapidly colonise the surface of the substratum. *Pseudomonas aeruginosa* therefore could provide the base layer of biofilms with other more rapidly growing bacteria producing surface microcolonies (Donlan, 2002).

1.3.3 Synergistic interactions

It is thought that synergistic mechanisms play a role in mixed species biofilms (Hofstad, 1992). Synergistic effects include oxygen consumption by aerobes thereby increasing the redox potential and allowing anaerobes to proliferate, as well as nutrient production and impairment of host immune response. Polymicrobial interactions may well play a crucial role for biofilms in delayed wound healing. For example, less invasive microorganisms can act synergistically with more virulent ones (Bowler, 2003).

1.3.4 Quorum sensing

Quorum sensing (QS) can be defined as a cell-to-cell communication system utilising chemical messages that allows a single bacterium to interact with its neighbours (Jones, 2005) and serves to regulate colonisation and virulence factor expression (Yarwood and Schlievert, 2003). Quorum sensing chemicals are often referred to as microbial pheromones or autoinducers (Jones, 2005). Bacteria release these pheromones into the environment and effectively the level released relates to the population density. Quorum sensing communication is coordinated by small, diffusible signal molecules. Gram-negative bacteria typically produce acylated homoserine lactones (AHLs, Fig. 1.4a) whereas Gram-positive bacteria use small cyclic peptides (Fig. 1.4b) (Donabedian, 2003; Smith and Iglewski, 2003).

In the natural environment, there are many different types of biofilm communities, all of which require the coordination of the constituent cells' behaviour. As environmental conditions can change rapidly, microorganisms need to respond quickly to adapt to these changes. Examples where such adaptations are needed include responding appropriately to the availability of nutrients and protecting against other 'in-coming' and potentially competitive microorganisms, host defence mechanisms or antimicrobial agents. The cell-cell signalling system helps bacteria to survive various stresses in a cell-density-dependent manner and regulates the expression of genes that also influence their ability to cause disease (Marsh, 2004).

a

R1

Basic structure of the AHL signal molecule

R2

R1=H, OH or O

R2=C_1-C_{18}

3-oxo-C12-homoserine lactone

C4-homoserine lactone

b

YSTCDFIM

S-C=O

GVNACSSLF

S-C=O

YINCDFLL

S-C=O

YSTCYFIM

S-C=O

A = alanine

C = cysteine

D = aspartic acid

F = phenylalanine

G = glycine

I = isoleucine

L = leucine

M = methionine

N = asparagine

S = serine

T = threonine

V = valine

Y = tyrosine
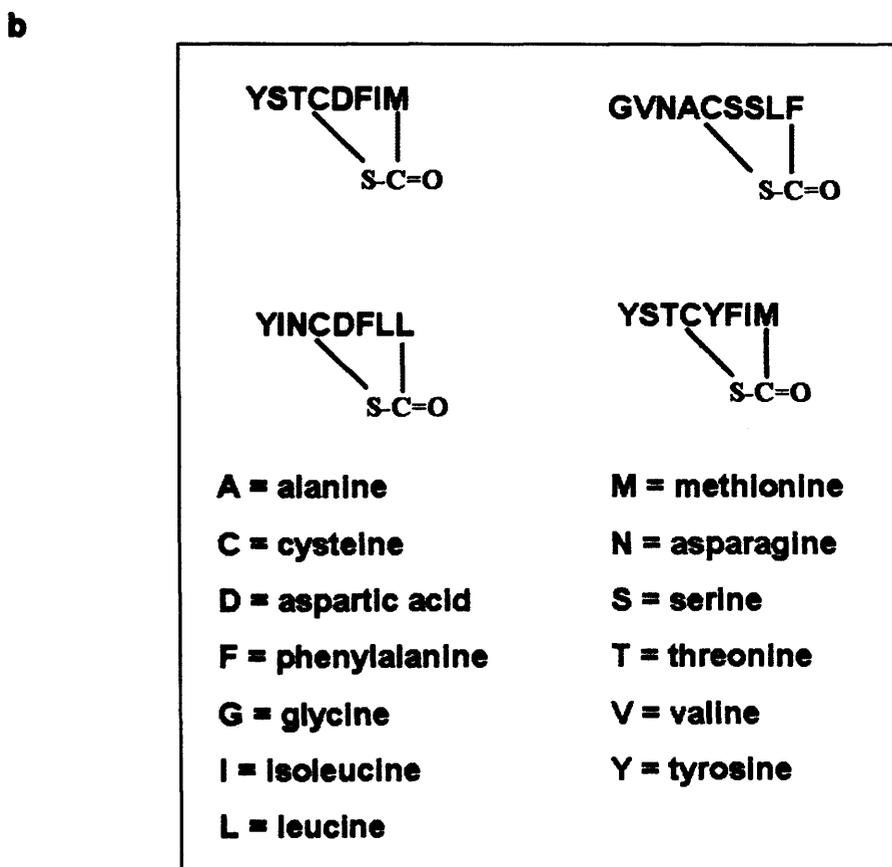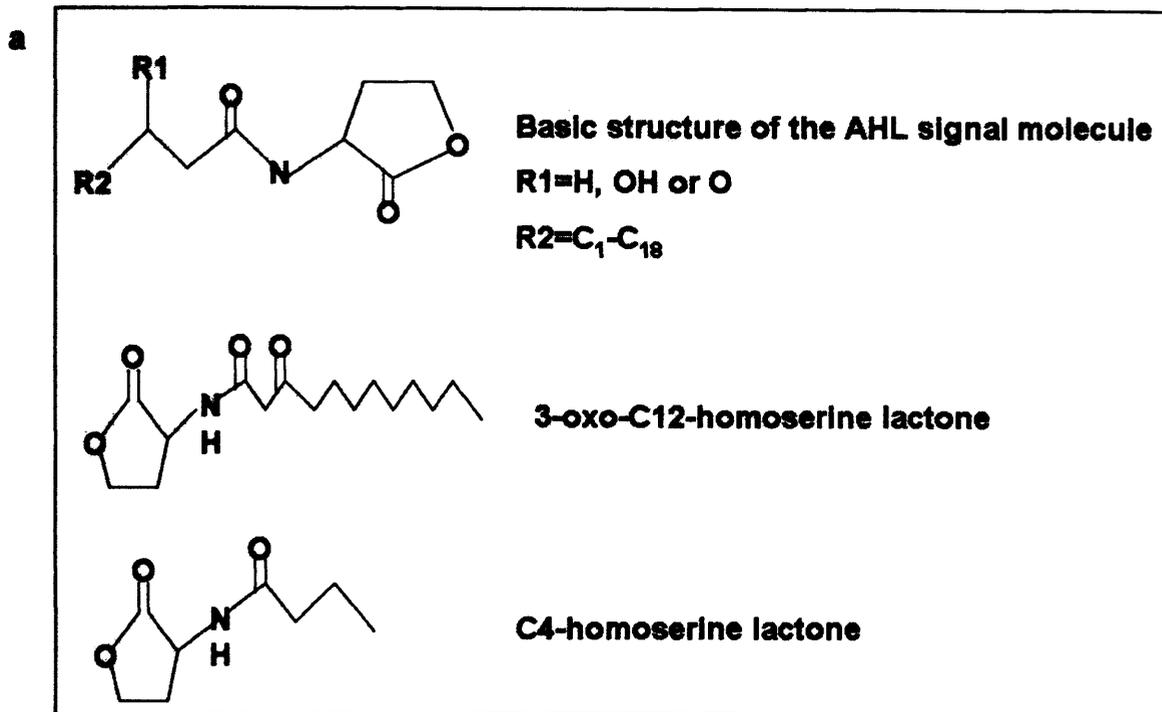
Fig. 1.4 Bacterial quorum sensing molecules a) homoserine lactones of *P. aeruginosa* and b) cyclic peptides of *S. aureus* (adapted from Donabedian, 2003; Rasmussen and Givskov, 2006).

For pathogenic bacteria to cause infection, coordination of virulence factors may be required to avoid associated host immune responses.

1.3.4.1 Quorum sensing by *Pseudomonas aeruginosa*

Pseudomonas aeruginosa has at least two quorum sensing systems which interact with each other, the LasR-LasI and the RhlR-RhlI with the cognate signal molecules N-(3-oxo-dodecanoyl)-L-homoserine lactone (3-oxo-C12-HSL; HSL for homoserine lactone usually referred to as PAI-1) and N-butanoyl-L-homoserine lactone (C4-HSL) (Donabedian, 2003; Rasmussen and Givskov, 2006). It has been shown that the gene product of *lasI* produces a diffusible molecule (PAI-1) which could activate the LasR protein (Donabedian, 2003). The two systems are linked with the *las* system being the first part of the signalling cascade positively regulating expression of both *rhlR* and *rhlI* (Fig. 1.5).

A third non-AHL dependent QS system has been identified which relies on the signal molecule heptyl-3-hydroxy-4-quinolone (PQS) and is interspaced between the *las* and the *rhl* system.

Pseudomonas aeruginosa produces many extracellular virulence factors including alkaline protease, toxin A, and two different elastases (as the products of the *lasA* and *lasB* genes). The LasR protein enhances the transcription of the genes which encode those virulence factors. *LasI* is necessary for the production of the same virulence factors. A normal and dense biofilm formation of *P. aeruginosa* depends on the synthesis of PAI-1. Bacteria lacking the *lasI* gene produce a loose and easily disrupted biofilm (Donabedian, 2003). As expected for a density-dependent gene system, the expression of *lasI* and *rhlI* is greatest at the base of the biofilm and decreases as the height of the biofilm increases (De Kievit et al., 2001a). *Pseudomonas aeruginosa* isolated from urinary catheters (Stickler et al., 1998), contact lenses (Zhu et al., 2001) and cystic fibrosis (Singh et al., 2000) all produce these quorum-sensing molecules.

The *las* regulon

Other regulators

LasR | LasI → LasI

- Exoenzymes: elastase, alkaline, proteases
- Exotoxin A
- Biofilms
- Haemolysin
- Siderophore
- Secretion apparatus (Xcp)

Multiple gene regulation

LasR ← 3-oxo-C12-HSL (PAI-1)

+

The *rhl* regulon

RhlI ← *rhlI* | *rhlR*

C4-HSL (PAI-2) → RhlR → Multiple gene regulation

- Exoenzymes: elastase
- Lectins
- Hydrogen cyanide
- Rhamnolipids
- Siderophore
- Secretion apparatus (Xcp)

Fig. 1.5 Hierarchical organisation of *P. aeruginosa* quorum sensing system. LasR activates the expression of *lasI* to generate 3-oxo-C12-HCL (also known as PAI-1). The LasR/3-oxo-C12-HSL complex drives the expression of multiple target genes. The RhlR protein drives the expression of *rhlI*, and hence C4-HSL (also known as PAI-2) and the RhlR/C4-HSL complex is responsible for controlling multiple target genes. LasR/3-oxo-C12-HSL also regulates the expression of *rhlI*.

A study using the burn mouse model showed that QS plays a significant role in wound infections (Rasmussen and Givskov, 2006). However, if the mice were infected with QS mutants (deletion of synthase genes in *P. aeruginosa*, e.g. *lasI* deletion mutant, *lasI rhlI* deletion mutant), the immune response appeared to develop quicker, the polymorphonuclear leukocytes (PMLs) responded with the development of stronger oxidative bursts and antibodies accumulated faster at the infected lung site (Bjarnsholt et al., 2005; Smith et al., 2002; Wu et al., 2001). In addition to the role of QS in the virulence and pathogenesis of *P. aeruginosa*, other pathogenic organisms, including *Serratia liquefaciens* and *Chromobacterium violaceum* (both of which are human pathogens), and *Agrobacterium tumefaciens* and *Erwinia carotovora* (both of which are plant pathogens) have also been shown to encode QS signalling pathways (Rasmussen and Givskov, 2006; Sheng and Citovsky, 1996; Whitehead et al., 2002).

1.3.4.2 Quorum sensing by *Staphylococcus aureus*

In the case of *S. aureus*, the accessory gene regulator (*agr*) controls the coordinated production of virulence factors. The *agr* signalling system (Fig. 1.6) consists of a classical two-component system; AgrC as the signal receptor and AgrA as the response regulator. The system serves as a QS regulon to autoinduce RNAIII, the principal effector of the *agr* response. An autoinducing peptide (AIP) encoded within the *agrD* drives the *agr* signalling system (Ben Ayed et al., 2006; Shopsin et al., 2003). The peptide quorum sensing molecule is actively secreted by an ATP-binding cassette exporter. The receptor for the peptide is a histidine protein kinase molecule which spans the staphylococcal cell membrane. The *agrD* gene codes for a peptide which is cleaved by the AgrB protein to form the cyclic octapeptide. The AgrC protein acts as the transmembrane receptor for the secreted peptide. The receptor-ligand pair then phosphorylates the product of the *agrA* gene which in turn allows the transcription of many staphylococcal virulence genes, such as alpha, beta and delta toxins (Donabedian, 2003). Table 1.4 summarises factors which regulate quorum sensing.
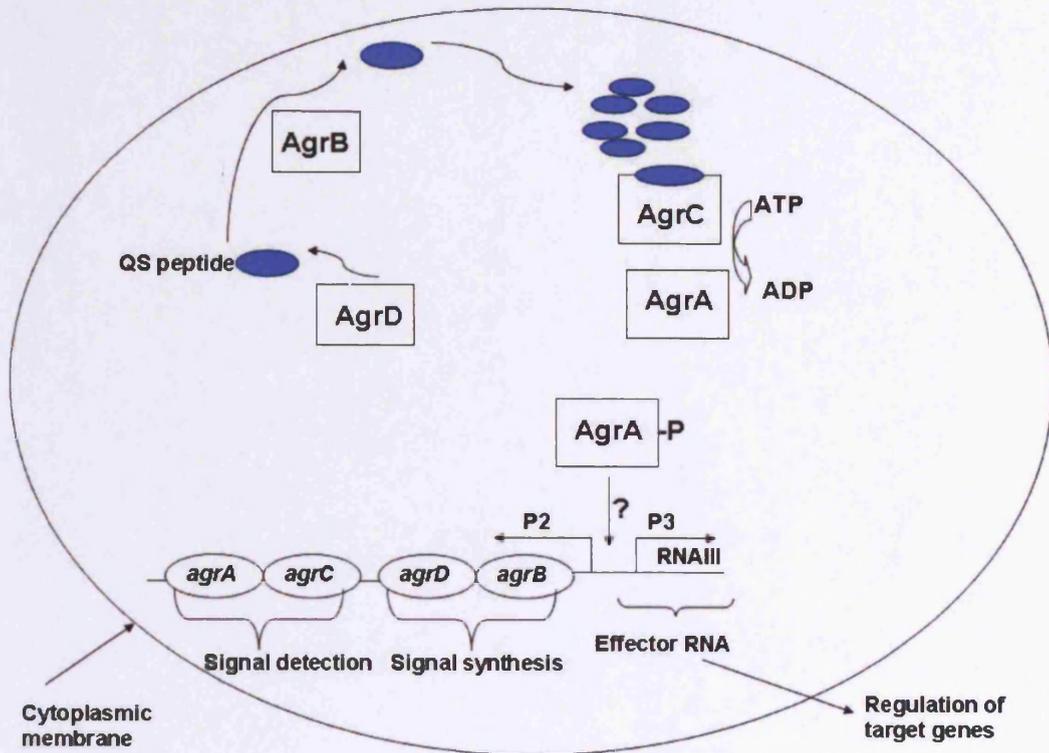
Fig. 1.6 Quorum sensing in *S. aureus.*

The *agr* locus consists of two divergent transcriptional units. The left operon is expressed from the P2 promoter and consists of agrABCD, which encode the proteins that are responsible for generating and sensing the peptide signal molecule; the right RNAIII operon is transcribed from the P3 promoter. The QS peptide is derived from the gene product of *agrD* by the action of the AgrB protein, which is responsible for the export of the peptide out of the cell. Once a critical concentration is reached, the peptide interacts with the AgrC membrane associated sensor protein, causing it to autophosphorylate. The phosphate is then transferred to the response regulator and in its phosphorylated state AgrA then activates expression of RNAIII, which mediates the changes in expression of multiple target genes. Since the genes responsible for peptide production (agrBD) are also activated, this results in the production of more peptide signal and the generation of an autoinduction feedback loop.

Table 1.4. Quorum sensing systems and the factors they regulate

Organism	Quorum Sensing Systems Used	Factors Regulated by Quorum Sensing	Reference
Pseudomonas aeruginosa	*lasI-lasR* via *rhlI-rhlR*. *Pseudomonas* quinolone signal (PQS).	Involved in • Biofilm formation and architecture • Virulence factor production, including elastase, alkaline protease, hydrogen cyanide, exotoxin A, catalase, pyocyanin, lectins, superoxide dismutase • Pathogenesis of *P. aeruginosa*	(Donabedian, 2003) (Rasmussen and Givskov, 2006) (Heurlier et al., 2006)
Staphylococcus aureus	*agr* (accessory gene regulator) quorum sensing system, shown to be expressed in several infection models.	Involved in • Virulence factor production, attachment and dispersal • Tissue colonisation and enhancement in biofilm formation	(Donabedian, 2003) (Camara et al., 2002) (Vuong et al., 2003)
Vibrio cholera	*HapR* and *CqsA*	Involved in • Expression of genes, when density of its species is sufficiently high • Depth of biofilm	(Zhu and Mekalanos, 2003) (Donabedian, 2003)

1.4 BIOFILM STABILITY

1.4.1 Resistance of biofilms against host defence mechanisms

Bacteria within a biofilm generate a protected microenvironment through the production of an extracellular biofilm matrix. This matrix is composed of a mixture of components, including EPS, proteins and nucleic acids. The biofilm matrix is a complex structure encompassing of water channels that permit transfer of nutrients and waste products (Fig. 1.3a). The most widely studied of these components is EPS (Table 1.5). Bacterial generated EPS would appear to be the key component in affording biofilm protection against host defences (Davey and O'Toole, 2000). The fact that biofilm-based infections are rarely resolved, even in individuals with competent innate and adaptive immune responses, highlights the high degree of resistance exhibited (Stewart and Costerton, 2001) . It has been shown that biofilms are resistant against the host cellular defence system (the phagocytic and immune responses) (Todar, 2005) and the adaptive and innate immunity (lysozyme, lactoferrin, Toll-like receptors) (Hiemstra, 2001).

1.4.2 Resistance of biofilms against antimicrobial agents

The nature of the biofilm structure and the physiological attributes of microorganisms within the biofilm create an intrinsic resistance to antimicrobial agents (antibiotics, disinfectants, germicides or antifungals). Mechanisms responsible for resistance may be by one or more of the following: a) reduced penetration of the antimicrobial agent into the biofilm matrix, b) altered growth rate of biofilm organisms and c) other physiological changes due to the biofilm mode of growth (Fig.1.7); (Donlan and Costerton, 2002).

Table 1.5 Typical components of a biofilm matrix.

Component	Percentage of matrix (%)
Water	up to 97
Microbial cells	2-5 (polymicrobial)
Polysaccharides (monosaccharides, heteropolysaccharides)	1-2 (neutral & polyanionic)
Proteins (extracellular & resulting from lysis)	< 1-2 (including enzymes)
Nucleic acids	< 1-2 (from lysed cells)
Ions	Bound and free

Adapted from Sutherland 2001.
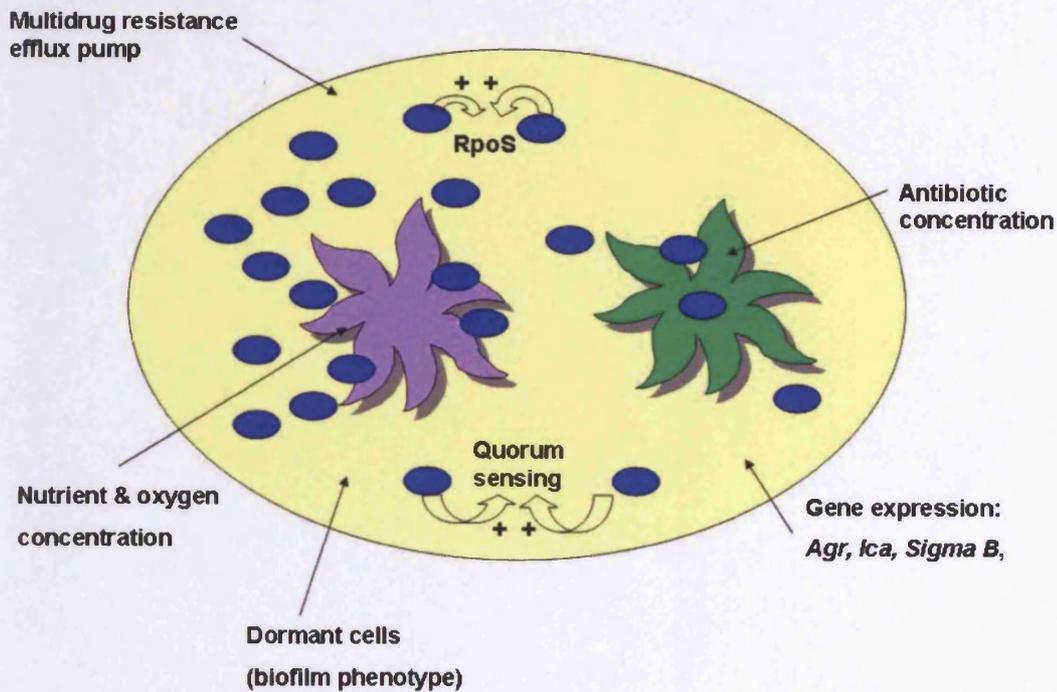
Fig. 1.7 A schematic drawing of mechanisms that can contribute to the resistance of biofilm-grown bacteria to antimicrobial agents.
The EPS is represented in yellow and the bacteria as blue ovals. In general, biofilms are characterised by their heterogeneity which includes nutrients, waste products and oxygen (illustrated in violet and green areas). Resistance include increased cell density and physical exclusion of the antimicrobial agent. The microorganism in the biofilm can undergo physiological changes to improve resistance (biofilm phenotype, induction of stress response rpoS, increasing expression of multiple drug resistance pumps and activation of the QS system).

Biofilm-grown bacteria can be up to 1000 times more resistant to antibiotics than their planktonic equivalents (Hoyle and Costerton, 1991; Mah and O'Toole, 2001). Whilst biofilm cells themselves employ a variety of mechanisms to resist the action of antimicrobial agents (Mah et al., 2003) studies have also shown that a number of other factors could contribute to the antimicrobial resistance properties of biofilms (Percival and Bowler, 2004). Some details of these mechanisms are discussed below.

1.4.2.1 Reduced penetration of an antibiotic into the biofilm matrix

Enhanced antimicrobial resistance by biofilms could relate to a reduced ability of the antimicrobial to gain access to the microorganisms located within the matrix of the biofilm. A reduced penetration of the agent through the matrix could arise through chemical interaction with extracellular biofilm components or adsorption to anionic polysaccharides. It has been shown that β-lactamase producing organisms increase enzyme production in response to antibiotic exposure (Bagge et al., 2004a). β-lactamase also accumulates in the biofilm matrix due to secretion or cell lysis, deactivating β-lactam antibiotics in the surface layers more rapidly than the rate they diffuse into the biofilm (Anderl et al., 2003). A recent study identified that biofilm penetration of positively charged aminoglycosides is retarded through binding to negatively charged matrices, such as alginate produced by P. aeruginosa biofilms (Walters III et al., 2003). It has been suggested that this retardation could provide more time for bacteria to conduct adaptive stress responses (Fux et al., 2005). Another study showed that extracellular slime derived from coagulase-negative staphylococci reduced the effect of glycopeptide antibiotics, even in planktonic cultures (Fux et al., 2005; König et al., 2001). In addition, Mah et al (2003) showed that bacteria within a biofilm may actively employ distinct mechanisms such as sequestering antibiotics, such as tobramycin, in the periplasm to prevent them from reaching their target sites. Whilst this may occur, it is known that the biofilm matrix does not form a completely impenetrable barrier to antimicrobial agents (Percival and Bowler, 2004).

1.4.2.2 Growth rate of bacteria in biofilms

Antibiotics are generally more effective in killing actively reproducing cells. Hence, reduced activity of microorganisms within a biofilm could render these cells less susceptible to antimicrobial agents. It has been known for sometime that non-dividing bacteria escape the killing effects of antibiotics targeted against growth-specific factors (Davies, 2003). Antibiotics, such as ampicillin and penicillin, which target cell-wall synthesis, are not able to kill non-dividing cells (Costerton et al., 1999; Stewart and Costerton, 2001). Whilst cephalosporins and fluoroquinolones, are cidal to non-growing cells, they do have greater activity against rapidly growing bacteria. Therefore, the presence of bacteria that are growing slowly in a biofilm state may contribute to their reduced susceptibility to antibiotics (Costerton et al., 1999). The likely location of slow-growing bacteria in a biofilm is in the lower region of the biofilm (Davies, 2003). Cells deep within the biofilm are more likely to be metabolically inactive, because of reduced access to essential nutrients and gaseous exchange. Embedded biofilm cells are at the stage of dormancy and these cells are phenotypically equipped to persist even in the most hostile environments (Anwar et al., 1992b; Rhoads et al., 2007). These 'persister cells' are defined as a small and slow-growing subpopulation of the biofilm which have differentiated into an inactive, but highly protected state (Roberts and Stewart, 2005). It has been estimated that these subpopulations represent 0.1-10% of all cells in the biofilm and it has been hypothesised that they can survive an enormous antimicrobial challenge, subsequently allowing this subpopulation to reseed the biofilm once use of antibiotics is ceased (Harrison et al., 2005; Roberts and Stewart, 2005).

1.4.2.3 Induction of a biofilm phenotype to resist antimicrobials

Once microorganisms attach a surface they may express a general and more virulent biofilm phenotype compared to planktonically grown identical cells (Mah and O'Toole, 2001; Saye, 2007). Gilbert *et al* (1997) suggested that a biofilm-specific phenotype may be induced in a subpopulation of the biofilm. These subpopulations have been shown to express active mechanisms to reduce the efficacy of antibiotics, such as the expression of bacterial

periplasmic glucans to bind to and physically sequester antibiotics (Gilbert et al., 1997; Maira-Litran et al., 2000; Percival and Bowler, 2004). Once microorganisms attach a surface some genes are turned on whilst others are turned off (Stewart and Costerton, 2001).

It has been documented that altered gene expression by organisms within a biofilm or a general stress response of a biofilm could reduce susceptibility to antimicrobial agents (Brown and Barker, 1999). In theory, the bacterial cell has a small number of target sites for antibiotics. It could therefore be that biofilm cells use specific genes to phenotypically alter these target sites to protect themselves (Saye, 2007). Exact details about the physiological changes that occur during the transition from a planktonic to a biofilm state is not known. It has been suggested that multidrug resistance efflux pumps (MDR) may substantially contribute to the resistance of biofilms (De Kievit et al., 2001b; Mah and O'Toole, 2001). However, studies have shown that expression of well characterised multidrug efflux (MDR) pumps does not increase in *P. aeruginosa* biofilms (De Kievit et al., 2001b).

1.5 GENE EXPRESSION ASSOCIATED WITH BIOFILM DEVELOPMENT

For the initiation of biofilm formation, microorganisms must be able to attach to and move on surfaces, sense their cell density and generate a 3-dimensional structure of cells encased in exopolysaccharides (O'Toole et al., 2000a). These processes are all thought to be controlled via differential gene expression (Table 1.6), which differs markedly from that of planktonic microorganisms (Beloin and Ghigo, 2005). A pioneering study showed that compared to planktonic cultures maintained in steady state, *P. aeruginosa* biofilms (6 day old biofilm) differed in gene expression by 1% (Stanley et al., 2003; Whiteley et al., 2001).

Table 1.6 Genes involved in biofilm formation (Adapted from Beloin and Ghigo 2005)

Regulatory genes	Function	Bacterial species
Gram positive bacteria		
icaADBC	Mediates cell-cell adhesion	*S. aureus*
Accessory gene regulator (*agr*)	Regulates virulence factors /	*S. aureus*
	Involved in detachment process	*S. epidermidis*
Staphylococcal accessory gene regulator (*sarA*)	Production of surface associated & secreted virulence factors	*S. aureus*
SigmaB	Activates biofilm formation	*S. epidermidis*
Gram negative bacteria		
rpoS	Reduces/increases depth of biofilm	*E. coli*
rpoS	Reduces depth of the biofilm	*P. aeruginosa*
Crc	Activation of type IV motility	*P. aeruginosa*
mvaT	Reduces adhesion via *cup* gene repression	*P. aeruginosa*
rpoN	Role in initial adhesion & biofilm formation	*P. aeruginosa*

1.5.1 Gene expression in *S. aureus* biofilms

Staphylococcus aureus is a significant bacterial pathogen of humans responsible for a wide variety of infections and a major source of patient morbidity (Beenken et al., 2004; Yazdani et al., 2006). Biofilm formation by staphylococci develops in two stages: primary attachment followed by cell-cell adhesion and accumulation (Gotz, 2002). The second phase of biofilm formation requires the polysaccharide intercellular adhesin (PIA) (Vuong et al., 2003).

Staphylococcus aureus adheres to surfaces through the production of a capsular polysaccharide/adhesin (PS/A). A multilayer biofilm is formed following *S. aureus* multiplication, which also results in the production of a PIA (Beenken et al., 2004; Rohde et al., 2005; Yazdani et al., 2006). PIA is a homoglycan consisting of β-1,6-linked polymeric N-acetylglucosamine residues (Beenken et al., 2004; Mack et al., 1996). Control of PIA production is dependent upon the genes within the *icaADBC* operon (Heilmann et al., 1996; Yazdani et al., 2006) which is also known as a key virulence determinant for *S. epidermidis* (Arciola et al., 2001; Conlon et al., 2002a; Conlon et al., 2002b).

Whilst there are strain dependent differences (Beenken et al., 2004; Cramton et al., 1999; Rohde et al., 2001), most *S. aureus* strains appear to contain the entire *ica* operon (Cramton et al., 1999; Fowler et al., 2001; Rohde et al., 2001). Vandecasteele *et al.* (2003) showed that *ica* expression was associated with the initial colonisation of *S. epidermidis* in an infection model but not with long term persistence. Frebourg *et al.* (2000) have indicated the possible value of targeting the *ica* locus as a diagnostic marker to distinguish between invasive and contaminating isolates of *S. epidermidis*. Regulation of *ica* expression and the ability to form a biofim also involves other regulatory elements. Among these additional regulatory loci are the accessory gene regulator (*agr*, section 1.3.4.2) and the staphylococcal regulator (*sarA*) (Beenken et al., 2004; Patti et al., 1994). It has been suggested that *sarA* modulates biofilm production. Studies have confirmed that *sarA* mutants

have a reduced capacity to form a biofilm. Hence, *sarA* would presumably be required for production of an activator of biofilm formation or a required effector molecule (Beenken et al., 2004; Patti et al., 1994).

In *S. aureus* proteolysis also plays a pivotal role in its pathogenicity (Rohde et al., 2005). The expression of staphylococcal proteases is under environmental control of quorum sensing circuits like *agr* and *sar* (Lindsay and Foster, 1999; Rohde et al., 2005), potentially linking *Aap* (accumulation associated protein) processing with these global regulators of staphylococcal virulence. The differential expression of protease activity contributes to the adaptation of the pathogen to a changing environment by altering the adhesive properties to extracellular matrix proteins (Karlsson et al., 2001; McAleese et al., 2001; McGavin et al., 1997; Rohde et al., 2005; Savolainen et al., 2001).

Staphylococcus aureus adherence is also promoted by cell wall adhesins (Fluckiger et al., 2005). More specifically, a group of surface-exposed proteins collectively referred to as MSCRAMMs (microbial surface components recognising adhesive matrix molecules) interact with a number of host proteins, such as fibronectin, fibrinogen, collagen, vitronectin and laminin (Beenken et al., 2004; Harris et al., 2002; Patti et al., 1994).

1.5.2 Gene expression in *P. aeruginosa* biofilms

Pseudomonas aeruginosa is a commensal of humans and a recognised opportunistic pathogen with great adaptability and metabolic versatility in individuals whose defence systems are impaired (Bagge et al., 2004b; Wilson, 2001). This organism is particularly associated with morbidity and mortality in patients with cystic fibrosis (CF). Alginate expression by *P. aeruginosa* is considered as an important virulence factor of this bacterium, as it is thought to have a protective function against an environment that is high in oxidative stress and against attack by immune system components (Krieg et al., 1988; Simpson et al., 1988; Simpson et al., 1989). Possible roles of alginate could therefore be to serve as a barrier to phagocytosis and

as a substance that neutralizes oxygen radicals (Edwards and Saunders, 2001). Alginate is a viscous exopolysaccharide, consisting of D-mannuronic and L-guluronic acids (Hershberger et al., 1995). Studies have shown that *P. aeruginosa* strains isolated from the lungs of CF patients, have a mucoid phenotype and exhibit production of the exopolysaccharide alginate (Edwards and Saunders, 2001).

Pseudomonas aeruginosa can also express genes coding for extracellular appendages, such as flagella and type IV pili, which play a role in biofilm formation (section 1.2.1.1), in bacterial conjugation for plasmid transfer and other pathogenic adaptations (Finelli et al., 2003; Goodman and Lory, 2004; O'Toole and Kolter, 1998a; Vallet et al., 2004). Flagellar motility appears to be required for approaching surfaces and counteracting repulsive forces. Twitching motility is mediated by type IV pili and is required for subsequent spreading over a surface (Vallet et al., 2004). It has been shown that type IV pili play a role in microcolony formation (O'Toole and Kolter, 1998a) and in a recent study 3 genes from different chromosomal regions involved in type IV pilus production (*pilB, pilY2* and *pilP*) were up-regulated in 8h developing biofilms (Waite et al., 2005). Using infected animal models such, appendages are directly implicated in virulence and serve as pattern recognition molecules for host defences (Mah et al., 2003; Van Bambeke et al., 2003).

Biofilm formation is controlled by a number of different regulators. Vallet *et al.* (2004) identified gene clusters, namely the *cup* (chaperone-usher pathways), consisting of *cupA, cupB* and *cupC*, which may be involved in the assembly of novel *P. aeruginosa* fimbrial structures (Croft et al., 2000; Vallet et al., 2001). The *cupA* gene cluster is required for adhesion to inert surfaces and likely to be important in the initiation of biofilm formation. The two additional gene clusters *cupB* and *cupC* have no obvious influence on surface attachment (Vallet et al., 2004; Vallet et al., 2001). Another regulatory factor, Crc (catabolite repression control) protein which plays a role in the regulation of carbon metabolism is necessary for biofilm formation

(O'Toole et al., 2000b). It has been suggested that Crc controls the transcription of genes required for type IV pilus biogenesis. Therefore, Crc may be part of a signal transduction pathway; it can sense and respond to nutritional signals and may play a role in the organisms' transition from planktonic to biofilm growth. However, additional work is needed to determine the precise mechanism by which Crc is regulated (Finelli et al., 2003; O'Toole et al., 2000b).

Friedman and Kolter (2004) identified the *pel* (pellicle formation) gene cluster and revealed its involvement in the formation of biofilms by demonstrating that *pel* mutants were unable to form a robust and shear force resistant biofilm. It was hypothesised that the *pel* genes are involved in the production of a novel glucose rich polysaccharide component that contributes to the formation of a matrix material that encases the cells and holds them together in a resistant biofilm. Another study by Vasseur *et al.* (2005) showed that the *pel* genes probably influence the initiation of the biofilm formation process and the irreversible attachment on abiotic surfaces.

The stationary sigma factor *rpoS* (Table 1.6) is thought to play a central role in development of starvation-mediated general resistance in *Escherichia coli* (Lange and Hengge-Aronis, 1994; Xu et al., 2001). It is believed that it could also contribute *to P. aeruginosa* biofilm resistance (Fig. 1.7); (Jorgensen et al., 1999; Xu et al., 2001).

It is important to highlight that bacteria are subject to an array of stresses within their natural environment and it has been demonstrated that stationary phase (starving) cells survive better than their exponential phase counterparts (Xu et al., 2001). Bacteria in a biofilm and planktonic state are capable of turning on stress-response genes and switching to more environmental tolerant phenotypes upon thereby resisting environments poor in nutritional quality, high cell density, and extremes in temperature, pH or osmolarity (Novick, 2003).

It is thought that the control of key biofilm gene patterns present promising targets to characterise the biofilm mode of growth and offer excellent opportunities to overcome antibiotic tolerance in biofilm-related infections.

To compare gene expression patterns in biofilms with those in planktonic cultures, a multitude of strategies have been used, such as microarray studies and real time PCR (Fux et al., 2005). Such strategies are discussed in more detail in Chapter 6.

1.6 ROLE OF BIOFILMS IN NON ORAL INFECTION

1.6.1 Native Valve Endocarditis (NVE)

Native valve endocarditis is a condition that results from the interaction of bacteria, the vascular endothelium and pulmonic valves of the heart. The causes of NVE are varied, but the condition is frequently associated with streptococci, staphylococci, Gram-negative bacteria and also fungi (Braunwald, 1997). These microorganisms gain access to the blood and the heart via the oropharynx, gastrointestinal and urinary tract. Once the intact endothelium is damaged, microorganisms adhere to it and non-bacterial thrombotic endocarditis (NBTE) develops at the injury. At the point of injury, the thrombus develops, which is an accumulation of platelets, fibrin and red blood cells (Donlan and Costerton, 2002). Treatment is less effective due to a combination of mass transfer limitations and inherent resistance of biofilm organisms (Donlan and Costerton, 2002).

1.6.2 Otitis Media

Otitis media (OM) is an infection or inflammation of the middle ear. This inflammation often begins when infections that cause sore throats, colds, or other respiratory or breathing problems spread to the middle ear. OM infections can be of viral or bacterial origin and involve the inflammation of the mucoperiosteal lining. The bacteria that cause OM include *Streptococcus pneumoniae, Haemophilus influenza, Moraxella catarrhalis*, group A β-haemolytic streptococci, enteric bacteria, *S. aureus, S. epidermidis*

and *P. aeruginosa* (Ferguson et al., 1986). Although most effusions from acute OM are culture-positive for bacteria, the majority of chronic effusions are culture-negative and resistant to antibiotic treatment (Ehrlich et al., 2002). Tympanostomy tubes are implanted to alleviate pressure build-up and hearing loss. Unfortunately, these too are subject to contamination and biofilms build up (Donlan and Costerton, 2002).

1.6.3 Cystic Fibrosis

Cystic Fibrosis (CF) is a relatively common disease caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR), a phosphorylation-regulated Cl⁻ channel in the apical membrane of involved epithelia (Hilman, 1997; Mathee et al., 1999; Sferra and Collins, 1993). In CF patients, the constant flow of mucus which removes debris and bacteria is excessively thick and sticky. It is an ideal environment for bacterial growth, resulting in frequent bacterial chest infection and pneumonia (BUPA, 2006).

The bacterium most frequently responsible for the morbidity and mortality seen in CF patients, is *P. aeruginosa,* although other bacterial species have also been implicated *e.g. Burkholderia cepacia* (Bagge et al., 2004b; Hershberger et al., 1995; Mathee et al., 1999; Stapper et al., 2004). In CF, bacteria colonise and infect the chronic airway, which progressively destroys the lung and often leads to respiratory failure (Mathee et al., 1999). The colonisation of the CF airways occurs early in the lifetime of the individual (Hoiby, 1974). The environment in the CF lungs causes *P. aeruginosa* to switch to a mucoidy phenotype (Doggett, 1969; Hentzer et al., 2001; Iacocca et al., 1963). *Pseudomonas aeruginosa* adapts to the inflammatory defence system of CF patients and occupies specific niches, forming mucoid biofilms or microcolonies that reflect particular conditions.

The mucoid phenotype of *P. aeruginosa* is caused by an overproduction of alginate, which is an exopolysaccharide consisting of mannuronic acid and guluronic acid monomers (Hentzer et al., 2001). Alginate has a protective

function and the switch to mucoidy is also thought to promote persistence of *P. aeruginosa* (Hentzer et al., 2001; Krieg et al., 1988; Simpson et al., 1988; Simpson et al., 1989). Evidence to support the case that CF is a biofilm disease include its innate resistance to antibiotics, demonstration of aggregates of bacteria embedded in EPS and, also that CF sputum contains the two principal *P. aeruginosa* quorum sensing signal molecules, 3OC12-HSL and C4-HSL (Fergie et al., 2004; Singh et al., 2000). It has been shown that *P. aeruginosa* in CF patients also carry mutations in the *mucA* gene which directs bacteria to produce alginate, which is an oxygen scavenger. The phenotype change protects *P. aeruginosa* against phagocytosis and other inflammatory host defence mechanisms (Stapper et al., 2004).

1.7 INDWELLING MEDICAL DEVICE RELATED INFECTIONS

Microbial biofilms often develop on, or within indwelling medical devices *e.g.* contact lenses, central venous catheters, mechanical heart valves, pacemakers, peritoneal dialysis catheters, prosthetic joints, urinary catheters and voice prostheses (Table 1.7) (Donlan, 2001). There are a number of key organisms that cause biofilms on these surfaces. *Staphylococcus* spp. (*S. aureus*, *S. epidermidis*) which are frequent members of the commensal microflora of the skin, can form biofilms on implantable medical devices, such as intravenous catheters, and hip and knee joint prostheses (Bayston, 1999; Habash and Reid, 1999; Khardori and Yassien, 1995). *Staphylococcus epidermidis*, *S. aureus*, *Streptococcus* spp., *Escherichia coli*, *Proteus mirabilis*, *Enterococcus faecalis*, *P. aeruginosa*, *K. pneumoniae,* other Gram-negative bacteria and *Candida* spp. are the primary organisms responsible for central venous catheter, mechanical heart valve or urinary catheter biofilms. These organisms may originate from a patient's normal skin microflora, from the environment, from an invasive procedure such as dental work or other indwelling devices.

Microorganisms usually gain access to the catheter or medical device by migration externally from the skin along the exterior catheter surface or internally from the catheter hub or port (Maki, 1994).

Table 1.7 Biofilms on medical devices.

Category	Biofilm on medical devices	Reference
Category 1	Prosthetic heart valves	(Donlan and Costerton, 2002)
	Endotracheal tubes	(Ramage et al., 2006)
	Prosthetic joints	(Ramage et al., 2006)
	Dentures prostheses	(Ramage et al., 2006)
	Neurosurgical shunts	(Ramage et al., 2006)
Category 2	Central venous catheters	(Donlan and Costerton, 2002; Ramage et al., 2006)
Category 3	Urinary catheters	(Donlan and Costerton, 2002; Moore et al., 2002)
	Contact lenses	(Donlan and Costerton, 2002; Stickler, 2005; Stickler et al., 1998)
	Voice box prostheses	(Ramage et al., 2006)

According to their degree of implantation 3 categories of prosthetic devices are established. Category 1: totally implanted; Category 2: partially implanted and Category 3: not implanted.

Implantation of mechanical heart valves causes tissue damage, and circulating platelets and fibrin tend to accumulate where the valve has been attached. Once the tissue is damaged, it facilitates a greater tendency for microorganisms to colonise these locations (Anwar et al., 1992a). The resulting biofilms develop on the heart tissue (Carrel et al., 1998; Illingworth et al., 1999) surrounding the prosthesis or the sewing cuff fabric used to attach the device to the tissue (Donlan, 2001; Karchmer and Gibbons, 1994).

1.7.1 Urinary Catheters

Urinary catheters are used to remove and monitor urine production from impaired patients. Urinary catheters also facilitate repair of the urethra after surgical procedures and are used to manage urinary retention and incontinence in the elderly and disabled patients (Moore and Lindsay, 2001; Stickler, 2005). Biofilms associated with urinary catheters are particularly important because they cause infections in 10-50% of patients who undergo catheterisation (Mulhall et al., 1988; Stickler, 2002). *Proteus mirabilis, Morganella morganii, P. aeruginosa, K. pneumoniae* and *Proteus vulgaris*, are commonly found in urinary catheter biofilms. A number of these bacteria (*e.g. P. mirabilis*) express urease, an enzyme which hydrolyses urea found in the urine, resulting in the production of ammonia. Ammonia causes an increase in the pH of the urine, which in turn allows mineral precipitation including that of calcium and magnesium phosphates, leading to blockage of the catheter and infection (Stickler et al., 1999; Tunney et al., 1999). The problem is compounded because these organisms tend to be antibiotic resistant and as a biofilm forms within the catheter, they are less susceptible to host defences and antimicrobial treatments (Trautner and Darouiche, 2004).

Unfortunately, urinary catheters also provide a passageway for bacteria from a heavily contaminated external skin site to a vulnerable body cavity. Polymicrobial communities develop as time goes by, but the initial infections are usually by single bacterial species (Stickler, 2005).

1.7.2 Ventilator associated pneumonia

Patients requiring airway management can develop ventilator-associated pneumonia (VAP). VAP involves infection of the lower respiratory tract and can cause morbidity and mortality in intensive care settings (Brennan et al., 2004). Biofilms occur on the endotracheal tubes which are an important factor promoting the occurrence of VAP (Iregui and Kollef, 2002). Microorganisms associated with VAP are Gram negative organisms including *P. aeruginosa*, *Enterobacteriaceae*, *Haemophilus*, and *Acinetobacter* spp. Gram-positive organisms include *S. aureus*, *Streptococcus* spp., *Streptococcus pneumoniae* and methicillin resistant *S. aureus* (MRSA) strains. It has been suggested that VAP associated pathogens have differing levels of virulence, with the highest mortality associated with *P. aeruginosa* and MRSA (Brennan et al., 2004).

1.7.3 Contact lens infections

Bacterial biofilms on contact lenses and storage cases may be a risk factor for corneal infection (Dyavaiah et al., 2007; McLaughlin et al., 1998). In several studies, such biofilms contained multiple species of bacteria and fungi including *P. aeruginosa, S. aureus, S. epidermidis, E. coli, Proteus, Candida* and *Fusarium* spp. (Donlan and Costerton, 2002; Dyavaiah et al., 2007). It has been suggested that improper lens cleaning regimens are contributing factors for corneal infections (Dyavaiah et al., 2007; Ifejika et al., 2000).

1.7.4 Denture-based infections

Candida associated denture stomatitis is a common and multifactorial disease (Ramage et al., 2006), associated with poor denture fitting, denture cleanliness, trauma, oral bacteria, immune defects and the presence of *C. albicans*. Attachment and colonisation of *C. albicans* is supported by the roughness of the surface and by cracks within the acrylic (Ramage et al., 2006).

1.8 ORAL BIOFILMS AND THEIR ROLE IN INFECTION

1.8.1 The oral microflora

The oral microflora is regarded to be the most diverse microbial population in humans and contains at least 350 different cultivable bacterial genera. It has been estimated that many more bacterial groups remain uncultivable from this environment (Bagg et al., 2003). The multiplicity of microbial groups reflects the diversity between these habitats. In the oral cavity, there are unique habitats for bacterial colonisation, including the teeth, mucosal surfaces and gingival crevices.

Normally there exists a harmonious relationship between the normal oral microflora and the host, which can be disturbed by changes affecting the stability of the bacterial population. For example, the use of broad spectrum antibiotics, a carbohydrate rich diet and poor oral hygiene can all influence the composition of the microflora. Changes may lead to mucosal infection and increase the prevalence of both dental caries and periodontal disease (Bagg et al., 2003). The normal oral flora maintains a balance within the oral cavity, helping to prevent oral infections such as candidiosis and periodontal diseases.

1.8.2 Composition of the oral microflora

Microorganisms present in the mouth are commonly referred to as commensal organisms but can be responsible for two of the most frequent forms of human diseases, namely dental caries and periodontal disease.

There is evidence that overall some bacteria (*S. mutans*, *Lactobacillus* and perhaps *Actinomyces* spp.) are more important than others in dental caries, purulent oral infections and infective endocarditis.

1.8.3 Dental plaque

Plaque is a complex, metabolically interconnected, highly organised bacterial ecosystem (Marsh and Martin, 1992). Plaque probably represents the most widely studied naturally occurring biofilm. As a result of bacterial attachment

to the tooth surface and subsequent proliferation of these cells dental plaque is formed. Plaque (Fig. 1.8) is comprised of plaque bacteria (60-70% of the plaque volume), salivary polymers and bacterial extracellular products. Primary plaque formers such as *Streptococcus* spp. are the first to colonise the salivary pellicle that is initially present on the tooth surface. Once a streptococcal layer has formed, secondary plaque formers such as *Actinomyces* spp. colonise the developing biofilm (Kolenbrander et al., 2006). Bacteria within plaque may reach a thickness of 300-500 cells on the surface of teeth and the close proximity to dental and supporting tissues may promote oral disease. It has been estimated that undisturbed plaque is suitable for the growth of anaerobes (Kenney and Ash, 1969). *Fusobacterium nucleatum* bacteria act as coaggregation bridges between non-coaggregating aerobes and anaerobes (Kolenbrander et al., 2006). Plaque is most prominent on the back molars, just above the gingival line on all teeth and at the edges of restorations.

There is a high species diversity in plaque that can be explained by the spatial heterogeneity within the biofilm allowing bacteria to co-exist at the same site where they would not normally be able to. Plaque formation is initiated by the weak attachment of the streptococcal cells to salivary glycoproteins, forming a pellicle on the surface of the teeth. This is followed by a stronger attachment by means of 'sticky' extracellular polymers of glucose (glucans) which are synthesised by bacteria from dietary sugars (principally sucrose). Glycosyl transferase is an enzyme on the surface of *S. mutans* which converts sucrose to dextran polymers (glucans) and promotes the attachment of bacterial cells to the tooth surface. As the complexity of the plaque increases, the niches available for colonisation decrease and the community becomes relatively stable, reaching a steady state. This stability is thought to be important in maintaining the balance between periodontal health and disease (Socransky, 1970).
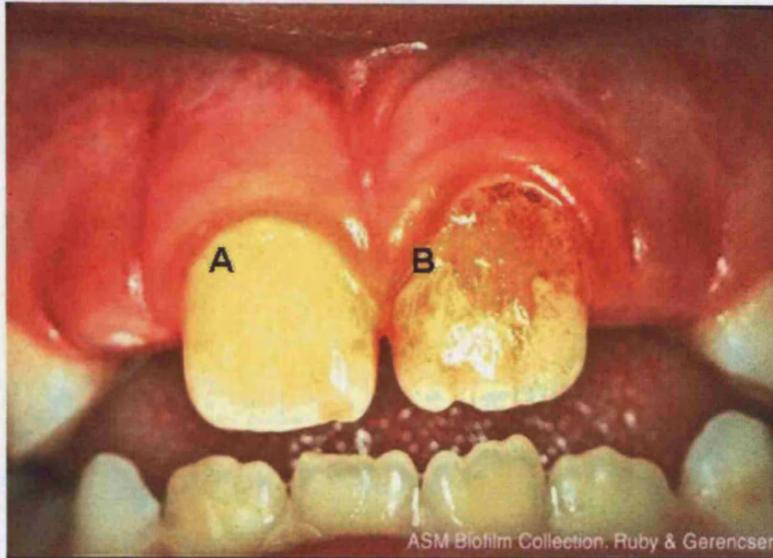
Fig. 1.8 An oral plaque biofilm on teeth A) unstained and stained with iodine B). Adapted from the Centre for Biofilm Engineering, Montana University, Bozeman; www.erc.montana.edu.

1.8.4 Dental plaque and caries

Microorganisms are a prerequisite for the development of dental caries (Samaranayake, 2006a). Dental caries is defined as the localized dissolution of the enamel or root surface by acid derived from the microbial degradation of dietary carbohydrates. It is a multifactorial plaque related chronic infection of the enamel, cementum or dentine. The initial caries lesion is characterised by a "white spot", and represents enamel damage by lactic acid and other organic acids which accumulate in dental plaque. Acidogenic bacteria in the plaque produce lactic acid from the fermentation of sugars and other carbohydrates in the diet of the host. *Streptococcus mutans* is primarily associated with the initiation of dental caries, but other lactic acid bacteria, such as *Lactobacillus* spp. are also implicated (Todar, 2006).

1.8.5 Dental plaque and periodontal disease

Periodontal disease can be defined as the bacterial mediated destruction of supporting structures of the teeth including the gingivae, periodontal ligament and the alveolar bone, and can be classified into gingivitis and periodontitis (Samaranayake, 2006b). Periodontal disease is an inflammatory condition of the periodontum and develops from a pre-existing gingivitis. However, not every case of gingivitis develops into periodontitis. It causes bleeding in the gingiva, mobility of the teeth and ultimately leads to teeth loss (You et al., 2004).

Microbiological evidence suggests that there is not one single pathogen associated with the destructive periodontal disease, moreover groups of bacteria may be associated and acting synergistically (Socransky et al., 1998). To elucidate the pathogenesis of periodontal disease, an understanding of ecological factors such as microbial diversity, microbial succession, microbial interactions and conditions for maintaining a balance between health and disease is required. However, the complex nature of these ecological factors prevents straightforward analysis and thus progress is slow. The bacteria that have been associated with periodontal destruction are *Porphyromonas gingivalis, Bacteroides forsythus, Prevotella intermedia,*

Peptostreptococcus (Micromonas) micros, Eubacterium nodatum, Capnocytophaga spp., *Eikenella corrodens*, and *Fusobacterium nucleatum* (Socransky et al., 1998).

1.8.6 Oral candidosis

The yeast *Candida albicans* is a common, human commensal organism that inhabits the oral cavity, gastrointestinal tract, female genital tract and the skin (Jayatilake et al., 2005; Samaranayake, 2006a). *Candida albicans* is an important cause of superficial infection of mucosal membranes and systemic disease in humans (Sherman et al., 2002). Four clinical forms of primary oral candidosis are traditionally recognised (Fig. 1.9); pseudomembranous candidosis, acute erythematous candidosis, chronic erythematous candidosis and chronic hyperplastic candidosis (CHC). Other oral infections associated with *Candida* include angular cheilitis and median rhomboid glossits

Candida albicans possesses a number of virulence attributes, including the ability to adhere to host tissues, to produce extracellular proteinases, phospholipases and lipases and to reversibly switch between the two morphology forms, a unicellular yeast form and a filamentous, growth form, *e.g.* hyphal (Calderone and Fonzi, 2001; Jayatilake et al., 2005; Samaranayake, 2006a).

1.8.6.1 Pseudomembranous candidosis

Pseudomembranous candidosis (Fig. 1.9a) is also commonly referred to by the generic term, 'oral thrush'. This infection classically presents as white patches on the surface of the oral mucosa or the tongue and is characterised by being amenable to removal following gentle scraping. The removed white pseuodmembranes consist of desquamated epithelial cells, fibrin and fungal hyphae (Akpan and Morgan, 2002). This form of infection is commonly found in infants, the elderly and terminally ill patients, particularly with underlying conditions such as leukaemia and HIV (Farah et al., 2000).
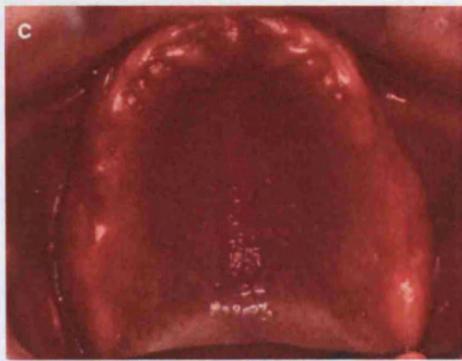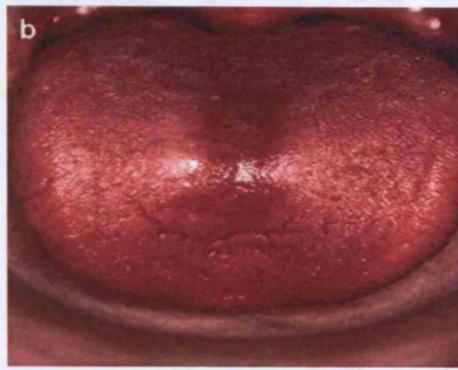
Fig. 1.9 Oral candidosis infections (a) acute/chronic pseudomembranous candidosis (b) acute erythematous candidosis, (c) chronic erythematous candidosis, (d) chronic hyperplastic candidosis. Courtesy of Prof. M. Lewis at the Cardiff Dental School.

1.8.6.2 Acute erythematous candidosis (acute atrophic candidosis)

Acute erythematous candidosis (Fig. 1.9b) is associated with the use of broad spectrum antibiotics or steroid therapy. Clinically it presents as a reddening of the oral mucosa similar to that seen in patients with low serum levels of vitamin B12, low folate and low ferritin (Akpan and Morgan, 2002). It is believed that the antimicrobial therapy alters the normal oral bacterial community and allows *C. albicans* numbers to increase (Farah et al., 2000).

1.8.6.3 Chronic erythematous candidosis (chronic atrophic candidosis)

Chronic erythematous candidosis (Fig. 1.9c) is associated with the wearing of denture prostheses and results in mucosal edema of the palate. In this infection, other factors such as bacterial accumulation reduced salivary protection and mechanical irritation may be implicated. A poor fitting denture and poor oral hygiene is associated with this type of infection (Akpan and Morgan, 2002). The patient usually does not have any symptoms but may complain of slight soreness (Farah et al., 2000). It presents in approximately 50% of all denture wearers (Farah et al., 2000).

1.8.6.4 Chronic hyperplastic candidosis (CHC)

CHC (Fig. 1.9d) presents as white lesions on the oral mucosa. It is distinct from the other forms of oral candidoses as it is characterised by hyphal penetration of the oral epithelium (Sitheeque and Samaranayake, 2003) and the presence of an associated inflammatory cell infiltrate (Williams et al., 1997).

Importantly, CHC is also implicated in the development of squamous cell carcinoma (SCC) at the lesional site (Banoczy, 1977; Krogh et al., 1987; Williams et al., 2001). The host factors implicated in CHC include tobacco smoking, denture wearing, and regular alcohol intake (Arendorf et al., 1983). However, it is also possible that *Candida* express specific virulence attributes that promote this infection. Since hyphal invasion of the epithelium is a distinguishing feature of CHC, expression of certain *C. albicans* virulence factors may be enhanced for CHC strains compared with others.

1.8.6.5 Angular cheilitis

Angular cheilitis is a clinical term used to describe painful erythematous lesions affecting the angles of the mouth. The condition seems to be associated with the wearing of removable dentures (Samaranayake and MacFarlane, 1990a), and is often accompanied by the presence of chronic erythematous candidosis (Farah et al., 2000). Other microorganisms are frequently present in this infection, such as *Staphylococcus aureus* and *Streptococcus* spp. (Akpan and Morgan, 2002).

1.8.6.6 Median rhomboid glossits

Median rhomboid glossits presents on the tongue as a chronic symmetrical shaped area consisting of atrophic filiform papillae. Recovery of *Candida* from this area is high. The exact cause of this infection is not known, although hormonal links, tobacco smoking and the use of inhaled steroids have been suggested (Samaranayake and MacFarlane, 1990b).

1.8.7 Predisposing host factors to oral candidosis

Oral candidosis has frequently been described as a 'disease of the diseased'. Whilst the incidence of normal commensal carriage of *Candida* in the mouth is high, such individuals will not suffer infection unless otherwise debilitated. The transition of *Candida* from a harmless commensal member of the microflora to a disease causing organism is complex, involving a variety of both local and systemic host factors. Several of these factors are discussed below, and presented in Table 1.8.

1.8.7.1 Damage to the oral mucosal

In the mouth the oral mucosa provides a primary host defence mechanism by acting as a mechanical barrier to *Candida*. However, damage to the oral mucosa can occur through denture wearing, which has an association with chronic erythematous candidosis and CHC (Arendorf et al., 1983). It has also been shown that the wearing of dentures produces an anaerobic microenvironment beneficial to the growth of *Candida* due to its associated low oxygen levels and low pH (Ruby and Barbeau, 2002).

Table 1.8 Host related factors associated with the development of oral candidiosis.

Local factors	Systemic factors	Miscellaneous factors
Mucosal damage, denture wearing	Extremes of age	Tobacco smoking
Saliva reduced flow of saliva, acidic pH reduced antibodies	Hormonal changes diabetes mellitus	Nutritional status vitamin deficiency mineral deficiency carbohydrate rich diets
Commensal flora, inhibition of candidal adherence by oral bacterial flora	Immune status immunodeficiency immunosuppression	Antibiotic therapy Corticosteroid therapy Cytotoxic drugs and radiotherapy

Furthermore oral hygiene levels in many denture wearers can be poor, due to inadequate cleaning regimes, particularly for individuals who wear their dentures through the night (Eppstein, 1990; Guida, 1988).

1.8.7.2 Effect of saliva

The constant flushing action of saliva can remove *Candida* cells prior to their attachment to oral surfaces. Furthermore, antimicrobial proteins in saliva such as lactoferrin, sialoperoxidase, lysozyme, histidine-rich polypeptides, and specific anti-*Candida* antibodies, can contribute to the killing and removal of organisms from the mucosa and prevent overgrowth of *Candida*. Therefore, drugs that reduce salivary secretions or radiotherapy of the head and neck can lead to an increased risk of oral candidosis (Akpan and Morgan, 2002). Sjögren's syndrome is one of the most common systemic conditions associated with xerostomia and salivary dysfunction (Turner and Ship, 2007). It has been shown that this condition favours increased oral carriage of *Candida* (Sitheeque and Samaranayake, 2003). Colonisation of the denture acrylic surface by *Candida* is also favoured by the relatively low saliva flow under the surfaces of the denture fittings which limits removal of otherwise poorly attached yeast (Eppstein, 1990; Guida, 1988).

1.8.7.3 Commensal microflora

It is a well-documented problem that patients receiving broad spectrum antibiotics are highly likely to develop oral candidosis (Farah et al., 2000). A reduction in the bacterial component of the oral microflora reduces the competition for nutrients and adherence sites. Generally, the infection acute erythematous candidosis is the form of oral candidosis that follows (section 1.8.6.2).

1.8.7.4 Diabetes mellitus

Diabetes mellitus has been associated with the occurrence of high levels of candidal colonisation and oral candidosis (Manfredi et al., 2002; Sitheeque and Samaranayake, 2003). Several reasons for the high oral candidal carriage in diabetics have been suggested. Firstly, high salivary glucose

levels in diabetics may favour the growth of yeasts (Sitheeque and Samaranayake, 2003). Furthermore, adherence of *Candida* to buccal epithelial cells (BEC) from diabetics has been demonstrated to be greater than to cells originating from non-diabetic controls (Sitheeque and Samaranayake, 2003). This could be due to intrinsic qualitative changes in the cell surface receptors of diabetics that modulate yeast adhesion (Sitheeque and Samaranayake, 2003).

1.8.7.5 Nutritional status

Oral candidosis is also associated with mineral and vitamin deficiencies, and carbohydrate rich diets of infected patients (Sitheeque and Samaranayake, 2003). On the basis of animal models, deficiencies in vitamins A, B1 and B2 are generally implicated with predisposition to oral candidosis (Akpan and Morgan, 2002). Iron deficiency can cause epithelial abnormalities such as hyperkeratonisis and atrophy, through alterations in the kinetics of the rapidly dividing cells of the oral mucosa, which in turn, result from an impairment of iron-dependent enzymes (Sitheeque and Samaranayake, 2003). Iron-deficiency could also influence oral carriage of *Candida* through the impairment of iron-dependent host enzymes, depression of host cell-mediated immunity (CMI) or by reducing antibody production (Sitheeque and Samaranayake, 2003).

1.9 CHRONIC WOUNDS AND THE ROLE OF BACTERIA

Chronic wounds are defined as those wounds that do not heal within an expected time frame. These chronic wounds are a serious health concern with substantial morbidity and are a source of frustration to both physician and patient due to lengthy treatments, treatment failures and long periods of patient compliance.

Chronic wounds include chronic venous leg ulcers (CVLUs; Fig. 1.10), diabetic foot ulcers and pressure sores. CVLUs are non-healing wounds that occur due to a breakdown in the underlying physiology of the leg.

Fig. 1.10 Examples of Chronic Venous Leg Ulcers (CVLU).
Courtesy of the Wound Healing Research Unit Clinic at the University Hospital Wales, Cardiff.

The breakdown is associated with venous, arterial or metabolic factors (Bowler et al., 2001; Howell-Jones et al., 2005; Thomas and Harding, 2002). Treatment costs are almost £1 billion each year to the NHS, which will only increase with the increasingly aged population of the UK. Chronic wounds are characterised by oedema, severe pain, erythema and strong odour and affect more than 1% of the UK population (Thomas and Harding, 2002). These wounds tend to occur in individuals with an increased risk of bacterial invasion as a result of poor local factors (arterial insufficiency, venous hypertension, trauma) or systemic disease such as diabetes mellitus and rheumatoid arthritis (Falanga, 1993).

1.9.1 Course of normal wound healing

In healthy individuals the normal wound healing process involves a complex series of events including cell proliferation, migration, differentiation and remodelling that can be summarised into overlapping phases. However in chronic wounds, factors such as systemic illness, poor nutrition, vascular insufficiency and bacterial interference influence the normal wound healing process (Jones et al., 2004; Rhoads et al., 2007). The time of healing in chronic wounds is extended as shown in Fig. 1.11. The inflammatory phase begins at time of injury and serves to cleanse the wound of bacteria and debris; this phase can take up to 25 days.

The exposed extracellular matrix triggers the coagulation cascade, plasminogen cascade and others. These cascades along with the exposed extracellular matrix and microorganisms that find their way back into the wound; stimulate the innate immune system. Polymorphonuclear cells arrive first with preprogrammed apoptosis. Macrophages follow and finalise the cleanup of pathogens, remove devitalized tissue and clean host agents such as polymorphonuclear cells by phagocytosis and down-regulate acute inflammatory signals (Rhoads et al., 2007; Schultz et al., 2004). In the proliferative phase, a process of deposition between angiogenesis, fibroblasts, fibronectin and collagen takes place and can take up to 25 days to complete.
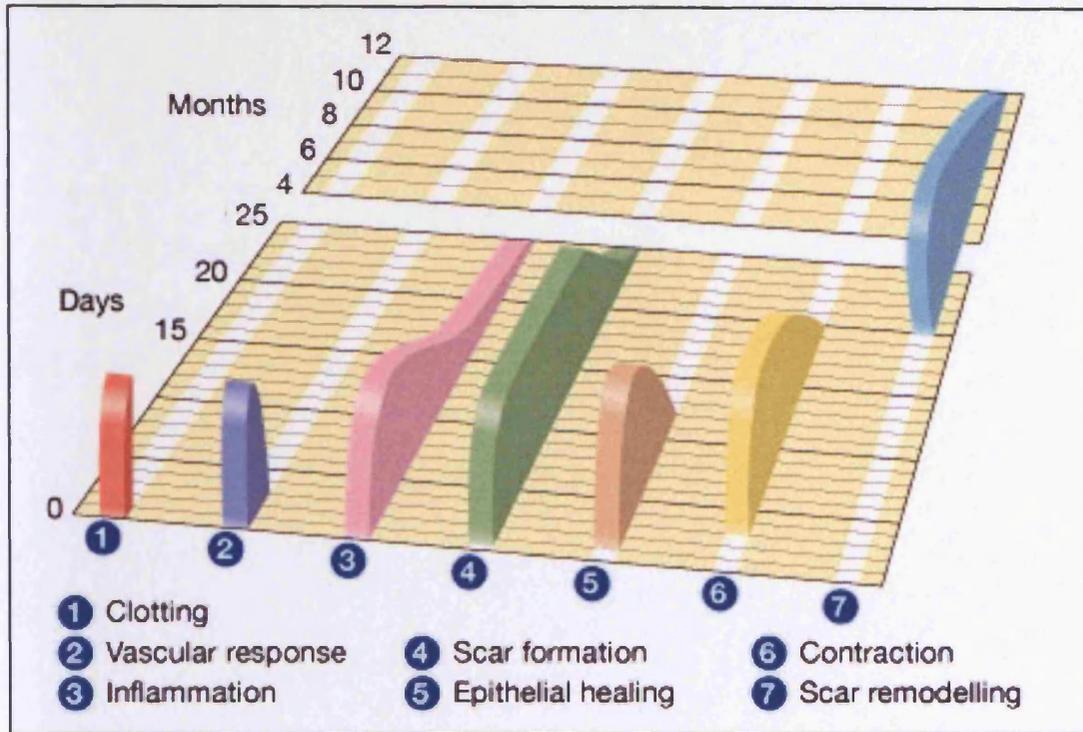
Fig. 1.11 Course of normal wound healing. Adapted from Asmussen and Soellner (1993).

Cytokines are controlling factors for granulation tissue formation. These small molecules direct the endothelial cell neovascularisation of the wound bed and also direct fibroblasts to deposit the new extracellular matrix materials. The remodelling phase which succeeds the proliferation phase, can take up to 12 months (Rhoads et al., 2007).

1.9.2 The significance of bacteria in chronic wounds

Open skin wounds are often colonised with microorganisms, having lost many of the protective defence mechanisms of intact skin (Edwards and Harding, 2004). Chronic wounds harbour a diverse, often dense and complex polymicrobial microflora of potentially pathogenic organisms (both aerobic and anaerobic species). The wound environment provides support for bacterial survival as it is both moist and warm plus there is a good nutrient supply from exuded wound fluid. As a result wounds are susceptible to microbial contamination from both exogenous and endogenous sources including the nose, skin, mouth and the gut (Percival and Bowler, 2004).

There is evidence that microorganisms in chronic wound biofilms contribute to the non-healing phenotype (Hill et al., 2003). Cultural analyses have documented that pseudomonads, staphylococci (present in >70% of wounds) and anaerobic species such as *Peptostreptococcus* spp. are the bacterial groups most frequently recovered from chronic wounds (Hansson et al., 1995; Hill et al., 2003). Polymicrobial interactions may play a crucial role in delayed wound healing. Moreover, less invasive microorganisms may act synergistically with more virulent ones (Bowler, 2003). However, although significant in inhibiting the wound healing process, the importance of individual species or microbial density in relation to healing is unclear. Nevertheless, bacteria play an important role in wounds and possess indirect and direct mechanisms to impair wound healing (Stephens et al., 2003).

It has been shown that all wounds are colonised by bacteria, although not all wounds are infected (Bowler, 2003). One of the most difficult clinical judgements facing the clinician is determining whether a wound is purely

colonised by bacteria or whether there are signs of infection. However, often the lines between colonisation and the beginnings of infection are blurred and correct diagnosis is wholly dependent upon the experience and skill of the clinician.

To reduce the bioburden (the bacterial load in chronic wounds) and improve the wound healing process, the use of antimicrobial therapies are necessary. It is important to note that many patients with chronic wounds are often elderly and hence may also have underlying conditions which make them immunocompromised. Howell-Jones *et al.* (2006; 2005) showed that over-prescribing of antibiotics seems typical in this particular group of patients. Therefore, an understanding of the physiology and interactions within multi-species biofilms may aid the development of more effective methods of treating infected and poorly healing wounds (Edwards and Harding, 2004).

1.9.3 Biofilms and wounds

There is now evidence to show that bacteria are able to form biofilms within the wound environment (James et al., 2007; Mertz, 2003; Percival and Bowler, 2004). It has recently been suggested that both acute partial thickness wounds (Serralta et al., 2001) and chronic human wounds (Bello et al., 2001) may harbour bacterial biofilms growing on the wound surface. In acute wounds, a study involving a pig model inoculated with a laboratory strain of *P. aeruginosa* showed that two distinct bacterial populations were present in the wound (Serralta et al., 2001). The first population was loosely associated with the wound and was readily washed off. In contrast, the second population was more firmly anchored and could only be removed by significant scrubbing with detergent. The authors suggest that the loosely associated wound population were planktonically growing cells, whereas those that could only be removed by mechanical and chemical means were biofilm associated.

A second study showed that *in vitro* biofilms could easily be grown using wound isolates. For example, *P. aeruginosa* from a human burn wound

formed mature biofilms within 10h as visualised by light microscopy (Harrison-Balestra et al., 2003). Scanning electron micrograph (SEM) images of *S. aureus* biofilms in acute wounds have also been produced (Welsh et al., 2005). In contrast, fewer studies have been done with chronic wounds. EPS has been visualised by epifluoresence and light microscopy on chronic wound smears (Bello et al., 2001; Ricotti et al., 2003) and a biofilm has been demonstrated on a dermal model (Mertz, 2003). Moreover, Akiyama *et al.* (2002) demonstrated that *S. aureus* can assemble a glycocalyx on the surface of traumatic wounds in mice and that the biofilm could be visualised using confocal laser scanning microscopy.

From the above studies it is clear that bacteria can persist within a wound and establish a biofilm community which can overcome the host's natural defences and antimicrobial therapy. Whilst the impaired immune system tries to prepare the wound for proliferation of healing, the body cannot successfully complete the inflammatory phase. Therefore, the wound remains in the inflammatory phase, unless successful therapeutic strategies can be applied to eliminate the infection (Rhoads et al., 2007; Schultz et al., 2004).

1.10 MANAGEMENT OF BIOFILM INFECTIONS

Viewing bacteria from the perspective of multicellular behaviour is altering our view of microbiology and of Koch's postulates. It is evident that 99.9% of organisms prefer attachment, and that bacterial cells have the ability to aggregate into particular three-dimensional assemblages (Davey and O'Toole, 2000). Biofilms have been recognised as being important in human disease and the number of biofilm-associated diseases seems to be increasing (Davies, 2003). It is important to understand the characteristics of the biofilm mode of growth and the various aspects of biofilm formation. To successfully treat biofilm infections, knowledge of the phenotype of the bacterial population is required. This is important as antibiotic treatments may not be totally effective if more than one phenotype exists. Some cells might, remain intact which are able to re-colonise a wound and debilitate the

host once the antimicrobial treatment has finished (Brooun et al., 2000; Davies, 2003; Spoering and Lewis, 2001).

A key factor to combating biofilm infections is to understand the physiology of biofilm development. Davies (2003) suggested that chemotherapeutic agents could be developed to promote or prevent transition from one stage of biofilm maturation to the next by targeting unique biofilm regulatory or signalling molecules. Specific agents might be discovered or developed which will interfere with the production of virulence factors, or promote or inhibit the shedding of biofilm bacteria (Davies, 2003).

As mentioned before (section 1.4.2), biofilm resistance depends on aggregation of bacteria into multicellular communities. Therefore, one antimicrobial strategy might be to develop therapies to disrupt the multicellular structure of the biofilm. It could be that host defences might be able to resolve the infection once the multicellularity of the biofilm is reduced, and then the effectiveness of antibiotics might be restored (Stewart and Costerton, 2001). Other potential therapies include enzymes that dissolve the matrix polymers of the biofilm, chemical reactions that block biofilm matrix synthesis, and analogues of microbial signalling molecules that interfere with cell-to-cell communication, required for normal biofilm formation (Nemoto et al., 2000; Parsek and Greenberg, 2000; Yasuda et al., 1993). Already, a number of QS inhibitors have been identified such as the inhibitory peptide RNAIII, which inhibits the *agr* system of Gram-positive bacteria (Rhoads et al., 2007). In *P. aeruginosa*, furanones derived from plants have been demonstrated to block AHL pathways (Heurlier et al., 2006).

For *in vivo* indwelling device-associated infections, effective, preventive and therapeutic strategies still need to be developed. One such therapy could be the production of materials with anti-adhesive surfaces, for example heparin (Tenke et al., 2004). Tenke *et al.* (2004) showed that on heparin-coated catheter stents, no biofilm formation was evident between 6-8 weeks, whereas uncoated tubes got obstructed within 2-3 weeks. Heparin coating

seems one possible solution, but further development of materials resisting bacterial colonisation is needed (Tenke et al., 2004).

Progress has already been made, but the future of biofilm research and management relies upon collaborative efforts to fully explore these complex systems of the microbial world.

1.11 RESEARCH INTO BIOFILMS

Biofims have a great importance for public health because of their significant role in certain infectious diseases and in medical device-related infections. A greater understanding of biofilm processes will hopefully lead to novel and effective strategies for biofilm control resulting in improvement in patient management (Donlan, 2002). To enable easier study of bacterial attachment and colonisation, a variety of direct and indirect experimental observation methods have been developed. Direct methods mainly consist of microscopy techniques, which permit visualisation of the biofilms, while indirect methods estimate the numbers of adherent microbes *in situ* (Djordjevic et al., 2002; Jin et al., 2003). Standard plate counts, roll techniques and sonication are indirect methods that first detach the microorganisms from the surface prior to enumeration. Other indirect methods (radio-labelled bacteria, enzyme-linked immunosorbent assays (ELISAs), biologic assays, stained bacterial films and microtitre plate procedures) estimate the number of attached organisms *in situ* by measuring some attribute of the attached organism (An and Friedman, 2000; Djordjevic et al., 2002).

1.11.1 Microtitre plate assays

A microtitre plate assay is typically an indirect method for the estimation of bacteria *in vitro* and can be modified for various biofilm formation procedures (An and Friedman, 2000). Typically, this semi-quantitative method for measuring biofilm formation involves growing bacterial cultures in individual wells of a 96-well polystyrene plate before fixing and staining with 1% crystal violet solution. It is a simple and sensitive assay, which permits the simultaneous quantification of an organism. It is also widely used for

susceptibility testing of biofilms (Casey et al., 2004; McBride et al., 2005).

1.11.2 Colorimetric methods using XTT

In the XTT assay, a microtitre plate assay is often used and the biofilm quantified by reduction of the tetrazolium salt 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT). XTT is reduced to the XTT formazan product by the mitochondrial dehydrogenases of metabolically active cells. The colorimetric change produced is proportional to the number of living cells which therefore can be indirectly quantified (Hawser and Douglas, 1994; Jin et al., 2003; Kuhn et al., 2002a).

1.11.3 Flow cells

Flow cells are small continuous-flow systems with a viewing port that allows direct observation of the biofilm using microscopy and image analysis without disrupting the community (Davey and O'Toole, 2000). Biofilms are usually grown on microscope glass coverslips in three or more channel chambers. The inoculum is added to each channel and the cells are allowed to attach to the substratum for a specific time before a fluid is injected covering the surface (Hancock and Klemm, 2006). Fresh medium enters the system, passes through the cell and is collected as waste. The medium is not recycled through the flow cell. Slides can be removed without stopping the flow, thus, diminishing the possibility of contamination and disturbance of the biofilm (Battin et al., 2003; Caiazza and O'Toole, 2003; Davey and O'Toole, 2000; Davies et al., 1998; Pereira et al., 2000; Sauer et al., 2002).

1.11.4 Constant Depth Film Fermenter (CDFF)

In recent years, complex instruments have been developed to generate biofilms in vitro. The constant depth film fermenter (CDFF; Fig. 1.12) was originally developed to model dental plaque biofilms (McBain et al., 2003; Wilson, 1999) and has since been used to model water system bacteria (Allan et al., 2002).

The CDFF allows multiple biofilms of uniform depth to be produced for sequential analysis whilst maintaining key parameters including nutrient source, temperature, oxygen availability and substrata. Other advantages of the CDFF include, the adaptation to use any substratum material, controlling medium flow rate and the addition of supplements or antimicrobials to examine their effects on biofilm formation.

The rotating turntable of the CDFF typically holds 15 polytetrafluoroethylene (PTFE) pans located flush around the rim, with each pan holding five cylindrical holes containing recessed PTFE plugs. The plugs are recessed to a uniform depth, on top of which the bacteria are able to form biofilms. After inoculation, sterile media flows over the biofilms, and excess growth is removed by scraper blades which sit flush with the top of the pans, hence keeping the biofilms at a constant depth.

1.11.5 Confocal laser scanning microscopy (CLSM)

Studies of microbial biofilms and their formation have been performed by using light, scanning and transmission electron microscopy. Besides these established techniques, CLSM offers the possibility of detailed visualisation of "thick" and "organised" microbiological samples in cases in which the application of conventional phase contrast or fluorescent microscopy is limited (Auschill et al., 2002; Auschill et al., 2001; Wood et al., 2000). CLSM allows the elimination of 'out-of-focus' light as well as horizontal and vertical optical sectioning. More recently, CLSM has become standard in microbiology for the assessment of undisturbed biofilms (Costerton et al., 1995; Lawrence et al., 1991; Wagner et al., 1994). CLSM now permits the acquisition of high resolution images and, by generating focused images through thick specimens at various depths, 3-D reconstructions can also be achieved (Fig.1.13).
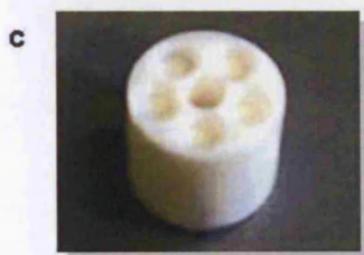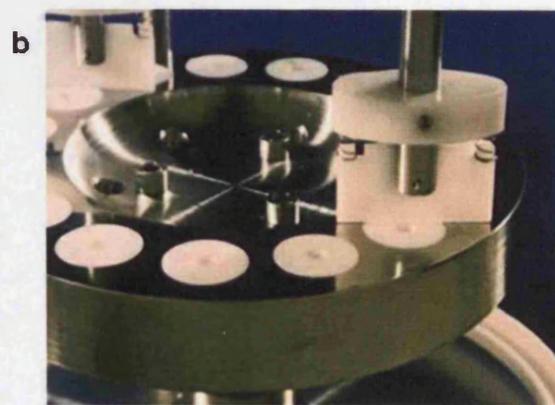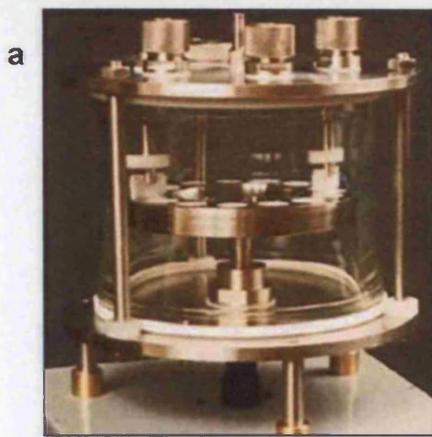
Fig. 1.12 a) The Constant Depth Film Fermenter (CDFF); b) close up of the rotating turntable; c) PTFE pan with 5 recessed plugs; d) close-up of the scrapper blade

8. Detector

7. Detector pinholes

3. Laser pinhholes

1. Laser

4. Beam splitter

2. Lens

5. Objective

6. Sample
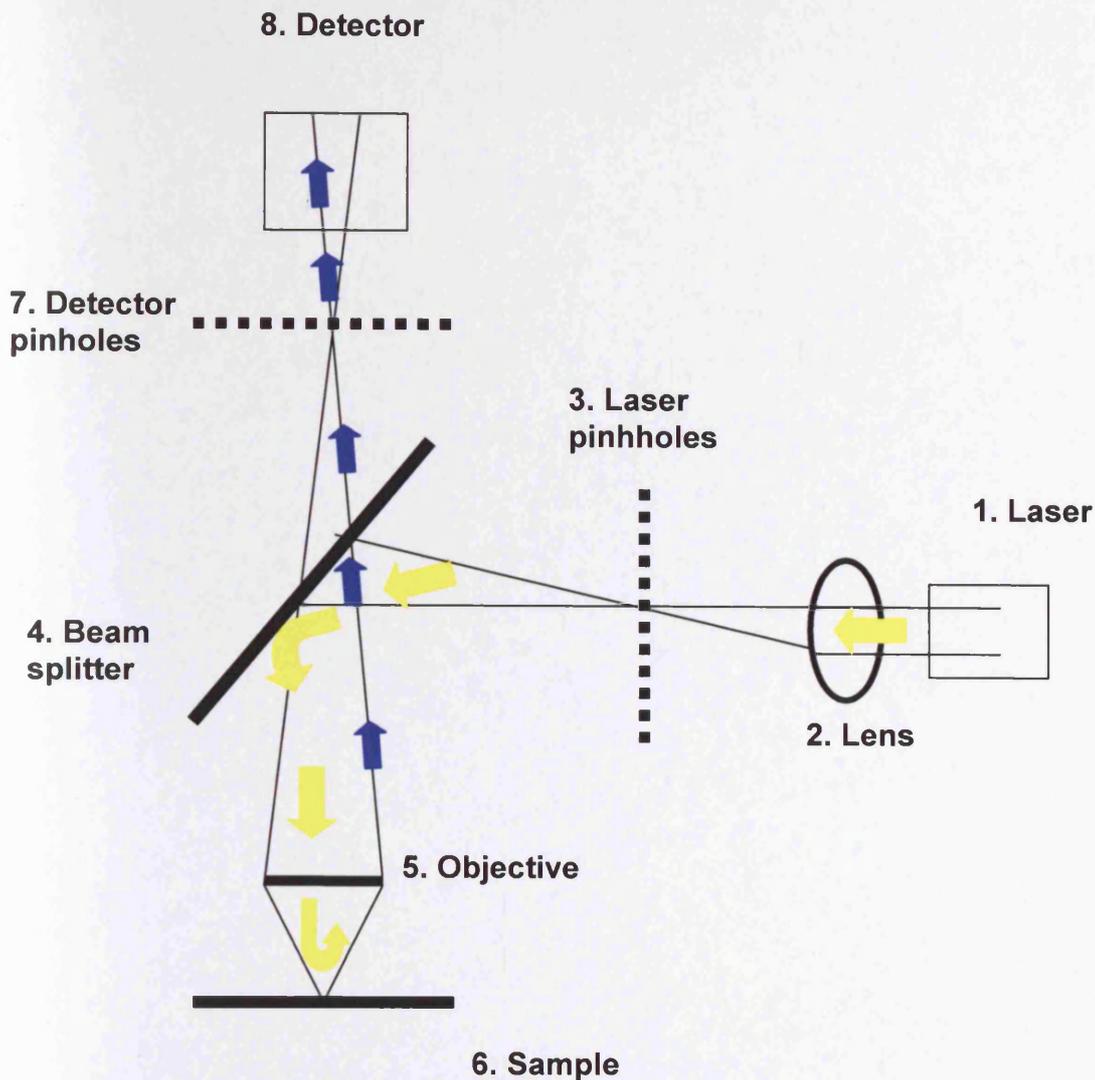
Fig. 1.13 Confocal Laser Scanning Microscopy
Adapted from Leica, www.leica-microsystems.com.

For the CLSM imaging a laser is used as a light source (1). The laser beam (1) is focused by using a lens (2) and a laser pinhole (3). The laser beam is reflected by a beam splitter (4) through an objective lens (5) and onto the sample (6) in the focal plane. The beam splitter (4) separates the laser light from the emitted light coming from the sample. When the sample (6) is exposed to a laser of a particular wavelength, substances (e.g. Alexa 594) in the RHE are excited and emit a response emission of different wavelengths. Then the beam splitter (4) allows only that wavelength which corresponds to the response emission to reach the detector pinhole (7). This emitted light passes through the pinhole (7) and is then recorded by the detector (8).

1.11.6 Fluorescence *in situ* hybridisation (FISH)

FISH is a frequently used and culture – independent approach for detecting and visualising microorganisms. Bacteria and Archaea contain 5S, 16S and 23S ribosomal Ribonucleic acid (rRNAs) with lengths of approximately 120, 1500 and 3000 nucleotides. In the vast majority of applications FISH probes have been designed to specifically target 16S rRNA (Amann, 2001). A typical FISH protocol includes fixation and permeabilisation of the sample, hybridisation of the probe, a washing step to remove unbound probe and the detection of labelled cells by microscopy (Amann, 2001).

Peptide nucleic acid (PNA) molecules are pseudopeptides that obey Watson-Crick base-pairing rules for hybridisation to complementary nucleic acid targets (Egholm et al., 1993; Nielsen and Haaima, 1997). PNA probes are extremely efficient at hybridisation (having high specificities, strong affinities, rapid kinetics) due to their uncharged, neutral backbones (Oliveira et al., 2002). PNA probes penetrate the hydrophobic cell wall of bacteria to a greater extent than that of DNA oligonucleotides, because of their relatively hydrophobic character. The FISH technique and application is discussed in more details in Chapter 5.

1.12 SUMMARY

Microorganisms are capable of growing in both a free form (planktonic) or as biofilms attached to solid surfaces. Biofilms are defined as communities of microorganisms, often adhered to a surface and encased within an extracellular polysaccharide matrix (Kumamoto and Marcelo, 2005). Examples of surfaces supporting biofilm growth include inanimate environmental materials, biomaterials interfacing with host tissue and systems, or the host tissue itself. The behaviour and phenotype of microbes existing in either planktonic and biofilm states is known to differ significantly and this is perhaps best exemplified by studies on antimicrobial efficacy against the different growth phases (Hill et al., 2003).

In the oral environment candidal biofilms on prostheses and the oral mucosa have been associated with infection (Kumamoto and Marcelo, 2005). Intra-oral biofilms can develop on the tooth enamel, oral mucosa or on introduced oral prostheses and these can all provide a reservoir of potentially pathogenic organisms promoting dental caries and periodontal disease. Furthermore, in the case of oral tissue, certain microorganisms such as yeast of the genus *Candida* have been shown to actually invade the tissue, invoking a pathogenic effect. Similarly, within a chronic wound, it may well be the case that the occurrence of a biofilm results in clinical problems due to existence of the microbes in a more persistent state. Examples of clinically, important biofilms include *Proteus mirabilis* on urinary tract catheters (Stickler, 2005), *Candida* spp. on denture surfaces and bacteria (polymicrobial) within chronic wounds (Hill et al., 2003). Significantly, when biofilms are present on the surface of medical devices, a failure of the device can occur, as encountered in the blocking of urinary catheters and the obstruction of airways within artificial voice box prostheses (Douglas, 2003; Van der Mei et al., 2000). Furthermore, biofilms tend to exhibit heightened resistance to antibiotics, possibly by diffusion limitation or the presence of biochemically inert microbes within the biofilm (Donlan and Costerton, 2002).

The clinical significance of biofilms provides the basis for this PhD and consequently the aim was to characterise specific biofilms that are involved in human infection, specifically those biofilms associated with chronic wounds and oral candidosis.

1.13 AIMS AND OBJECTIVES OF STUDY

The aims of this study were to examine the *in vitro* properties of *C. albicans* biofilms and also biofilms of bacteria recovered from the chronic wound environment. The underlying hypothesis of this work was that clinically important biofilms associated with infections have characteristics and features that promote their virulence and resistance to both host defences and administered antimicrobial agents. Specifically the aims were:

1. To determine the structural properties of *C. albicans* biofilm generated in an *in vitro* model of oral candidosis.

2. To analyse *C. albicans* virulence gene expression within developed biofilms.

3. To examine the relative *in vitro* biofilm-forming ability of selected bacterial strains originating from chronic wound environments.

4. To investigate the spatial distribution and structure of *in vitro* chronic wound biofilms.

5. To look at gene expression of chronic wound biofilm organisms.

Chapter 2

Biofilm formation and oral epithelium infection by *Candida albicans*

2.1 Introduction

The genus *Candida* comprises of over 150 species of 'yeast-like' fungi, several of which can exist as either harmless commensals or as opportunistic pathogens of humans. *Candida* infection (candidosis, sing; candidoses, pl.), has been reported at most body sites, with superficial infections of the oral and vaginal mucosa the most frequently encountered. Although, *Candida albicans* is generally isolated from 80% of all oral candidoses, this organism is also prevalent as a harmless member of the oral microflora. It is estimated that commensal carriage rate in humans is between 35 and 55%. Whilst *C. albicans* is the most frequent cause of human fungal infection resulting in a mortality rate of 30-50% in immunocompromised patients with systemic infection, the pathogenesis of this organism remains unclear (Mukherjee et al., 2003; Odds, 1988).

2.1.1 Non-oral infection

Candidoses are primarily superficial infections, occurring on moist and warm mucosal membranes such as in the mouth and vagina. In women using oral contraceptives, candidal vulvovaginitis is common. Vulvovaginitis is accompanied by a thick discharge, vaginal itching and discomfort (Samaranayake, 2006). Candidosis can also develop at a variety of other body locations including the skin, in the gut, bone joints, nails and urinary tract (Table 2.1).

2.1.1.1 *Candida* and implantable medical device associated infection

Introduction of artificial materials at various body locations has been accompanied by the ability of microorganisms, including *Candida* spp. to colonise and form biofilms (Ramage et al., 2006). Devices such as shunts, artificial prostheses, stents, implants, endotracheal tubes, pacemakers and various catheter types have been shown to support *Candida* colonisation and biofilm formation (Ramage et al., 2006).

Table 2.1 Body locations for *Candida* infection and specific candidoses.

Candidosis location	Disease
Genitalia	Vulvovaginal candidosis
	Candida balanoposthitis
Skin	*Candida* onychia
	Candida paronychia
Gastrointestinal tract	Oesophageal candidosis
Respiratory tract	Laryngeal candidosis
	Pulmonary candidosis
Urinary tract	*Candida* cystitis
	Candida urethritis
Eye	*Candida* infections of the cornea
	Candida conjunctivitis
Bones and joints	*Candida* arthritis
	Candida osteomyelitis
Oral mucosa	Oral candidosis

2.1.1.2 Central venous catheters

Yeasts, mainly *Candida*, are the third leading cause of intravascular catheter-related infections, with the second highest colonisation to infection rate and the overall highest crude mortality rate in the USA and Western Europe (Ramage et al., 2006). Studies have shown that biofilms are present in all central venous catheters, on both the inner lumen and outside the catheter (Raad, 1998).

2.1.1.3 Voice box prostheses

A voice box prosthesis is a device fitted following a total laryngectomy. A total laryngectomy involves the removal of the larynx from the throat and is performed as part of the surgical treatment for laryngeal cancer. The procedure leads to loss of natural speech, is very traumatic for the patient and presents a lifelong challenge for communication (Dijk et al., 2000). Silicone rubber voice prostheses fitted in patients are subject to rapid microbial colonisation and biofilm formation, mainly by *Candida spp.* The problem is exacerbated due to the lack of salivary flow to remove loosely attached organisms. The biofilm causes malfunction, increased airflow resistance and leakage of the valves of the prosthesis, which then needs to be surgically replaced (Ell, 1996; Neu et al., 1994; Ramage et al., 2006).

2.1.2 *Candida* and oral candidosis

As already mentioned, *C. albicans* is an important cause of superficial infection of mucosal membranes and systemic disease in humans (Sherman et al., 2002). Four clinical forms of primary oral candidosis are recognised; acute/chronic pseudomembranous candidosis, acute erythematous candidosis, chronic erythematous candidosis, and chronic hyperplastic candidosis (CHC; Chapter 1, Fig. 1.9, section 1.8.6) (Axell et al., 1997).

2.1.2.1 Pseudomembranous candidosis

Pseudomembranous candidosis (PMC) is also synonymous with the term "oral thrush" and is traditionally regarded as an acute infection characterised by the presence of non-adherent white pseudomembranes on the oral

mucosa. Primarily, PMC occurs with extremes of age *e.g.* in the new-born and elderly, or in patients suffering from a systemic debilitating disease. The persistence of PMC in HIV-infected or otherwise debilitated individuals has led to the occurrence of chronic PMC. The lesions of PMC can be scraped off with a swab to expose the underlying erythematous mucosa. The removed white pseudomembranes consist of desquamated epithelial cells, fibrin and fungal hyphae. Diagnosis is usually straightforward and can be confirmed microbiologically either by staining a smear from the affected area or by culturing a swab or an oral rinse (Akpan and Morgan, 2002).

2.1.2.2 Acute erythematous candidosis

Acute erythematous candidosis is most frequently seen on the dorsum of the tongue in patients receiving prolonged antibiotic therapy. It presents as a red, painful area of the mucosa and may also be seen with patients in conjunction with low serum vitamin B12, low folate and low ferritin concentrations (Akpan and Morgan, 2002). It is believed that broad spectrum antibiotic therapy reduces the normal numbers of the oral bacterial community and allows *C. albicans* to overgrow in the mouth (Farah et al., 2000). Diagnosis of acute erythematous candidosis can be confirmed through use of a swab from the tongue or buccal mucosa and confirmation of *Candida* presence by culture and identification (Akpan and Morgan, 2002).

2.1.2.3 Chronic erythematous candidosis

Chronic erythematous candidosis (denture stomatitis) is the most frequently occurring form of oral candidosis and is present in up to 65% of denture wearers (Akpan and Morgan, 2002). This form of oral candidosis is believed to be promoted through the occurrence of tissue damage caused by frictional irritation of the palatal mucosa by the denture. A poor fitting denture and poor oral hygiene is associated with this infection. Diagnosis requires isolation and culture of *Candida* from the denture surface (Akpan and Morgan, 2002).

2.1.2.4 Chronic hyperplastic candidiosis

Chronic hyperplastic candidiosis (CHC) is a distinct form of oral candidosis and is characterised by hyphal penetration of the oral epithelium (Sitheeque and Samaranayake, 2003) and the presence of an inflammatory cell infiltrate (Williams et al., 1997). Of particular concern with CHC is the potential risk of squamous cell carcinoma (SCC) development at the lesional site (Banoczy, 1977; Krogh et al., 1987a; Williams et al., 2001). The presence of *Candida* is associated with an increased incidence of malignant transformation. The exact role of *Candida* in the development of oral cancer is unclear but might be related to the organism's ability to generate carcinogenic nitrosamines from salivary precursor molecules (Krogh et al., 1987b; Sitheeque and Samaranayake, 2003). In addition, specific *C. albicans* strains are known to promote neoplastic change, which highlights the potential role of these organisms in malignant transformation (Krogh et al., 1987a). It is also important to note that the lifestyle for both promotion of *Candida* carriage and oral cancer are similar which makes establishing an association difficult (Sitheeque and Samaranayake, 2003).

2.1.3 *Candida* virulence factors associated with oral candidosis

In addition to host factors (Chapter 1, section 1.8.7) *Candida* itself expresses specific virulence attributes that promote oral infection. No single predominant virulence factor has been associated with *Candida*, although many factors have been implicated in its pathogenicity (Table 2.2).

2.1.3.1 Adherence

Adherence of *Candida* to the oral mucosa is an important initial step in the establishment of infection and therefore, candidal adhesion to buccal epithelial cells (BECs) (Nair and Samaranayake, 1996; Tsang and Samaranayake, 1999; Verran et al., 2007), to denture acrylic (Bulad et al., 2004; Verran and Maryan, 1997) and silicone rubber (Price et al., 2002; Price et al., 2005) have been extensively studied.

Table 2.2 Virulence factors associated with *Candida albicans*.

Virulence factor	Effect
Adherence	Promotes retention in the mouth
Expression of cell surface adhesion molecules	
Evasion of host defences	Promotes retention in the mouth
High frequency phenotypic switching Hyphal development Secreted aspartyl proteinases (SAP)	
Invasion and destruction of host tissue	Enhances pathogenicity and promotes retention in the mouth
Hyphal development Secreted aspartyl proteinase production (SAP) Phospholipase production	

Candida albicans must first adhere to the epithelium to colonise, infect or invade tissues (Vargas et al., 1994). Attachment of *Candida* cells can be mediated by both non-specific and specific factors (Table 2.3). Non specific factors include relative cell surface hydrophobicity and electrostatic forces (Ramage et al., 2005). Specific factors include adhesins, such as serum proteins (fibrinogen and fibronectin) and salivary factors (Ramage et al., 2005). Hence, specific *C. albicans* adherence is mediated by both protein and carbohydrate factors and is a complex phenomenon, with strain variation evident (Ramage et al., 2005).

2.1.3.2 Morphology

Candida albicans has the ability to grow in different morphological forms including budding yeast cells, pseudohyphae and true filamentous hyphae (Fig. 2.1). Once *C. albicans* attaches to host surfaces, it can switch from its yeast morphology to a filamentous form which may promote adhesion and penetration of the epithelium and increased resistance to phagocytosis by host immune cells. It has also been suggested that filamentous forms of *Candida* secrete higher quantities of hydrolytic enzymes (Senet, 1998) (section 2.1.3.4). It is generally believed that the budding yeast form is predominant in commensal carriage whilst the hyphal form predominates in tissue penetration. Although others have demonstrated that both forms can be associated with commensalism and disease (Soll, 2002). It has been observed that hyphae grow by tip extension and generate significant pressure for tissue invasion (Gow et al., 2002).

Table 2.3 Adhesion factors associated with *Candida albicans*.

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Non-specific factors	Receptor	Reference
Electrostatic interactions		(Ramage et al., 2005)
Hydrophobic interactions		(Chaffin et al., 1998; Ramage et al., 2005)
Specific factors		
Proteins (mannoproteins, fibrinogen, fibronectin, Fibrillar adhesins) Proteins of the ALS gene family	Binds to complement receptors and membranes on host cells and to fucosyl receptors	(Chaffin et al., 1998; Holmes et al., 1996; Hostetter, 1994; Ramage et al., 2005)
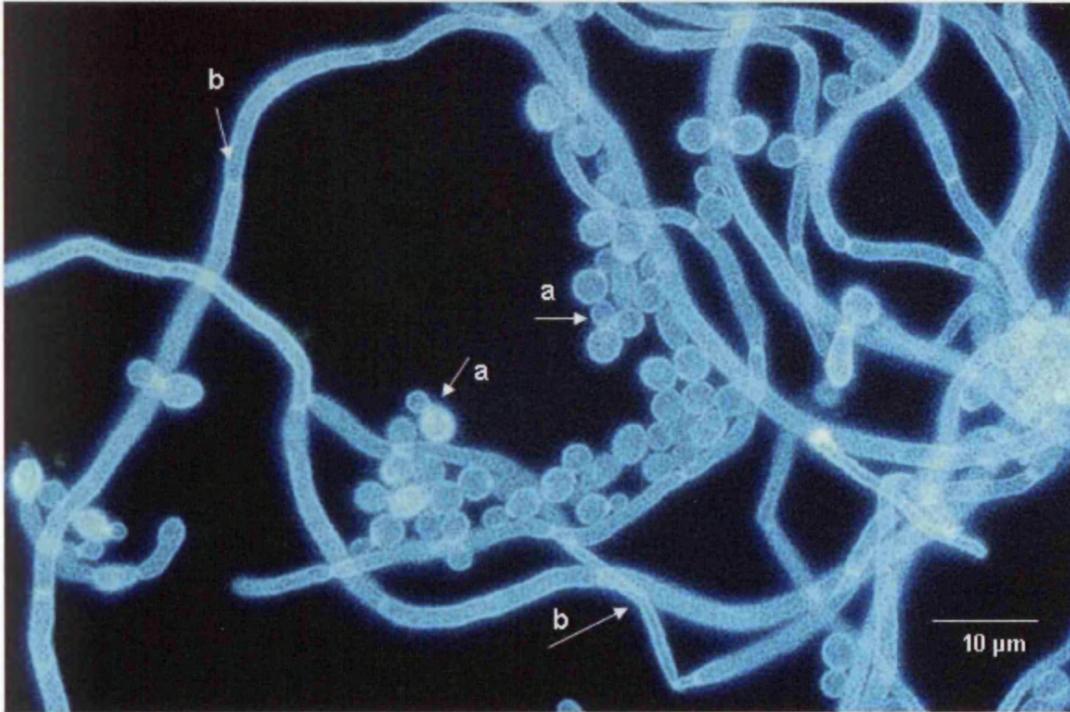
Fig. 2.1 *Candida albicans* stained with calcofluor white illustrating the different morphological forms including yeast (a) and hyphae (b). Courtesy of Dr D Williams at the Cardiff Dental School.

2.1.3.3 Phenotypic switching

Candida albicans has the ability to rapidly and reversibly switch between the yeast budding form and a hyphal growth form (Gow, 1997; Soll, 1986). Various studies have demonstrated that high frequency switching affects a variety of virulence features, including the bud-hypha transition (Anderson et al., 1989), antigenicity (Anderson et al., 1990), adhesion (Kennedy et al., 1988; Vargas et al., 1994), sensitivity to neutrophils and oxidants (Kolotila and Diamond, 1990), secretion of proteinase (Soll et al., 1989; Vargas et al., 2000) and drug susceptibility (Soll et al., 1989; Vargas et al., 2000). The morphogenic transition between yeast and hyphal forms is often stimulated by growth in the host, correlated with invasion and associated with pathogenesis (Gow et al., 2002). *In vitro* high frequency switching can be detected by colony morphologies of strains grown on amino acid-rich Lee's medium supplemented with arginine and a limiting concentration of zinc (Soll, 1992).

2.1.3.4 Production of hydrolytic enzymes

Destruction of host tissues by *C. albicans* is believed to occur through the activity of hydrolytic enzymes produced by the organism. Most of these enzymes are extracellularly secreted by *C. albicans*. The most frequently studied of these enzymes are secreted aspartyl proteinases (SAPs) (Hube and Naglik, 2001; Naglik et al., 2004). Hydrolytic enzymes contribute to host tissue invasion by digesting or destroying cell membranes and by degrading host surface molecules. There is some evidence that hydrolytic enzymes are able to damage cells and molecules of the host immune system thereby avoiding or resisting antimicrobial activity (Schaller et al., 2005a).

In addition to SAPs, phospholipases (PLs) contribute to the pathogenicity of *C. albicans* by damaging host-cell membranes, which aids the fungus by facilitating host tissue invasion (Borst and Fluit, 2003). Details on *Candida* hydrolytic enzymes and associated genes are discussed in detail in Chapter 3, section 3.1.1.

2.1.4 Models to assess *Candida* biofilm formation

In this present study the primary aim was to compare the ability of different *C. albicans* strains to grow as biofilms and determine whether any observed differences with respect to the biofilm growth related to their origin of infection. A suitable model for this had therefore to be adopted.

In recent years a large number of experimental model systems have been designed to study *Candida* grown in the biofilm state (Table 2.4). Many of these have already been highlighted in the Literature Review (section 1.11) of this thesis. The availability of well-characterised, reproducible biofilm models is essential to understand the nature of *Candida* biofilms and to perform studies of biofilm formation and antifungal drug resistance (Chandra et al., 2001a). Many of the models outlined in Table 2.4 are sufficient for device infection studies but not for tissue infections. However, the recent use of a reconstituted human oral epithelium system is adequate to study tissue infections and closely resembles the healthy human epithelium (Jayatilake et al., 2005).

2.1.4.1 Reconstituted human oral epithelium (RHE)

The human oral mucosa is composed of an outermost layer of stratified squamous epithelium similar to the stratified squamous epithelia found in the rest of the body. It has a mitotically active basal cell layer, a number of differentiating intermediate layers and a superficial layer, where cells are shed from the surface of the epithelium (Giannola et al., 2007). SkinEthic Laboratories cultivate a 0.5 cm^2 reconstituted human epithelium (RHE) *in vitro* on a polycarbonate filter at the air liquid interface in chemically defined medium lacking antibiotics for 5 days (Fig. 2.2). The transformed human keratinocytes of the cell line TR146, from a squamous cell carcinoma of the buccal mucosa form an epithelial tissue devoid of stratum corneum, yet histologically resembling the mucosa of the oral cavity.

Table 2.4 Models and methods to assess *Candida* biofilm formation.

Models	References
Adherence assay (buccal and vaginal epithelial cells)	(Abu-Elteen, 2000; Nikawa et al., 2006; Skoutelis et al., 1995)
Microtitre plate assay	(Ramage et al., 2001a; Ramage et al., 2001b)
Discs	(Hawser and Douglas, 1994)
Perfused biofilm fermenter	(Baillie and Douglas, 1998; Gilbert et al., 1989)
Microfermenter model	(Garcia-Sanchez et al., 2004)
Polymethylmethacrylate and silicone elastomer	(Chandra et al., 2001a; Chandra et al., 2001b)
Hydroxyapatite and acrylic	(Chandra et al., 2001b; Henriques et al., 2003; Ramage et al., 2001a)
Constant Depth Film Fermenter	(Lamfon et al., 2005; Lamfon et al., 2003; Lamfon et al., 2004; Pratten et al., 2003)
Reconstituted Human Oral Epithelium (RHE)	(Giannola et al., 2007; Jayatilake et al., 2005; Schaller et al., 2001; Schaller et al., 2005b)
Animal models	(Adams and Jones, 1971; Budtz-Jorgensen, 1971; Garcia-Sanchez et al., 2004; Jones and Adams, 1970; Samaranayake and Samaranayake, 2001; Shakir et al., 1981; Shakir et al., 1986)
Methods	
Roll plate method	(Maki et al., 1997; Storti et al., 2003)
Enumeration of viable cells	(Burton et al., 2007; Djordjevic et al., 2002; Jin et al., 2003; Li et al., 2003)
Total biomass determination	(Hawser and Douglas, 1994)
Microscopical analysis	(Caddy et al., 2003; Chandra et al., 2001a; Harrison et al., 2007; LaFleur et al., 2006; Mendez-Vilas et al., 2007)
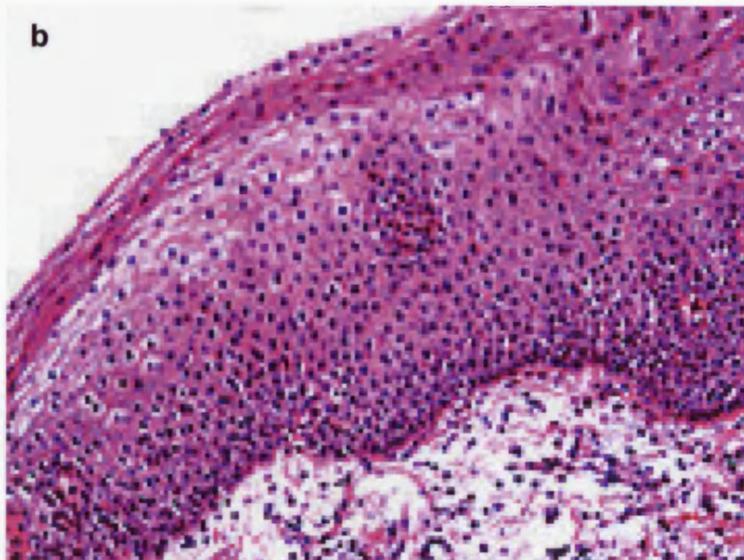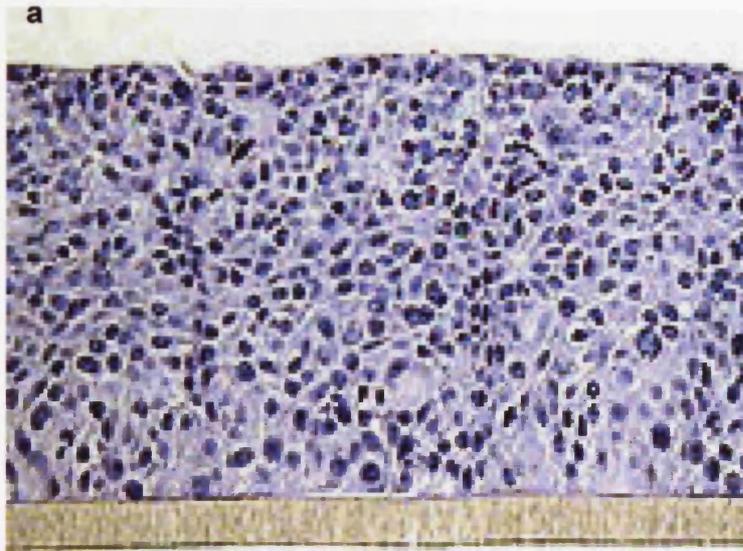
Fig. 2.2 a) Reconstituted human oral epithelium (SkinEthic Laboratories, Nice, France); b) Oral epithelium *ex vivo* (SkinEthic Laboratories, Nice, France).

In recent years, this RHE (SkinEthic Laboratories, Nice, France) has successfully been used to investigate mechanisms of tissue degradation by *Candida* (Jayatilake et al., 2005; Schaller et al., 2001; Schaller et al., 1999; Schaller et al., 1998) and examine the response of tissue to infection (Schaller et al., 2005b). Indeed, previous studies using Periodic Acid Schiff (PAS) staining to profile *C. albicans* RHE infection have suggested that strain differences exist between *C. albicans* isolates with respect to tissue invasion (Bartie et al., 2004).

In general, this approach provides a number of advantages compared to other *in vitro* models. The stratified cultured TR146 cell layers are analogous to normal human buccal epithelium with the cells are able to grow on the polycarbonate permeable inserts to form cell layers resembling the stratified human buccal tissue. So overall, the model has similar morphology, ultra-structure and permeability barrier properties to intact buccal mucosa (Jacobsen et al., 1999). The RHE has also been used to perform permeability studies of drugs in a well-defined environment (Giannola et al., 2007; Jacobsen et al., 1999). Importantly, when using any model system, one has to be aware of its limitations *e.g.* in the case of the RHE, the epithelium cannot recover after destruction nor can it elicit a full immune response.

2.1.5 Methods of analysing the developed *Candida* biofilms

Once a biofilm has been generated, it can be characterised using a variety of approaches including the assessment of cell number either through counting the total number of cells (live or dead) or by viable count estimation. Other characteristics that can be measured include the analysis of biofilm associated gene expression (Chapter 3), or investigating the actual architecture of the biofilm that develops. In this present Chapter, the aim was the latter and therefore a microscopical analysis was selected. Several options were available as outlined below.

2.1.6 Microscopical analysis

Scanning electron microscopy (SEM) techniques have been used to observe biofilms (or more often adherent cells) because of the high magnification and image contrast that can be achieved. However, sample preparation (dehydration of the samples) may cause problems leading to false artifactual observations (Caddy et al., 2003). Another disadvantage of SEM is that detail on the exact architecture of biofilms is difficult to obtain.

Inverted light microscopy has also been used, e.g. to measure adherence of C. albicans to acrylic (Williams et al., 1998). Recently, Harrison et al. (2007) used the inverted light microscope to examine C. albicans and C. tropicalis biofilms cultivated at the bottom of microtitre plate wells (Harrison et al., 2007). The benefits of such an approach are that visualisation of the biofilm in a liquid environment can be undertaken without disturbing the architecture.

The atomic force microscope (AFM) has become an important tool for investigating biological materials such as tissues, yeast, bacteria and their biological constituents. It can provide real-time in situ quantitative morphological information, as well as measure the interaction forces between a sharp tip and the surface of interest. The probe of interest should be positioned on the stage of the AFM to be scanned and has to be fixed by chemical treatment so force applied by the AFM tip does not induce movement of the sample. Surface modifications include polyethyleneimine (PEI), silanisation or poly-L-lysine treatment to immobilise microbes onto the surface (Mendez-Vilas et al., 2007; Schar-Zammaretti and Ubbink, 2003; Velegol and Logan, 2002). However, one of the limitations of the AFM is that all these methods can alter the surface physiochemical properties of the cells (Mendez-Vilas et al., 2007).

Fluorescent microscopy can be used to evaluate the morphology of biofilms (Chandra et al., 2001a; Kuhn et al., 2002b), and this is of particular value for live/dead determination of biofilm cells and the specific detection of individual group members in biofilm consortia (LaFleur et al., 2006). Furthermore, cells

can be detected when present on an opaque surface. The disadvantage of standard fluorescent microscopy is that it cannot generate focused images through very thick specimens.

Confocal laser scanning microscopy is a microscopy method that permits the acquisition of high resolution images and 3-D reconstructions. This technique has the advantages of fluorescence microscopy but significantly can also generate focused images through thick specimens at various depths. The CLSM procedure involves images being taken 'point-by-point' and then reconstructed using a computer, rather than projected through an eyepiece. Particulars of this method are discussed in detail in Chapter 1, section 1.11.5. Due to the significant advantages of CLSM over other microscopical methods, it was chosen to study the *Candida* biofilms generated in this study.

2.2 Aims

With regards to oral candidosis, the question that remains to be answered is why individuals present with such different infections, which are all seemingly caused by the same fungus. It is evident that *C. albicans* is an extremely heterogeneous species whose strains differ phenotypically and genotypically. Strain variation could determine which type of infection develops and whether the host actually manages to combat the colonising strain or whether it is retained as a commensal strain.

The hypothesis of this study was that different strains of *C. albicans* develop specific biofilm growth forms on a Reconstituted Human Oral Epithelium which are directly associated with their origin of infection. For example, if isolates originated from an invasive candidosis such as CHC, the resulting biofilm/infection would differ compared to another strain from a more superficial mucosal infection such as PMC. To test this hypothesis, the specific aims were:

1. To develop a suitable *in vitro* biofilm model for *C. albicans* infection using the RHE.

2. To infect this model with a range of *C. albicans* strains isolated from various forms of oral candidosis.

3. To compare the following parameters of RHE infection: extent of RHE colonisation; type of candidal morphology involved in the infection; degree of RHE tissue invasion exhibited and the pattern of tissue invasion.

2.3 Materials and Methods

2.3.1 Identification of test isolates

Nineteen isolates of *C. albicans* from four distinct clinical conditions (normal oral mucosa, n=4; non-CHC oral candidoses, n=4; CHC, n=8; SCC (squamous cell carcinoma), n=3) were obtained either by imprint culture from patients with defined lesions or concentrated rinse culture for isolates from non-defined infection sites. The strains were collected between 1993 until 2001 as part of the routine microbiological service at the Cardiff Dental School. Details of these isolates are presented in Table 2.5. Identification of *C. albicans* was based on germ-tube formation and biochemical profiling using the API 32C system (bioMérieux, Basingstoke, UK).

2.3.2 *In vitro* RHE infection model

2.3.2.1 Preparation of *Candida albicans*

Candida albicans strains were cultured on Sabouraud Dextrose Agar (SDA; LAB M, Bury, UK) at 37°C for 24 h. The resulting growth was used to inoculate 10 ml of Yeast Nitrogen Base (YNB; BD Diagnostics, Cowley, UK) medium supplemented with 0.5% glucose (w/v). This medium was then incubated for 12 h at 37°C. Yeast cells were harvested by centrifugation and washed (×3) with phosphate buffered saline (PBS). The resulting yeast cells were enumerated using a haemocytometer and a total of either 4×10^7 yeast cells or 2×10^6 yeast cells was added to the RHE (SkinEthic Laboratories, Nice, France; section 2.3.2.2). The initial morphology of the inoculum was determined using a light microscope at original magnifications of x400 and x600 (Carl Zeiss Ltd., Welwyn Garden City, UK).

2.3.2.2 Preparation of RHE

The RHE was supplied by SkinEthic Laboratories (Nice, France) and was generated by culturing transformed human keratinocytes derived from the cell line TR146 originally obtained from a squamous cell carcinoma of the buccal mucosa.

Table 2.5 *Candida albicans* isolates and source.

Candida albicans strain	Isolate source
135BM2/94	CHC-SCC, buccal mucosal
970/00	CAC, oral mucosa
PTR/94	CHC, buccal mucosa
324LA/94	CHC, commissure
1190/97	CHC, buccal mucosa
705/93	CHC, buccal mucosa
DW1/93	Normal oral mucosa
PB1/93	Normal oral mucosa
243/00	Lichen planus
455rgh/94	CHC, tongue
LR1/93	Normal oral mucosa
289T/00	SCC, tongue
480/00	SCC, oral mucosa
819/99	Keratosis, sublingual
408/99	SCC, tongue
848/99	CHC, tongue
458R/94	CHC, buccal mucosa
WK1/93	Normal oral mucosa
40/01	PMC, palate

CHC, Chronic hyperplastic candidosis; SCC, Squamous cell carcinoma; CAC, Chronic atrophic candidosis; PMC, Pseudomembranous candidosis.

Cultures were incubated in serum-free conditions in a chemically defined MCDB-153 medium containing 5 μg ml^{-1} insulin and 1.5 mM calcium chloride on a 0.5 cm^2 microporous polycarbonate filter for 5 days. The MCDB-153 medium comprised of essential amino acids, vitamins, inorganic salts, organic compounds and trace elements. The RHE *in vitro* model and all culture media were prepared for this study without antibiotics and antimycotics.

Immediately after receipt of the RHE from the supplier, the tissue was removed from the agarose-nutrient solution in the shipping multiwell plate and placed in a fresh 6-well tissue culture plate previously filled with 100 μl of SkinEthic maintenance medium.

2.3.2.3 Culture of infected RHE

Infected RHE was incubated for 24 h (for 4×10^7 yeast cells) or 12 h (for 2×10^6 yeast cells) at 37°C in a humidified atmosphere enriched with 5% CO_2. After incubation, the tissue was bisected; one portion was used for CLSM analysis and the other for RT-PCR (Chapter 3, section 3.3.4). A non-infected control was included for comparison and in all cases, the experiments were repeated on a minimum of two occasions.

2.3.2.4 RHE processing for Confocal Laser Scanning Microscopy (CLSM)

Three processing protocols were assessed in this study and included the analysis of fresh (unprocessed) tissue, frozen fixed tissue and formalin fixed tissue.

2.3.2.4.1 Fresh tissue

The fresh tissue portion used for CLSM was placed on a microscope slide, and overlaid with 20 μl concanavalin A lectin (0.025% w/v in PBS) conjugated with the fluorescent tag, Alexa 594 (Sigma, Poole, UK). After 30 min incubation at room temperature, the lectin solution was removed and the tissue washed once using distilled H_2O. Finally, the tissue was overlaid with

5 μl of vectashield (Vector Laboratories Ltd, Peterborough, UK). A coverslip was placed on top of the preparation and sealed with nail varnish.

2.3.2.4.2 Frozen fixed tissue

Specimens of infected tissue were also frozen using isopentane (100%) cooled in liquid nitrogen. Fresh specimens were placed in aluminium foil and lowered into the isopentane solution for 30 s until frozen. The tissue in the foil was then stored at -80°C in cryotubes until required for staining and CLSM analysis. To visualise the infecting *C. albicans*, sections were directly labelled with 20 μl of Alexa 594-conjugated concanavalin A lectin (Molecular Probes) at 0.025% w/v in PBS for 20 min at room temperature.

2.3.2.4.3 Formalin fixed tissue

Infected tissue was fixed in 2% (v/v in H_2O) paraformaldehyde solution for 24 h and embedded in paraffin wax using standard histological techniques. Sections (20 μm) were cut and placed on to Histobond+ coated microscope slides (Raymond A Lamb, East Sussex, UK), de-waxed and processed through xylene and ethanol to water before fluorescent labelling.

2.3.2.4.4 Immunohistochemistry on fixed tissue

To identify epithelial cells within the RHE, sections were also labelled with a pan-cytokeratin antibody using an indirect immunofluorescent labelling procedure. Sections were first heated in citrate buffer for 10 min at 95°C, washed in PBS and blocked with normal goat serum (Sigma-Aldrich, Dorset, UK) using a 1:20 dilution in PBS for 30 min, prior to primary antibody staining. Sections were then incubated overnight at 4°C with a mouse monoclonal pan-cytokeratin primary antibody (Santa Cruz Biotechnology, Inc; Heidelberg, Germany) used at a 1:50 dilution in PBS. After washing, sections were fluorescently labelled with Alexa 488-conjugated goat anti-mouse secondary antibody (5 μg ml^{-1}; Invitrogen) for 30 min at room temperature.

For nuclear context, the sections were counterstained with Hoechst 33258 (Sigma) 1µg ml^{-1} for 20 min, before washing in PBS. Tissues were then mounted using vectashield fade-retarding mountant .

2.3.2.5 Confocal Laser Scanning Microscopy (CLSM)

Stained sections were viewed and analysed by CLSM using a Leica TCS SP2 AOBS spectral confocal microscope (Leica, Wetzlar, Germany). The sections were scanned through the full depth of the tissue using appropriate settings for single, double or triple channel fluorescence recordings of Alexa 594 alone; Alexa 594 and Hoechst 33258; or Alexa 594, Hoechst 33258 and Alexa 488 as detailed in Table 2.6. For multi-channel recordings, fluorochromes were scanned sequentially to eliminate spectral overlap between probes. Selected images were presented either as single confocal optical sections or maximum intensity type reconstructions.

2.3.2.6 Criteria of biofilm parameters examined

The parameters of the biofillms examined were I) the level of *C. albicans* colonisation on the RHE, II) the morphology of the infecting *C. albicans* and, III) the extent and pattern of any tissue invasion by the organism. "High invaders" were defined as those strains that readily penetrated the RHE within the incubation time period. These were in contrast to "low invaders" where only superficial invasion on the RHE was noted. "Non-invaders" did not display any evidence of tissue penetration.

Table 2.6 Excitation and Emission spectra for the 3 fluorochromes used for CLSM.

Fluorochrome	Laser Excitation Line (nm)	Emissions Detected (nm)
Hoechst 33258	405	410-480
Alexa 488	488	500-550
Alexa 594	546	600-660

2.4 Results

2.4.1 Infection of the RHE model

Preliminary studies with the two inoculum levels and incubation times showed that a high inoculum ($4x10^7$) and longer incubation time (24 h) caused an excessive level of tissue destruction with many of the tested strains. As a consequence, the lower inoculum ($2x10^6$) level and shorter incubation time (12 h) proved more suitable for strain comparisons with respect to the chosen measured parameters. The following results are based on these optimal infection conditions. Furthermore, detection of *Candida* was achieved with all three processing methods with no apparent differences in staining quality or alterations in tissue morphology. Formalin fixed tissue was however easiest to handle and to store, and so the reported results are also based on this tissue processing approach.

2.4.2 Comparison of planktonic morphology and biofilm morphology

For the majority of strains tested, the morphological form of the *Candida* strain was the same regardless of whether culture occurred in the presence (biofilm form) or absence (planktonic form) of the RHE. Six strains (324LA/94, PB1/93, 455rgh/94, LR1/93, 819/99 and 40/01) out of 19 exhibited a different morphology between the two different culture conditions (Table 2.7).

2.4.3 *In vitro* RHE infection

2.4.3.1 Extent of colonisation of the surface of the RHE

The majority of *C. albicans* strains (n=17/19) invaded the RHE (Table 2.7) and CLSM showed the presence of both yeast and hyphae on the RHE surface. The results were consistent between the three separately repeated experiments.

Table 2.7 *Candida albicans* invasion of reconstituted human oral epithelium (RHE) as determined by confocal laser scanning microscopy (CLSM).

Candida albicans strain	Isolate source	Surface colonisation (biofilm formation)	Strain morphology in presence of tissue (without tissue*)	Invasion
135BM2/94	CHC-SCC, buccal mucosal	+++	Yeast and hyphae	high
970/00	CAC, oral mucosa	+++	Predominantly hyphae	high
PTR/94	CHC, buccal mucosa	+++	Yeast and hyphae	high
324LA/94	CHC, commissure	+++	Yeast and hyphae (predominantly yeast)	high
1190/97	CHC, buccal mucosa	+++	Yeast and hyphae	high
705/93	CHC, buccal mucosa	+++	Yeast and hyphae	low
DW1/93	Normal oral mucosa	++	Yeast and hyphae	low
PB1/93	Normal oral mucosa	++	Yeast and hyphae (predominantly yeast)	low
243/00	Lichen planus	++	Yeast and hyphae	low
455rgh/94	CHC, tongue	++	Predominantly yeast (yeast and hyphae)	low
LR1/93	Normal oral mucosa	++	Predominantly yeast (yeast and hyphae)	low
289T/00	SCC, tongue	++	Predominantly yeast	low
480/00	SCC, oral mucosa	++	Yeast and hyphae	low
819/99	Keratosis, sublingual	++	Predominantly yeast (yeast and hyphae)	low
408/99	SCC, tongue	++	Predominantly yeast	low
848/99	CHC, tongue	++	Predominantly yeast	low
458R/94	CHC, buccal mucosa	++	Predominantly yeast	low
WK1/93	Normal oral mucosa	+	Predominantly yeast	none
40/01	PMC, palate	+	Predominantly yeast (yeast and hyphae)	none

CHC, Chronic hyperplastic candidosis; SCC, Squamous cell carcinoma; CAC, Chronic atrophic candidosis; PMC, Pseudomembranous candidosis; +, sparsely colonised; ++, moderately colonised; +++, extensively colonised. *Highlighted morphological findings indicate differences observed between the two different culture environments.

Figure 2.3a shows a more extensive surface colonisation with more hyphal development and Figure 2.3b shows an example of sparse surface colonisation of the RHE.

In some cases, hyphal and yeast growth degraded the epithelial layers of the RHE and, therefore, the cell numbers and the incubation time was reduced as highlighted earlier.

2.4.3.2 Invasion of the biofilm in the RHE

By decreasing the yeast inoculum level and reducing the incubation time, a better analysis of the direction of hyphal penetration and determination of any budding occurring within the tissue was possible. Figures 2.4a-2.6a illustrate the different forms of *Candida* infection (24h and a high inoculum 4×10^7) of the RHE and depict variation in the extent of both tissue invasion and the actual pattern of hyphal invasion within the tissue.

The 19 *C. albicans* strains were categorised as either "high invaders", "low invaders" or "non-invaders" (Table 2.7). CLSM showed the presence of both yeast budding and hyphae on the RHE surface and within the tissue. Hyphal growth forms were most frequently detected within the tissues. Figures 2.4a-2.6a illustrate infection of RHE (24h, with a high inoculum 4×10^7 level) and Fig. 2.4b-2.10 illustrate infection of the RHE (12h, with a lower inoculum 2×10^6 level) and the pattern of hyphal invasion. Five strains out of 19 were 'high invaders', where extensive surface colonisation and large numbers of hyphae occurred within the tissue (Fig. 2.4b and 2.5b). This contrasted with 'low invader' or 'none invader', where yeast predominated for 10 of the 14 isolates and little or no invasion was evident (Fig. 2.6b and 2.7a and b). The RHE was approximately 50-60 μm thick and the 'high invaders' penetrated through the whole thickness of the tissue. The extent of RHE invasion was therefore isolate-dependent and consistent over repeat experiments for each given isolate (Table 2.7).
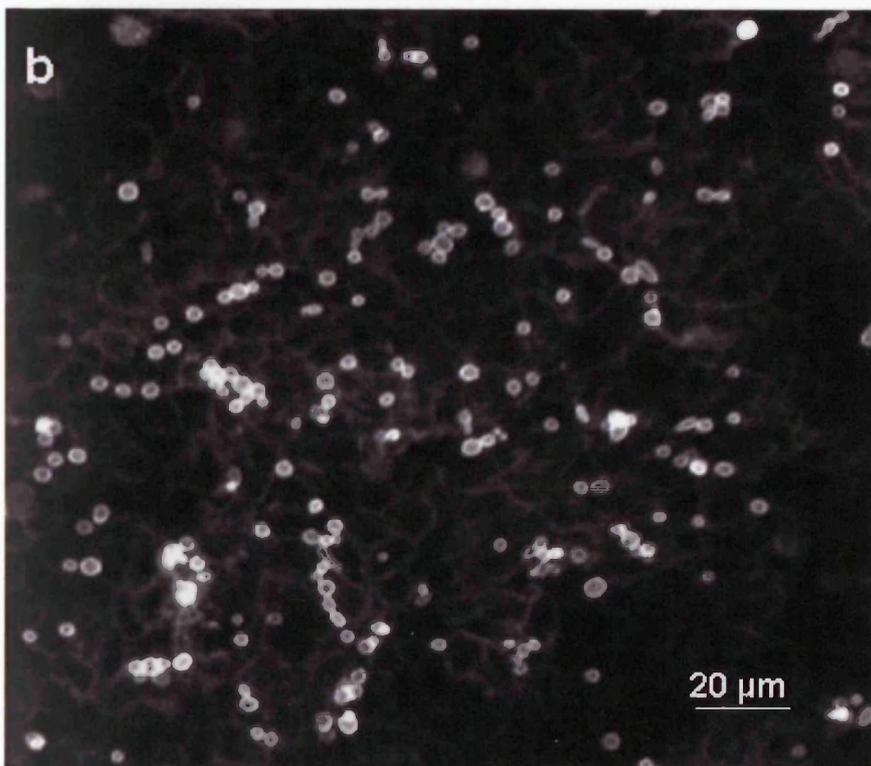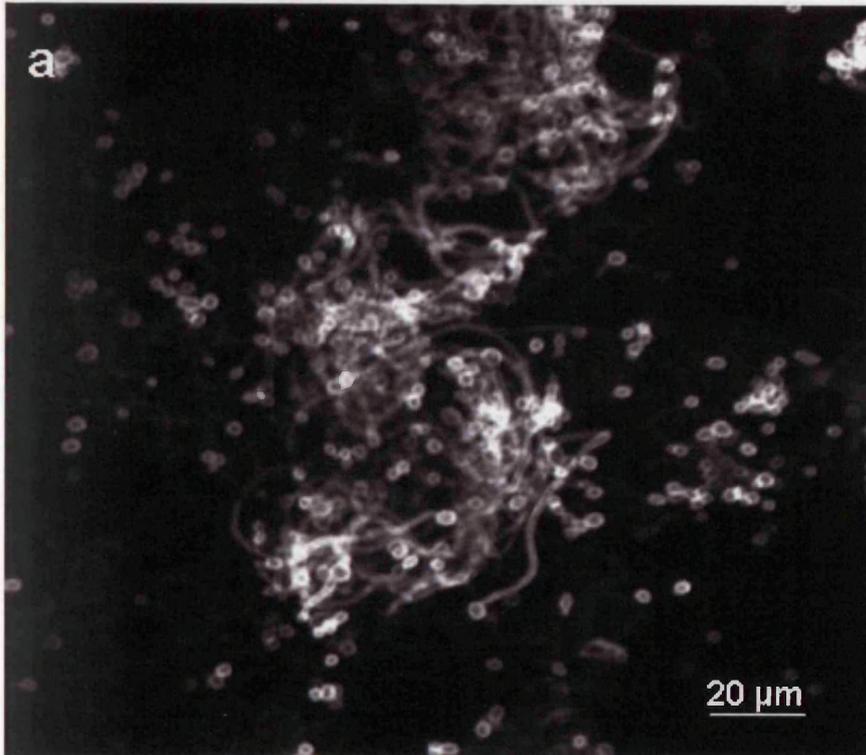
Fig. 2.3 Surface colonisation of two strains of *C. albicans* on RHE tissue
a) strain 455rgh/94 illustrating an extensive surface colonisation, yeast and hyphae are evident, and b) strain WK1/91 illustrating a sparse surface colonisation, primarily only yeast.
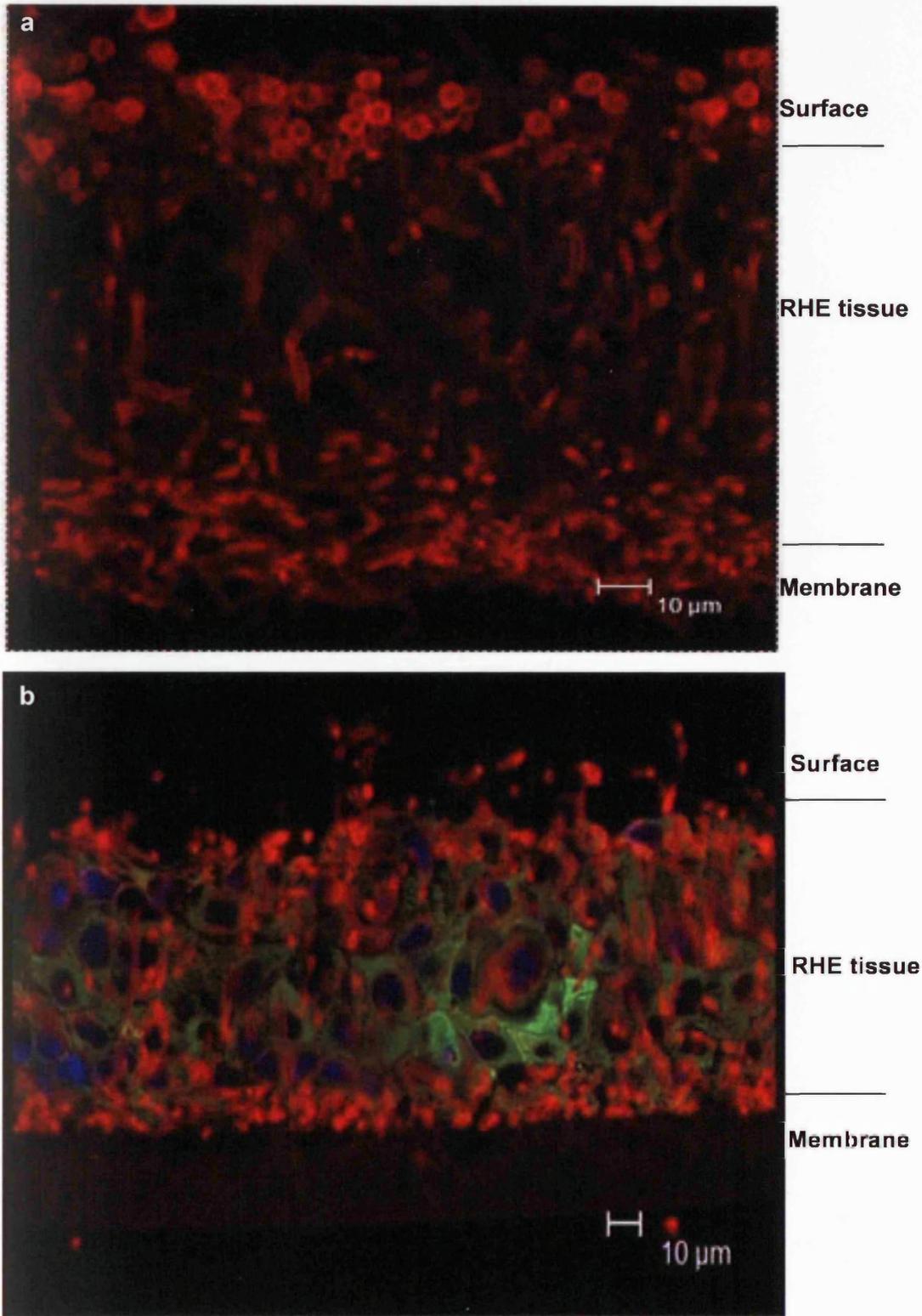
Fig. 2.4 Infection of RHE by *C. albicans* high invader strain (135BM2/94) and visualised using CLSM a) 24h incubation, stained with ConA-Alexa 594 (red) and b) 12h incubation, triple stained The nuclei are stained with Hoechst (blue), the epithelial cells are stained with pan-cytokeratin antibody – Alexa 488 (green) and the *Candida* are stained with ConA-Alexa 594 (red) showing the pattern of hyphal invasion and surface colonisation.
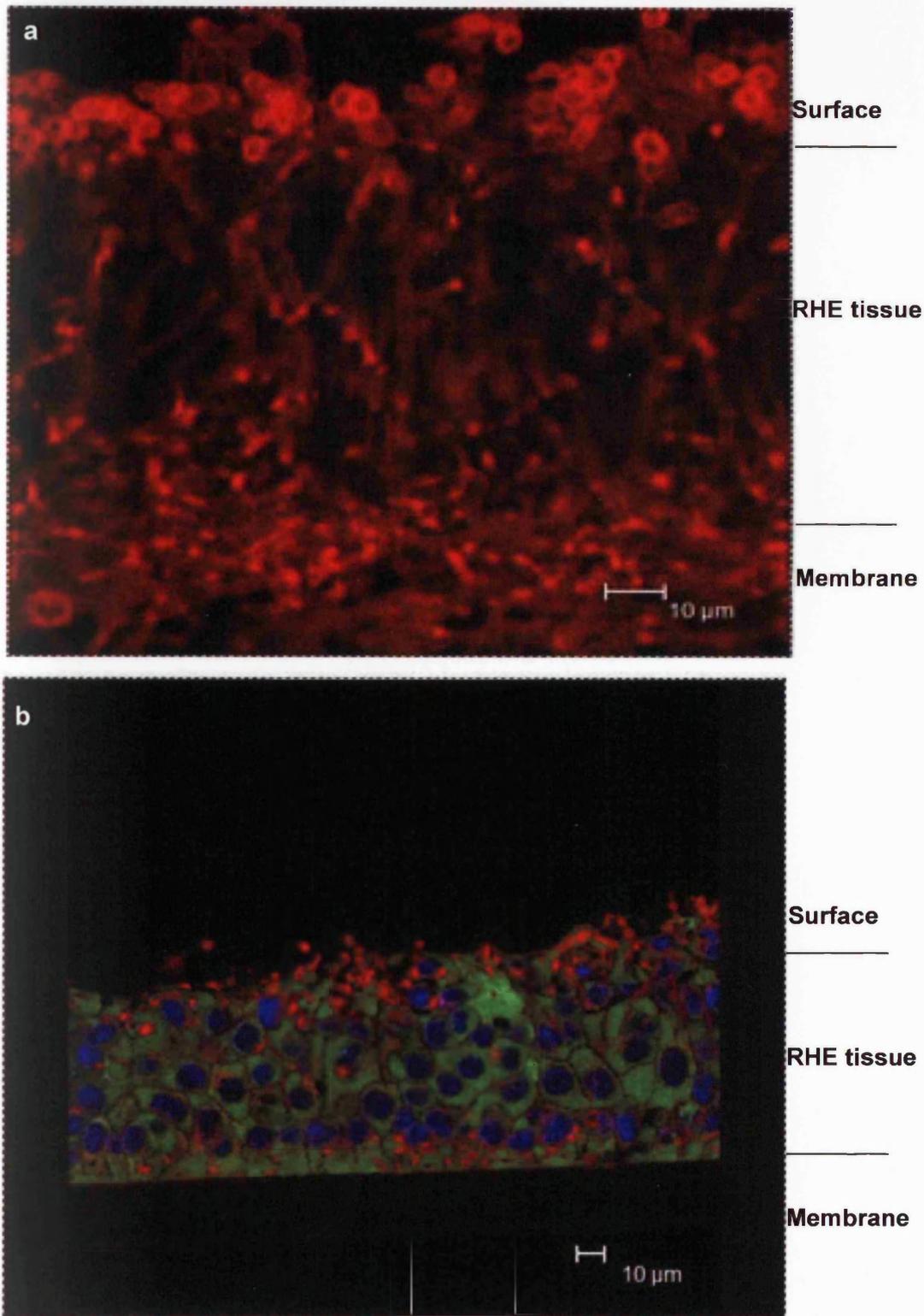
Fig. 2.5 Infection of RHE by *C. albicans* high invader strain (PTR/94) and visualised using CLSM a) 24h incubation, stained with ConA-Alexa 594 (red) and b) 12h incubation, triple stained The nuclei are stained with Hoechst (blue), the epithelial cells are stained with pan-cytokeratin antibody – Alexa 488 (green) and the *Candida* are stained with ConA-Alexa 594 (red) showing the pattern of hyphal invasion and surface colonisation.
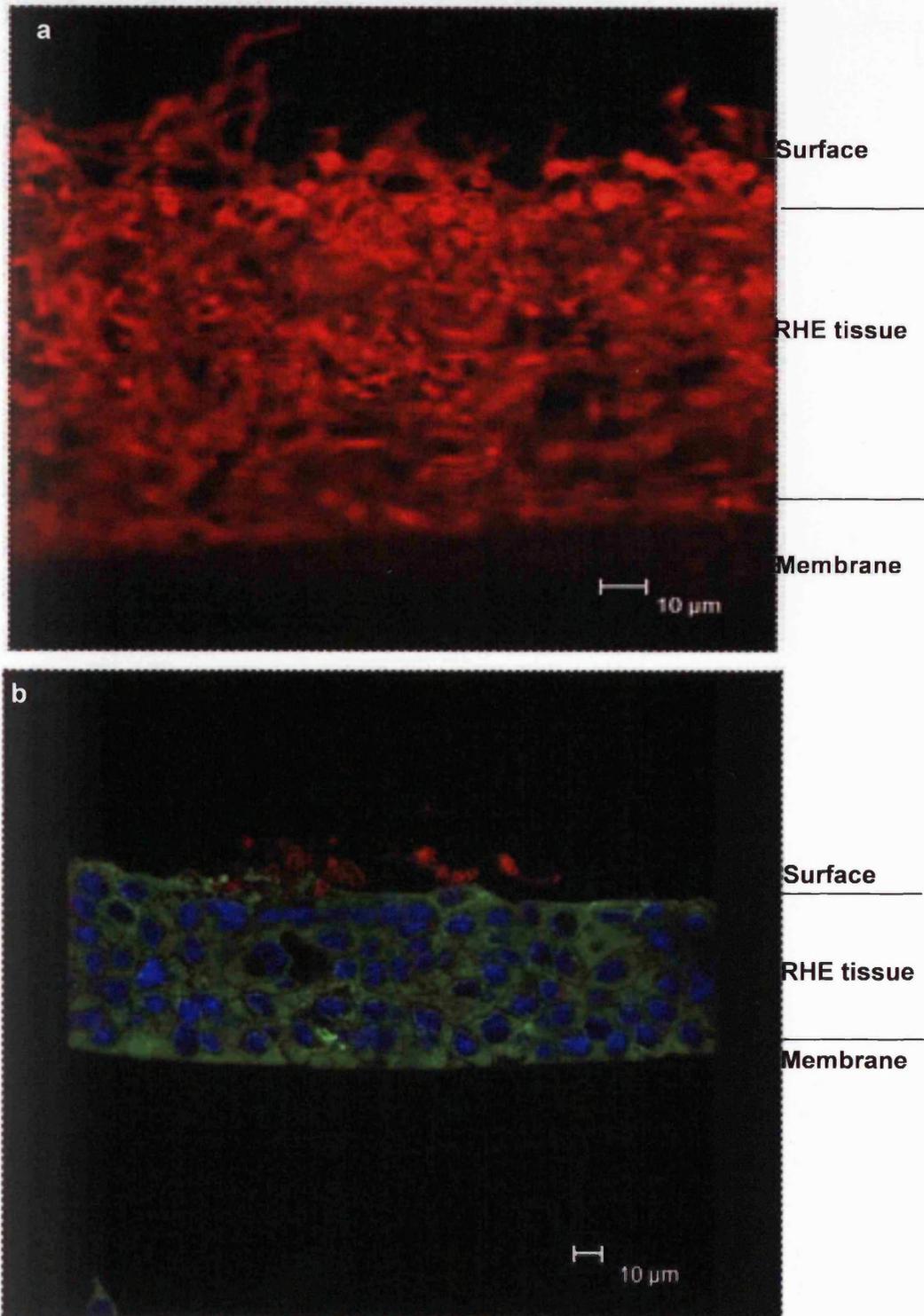
Fig. 2.6 Infection of RHE by *C. albicans* low invader strain (705/93) and visualised using CLSM a) 24h incubation, stained with ConA-Alexa 594 (red) and b) 12h incubation, triple stained. The nuclei are stained with Hoechst (blue), the epithelial cells are stained with pan-cytokeratin antibody – Alexa 488 (green) and the *Candida* are stained with ConA-Alexa 594 (red) showing the pattern of hyphal invasion and surface colonisation.
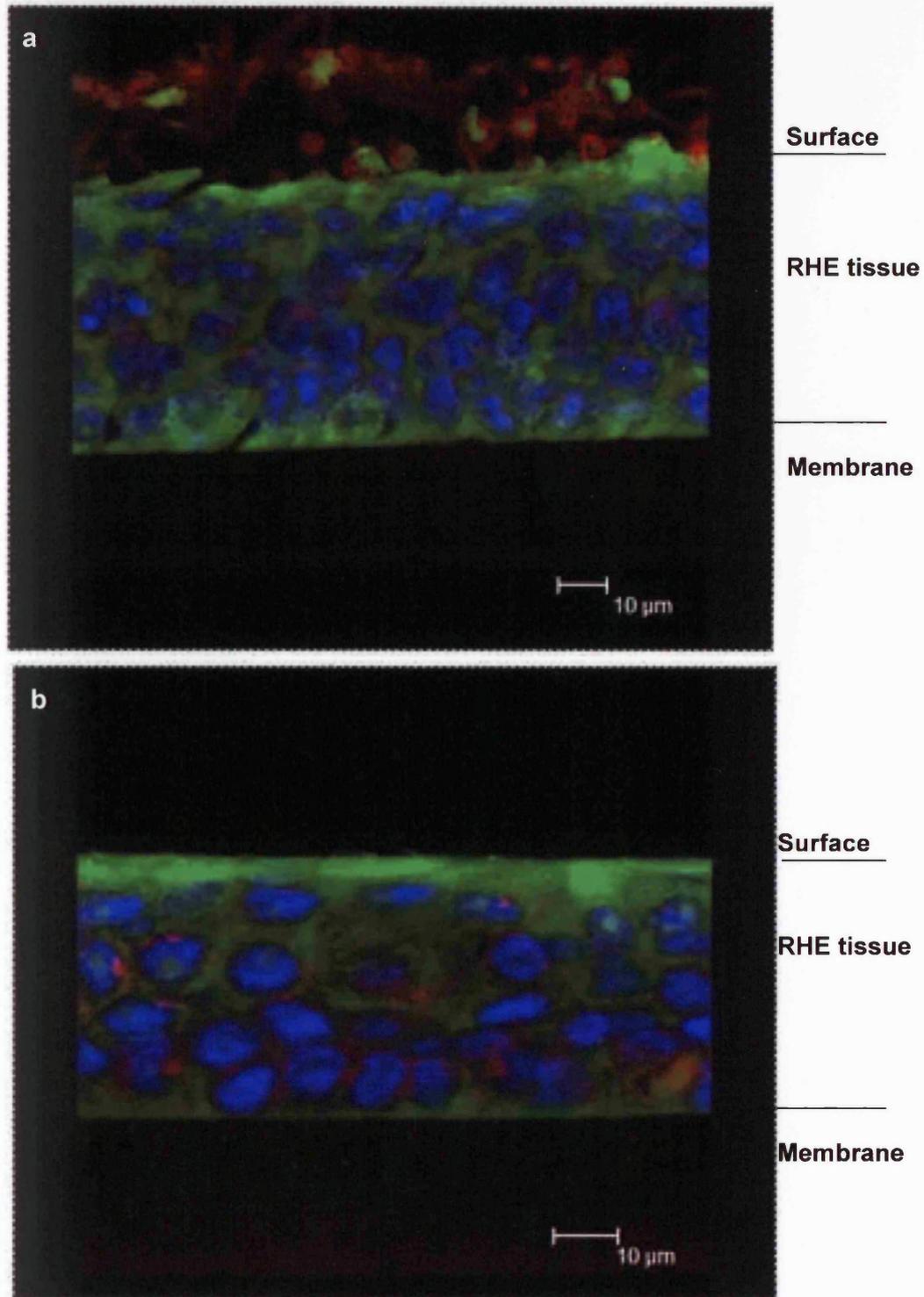
Fig. 2.7 *Candida albicans* infected oral epithelium showing the pattern of hyphal invasion; a) low invasion by strain PB1/93; b) no-invasion by strain 40/01 The nuclei are stained with Hoechst (blue), the epithelial cells are stained with pan-cytokeratin antibody – Alexa 488 (green) and the *Candida* are stained with ConA-Alexa 594 (red).

2.4.3.3 Pattern of tissue invasion

CLSM analysis of triple stained tissue shows that hyphae were actually capable of moving both around and directly through individual epithelial cells (strains 135BMS2/94 and PTR/94; Fig. 2.8) respectively. CLSM analysis revealed that hyphal penetration of the RHE tissue occurred both vertically and horizontally within the RHE. Hyphae that were seen to pass through all the epithelial layers subsequently spread and grew horizontally across the bottom of the RHE (Fig. 2.9). For example, strain 243/00 (Fig.2.9a) demonstrated a 'horizontal pathway' of hyphal invasion whilst strain 135BM2/94 (Fig. 2.9b) exhibited a vertical path of hyphal invasion. These strains reverted to the yeast morphology once total tissue invasion had occurred. In the case of 'high invading' isolates (e.g. 135BM2/94, PTR/94 and 1190/97), a more direct and vertical invasion profile occurred (Fig. 2.9b), possibly suggesting that these strains were less impeded by the tissue. Significantly, the latter three isolates all originated from cases of CHC. Furthermore, it was also evident that 4 of the 5 strains classed as 'high invaders' originated from CHC infection. It was also apparent for some strains that yeast growth occurred within the RHE, with growth originating from the budding of hyphal branches (Fig. 2.10a and 2.10b).
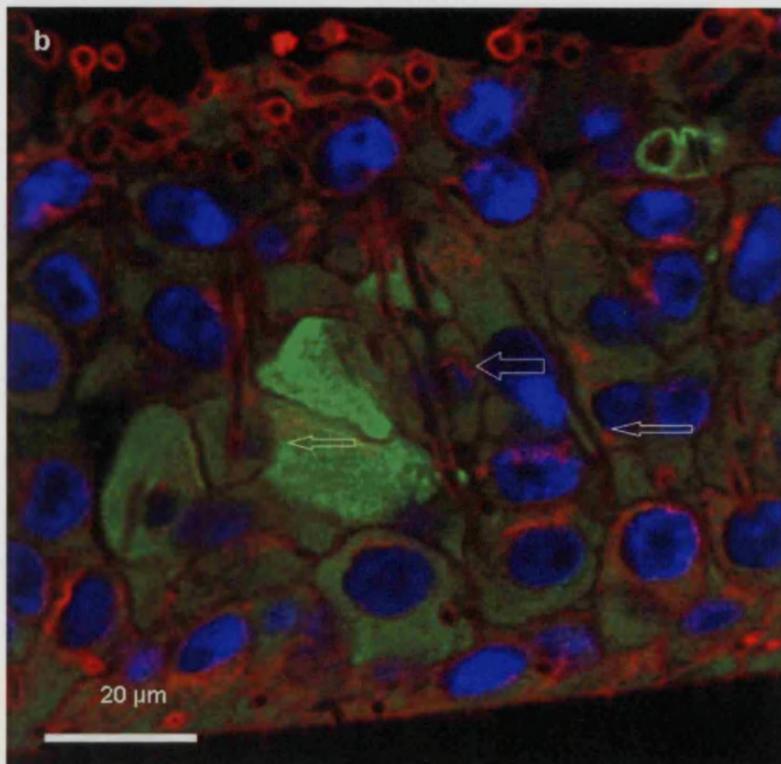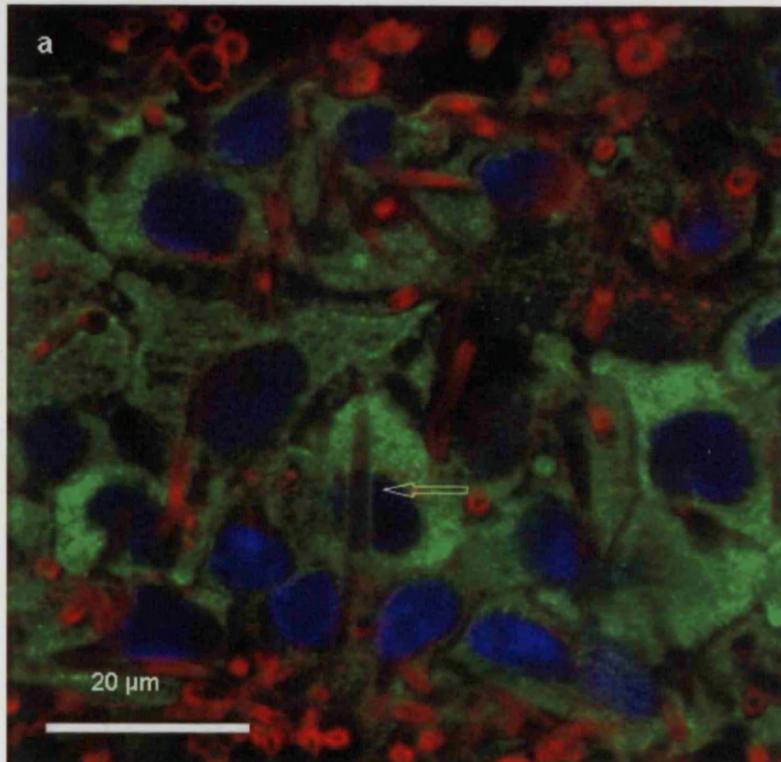
Fig. 2.8 Tracking of invading *C. albicans* hyphae a) strain 135BM2/94 and b) strain PTR/94 during infection of RHE, illustrates hyphae directly penetrating epithelial cells as well as circumventing them. Arrows indicate an example of hyphal invasion. The nuclei are stained with Hoechst (blue), the epithelial cells are stained with pan-cytokeratin antibody – Alexa 488 (green) and the *Candida* are stained with ConA-Alexa 594 (red).
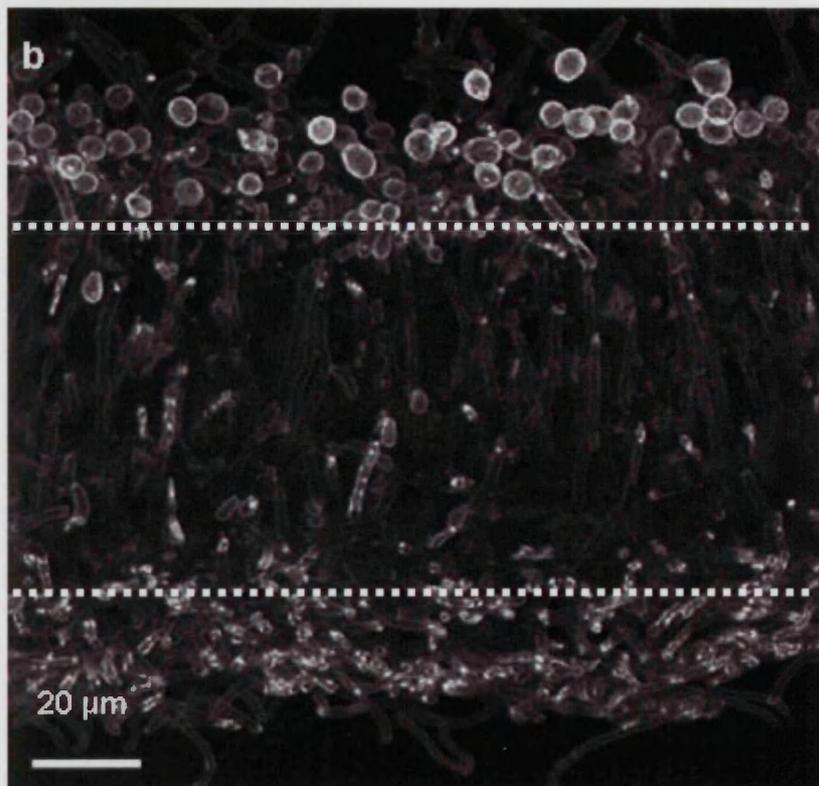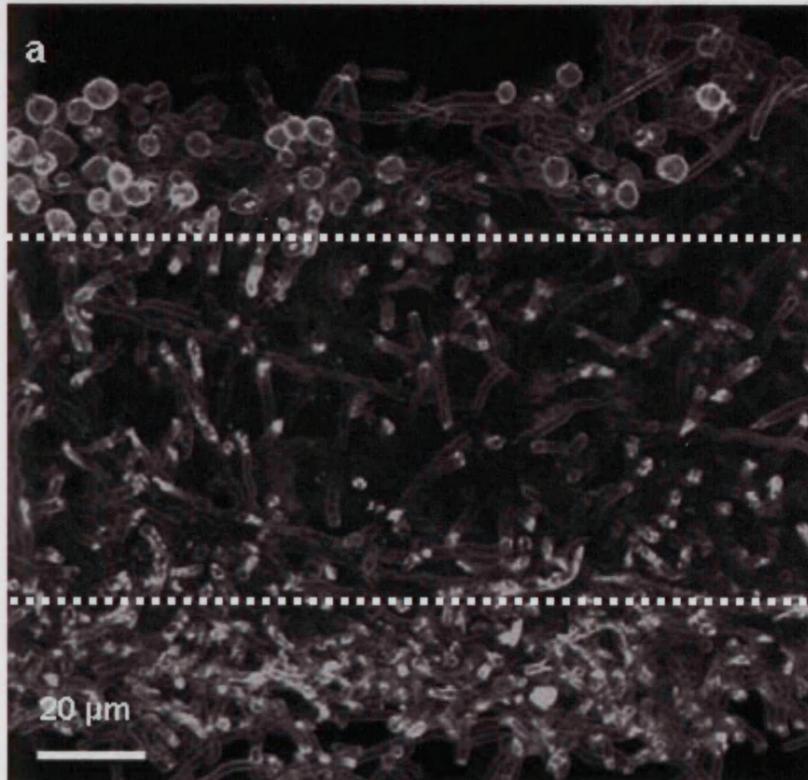
Fig. 2.9 Pattern of *Candida albicans* hyphal invasion of infected RHE a) heterogenous hyphal network during *C. albicans* infection by strain 243/00; b) vertical hyphal invasion during *C. albicans* infection by strain 135BM2/94.
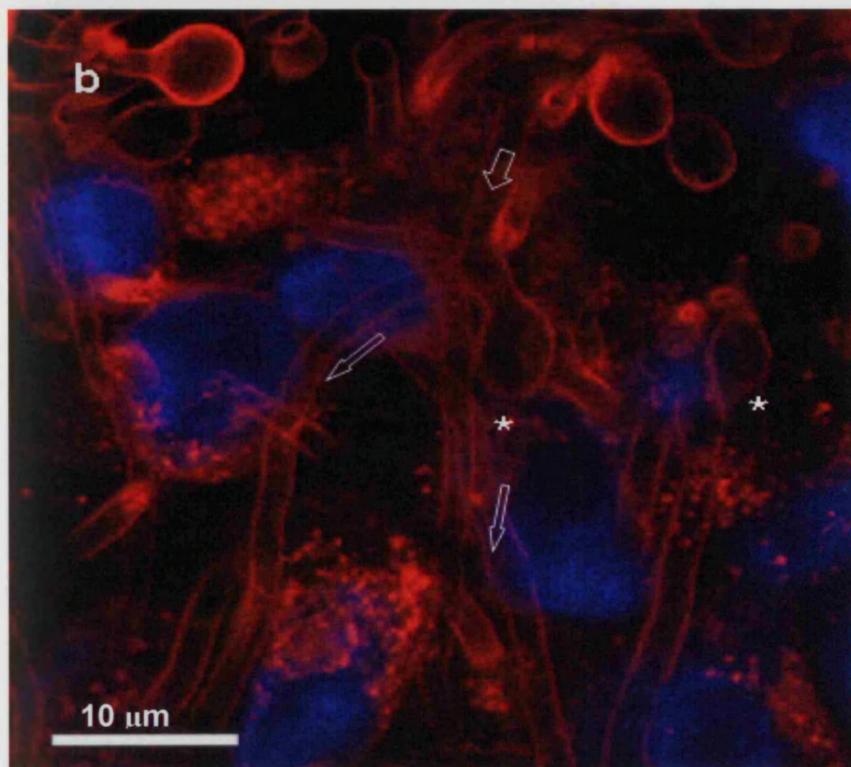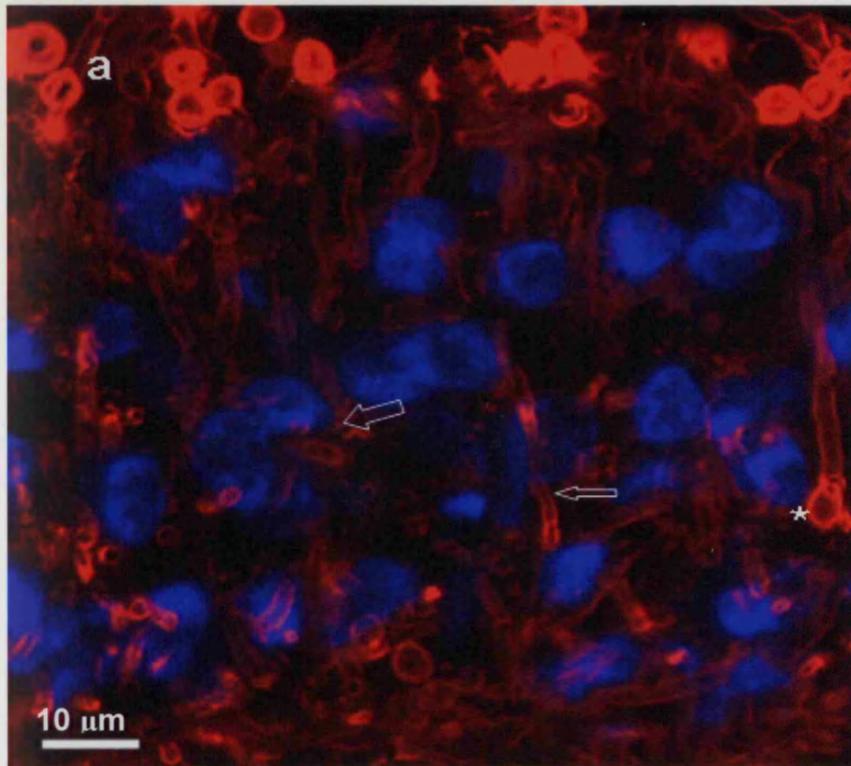
Fig. 2.10 (a) Nuclear penetration by hyphae of strain 1190/97, (b) nuclear penetration and budding of yeast from hyphal branch by strain 135BM2/94. Arrows indicate the example of hyphal invasion type; * indicates budding.

2.5 Discussion

The pathogenic behaviour of opportunistic *Candida* species is dependent on expression of certain virulence factors (*e.g.* hydrolytic enzymes) (Schaller et al., 2002). Recently, the organisms ability to form biofilms has been recognised as one of these factors (Ramage et al., 2005; Richard et al., 2005). *Candida albicans* is dimorphic, (*i.e.* it is capable of reversible transition between a yeast and hyphal form) and the morphogenic transition between these forms is often stimulated by growth in the host and associated with host tissue invasion (Gow et al., 2002). It has been suggested that hyphae grow by tip extension and can generate significant tip pressures for penetration (Gow et al., 2002). The underlying hypothesis for this present study was that different strains of *C. albicans* from specific types of oral infection would produce distinct characteristics of RHE infection and biofilm formation. To compare biofilm formation and subsequent tissue invasion by *C. albicans,* the use of the RHE model together with CLSM was chosen. Previous studies using the multilayered RHE model have shown its histological similarity with healthy oral epithelium and also described the biofilm-like infection of the tissue by *C. albicans* (Jayatilake et al., 2005).

Compared with standard light microscopy, CLSM provides much more detailed information on *C. albicans* tissue invasion. Previous use of CLSM to assess *C. albicans* interaction with oral epithelium has been limited. Studies involving infection of the FaDu oropharyngeal epithelial cell line (isolated from a pharyngeal squamous cell carcinoma, American Type Culture Collection) with *C. albicans* have previously described the use of CLSM in determining fungal-epithelial cell interaction mechanisms (Park et al., 2005). Additional work examining endothelial infection by *C. albicans* using CLSM has elegantly revealed a self-induced endocytotic mechanism exhibited by candidal hyphae as a means of tissue invasion (Phan et al., 2005).

The commercially available RHE employed in this present study has been used with success as a basis for *in vitro Candida* infection models. A possible limitation of the RHE system is the lack of certain host immune

responses. It has however been shown that pro-inflammatory cytokine responses by *in vitro* epithelial cell lines including RHE infected with *Candida*, appear to be maintained, and have actually been shown to vary with *C. albicans* of different invasive potential and virulence (Schaller et al., 2002; Villar et al., 2005). This present Chapter describes for the first time the CLSM based characterisation of actual tissue infection and biofilm formation by *Candida*.

Using conventional microscopy procedures, it has previously been difficult to ascertain the manner by which hyphae migrate through tissue. The CLSM approach used in this study, conclusively demonstrated that, in addition to being able to move between epithelial cell junctions, hyphae can track directly through keratinocytes. Epithelial cell penetration was also more strongly associated with strains that demonstrated the vertical invasion profile depicted in Fig. 2.9b compared with the less direct invasion profile seen in Fig. 2.9a. The exact mechanism of hyphal penetration remains unclear. It may occur either through pressure exerted from the growing tip of the hyphae or as mentioned earlier by inducing an endocytosis process, alternatively, invasion may be facilitated by extracellular hydrolytic enzymes promoting the passage of hyphae.

It is also important to mention that biofilm structure and the proportions of yeast- and hyphal- cells are strongly influenced by parameters such as medium composition, temperature and the nature of the substratum (Kumamoto, 2002). The key component of invasive growth is hyphal penetration. The RHE model used in this study showed strain differences between the 19 strains and was highly suitable for characterisation.

The levels of candidal invasion reported in this present study were largely comparable to those previously found by the research group at the Cardiff Dental School using standard light microscopy and PAS histological staining analysis (Bartie et al., 2004). This was reassuring, particularly as the methods of assessment between the two studies were different and were

performed by separate operators. All isolates previously deemed to be 'high invaders' (Bartie et al., 2004) were also identified as such in this present study. In addition, three of the four isolates originating from normal healthy oral mucosa exhibited low RHE invasion and two of these were also previously considered to be low invaders (Bartie et al., 2004). This latter finding for commensal strains could indicate a limited ability of such isolates to produce the necessary virulence attributes required for invasion. This was also reflected by the relatively low level of RHE colonisation by these isolates, together with an apparent reduced ability to produce hyphal forms. It is, however, worth noting that invasion, albeit at a low level, did occur with three of the four commensal isolates and it is therefore possible that given an appropriate oral environment, these 'commensal' isolates could instigate infection. Overall, the ability of *C. albicans* to invade appeared to correlate with the isolates' capacity to exhibit yeast/hyphal transition.

Interestingly, in all but one case, the 'high invaders' revealed a predominance of hyphae within the tissue. This was not surprising, as movement of yeast from the surface of the RHE would have been physically difficult, as traditionally, yeast are considered to lack the migratory ability possessed by hyphal elements (Gow et al., 2002). For a number of tissues, yeast predominated on the surface of the RHE, whilst hyphae predominated within and below the tissue. This would suggest that either the epithelium provided a trigger for morphological transition following contact or that only hyphae penetrated the tissue and thus predominated due to this selective process. To support the latter, previous work using CLSM to study endothelium invasion by *C. albicans* has shown that hyphae can induce their own endocytosis, an ability not possessed by yeast (Phan et al., 2005). For most strains used in the present study, the same morphological type was evident in either the presence or absence of tissue (Table 2.7) and would suggest that hyphal morphology was not induced through contact with the epithelium and supports the view that hyphae are the principle invasive forms of *C. albicans*.

Four of the five strains classed in this study as 'high invaders' (135BM2/94, PTR/94, 324LA/94, 1190/97) originated from CHC and this might suggest an inherently greater ability of these strains to invade the oral epithelium. A particular pathogenic feature of *C. albicans* that is essential to the development of CHC is the ability to invade the oral mucosa. Furthermore, this feature of mucosal invasion is notably distinct from other forms of oral candidosis and indeed serves as a diagnostic marker of CHC (see section 2.1.2.4). The fact that four other CHC strains were 'low invaders' again highlights the undoubted importance of additional host factors in the development of CHC infection. Indeed, studies performed by Soll (2002) emphasise the importance of candidal adaptation to host environments as being critical in determining whether commensalism or virulence results. Evidence from complex DNA profiling of *C. albicans* populations currently suggests the absence of true commensal and pathogenic strains, and advocates rapid phenotypic adaptation as a key component in promoting a particular oral status (Soll, 2002). The advantage of the current model is that strain differences can be ascertained under the standardised conditions used. An ability to associate invasive features with the clinical origin of the isolates would be a significant advantage, as it would enable the early identification of strains with the potential to cause CHC, and possibly influence patient management.

2.6 Summary

A suitable method of assessing biofilm formation and tissue infection by *C. albicans* using an *in vitro* tissue model using CLSM has been developed. Using this RHE model we have shown that strain differences exist and may relate to the clinical origin of *Candida*. The differences evident related to adhesion, invasion and hyphal formation. *Candida* hyphae were seen to develop as a complex network of filaments within the tissue and were seen to physically penetrate cells and exhibit budding within the tissue. In summary:

1) A suitable *in vitro* biofilm model for *C. albicans* infection was established using the RHE.

2) Seventeen out of 19 *C. albicans* strains invaded the RHE. Using this model it has been shown that strain differences exist and may relate to the clinical origin of the *Candida*. Factors associated with *C. albicans* in addition to those of the host could therefore be important in the progression of oral infections such as CHC.

3) Strain variation was evident based on the level of adherence, tissue penetration and pattern of invasion. Heterogeneous tissue invasion was evident. *Candida* hyphae develop as a complex network of filaments within the tissue and are seen to physically penetrate the cells (nuclei) and exhibit budding within the tissue.

Chapter 3

Expression of *Candida albicans* virulence genes in an *in vitro* tissue model

3.1 Introduction

Candida albicans is an opportunistic fungal pathogen of humans and as a result causes infection only in suitably debilitated individuals. It is not surprising therefore that no single predominant virulence factor is currently recognised for this normally harmless organism. However, a number of putative virulence factors have been proposed.

Candida albicans produces a variety of secreted hydrolytic enzymes (Hube et al., 1994; Hube et al., 2000; Ibrahim et al., 1995; Monod et al., 1998) that may enable it to persist and cause disease within a suitably compromised host (Schofield et al., 2003). Differences in such gene expression may be key to the pathogenic potential of a given strain. Furthermore, even for individual strains, gene expression can give rise to phenotypic differences as seen between biofilm and planktonic cells. *Candida albicans* infections (see Chapter 2, section 2.1.2) are common, debilitating and an important clinical problem (Naglik et al., 2004). In the case of oral infections, candida biofilm formation is considered to be a key factor in the cause of the infection, *e.g.* denture stomatitis is associated with candidal biofilms on the acrylic surfaces of the denture (Douglas, 2003). The adherence of *Candida* to oral mucosal surfaces is critical for the persistence of the organism in the mouth (Sitheeque and Samaranayake, 2003). Pseudomembranous candidosis could be considered to be a complex biofilm, although this concept has still not been well recognized (Jin et al., 2003).

It remains unclear whether 'biofilm-like' growth imposes a significant change in *C. albicans* physiology and whether this change is reflected in a particular pattern of gene expression, although some differential properties of biofilms have been observed and several gene expression changes have been reported. Genome-wide approaches are now available that allow this question to be more fully investigated (Berman and Sudbery, 2002; Garcia-Sanchez et al., 2004). Previous studies, using microarray technology, assessed *C. albicans* biofilm transcriptional profiles to identify which genes were involved in the biofilm development process (Cao et al., 2005; Garcia-

Sanchez et al., 2004; Murillo et al., 2005). The data from this previous study suggest that the sulphur-amino acid biosynthesis pathway was an important feature of *C. albicans* biofilms (Garcia-Sanchez et al., 2004).

3.1.1 *Candida* virulence factors and associated gene families

3.1.1.1 Secreted aspartyl proteinase (SAP) production

Proteinases are classified on the basis of their catalytic mechanism and at present four classes are recognised: I) serine, II) cysteine, III) metallo and, IV) aspartyl proteinases.

Aspartyl proteinases are ubiquitous in nature and are involved in a myriad of biochemical processes. Aspartyl proteinases include HIV aspartyl proteinase, and pepsin and rennin in humans (Naglik et al., 2004). It is these aspartyl proteinases that are associated with *C. albicans* virulence. Candidal-proteinases all have a broad spectrum of activity, and salivary proteins such as mucin, lactoferrin and secretory immunoglobulin A (sIgA) are all degraded by these enzymes. Based on current data, the main roles of candidal aspartyl proteinases are to provide nutrition for the cells, to aid penetration and invasion and to evade immune responses (Naglik et al., 2003). MacDonald and Odds (1983) first demonstrated the relationship between proteinase production and pathogenicity using chemically induced mutants of *C. albicans* with decreased proteolytic activity. These mutants were shown to be less virulent in murine models compared with the wild type strains. Figure 3.1 shows a hypothetical model for the correlation of *SAP* gene expression with function in *C. albicans* pathogenicity.

In recent years there has been a relatively large amount of work aimed at studying SAPs of *C. albicans*. At present, 10 different genes have been identified that encode for *C. albicans* SAPs (Hube, 1996; Hube et al., 1997; Sanglard et al., 1997). One explanation for the existence of the 10 different *SAP* genes may be the necessity for specific and optimised proteinase production during different stages of an infection (Hube and Naglik, 2001).

Biofilm formation
SAP5, SAP6 and SAP9
are more highly expressed
in biofilms compared with
planktonic ells

Adhesion
SAP1 to SAP3 are
involved in
adhesion to BECs

Cavitation
SAP1 possesses the ability to
cavitate skin; pepstatin A
inhibits cavitation

Phenotypic switching
SAP1 and SAP3 are
expressed in opaque but
not in white cells (WO-1
strain). Other SAP specific
expression profiles in other
switching strains are highly
likely

Invasion / Tissue damage
Sap1-3 are critical for
mucosal infection and
probably contribute to
systemic infections
Sap4-6 contribute to systemic
infections

Sap gene
family

Dimorphism
Expression of SAP4,
SAP5 and SAP6 is hyphal
specific

Interaction with the host immune system
SAP2 hydrolyses proteins of the immune
system. SAP4-6 are necessary for survival
and escape from macrophages

Nutrient acquisition
Secreted proteinases hydrolyse
complex protein and provide
nitrogen for the cells

Fig. 3.1 Correlation of *SAP* gene expression with function in *C. albicans* pathogenicity. (Adapted from Naglik *et al.* (2004).

The SAPs are essential for growth when protein is the sole nitrogen source (Hube et al., 1994; Naglik et al., 2004). SAPs are characterised by functioning in an acidic environment and being inhibited by pepstatin A, a hexapeptide from *Streptomyces* (Hoegl et al., 1996; Watts et al., 1998). It has been reported that pepstatin A blocks invasion of host tissue by hyphal cells at neutral pH and reduces the adherence of yeast cells to various cell types (Watts et al., 1998). *Candida albicans* is not the only *Candida* species known to produce extracellular proteinases. *Candida tropicalis* is thought to possess four *SAP* genes (Zaugg et al., 2001), whereas *C. parapsilosis* possesses at least two *SAP* genes (De Viragh et al., 1993). Proteolytic activity has also been found *in vitro* by *Candida dubliniensis* (Monod and Borg-von, 2002).

One approach to study the role and expression of SAPs involves the proteolytic degradation of bovine serum albumin (BSA) which is spectrophotometrically assessed by determining the generation of trichloroacetic acid (TCA) soluble BSA fragments (Hube et al., 1997; Kuriyama et al., 2003). Reverse-transcriptase polymerase chain reaction (RT-PCR) has also been used to detect mRNA products from *SAP* genes (section 3.3.6).

3.1.1.2 Phospholipase (*PL*) expression

Phospholipases represent a heterogeneous group of enzymes with the ability to hydrolyse phospholipids into fatty acids and other lipophilic products. Besides *C. albicans*, PLs are also considered to be virulence factors for other fungi, *e.g. Aspergillus fumigatus* or *Cryptococcus neoformans* and for many pathogenic bacteria, *e.g. Pseudomonas* species, *Staphylococcus aureus* etc. (Schaller et al., 2005).

Candida albicans can exhibit lipolytic activity through the production of four classes of phospholipases termed A, B, C and D (Barrett-Bee et al., 1985). As these enzymes degrade phospholipids to release free fatty acids, damage to host cell membranes and membrane bound vesicles can therefore theoretically occur, which could promote cell lysis or expose receptors to

facilitate adherence. Samaranayake *et al* (1984) examined 41 oral isolates of *Candida* species, and found that 79% of *C. albicans* isolates produced extracellular phospholipase, which was not detectable in isolates of *C. tropicalis*, *C. glabrata* or *C. parapsilosis*. However, subsequent studies have shown that *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *C. lusitaniae* and *C. krusei* can all secrete phospholipases (Schaller et al., 2005).

It is important to note that phospholipase activity is inversely proportional to the glucose or galactose concentration in the growth medium (Samaranayake et al., 1984) and appears to be greatest at the tip region of hyphae (Pugh and Cawson, 1975). In addition, high phospholipase activity positively correlates with enhanced adherence of *C. albicans* buccal epithelial cells (BEC) and increased virulence in mice (Barrett-Bee et al., 1985). Moreover, it has been demonstrated that blood isolates of *C. albicans* from patients with systemic candidosis produce significantly more phospholipase than commensal strains and are more invasive in mouse models (Ibrahim et al., 1995).

A study by Price *et al* (1982) demonstrated extracellular phospholipase production by *C. albicans* isolated from urine, blood and wounds, involving a simple egg-yolk agar plate assay. In this method, *Candida* were cultured on egg yolk agar, incubated for 48h at 37°C and any hydrolysis of lipid substrates present in egg-yolk resulted in the formation of a calcium complex with fatty acids released by the action of the secreted enzymes (Price et al., 1982). Furthermore, molecular techniques (such as RT-PCR, Northern- and Western- blot analysis) can also be used to detect phospholipase gene expression in *C. albicans*.

3.1.1.3 Agglutinin-like-sequence (*ALS*) gene expression

Candida albicans, like many fungi, contain a family of cell-wall glycoproteins, known as adhesins that confer unique adhesion properties (Guar and SA, 1997; Hoyer, 2001; Verstrepen et al., 2004). Eight *Candida ALS* genes (*ALS1* to *ALS7* and *ALS9*) encoding large glycoproteins have been shown to promote candidal adhesion to host surfaces (Guar and SA, 1997; Hoyer,

2001). Although these proteins share a similar three-domain structure, sequence differences between the ALS proteins can be large, suggesting that the proteins may have different functions (Hoyer, 2001). The gene family is called *ALS* due to the resemblance of domains of its encoded proteins to α-agglutinin, a cell-surface adhesion glycoprotein in *Saccharomyces cerevisiae* (Hoyer et al., 1995). In *S. cerevisiae*, heterologous expression of *ALS* confers an adherence phenotype, suggesting ALS proteins function in adhesion to host surfaces, which could correlate with *Candida* pathogenesis (Calderone and Braun, 1991; Fu et al., 1998; Guar and SA, 1997). Similar to the *SAP* gene family, *ALS* genes are differentially expressed under various conditions and growth phases including morphological switching, growth medium composition and strain type of *C. albicans* (Hoyer et al., 1998a; Hoyer et al., 1998b; Hube et al., 1994).

It has been suggested that the *ALS* gene family is important in *C. albicans* pathogenesis (Hoyer, 2001). Green *et al.* (2004) used an RT-PCR assay for *ALS* genes to analyse *C. albicans* RNA expression from *in vitro* models of oral candidosis and denture and catheter biofilms. In the study by Green *et al.* (2004) an infected RHE model was used and consistent expression of *ALS1*, *ALS2*, *ALS3* and *ALS4* was found as destruction of the RHE progressed in contrast to the more sporadic detection of *ALS6* and *ALS7* gene. Another study focused on human clinical vaginal specimens and two models of vaginal candidosis (Cheng et al., 2005). In this work *ALS1, ALS2, ALS3* and *ALS9* were detected most frequently and expression of *ALS4* and *ALS5* were detected least frequently (Cheng et al., 2005). It has been proposed that *Candida* virulence properties may act synergistically to enhance the virulence properties of *C. albicans* (Felk et al., 2002; Korting et al., 2003; Schroppel et al., 2000; Schweizer et al., 2000; Srikantha et al., 2000; Staib et al., 2002).

3.1.1.4 Morphology controlling genes
The morphological flexibility of *C. albicans* is regulated by a number of possible cross-talking signal transduction pathways (Ernst, 2000; Felk et al.,

2002). Efg1 and Cph1 are key transcriptional factors that regulate the yeast-to-hypha transition and are therefore considered to be important virulence attributes (Felk et al., 2002; Lo et al., 1997). It is also believed that these factors, and especially Efg1, regulate the expression of hyphal-associated genes, *e.g. SAP4-SAP6*. The relevance of these genes for *Candida* infections was studied using strains which lacked functional Efg1, Cph1 or both factors during interaction of the oral epithelium and polymorphonuclear neutrophils (PMNs). These studies confirmed that Efg1 was a key regulator for virulence during interaction with human epithelial cells and PMNs by regulating dimorphism and by influencing the expression of hyphal-dependent and hyphal-independent aspartyl proteinase genes during experimental oral infection (Korting et al., 2003).

3.1.2 Reconstituted human epithelium

In the present Chapter, a human *in vitro* tissue model was used to investigate the expression of *SAPs*, *PL* and *ALS* by *C. albicans* strains. The RHE used is a 0.5 cm² epithelium reconstituted by airlifted culture of transformed human keratinocytes derived from a squamous cell carcinoma (SCC) of the buccal mucosa (as previously described in Chapter 2). Previous investigations have used *in vitro* models of oral candidosis based on the RHE model. *SAP* gene expression has previously been examined in this commercially available RHE to assess the role of the genes in tissue invasion (Schaller et al., 1998). These experiments showed temporal expression of *SAP* genes (*SAP1*, *SAP3*, *SAP6*, *SAP8*, and *SAP2*) for *C. albicans* SC5314. Although the hydrolytic enzymes *in vivo* have not been well-defined, possible roles for them include the acquisition of nutrients (Hube et al., 1994), promotion of adherence (Watts et al., 1998) and degradation of gastric mucosa (Colina et al., 1996; Mukherjee et al., 2001). *SAPs* have also been implicated in both the evasion and destruction of host immune system molecules (Hube, 1996).

The RHE model has previously proved to be very effective for the direct observation of *C. albicans* virulence gene expression but nevertheless the data has to be interpreted carefully. The model does not wholly represent the *in vivo* situation in human patients. Parameters such as pH changes,

nutrient limitation, competition with the oral flora, and cell-mediated and cytokine responses are likely to have a profound effect on the ability of *C. albicans* to grow unchallenged on the mucosal surface (as in the models) and also to provide strong local pressures that may influence the differential expression of the *SAP* gene family (Naglik et al., 2003). However, studies have shown that proinflammatory cytokine responses by *in vitro* epithelial cell lines, including RHE infected with *Candida*, appear to be maintained and actually have been shown to vary with *C. albicans* strains with different invasive potential and virulence (Schaller et al., 2002; Villar et al., 2005).

3.2 AIMS

The RHE provides an *in vitro* model that allows study of the organism in an environment closer to that of the *in vivo* situation than previously used approaches. Culture of *Candida* on the RHE results in a "biofilm"-like appearance of growth as evident in Chapter 2 of this thesis and by other studies (Schaller et al., 1999; Schaller et al., 1998). The hypothesis of this study was that mRNA transcripts of specific virulence genes correlated with the type of infection and colonisation profiles of *C. albicans* in the RHE model as described in Chapter 2. To test this hypothesis, the specific aims were:

1. To investigate whether candidal biofilms on the RHE exhibited a different profile of virulence gene expression compared with equivalent cultures without the RHE.

2. To determine whether the presence of mRNA from the gene families correlated with the profile of infection and biofilm formation for the different strains.

3. To compare a range of *C. albicans* strains from different oral conditions, to ascertain whether inherent strain differences exist with respect to gene expression.

3.3 Materials and Methods

3.3.1 Preparation of *C. albicans* for infection of RHE

Candida albicans strains (n=19) were cultured on Sabouraud Dextrose Agar (SDA; LAB M, Bury, UK) at 37°C for 24 h. Subcultures were used to inoculate 10 ml of Yeast Nitrogen Base (YNB; BD Diagnostics, Cowley, UK) medium supplemented with 0.5% glucose (w/v). This medium was then incubated for 12 h at 37°C. Yeast cells were harvested by centrifugation and washed (×3) with phosphate buffered saline (PBS). The resulting yeast cells were enumerated using a haemocytometer and a total of 4×10^7 yeast cells in maintenance medium were added to a microtitre plate well containing RHE in a CO_2 humidified atmosphere and incubated at 37°C for 24 h. The preparation of RHE for use in these experiments was as previously described in Chapter 2 (sections 2.3.2.1; 2.3.2.3 and 2.3.2.4).

3.3.2. Preparation of *C. albicans* for control gene expression comparison (without RHE)

In addition to *C. albicans* infecting the RHE, gene expression was also assessed for strains of *C. albicans* cultured in the tissue maintenance medium under an identical culture environment, but in the absence of the RHE tissue.

3.3.3 RNA extraction protocols for *C. albicans*

Two basic approaches were initially evaluated for RNA extraction from *C. albicans*. These were the use of Ultraspec (Biotecx Laboratories, Texas, USA) reagent and also the use of the RNeasy mini kit (Qiagen, West Sussex, UK) with physical disruption using a bead-beater. To assess these RNA extraction protocols a standardised preparation of *C. albicans* was prepared that would induce mRNA expression of *SAP*, *PL* and *ALS* genes. Briefly, these cells were prepared as follows.

All *C. albicans* isolates (n=19) were cultured on Sabouraud Dextrose Agar (SDA) at 37°C overnight, then subcultured into yeast extract peptone dextrose (YEPD) broth (1% w/v yeast extract, Oxoid; 2% w/v peptone, Oxoid; 2% w/v dextrose) at 37°C for 24 h. A 200-µl volume of the suspension was inoculated into 5 ml of yeast carbon base-bovine serum albumin (YCB – BSA) medium (1.17% w/v yeast carbon base; Becton Dickinson, Oxford, UK; 1% w/v glucose; 0.5% w/v bovine serum albumin; Sigma, Poole, UK; pH 5.6). Cultures were incubated at 37°C for 24 h.

For all RNA extraction protocols diethyl-pyrocarbonate (DEPC) treated water was used which was prepared by treating 1 L of distilled H_2O, with 2 ml of DEPC and incubating overnight at 37°C. After incubation the DEPC H_2O was autoclaved at 126°C for 30 min, to remove the DEPC.

3.3.3.1 Ultraspec RNA extraction

A 1.5-ml volume from the overnight YCB-BSA culture was centrifuged, the broth was removed and 1 ml of Ultraspec (Biotecx Laboratories Inc., Houston, USA) and 1.5-ml volume of sterile glass beads (0.5 mm diameter; Sigma, UK) were added and vortexed for 30 min at 4°C. The supernatant was transferred to a fresh 1.5 ml Eppendorf tube containing chloroform (200 µl) and the mixture was shaken vigorously. The preparation was placed on ice for 5 min, centrifuged at 12,000 *g* for 3 min and the RNA (top aqueous layer) was removed into a fresh Eppendorf tube. An equal volume of isopropanol (Sigma, UK) was added and the preparation centrifuged at 12,000 *g* for 10 min. The supernatant was decanted and the RNA pellet was washed twice with 75 % ethanol and air-dried in an incubator. The RNA pellet was suspended in 30 µl of DEPC treated water.

3.3.3.2 RNeasy RNA extraction

For this protocol, sterile glass beads were initially placed in a small bottle to which 100 ml DEPC H_2O was added and incubated overnight at 37°C. After incubation, the water was removed and the beads dried in an oven at 150°C.

Subsequently RLT extraction medium (Qiagen, Crawley, UK) and prepared glass beads (approx. 500 µl) were added to the prepared *C. albicans* and the mix was homogenised twice for 30 s using a Mini-BeadBeater-8 (Stratech Scientific Ltd., Soham, UK; Fig. 3.2). After cell disruption, the RNeasy Mini Kit (Qiagen) was used to complete total RNA extraction according to the manufacturers' recommended protocol.

3.3.4 Processing of infected RHE tissue for RNA extraction

After 24 h infection the RHE for RT-PCR was stored in a 2 ml microtube with RNAlater® (Ambion Europe Ltd, Huntington, UK) solution before RNA extraction and was maintained at -80 °C, until required. RNA extraction was then undertaken using one of the two protocols outlined above.

3.3.5 cDNA synthesis from extracted RNA

Potential DNA contamination was first removed from samples using RNase-free DNase I treatment following the manufacturer's instructions (Qiagen). cDNA synthesis involved the use of an oligo dT primer (250 ng, 5 µl per reaction; Promega, Southampton, UK), with Moloney Murine Leukemia Virus-Reverse Transcriptase (400 U, 2 µl per reaction; MMLV-RT, Promega). After incubation at 42°C for 1 h, the cDNA-RNA hybrid was denatured at 95°C for 5 min.

3.3.6 PCR on cDNA samples

PCR targeted a number of virulence gene families including secreted aspartyl proteinase (*SAPs*), phospholipases (*PLs*), and agglutinin-like sequence (*ALS*) genes. For each gene a specific primer pair was used to yield an amplicon of distinct size (Table 3.1a-c). The reaction conditions (Table 3.2) were as previously described for *SAPs* (Monod et al., 1998; Monod et al., 1994), *PLs* (Niewerth and Korting, 2001; Sugiyama et al., 1999), and *ALS* genes (Green et al., 2004). Control S14 (Cairns et al., 1997) primers were also used to detect human rRNA and additional primers were employed for the amplification of the candidal elongation factor 1β (EFB1) RNA (Schofield et al., 2003). The S14 control was used to confirm RNA recovery from the

tissue, whilst *EFB1* provided an ideal control gene for candidal RT-PCR as it is believed to be universally expressed by all forms of *C. albicans* (Schofield et al., 2003).
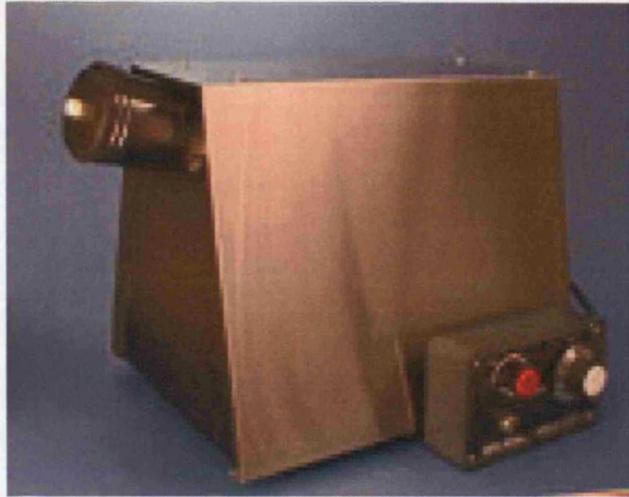
Fig. 3.2 Mini-BeadBeater-8 (Stratech Scientific Ltd.)

Table 3.1a Primers used to amplify hydrolytic enzyme (SAPs, PLs and ALS) gene sequences.

Primer	Sequence (5'-3') Forward/Reverse	Target	Expected product size (bp)
SAP1a F	ATG CCT TCC AAG CTG AAT TG	Secreted aspartyl	150
SAP1a R	CGT AGC TCA ACG GAG CAG TA	proteinases gene 1	
SAP1b F	GAC AAA TCA TTT TCG GTG GG	Secreted aspartyl	527
SAP1b R	GCA GCA ATG TTT GAA GCA GA	proteinases gene 1	
SAP2a F	CAA TGA AGC CGG TGG TAG TT	Secreted aspartyl	140
SAP2a R	CAT TAT CAA CCC CAC CGA AA	proteinases gene 2	
SAP2b F	CAA TGA AGC CGG TGG TAG TT	Secreted aspartyl	459
SAP2b R	AAA GAA GCA GCA AAT TCG GA	proteinases gene 2	
SAP3a F	CAA GGT GTT GCT GAT CAA	Secreted aspartyl	152
SAP3a R	GTG TCA GAA GCT GGA ACG GAA AT	proteinases gene 3	
SAP3b F	TGT GAG AAC CTC CTT CCC AG	Secreted aspartyl	365
SAP3b R	TCT CTT GAC GTT GAC GTT GG	proteinases gene 3	
SAP4 F	AAT GAT GTG GGC AAA AGA GG	Secreted aspartyl	329
SAP4 R	ACA CCA CCA ATA CCA ACG GT	proteinases gene 4	
SAP5 F	ATT AAT TGA TGC GGC TCC AG	Secreted aspartyl	498
SAP5 R	ACA CCA CCA ATA CCA ACG GT	proteinases gene 5	
SAP6 F	TCC AAA CCA ACG AAG CTA CC	Secreted aspartyl	445
SAP6 R	GCA GGA ACG GAG ATC TTG AG	proteinases gene 6	
SAP7 F	AGT GCA CAT TTC CCT AAC GC	Secreted aspartyl	434
SAP7 R	GAG CCA GGA GCA GTT GAT TC	proteinases gene 7	
SAP8 F	TTT GAC CGC ATT ACC AAT CA	Secreted aspartyl	161
SAP8 R	CAG CAG CCA ATT TAT CAG CA	proteinases gene 8	
SAP9 F	CCA TAG AAA CCG ACG AGG AA	Secreted aspartyl	153
SAP9 R	CAC CAG AAC CAC CCT CAG TT	proteinases gene 9	

Table 3.1b Primers used to amplify hydrolytic enzyme (PLs & controls) gene sequences.

Primer	Sequence (5'-3') Forward/Reverse	Target	Expected product size (bp)
PLB1 F	CAA CGA AGC GGT GTT GTC TA	Phospholipase	150
PLB1 R	TTG CTG CCA GAA CTT TTG AA	gene B1	
PLB2 F	GGC CAG ATG GAT CAG CTT TA	Phospholipase	154
PLB2 R	AAG TTC TGG GCA TCA CAT CC	gene B2	
PLC F	GGT TCA ATT CGG CTG GTG AT	Phospholipase	150
PLC R	TTG TTC ACC GGA ATG TCA AA	gene C	
PLD F	GAC AAA GCA GCG CCT AAA TC	Phospholipase	148
PLD R	CCC TTT TCC TGG TTT TGG AT	gene D	
EFB1 F	GAA CGA ATT CTT GGC TGA C	*C. albicans*	242
EFB1 R	CAT CAG AAC CGA ACA AGT C	elongation factor 1β	
S14 F	CAG GTC CAG GGG TCT TGG TCC	Human control	143
S14 R	GGC AGA CCG AGA TGA ATC CTC A	ribosomal RNA	

Table 3.1c Primers used to amplify hydrolytic enzyme (ALS) gene sequences.

Primer	Sequence (5'-3') Forward/Reverse	Target	Expected product size (bp)
ALS1 F ALS1 R	GAC TAG TGA ACC AAC AAA TAC CAG A CCA GAA GAA ACA GCA GGT GA	Agglutinin-like sequence gene 1	318
ALS2 F ALS2 R	CCA AGT ATT AAC AAA GTT TCA ATC ACT TAT TCT CAA TCT TAA ATT GAA CGG CTT AC	Agglutinin-like sequence gene 2	366
ALS3 F ALS3 R	CCA CTT CAC AAT CCC CAT C CAG CAG TAG TAA CAG TAG TAG TTT CAT C	Agglutinin-like sequence gene 3	342
ALS4 F ALS4 R	CCC AGT CTT TCA CAA GCA GTA AAT GTA AAT GAG TCA TCA ACA GAA GCC	Agglutinin-like sequence gene 4	356
ALS5 F ALS5 R	TGA CTA CTT CCA GAT TTA TGC CGA G ATT GAT ACT GGT TAT TAT CTG AGG GAG AAA	Agglutinin-like sequence gene 5	318
ALS6 F ALS6 R	GAC TCC ACA ATC ATC TAG TAG CTT GGT TT CAA TTG TCA CAT CAT CTT TTG TTG C	Agglutinin-like sequence gene 6	152
ALS7 F ALS7 R	GAA GAG AAC TAG CGT TTG GTC TAG TTG T TGG CAT ACT CCA ATC ATT TAT TTC A	Agglutinin-like sequence gene 7	206
ALS9 F ALS9 R	CCA TAT TCA GAA ACA AAG GGT TC AAC TGA AAC TGC TGG ATT TGG	Agglutinin-like sequence gene 9	198

Table 3.2 RT-PCR conditions used for the gene targets.

	SAPs, PLB1, PLD, S14, EFB1	SAP3, PLB2, PLC	ALS
Denaturing step	95°C (5 min)	95°C (5 min)	95°C (5 min)
Cycles	40	40	40
Denaturing temp.	94°C (1 min)	94°C (1 min)	94°C (30 s)
Annealing temp.	55°C (1 min)	48°C (1 min)	58°C (30 s)
Extension temp.	72°C (1 min)	72°C (1 min)	72°C (30 s)
Final extension	72°C (10 min)	72°C (10 min)	72°C (7 min)

Controls for each reaction included tissue extracts not treated with reverse transcriptase (to establish that genomic DNA amplification was not occurring) and a water template control.

3.3.7 Gel electrophoresis of PCR products

PCR products (10 µl) were visualised following standard gel electrophoresis in a 0.75 % agarose gel (Sigma, UK) with ethidium bromide staining (10 µg µl^{-1}). Gel images were obtained using a Gel-Doc 2000 (Bio-Rad, Hemel-Hempstead, UK) and the average band densities for each PCR product were measured using the Discovery Series™ Quantity One® (Bio-Rad) software. Estimation of semi-quantitative values involved defining lanes and bands on the gel image following the Quantity One user guide. After defining lanes, a lane-based background subtraction was performed with bands identified using the Detect Bands command and then the values of a standard (a quantitative MassRuler DNA Ladder; low range, Fermentas, York, UK) being entered. The values of the experimental bands were then determined using this standard. The DNA concentration of each PCR product was then expressed as a percentage relative to that of the matched housekeeping gene (*EFB1*) amplified for each strain.

3.4 Results

3.4.1 RNA extraction-comparison of the methods

It was not possible to extract RNA from *C. albicans* using the ultraspec method. For this study RNA extraction was most consistently obtained using physical cell disruption and the RNeasy mini kit method (section 3.3.3). Subsequent results are therefore based on the latter RNA extraction protocol.

3.4.2 Control gene expression

For all RT-PCRs, control gene expression was routinely detected by EFB1 and S14 primers (Fig. 3.3 - 3.4).

3.4.2.1 *SAP* gene expression by *C. albicans* cultures without RHE (maintenance medium alone)

Sixteen out of 19 strains expressed *SAP1-3*. The non-invasive strains (WK1/93; 40/01) did not express *SAP1-3*. *SAP4-6* was expressed by 10 out of 19 strains. The non-invasive strains (WK1/93; 40/01) did not express *SAP4-6*. None of the tested strains expressed *SAP7*. *SAP8* was expressed by all strains except non invading strain WK1/93. *SAP9* was expressed by 7 strains out of 19 (Table 3.3).

3.4.2.2 *PLs* gene expression by *C. albicans* cultures without RHE (maintenance medium alone)

PLs gene expression (Table 3.3) appeared to be more frequently detected in *Candida* isolates without the RHE. Six strains out of 19 strains expressed *PLB1*, *PLB2* and *PLD*, whilst 6 expressed *PLC*. The non-invasive strains (WK1/93 and 40/01) did not express *PLB2* and *PLC*. When cultured without the RHE, 44 *PL* amplicons were evident (with multiple genes expressed by individual strains).
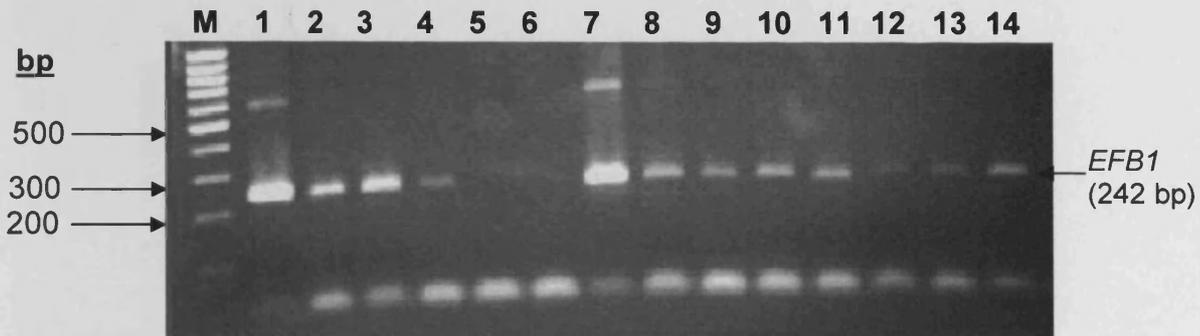
Fig. 3.3 Agarose gel showing typical RT-PCR products of *EFB1 Candida* control gene expressed during RHE infection.
Lanes: M, low range MassRuler DNA ladder (Fermentas, UK); RHE infected with *C. albicans* strains: 1, DW1/93; 2, PB1/93; 3, WK1/93; 4, LR1/93; 5, H_2O; 6, pure RNA; 7, 289T/00; 8, 480/00, 9, 819/99; 10, 970/00; 11, 40/01; 12, 705/93, 13, 458R/94; 14, 324LA/94.
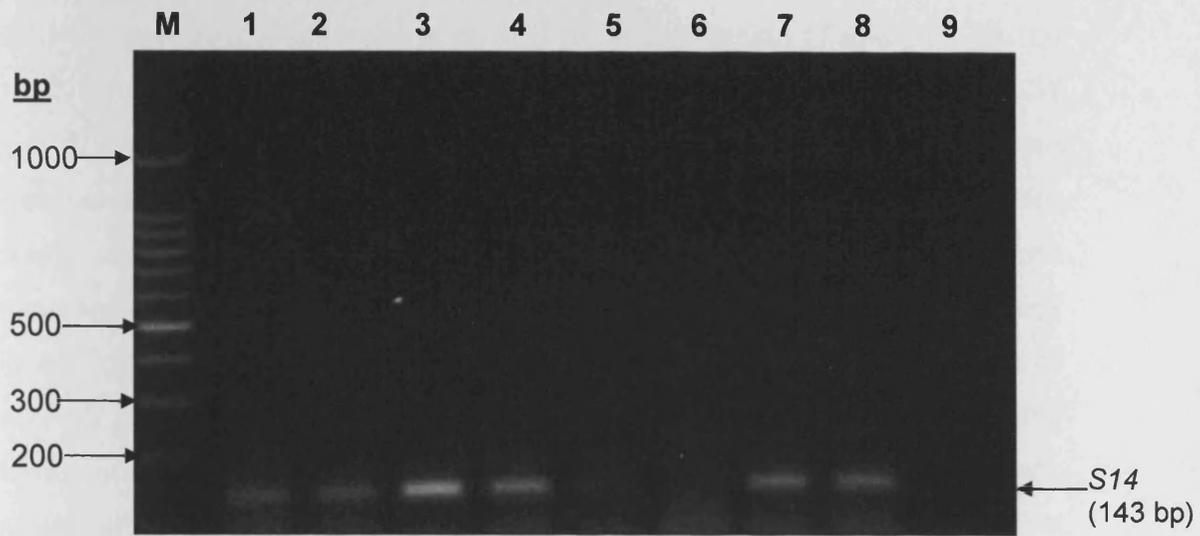
Fig. 3.4 Agarose gel showing typical RT-PCR products of S14 human control gene expressed during RHE infection.
Lanes: M, 100-bp ladder; RHE infected with *C. albicans* strains: 1, 135BM2/94; 2, 819/99; 3, 970/00; 4, 40/01; 5, 848/99; 6, 458R/94; 7, 324LA/94; 8, 1190/97; 9, H_2O.

3.4.2.3 *ALS* gene expression by *C. albicans* cultures without RHE (maintenance medium alone)

RT-PCR revealed a wide range of *ALS* gene expression (Table 3.3) for the 19 *C. albicans* strains without the RHE. Seventeen strains expressed *ALS1* and *ALS3*. Sixteen strains expressed *ALS4* and eleven strains *ALS2*. There was no expression detected for *ALS5*, whilst *ALS6*, *ALS7* and *ALS9* were rarely encountered. It was apparent that when compared with gene expression in the RHE medium (section 3.4.3), a large number of differences in *ALS* gene expression were encountered. These included 36 instances of no *ALS* gene expression and 11 cases of ALS gene expression without the RHE, mostly *ALS4*, *ALS6*, *ALS7* and *ALS9*. Detection of these genes was mostly attributable in three strains PTR/94, 243/00 and 408/99 which did not correlate to their respective invasiveness (Chapter 2).

3.4.3 Gene expression within the RHE model

3.4.3.1 *SAP* gene expression within the RHE model

RT-PCR analysis (Table 3.4; Fig. 3.5 - 3.6) revealed a wide range of *SAP* gene expression for the 19 *C. albicans* strains tested in the RHE infected model. *SAP1-3*, *SAP4-6* and *SAP8* transcripts were frequently detected, whilst *SAP7* (3 out of 19) was rarely encountered. *SAP1-3* and *SAP8* were produced by 18 out of 19 strains in the presence of RHE. However, a less consistent expression of *SAP4-6* (13 out of 19) and *SAP9* (6 out of 19) occurred. Expression of *SAP4-6* with all high invading strains was evident. These genes were not however expressed by the two non-invasive strains (WK1/93 and 40/01).

3.4.3.2 *PL* gene expression within RHE model

Phospholipase gene expression in the RHE model (Table 3.4; Fig. 3.7) did not appear to readily correlate with the level of RHE invasion and was detected in 11 out of the 19 strains tested in the infection model. When cultured in the presence of RHE, 23 *PL* amplicons were evident (multiple genes expressed by individual strains).

Table 3.3 Putative virulence gene expression (*SAP, PLs, ALS*) for *C. albicans* cultured in tissue maintenance medium alone.

Candida strain	SAP genes 1-9					Phospholipase genes (B-D)				Agglutinin-like sequence genes (ALS1-9)							
	1-3	4-6	7	8	9	B1	B2	C	D	ALS1	ALS2	ALS3	ALS4	ALS5	ALS6	ALS7	ALS9
135BM2/94[a]	+	+	+	+	+	+	-	-	+	+	+	+	+	-	+	+	+
970/00[a]	+	+	-	+	-	+	+	+	+	+	+	+	+	-	+	+	+
PTR/94[a]	+	+	-	+	-	-	+	+	-	+	+	+	+	-	-	+	-
324LA/94[a]	+	+	-	+	-	+	+	-	+	-	+	+	-	-	-	-	-
1190/97[a]	+	+	-	+	+	-	-	+	+	+	+	+	+	-	+	+	+
705/93[b]	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+
DW1/93[b]	-	+	-	+	+	+	+	-	+	+	+	+	+	-	+	+	+
PB1/93[b]	+	+	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+
243/00[b]	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+
455rgh/94[b]	+	+	-	+	+	+	+	-	+	+	+	+	+	-	-	-	-
LR1/93[b]	+	+	-	+	-	+	+	+	+	+	+	+	+	-	+	+	+
289T/00[b]	+	+	+	+	-	+	-	-	-	+	+	+	+	+	-	+	+
480/00[b]	+	+	-	+	-	-	+	-	-	+	+	+	+	+	-	+	+
819/99[b]	+	-	-	+	+	+	+	-	+	+	+	+	-	-	-	+	+
408/99[b]	+	+	-	+	+	+	+	+	-	+	+	+	+	-	-	+	+
848/99[b]	+	-	-	+	+	+	+	-	-	+	+	+	+	-	-	+	-
458R/94[b]	+	-	-	+	-	+	+	-	-	+	+	+	+	-	-	+	-
WK1/93[c]	+	-	+	+	-	+	-	-	-	+	+	+	+	-	+	+	-
40/01[c]	+	-	-	+	+	+	-	-	+	+	+	+	+	+	-	+	+

+ Detected; - not detected; [a]Highly invasive strain; [b]Low invading strain; [c]Non-invasive strain; for all RT-PCRs control gene expression was routinely detected. Values highlighted in pink show where differences in gene expression occurred compared to strains grown in the RHE model (Table 3.4).

Table 3.4 Putative virulence gene expression (*SAP*, *PLs*, *ALS*) for *C. albicans* cultured during RHE infection.

Candida strain	SAP genes 1-9					Phospholipase genes (B-D)				Agglutinin-like sequence genes (ALS1-9)							
	1-3	4-6	7	8	9	B1	B2	C	D	ALS1	ALS2	ALS3	ALS4	ALS5	ALS6	ALS7	ALS9
135BM2/94[a]	+	+	+	+	+	+	-	-	+	+	+	+	+	-	+	+	+
970/00[a]	+	+	-	+	-	+	+	+	+	+	+	+	+	-	+	+	+
PTR/94[a]	+	+	-	+	-	-	+	+	-	+	+	+	+	-	-	+	-
324LA/94[a]	+	+	-	+	-	+	+	+	+	-	+	+	-	-	-	-	-
1190/97[a]	+	+	-	+	+	-	+	+	+	+	+	+	+	-	+	+	+
705/93[b]	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+
DW1/93[b]	-	+	-	+	+	+	+	-	+	+	+	+	+	-	+	+	+
PB1/93[b]	+	+	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+
243/00[b]	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+
455rgh/94[b]	+	+	-	+	+	+	+	-	+	+	+	+	-	-	-	-	-
LR1/93[b]	+	+	-	+	-	+	+	+	+	+	+	+	+	-	-	+	+
289T/00[b]	+	+	+	+	-	+	-	-	-	+	+	+	+	+	-	+	+
480/00[b]	+	+	-	+	-	-	+	-	-	+	+	+	+	+	-	+	+
819/99[b]	+	-	-	+	+	+	+	-	+	+	+	+	+	-	-	+	-
408/99[b]	+	-	-	+	+	+	+	+	-	+	+	+	+	-	-	+	+
848/99[b]	+	-	-	+	+	+	+	-	-	+	+	+	+	-	-	+	-
458R/94[b]	+	-	-	+	-	+	+	-	-	+	+	+	+	-	-	+	-
WK1/93[c]	+	-	+	+	-	+	-	-	-	+	+	+	+	-	+	+	-
40/01[c]	+	-	-	+	+	+	-	-	+	+	+	+	+	+	-	+	+

Detected; - not detected; [a]Highly invasive strain; [b]Low invading strain; [c]Non-invasive strain; for all RT-PCRs control gene expression was routinely detected. Values highlighted in pink show where differences in gene expression occurred compared to the same strains cultured in maintenance medium (Table 3.3).
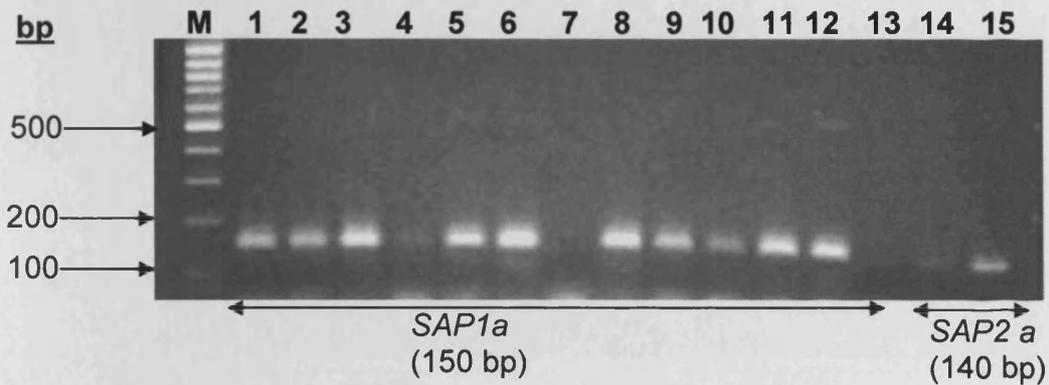
Fig. 3.5 Agarose gels showing typical RT-PCR products of candidal virulence genes (*SAP1* and *SAP2*) expressed during RHE infection.
Lanes M, low range MassRuler DNA ladder; RHE infected with *C. albicans* strains: 1, PB1/93; 2, LR1/93; 3, 408/99; 4, 819/99; 5, 970/00; 6, 243/00; 7, PTR/94; 8, 705/93; 9, 848/99; 10, 324LA/94; 11, 455rgh/94; 12, 1190/97; 13, H₂O; 14, PB1/93; 15, 408/99.

For each strain, band density was expressed as a percentage relative to that of the matched *EFB1* amplicon (Fig. 3.3).
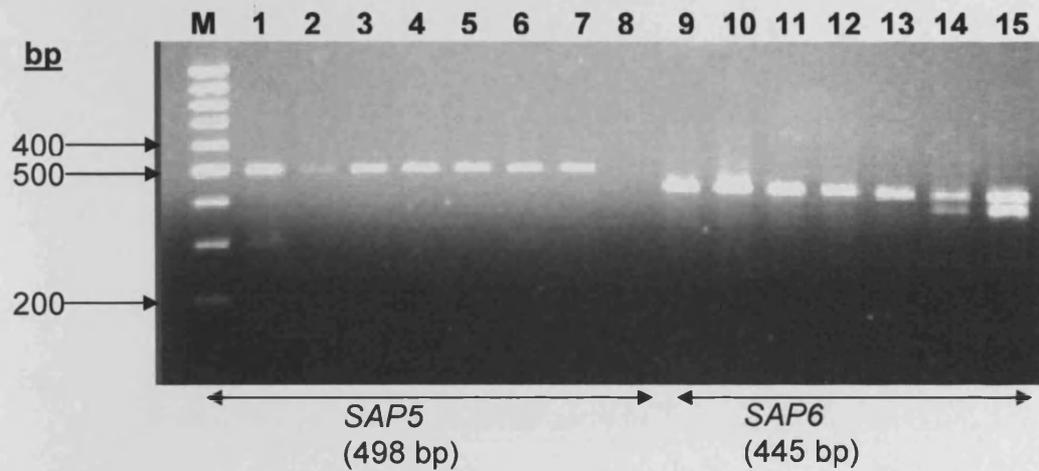
Fig. 3.6 Agarose gels showing typical RT-PCR products of candidal virulence genes (*SAP5*, and *SAP6*) expressed during RHE infection.
Lanes M, low range MassRuler DNA ladder; RHE infected with *C. albicans* strains: 1, DW1/93; 2, LR1/93; 3, 408/99; 4, 243/00; 5, PTR/94; 6, 455rgh/94; 7, 1190/97; 8, H_2O; 9, DW1/93; 10, PB1/93; 11, LR1/93; 12, 408/99; 13, 243/00; 14, 705/93; 15, 455rgh/94.

For each strain, band density was expressed as a percentage relative to that of the matched *EFB1* amplicon (Fig. 3.3).
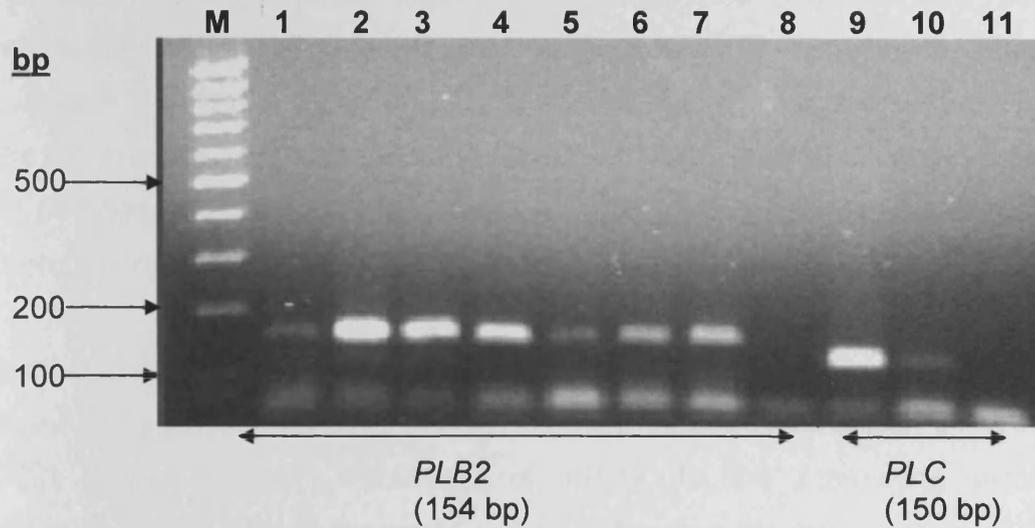
Fig. 3.7   Agarose gels showing typical RT-PCR products of candidal virulence genes (*PLB2*, and *PLC*) expressed during RHE infection.
Lanes M, low range MassRuler DNA ladder; RHE infected with *C. albicans* strains: 1, DW1/93; 2, PB1/93; 3, LR1/93; 4, 408/99; 5, 970/00; 6, 324LA/94; 7, 455rgh/94; 8, H_2O; 9, PB1/93; 10, LR1/93; 11, H_2O.

For each strain, band density was expressed as a percentage relative to that of the matched *EFB1* amplicon (Fig. 3.3).

3.4.3.3 *ALS* gene expression within the RHE model

As seen with PL genes expression, associations between *ALS* gene expression in the RHE model (Table 3.4; Fig. 3.8–3.10) and invasion showed that four *ALS* genes (*ALS1, ALS2, ALS3* and *ALS4*) were detected in 14 of the 19 infected RHE samples. Detection of *ALS5* (and to a certain extent *ALS6*) was however noticeably lower in infected RHE compared with other members of the gene family.

3.4.4 Semi-quantitative comparisons of gene expression using RT-PCR

Semi-quantitative analysis of (Tables 3.5a and b) gene expression using RT-PCR indicated varying quantities of mRNA for the expressing strains, although these differences could not be related to the previously reported (Chapter 2) levels of *Candida* invasion within the model.

Between the two growth phases (in the presence of RHE - biofilm state and without the RHE) there seemed to be a greater incidence of up-regulation of *PLs* gene expression without the RHE, whilst the converse was evident for *SAPs* and *ALS* gene expression in the biofilm phase. There also appeared to be up-regulation of *ALS* gene expression in high invading strains and more down regulation of *ALS* in low invading and non-invading strains in the absence of the RHE.
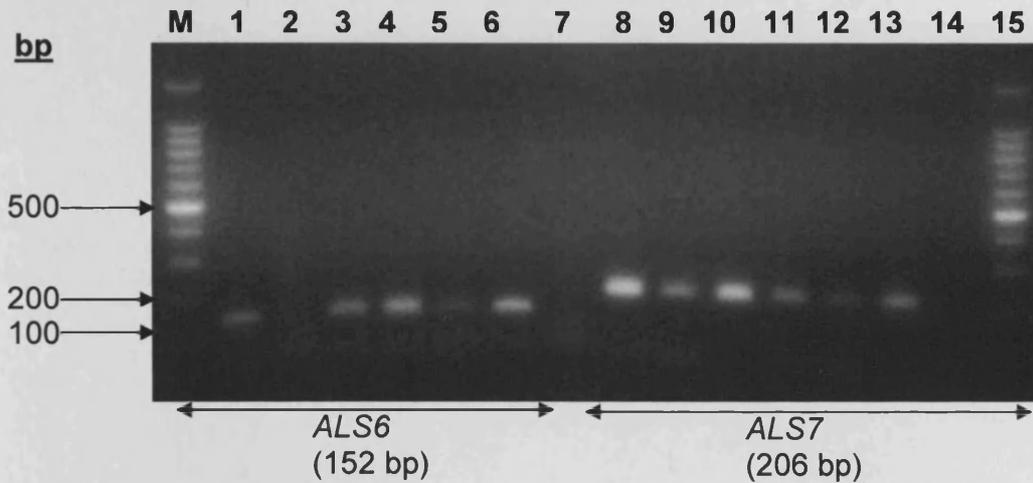
Fig. 3.8 Agarose gels showing typical RT-PCR products of candidal virulence genes (*ALS6*, and *ALS7*) expressed during RHE infection.
Lanes M, low range MassRuler DNA ladder; RHE infected with *C. albicans* strains: 1, DW1/93; 2, PB1/93; 3, 135BM2/94; 4, 970/00; 5, 705/93; 6, 1190/97; 7, H$_2$O; 8, DW1/93; 9, PB1/93; 10, 135BM2/94; 11, 970/00; 12, 705/93; 13, 1190/97; 14, H$_2$O; 15, low range MassRuler DNA ladder.

For each strain, band density was expressed as a percentage relative to that of the matched *EFB1* amplicon (Fig. 3.3).
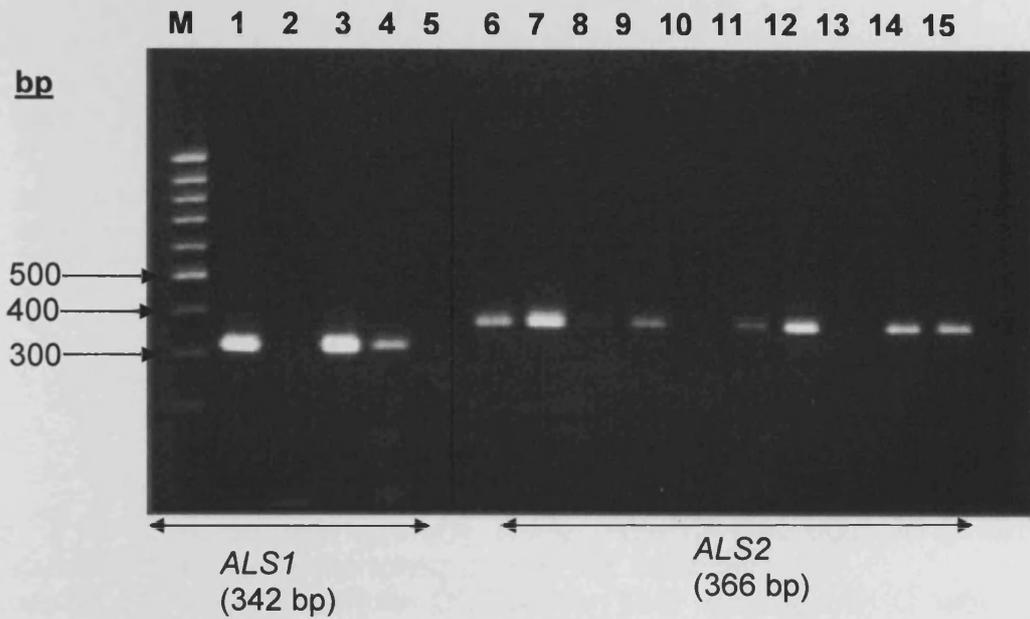
Fig. 3.9 Agarose gels showing typical RT-PCR products of candidal virulence genes (*ALS1*, and *ALS2*) expressed during RHE infection.
Lanes M, low range MassRuler DNA ladder; RHE infected with *C. albicans* strains: 1, 458R/94; 2, 324LA/94; 3, 455rgh/94; 4, 1190/97; 5, H$_2$O; 6, DW1/93; 7, PB1/93; 8, WK1/93; 9, LR1/93; 10, 408/99; 11, 135BM2/94; 12, 289T/00; 13, 480/00; 14, 819/99; 15, 970/00.

For each strain, band density was expressed as a percentage relative to that of the matched *EFB1* amplicon (Fig. 3.3).
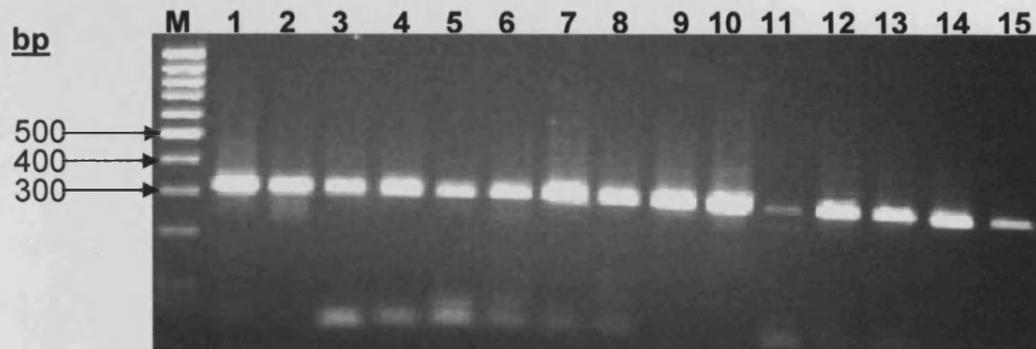
Fig. 3.10 Agarose gels showing typical RT-PCR products of candidal virulence genes (*ALS1*) expressed during RHE infection.

Lanes M, low range MassRuler DNA ladder; RHE infected with *C. albicans* strains: 1, DW1/93; 2, PB1/93; 3, WK1/93; 4, LR1/93; 5, 408/99; 6, 135BM2/94; 7, 289T/00; 8, 480/00; 9, 819/99; 10, 970/00; 11, 40/01; 12, 243/00; 13, PTR/94; 14, 705/93; 15, 848/99.

For each strain, band density was expressed as a percentage relative to that of the matched *EFB1* amplicon (Fig. 3.3).

Table 3.5a Detection of gene families associated with pathogenesis during planktonic culture using RT-PCR.

Candida albicans	Secreted aspartyl proteinase (*SAP*1-9)					Phospholipase genes (B-D)				Agglutinin-like sequence genes (*ALS1-7, ALS9*)							
	1-3	4-6	7	8	9	B1	B2	C	D	1	2	3	4	5	6	7	9
135BM2/94[a]	0	19	0	80	51	49	0	0	48	87	89	121	107	0	0	19	0
970/00[a]	351	176	0	214	0	150	44	26	27	192	149	326	230	0	0	422	264
PTR/94[a]	779	243	0	57	0	0	48	80	0	217	0	406	179	0	0	45	0
324LA/94[a]	162	201	0	96	0	154	106	0	0	0	0	60	0	0	0	0	0
1190/97[a]	642	233	0	85	0	0	0	0	0	664	136	467	312	0	0	0	0
705/93[b]	842	348	0	504	0	245	96	605	95	490	178	1040	137	0	0	120	0
DW1/93[b]	189	0	0	5	0	32	0	0	68	123	82	398	75	0	0	0	422
PB1/93[b]	1157	414	0	717	704	292	116	378	397	976	0	925	359	0	0	0	0
243/00[b]	143	84	0	102	49	85	39	94	27	67	72	105	77	0	56	29	125
455rgh/94[b]	828	320	0	8	278	145	62	0	0	476	99	363	164	0	0	0	0
LR1/93[b]	81	27	0	66	0	41	14	24	14	56	0	111	14	0	0	0	0
289T/00[b]	45	59	0	20	0	0	0	0	0	23	0	69	24	0	0	0	0
480/00[b]	56	0	0	2	0	0	0	0	0	141	113	199	172	0	0	121	0
819/99[b]	67	55	0	96	36	76	30	0	38	30	0	0	0	0	0	0	0
408/99[b]	140	43	0	81	0	25	0	57	0	68	60	125	87	0	0	14	0
848/99[b]	381	0	0	180	37	76	59	0	0	174	103	265	45	0	0	30	0
458R/94[b]	0	21	0	42	0	9	24	0	0	91	101	145	182	0	0	38	0
WK1/93[c]	0	0	0	0	0	8	0	0	0	9	0	0	0	0	0	0	0
40/01[c]	56	49	0	91	42	25	0	0	8	0	0	52	0	0	0	0	0

For all RT-PCRs control gene expression was routinely detected. The numbers represent the relative quantity (as a percentage) of PCR amplicon compared with the housekeeping gene product (EFB1). [a]Highly invasive. [b]Low invading strain. [c]Non-invasive. Percentages highlighted in pink show where up-regulation in gene expression occurred compared to the RHE model. Percentages highlighted in yellow show where down regulation in gene expression occurred compared to the RHE model.

Table 3.5b Detection of gene families associated with pathogenesis during RHE infection using RT-PCR.

Candida albicans	Secreted aspartyl proteinase (*SAP*1-9)					Phospholipase genes (B-D)				Agglutinin like sequence genes (*ALS1-7, ALS9*)							
	1-3	4-6	7	8	9	B1	B2	C	D	1	2	3	4	5	6	7	9
135BM2/94[a]	47	150	37	1	0	0	0	0	0	53	17	95	182	0	72	121	32
970/00[a]	249	28	0	92	0	30	20	0	0	245	126	311	707	0	78	192	561
PTR/94[a]	34	108	0	57	0	0	0	0	0	60	22	143	0	0	0	0	0
324LA/94[a]	433	1384	0	134	0	122	70	0	14	0	22	137	0	0	0	0	0
1190/97[a]	163	211	0	111	569	0	0	0	0	108	21	196	172	0	38	205	219
705/93[b]	279	104	0	144	156	319	0	0	66	237	209	616	820	0	148	293	267
DW1/93[b]	0	110	0	62	48	0	6	0	0	54	40	85	125	0	53	91	56
PB1/93[b]	109	82	0	0	43	96	21	85	53	111	192	203	293	0	0	137	242
243/00[b]	201	717	0	207	1010	310	0	0	0	388	452	680	0	0	0	0	0
455rgh/94[b]	31	56	0	29	0	1	15	0	1	62	3	46	7	0	0	0	0
LR1/93[b]	236	768	0	345	0	61	210	18	35	254	132	403	832	0	0	263	106
289T/00[b]	23	22	51	19	0	5	0	0	0	60	60	71	172	20	0	60	67
480/00[b]	218	70	0	46	0	0	0	0	0	116	0	156	282	57	0	43	64
819/99[b]	147	0	0	96	0	0	0	0	0	240	154	325	630	0	0	249	0
408/99[b]	60	112	0	11	80	0	48	0	0	41	74	13	0	0	0	0	0
848/99[b]	127	0	0	181	0	110	0	0	0	27	77	336	631	0	0	0	0
458R/94[b]	216	0	0	177	0	0	0	0	0	687	367	628	595	0	0	377	0
WK1/93[c]	69	0	36	6	0	0	0	0	0	71	4	143	26	0	11	6	0
40/01[c]	580	0	0	197	0	0	0	0	0	104	52	113	277	30	0	49	42

For all RT-PCRs control gene expression was routinely detected. The numbers represent the relative quantity (as a percentage) of PCR amplicon compared with the housekeeping gene product (EFB1). [a]Highly invasive. [b]Low invading strain. [c]Non-invasive. Percentages highlighted in pink show where up-regulation in gene expression occurred compared to the RHE model. Percentages highlighted in yellow show where down regulation in gene expression occurred compared to the RHE model.

3.5 Discussion

Compared to bacteria, there has been little work looking at *C. albicans* gene expression, or, indeed, at gene expression in fungal biofilms generally (Douglas, 2003). However, it has been shown that *Candida albicans* expresses several virulence factors that contribute to pathogenesis (Calderone and Fonzi, 2001). Genes associated with virulence factors include those linked with adhesion to the host tissue (*e.g. ALS* genes), morphogenesis, and hydrolytic enzymes, such as *SAPs*, and *PLs* (Jayatilake et al., 2005; Schaller et al., 2005). The main role of the hydrolytic enzymes is to provide nutrition for the cells, to aid penetration and invasion and to evade immune responses (Naglik et al., 2003) by degrading cellular substrates.

In various *Candida* infections, these gene families may be differentially regulated and prevention and control of expression might be achieved by pharmacological or immunological tools specifically designed to modulate or inhibit these virulence factors (Schaller et al., 2005). This present Chapter compared the expression of specific candidal virulence factors in an *in vitro* RHE model and, as a control, devoid of tissue.

It was apparent in this study that those strains previously categorised as 'high invaders' (Chapter 2) consistently expressed *SAPs4-6* despite the level of expression being strain variable. It is important to note that 4 "high invading" strains (135BM2/94, PTR/94, 324LA/94, 1190/97) and 4 "low-invading" (705/93, 455rhg/94, 848/99 and 458R/94) that originated from CHC infections all expressed *SAPs1-3,* and six of them expressed *SAPs4-6* (135BM2/94, PTR/94, 324LA/94, 1190/97, 705/93 and 455rhg/94). These findings may suggest that *SAPs* play a role in oral *Candida* infections, such as CHC. However, some of the "low invaders" also expressed *SAP1-3* and *SAP4-6* which may suggest that host factors, including tobacco smoking, denture wearing and regular alcohol intake, also play an important role in the development of CHC (Bartie et al., 2004). Furthermore, *SAP* genes 4-6 were not detected for the two non-invading strains (WK1/93 and 40/01). *SAP* genes 4-6 are believed to be important in controlling the yeast to hyphal

transition (Naglik et al., 2003) and so an indirect role for these enzymes in tissue invasion can readily be envisaged. These results might be considered to contrast with those of Korting *et al* (2003), who used *efg1/cph1* mutants of *C. albicans* SC5314 (which are known to exhibit reduced expression of the hyphal-associated genes *SAP4-SAP6*) to demonstrate that *SAP1 and SAP3* were important causes of epithelial cell damage in the same RHE model. In those studies, epithelial cell injury was determined by lactate dehydrogenase (LD) release and this correlated with the presence of *SAP1* and *SAP3* transcripts (Korting et al., 2003). Unfortunately, the model of RHE invasion used in this study did not incorporate the LDH release assay, and so any associations between *SAP* expression, invasion and tissue damage could not be confirmed. Since *SAP1-3* transcripts were detected in the majority of strains, it may be the case that tissue damage was still occurring, even for strains where limited RHE invasion was detected. In addition, gene expression was also assessed for *C. albicans* strains cultured in the tissue maintenance medium in an identical culture environment but in absence of the RHE tissue. A comparison of gene expression revealed a largely consistent expression of *SAPs* between the two growth environments. However, there were some notable differences, with an up-regulation of some *SAPs* evident in the presence of the RHE, which may suggest that external factors such as the growth environment play an important role.

A relationship between RHE invasion and agglutinin-like sequence (*ALS*) gene expression was not evident. However, there may have been up-regulation of these genes for some *C. albicans* strains in the presence of the RHE; in particularly this was seen with *ALS2, ALS6, ALS7* and *ALS9*. The *ALS* gene family designated *ALS1-ALS7* and *ALS9* has recently been implicated with facilitating candidal adherence to host tissues (Green et al., 2004; Hoyer, 2001). Garcia-Sanchez *et al* (2004) also looked at differences in *ALS* gene expression between biofilm and planktonic populations and contrary to this present work *ALS1* was dramatically over-expressed in all the biofilm populations and *ALS7* was under-expressed compared to planktonic ones. However, those differences could be explained by different culture

conditions and these investigators were not looking at gene expression in conjunction with the RHE model. However, the expression of *ALS* genes in this study, agreed in part, with those reported previously by Green *et al.* (2004), who also used a similar RHE infection model. Green *et al.* (2004) found consistent expression of *ALS1*, *ALS2*, *ALS3* and *ALS4*, in contrast to the more sporadic detection of *ALS6* and *ALS7* genes. In this present investigation, a lower incidence of *ALS5* and *ALS6* was found (3/19 and 6/19 strains respectively). However, only four *C. albicans* strains were used by Green *et al.* (2004) as opposed to the 19 strains from specifically-defined oral infections used in this study, which may account for these apparent differences.

Phospholipase expression similarly did not appear to correlate with RHE invasion (Table 3.4), as exemplified by the fact that three highly invasive strains did not produce mRNA for any *PL*s, whilst the poorly invasive *C. albicans* PB1/93 and *C. albicans* LR1/93 were positive for expression of all four *PL* genes. Furthermore, *PL* gene expression actually seemed to be higher in the absence of the RHE which might suggest the possible induction of *PL* genes through a starvation trigger as opposed to contact with tissue. Damage of the RHE during infection may negate this 'starvation' trigger through the release of nutrients for the infecting organism.

Phenotypic differences between biofilm and planktonic organisms are often regarded as important pathogenic determinants. These phenotypic changes may manifest through differential gene control arising from environmental triggers. For example, adherent organisms within a biofilm may express a pattern of genes different from that of their planktonic counterparts (Becker et al., 2001). It has been demonstrated with *Escherichia coli* that levels of gene expression between biofilm and planktonic populations differ significantly (Becker et al., 2001). Understanding gene expression patterns might therefore be useful for determining when certain proteins are generated in *C. albicans* and enhance our understanding on the pathogenicity of this organism (Green et al., 2004). This study showed that some of the strains

which are from conditions such as CHC express *SAPs* 4-6 (important for hyphal development). Potentially, screening of clinical isolates for these or other expressed virulence genes could maybe indicate the patient's possible risk of CHC and other invasive diseases.

Previous studies have assessed *C. albicans* biofilm transcriptional profiles to identify genes that were involved in the biofilm development process (Yeater et al., 2007). Garcia-Sanchez *et al.* (2004) used microarray transcript profiling to investigate a gene expression pattern specific to *C. albicans* biofilms. Genes involved in protein synthesis were over expressed in biofilms as well as genes that encoded proteins with secretion signals and adhesion-like domains (Garcia-Sanchez et al., 2004). Another study identified genes that were differentially expressed during biofilm development using microarray analysis (Yeater et al., 2007). Yeater *et al.* (2007) showed that genes were differentially expressed in the biofilm state compared to planktonic cells independent of the strain of *C. albicans* used or the substrate on which the biofilm was grown. *Candida albicans* biofilms expressed changes in gene expression by the expression of specific subsets of genes at different developmental stages as a result of adjusting to diverse requirements during development (Yeater et al., 2007).

A combination of real-time PCR and microarray analysis could be used to verify the findings in this present study. It would be also interesting to look at *C. albicans* strains from different body locations and to detect any relevant gene expression pattern compared to oral strains. This could give a link to pathogenicity of *C. albicans* strains in general.

3.6 Summary

In summary, this study and previous work on the *in vitro* RHE model illustrated that sufficient material can be obtained for the molecular analysis of gene expression (Schaller et al., 1998). Our findings support the hypothesis that *C. albicans* has a number of virulence factors associated with

its pathogenicity and that tissue infection is a result of the interaction of physical, mechanical and enzymatic processes (Jayatilake et al., 2005).

1) The *in vitro* expression of *C. albicans* virulence factors was largely consistent between the two growth phases (without the RHE and RHE biofilm state). Importantly there were some notable differences, *e.g.* up-regulation of certain *SAPs* and *ALS* expression in the presence of the tissue, and up-regulation of *PLs* expression without the RHE; implying that the culture environment was an important factor in phenotype determination. In the case of *PLs*, a starvation response might be occurring in the presence of maintenance medium alone. However, in the case of *SAPs*, a starvation response was not evident. The interaction with the RHE may trigger *SAP* gene expression which could induce hyphae production.

2) It was evident though, that a heterogeneous behaviour with regards to gene expression existed for isolates of *C. albicans*, from a variety of clinical origins. All strains associated with CHC expressed SAPs 1-3 (important for tissue damage) and some of them expressed SAPs 4-6 (important for hyphae development).

3) Some strain types (high invaders) were consistent producers of *SAPs* 4-6 and may possibly be equipped to have enhanced adherence and invasion. It is appropriate to conclude that isolate-specific differences exist in the invasive ability of *C. albicans* and the expression of certain putative virulence genes.

Chapter 4
Development of *in vitro* biofilm models for chronic wound bacteria

4.1 Introduction

4.1.1 Chronic wounds

Chronic wounds can be defined as wounds that do not heal within the normal expected time-frame. These wounds are considered to be a worldwide health problem with an estimated incidence of 1% of the world's population suffering from them (James et al., 2007). Chronic wounds are often an unrecognised cause of disease and disability of the elderly population despite having a great impact on the quality of life of the patients affected (Davies et al., 2007). Chronic wounds are also a source of frustration to both physician and patient due to requirements for lengthy treatment, a high incidence of treatment failure and long periods of patient compliance.

Recently, much attention has been focused on the ability of bacteria within chronic wounds to form and exist in a biofilm (James et al., 2007). Moreover, the wound environment is capable of supporting, and indeed, favours the growth of biofilms, in that such wounds tend to be both moist and warm.

There is evidence to show that bacteria are able to form biofilms within the wound environment (Mertz, 2003; Percival and Rogers, 2005). Microscopic studies of chronic wound specimens have revealed the presence of densely aggregated colonies of bacteria often surrounded by an extracellular matrix (James et al., 2007).

It is important to recognise that bacteria in chronic wounds exist within a polymicrobial environment. Therefore, treatment regimes need to be targeted against all the microorganisms present, not just to individual species or genera of bacteria when the wound is critically colonised (Percival and Bowler, 2004; Percival et al., 2005) or infected (Hansson et al., 1995). To study biofilms effectively, a thorough characterisation of the individual bacteria in these wound environments is required.

4.1.2 Bacterial infection of chronic wounds

All skin wounds are colonised by bacteria. The key factors that determine whether skin wounds colonised with bacteria become infected are, as yet, still not completely understood (Edwards and Harding, 2004). The definition of bacterial infection is the process by which microorganisms bind to tissue, multiply and then invade viable tissue and elicit a marked host response (Bowler et al., 2001). In patients with chronic wounds, the risk of infection is increased due to underlying systemic conditions such as possible receipt of steroid therapy, old age and poor vascularisation; all of which reduce immune function and hence increase the risk of bacterial invasion (Bowler et al., 2001; Davies et al., 2001). Actual wound infection is defined as the presence of replicating organisms within a wound with subsequent induction of host injury (Jones et al., 2004a).

4.1.3 Biofilm formation in chronic wounds

Moist environments such as those found in chronic wounds are optimal for the formation of biofilms. In chronic wounds, it is apparent that the biofilm is not eradicated through host defence mechanisms nor are they necessarily removed following administration of systemic or topical antibiotics.

Serralta et al. (2001) investigated the ability of P. aeruginosa to assemble a biofilm in an experimental pig dermal wound model. Their data showed that two bacterial populations were present; a population of bacteria adherent to the wound that required excessive scrubbing to be removed and a planktonic population that was easily removed. Akiyama et al. (2002) also showed that S. aureus could produce a glycocalyx on the surface of traumatic wounds in mice and this biofilm could be visualised by Confocal Laser Scanning Microscopy (CLSM). Davis et al. (2008) used a porcine model to create a biofilm associated wound. Partial thickness wounds were inoculated with a S. aureus wound isolate and treated with topical antimicrobial agents. They showed aggregates of microcolonies of bacteria embedded within and attached to the wound bed and increased antimicrobial resistance of these biofilm communities was also demonstrated (Davis et al., 2008). Another

investigation revealed by microscopic analysis aggregated colonies of bacteria in chronic wounds which seemed to be coated with EPS. Polymicrobial communities and anaerobic bacteria were also identified by denaturing gradient gel electrophoresis (DGGE) (James et al., 2007).

4.1.4 Treatment of chronic wounds

The aetiology of a chronic wound is likely to be multi-factorial. Many of the patients who have chronic wounds are elderly, have contemporaneous medical problems and other factors such as poor diet, poor vascular supply or other systemic factors which play a role in poor wound healing (Hill et al., 2003; Jones et al., 2004b). The clinical approach to treatment of chronic wounds needs to consider all of these factors for the best outcome. It is vitally important to establish an accurate diagnosis, as clinically distinct wound types (e.g. venous, arterial, pressure) are treated in different ways. Typical treatments involve decreasing the infection risk (wide variety of dressings are available) and stimulating an acute wound healing response. For instance, debridement reduces the bacterial bioburden along with any secreted toxins and also clears devitalized tissue, reducing the source of nutrients for remaining bacteria (Bowler et al., 2001; McGuckin et al., 2003). The formation of new blood vessels (angiogenesis) to supply nutrients and oxygen is also essential to allow growth of new tissue (Li et al., 2007). A wound presenting with a clinical infection (characterised by erythema, bleeding tissue, pain, oedema, and odour) may require both topical antimicrobial and systemic antibiotic therapy.

Iodine (polyvinylpyrrolidone iodine complex, PVP1) is a bacteriostatic antimicrobial that is commonly used as a topical agent in both the treatment and prophylaxis of wound infection (Mertz et al., 1999). Early iodine-based products caused allergic responses manifested in the form of pain, irritation and skin discolouration. However, the development of iodophores (povidone-iodine and cadexomer iodine) post 1949, has yielded safer and less irritating formulations. Iodophors are iodine-carrying complexes in which iodine is solubilised by surface active agents whilst retaining its bactericidal activity

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and losing its undesirable properties (Russell, 2003). Overall observations from animal models have indicated a degree of cytotoxicity by iodine (at a concentration of approximately 1%) against granulocytes and monocytes (Van den Broek et al., 1982), keratinocytes (Lineaweaver et al., 1985) and fibroblasts (Lineaweaver et al., 1985). However, no studies have looked at the PVP1 levels required to kill bacteria when in the biofilm state.

4.1.5 Analysis of bacterial biofilms

There are different methods available to analyse wound biofilms (Table 4.1). In this study, an *in vitro* microtitre plate assay and the use of the Constant Depth Film Fermenter (CDFF) were evaluated (section 4.1.5.1 and section 4.1.5.2). A simple model of bacterial attachment was used in our preliminary studies using a microtitre plate assay. To overcome some recognised limitations of the microtitre plate assay, a more established and complex laboratory model, the CDFF was also used.

4.1.5.1 Microtitre plate assay system

The microtitre plate assay is one of the most frequently employed *in vitro* methods to measure the biofilm forming ability of bacterial and fungal isolates (Kennedy and O'Gara, 2004; Ramage et al., 2005; Ramage et al., 2001). This semi-quantitative method for measuring biofilm formation involves growing microbial cultures in individual wells of 96-well polystyrene plates before fixing the developed biofilm and staining with 1% (w/v) crystal violet solution. The extent of biofilm formation can then be measured indirectly by the degree of crystal violet staining. It is a simple and sensitive assay, which permits the semi-quantification of biofilm formation and a high throughput of samples is possible. The major disadvantage of the crystal violet staining is that it is an indirect indication of the level of biofilm produced and it is a closed batch model (Djordjevic et al., 2002).

Table. 4.1 Methods of analysing bacterial biofilms.

Indirect methods	Reference
Standard plate counts	(An and Friedman, 2000; Djordjevic et al., 2002)
Sonication	(An and Friedman, 2000; Djordjevic et al., 2002)
Radiolabelled Bacteria	(An and Friedman, 2000; Djordjevic et al., 2002)
Enzyme-Linked Immunosorbent Assay	(An and Friedman, 2000; Djordjevic et al., 2002)
Direct methods	
Confocal Laser Scanning Microscopy Light and Transmission Electron Microscopy	(Costerton et al., 1995, Lawrence et al., 1991, Wagner et al., 1994)
Scanning Electron Microscopy	(Caddy et al., 2003)

4.1.5.2 Constant Depth Film Fermenter (CDFF)

The CDFF (Chapter 1, Fig. 1.12) allows reproducible biofilms of fixed thickness to be grown *in vitro*. The instrument houses 15 polytetrafluoroethylene (PTFE) pans located within a rotating carousel in the main body of a fermenter. Each PTFE pan contains five recessed PTFE plugs. The top stainless steel plate contains ports allowing entry of inoculum, sterile medium and gas to the chamber. The sample can be lifted free from the turntable under aseptic conditions. Media is pumped into the CDFF via the inlet port using a peristaltic pump (30 ml min^{-1} flow rate). The PTFE scraper blade situated above the rotating turntable distributes the media over the surface of the PTFE discs and also controls the depth of the biofilm by constantly shaving the uppermost layer. The bottom stainless steel plate contains a port for effluent drainage and has a site for the attachment of the motor to drive the turntable.

The CDFF has previously been used to model biofilms of bacteria from non-wound environments *e.g.* oral and waste water biofilms (Allan et al., 2002, McBain et al., 2003, Wilson, 1999) and has also been used for testing antimicrobials such as chlorhexidine, sodium hypochlorite, tetracycline and silver on biofilm growth (Deng et al., 2004; Mulligan et al., 2003; Norwood and Gilmour, 2000; Ready et al., 2002b). The CDFF has also been used to study aspects of biofilm physiology such as transport, structure and stability, bacterial immigration and emigration, cell viability and viability mapping, and gene transfer (Al-Bakri et al., 2004, Hope and Wilson, 2003, Hope and Wilson, 2006).

The CDFF is unable to reproduce a number of physiological processes seen *in vivo*, for example, a host defence mechanism cannot obviously be incorporated. However since the CDFF is a simplified system with few variable factors; it has great merit in the initial evaluation of biofilms. The use of the CDFF is of great value in allowing the investigation of specific aspects of antimicrobial action and therapeutic interventions in isolation from other factors.

Initial experiments in this present study using the CDFF were designed to determine whether biofilms could be produced and maintained using bacterial isolates from chronic wound patients. Once biofilms were successfully produced, analysis of an antimicrobial wound therapeutic agent was tested *in vitro*. The antimicrobial examined was povidone-iodine (PVP1), commonly prescribed for chronic wound patients. Iodine is one of the most frequently employed therapeutic constituents of wound dressings, *e.g.* Inadine (Johnson & Johnson) and is also used in antiseptic pre-surgical scrubs and other antiseptics.

4.2 Aims

The aims of this study were:

1. To confirm the species identity of chronic wound isolates to be used in these studies using 16S rRNA gene sequencing.
2. To develop *in vitro* models of biofilm formation for the identified wound bacterial isolates.
3. To compare the ability of these wound bacteria to produce biofilms and determine whether synergism or antagonism occurred in mixed biofilm populations.
4. To assess the susceptibility of the wound bacterial biofilms to PVP1.

4.3 Methods

4.3.1 Preparation of bacterial species

Bacterial species were selected from a previous prospective study of 70 patients with chronic venous leg ulcers (Davies et al., 2007, Davies et al., 2004) and included representation of four species most frequently encountered and with distinctive colony morphologies namely: *Staphylococcus aureus, Pseudomonas aeruginosa, Micrococcus luteus* and *Streptococcus oralis.* Isolates were routinely grown on blood agar (BA) supplemented with 5% (v/v) sheep blood. BA plates were incubated aerobically at 37°C for 24 h. Confirmation of identification of these isolates in this present study was through 16S rRNA gene sequencing (section 4.3.2).

4.3.2 DNA extraction from test bacterial isolates

16S rRNA gene sequencing was used to confirm the identification of the four isolates selected for this study. The previous presumptive identification of these isolates, which had been based upon biochemical profiling using API ID strips (BioMérieux sa, Marcy L'Etoile, France), was *S. aureus, P. aeruginosa, M. luteus* and *S. oralis.* DNA was extracted from an overnight culture in Brain Heart Infusion (BHI) broth of each pure bacterial isolate using a Puregene® DNA isolation kit (Gentra Systems, Minneapolis, USA) by following the "DNA Isolation From 1 ml Gram-positive Bacteria Culture Medium" protocol (http://www.gentra.com/technical_assistance /protocols.asp).

4.3.3 Amplification of 16S rRNA genes from bacterial isolates

Extracted DNA was quantified using the Nanodrop ND-1000 Spectrophotometer (NanoDrop Technologies, Fisher, UK) and stock DNA concentrations of 200 µg ml^{-1} were prepared. PCR was used to amplify targeted sequences of the 16S rRNA using the universal bacterial primer pair of 27F and 1492R (0.5 µM of each; oligonucleotides synthesized by Invitrogen Ltd. Paisley, UK), both sequences of which were specific for the domain *Bacteria* (Dymock et al 1996; Table 4.2).

Table 4.2   Oligonucleotide primers used for sequencing of bacterial DNA extracted from wound isolates.

Primer	Nucleotide sequence	Reference
27F	5'-GTGCTGCAGAGAGTTTGATCCTGGCTCAG-3'	(Dymock et al.1996)
1492R	5'-CACGGATCCTACGGGTACCTTGTTACGACTT-3'	(Dymock et al. 1996)
357F	5'-CTCCTACGGGAGGCAGCAG-3'	(Lane,1991)

PCR was performed in a total reaction volume of 50 μl and used 1.5 U of *Taq* DNA polymerase, 200 μM of each deoxynucleotide, 0.5 μM of each primer, and the working concentration of appropriate magnesium-free buffer (Promega, Southampton, UK). Additionally, 2.0 mM of $MgCl_2$ (Promega) was used, with 5 μl of DNA extract as template.

A touchdown PCR protocol was used, whereby, in the first cycle, denaturation at 95°C for 1 min was followed by primer annealing at 65°C for 1 min, and primer extension at 72°C for 2 min. This cycle was then followed by denaturation 1 min with the annealing temperature subsequently decreased by 2°C for each cycle (11 cycles), after which 25 cycles were performed under the same primer annealing conditions (45°C). In the final cycle, primer extension was performed for 12 min. PCR was undertaken using a PTC-200 thermal cycler (MJ Research Ltd., GRI, Braintree, UK).

PCR products were purified by precipitation and washed with pure ethanol. Firstly, 15 μl of 5 M NaCl (Sigma-Aldrich, Steinheim, Germany) and 15 μl of 40% polyethylene glycol (Mol. Wt. 8000; Sigma) were added to each PCR tube. This mix was centrifuged (16,000 *g* for 15 min) and the supernatant aspirated and replaced with 200 μl of 70% ethanol (v/v in water; Fisher Scientific, Loughborough, UK). Centrifugation, aspiration and ethanol washing steps were repeated (3× in total). Following another centrifugation step, the PCR products were dried under a fume hood (EDF1200, Envair, Rossendale, UK) overnight, and resuspended with DNA-free and nuclease-free water (30 μl). Products were then stored at -20°C until required.

4.3.4 Sequencing of 16S rRNA genes

Purified and cleaned PCR products were sequenced using ABI Prism BigDye terminator cycle sequencing ready reaction kits (Applied Biosystems, Warrington, UK). A total reaction volume of 20 μl was used and contained the working concentrations of BigDye Sequencing Buffer and Ready Reaction Premix (Applied Biosystems), 0.5 μM of an oligonucleotide primer (Table 4.2, synthesized by Invitrogen), and 6 μl of purified PCR-product

(approx. 5-20 ng). For all isolates, sequencing was performed using the universal bacterial primer 357F (Lane, 1991; Table 4.2). Reactions were performed in a PTC-200 thermal cycler (MJ Research Ltd.) and comprised of 1 min of denaturation at 94°C, followed by 30 cycles of denaturation at 94°C (15 s), annealing at 50°C (30 s) and extension at 60°C (5 min).

Extension products were purified, firstly by precipitating via the addition of 1 μl sodium acetate (3M; Sigma) and 1 μl ethylenediaminetetraacetic acid (EDTA, pH 8.0, 0.5 M; Sigma), and then by adding 80 μl of chilled absolute ethanol, to each reaction mix. The supernatant was aspirated following centrifugation (16,000 g, 15 min), and replaced with chilled 70% ethanol (200 μl). This was repeated once and after a final centrifugation and drying under vacuum, products were re-suspended in 100% (v/v) formamide (30 μl) and run on an automated DNA sequencer (ABI PRISM 3100 Genetic Analyser; Applied Biosystems). This procedure was expected to generate reliable sequences of greater than 500 base pairs (bp) that could be compared to sequences in the Genbank and EMBL public databases, which is sufficient for species level identification (Clarridge, 2004).

4.3.5 Identification of bacterial isolates by 16S sequence analysis
Sequences obtained in this study were identified by comparison to the GenBank DNA sequence database EMBL (Benson et al 2004) using the FASTA sequence homology search program (http://www.ebi.ac.uk/services/index.html; Pearson, 1990).

A >99% homology to the 16S rRNA gene sequence of the type strain, or other suitable reference strain, was the criterion used to identify an isolate to the species level. Where more than one reference species exhibited >99% sequence homology, the match with the greatest homology was taken as the identity, wherever the sequence was shown to be reproducible and reliable.

4.3.6 *In vitro* biofilm formation by chronic wound bacteria

Preliminary studies were undertaken to identify a culture medium and incubation time that allowed growth and biofilm formation by all isolates to be studied. A total of four different culture media were evaluated. These included Brain Heart Infusion broth (BHI; Oxoid, Hampshire, UK), Tryptone Soya Broth (TSB; Oxoid), BM medium (Appendix I) (McKee et al., 1985) and a simulated wound fluid (Appendix I) previously described by Addison *et. al* (2004). The latter medium was modified by adding 2 g l^{-1} glucose to provide an additional carbon source.

4.3.7 Preparation of wound cultures for an *in vitro* microtitre plate assay

Micrococcus luteus (B81), *S. oralis* (B52), *P. aeruginosa* (D40) and *S. aureus* (D76) were cultured at 37°C for 24 h in 10 ml of BHI, TSB, BM or simulated wound fluid medium.

Standardised bacterial preparations were then made by adjusting the culture concentration to an optical density of 0.4 (absorbance measured at 600 nm) in PBS. After the required dilution for this OD measurement was identified using PBS, fresh medium was added to the overnight cultures, to adjust the bacteria to the required level.

4.3.8 Microtitre plate biofilm assay

Microtitre plate biofilm formation involved growing bacterial cultures in individual wells of 96-well microtitre plates (flat bottomed, Greiner, UK). A range of culture concentrations were tested in separate plates by performing two-fold serial dilutions (in appropriate culture medium) of the standardised bacterial preparation. A 100-μl volume of bacterial culture was then added to the wells of the microtitre plate. The microtitre plates were then sealed with parafilm to prevent dehydration (American National Can Group, USA) and incubated at 37°C for 24 h, 48 h, 72 h and 96 h.

Biofilm formation was subsequently assessed by one of 2 methods: a) crystal violet staining or b) total viable count estimation.

4.3.9 Semi quantification of microtitre plate biofilms by crystal violet staining

After the appropriate incubation period, culture medium was removed and the microtitre plate wells washed three times with sterile distilled water to remove loosely associated bacteria. Plates were dried at 56°C for 45 min and each well was stained with 150 μl of 1% v/v crystal violet solution (in water) for 45 min. After staining, plates were again washed three times with sterile distilled water. At this point, biofilms were visible by purple staining formed on the side of each well. The quantitative analysis of biofilm production was then performed by adding 200 μl of 95% ethanol to destain the cells. After 5 min, 100 μl from each well was transferred in a new microtitre plate and the level of the crystal violet present in the de-stain solution was measured at 540nm. This method has previously been described by Djordjevic *et al.* (2002).

4.3.10 Statistical analysis of the crystal violet microtitre plate assay

Statistical analyses were done on the data obtained from the crystal violet microtitre plate assays. The Friedman, Wilcoxon and Kruskal-Wallis tests are all non-parametric statistical tests. The Friedman test and the Wilcoxon Signed Rank test were used to measure any differences in biofilm formation when different starting inocula were used. The Kruskal-Wallis test is an extension of the Wilcoxon rank sum test. All statistical analysis was done using the Statistical Package for the Social Sciences (SPSS) software.

4.3.11 Assessment of biofilm formation by single and mixed bacterial isolates using the microtitre plate assay and viable count estimation

The ability of the test isolates to produce biofilms in both single species and mixed species population was also determined using the microtitre plate assay. Using the identified optimal growth conditions, single and mixed species biofilm experiments were undertaken to determine whether any synergy or antagonism between species occurred with regards to biofilm formation.

Briefly, in the case of mixed species biofilms, equal volumes of isolate combinations (total final volume of 100 µl) were mixed, added to the wells of the microtitre plate and incubated for 24 and 48 h in BHI medium.

After incubation the planktonic phase of growth was carefully removed from the microtitre plates. A 100-µl volume of PBS was added to each well to re-suspend the biofilms. The biofilms of 6 wells were then pooled and serially diluted in PBS. Dilutions (100 µl) were plated on to BA plates which were incubated aerobically at 37°C for 24 h. Enumeration of each species was confirmed by visual inspection of the plates and representative colonies of each species recorded at 24-48 h.

4.3.12 Use of the CDFF to develop biofilms using chronic wound bacteria

In the present study, the CDFF was fully assembled with the PTFE plugs recessed within the pans to a depth of 400 µm using a gauged recessing tool. The CDFF was then autoclaved at 121°C for 45 min, and allowed to cool. For the duration of each experiment, the CDFF was maintained in an incubator at 37°C and ambient air was allowed to diffuse into the CDFF via sterile air filters. For all CDFF experiments the turntable speed was operated at 20 revs min^{-1}. Initial experiments in the CDFF were performed to determine whether a stable biofilm wound model could be produced using bacterial isolates from chronic wound patients.

4.3.12.1 Inoculation of chronic wound bacteria for the *in vitro* biofilm model using the CDFF and preparation of planktonic cells

Chronic wound bacteria were routinely grown on BA and incubated aerobically at 37°C, as described previously (section 4.3.1). Isolates were then subcultured in 10 ml BHI broth for 24 h at 37°C in a shaking incubator. Isolates were tested for their ability to form biofilms in both single and mixed inocula. Five ml of each wound bacterial culture (section 4.3.1) was added to 1 L of BHI or BM broth and this preparation was recirculated through the CDFF for 24 h to 'seed' the inoculum in the system. After this time, the

inoculum was disconnected and fresh media was fed into the CDFF without recirculation and the CDFF waste was collected in an effluent bottle. Medium was delivered at a flow rate of 30 ml min^{-1} using a peristaltic pump (Watson-Marlow, Falmouth, UK). Purity plates were performed at the time of inoculation to ensure that no contamination occurred in the CDFF inoculum.

Planktonic mixes of the bacteria were also assessed in parallel to the biofilms. For planktonic cell preparation 100 μl from the overnight culture of each organism was added to fresh 19.9 ml BHI medium. Seven replicate broths were incubated with shaking at 37°C for 7 d. Each day the cells were centrifuged, the supernatant removed and new media was added. At each 24 h time point, 100 μl of one of the replicates suspensions was serial diluted in PBS and plated onto BA plates for viable counts of planktonic growth. The remainder of the suspension was centrifuged and the pelleted cells were stored in RNAlater® (Ambion, UK) for subsequent investigation of gene expression profiles (see Chapter 6).

4.3.13 Recovery of biofilms established in the CDFF

A single pan holding 5 plugs was aseptically removed at each sampling time point from the CDFF for microbial analysis of the wound microflora. The samples were lifted from the turntable with a dedicated tool under aseptic conditions every 24 h for 7 d. Individual plugs were then removed aseptically from the pan. The 5 plugs were each placed in 2.5 ml of PBS and serial decimally diluted. Dilutions (100 μl) were plated on to BA plates and the plates incubated aerobically at 37°C for 24 h.

4.3.14 PVP1 susceptibility of CDFF generated biofilms

Antimicrobial testing of the biocide, povidone-iodine (PVP1) in the CDFF was performed against a mixed biofilm consortia. Bacteria used for the inoculum were *S. aureus* (D76), *P. aeruginosa* (D40), *M. luteus* (B81) and *S. oralis* (B52) cultured for 24 h in BM medium at 37°C. In addition to these aerobic species, *Bacteroides fragilis* (B11) and *Peptostreptococcus anaerobius* (B12) were prepared in fastidious anaerobe broth (FAB; LabM Ltd., UK) for 72 h in

an anaerobic cabinet (10% v/v CO_2, 20% v/v H_2, 70v/v N_2; Don Whitley Scientific Ltd., Shipley, UK) at 37°C. The inoculum for the biofilm consisted of 5 ml of each of these bacterial species, which were added to the growth medium and used to inoculate the CDFF as previously described (4.3.12.1). PVP1 (final concentration of 1% w/v in BM) was added to the medium of the CDFF once a fully mature biofilm had been established (after 5 d incubation). The PVP1 concentration of 1 % used was thought to be representative of the concentration of iodine that is effectively available to the wound bed in commonly used commercially available dressings which contain 10% PVP1 (http://www.dressings.org/dressings/inadine.html).

4.3.15 Biofilm recovery from the CDFF during PVP1 testing

The CDFF was sampled every 48 h over a total experimental run of 29 d. A single pan containing 5 plugs was again removed from the CDFF at each sampling point. Two sets of two plugs were taken from each pan and separately vortexed in 5 ml PBS for 2 min to disperse the biofilm. Serial dilutions were then made of each of the resulting bacterial suspensions to a dilution factor of 10^7 fold. Then, 100 µl of each dilution was plated onto BA and FAA plates which were incubated at 37°C under aerobic and anaerobic conditions, respectively. Colony counts of each species on the plates after 24-48 h incubation were then recorded.

Resulting growth from the 'biofilm' cultures were also subsequently tested for susceptibility to PVP1 using a broth culture minimum bactericidal concentration (MBC) test (see section 4.3.16). This enabled an assessment to be made on possible acquired resistance development of these isolates during prolonged PVP1 exposure in the CDFF.

4.3.15.1 Live/dead staining of biofilms treated with PVP1

In addition to total viable count estimation, a smear of the biofilm was also stained with the Live/Dead® BacLight™ stain (Live/Dead® BacLight™ Bacterial Viability Kit, Invitrogen Ltd). The live/dead stain consisted of two nucleic acid stains: the green fluorescent SYTO9 and the red-fluorescent propidium

iodide stains are used in combination to discriminate live and dead bacteria in a mixed population. Bacterial cells with a compromised membrane that are considered to be dead or dying stain red, whereas cells with an intact membrane appear green.

Three μl of each stain was added to 300 μl PBS, mixed and 20 μl added to the biofilm smear. The preparation was left for 1 min and then viewed by standard fluorescence microscopy (Olympus Provis AX70 Digital Microscope; Olympus U.K. Ltd., Middlesex, U.K.), at a total magnification of x600.

4.3.16 Planktonic cell susceptibility to PVP1

In order to determine whether an elevated resistance to PVP1 occurred in chronic wound biofilms it was also necessary to establish the susceptibility of planktonic equivalent cells. These experiments were undertaken using a microtitre plate assay susceptibility test based on the CLSI (Clinical Laboratory Standards Institute; previously known as the National Committee for Clinical Laboratory Standards; NCCLS) guidelines for broth microdilution testing with some modifications. Tests were undertaken using cation-adjusted Mueller-Hinton broth (CAMHB; Lab114, LabM, Bury, England) or BM medium. PVP1 was diluted in the culture medium and serial two-fold dilutions of PVP1 ranging from 2% w/v to 0.004% w/v were used.

Bacterial isolates were grown on BA plates at 37°C overnight and then used to inoculate 10 ml BM broth which was incubated at 37°C overnight. The bacterial suspension was then washed by centrifugation. The cell pellet was re-suspended in PBS and the turbidity was adjusted to a 0.5 McFarland standard (approximately 10^8 CFU ml^{-1}). Based on the required dilution to establish the 0.5 MacFarland standard, an equivalent dilution preparation was made in the appropriate growth medium for study. A proportion of the standardised suspension was diluted 1:100 (10^6 CFU ml^{-1}) with BM broth or Mueller-Hinton broth. One hundred μl of the bacterial suspension was added to a microtitre plate well already containing 100 μl of diluted PVP1. A volume (100 μl) of the inoculum was plated on to BA to check for purity and inoculum

density. Broth not containing PVP1 was inoculated as a control for organism viability, and a further control of broth without inoculum was also included. The microtitre plate was sealed in parafilm and incubated at 37°C for 24 h, 48 h, 72 h and 96 h before minimum bactericidal concentrations (MBCs) were determined.

The lowest concentration of PVP1 found to completely inhibit visible growth of the organism was recorded as the MBC at all time points. This was confirmed by the total absence of growth on BA plates after sub-culturing a loopful of broth from the identified MBC dilution.

4.4 Results

4.4.1 16S rRNA gene sequence identification of test bacterial isolates

Results for the highest sequence match from 16S rRNA gene sequencing of bacterial wound isolates used in this study are shown in Table 4.3. All isolate sequences showed a high homology (> 99.4%) to at least one database sequence (actual sequences, Appendix I).

4.4.2 Comparison of biofilm formation by test species using the microtitre plate assay (crystal violet)

Figures 4.1 - 4.3 demonstrate the levels of biofilm formed by the test species in BHI, TSB and BM media respectively. In simulated wound fluid (with or without glucose) the bacteria were not found to produce biofilms. There were no statistical differences for each species in the biofilm level generated at each time point when different starting inocula were used (Fig. 4.1 - 4.3).

The level of biofilm formation by the test bacterial species was, however, found to vary with different growth media (BHI and TSB) over a time period of 24-96 h.

Table 4.3 16S rRNA gene sequencing: Identification of wound isolates.

Isolate	Species match	Accession number	% homology to 16S rRNA gene sequence in database
B52	*Streptococcus oralis* ATCC 35037;	AY485602	99.8
	Streptococcus sanguinis ATCC 29667;	AY281085	98.9
B81	*Micrococcus luteus*; DSM 20030	AJ536198	99.6
D40	*Pseudomonas aeruginosa* DSM 50071	Z76672	99.4
D76	*Staphylococcus aureus* ssp. *anaerobius* ATCC 35844;	D83355	99.8
	S. aureus ssp. *aureus* ATCC 12600;	X68417	99.8
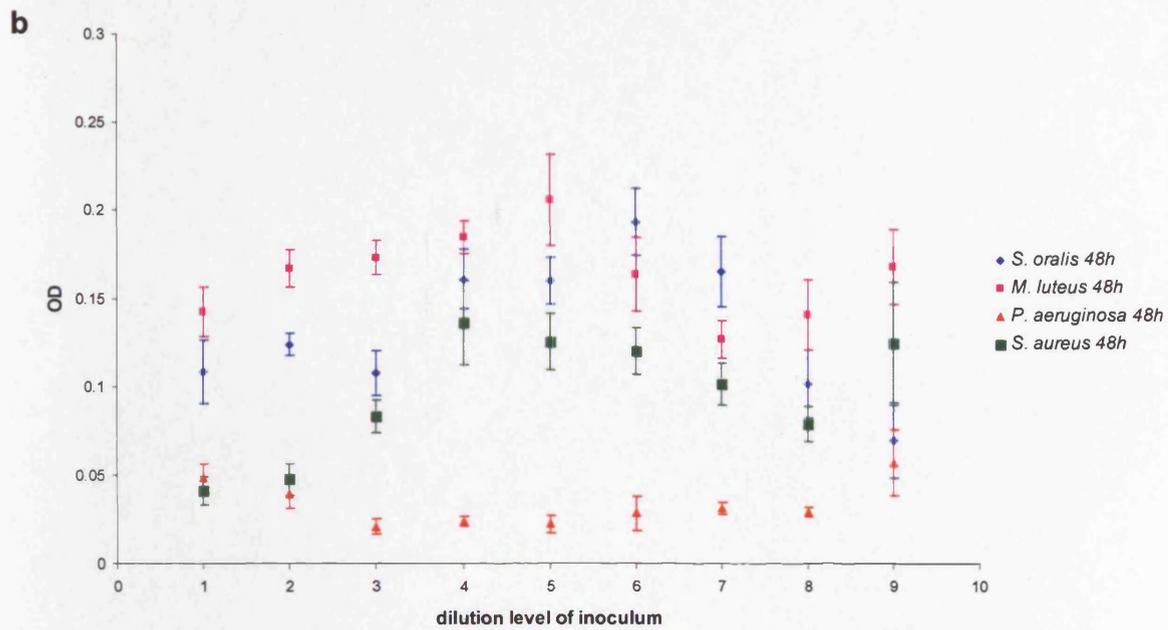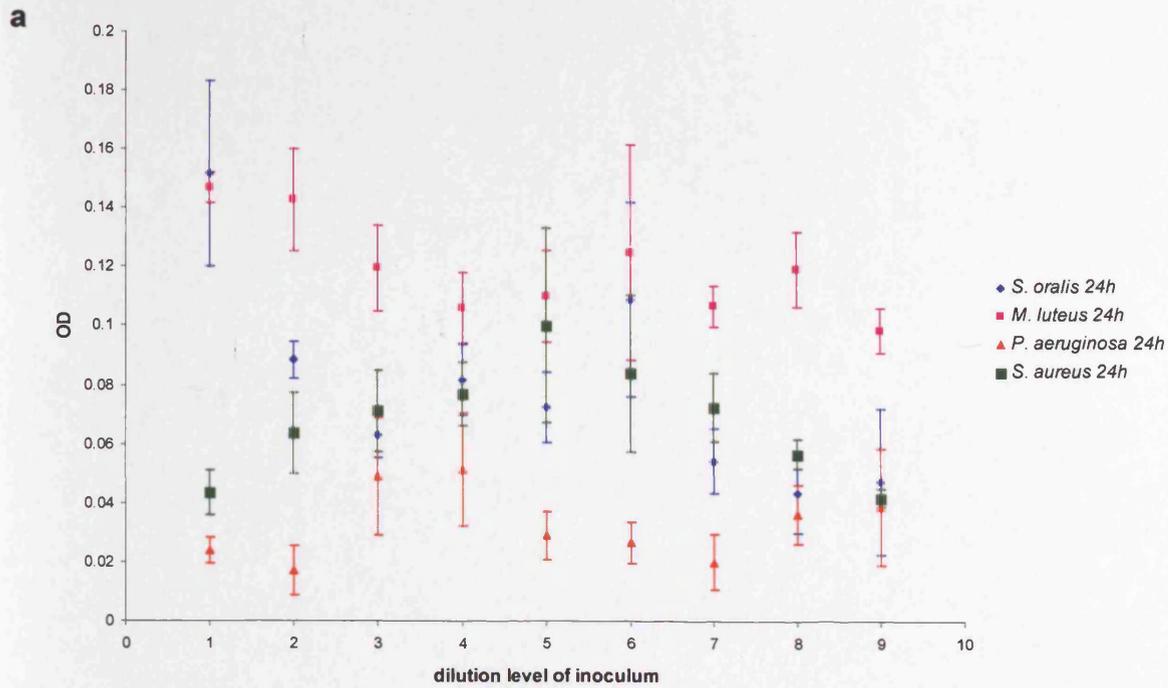
Fig. 4.1 Relative biofilm formation for different inocula levels of individually grown wound bacteria using the microtitre plate assay and BHI medium for a) 24h, b) 48h, c) 72h and d) 96h. Error bars represent standard deviation of 8 replicates.

Fig. 4.1 continued

c

d

165
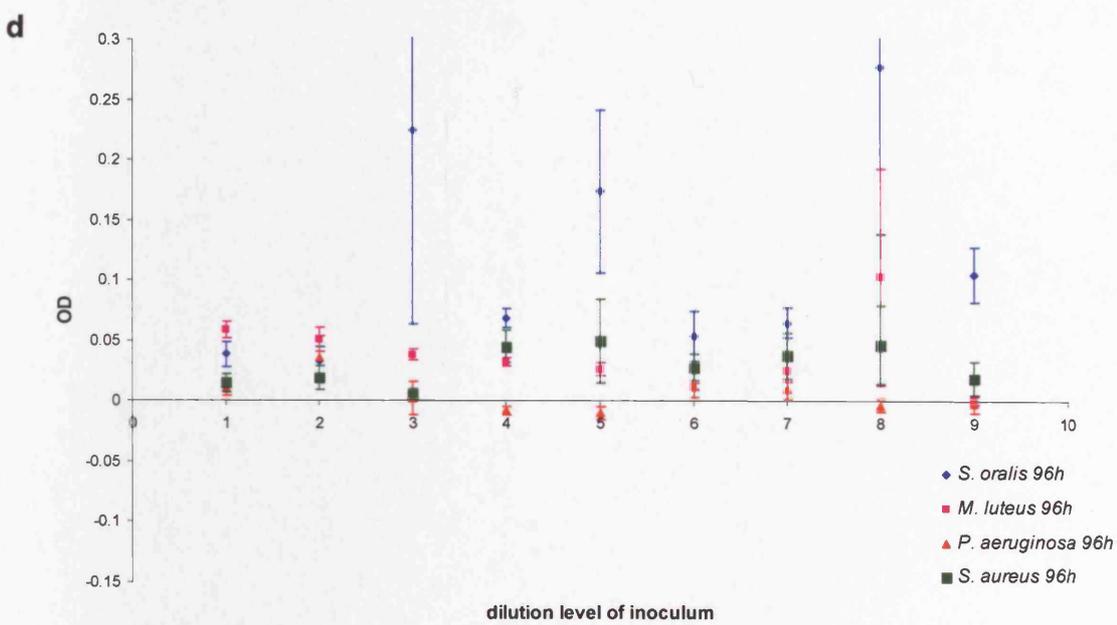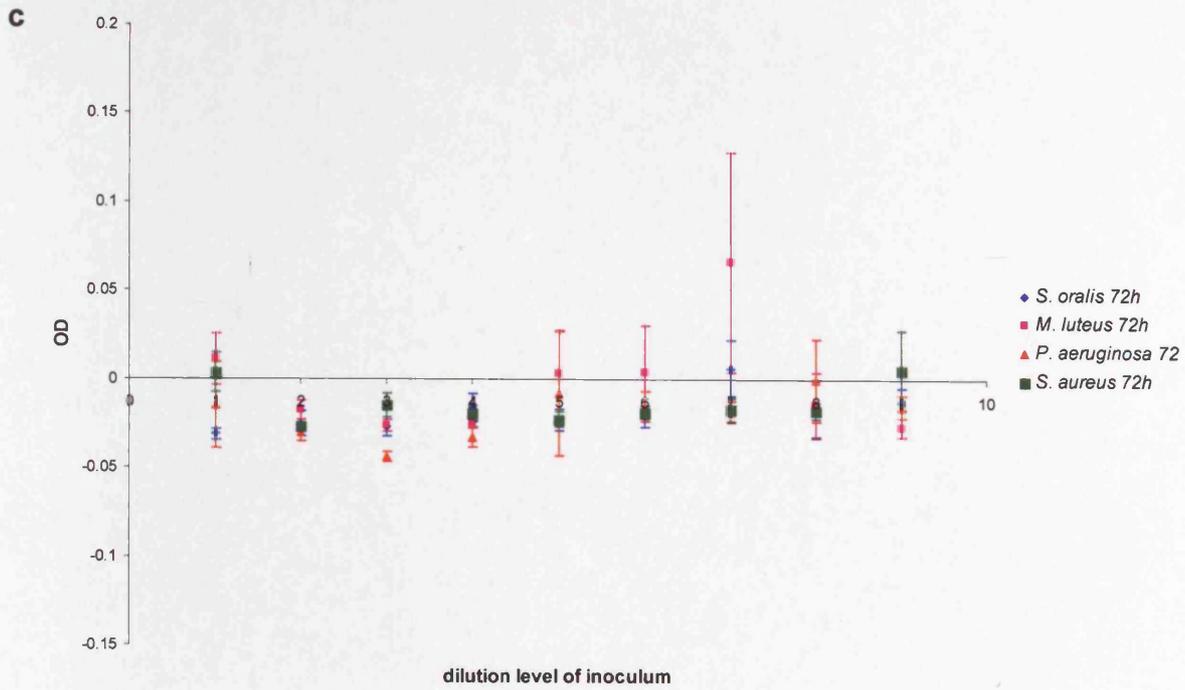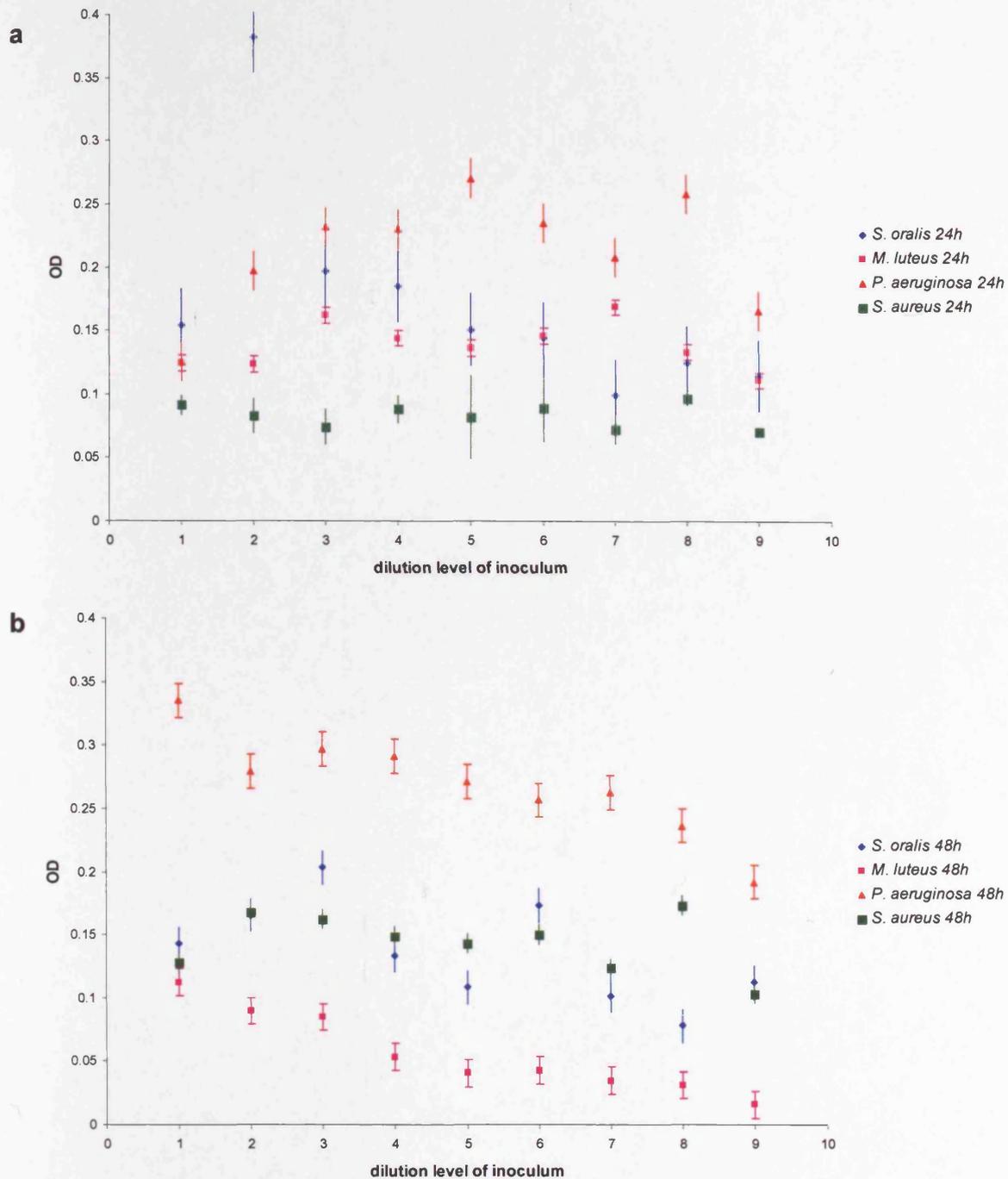
Fig. 4.2 Relative biofilm formation for different inocula levels of individually grown wound bacteria using the microtitre plate assay and TSB medium for a) 24h, b) 48h, c) 72h and d) 96h. Error bars represent standard deviation of 8 replicates.

166

Fig. 4.2 continued

c

dilution level of inoculum

Legend:
- S. oralis 72h
- M. luteus 72h
- P. aeruginosa 72h
- S. aureus 72h

d

dilution level of inoculum

Legend:
- S. oralis 96h
- M. luteus 96h
- P. aeruginosa 96h
- S. aureus 96h

167
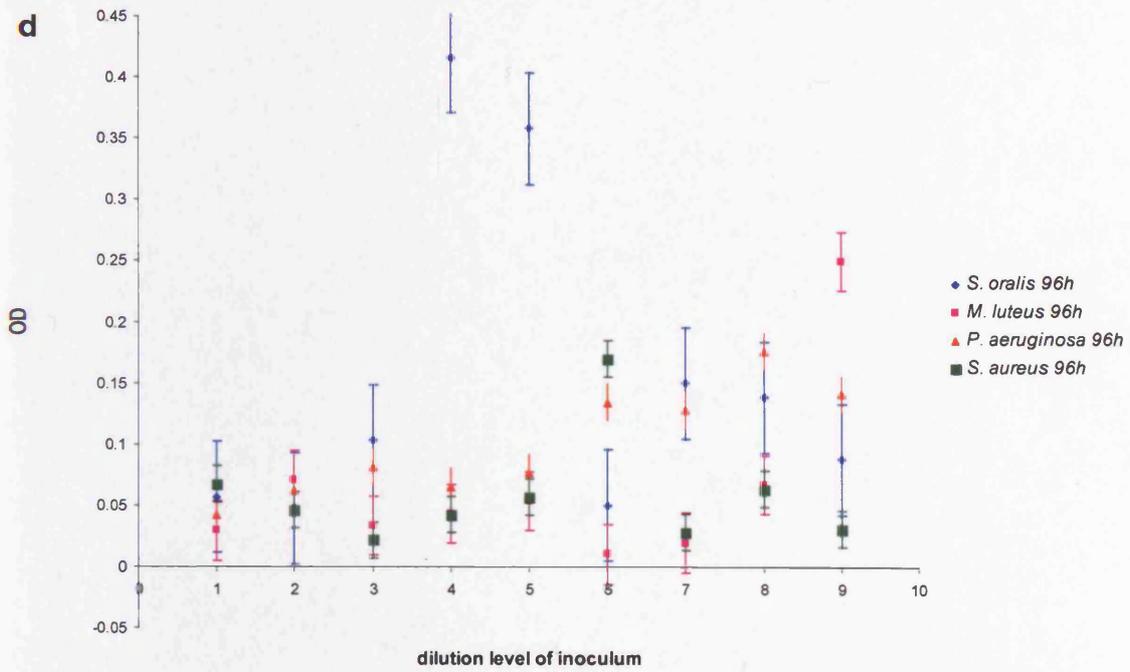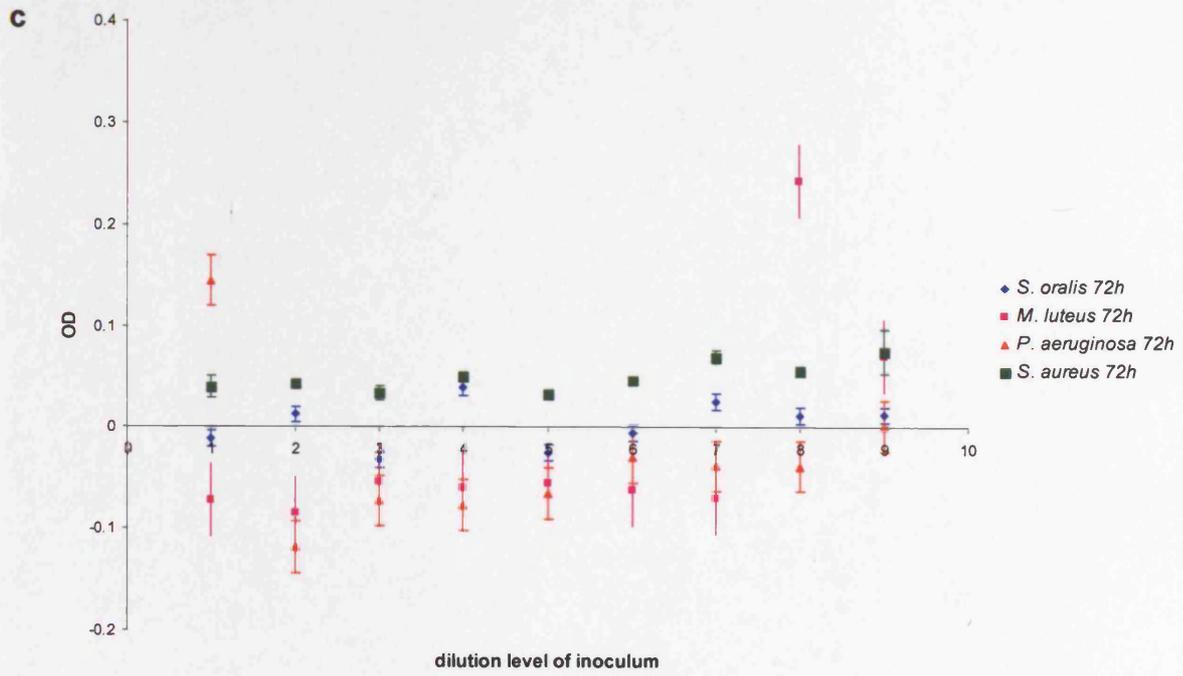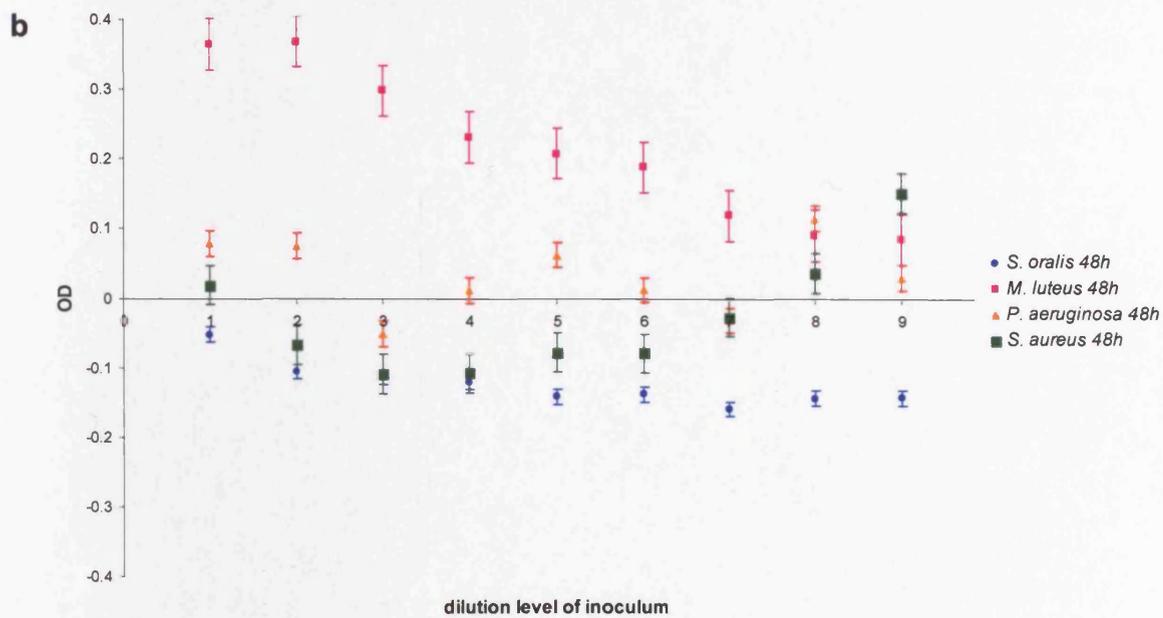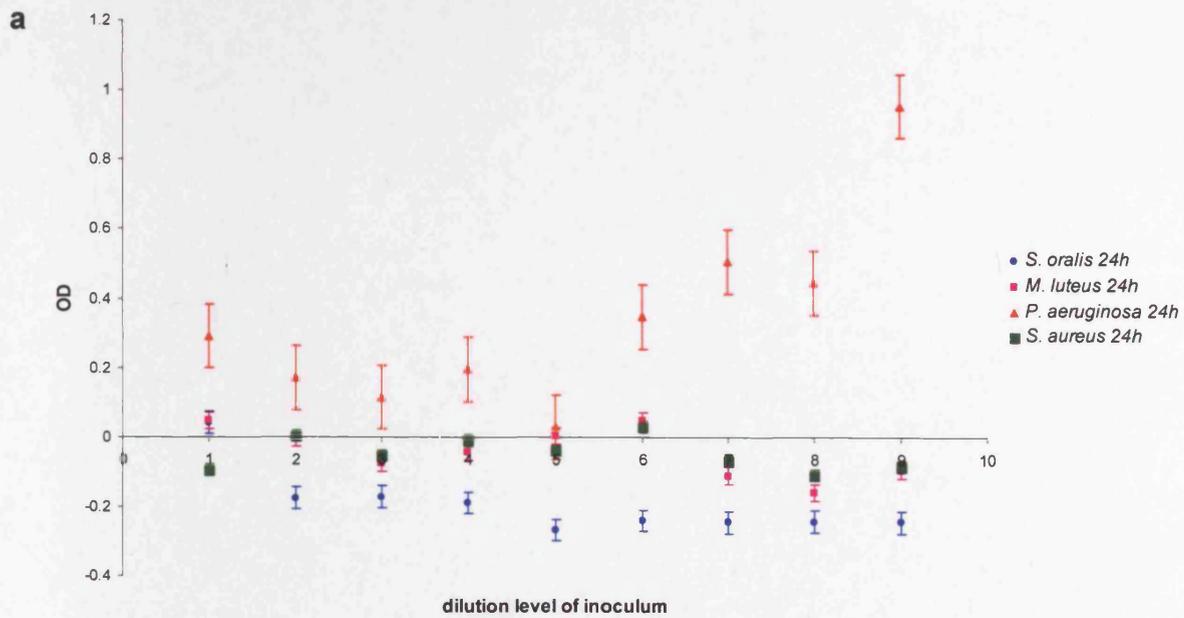
Fig. 4.3   Relative biofilm formation for different inocula levels of individually grown wound bacteria using the microtitre plate assay and BM medium for a) 24h, b) 48h, c) 72h and d) 96h. Error bars represent standard deviation of 8 replicates.

Fig. 4.3 continued

c

d
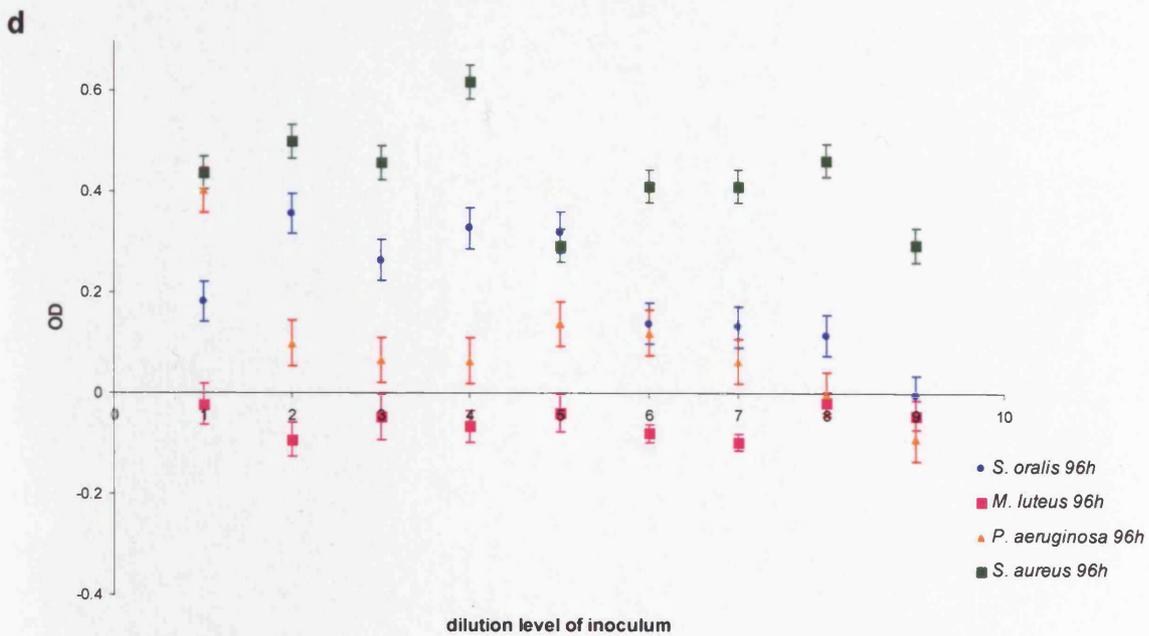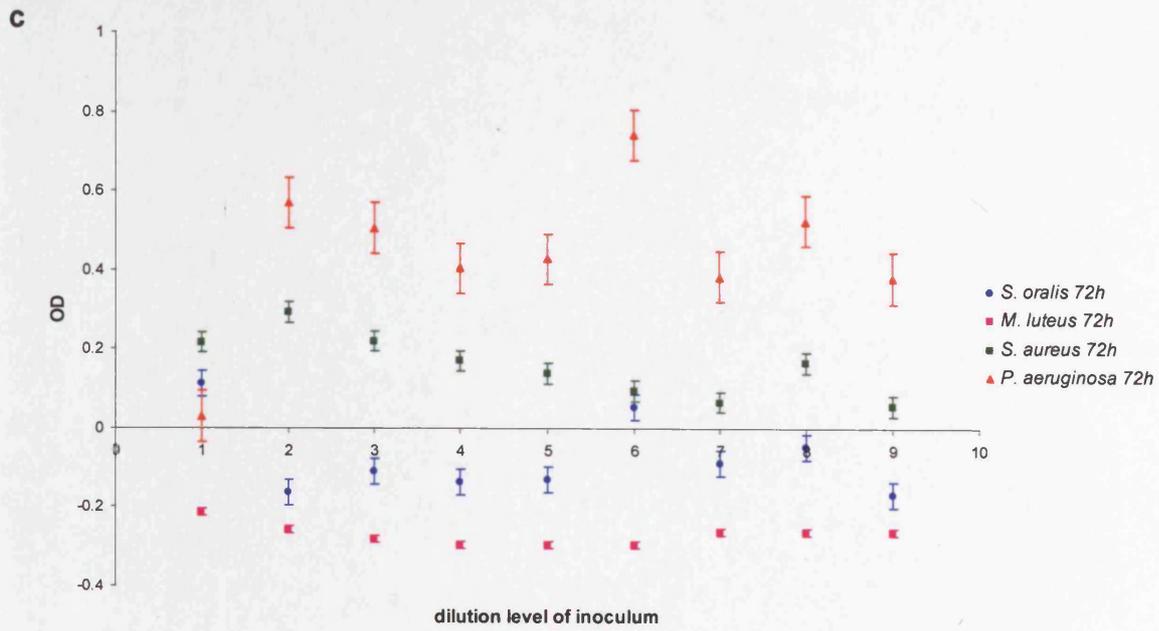
Using BHI, all the test species produced biofilms, although after 48 h the extent of biofilm formation decreased dramatically (Fig. 4.1). In BHI, the hierarchy of biofilm formation (as determined by OD) over 24-48 h was *M. luteus* > *S. oralis* ≥ *S. aureus* > *P. aeruginosa* with *M. luteus* producing a much thicker biofilm than the other organisms.

In experiments using TSB (Fig. 4.2), the most consistent levels of biofilms were again evident over 24-48 h, however the relative hierarchy of biofilm formation was found to change compared to that previously evident in BHI. In TSB medium, *P. aeruginosa* produced the greatest quantity of biofilm followed by *S. oralis*, *S. aureus* and *M. luteus*. All results were consistent, even when different starting inocula were used. In experiments using BM medium (Fig. 4.3) the levels of biofilm formation were lower, with no relative hierarchy of biofilm formation found between the species over 24-96 h incubation. *Pseudomonas aeruginosa* appeared to do best at 24 h, whilst *M. luteus* was predominant at 48 h and *S. aureus* at 96 h, presumably reflecting slower growth rates in this more defined medium.

Based on crystal violet staining results, subsequent microtitre experiments aimed at examining mixed species biofilms used a starting inoculum of 10^8 cells ml^{-1} with analysis after 24 h (Figs. 4.4 - 4.6). Figure 4.4 demonstrates the relative ability of single bacterial species to produce biofilms in BHI and TSB. As previously shown in Figs. 4.1 and 4.2, the relative hierarchy of the different species can clearly be seen.
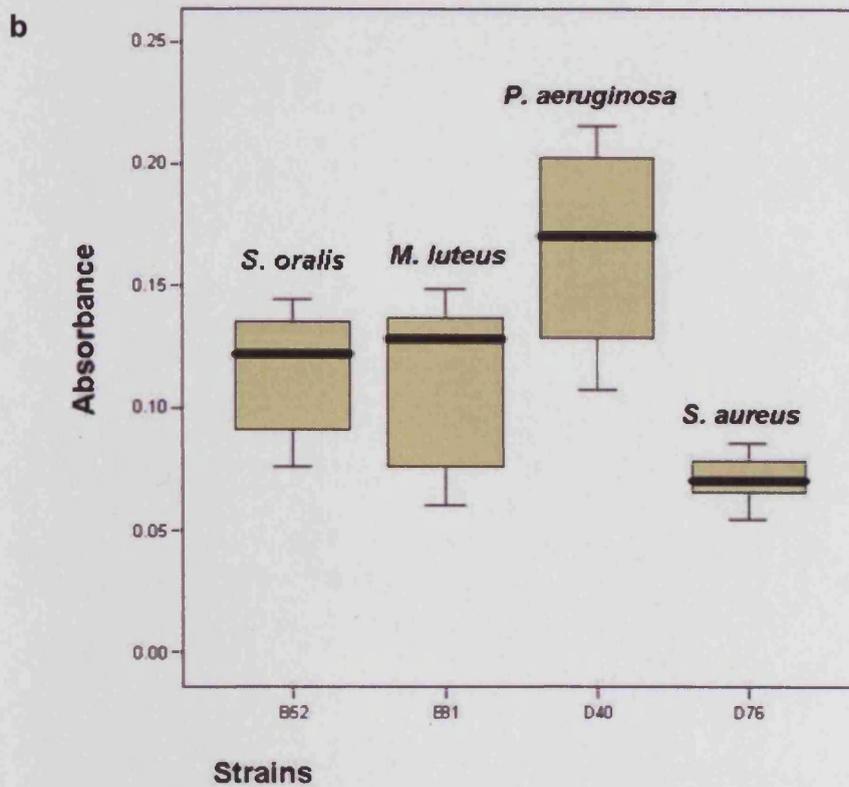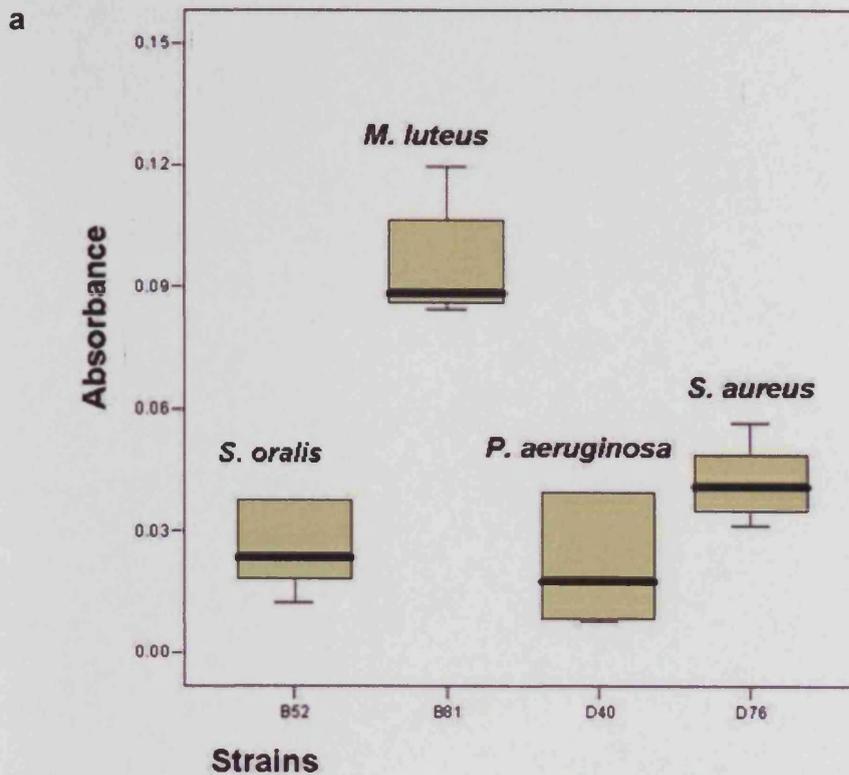
Fig. 4.4 Relative biofilm development for wound bacteria in the microtitre plate assay for 24h a) BHI and b) TSB medium. The box represents the data distribution between the 25th and 75th percentiles. The thick black horizontal line is the median. The median is described as the number separating the higher half of a sample from the lower half.
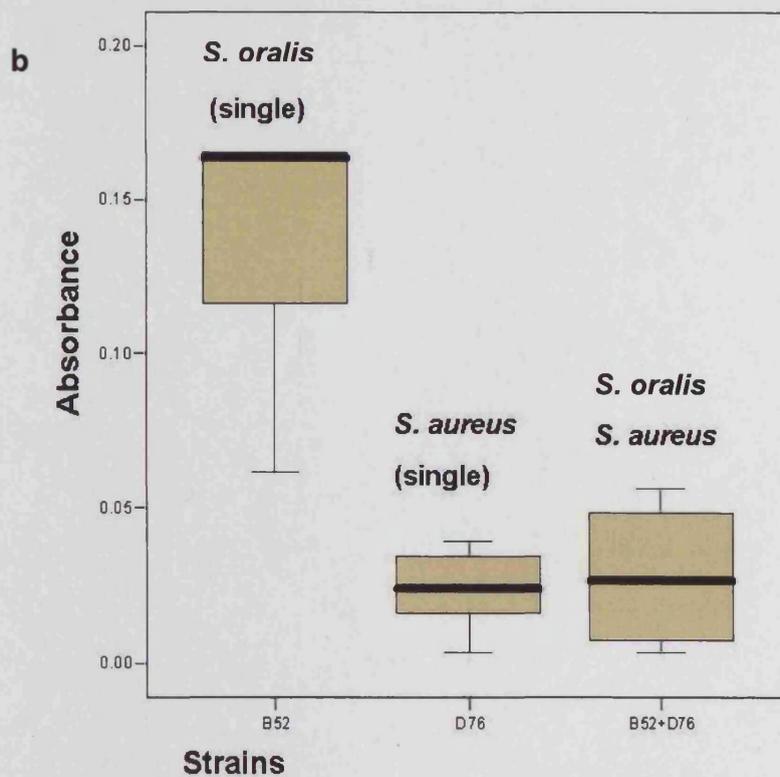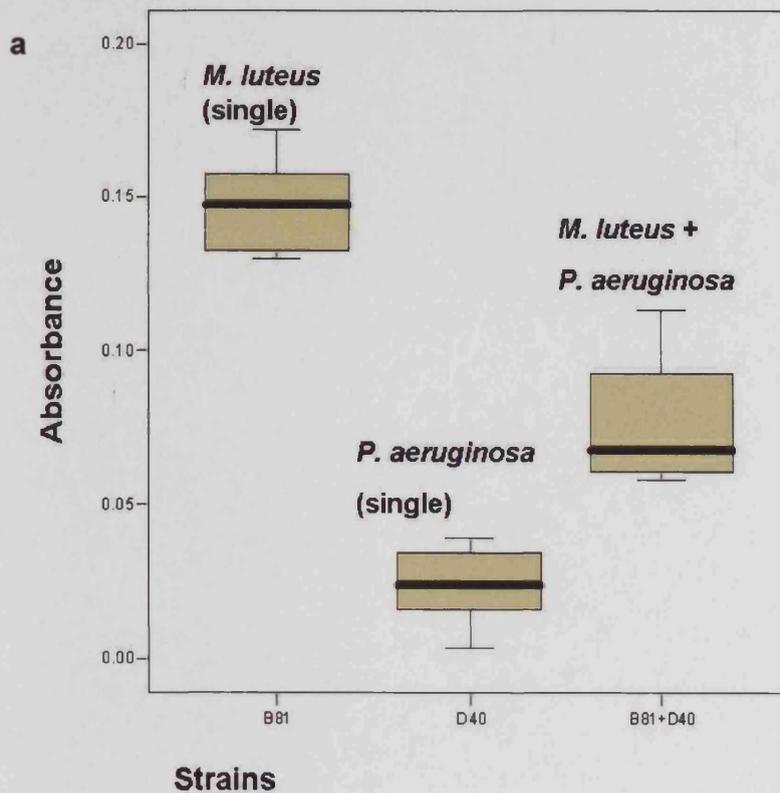
Fig. 4.5   Relative biofilm development for individual and mixed wound
bacteria in the microtitre plate assay for 24h in BHI medium, a) *M. luteus* and
P. aeruginosa; b) *S. oralis* and *S. aureus*.   The box represents the data
distribution between the 25th and 75th percentiles.   The thick black horizontal
line is the median.   The r   dian is described as the number separating the
higher half of a sample from the lower half.
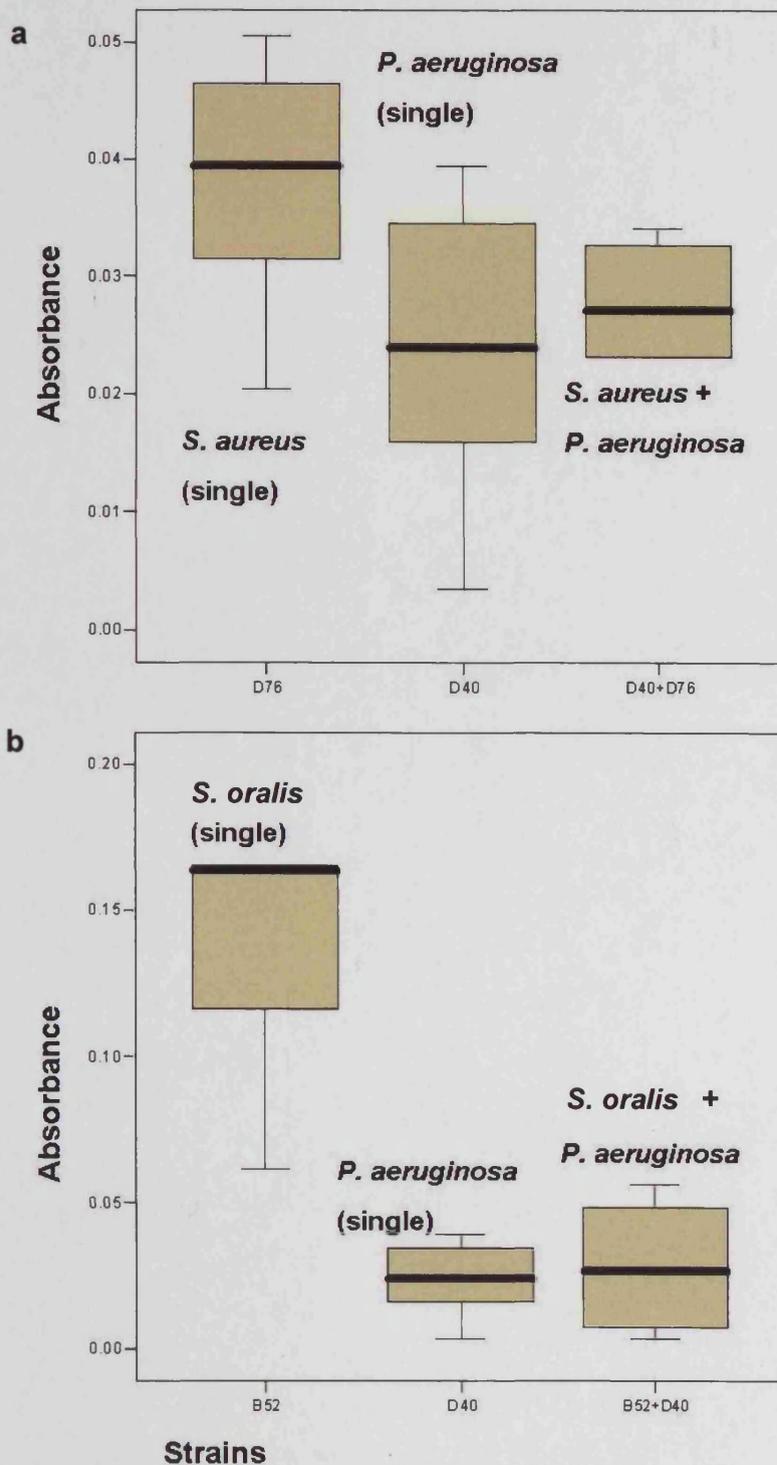
Fig. 4.6 Relative biofilm development for individual and mixed wound bacteria in the microtitre plate assay for 24h in BHI medium, a) *S. aureus* and *P. aeruginosa*; b) *S. oralis* and *P. aeruginosa*. The box represents the data distribution between the 25th and 75th percentiles. The thick black horizontal line is the median. The median is described as the number separating the higher half of a sample from the lower half.

Experiments investigating the extent of biofilm formation by different combinations of bacterial species were undertaken to assess whether synergism or antagonism occurred. Figures 4.5 and 4.6 demonstrate the findings of mixed species biofilm formation in BHI when compared with those of the single species. When *M. luteus* and *P. aeruginosa* were combined (Fig. 4.5a), a biofilm level intermediate to that generated by the respective single species was evident. Mixing of *S. oralis* and *S. aureus* (Fig. 4.5b) in the same growth medium resulted in a significant reduction of biofilm formation when compared to single *S. oralis* biofilm studies. *Pseudomonas aeruginosa* was also found to reduce the level of biofilm formation of *S. aureus* when the two species were cultured in combination (Fig. 4.6a). Similarly, *P. aeruginosa* was also found to greatly reduce the biofilm forming ability of *S. oralis* (Fig. 4.6b).

To obtain information on the composition of these biofilms, viable counts were obtained at 24 and 48 h (Table 4.4). These studies showed that, for example, *M. luteus* only grew in combination with *S. oralis*. When combined with either *P. aeruginosa* or *S. aureus*, *M. luteus* did not grow. *Streptococcus oralis* also did not compete well with *P. aeruginosa* in these biofilm studies without any growth again being recorded. The viable counts of the *P. aeruginosa* and *M. luteus* mixed culture (*M. luteus* no growth, *P. aeruginosa* 8.7×10^8 cfu ml^{-1}) support the idea of elevation of *P. aeruginosa* numbers in this mixed inoculum biofilm. In the case of *S. oralis* and *S. aureus*, the data suggest that there has been a decrease in the numbers of the former species (Fig. 4.5b).

4.4.3 Comparison of biofilm formation by test species using the CDFF

CDFF biofilm formation was evident for all four wound bacterial species when each was used as single species inocula (Fig. 4.7). In terms of cfu ml^{-1} from the recovered biofilms, the highest growth appeared to occur with *S. aureus* and *P. aeruginosa* throughout the 7 day incubation period (about 10^9 cells). Similar growth levels were detected for *S. oralis* and *M. luteus* (about 10^7 cells) which were much lower than those for *P. aeruginosa* and *S. aureus*.

Table 4.4 Analysis of a) single and b) mixed biofilms by total numbers in the microtitre plate assay in BHI medium for 24h and 48h.

a)

single species	24h; (cfu ml^{-1}) mean (n=3)	48h; (cfu ml^{-1}) mean (n=3)
P. aeruginosa (D40)	3.2×10^8	7.3×10^8
S. aureus (D76)	9×10^8	5.4×10^8
M. luteus (B81)	1.1×10^8	1.3×10^7
S. oralis (B52)	2.5×10^8	1.3×10^8

b)

Mixed species	24h; (cfu ml^{-1}) mean (n=3)	48h; (cfu ml^{-1}) mean (n=3)
M. luteus + *S. oralis*	B81: 2×10^7 B52: 3.7×10^8	B81: 1.8×10^6 B52: 4.8×10^8
M. luteus + *S. aureus*	B81: 0 D76: 5.6×10^8	B81: 0 D76: 5.7×10^8
M. luteus + *P. aeruginosa*	B81: 0 D40: 8.7×10^8	B81: 0 D40: 4.7×10^8
S. oralis + *S. aureus*	B52: 3.4×10^8 D76: 4.3×10^8	B52: 3.2×10^8 D76: 1.1×10^9
S. oralis + *P. aeruginosa*	B52: 0 D40: 1×10^8	B52: 0 D40: 2.2×10^8
S. aureus + *P. aeruginosa*	D76: 2.3×10^8 D40: 5×10^8	D76: 1.2×10^8 D40: 5×10^7

Data shown is the mean of 3 replicate experiments.
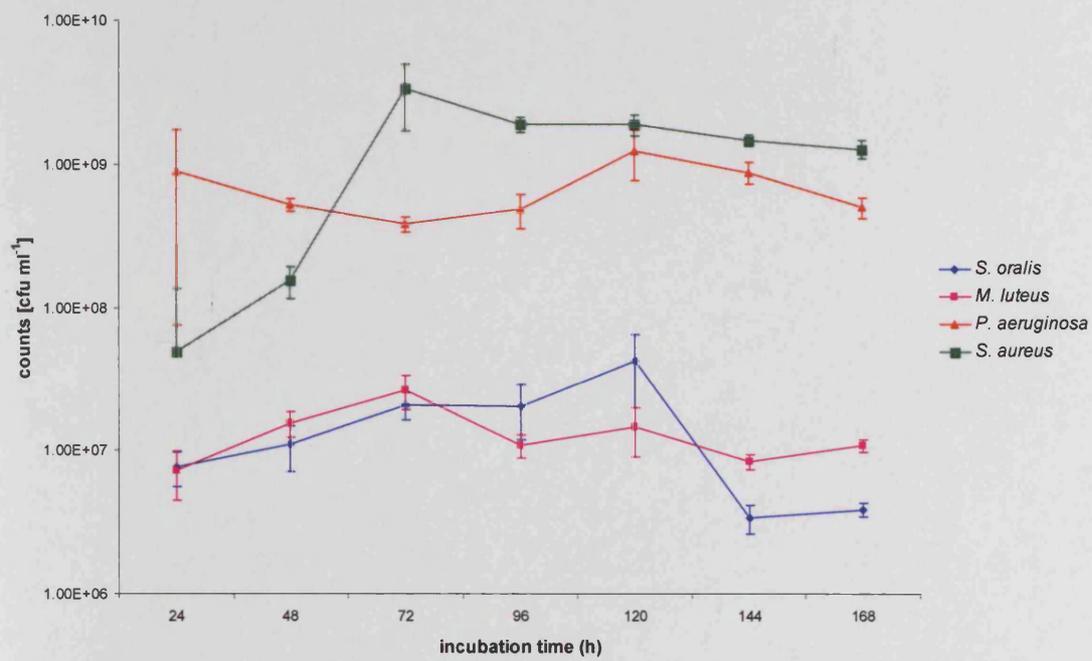
Fig. 4.7 Relative biofilm formation in the CDFF for 7 days in single species biofilms.

Figure 4.8 illustrates the ability to differentially enumerate the recovered bacterial species from CDFF generated biofilms. It was also noted that subcultures of *P. aeruginosa* from biofilms (Fig. 4.8) appeared to have different colony morphology compared to their planktonic equivalents (Fig. not shown). Using mixed species biofilms (Fig. 4.9), similar numbers of both *S. aureus* and *P. aeruginosa* were detected as previously reported in the single species experiments (10^8–10^9 cfu ml^{-1}). However, there was an apparent increase in the numbers of *S. oralis* compared with its single culture equivalent. In contrast, no growth of *M. luteus* in these mixed species biofilms was again detected throughout the experimental period. Interestingly, a similar loss of *M. luteus* was also noted in control mixed planktonic cultures of these isolates over 7 d incubation (Fig. 4.10). The viable count numbers for *S. aureus* and *S. oralis* were similar in both biofilm and planktonic mixed cultures, whereas counts for *P. aeruginosa* were at least one order of magnitude lower when grown in the planktonic state.

4.4.4 Planktonic test species' susceptibility to PVP1

The MBCs of planktonically cultured isolates against PVP1 were determined as the total inhibition of visible growth and the absence of viable bacteria growth. The latter was determined by taking a loopful of test solution and plating it onto BA. An example of a *P. aeruginosa* MBC using a microtitre plate assay is shown in Fig. 4.11. The MBC of PVP1 against *P. aeruginosa* and *S. aureus* was 1%, against *S. oralis* and *M. luteus* it was 0.25 % and 0.03%, respectively.
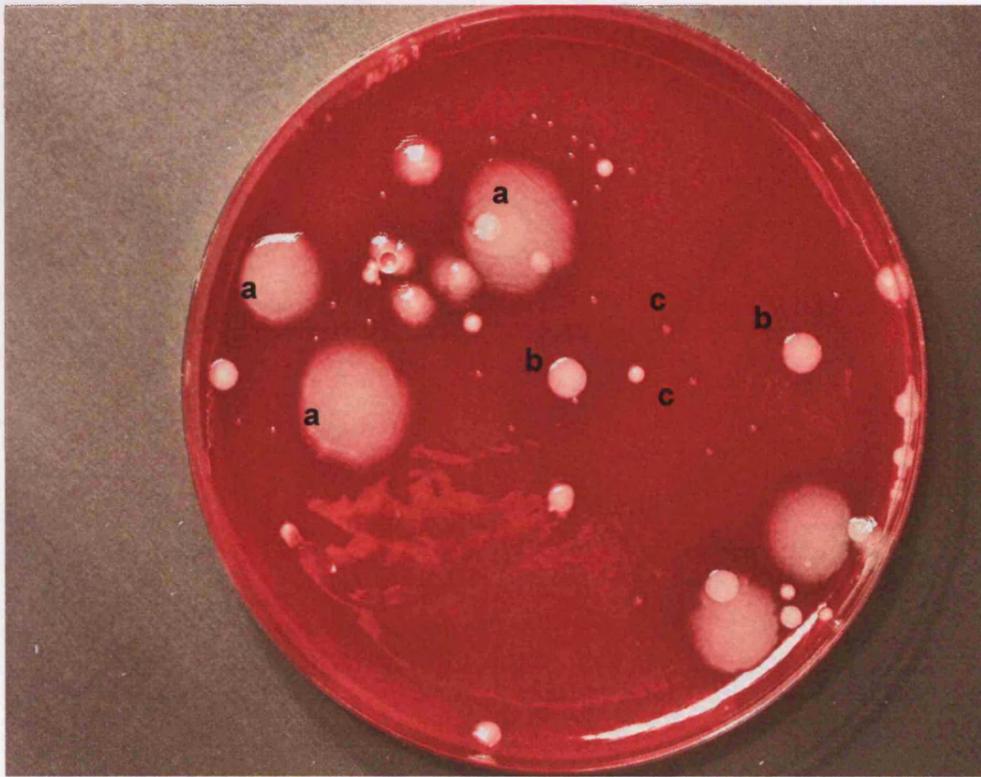
Fig. 4.8 Suspension of biofilms of chronic wound flora generated in the CDFF, shows colonies of a) *P. aeruginosa* (large colonies), b) *S. aureus* (smaller white discrete colonies) and c) *S. oralis* (small, haemolytic).
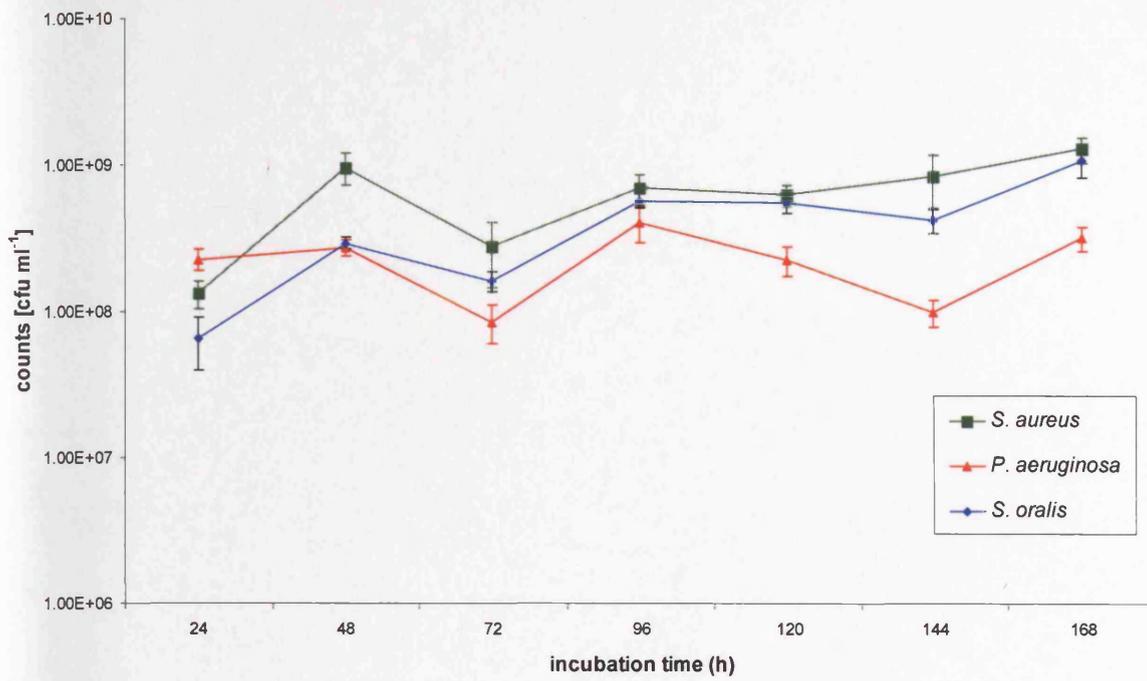
Fig. 4.9 Relative biofilm formation of four bacterial species in the CDFF for 7 days when cultured together as a biofilm; *M. luteus* not detected.
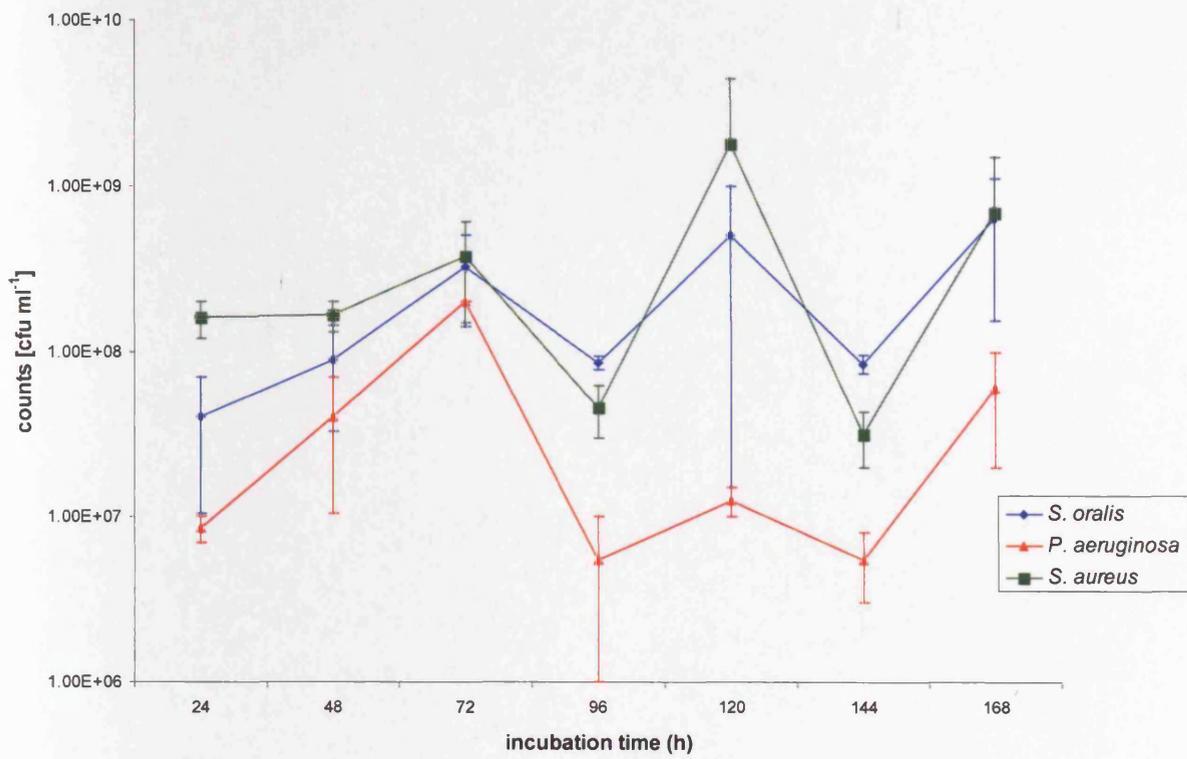
Fig. 4.10 Relative growth of four bacterial species in mixed planktonic culture; *M. luteus* not detected.
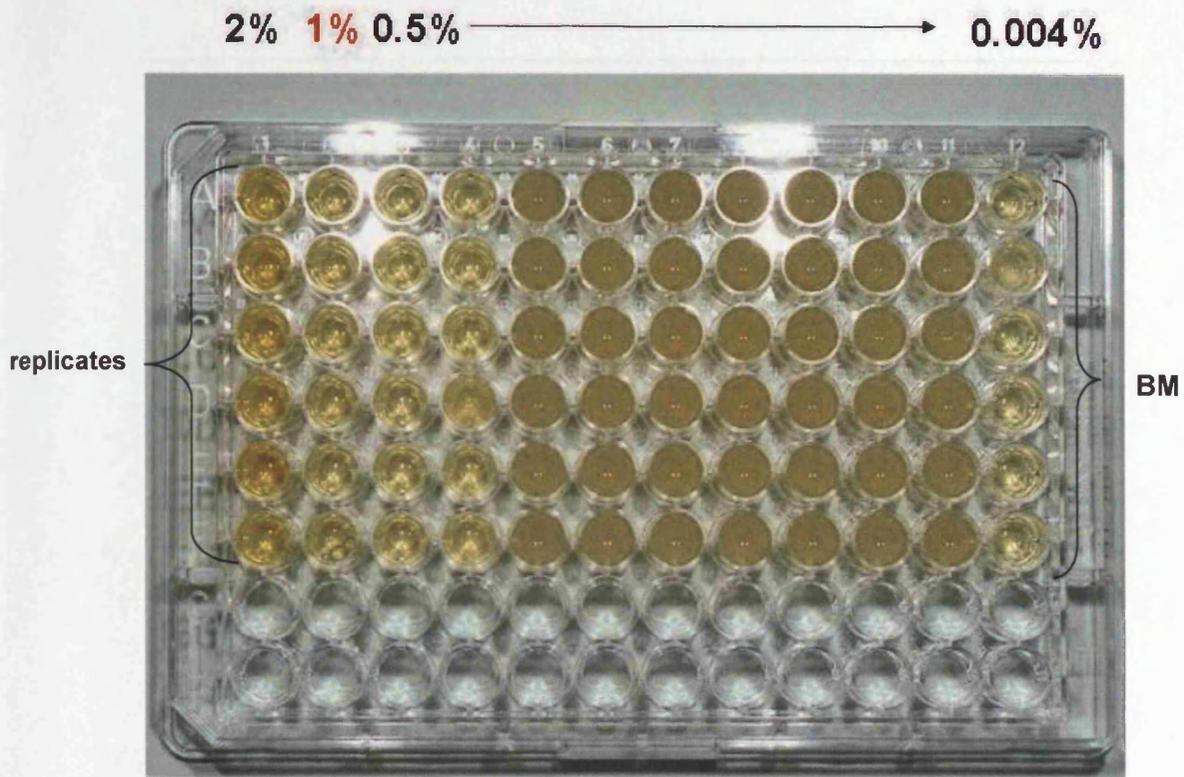
Fig. 4.11 Minimum bactericidal concentration (MBC) of planktonic *P. aeruginosa* to PVP1 in BM medium using a microtitre plate assay.
The MBC of *P. aeruginosa* was 1% PVP1. Even though the 0.5% PVP1 dilution seems clear, growth on BA plates was evident.

4.4.5 Susceptibility of CDFF generated biofilms to PVP1

Figure 4.12 shows the effect of 1% povidone-iodine (PVP1) on an established CDFF biofilm. Here, only two (*P. aeruginosa* and *S. aureus*) of the six bacterial species (*P. aeruginosa, S. aureus, M. luteus, S. oralis, B. fragilis,* and *P. anaerobius*) were detected at any sampling stage after initial inoculation. Addition of iodine to the medium between 6 and13 d resulted in a reduction in the numbers of *S. aureus* and *P. aeruginosa*. Between days 14-20, when PVP1 addition was stopped, the bacterial numbers in the biofilm rapidly returned to pre-treatment levels. PVP1 was again added for the same period of time, (between days 21 and 28), and as encountered previously there was again a decrease in the growth of both *S. aureus* and *P. aeruginosa* (one order of magnitude). However, within 1-2 days of terminating PVP1 treatment, the counts of these bacteria had returned to pre-treatment levels. The experiment was repeated twice and interestingly, *M. luteus*, *S. oralis* and the anaerobic species *B. fragilis* and *P. anaerobius* were not detected at any stage within the biofilm consortia.

After each PVP1 experiment a single colony of both *S. aureus* and *P. aeruginosa* was taken from a BA colony count plate and was inoculated into fresh BM medium and retested for PVP1 susceptibility. These planktonically grown cells were shown to be still susceptible to PVP1 at the original levels, with the MBC for both *S. aureus* and *P. aeruginosa* again being determined as 1%.

4.4.5.1 Live/dead stain of CDFF biofilms treated with PVP1

Figure 4.13 illustrates the viability of biofilm cells after PVP1 exposure. Biofilm cells were tested on day 27, after 6 d PVP1 treatment. Using this stain, dead cells appeared red and viable cells green. It was evident from analysing the PVP1 treated biofilms that the majority of cells in the biofilm preparation were red staining.
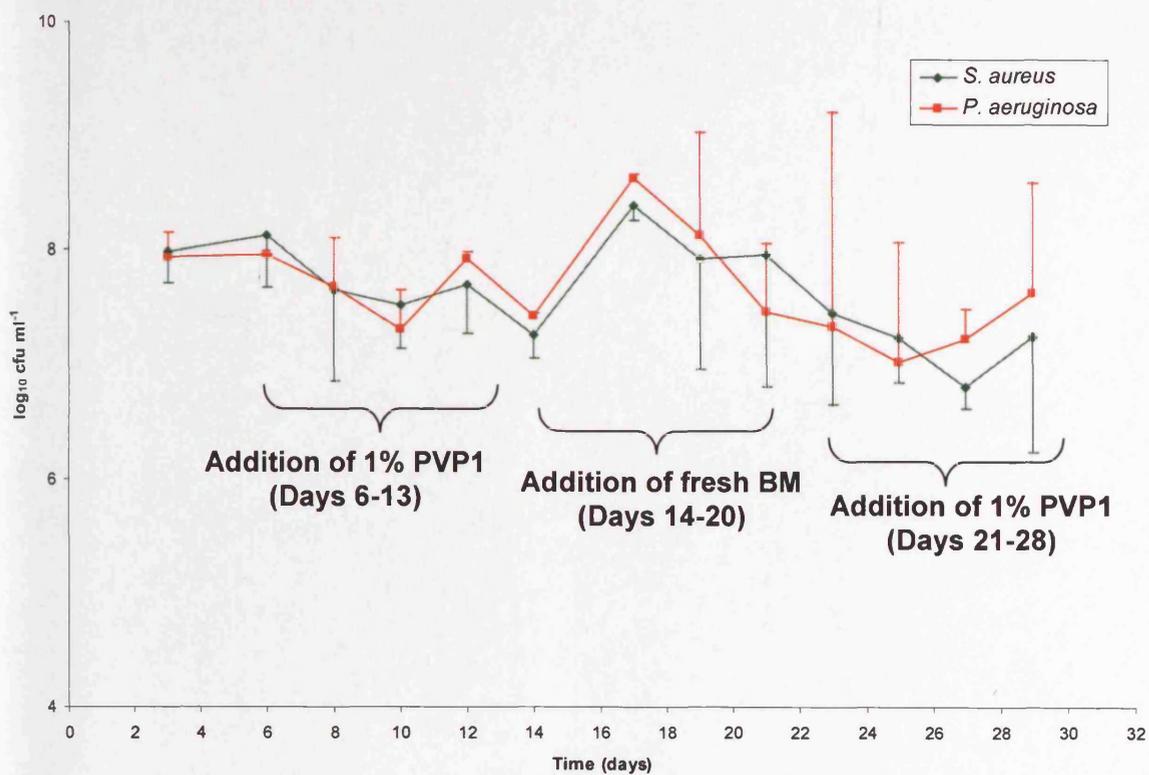
Fig. 4.12 Effect of PVP1 on a mixed six member wound bacteria consortium grown in BM. Error bars represent standard deviations (n=2). (*M. luteus* and *S. oralis* not detected).
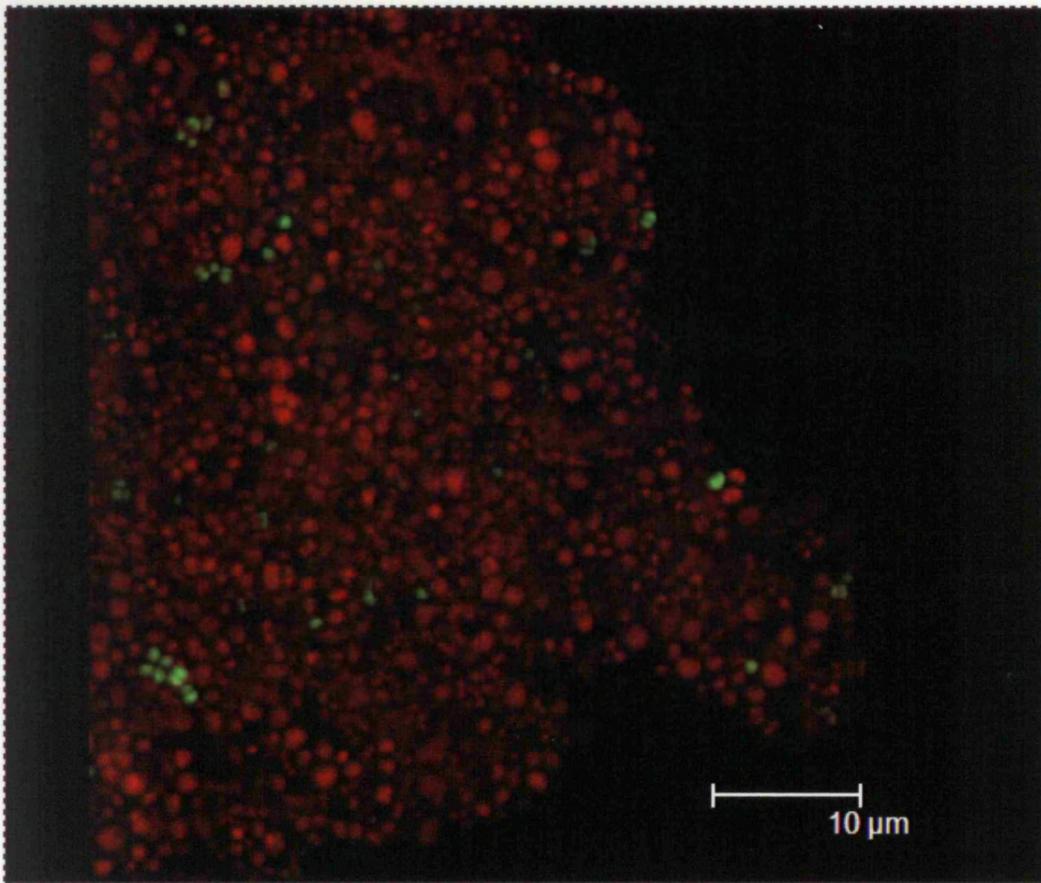
Fig. 4.13 Live/Dead® staining of a PVP1 treated biofilm smear using the *Bac*Light™ bacterial viability stain. Live cells appear green and dead cells appear red.

4.5 Discussion

A biofilm is often described as a community of microorganisms attached to a surface and encased within an extracellular polysaccharide matrix (EPS). In the laboratory, the microtitre plate assay is one of the most frequently used methods to measure the biofilm-forming ability of bacteria (Kennedy and O'Gara, 2004). In this present study, the microtitre plate assay was used to examine the *in vitro* biofilm formation of chronic wound bacterial species and in particular, to directly compare biofilm development of single (Fig. 4.4a and b) and mixed wound bacterial species (Fig. 4.5-4.6a and b).

The benefits of using the microtitre plate assay were that it enabled the rapid analysis of biofilm formation of multiple bacterial strains in a way that was inexpensive and relatively simple to use. The microtitre plate biofilm model does however have limitations. Principal amongst these is that it is a closed model (batch system) which ultimately limits the level of biofilm formation through depletion of nutrients and accumulation of waste products. However, this could partially be overcome in any future experiments by replenishing the growth medium at specific time intervals. The microtitre plate assay was also labour intensive to prepare, particularly when many isolates were being tested. Furthermore, inclusion of more than one species per plate can promote contamination problems between neighbouring wells.

Throughout the study, species differences in the level of biofilm generated in the microtitre plate assay were evident, and consistent for any given medium. The hierarchy of biofilm formation altered with the medium and this highlights the importance of the environmental milieu on biofilm development, which is consistent with ideas presented by Kennedy *et al.* (2004). These differences in the biofilm forming ability between the species could either be a result of the different growth rates of the species in the chosen media, or a reflection of the adherence properties of the species to the microtitre plate surface. Previous work has indeed demonstrated that bacteria adhere more favourably to some materials than others (Simões et al., 2007a). Furthermore, differences in the adherence capability to polystyrene microtitre

plates has been found between strains of some species, including *S. aureus* (Stepanovic et al., 2000).

For all subsequent experiments using mixed species analysis, BHI, was used as the growth medium, since this was found to be the most consistent of the tested media in supporting biofilm formation for all the bacterial species. BHI has also been widely used and validated in previous work (Fitzpatrick et al., 2005; Kennedy and O'Gara, 2004; Parsek and Singh, 2003).

It was interesting to note that in mixed species biofilms, both *P. aeruginosa* and *S. aureus* appeared to antagonise the biofilm forming ability of *S. oralis* and *M. luteus*. Such an effect could occur for a variety of reasons including occurrence of inter-species competition for surface attachment sites, competition for nutrients within the growth medium or by production of a less stable biofilm architecture. Furthermore, a motile organism like *P. aeruginosa* can readily be envisaged as having an advantage over non-motile counterparts within a biofilm environment. As the biofilm matures it is inevitable that areas will develop that are restricted with respect to nutrient and gaseous availability. In theory, a motile organism could migrate to areas of the biofilm that are more suitable for its growth.

Simoes *et al.* (2007b) also reported antagonism between bacterial species isolated from drinking water in biofilms generated within 96 well microtitre plates. The microbial interactions were compared between single and dual biofilms. Weak biofilm production was found between *Sphingomonas capsulate* and *Mycobacterium mucogenicum*, *S. capsulate* and *Acinetobacter calcoaceticus* and between *M. mucogenicum* and *Staphylococcus* spp. (Simoes et al., 2007b). Kreth *et al.* (2005) also showed that *S. mutans* and *S. sanguinis* engage in antagonistic interactions in dental biofilms.

The predominant microorganisms in chronic wounds have been determined as *P. aeruginosa* and *Staphylococcus* spp. found in 34.8% (surface), 31.8% (tissue), and 71.2% (both surface and tissue) of patients (Davies et al.,

2007). However, it is still unclear whether all four of the microorganisms used in this study occur simultaneously within the same wound and therefore whether the relative proportions of these species within a wound environment reflects that found in these *in vitro* studies. Further clinical research of chronic wounds is required to understand the relationship between biofilm communities and the wound healing process. Extrapolating the results in the present *in vitro* study to wounds might suggest that *P. aeruginosa* would be expected to predominate over organisms such as *M. luteus*. Chronic wounds do show considerable diversity in the species of bacteria present, although *S. aureus* and *P. aeruginosa* do appear to be the predominant organisms encountered (James et al., 2007; Jones et al., 2004b).

Generally a lack of synergy between the bacterial species in the microtitre plate assay and in the CDFF was observed with the exception of *P. aeruginosa* which appeared to perform much better in the presence of *M. luteus* (although this was to the detriment of the *Micrococcus* species; Fig. 4.5a). The ability of strains to compete in this *in vitro* environment could theoretically be related to the relative bacterial numbers encountered in a wound environment. Davies *et al.* (2007) in a study examining the relationship between bacterial load and wound status, quantified both *S. aureus* and *P. aeruginosa* from the same chronic wounds and showed that the relative proportions of these two species was variable depending on patients (results not shown). Equivalent results for *Micrococcus* species were not however available.

The inability to extrapolate the *in vitro* findings of this present study to those obtained from the work of Davies *et al* (2007) highlight the role of other factors that are present in the wound, including the presence of other microorganisms and host defence processes. In all microbial communities, a degree of competition between species is expected despite the fact that a stable community exists.

It could be hypothesised that *M. luteus* represents a wound contaminant from the skin with only a transient occurrence in chronic wounds, whilst the presence of *P. aeruginosa* and/or *S. aureus* could be indicative of the pathogenic state. However, *Micrococcus* spp. were found to be present in 64% of patients (Davies et al., 2007) suggesting that this species is a true coloniser of chronic wounds. In this regard, treatment options targeting *P. aeruginosa* would be advocated. Further work is still needed on clinical samples to confirm this hypothesis.

Due to the previously mentioned limitations of the microtitre plate assay the Constant Depth Film Fermenter (CDFF) was also used to compare wound bacterial biofilms. The advantages of the CDFF over the microtitre plate system included, 1) the medium could be constantly replenished and the waste components of the medium removed; 2) many replicates of equivalent biofilms could be obtained; 3) the flow rate of the medium and any supplements could be introduced as required; 4) almost any material could theoretically be incorporated into the CDFF and serve as the biofilm substratum. The CDFF model also has the potential to be adapted to simulate the wound environment further, for example by the addition of fixed volumes of collagen or fibronectin. However, the model also has limitations, in that it is not able to simulate the effects of host factors, it is an abiotic surface and lacks the presence of components of the host immune system (Chapter 5 describes the use of a skin equivalent system that addresses some of these limitations). Additionally, it is important to be aware of the potential risk of contamination from airborne bacteria being introduced when opening up the system daily to take samples. Nevertheless, it remains important to gain an understanding of biofilm physiology by studying various aspects of a biofilm in isolation, and the CDFF is an ideal model for such analysis.

Results from the CDFF again showed that all four bacterial species grown individually in the CDFF were able to form biofilms and that these biofilms were readily maintained (Fig. 4.7). Interestingly, the relative species

hierarchy of biofilm formation using BHI medium was different between the microtitre plate assay and the CDFF. In the microtitre plate assay, the hierarchy of biofilm formation was *M. luteus* > *S. oralis* ≥ *S. aureus* > *P. aeruginosa*. In the CDFF, *S. aureus* produced the highest level of biofilm followed by *P. aeruginosa, S. oralis* and *M. luteus.* The explanation for these differences in biofilm hierarchy could be that the CDFF provided an open system where fresh media was being constantly replenished and this may have been of greater benefit to those species with higher growth rates. In addition, the actual surface for attachment of the bacteria was different in the the two models which could influence initial adherence and subsequently the relative growth of the respective species.

Whilst it was evident that multispecies biofilms of chronic wound bacterial species were able to be established and maintained within the CDFF model, it was apparent that anaerobes could not be detected when included in mixed species biofilm consortia. This could be because the CDFF did not provide a truly anaerobic environment since anaerobic gas was not infused into the fermenter for these experiments. Bradshaw *et al.* (1998) did however find that anaerobes can flourish in a primarily aerobic environment as they utilise specific anaerobic pockets within the biofilm. For this to occur however, the anaerobic niches have to develop prior to loss of the anaerobic bacteria within the system. Furthermore, it is also possible that in this present study the anaerobes required a richer growth medium to be supported instead of Brain Heart Infusion (BHI) medium. BHI had been selected based on the previous microtitre plate experiments which did not include anaerobic bacterial species.

In this present study, all bacterial species were added simultaneously and it is important to note that under such situations the anaerobes would have had to compete with aerobic organisms in an entirely aerobic environment. It has been suggested that in a wound environment, the early contaminants are most likely to be skin flora (e.g. *Staphylococcus epidermidis* and *Micrococcus* species), which adhere to the wound, proliferate and synthesise EPS

(Percival and Bowler, 2004). These pioneer colonisers are then followed by Gram-negative bacilli which populate the biofilm, utilise oxygen and provide growth factors for anaerobes to establish themselves within the biofilm (Percival and Bowler, 2004). Future work could therefore attempt to create such a succession of colonisation of the biofilm through a later introduction of the anaerobic bacteria into the system.

In the CDFF experiments that combined all four bacterial species, *M. luteus* appeared to be 'out-competed' by the other organisms present (Fig. 4.10). These results would therefore confirm those earlier findings of the microtitre plate assay (section 4.4.2). The analysis of viable counts from biofilm cultures also showed that *M. luteus* did not grow in combination with both *S. aureus* and *P. aeruginosa* (Table 4.6 b). Since *M. luteus* was able to form biofilms in single culture it is possible that this organism can rapidly colonise the substratum used and thus as a non motile organism provide the base of the maturing biofilm. Given such a location in the biofilm structure and the associated limitations in terms of nutrients and oxygen at this site it is possible that a dormant state or even a completer loss of viability might occur for these organisms (Hill et al., 2003). However, the fact that *M. luteus* also appeared to be 'out-competed' within a planktonic (Fig. 4.11) mixed culture does support the likelihood of a true competition for nutrients with the other bacteria as opposed to a strictly biofilm related factor.

It is also worth noting that subcultures of *P. aeruginosa* from the mixed biofilms generated in the CDFF appeared to have a different colony morphology compared to those of planktonic origin, suggesting that there were phenotypic variations occurring for the *P. aeruginosa* in the two different growth phases (Fig. 4.8). This phenotypic switch not only highlights the successful biofilm development in the CDFF but also validates the importance of subsequent investigations into any associated gene expression changes within these biofilms (Chapter 6). It has been suggested that under stress conditions, bacteria might possess diverse mechanisms that may lead to an increase in genetic and phenotypic variability (Webb et

al., 2004). These mechanisms include adaptive mutation (Bjedov et al., 2003; Oliver et al., 2000; Taddei et al., 1995), phase variation (Drenkard and Ausubel, 2002) and enhanced gene transfer (Hausner and Wuertz, 1999; Molin and Tolker-Nielsen, 2003).

In the present study, the CDFF was also used to model the sensitivity of a biofilm comprising chronic wound bacteria to PVP1. PVP1 is a bacteriostatic antimicrobial which is commonly used as a topical agent in the treatment of wound infections (Mertz et al., 1999). Similar studies with antimicrobials have been done previously using the CDFF. Ready *et al.* (2002a) used the CDFF to examine the effect of tetracycline on the composition and antibiotic resistance profile of dental plaque. However, that study examined the effect of a single pulse of antibiotic rather than continuous administration (Ready et al., 2002a). Also, their group did not compare the sensitivities of bacteria recovered from the biofilm to the antibiotic when subsequently grown in the planktonic state

Initial experiments with PVP1 in the present study, showed that planktonic organisms were susceptible to a 1% concentration of PVP1. This is the concentration that is believed to be available to the wound surface from PVP1 impregnated dressings such as Inadine (Kumar et al., 2004). In contrast, results showed that 1% PVP1 had only a slight effect on CDFF generated bacterial biofilms. However, when colonies from *S. aureus* and *P. aeruginosa* were recovered from the CDFF and then grown planktonically, they remained susceptible to this 1% PVP1 concentration. This suggests that the PVP1 resistance of the CDFF biofilms was associated with the biofilm phenotype rather than being the result of an acquired resistance by individual organisms exposed to the agent. The data presented in this study concurs with that of Anderl *et al.* (2003) who suggested that planktonic cells in stationary phase rapidly regain antibiotic susceptibility upon dilution in fresh medium, which provides nutrients and also might dilute protective cell-cell signalling pathways (Anderl et al., 2003). This reversibility in

antimicrobial sensitivity indicates that biofilm tolerance of antimicrobials represents a phenotypic rather than a genotypic alteration (Fux et al., 2005).

One mechanism of biofilm resistance to antimicrobials could be explained by the 'persister cell' theory. As previously mentioned, bacteria embedded deep within a biofilm are likely to be less metabolically active because of poor access to essential nutrients, including oxygen, and may have problems with the accumulation of waste metabolites in their surroundings. This reduced activity has been linked to the finding that surface biofilm cells are normally large in size whilst the biofilm embedded cells are often smaller (Anwar et al., 1992). Embedded cells, or 'persister cells' are at a stage of 'dormancy' thereby making them phenotypically better equipped to persist in hostile environments (Anwar et al., 1992; Rhoads et al., 2007). It was interesting to note that live/dead staining of PVP1 biofilms indicated a majority of 'dead' cells. This could be indicative of 'persister cells' existing in the established biofilm, which are dormant but culturable as seen by the viable counts. Another reason could be that PVP1 does not actually kill the bacteria but might only damage the cell membrane which is then reflected as the bacteria staining 'dead' by the live/dead stain. It is important to note that on withdrawal of treatment with PVP1, the level of bacterial numbers within the biofilm rapidly reverted to those seen before treatment. This observation might have important clinical implications in terms of the duration of treatment with topical PVP1.

The antimicrobial activity of an agent on a biofilm can occur by two mechanisms. Firstly, there is the direct effect of the agent on the microbial cells themselves. PVP1 clearly demonstrates effective killing against the cells used in this study as seen from the planktonic cell inhibition work. It has also been proposed that topical antimicrobials enhance the recruitment of polymorphonuclear neutrophils (PMNs) to the site of biofilm occurrence (Akiyama et al., 2003). For example, treatment of S. aureus skin infections with 2% fusidic acid ointment resulted in 100 times lower numbers of bacteria in normal mice than in neutropenic mice 12 h after treatment (Akiyama et al.,

2002). As a consequence, whilst 1 % PVP1 appears to have limited value in combating 'wound biofilms' *in vitro*, the *in vivo* effects may be higher through synergism with patient host defence mechanisms.

This present study showed that the CDFF was an effective and reliable model to generate *in vitro* sustainable growth of multispecies biofilms from chronic wound bacteria, as evident by viable count determination (and later by CLSM images of the biofilms; see Chapter 5). The PVP1 experiments highlight the value of the CDFF for future *in vitro* experiments examining both conventional and novel therapeutic interventions. The CDFF model also has great potential to be adapted to allow investigation of a wide range of other medically important biofilm-related infections, with wide reaching implications.

4.6 Summary

1. This study demonstrated the development of *in vitro* biofilm models by identified wound bacterial species.

2. Wound bacteria were able to produce biofilms in both a microtitre plate assay and in the CDFF. The CDFF allowed multiple, identical and reproducible biofilms to be grown simultaneously. Species differences in terms of level of biofilm formation were evident for any given growth medium and species antagonism occurred in mixed species biofilms for certain bacterial combinations.

3. Susceptibility of the wound bacterial biofilms generated in the CDFF to PVP1 was limited compared with planktonic cell susceptibility and any effects observed rapidly returned to pre-treatment levels once PVP1 treatment was stopped.

Chapter 5

Structural analysis of biofilms

generated by wound bacteria

5.1 INTRODUCTION

Previous chapters of this thesis have described the use of *in vitro* models to construct biofilms using oral (*Candida albicans*) and wound (bacterial isolates) microorganisms. Although a lot of research has been done in the study of oral plaque biofilms (Gu et al., 2005), there have been limited studies undertaken on wound biofilms despite the recent proposed role of biofilm involvement in delaying wound healing (Davies et al., 2007; Mertz, 2003).

Sophisticated molecular techniques have been applied in many fields of microbiology. However, in the application of molecular techniques to biofilms, little information is generally obtained regarding biofilm morphology or architecture. For example, in the case of DNA/RNA extraction, cells are often pooled and any tissue/biofilm destroyed in the extraction process. Traditional microscopical analyses and bacterial stains, like the Gram or Ziehl-Neelsen stain (Moter and Gobel, 2000) are useful, but limited, as they often rely on prior cultivation of the bacteria. Whilst Gram staining provides information on the type of bacterial cell wall it does not allow for species identification (Moter and Gobel, 2000). It is important to highlight that microbial communities are fundamental in the functioning of all ecosystems (Tyson, 2004). Since cultivation techniques tend to work only for a minority of species (only 2-3% of bacteria are thought to be cultivable using currently available media), the development of alternative methods for the identification and visualisation of bacteria has become a necessity (Amann, 2001; Moter and Gobel, 2000).

5.1.1 Bacterial detection and distribution in a biofilm community

Biofilm formation is a dynamic process involving continuous changes in structure and composition through microbial attachment, detachment, growth of different species and the production of extracellular polysaccharides (EPS) (Gu et al., 2005). An ambitious goal in microbiology is to have rapid and accurate identification of pathogenic bacteria within biofilm communities. Effective monitoring of the organisms within a biofilm is a crucial step in

assessing the possible risk or the status of a disease (Gu et al., 2005). Marsh *et al.* (2003) established a connection between the proportions of certain bacterial species in the oral biofilm (plaque) and the absence or presence of dental caries. Marsh *et al.* (2003) suggested that changes in the environment (*e.g.* nutrition, oxygen, pH, long term use of medication) alter the pattern of gene expression of oral bacteria, so that the competitiveness of the species associated with disease is increased, while that of organisms that normally prevail in health is reduced (ecological plaque hypothesis). Dental caries is associated with an increase in the proportion of acidogenic and aciduric bacteria (*e.g. Streptococcus mutans*, *S. sobrinus* and lactobacilli) which demineralise the enamel, and in periodontal diseases anaerobic bacteria including Gram-negative proteolytic species are increased. These plaque mediated diseases could be prevented by interfering with the environmental factors as well as by inhibiting the putative pathogens (Marsh, 2003). The work on dental plaque therefore highlights the potential importance in analysing the diversity and distribution pattern of pathogenic organisms which reside within chronic wounds or any other biofilm-related infections.

5.1.2 Biofilm structure within a community

In the majority of natural habitats, microorganisms exist as biofilms, which usually comprise (although not exclusively) of a variety of species and genera. Biofilms are formed after attachment and growth of microorganisms on a broad range of surfaces (Thurnheer et al., 2004). Biofilms are comprised of water, cells, cell aggregates and microcolonies embedded in an EPS matrix (Costerton et al., 1995; Costerton et al., 1999). The biofilm architecture and the physiological status within this community is the main interest for clinical, industrial and environmental microbiology (Thurnheer et al., 2004). Further detail on biofilm structure is provided in the Literature Review (section 1.2.2).

5.1.3 Fluorescence *in situ* hybridisation

Fluorescence *in situ* hybridisation (FISH) is a relatively recent and now frequently used culture-independent approach for detecting and visualising microorganisms. This technique detects nucleic acid sequences within cells using fluorescently labelled probes that hybridize specifically to their complementary target sequences within intact cells (Moter and Gobel, 2000). FISH therefore combines the precision of molecular genetics with the visual imaging of fluorescent microscopy. Hence it permits visualisation and identification of individual cells within their natural habitat or diseased tissue (Moter and Gobel, 2000). The FISH procedure includes the following steps (Fig. 5.1): a) fixation of the specimen; b) preparation of the sample, including specific pre-treatment steps if required; c) hybridisation with probes for detecting the respective target sequences; d) washing steps to remove unbound probes; e) mounting and visualisation (Moter and Gobel, 2000).

More often, probes are used that target ribosomal RNA molecules as these are functionally conserved, abundant and present in all cells. The small subunit (16S and sometimes 23S) rRNA molecules are ideal targets for bacterial detection because they are stable and have high a copy number of molecules per cell (usually more than 10,000), which means that probing can be highly sensitive (Perry-O'Keefe et al., 2001b). As 16S rRNA sequence data is readily available for a wide variety of species, probes can be designed either to target signature sequences of specific species or chosen groups, or to hybridise with sequences conserved in all known bacteria (Amann, 2001). The probes used in FISH are typically 15 to 30 nucleotides in length and covalently linked at the 5' end to a single fluorescent dye molecule (Wagner et al., 2003b). Suitable fluorophors include carbocyanide dyes, tetramethylrhodamine and fluorescein (Amann, 2001).

One of the major disadvantages of FISH when using oligonucleotide probes is that sometimes insufficient penetration of probes into bacteria occurs due to their thick cell wall, *e.g.* the walls of Gram-positive bacteria.

a) cell fixation in 4% paraformaldehyde

bacteria

bacteria

b) preparation

c) hybridisation of probe

microscope slide

d) washing to remove excess or unhybridised probe

FISH probe

e) mounting

f) Visualisation by fluorescent microscopy
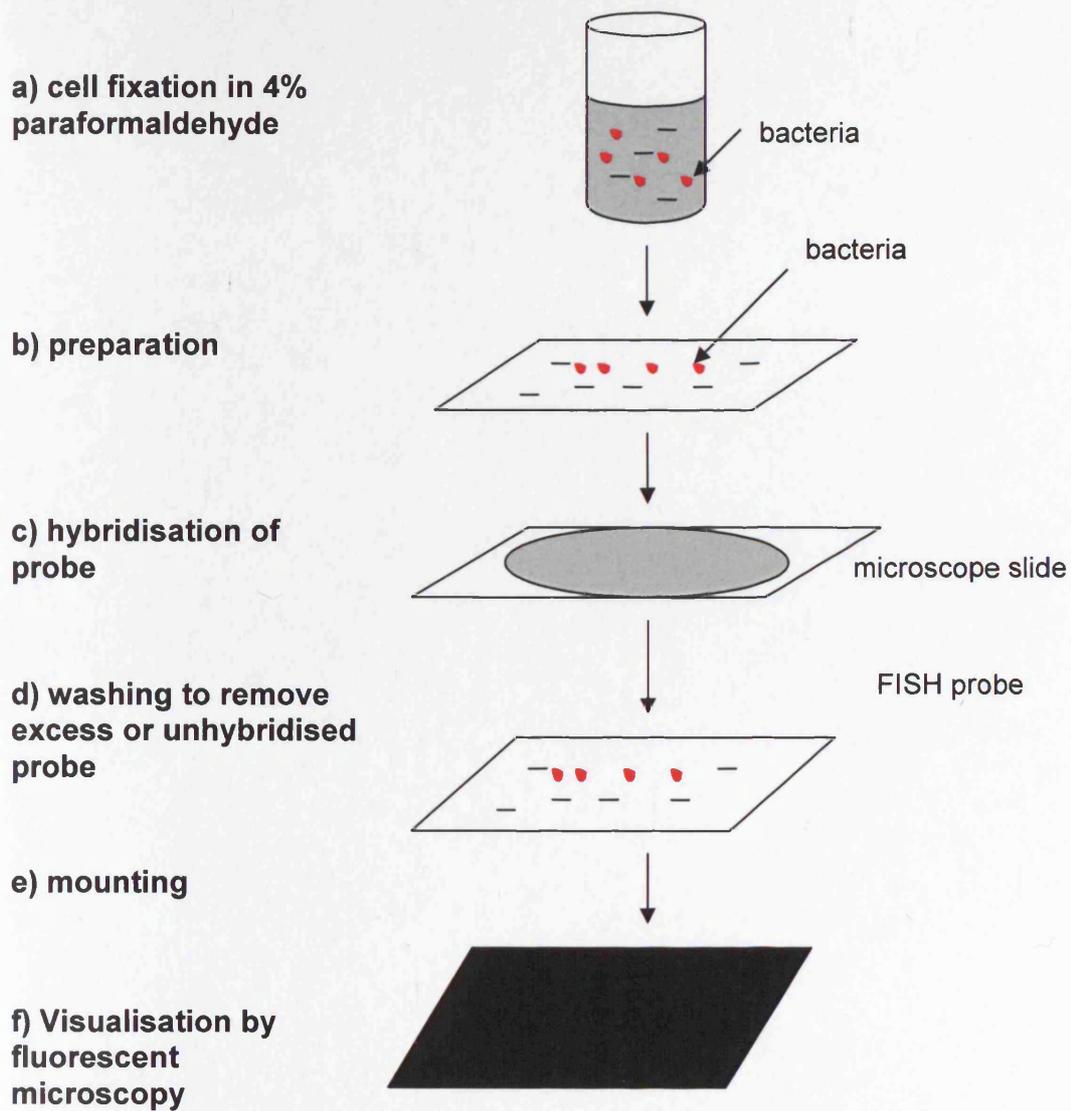
Fig. 5.1 A schematic diagram summarising the typical steps of a FISH protocol.

5.1.4 Peptide Nucleic Acid (PNA) probes

Recently, PNA probes have been incorporated into FISH applications. PNA probes are DNA-analogues (pseudopeptides) with an uncharged polyamide backbone instead of conventional sugar phosphates. PNA probes hybridise to complementary nucleic acid targets obeying the Watson-Crick base pairing rules (Moter and Gobel, 2000; Perry-O'Keefe et al., 2001b). Due to their uncharged backbone, PNA probes have superior hybridisation characteristics, including higher specificity and more rapid hybridisation kinetics. PNA probes also hybridise independently of the salt concentration and readily diffuse through hydrophobic cell walls, thus allowing detection without any pre-treatments (Moter and Gobel, 2000; Perry-O'Keefe et al., 2001b). As hybridisation with PNA probes can be performed in a low salt buffer, a decrease in the stability of the rRNA secondary structure can be induced, thereby allowing PNA probes to hybridise to otherwise less accessible targets (Perry-O'Keefe et al., 2001b). Hence PNA probes have considerable advantages over DNA probes, but unfortunately are significantly more expensive to synthesize.

5.1.5 Applications of FISH

FISH targeted towards 16S rRNA sequences has been applied to many diverse bacterial systems, often in conjunction with other molecular-based profiling approaches such as denaturing gradient gel electrophoresis (DGGE) or gene sequencing. The first uses of the FISH technique were in the detection and identification of bacteria in environmental specimens, including activated sludge granules (Sekiguchi et al., 1999) and marine sediments (Amann, 2001). In clinical samples, FISH has been applied to the study of potentially medically important bacteria, such as *Escherichia coli* in human faecal samples (Jansen et al., 1999; Vaahtovuo et al., 2005) and polymicrobial specimens from the oral cavity (Thurnheer et al., 2004). In the case of oral bacteria, FISH has been employed in the detection of organisms within samples of human carious dentine (Banerjee et al., 2002), dental plaque (Banerjee et al., 2002; Thurnheer et al., 2004; Thurnheer et al., 2001; Wecke et al., 2000), periapical endodontic lesions (Sunde et al., 2003) and

buccal epithelial cells (Rudney et al., 2001; Rudney et al., 2005). PNA FISH has been less extensively used, although in recent years the approach has been applied for fast visualisation and identification of clinically relevant mycobacteria in clinical specimens and tissue (Lefmann et al., 2006). Examples of bacteria detected by PNA probes include clinically relevant *Enterococcus* species (Wellinghausen et al., 2007), *Helicobacter pylori* in gastric biopsy specimens (Guimaraes et al., 2007) and the identification of *S. aureus* directly from positive blood culture bottles (Oliveira et al., 2002).

5.2 AIMS

The aims of the present study were:

1. To establish a reliable FISH methodology using both DNA and PNA probes to detect specific wound bacterial species.

2. To evaluate FISH and CLSM in examining the spatial organisation of bacteria in biofilm models and wound tissue.

5.3 Methods

To establish a reliable FISH methodology two separate FISH protocols were initially evaluated against planktonic bacteria and after validating these methods were extended to determine the spatial organisation in mixed species biofilm modes of growth. The two protocols were: A) detection of fixed bacteria using a universal DNA probe– EUB338 and B) PNA probe detection of bacteria (PNA FISH). The probes and their conditions are summarised in Table 5.1.

5.3.1 Universal DNA EUB338 probe

The universal bacterial DNA probe EUB338 is complementary to a portion of the 16S rRNA gene conserved in the domain *Bacteria*, and has been used to visualise the entire bacterial population of biofilms (Amann et al., 1990; Banerjee et al., 2002; Sunde et al., 2003). The probe EUB338, was synthesised commercially (MWG Biotech, Ebersberg, Germany) and at the 5' end-labelled with fluorescein isothiocyanate (FITC).

The NON338 probe (Amann et al., 1990) which is complementary to the universal bacterial probe EUB338 was used in this study as a negative control for non-specificity. As an additional negative control, RNase treated cells were also probed to confirm that the probes specifically targeted RNA.

5.3.2 Peptide nucleic acid probes (PNA)

Initially, PNA probe sequences to target two bacterial species (*P. aeruginosa*, *S. aureus*) and the Bac-Uni1 universal bacterial PNA probe were used (Table 5.1). The sequences of these probes were partially adapted from Perry-O'Keefe *et al.* (2001b). The probes were manufactured by Boston Probes (Foster City, USA) for Applied Biosystems (Warrington, UK) and were labelled either with FITC, cyanine 5 (CY5) or cyanine 3 (CY3) and designed to target the 16S rRNA. All probes were pre-validated using the PNA probe designer software (Applied Biosystems) before synthesis.

Table 5.1 Probes used and their conditions.

Probes	Target	Nucleotide sequence	Hybridisation conditions: temp. [°C] formamide [%]	Fluorescent label	Working Conc. [nM]	Excitation wavelengths [nm]	References
EUB338	Universal probe (Eubacteria)	5'-GCTGCCTCCCGTAGGAGT-3'	46°C no formamide	FITC	0.2-0.5 µM	488	(Amann et al., 1990)
Non EUB338	Non-sense EUB338	5'-CGACGGAGGGCATCCTCA-3'	46°C no formamide	FITC	0.2-0.5 µM	488	(Amann et al., 1990)
Psaer	**PNA** *P. aeruginosa*	5'-OOAACTTGCTGAACCAC-3'	55°C no formamide	FITC	300	488	(Coull and Hyldig-Nielsen, 2003)
Sta 16S03	**PNA** *S. aureus*	5'-OOGCTTCTCGTCCGTTC-3'	55°C 30% formamide	CY5	500	633	(Perry-O'Keefe et al., 2001)
Bac-Uni1	**PNA** Universal probe (Eubacteria)	5'OOCTGCCTCCCGTAGGA-3'	55°C no formamide	CY3	150	561	(Perry-O'Keefe et al., 2001)

5.3.3 Development of fluorescence *in situ* hybridisation (FISH) for planktonically grown bacteria

5.3.3.1 Protocol A: Detection of fixed planktonic bacteria using the universal bacterial DNA EUB338 probe

Bacteria were prepared and detected by EUB338 using two methods. The first of these involved culturing bacteria overnight (in BHI broth) at 37°C. The growth was pelleted by centrifugation (7000 g, 5 min at 4°C) and resuspended in 0.85% (w/v) saline. This procedure was repeated to produce a washed pellet. A portion of the suspension was taken (100 μl) and three volumes of 4% paraformaldehyde added (giving a final concentration of paraformaldehyde of 3%). The cell suspension was then vortexed and stored overnight at 4°C. The cells were then centrifuged (7000 g, 5 min at 4°C) and the bacterial pellet was again resuspended twice in 0.85% saline to its original volume, before dilution with paraformaldehyde. Subsequently, the suspension was incubated in 50 mM Tris-EDTA (pH 8; TE, Sigma) buffer containing lysozyme (10 mg ml^{-1}; Sigma) for 20 min at 37°C (60 μl). The cell suspension was centrifuged and resuspended in half its original volume (50 μl). An equal volume of 96% ethanol was then added. The suspension was vortexed for 30 s and stored at -20°C. Prior to staining with the DNA probe a portion of these fixed cells (10 μl) was deposited on to a microscope slide and air dried.

Pre-warmed hybridisation buffer (300 μl) containing 0.5 μM (in H_2O) of oligonucleotide probe EUB338 (FITC-labelled), was carefully applied to the slide. Following incubation for 3.5 h in a dark humid chamber at 46°C, slides were washed with distilled water, air-dried in the dark and subsequently mounted with FluoroSave™ (Calbiochem, Merck Biosciences Ltd., Nottingham, UK).

The second method of preparing and detecting bacteria involved the resuspension of a colony of the bacteria from an agar plate into PBS.

A smear was then made on a microscope slide that was air dried, flamed briefly to fix and then immersed in 4% paraformaldehyde overnight at 4°C. Slides were then washed with distilled water twice and dried at 37°C. To permeabilise the cells for entry of the oligonucleotide probe, slides were incubated in 50mM Tris-EDTA (pH 8) buffer containing lysozyme (10mg ml^{-1}) for 20 min at 37°C. Slides were washed with distilled water, air-dried and incubated in 50mM TE buffer (pH 8) containing proteinase K (7µg ml^{-1}) at 37°C for 20 min. Slides were then washed again with distilled water, air-dried and pre-incubated at 48°C for 20 min in a hybridisation buffer (300 µl) containing 0.9 M sodium chloride (Sigma, Poole, UK), 20mM Tris-HCl (pH 7.4, Sigma), and 0.5% SDS (Sigma). EUB338 hybridisation was then performed as described above.

5.3.3.2 Protocol B: Detection of fixed planktonic bacteria using the PNA probes

The first step was to confirm the specificity of the PNA probes on planktonic bacteria in a mixed fixed population using the three PNA probes; the universal probe (Bac-Uni1CY3), the *P. aeruginosa* specific probe (PsaerFITC) and the *S. aureus* specific probe (Sta16S03).

Cell fixation and PNA FISH was performed as described by Perry-O'Keefe *et al.* (2001a). Overnight bacterial cultures were pelleted by centrifugation (16,000 *g*, 5 min) and resuspended in PBS (7 mM Na$_2$HPO$_4$, 7 mM NaH$_2$PO$_4$, 130 mM NaCl). Cell suspensions were centrifuged again (16,000 *g*, 5 min), resuspended in PBS with 4% (w/v) paraformaldehyde (Sigma) and fixed for 1 h. The fixed cells were rinsed in PBS, resuspended in 50% (v/v) ethanol and incubated for at least 30 min at -20°C prior to use.

A 100-µl volume of fixed cells was pelleted by centrifugation, the cell pellet was rinsed with PBS and resuspended in 100 µl of hybridisation buffer (25 mM Tris-HCl, pH 9.0; 100mM NaCl; 0.5% (w/v) SDS) containing 150-500 nM fluorescently labelled PNA probe. The cells were incubated at 55°C for 30

min, centrifuged at 16,000 g for 5 min and resuspended in 500 µl of wash solution (10 mM Tris pH 9.0, 1 mM EDTA). After a further incubation at 55°C for 10 min the cells were pelleted by centrifugation. This was repeated twice for a total of three washes. Following the last wash, cells were resuspended in 100 µl wash solution and 2 µl of this cell suspension was spread on a clean microscope slide and allowed to air dry. Vectashield mounting medium was applied to the dried cells which were overlaid with a coverslip (Perry-O'Keefe et al., 2001a).

5.3.4 Development of biofilms for fluorescence *in situ* hybridisation (FISH) detection

Prior to bacterial detection and determination of bacterial spatial organisation in the biofilm mode of growth using FISH, biofilms had to be established using different models (CDFF, RHE). The preliminary steps for this are described below. The chronic wound biopsies were also processed as described below prior to FISH imaging.

5.3.4.1 Production of biofilms in the CDFF

Prior to FISH detection, biofilms were grown in the constant depth film fermenter (CDFF; Chapter 4, section 4.3.12.1). The temperature of the fermenter was maintained at 37°C by housing it in an incubator. The plugs were recessed to a depth of 400 µm, and the turntable speed of the CDFF was at 20 revs min⁻¹. Wound bacteria (*Staphylococcus aureus* D76, *Streptococcus oralis* B52, *Micrococcus luteus* B81, *and Pseudomonas aeruginosa* D40) were grown overnight at 37°C in 10 ml BHI, and five ml of each wound isolate was added to 1 L of BHI medium. This inoculum was recirculated through the CDFF for 24 h to seed the system. After this time, the inoculum was disconnected and fresh media fed into the CDFF. Biofilms were grown in the CDFF for 7 d. Fresh medium was delivered at a rate of 30 ml min⁻¹, using a peristaltic pump (Watson-Marlow, Falmouth, UK) and biofilm samples were taken for analysis over a period of 7 days every 24 h.

5.3.4.2 Fixation of CDFF generated biofilms

Biofilm samples (including the PTFE pin) generated in the CDFF were placed in molten agarose (2% w/v; Sigma) for 24h; fixed in 2% paraformaldehyde solution for 24 h and then embedded in paraffin wax using standard histological techniques.

5.3.4.3 Preparation of bacteria for infection of an *in vitro* Reconstituted Human Epidermidis (RHE) model

SkinEthic Laboratories cultivate a 0.5 cm^2 reconstituted human epidermis *in vitro* on a polycarbonate filter at the air liquid interface in chemically defined medium lacking antibiotics for 17 days. The RHE consists of normal human keratinocytes (human foreskin derived) with a well differentiated epidermis consisting of basal, spinous, granular layers and a stratum corneum. At least four viable cell layers are present. This RHE was different to the oral epithelium used previously in Chapter 2.

Bacterial wound species (*S. aureus* D76, *S. oralis* B52, *M. luteus* B81, *and P. aeruginosa* D40) were cultured on blood agar (LAB M, Bury, UK) at 37°C overnight. The resulting growth was used to inoculate 10 ml of BHI and incubated at 37°C for 24 h. Bacterial cells were then harvested by centrifugation and washed (×3) with PBS. The pelleted bacterial cells were resuspended in RHE tissue maintenance medium (supplied by SkinEthic Laboratories, Nice, France; chemically defined medium MCDB 153 containing 5 µg ml^{-1} insulin and 1.5 mM calcium chloride with no antibiotic) and 200 µl of the resulting bacterial suspension added to the RHE.

Additionally to simulate a skin wound, the surface of the RHE model was on occasion scratched with a sterile pipette tip prior to infection with bacteria.

5.3.4.4 Culture of infected RHE

Infected RHE was incubated for 24-28 h at 37°C in a humidified atmosphere, enriched with 5% CO_2 as described previously (Chapter 2, section 2.3.2.4).

5.3.4.5 Processing of infected RHE

After incubation, the whole RHE was fixed in 10% (v/v) formalin for 24 h and then embedded in paraffin wax using standard histological techniques (as previously described (Chapter 2, section 2.3.2.4.3).

5.3.4.6 Processing of biopsies from non- infected venous leg ulcers

Wound biopsies had previously been obtained with ethical approval and patient informed written consent from non-infected venous leg ulcer patients attending the Wound Healing Research Unit Clinic at the University Hospital of Wales, Cardiff between 1999 until 2002 and stored at -20°C until required (Davies et al., 2007). Biopsies were fixed in 10% (v/v) formalin for 24 h and then embedded in paraffin wax using standard histological techniques.

5.3.4.7 Preparation of biofilms (CDFF and RHE) and biopsies

Prior to FISH, all processed biofilm sections (20 µm; see above) were placed on to Histobond+ coated microscope slides (Raymond A Lamb, East Sussex, England), de-waxed and processed through xylene and ethanol to water before probe hybridisation. To hybridise the PNA/DNA probes to the fixed bacteria, sections were directly treated with 100 µl lysozyme (10 mg ml^{-1}; Sigma) and incubated at 37°C for 30 min. Sections were then briefly washed in pre-warmed wash solution (10 mM Tris pH 9.0, 1 mM EDTA) prior to probe hybridisation.

5.3.4.8 Hybridisation of RHE derived biofilms with the universal EUB338 DNA probe

Sections of RHE developed biofilms (RHE, 20 µM) were pre-incubated for 20 min at 48°C in hybridisation buffer (300 µl) containing 0.9 M sodium chloride (Sigma), 20mM Tris-HCl (pH 7.4, Sigma), and 0.5% SDS (Sigma). Pre-warmed hybridisation buffer (300 µl) containing 0.2-0.5 µM of FITC-labelled EUB338 oligonucleotide probe, (MWG Biotech, Ebersberg, Germany) was carefully applied to the RHE section. Following incubation for 3.5 h in a dark humid chamber at 46 °C, slides were washed with dH$_2$O, air-dried in the dark.

5.3.4.9 Hybridisation of PNA probes to bacteria in RHE and CDFF biofilms and wound biopsies

One hundred and fifty μl of pre-warmed hybridisation buffer (25 mM Tris-HCl, pH 9.0; 100mM NaCl; 0.5% (w/v) SDS) containing 150-400 nM of fluorescently labelled PNA probe (see Table 5.1), was added to each CDFF biofilm/RHE tissue and wound biopsy section (20 μM), which was placed in a dark, humidified chamber and incubated at 55°C for 90 min. The stringency was adjusted by adding 30% (v/v) formamide to the hybridisation buffer for the *S. aureus* specific probe (Sta16S03). After incubation, each slide was washed with pre-warmed wash solution using a magnetic stirrer for 30 min.

5.3.4.10 Staining of RHE keratinocytes

For nuclear context, sections were counterstained with Hoechst 33258 (Sigma) 2 μg ml^{-1} for 20 min, before washing in wash-solution and mounted using Vectashield fade-retarding mountant (Vector Laboratories Ltd., Peterborough, UK).

5.3.4.11 Haematoxylin and eosin

Additionally, biofilm and RHE sections were stained by the routine haematoxylin and eosin (H and E) procedure (Cross, 2004). Sections were processed using a Shandon Linistain machine (Shandon, Runcorn, UK) in the following order. Sections were first de-waxed, rinsed in 100% ethanol and then rinsed in H$_2$O. After the washing steps, sections were stained with Harris' haemotoxylin (Sigma) for 5-6 min, rinsed briefly in 1% acidic alcohol, followed by a brief rinse in Scott's tap water substitute (blue, Sigma, UK) and counterstained with 1% aqueous eosin for 30 s to 1 min. Sections were dehydrated in alcohol, cleared in saline and mounted with DPX mounting medium (Raymond A Lamb Ltd., Eastbourne, UK).

5.3.4.12 Gram stain

Sections were first de-waxed, rinsed in 100% ethanol and then rinsed in H$_2$O. After the washing steps, sections were stained with Crystal violet (0.5% w/v) in 25% alcohol for 3 min, rinsed briefly in H$_2$O and then stained with Gram's

iodine for 3 min. Sections were rinsed briefly in H_2O, acetone decolourised for few seconds and again briefly rinsed in H_2O. Subsequently sections were stained with neutral red for 3 min, dehydrated in alcohol, rinsed briefly in saline and mounted with DPX mounting medium.

5.3.5 Confocal Laser Scanning Microscopy (CLSM)

Stained sections were viewed and analysed by CLSM using a Leica TCS SP2 AOBS spectral confocal microscope (Leica, Wetzlar, Germany). The sections were scanned through the full depth using appropriate settings for single, double or triple channel fluorescence recordings of FITC, CY5, CY3 or Hoechst 33258 as detailed in Table 5.2. For multi-channel recordings, fluorochromes were scanned sequentially to eliminate spectral overlap between probes. Selected images were presented either as single confocal optical sections or maximum intensity type reconstructions.

Table 5.2   Scan parameters used for the simultaneous excitation and detection of the four probes used for CSLM.

Fluorochrome	Laser Excitation Line (nm)	Emissions Detected (nm)
Hoechst 33258	405	410-485
FITC	488	498-540
CY3	543	550-610
CY 5	633	635-700

5.4 RESULTS

5.4.1 Analysis of planktonic cell detection and RHE biofilms using the universal EUB338 probe

To establish a reliable FISH protocol the universal EUB338 probe was initially evaluated on planktonic and biofilm cells. Using the EUB338 probe, all the bacteria species could be detected (Fig. 5.2). However, strong autofluorescence of planktonic cells and the tissue and weak hybridisation signals were evident on RHE sections (Fig. 5.2c).

5.4.2 Bacterial specificity of PNA probes

The FISH protocol using PNA probes was established and widely used in this study on fixed and non-fixed bacteria. Figure 5.3 is a composite of three micrographs that show detection of all four test bacteria (*S. aureus* D76, *S. oralis* B52, *M. luteus* B81, and *P. aeruginosa* D40). Figure 5.3a illustrates detection of all bacteria using the PNA universal probe (Bac-Uni1CY3) with all cells visualised red. Figure 5.3b shows detection of *P. aeruginosa* only with the PsaerFITC. In this figure only a proportion of the population stain green, identifying *P. aeruginosa*. Cells which did not stain with the PsaerFITC also clearly had a distinct coccus type morphology. Figure 5.3c is an overlay of all the CLSM channels and shows detection of all bacteria stained with the three different fluorescent markers. *Pseudomonas aeruginosa* cells appear yellow in this overlay as a result of hybridising with both the Bac-Uni1CY3 and with the PsaerFITC probe. Blue/purple cells were *S. aureus*, as a result of hybridising with both the Bac-Uni1CY3 and the Sta16S03 probes. Red staining cells were either *M. luteus* or *S. oralis* as these hybridised only with the Bac-Uni1CY3 probe. Multiplex PNA staining demonstrated that these combined probe preparations remained effective for species identification.
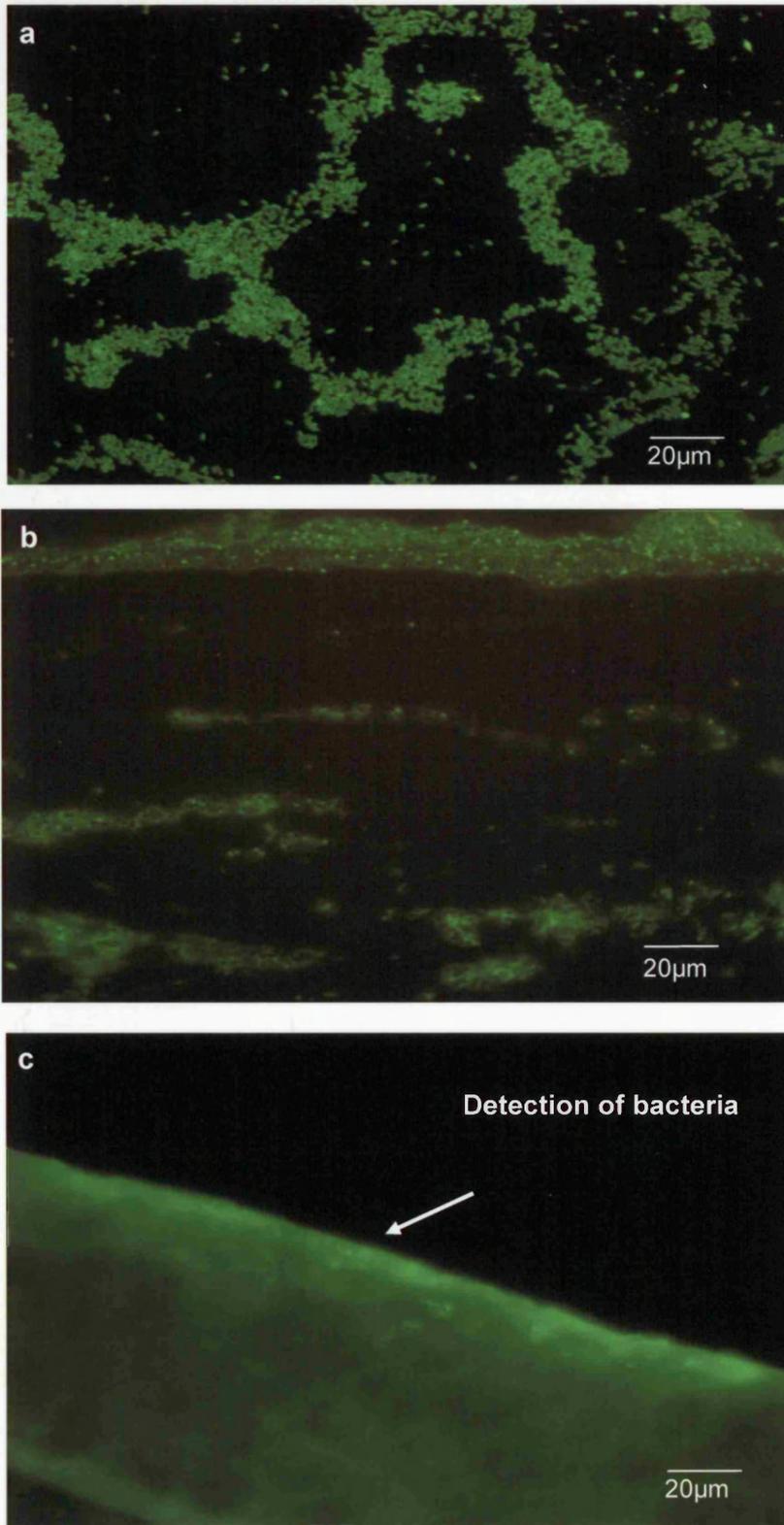
Fig. 5.2 Detection of bacteria by universal EUB338 probe on (a) on fixed planktonic bacteria (b) on a CDFF biofilm smear (c) on a RHE section; x 100 magnification.
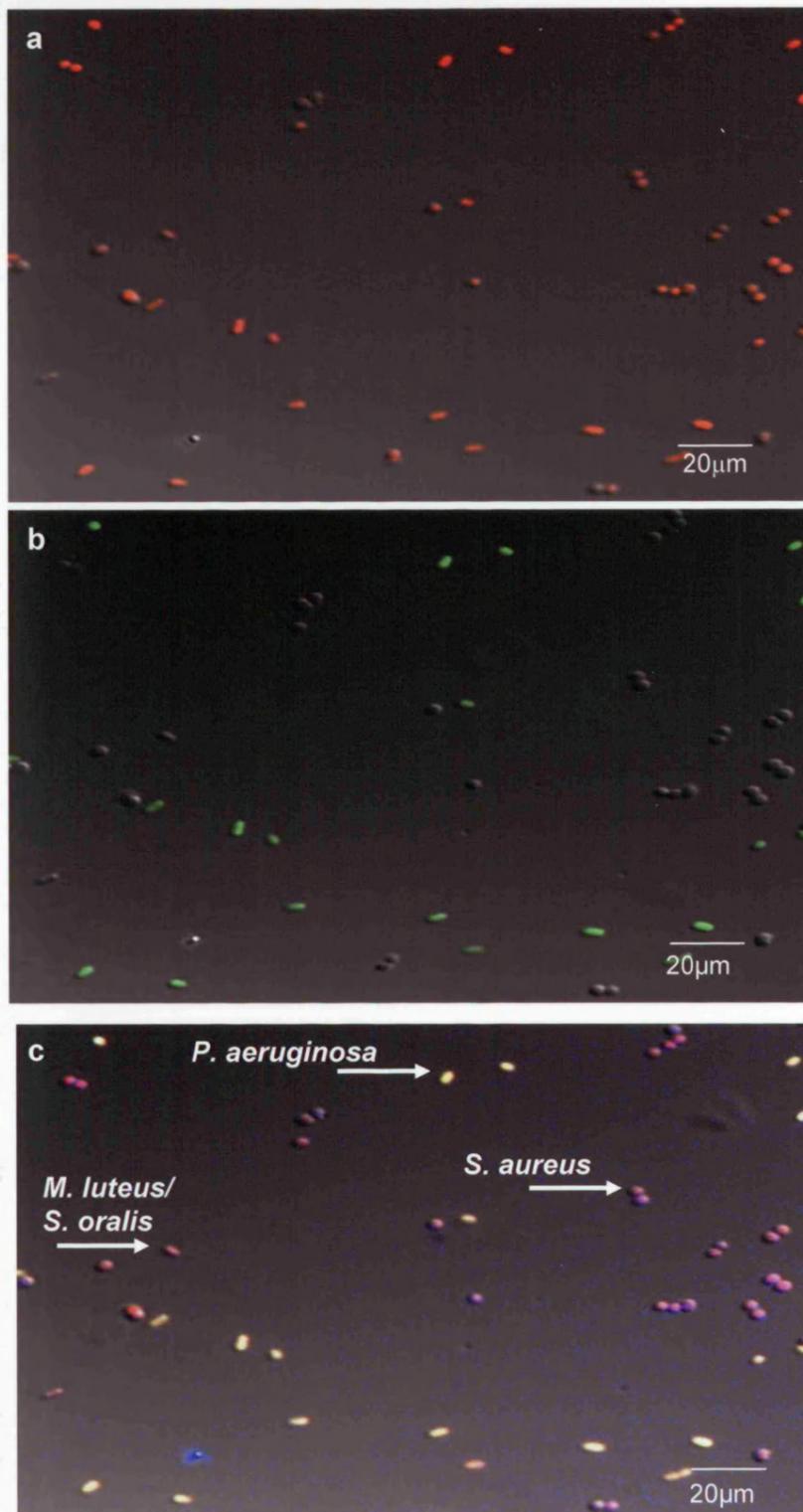
Fig. 5.3 Detection of mixed planktonic bacteria using three PNA probes.
a) Universal bacterial probe (Bac-Uni1CY3)-all bacteria stain red, b)
Pseudomonas aeruginosa probe (PsaerFITC)-only a proportion of the
population stain green c) Multiplex PNA staining *P. aeruginosa* (yellow cells
have hybridised with universal probe & *P. aeruginosa* specific probe). *S.
aureus* (purple cells have hybridised with universal & *S. aureus* probes;
Sta16S03CY5-blue). *Micrococcus luteus* and *Streptococcus oralis* (red only
cells have hybridised with the universal probe).

5.4.3 Analysis of CDFF generated biofilms using PNA FISH

The PNA FISH technique was applied to mixed species biofilm growth of the target bacteria. Biofilms generated in the CDFF (single and mixed) were successfully processed and stained with the PNA FISH probes. Using all three PNA probes, target bacteria could be detected in appropriate sections of the biofilms examined. Examples of biofilm sections prepared in the CDFF containing fluorescently-labelled bacteria are shown in Fig. 5.4.-5.7. In a mixed species biofilm hybridised with all three PNA probes, distinct zones of each bacterial population were observed, and Fig. 5.4 confirms that the mixed biofilm is heterogeneous with respect to the species composition. Within these mixed biofilms *P. aeruginosa* seemed to be the predominant organism (Fig. 5.4-5.7). The biofilm mass was principally of bacillus-shaped bacteria (*P. aeruginosa*), with cocci found in isolated pockets (Fig.5.4-5.7). *Pseudomonas aeruginosa* was detected throughout the biofilm whilst *S. aureus* was concentrated towards the surface of the biofilm (Fig. 5.4c). The other bacteria (*M. luteus* and *S. oralis*) used in this system appeared to concentrate in the middle and lower sections of the biofilm (Fig. 5.4d). A mixed biofilm section showing staining with the *P. aeruginosa* specific probe and *S. aureus* specific probe reveals that the biofilm is mostly comprised of *P. aeruginosa* which encircle the *S. aureus* cells (Fig. 5.7). Figure 5.8 illustrates spaces devoid of bacteria within the biofilm, suggestive of being water channels.

Analysis of biofilms over 7 days (day 3, day 5 and day 7) show how the mixed biofilms altered over time (Fig. 5.9). Figure 5.9a shows a 3 day old biofilm, where some of the bacteria are loose and the morphology of the community is clear. Figure 5.9b shows a 5 day old biofilm, which is very dense, especially on the top and on the base of the biofilm. Figure 5.9c shows a 7 day old biofilm which is very dense and features spaces devoid of bacteria characteristic of water channels.
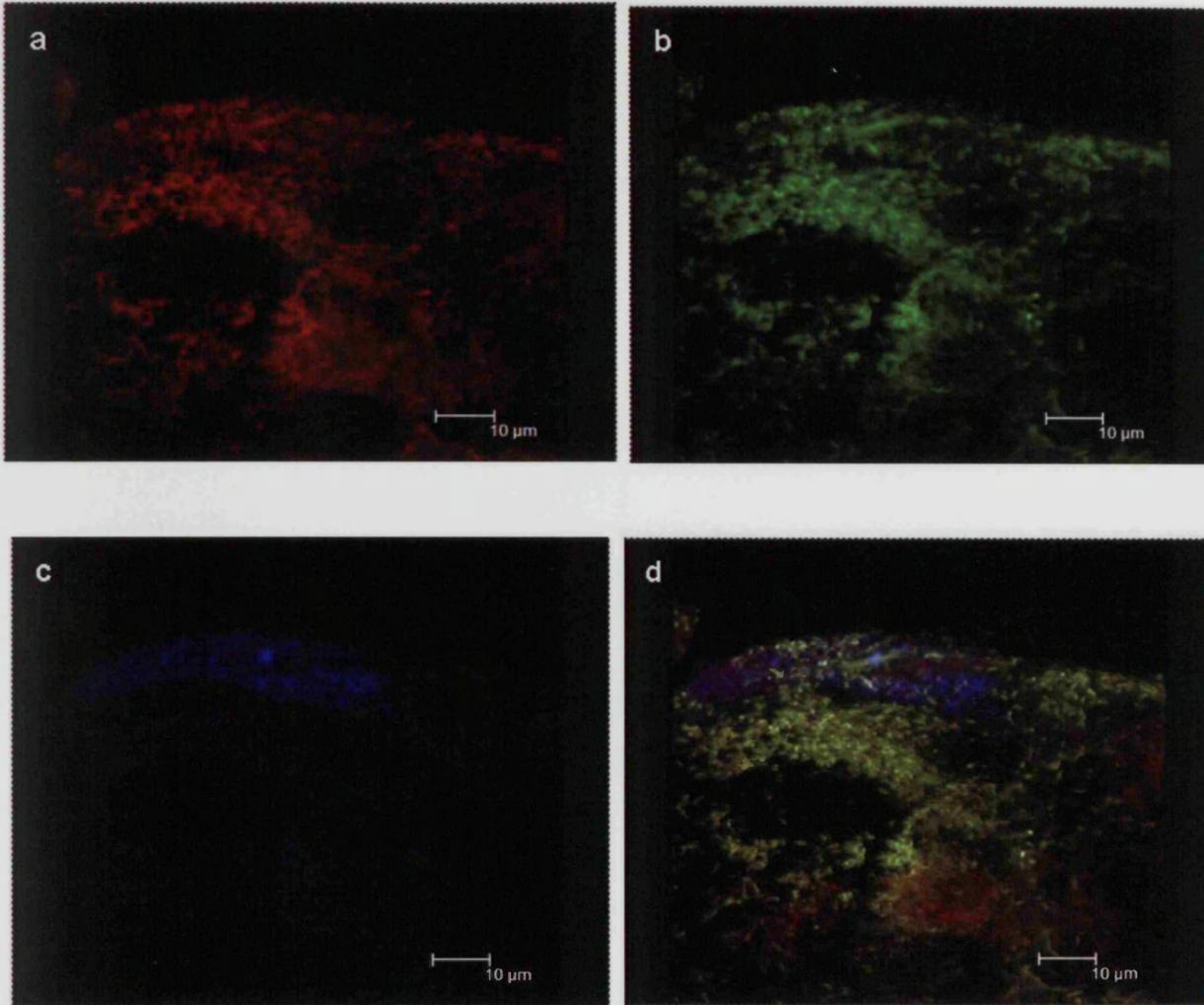
Fig. 5.4 PNA FISH on mixed bacterial species biofilms prepared in the CDFF, hybridised with 3 PNA probes a) universal bacterial probe (Bac-Uni1CY3-red), b) *P. aeruginosa* specific (PsaerFITC-green), c) *S. aureus* specific (Sta16S03CY5-blue) and d) all 3 channels. *P. aeruginosa* (yellow cells have hybridised with universal probe & *P. aeruginosa* specific probe). *S. aureus* (blue cells have hybridised with universal probe & *S. aureus* specific probe). *M. luteus* and *S. oralis* (red only, cells have hybridised with the universal probe).
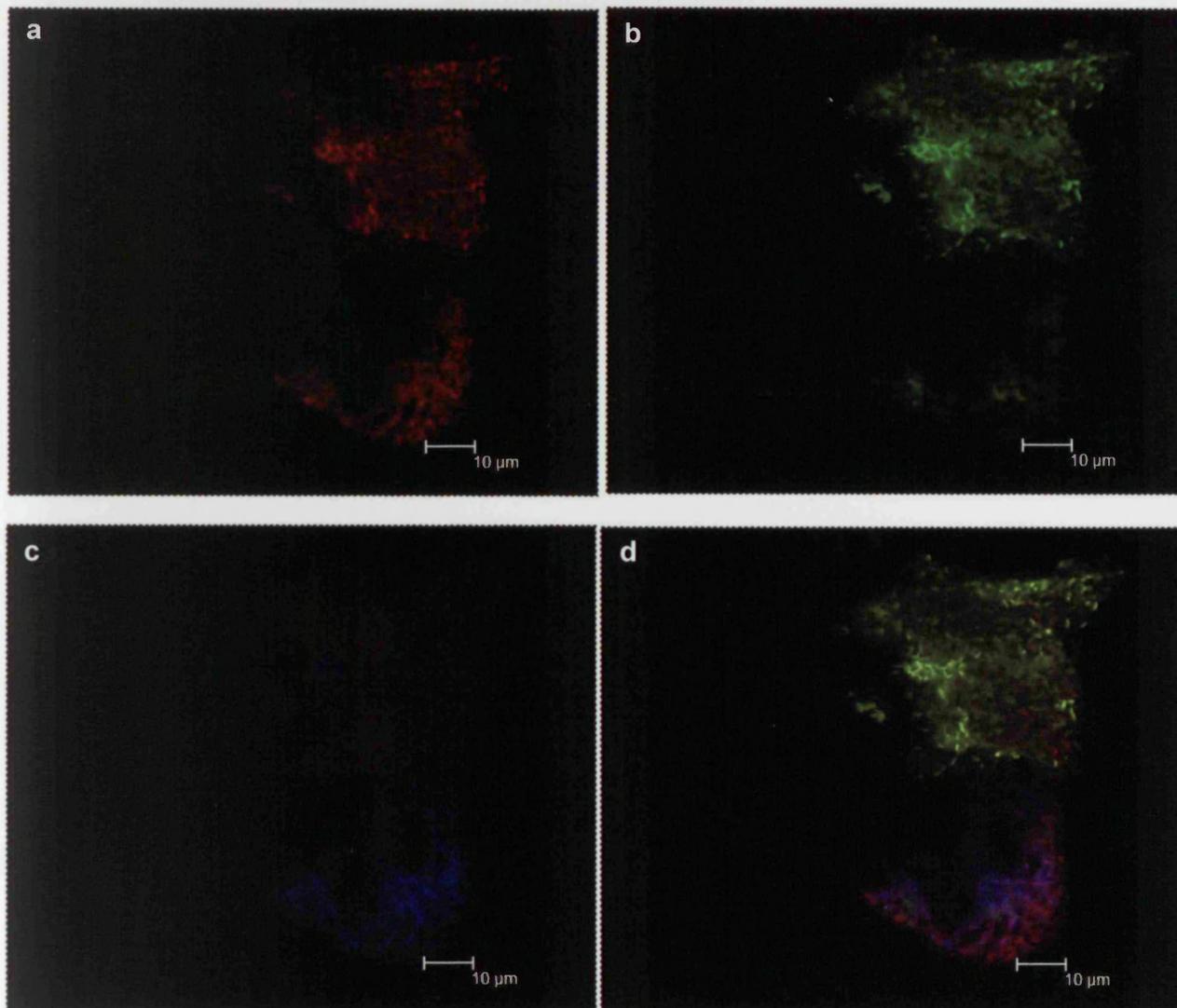
Fig. 5.5 PNA FISH on mixed CDFF biofilms using all 3 PNA probes a) universal bacterial probe (Bac-Uni1CY3-red), b) *P. aeruginosa* specific (PsaerFITC-green), c) *S. aureus* specific (Sta16S03CY5-blue) and d) overlay of all 3 channels. *P. aeruginosa* (yellow cells have hybridised with universal probe & *P. aeruginosa* specific probe). *S. aureus* (blue cells have hybridised with universal probe & *S. aureus* specific probe). *M. luteus* and *S. oralis* (red only, cells have hybridised with the universal probe).
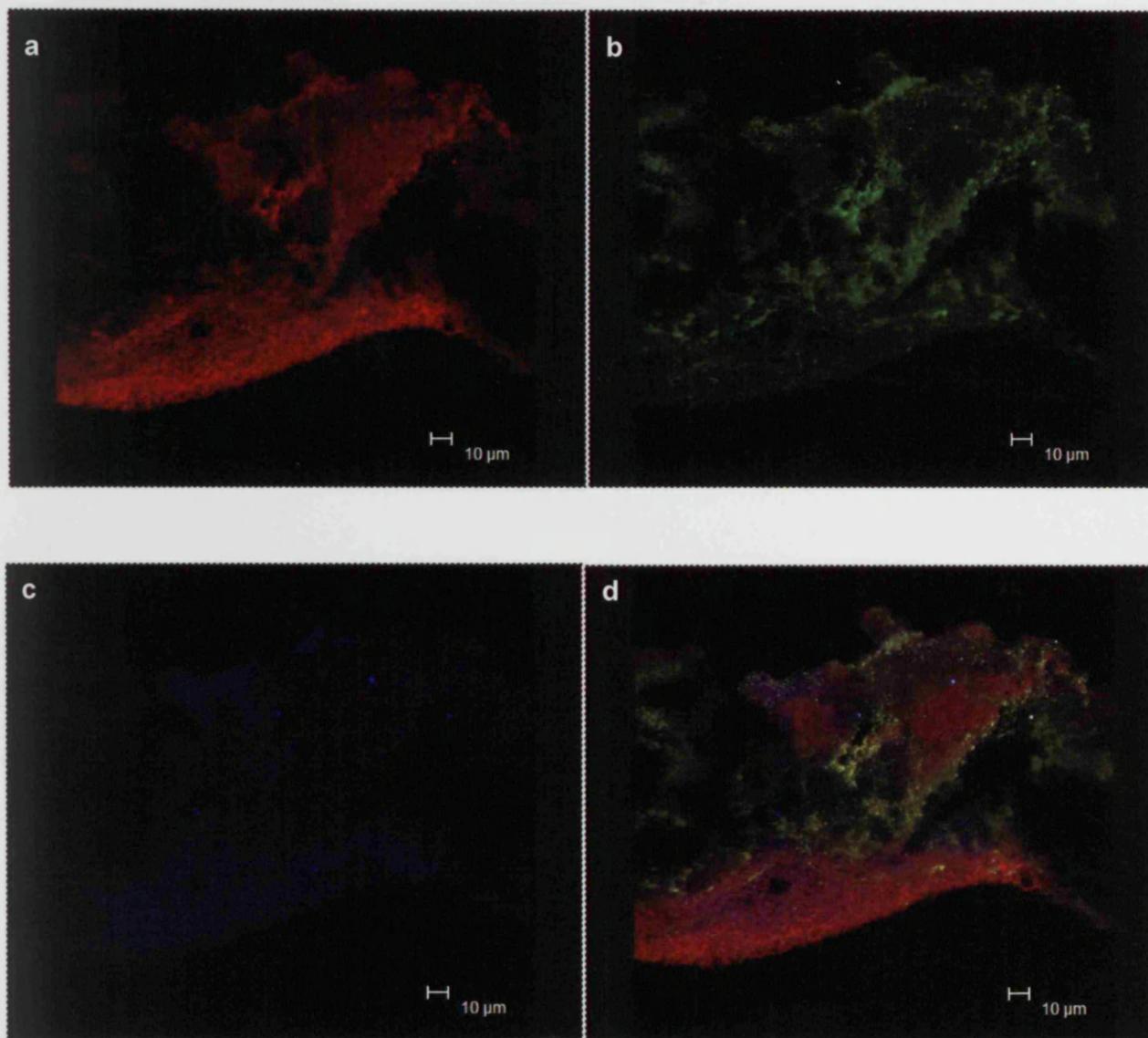
Fig. 5.6 PNA FISH on mixed CDFF biofilms using all 3 PNA probes a) universal bacterial probe (Bac-Uni1CY3-red), b) *P. aeruginosa* specific (PsaerFITC-green), c) *S. aureus* specific (Sta16S03CY5-blue) and d) overlay of all 3 channels. *P. aeruginosa* (yellow cells have hybridised with universal probe & *P. aeruginosa* specific probe). *S. aureus* (blue cells have hybridised with universal probe & *S. aureus* specific probe). *M. luteus* and *S. oralis* (red only, cells have hybridised with the universal probe).
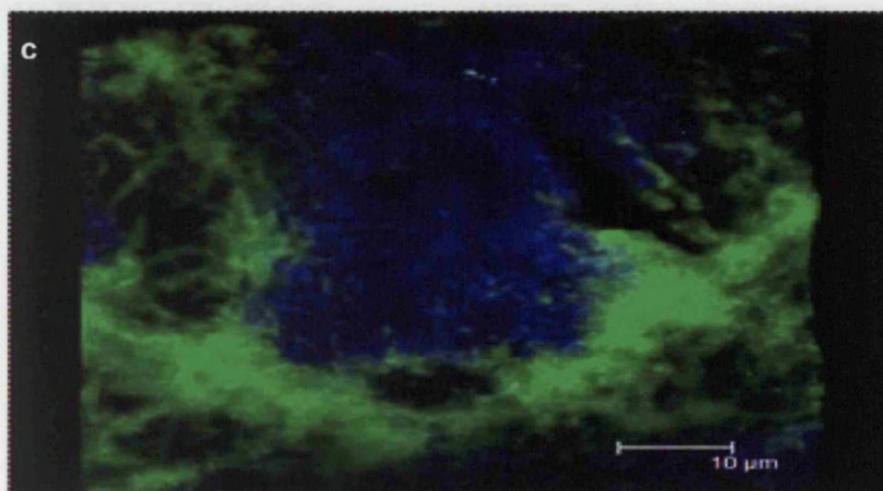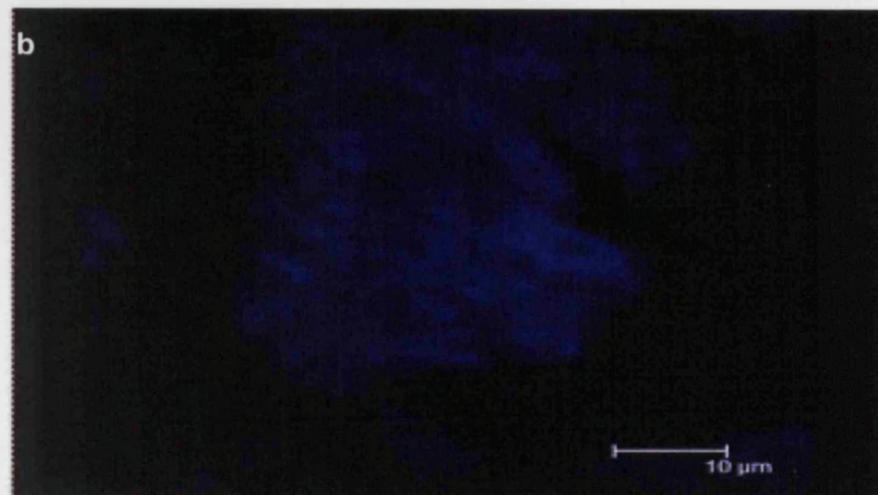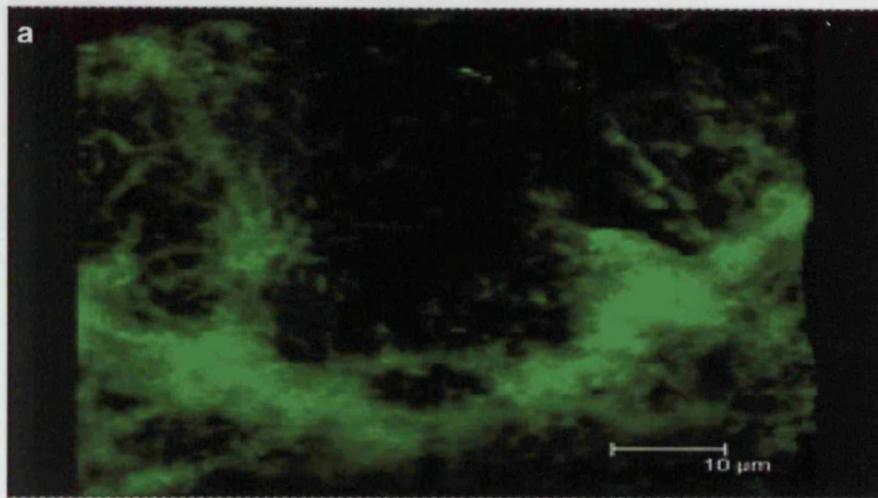
Fig. 5.7 PNA FISH on mixed CDFF biofilms using 2 PNA probes. a) *P. aeruginosa* specific (PsaerFITC-green), b) *S. aureus* specific (Sta16S03CY5-blue) and d) overlay of the 2 probes.
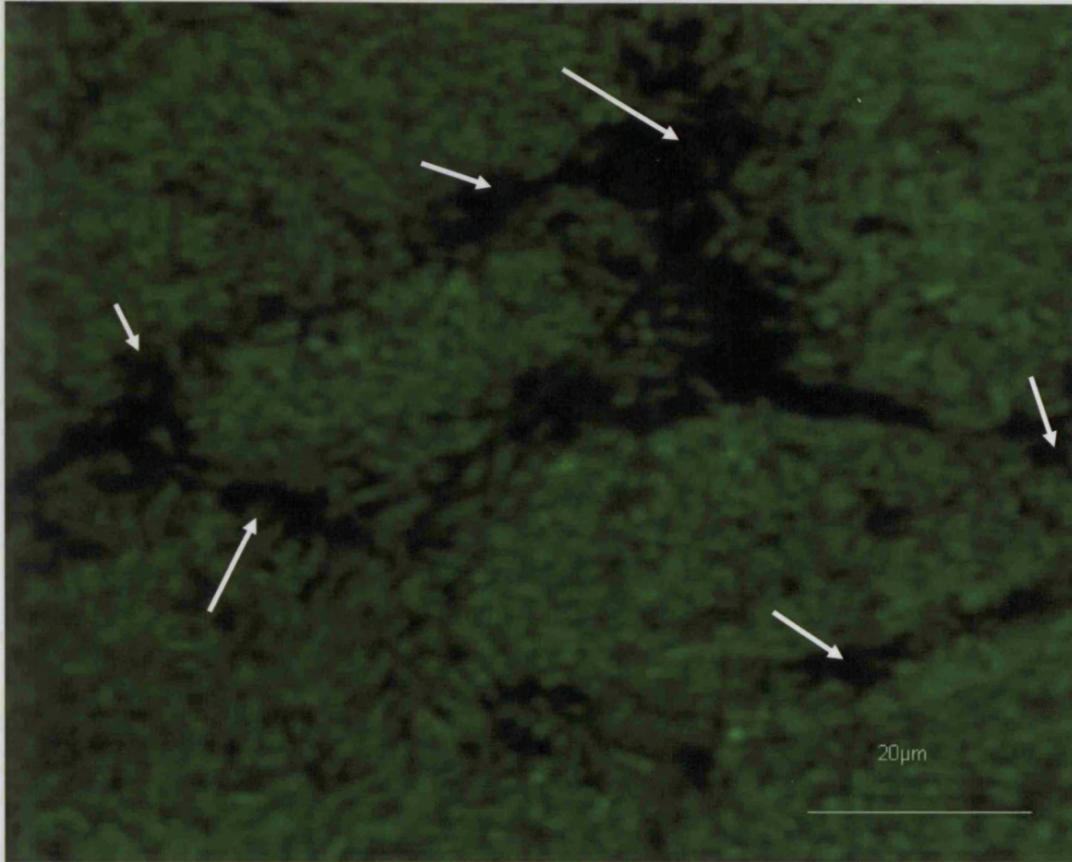
Fig. 5.8 PNA FISH on a *P. aeruginosa* CDFF biofilm using *P. aeruginosa* specific probe (PsaerFITC-green), spaces devoid of bacteria indicated in white arrows.
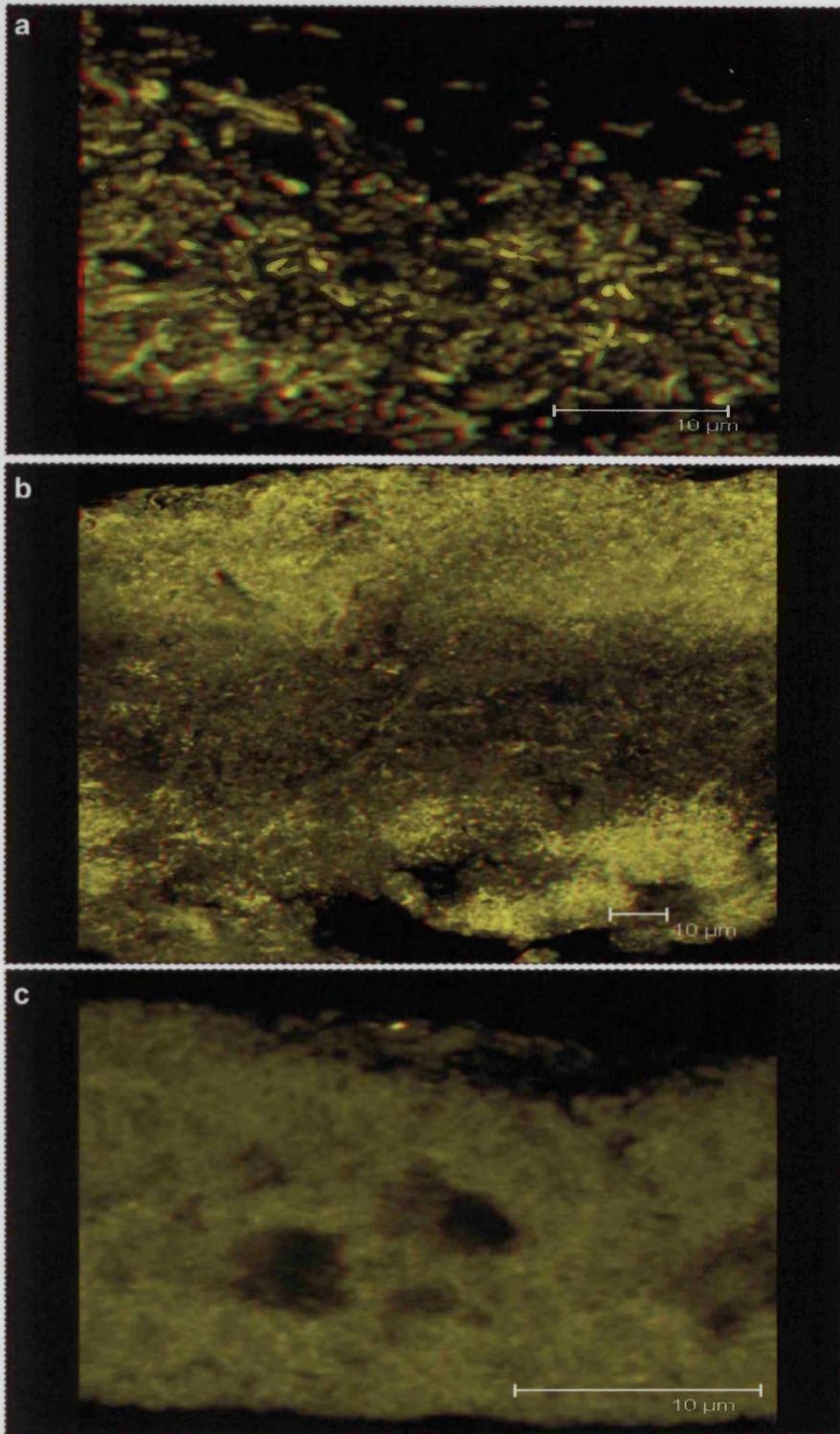
Fig. 5.9 A 3D reconstruction of a mixed biofilm generated on the CDFF and stained with universal PNA probe (Bac-Uni1CY3). Structure of a) early stage, 3 day [showing cells that are loose and in aggregates] b) middle stage, 5 day, [showing that cells are dense on the bottom of the biofilm and on the top. In the middle of the biofilm cells are more loosely structured] and c) late stage mixed biofilm, 7 day [showing spaces devoid of bacteria as well as dense areas with bacteria].

5.4.4 Analysis of RHE biofilms using PNA FISH

Biofilms generated on the RHE were successfully processed and stained by both standard Gram staining (Fig. 5.10) and with the PNA FISH probes (Fig. 5.11-5.17). The Hoechst stain, which was used to stain the nuclei of the RHE model, also stained the DNA of the bacterial organisms that were not detected by any of the species-specific PNA probes (i.e. non-*S. aureus* or *P. aeruginosa* organisms). Figure 5.10b shows a scratched RHE with limited bacterial invasion into the deep tissue.

5.4.5 Analysis of unfixed RHE biofilms using PNA FISH

In the infected (mixed population), unfixed RHE, bacteria were only detected on the tissue surface and not within the RHE. The detected bacteria tended to be present in clusters or aggregates on the surface of the RHE with no evidence of RHE invasion (Fig. 5.11-5.12). However, when a 3D motion video was created from the CLSM data, bacteria were seen as a multilayered community of cells deeper within the tissue (Fig. 5.12).

5.4.6 Analysis of fixed RHE biofilms using PNA FISH

Formalin fixed and paraffin-embedded RHE sections were also successfully processed and stained with the PNA FISH probes although only a few bacteria were found to be present (Fig. 5.13-5.15). These sections were infected with single species inocula. It was noticeable that bacterial colonisation was restricted to the surface of the RHE tissue with no bacterial invasion evident. Figures 5.16-5.17 confirm that bacteria actually invade the tissue when the tissue surface is damaged. It seemed that bacteria within the scratch wound tissue model were more abundant at the site of tissue damage compared with intact tissue. These results suggest that once the integrity is destroyed bacteria are able to move freely around and invade the tissue.

a

Bacterial cells, darkly
stained are surface
associated

RHE
Keratinocytes

20µm

insert
membrane

b

Bacterial cells, darkly
stained invade the tissue

RHE
Keratinocytes

insert
membrane

20µm
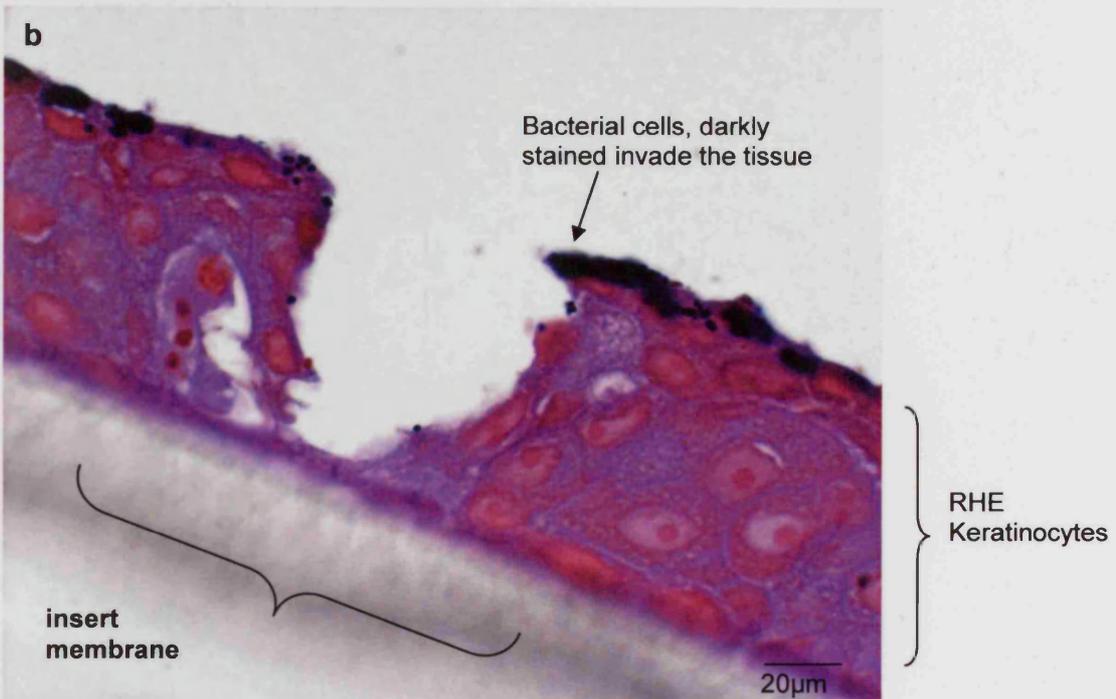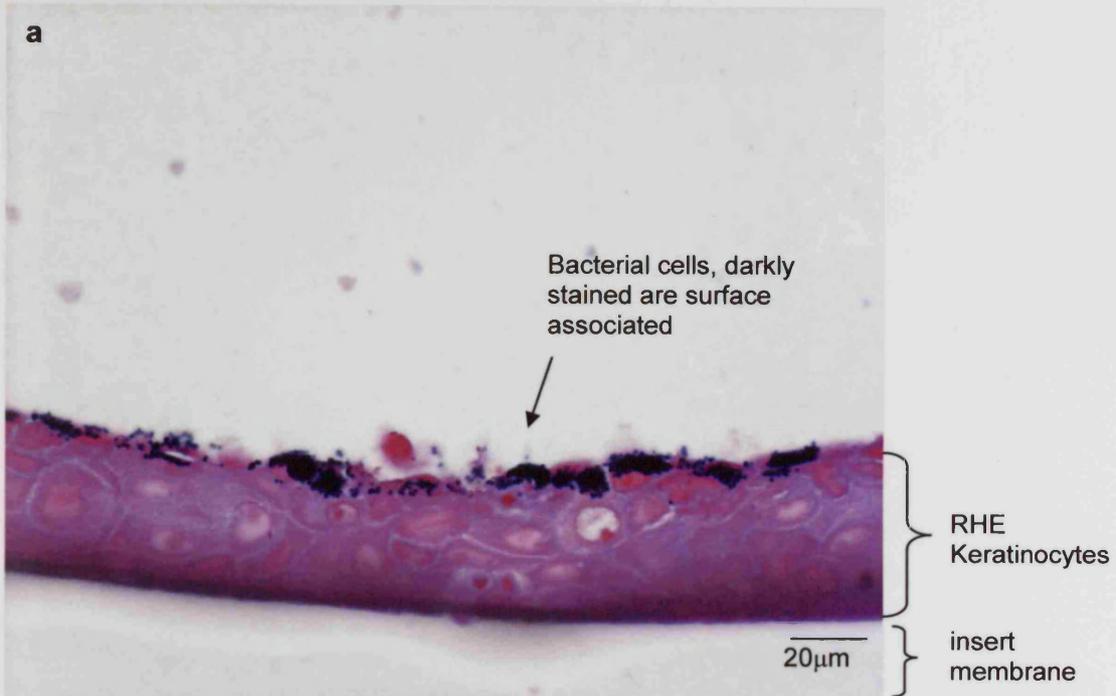
Fig. 5.10 Gram stain of RHE infected with two bacterial species (*P. aeruginosa* and *S. aureus*) a) x60 magnification, b) scratched RHE x100 magnification.
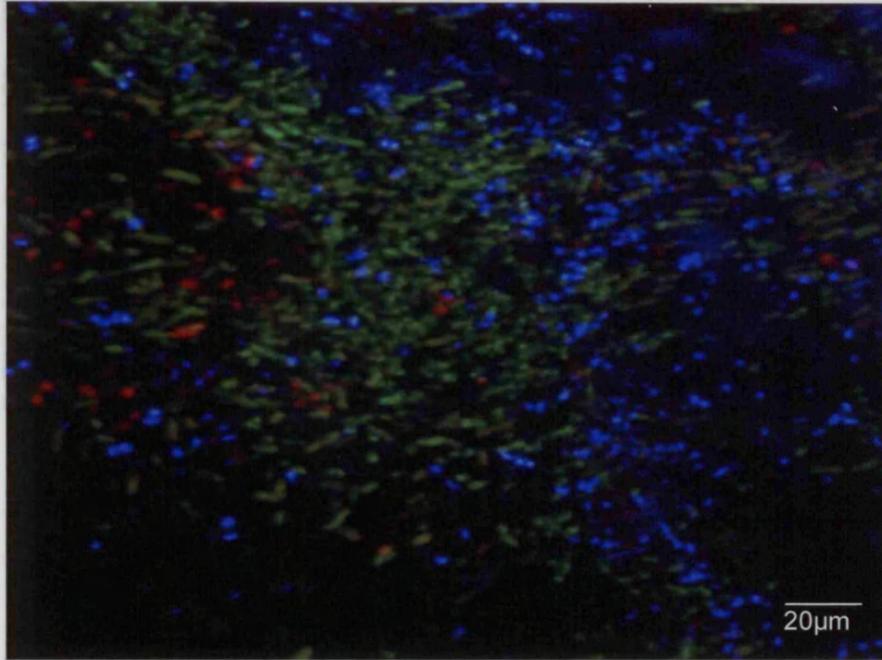
Fig. 5.11   Detection of a mixed population of bacteria in the infected RHE model (unfixed).   Stained with *P. aeruginosa* specific probe (PsaerFITC-green), with *S. aureus* specific probe (Sta16S03CY5-red) and counterstained with the Hoechst stain (blue).   *Pseudomonas aeruginosa* predominated with only a limited number of *S. aureus* seen.
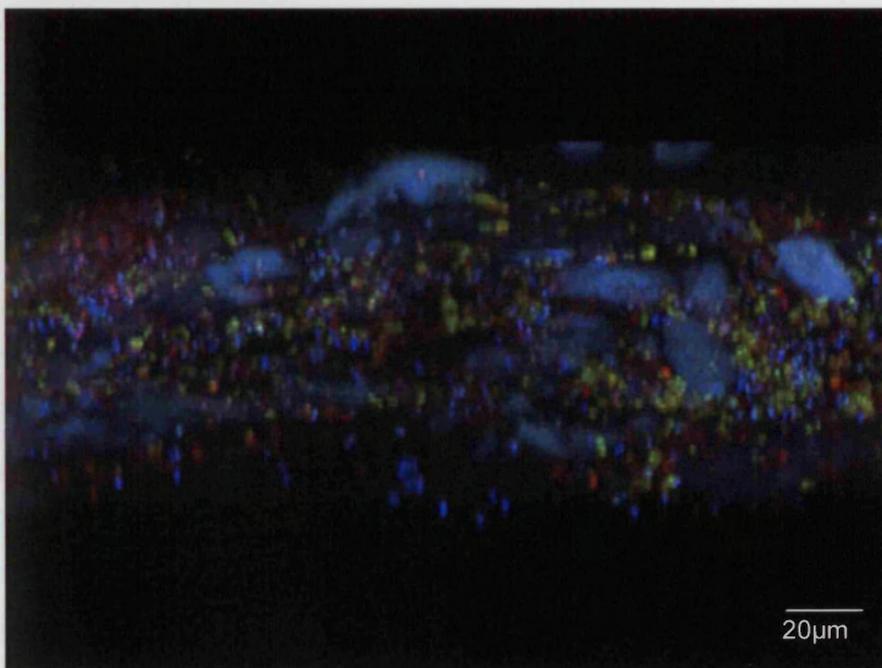
Fig. 5.12   Detection of mixed population of bacteria in infected RHE model (unfixed). Stained with *P. aeruginosa* specific probe (PsaerFITC-green), with *S. aureus* specific probe (Sta16S03CY5-red) and counterstained with the Hoechst stain (blue).

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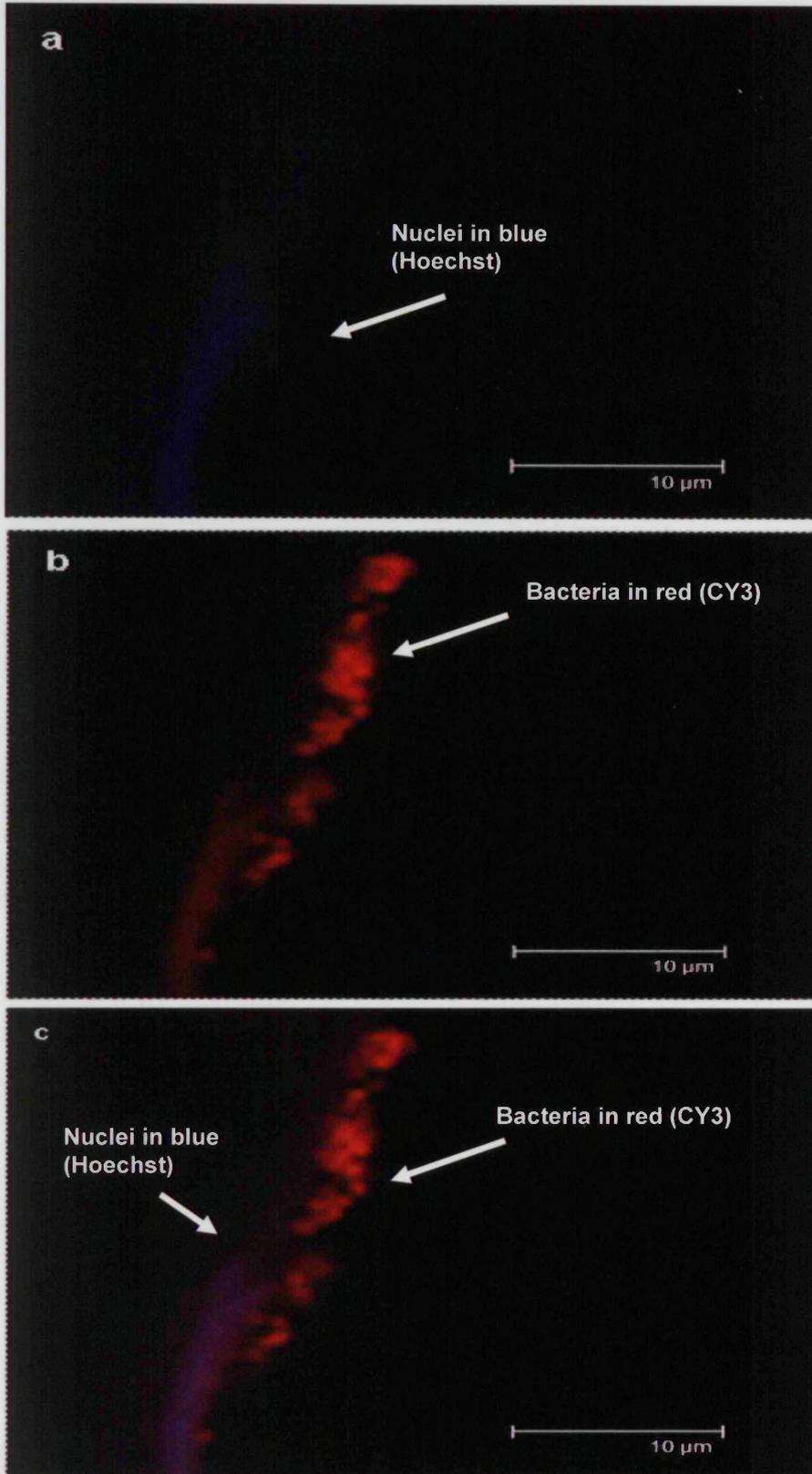
Fig. 5.13 Fixed RHE samples infected with *S. aureus* (D76) and stained with a) Hoechst, b) universal bacterial PNA probe (Bac-Uni1CY3-red) and c) overlay of both channels.
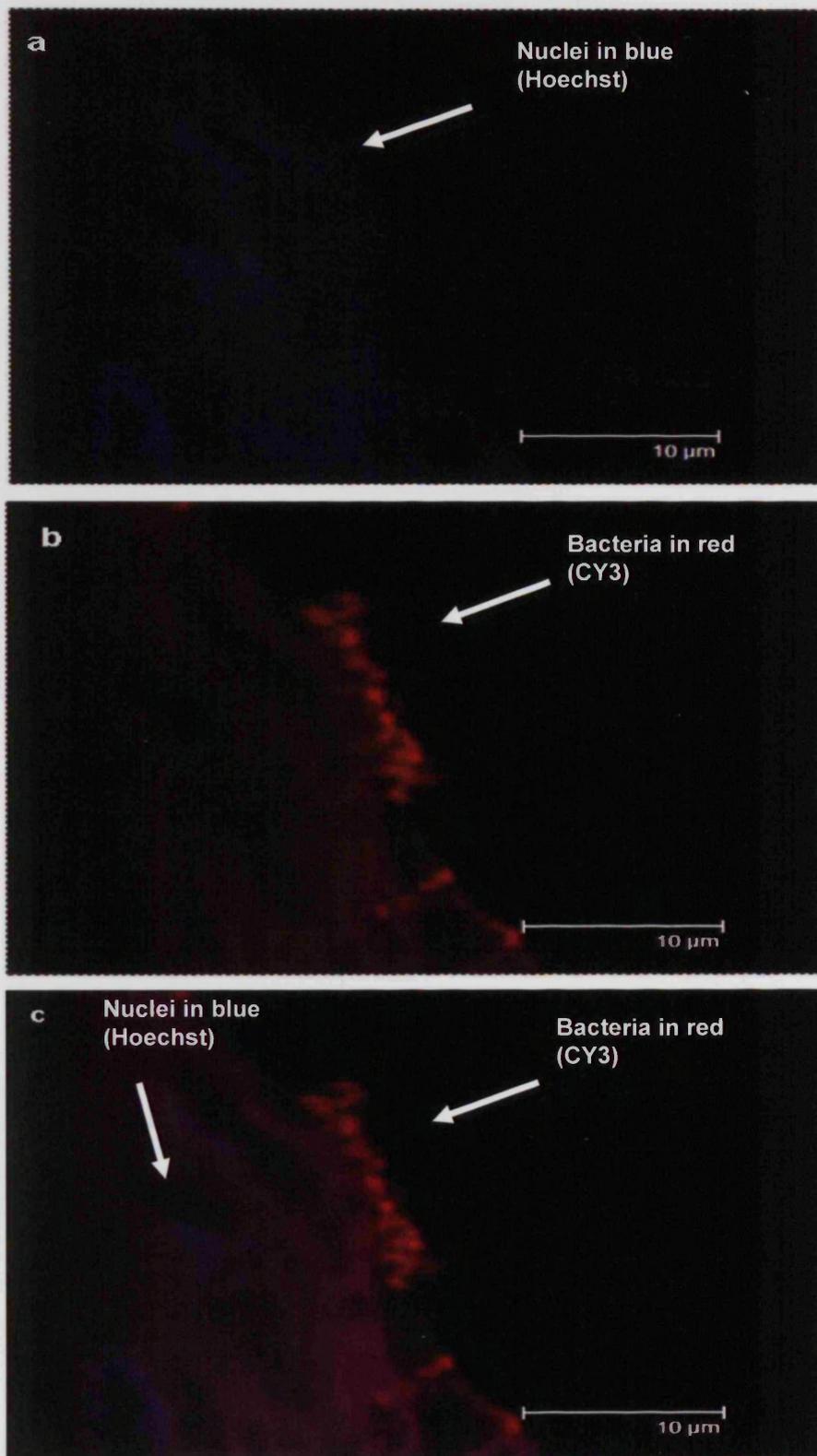
Fig. 5.14 Fixed RHE samples infected with *Streptococcus oralis* (B52) and stained with a) Hoechst, b) universal bacterial PNA probe (Bac-Uni1CY3-red) and c) overlay of both channels.
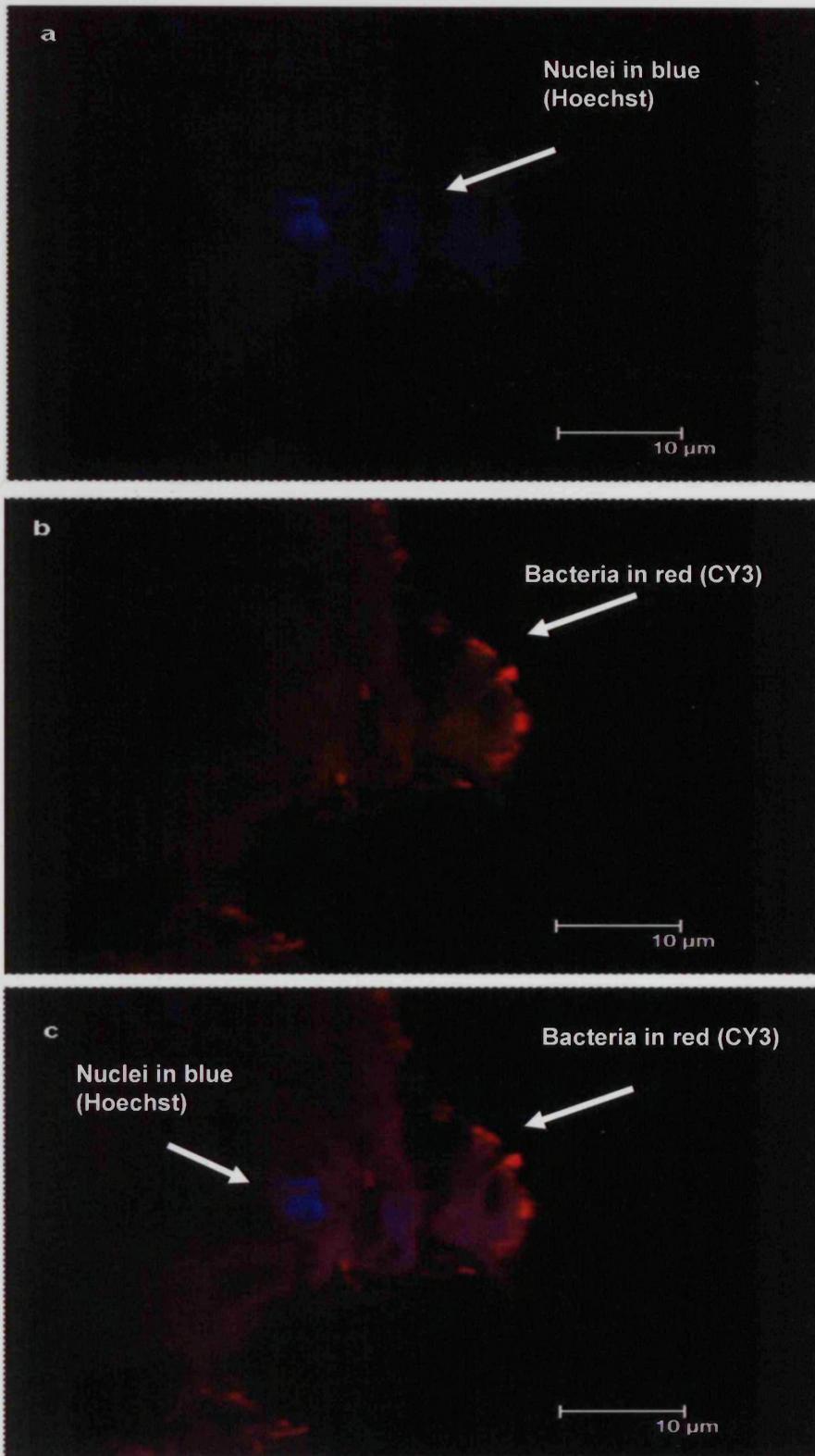
Fig. 5.15   RHE infected with *Micrococcus luteus* (B81) and stained with a) Hoechst, b) universal bacterial PNA probe (Bac-Uni1CY3-red) and c) overlay of both channels.
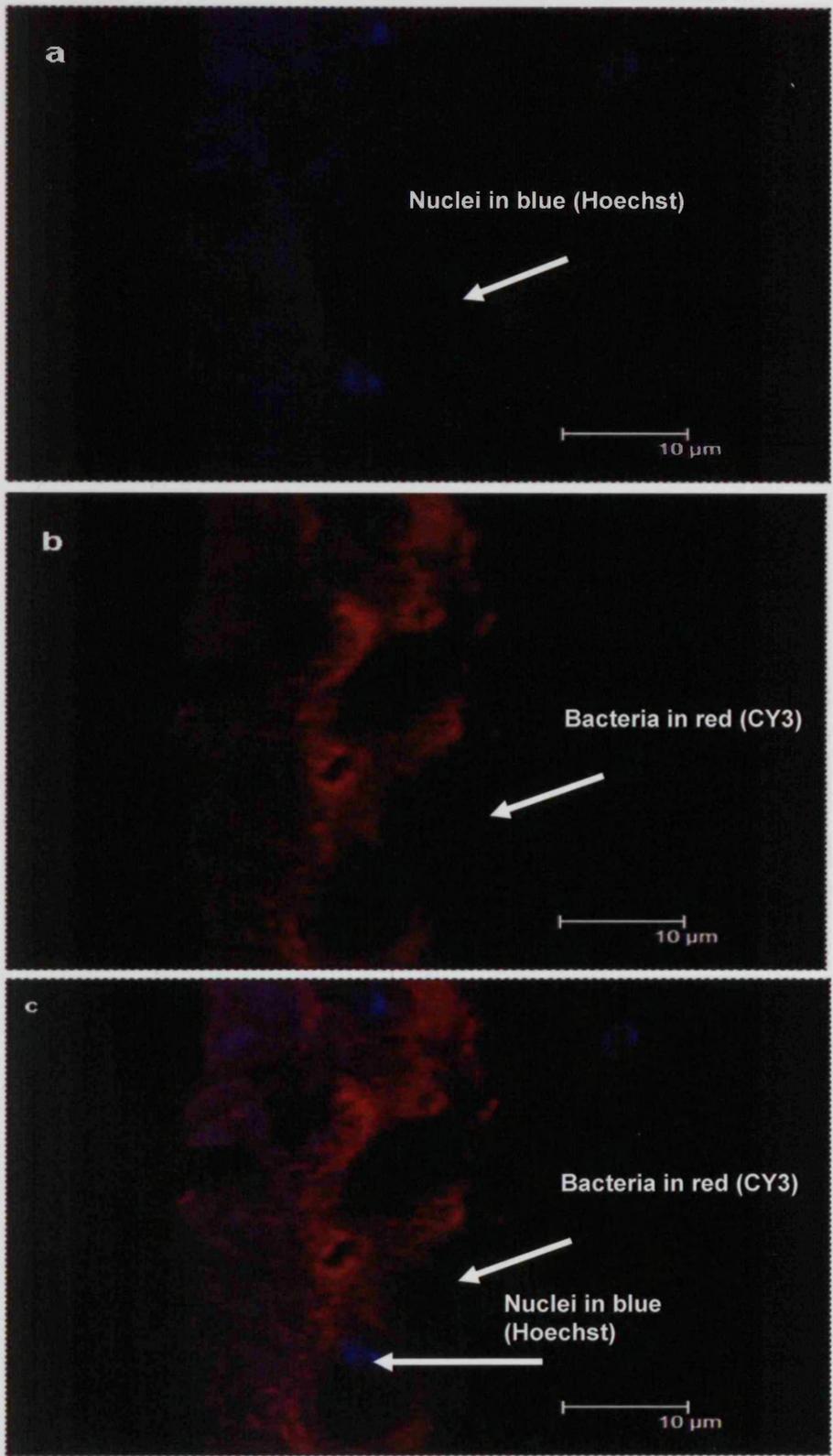
Fig. 5.16 Scratch wounded RHE tissue infected with *Streptococcus oralis* (B52), *Micrococcus luteus* (B81), *P. aeruginosa* (D40) and *S. aureus* (D76) and stained with a) Hoechst, b) universal bacterial PNA probe (Bac-Uni1CY3-red) and c) overlay of both channels.
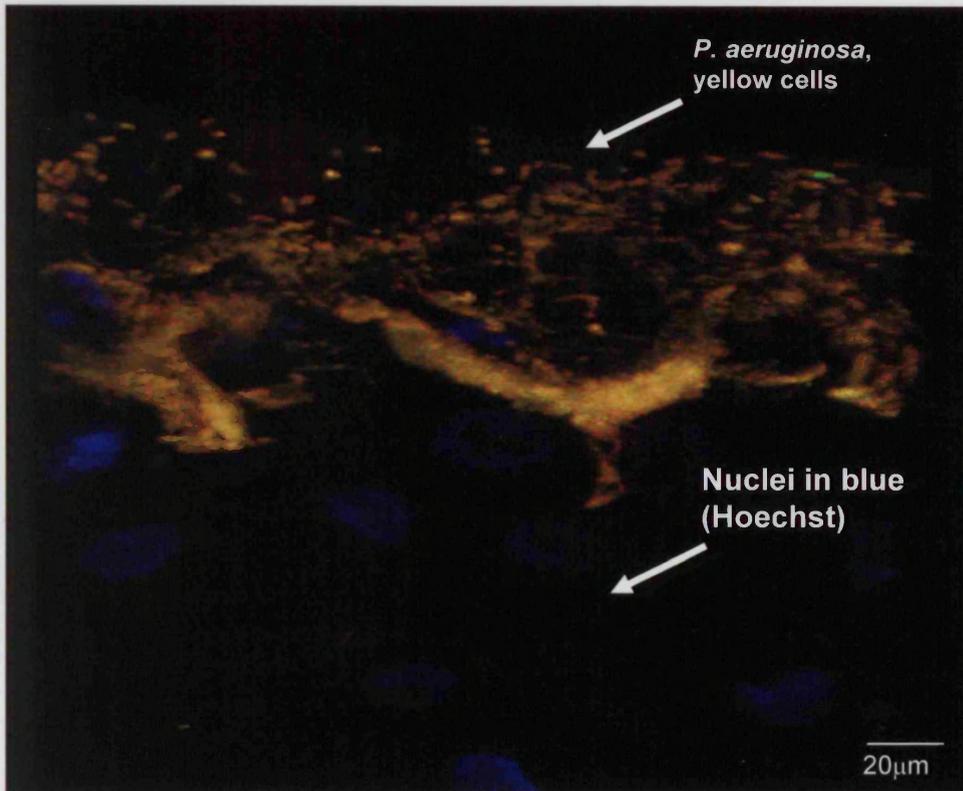
Fig. 5.17 Scratch wounded RHE specimen infected with *Streptococcus oralis* (B52), *Micrococcus luteus* (B81), *P. aeruginosa* (D40) and *S. aureus* (D76) and stained with Hoechst (nuclei-blue), universal bacterial PNA probe (Bac-Uni1CY3-red) and *P. aeruginosa* specific probe (PsaerFITC-green).
P. aeruginosa cells are yellow because they hybridise with universal probe and with *P. aeruginosa* specific probe. *Pseudomonas aeruginosa* appear to predominate with limited colonisation seen by the other organisms.

5.4.7 Analysis of wound biopsies using PNA FISH

Biopsy sections from chronic wounds were successfully processed and stained by both Gram-stain (Fig. 5.18) and with the universal bacterial PNA FISH probes. Using the universal PNA probe, bacteria could be detected within the biopsies sections. Examples of sections containing fluorescently labelled bacteria are shown in Fig. 5.19-5.21. The bacteria visualised appeared to be present both individually and in larger aggregates.

Sample 1 (Fig. 5.19) from a non-infected venous leg ulcer of a patient shows relatively few bacteria. This wound ultimately healed. Sample 2, a biopsy sections from a 73 year old female neurodiabetic patient with lower limb ischaemia and peripheral vascular disease were also successfully stained with universal PNA probe. Bacteria could again be detected (Fig. 5.20). A third sample, showing a biopsy section of a diabetic 71 year old female with lower leg ischaemia was taken from the pre-ulcer region showed bacteria on the surface of the section. Once the focal plane was moved inside the section, nuclei were detected but no bacteria, suggesting that they were surface associated (Fig. 5.21 animation on CD).
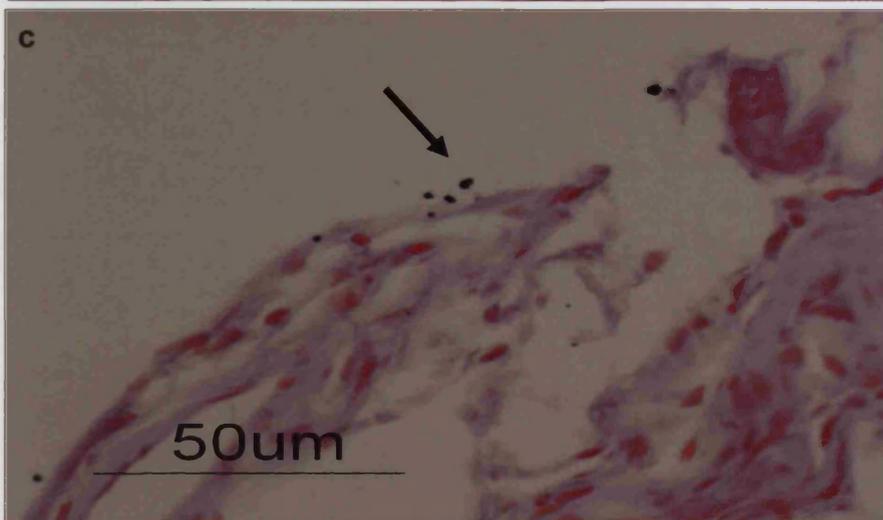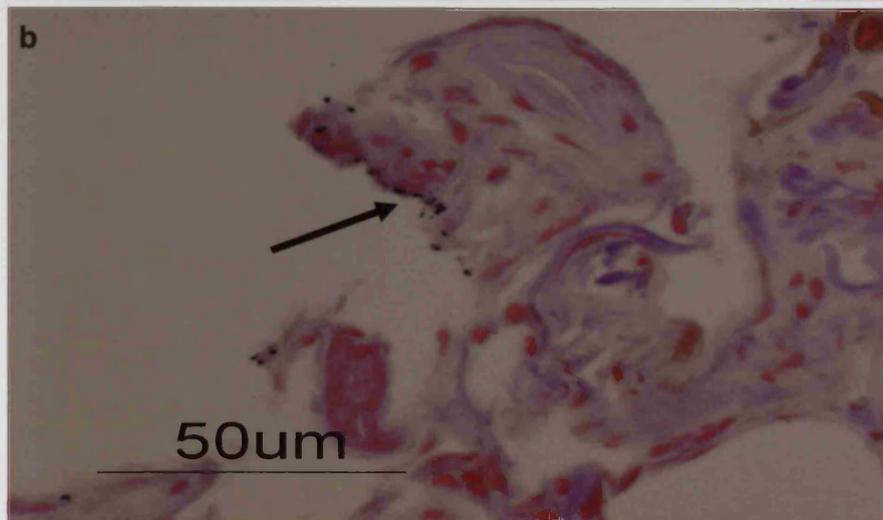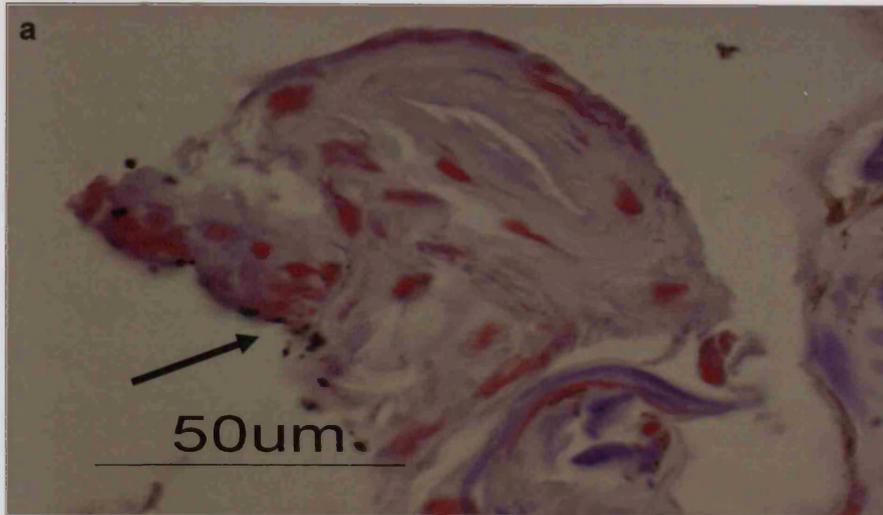
Fig. 5.18 Gram stain of Biopsy sections from non-infected chronic venous leg ulcers, a) x100 magnification; b-c) x 60 magnification; Bacteria are indicated by black arrows.
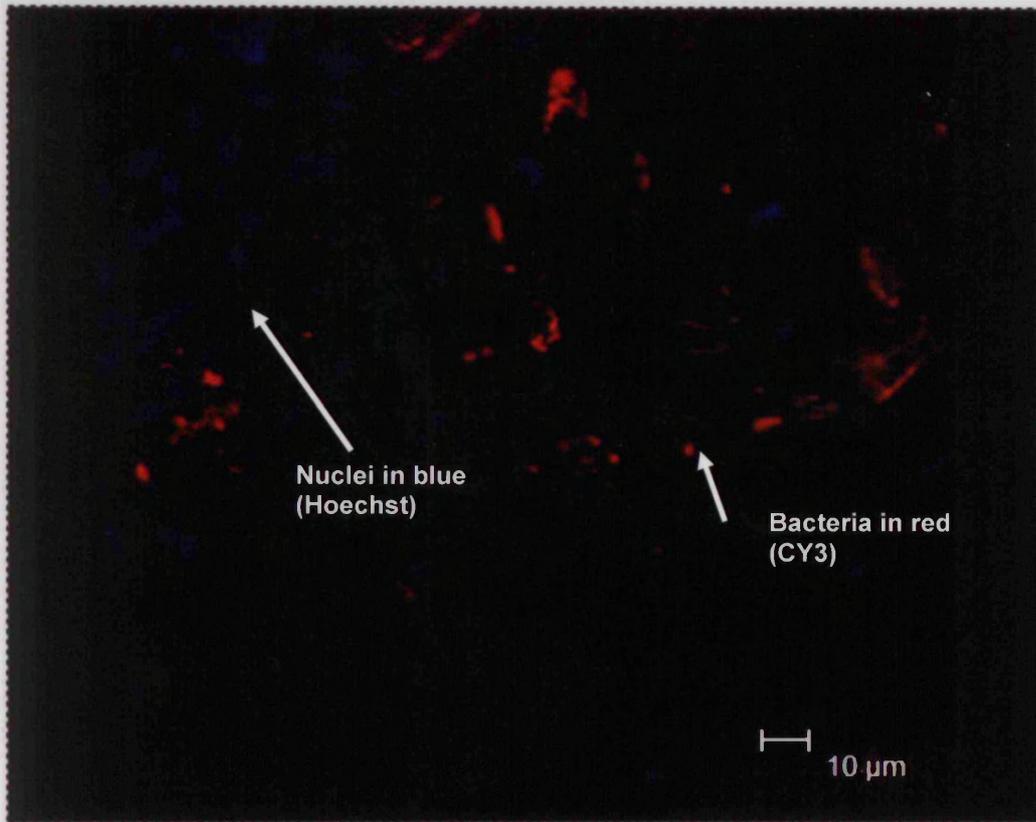
Fig. 5.19 Sections from a biopsy sample from a non-infected chronic venous leg ulcer patient (female, 64 year old) stained with the universal bacterial PNA probe (Bac-Uni1CY3-red) and the Hoechst stain.
The biopsy was taken from the centre of the wound bed.
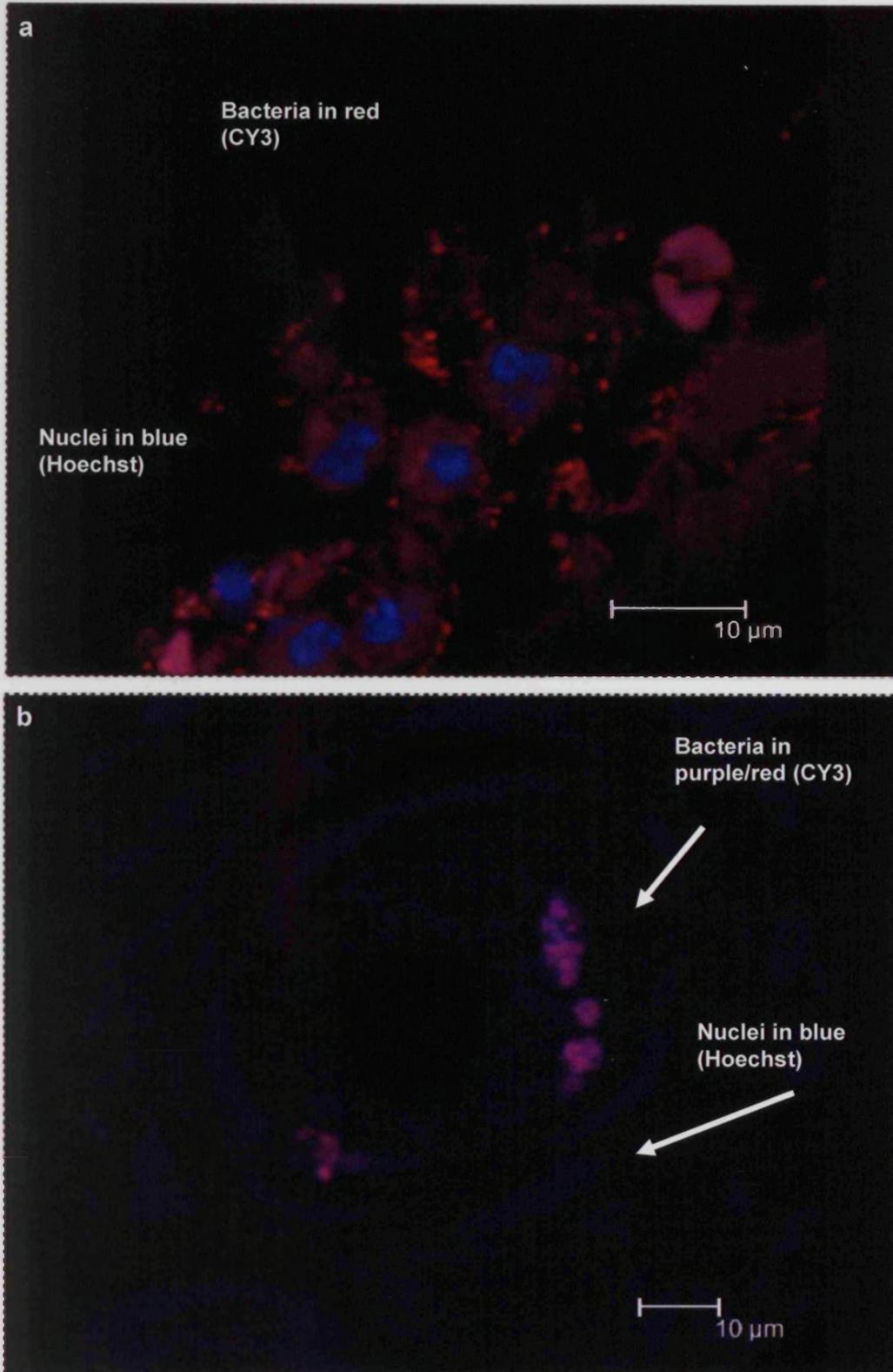
Fig. 5.20 Sections from a Biopsy sample from a 73 year old female, neurodiabetic with lower limb ischaemia and PVD. Ulcer present for 5 years. Section is stained with universal bacterial PNA probe (Bac-Uni1CY3-red) and the Hoechst stain (nuclei-blue).
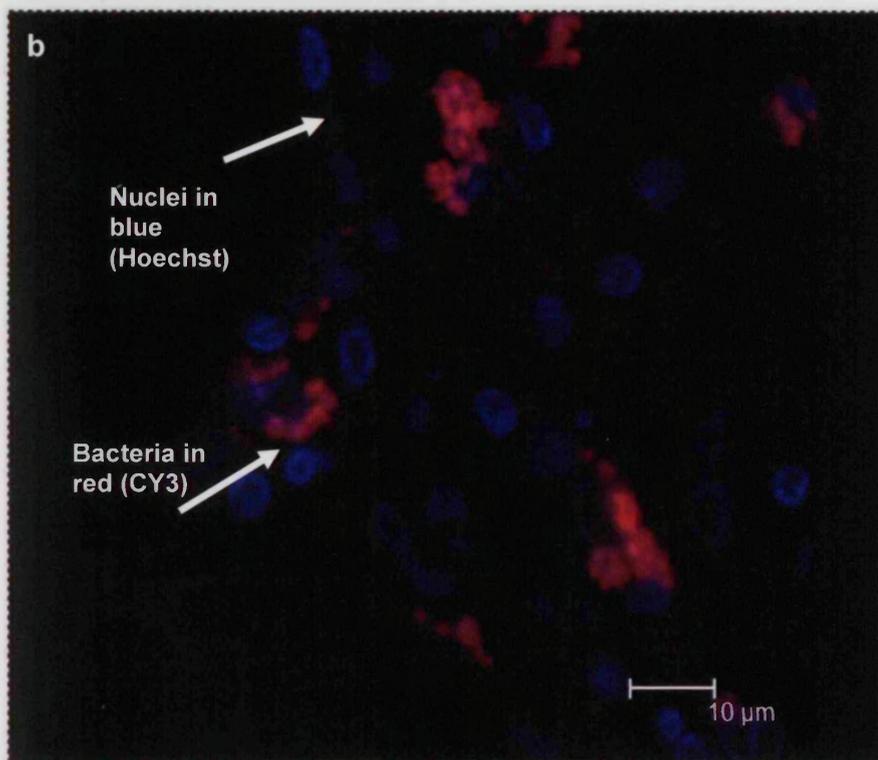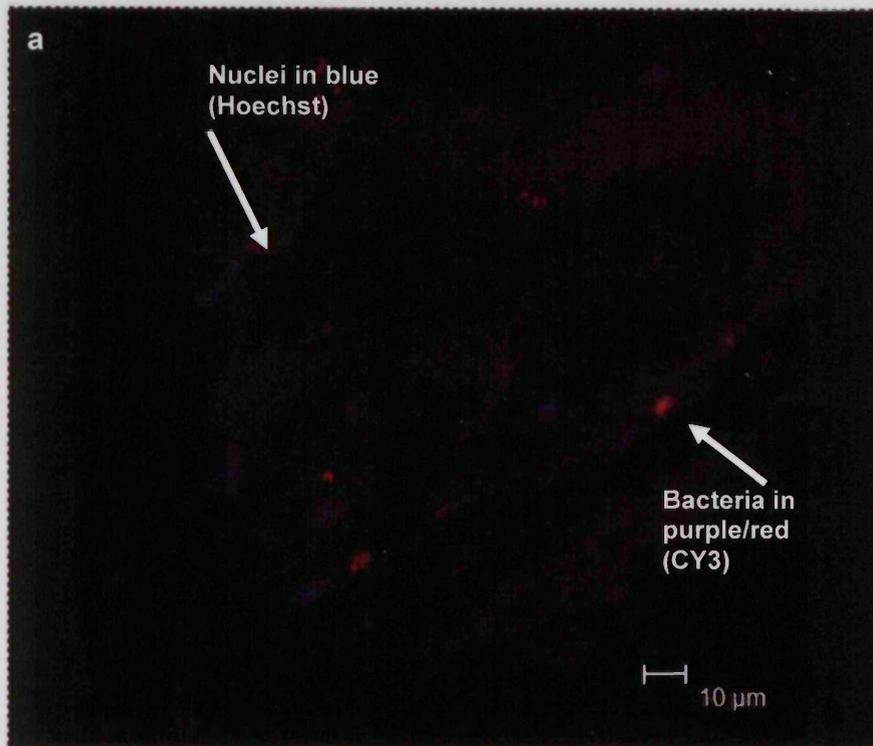
Fig. 5.21 Sections from a Biopsy sample from a 71 year old female, diabetic with lower limb ischaemia, a) biopsy was taken from a pre ulcer region, b) biopsy was taken 3 cm away from the ulcer region. Section is stained with universal bacterial PNA probe (Bac-Uni1CY3-red) and the Hoechst stain (nuclei-blue).

5.5 Discussion

The impact of microorganisms on wound healing is poorly understood but there is strong evidence to suggest that bacteria within chronic wounds delay the wound healing process (Edwards and Harding, 2004; Percival and Bowler, 2004; Percival and Rogers, 2005). The production of destructive enzymes and toxins by bacteria results in tissue damage and indirectly promotes an inflammatory state (Percival and Rogers, 2005; Stephens et al., 2003). Bacteria, such as *P. aeruginosa* release proteinases (*e.g.* matrix metalloproteinases) which are known to affect growth factors and many other tissue proteins necessary for wound healing (Kim et al., 2000; Percival and Rogers, 2005; Steed et al., 1996; Travis et al., 1995; Vaalamo et al., 1997). Removal of bacteria by host defence mechanisms and antimicrobial therapy is difficult in wound environments where biofilms have been established.

In this study, FISH was used to identify the presence of specific bacteria and to examine their spatial organisation within "*in vitro*" biofilms established using the CDFF and RHE models, as well as "*in vivo*" in actual chronic wound biopsy sections.

In the preliminary study, a universal eubacterial DNA probe, EUB338 was used to detect bacteria in planktonic suspension and in biofilms. One problem of using the EUB338 probe was its relatively low signal intensity, possibly as a consequence of insufficient penetration of the probe into the bacterial cells (Moter and Gobel, 2000). The enzymatic treatment of the sections in this study, with both lysozyme and proteinase K was employed in an effort to counter this problem. However, even after enzymatic treatment poor fluorescence of the target bacteria was evident.

In contrast, much stronger fluorescence of bacteria occurred when the PNA probes were used. Due to the neutral backbone of PNA probes they are able to diffuse readily through hydrophobic cell walls and membranes thereby allowing detection of bacteria without the need for any pretreatment to enhance cell permeabilisation. The high copy number of 16S rRNA in

replicating and metabolically active bacteria provides a sufficiently high target molecule for probes used in FISH. Potential problems of using RNA probing techniques are that the ribosomal content can differ between cells. Although normally abundant, the amount of rRNA is dependent on the physiological state of the bacterium and can vary significantly between bacterial species (Kemp et al., 1993; Moter and Gobel, 2000; Poulsen et al., 1993; Wallner et al., 1993). Another potential issue of targeting rRNA is the 3-dimensional structure of the rRNA. This means that not all sequences are equally accessible to probes (Moter and Gobel, 2000). These issues make probe design a critical factor for the successful application of PNA FISH.

In this study, a PNA - FISH methodology was developed and validated for three PNA probes to identify and visualise polymicrobial paraffin embedded biofilms and clinical specimens. To verify the PNA probe specificity, a planktonic preparation was again firstly analysed, before applying the probe to biofilms and biopsy sections. The use of PNA probes in this bacterial specific FISH protocol permitted the simultaneous identification of mixed bacterial species in planktonic suspension (Fig. 5.3). Subsequent application to bacterial biofilm populations using the differently labelled PNA probes revealed a much higher signal intensity compared to the previously used universal bacterial DNA probe. As a result, the PNA probes were used for later investigations of biofilms and wound biopsies. A significant advantage of PNA probes is the ability to hybridise under low salt concentrations, a condition that promotes the destabilisation of rRNA secondary structure and results in improved access to target sequences that can be elusive using conventional FISH (Perry-O'Keefe et al., 2001a; Perry-O'Keefe et al., 2001b). Hence, in this present study hybridisation was carried out under low salt concentration (100mM NaCl), high-temperature (55°C) and high pH (pH 9.0) conditions. Surprisingly, the *S. aureus* specific PNA probe (CY5 labelled) was very weak in fluorescence compared to the universal PNA bacterial probe (CY3 labelled) and the *P. aeruginosa* specific PNA probe (FITC labelled). To improve the fluorescence intensity, it was absolutely necessary to expose biofilms containing the Gram-positive organisms to lysozyme pre-

treatment for 15 min at 37°C and to add 30% formamide to the hybridisation buffer when using *S. aureus* specific PNA probe. Without lysozyme treatment and the addition of formamide, biofilms hybridised with the *S. aureus* specific probe (Cy5 labelled) showed none or only weak fluorescence. This has been previously observed in several other studies, (Hartmann et al., 2005; Lefmann et al., 2006; Wellinghausen et al., 2007). Perry-O'Keefe *et al.* (2001a) described a method using PNA probes for the simultaneous identification of Gram-positive and Gram-negative organisms. In their protocol, a 1 h paraformaldehyde fixation treatment was sufficient to permeablise Gram-positive cells (Thurnheer et al., 2004). This was not the case for the mixed biofilms in the present study.

Biofilms generated in the CDFF showed the characteristics of a typical biofilm architecture, *e.g.* the presence of confirmed spaces devoid of bacteria, which could potentially be water channels (Fig. 5.8). The function of channels is to facilitate and exchange nutrients and waste products (Hall-Stoodley et al., 2004). The 3D reconstruction of a mixed biofilm analysed by CLSM clearly shows the progression of biofilm establishment over a 7 d period. Initially, it is clearly apparent that on day 3 the bacterial community is composed of loosely associated cells compared to day 7. By day 5 the bacteria start to aggregate and the community is very dense on the top and on the base of the biofilm. On day 7 a typical biofilm structure is evident with spaces devoid of bacteria. Furthermore, use of the species-specific PNA probes showed that the spatial organisation of the organisms in the community grew in aggregates in distinct zones, suggesting that the organisms did not integrate well with one another under these conditions. The main constituent of these mixed species CDFF biofilms seemed to be *P. aeruginosa* followed by cocci bacteria.

In fixed RHE sections, few bacteria were detected on the surface and only a sparse biofilm was formed. This could be explained by the processing technique used, which could inadvertently have washed the bacteria off the tissue. However, in scratch wounded RHE sections, it was apparent that

bacteria started to penetrate the tissue and to migrate beneath the damaged surface layer. This suggests that once the integrity of the epithelial barrier has been disrupted (as would be encountered in a wounding situation) the bacteria were able to invade into the tissue. In fresh (unfixed) RHE sections, bacteria were restricted to the surface and the different species were not found to have integrated with one another. These data indicate that a well established biofilm was not produced in the RHE under the conditions applied in this study without wounding. In Chapter 2 the suitability of the oral mucosal as an *in vitro* model was illustrated and showed *Candida albicans'* ability to invade and form a biofilm. Unlike bacteria, however the fungus possesses the filamentous form, with hyphae actually able to penetrate the epithelium and multiply within the tissue.

A further limitation of the RHE system is the lack of certain host immune responses, although proinflammatory cytokine responses by *in vitro* epithelial cell lines, in a reconstituted oral epithelium model (Chapter 2), have been reported to be maintained (Schaller et al., 2002; Villar et al., 2005). Hence, the RHE has drawbacks as a chronic wound model but has to be considered as the closest related "*in vitro*" model to date for human epithelial cells, and has the benefit of avoiding the ethical issues that are associated with working with clinical human samples. Importantly, both models (CDFF and RHE) revealed heterogeneous biofilm structures with discrete clusters of bacterial species, with *P. aeruginosa* as the predominant organism. It is likely that the bacterial species within the biofilms compete with each other for space, attachment or nutrients. The high level of detection of *P. aeruginosa* by FISH in RHE and CDFF biofilms correlates closely with the cultural findings in Chapter 4. Two possible factors for the predominance of *P. aeruginosa* in these mixed biofilms could be its twitching and flagella-mediated motility, allowing *P. aeruginosa* to migrate to optimal growth localities as the biofilm matures as well as its faster growth rate. The other species used in this study were all non motile organisms. It seems that *P. aeruginosa* has adapted to use these surface structures, flagella and pili, to its own particular needs (Davey and O'Toole, 2000). Interestingly, *P. aeruginosa* did not

appear to do so well in single species biofilms (Chapter 4) and so seems to benefit synergistically from associations with other organisms.

Obviously, these experiments do not reflect exactly the *in vivo* situation within a chronic wound. *In vivo*, different microbial species could well be expected to colonise at different times with specific species acting as pioneer colonisers. In our experiments, all four bacteria species were added to the RHE simultaneously. It would therefore be interesting to add the species in a successive manner. For instance, *P. aeruginosa* could be added to the model first to form a base for the biofilm, with other species added subsequently to determine if they were able to grow to a greater extent on the biofilm surface. Since it was observed in this study that *P. aeruginosa* appeared to out-compete the other organisms it might also be of interest to see whether staging of the infection times allows the other species to grow at higher levels before *P. aeruginosa* is added to the system.

Analysis of chronic wound biopsies by FISH using the universal bacterial PNA probe showed the presence of bacteria. Unfortunately, maintaining the integrity of the biopsy tissue during processing and storage proved difficult even when the tissue had been paraffin-wax embedded. Also, establishing the orientation of the tissue was problematic, as it had been archived for a considerable time. Therefore, it was difficult to analyse the results from biopsy samples. Routine haematoxylin and eosin staining as well as PNA FISH detection confirmed that a few but not many bacteria were present in those biopsy sections. Previous work on chronic wound specimens has revealed the presence of densely aggregated colonies of bacteria surrounded by an extracellular matrix by microscopic studies (Bjarnsholt et al., 2008; James et al., 2007).

In future studies, it would be of interest to incubate the infected RHE for longer than 24 h to see if this would indeed result in greater infiltration of the bacterial cells within the RHE. The use of a different culture media that promotes bacterial growth as opposed to the tissue maintenance medium

could also be used. In addition, it would be interesting to detect the presence of any EPS matrix generated by the chronic wound bacterial biofilms. Due to time constraints EPS analysis was not pursued but it would be interesting to counterstain the EPS using for example, calcofluor white, after completion of FISH probing. Thurnheer *et al.* (2004) showed detection of organisms in a multispecies biofilm of supragingival plaque using FISH and combined this with EPS staining using calcofluor white.

Now that a basic protocol for using PNA FISH to examine biopsy sections has been established, the technique could be used in future studies on new 'fresh' chronic wound tissue. This could provide important information on the various species present in the chronic wound tissue and how they are spatially arranged on and within the tissue. Ideally, this approach could also be used to compare the species present within chronic wounds with those in healthy control tissue, either from the same patients, or from other, age-matched patients without chronic wounds.

PNA – FISH was shown to be a rapid and versatile tool for both research purposes and potentially for clinical microbiology diagnostics when used in conjunction with CLSM. CLSM has been established as a valuable tool for obtaining high-resolution images and three-dimensional reconstructions of fluorescently labelled biofilms and biological samples (Lopez et al., 2005; Sunde et al., 2003; Thurnheer et al., 2004; Wagner et al., 2003a). The future application of this technique to clinical biofilms from infected tissues or indwelling medical devices could facilitate the identification and estimation of the relative proportions of bacteria within a biofilm without the need for bacterial culture. This approach could be used to assess biofilm management strategies or evaluate the effectiveness of antimicrobials against members of the biofilm consortium. Importantly, this technique could potentially be applied to clinical samples for identifying significant bacterial infections.

6.6 Summary

1. This study showed that it was possible to simultaneously detect and identify several members of a four-species biofilm consortium, comprised of both Gram-positive and Gram-negative bacteria.

2. The spatial organisation of bacteria in biofilm models and wound tissue was assessed by PNA FISH and CLSM. This modified FISH technique enabled the visualisation of the arrangement of the microbial population as well as the typical architecture of the biofilms depicting spaces devoid of bacteria which could potentially be water channels. The different species did not appear to integrate well with each other, with distinct, discrete clusters of each species evident. The main constituent of the established biofilms was *P. aeruginosa*, which supports previous culture evaluations (Chapter 4).

Chapter 6

Gene expression in bacterial

biofilms

6.1 Introduction

The aetiology of chronic wounds is poorly understood, but evidence suggests that chronic wounds support a diverse microbial flora, which may contribute both indirectly and directly to the failure of the normal wound healing process. Bacteria can express numerous virulence factors that contribute to pathogenesis and host invasion. Typical examples of bacterial virulence factors include hydrolytic enzymes such as proteinases, DNAses, and phospholipases. In the past, evaluating the potential role of such agents in pathogenicity has involved detection of the protein or its activity in clinical specimens. However, with the advent of high technology molecular analysis it has become possible to detect expression of bacterial virulence factors through analysis of specific messenger RNAs.

The roles of bacterial virulence factors in promoting the chronic wound phenotype have, as yet, not been elucidated. Examples of *P. aeruginosa* virulence factors include the possession of pili, flagella, lipopolysaccharide, proteases, quorum sensing molecules, exotoxin A, exoenzymes and alginate, which all contribute to its pathogenic importance (Bjarnsholt et al., 2008; Lyczak et al., 2000). It has been suggested that proteases promote infection and dissemination, whilst elastase degrades collagen and non-collagen host proteins and disrupt the integrity of the host basement membrane (Lyczak et al., 2000). It can be readily envisaged how the expression of these enzymes that either degrade host tissue or destroy the protective immune cells or their products could contribute to delayed wound healing.

In recent years it has been postulated that bacterial biofilms may play a role in the prevention of the normal wound healing process (James et al., 2007). Biofilm bacteria are reported to be less susceptible to the immune system and also to administered antimicrobial agents and therefore a biofilm associated wound infection may persevere and progress from an acute to a chronic infection state (Percival and Rogers, 2005).

It has been hypothesised that the phenotypic changes observed in microorganisms as they attach to a surface are due to the differential expression of genes within biofilms (O'Toole et al., 2000a). However, it appears that biofilm development occurs differently according to nutritional and environmental conditions and consequently there may be many pathways and stages involved in biofilm development (Klausen et al., 2003).

6.1.1 The bacterial microflora of chronic wounds

The significance of microorganisms in chronic wounds has been extensively studied and different approaches used to elicit their possible role in non-healing wounds. Studies have included assessing the impact of microbial populations on clinical outcome (Madsen et al., 1996) and observations of the occurrence of particular species (Schraibman, 1990) or groups of organisms within specific wounds (Davies et al., 2007; Wall et al., 2002). Previous studies using molecular (Davies et al., 2004; Davies et al., 2001; Hill et al., 2003; James et al., 2007) and culture-based approaches (Bowler and Davies, 1999) have indicated the presence of polymicrobial bacterial communities in chronic wounds. The most frequent isolates include staphylococci, streptococci and *Pseudomonas* sp. (Davies et al., 2001). Cultural analysis has also shown *Acinetobacter* sp. and *Proteus* sp. to be prevalent in chronic wounds (Hill et al., 2003) and have also documented that staphylococci, streptococci, enterococci and facultative Gram-negative bacilli are the principle bacterial groups that are recovered from chronic venous leg ulcers (CVLU) (Davies et al., 2007; Hansson et al., 1995). In addition to these aerobic bacteria, anaerobic organisms have also been identified in chronic wounds, such as *Peptostreptococcus spp.*, *Bacteroides spp.* (Davies et al., 2007) and pigmented and non-pigmented *Prevotella* and *Porphyromonas* spp. (Bowler and Davies, 1999; James et al., 2007).

6.1.2 Genotypic differences between planktonic and biofilm populations

It is believed that microorganisms present in chronic wounds contribute to the non-healing phenotype and may exist within wounds as part of a biofilm community (Davies et al., 2007; Mertz, 2003; Serralta et al., 2001). Biofilm

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associated infections are typically persistent; they develop slowly, are rarely resolved by immune defences and respond transiently to antimicrobial therapy (James et al., 2007; Parsek and Singh, 2003). For example, Gram-negative bacterial components such as colonic acid (in *Escherichia coli*), alginate, glucose and mannose-rich Pel (product of the pellicle formation gene) and Pls (product of the polysaccharide synthesis locus) matrix components (in *P. aeruginosa*), cellulose and β-1-6-GlcNac polymer (in *Salmonella* and *E. coli*), have been reported to play important roles in biofilm formation (Beloin and Ghigo, 2005; Friedman and Kolter, 2004; Matsukawa and Greenberg, 2004).

As described previously (see section 6.1) and in the Literature Review (Chapter 1, section 1.9.2), *P. aeruginosa* and *S. aureus* are recognised key organisms in chronic wounds and consequently provide a major focus for this chapter.

6.1.3 Gene expression in *P. aeruginosa* and *S. aureus* biofilms
There are many genes whose expression has been associated with the virulence of *P. aeruginosa* and *S. aureus* (Table 6.1). Examples of genes important in biofilm formation are described below.

6.1.3.1 Periplasmic glucans synthesis in *P. aeruginosa*
Periplasmic glucans may contribute to antibiotic resistance by reducing the diffusion of antibiotics into the cell (extracellular sequestering) thereby allowing the bacteria additional time to adapt to an antibiotic challenge (Mah et al., 2003; Whiteley et al., 2001).

Table 6.1 Genes involved in biofilm formation

Regulatory genes	Function	Reference
Gram positive bacteria (*S. aureus, S. epidermidis*)		
icaADBC	Mediates cell-cell adhesion	(Arciola et al., 2001, Beenken et al., 2004, Conlon et al., 2002a, Conlon et al., 2002b, Mack et al., 1996, Rohde et al., 2005, Yazdani et al., 2006)
Accessory gene regulator (*agr*)	Regulates virulence factors / Involved in cell detachment	(Ben Ayed et al., 2006, Shopsin et al., 2003)
Staphylococcal accessory gene regulator (*sarA*)	Production of surface associated & secreted virulence factors	(Beenken et al., 2003, Beenken et al., 2004)
SigmaB	Activates biofilm formation	(Knobloch et al., 2004)
Gram negative bacteria (*P. aeruginosa*)		
Chaperone-usher pathways (*cupA, cupB, cupC*)	Assembly of novel fimbrial structures	(Croft et al., 2000, Vallet et al., 2001)
rpoS	Important for biofilm formation and antibiotic susceptibility	(Heydorn et al., 2002, Whiteley et al., 2001, Xu et al., 2001)
Crc	Activation of type IV motility	(O'Toole et al., 2000)
mvaT	Reduces adhesion via *cup* gene repression	(Vallet et al., 2004)
rpoN	Role in initial adhesion & biofilm formation	(Thompson et al., 2003)

The extracytoplasmic mode of antibiotic sequestration might be expected to interfere with the passage of antibiotics through the periplasmic space to their site of action in the cytoplasm. For the synthesis of periplasmic glucans *ndvB* is required. The *ndvB* gene encodes for a glucosyltransferase that is required for the synthesis of cyclic-β-(1, 3)-glucans (Mah et al., 2003; Whiteley et al., 2001). Mah *et al.* (2003) also predicted that *ndvB* is preferentially expressed in biofilm grown cells.

Cyclic glucans are circular polymers of glucose, which play a role in adaptation to low osmotic media, flagella-mediated motility and plant-microbe interactions. These glucans are located in the periplasm and also secreted into the extracellular medium (Breedveld and Miller, 1994; Mah et al., 2003). It has been suggested that the glucose polymers may prevent antibiotics from reaching their sites of action by sequestering antimicrobial agents in the periplasm (Breedveld and Miller, 1994; Mah et al., 2003).

The impermeability of bacteria to antibiotics involves several factors, including the *tolA* gene product (Rivera et al., 1988) and terminal electron transport proteins (Bryan and Kwan, 1983; Bryan et al., 1980; Whiteley et al., 2001). The *tolA* gene product affects the LPS structure, resulting in decreased aminoglycoside affinity for the outer membrane. Evidence indicates that *tolA* is an essential *P. aeruginosa* gene, but mutants that under-produce *tolA* are hypersensitive to aminoglycoside antibiotics (Rivera et al., 1988). Therefore, the *tolA* gene could contribute to biofilm resistance to aminoglycosides.

6.1.3.2 Alginate genes of *P. aeruginosa*

Alginate is a viscous exopolysaccharide, consisting of D-mannuronic and L-guluronic acids. Alginate production by *P. aeruginosa* is regarded as a virulence factor that appears to promote biofilm formation, thus enhancing host colonisation. Furthermore, alginate induced biofilms have also been shown to confer resistance to antibiotics (Hershberger et al., 1995), promote bacterial adherence, serve as a barrier to phagocytosis and provide a

mechanism to neutralise oxygen free radicals (Edwards and Saunders, 2001).

It has been suggested that alginates play an important role in *P. aeruginosa* infection of cystic fibrosis (CF) patients, urinary tract infections, the fouling of man-made materials and in biofilm formation on abiotic surfaces in the natural environment (Davies et al., 1993). Strains of *P. aeruginosa* isolated from the lungs of CF patients have been observed to have a mucoid phenotype with production of exopolysaccharide alginate (Edwards and Saunders, 2001). Alginate is thought to have a protective function in a relatively harsh environment in which the bacteria are continually subjected to oxidative stress and challenge by the immune system (Krieg et al., 1988; Simpson et al., 1988; Simpson et al., 1989).

Even though most *P. aeruginosa* strains have the ability to produce alginate, the majority of strains exhibit a non-mucoid phenotypes (Hershberger et al., 1995). The switch to the mucoid phenotype is also thought to promote persistence of *P. aeruginosa* (Hentzer et al., 2001). Mucoid production has further been shown to aid bacterial survival in nutrient poor environments (Hershberger et al., 1995). In a biofilm state or in a condition such as cystic fibrosis, *P. aeruginosa* must adapt to its environment and this is manifested by overproduction of alginate.

Alginate production is regulated by at least two mechanisms. Firstly, by a response regulator involved in a bacterial signal transduction system which is controlled by environmental factors. Secondly, by a gene cluster containing the sigma factor *algU* (Fig. 6.1) (Edwards and Saunders, 2001). The *algU* gene is autoregulated and has two negative regulators encoded by *mucA* and *mucB*. Inactivation of either *mucA* or *mucB* results in activation of *algD* through enhanced expression of *algU*, and so leads to overproduction of alginate (Edwards and Saunders, 2001). Although alginate production may not be required for biofilm formation, it undeniably affects the biofilm architecture (Stapper et al., 2004).
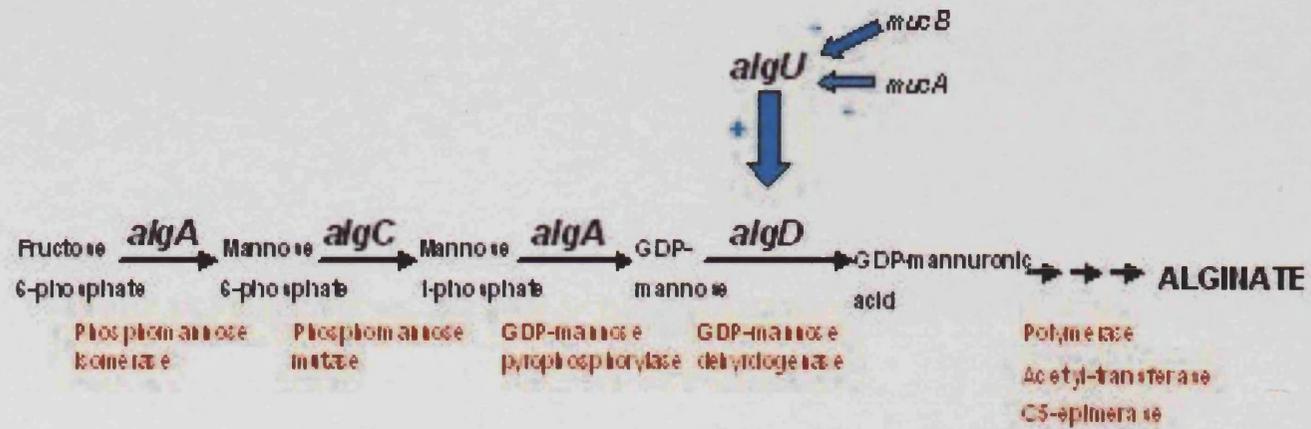
Fig. 6.1 Pathway leading to alginate production in *Pseudomonas aeruginosa*. (Adapted from Jain and Ohman (1998).

6.1.3.3 Accessory gene regulator (*agr*) of *S. aureus*

The *agr* system regulates the coordinated production of several virulence factors (Shopsin et al., 2003). The *agr* operon encodes a two-component signalling system (Chapter 1, Fig. 1.6; *agrC* is the signal receptor and *agrA* is the response regulator) which is driven by a quorum sensing autoinducing peptide, encoded by the *agrD* gene (Ben Ayed et al., 2006).

Staphylococcus aureus isolates can be classified into four (I-IV) specific *agr* groups on the basis of the autoinducing peptide specificity for its signal receptor (*agrC*) (Ben Ayed et al., 2006; Shopsin et al., 2003). It has been suggested that strains of one group can cross-activate the *agr* system of other strains in the same group to produce a response, *e.g.* an up regulation of virulence factors in other strains of the same group. Conversely, inhibition of the *agr* response of members of other groups (Camara et al., 2002) could also occur. It is important to note that these genotypes can also be correlated with specific phenotypes, *e.g.* most menstrual toxic shock syndrome strains belong to *agr* group III (Fischetti, 2006). The *agr* system coordinates the production of virulence factors during different phases of the cell cycle, which help with the organisms' ability to invade and its pathogenicity, *e.g.* it can down-regulate the production of cell wall-associated proteins and up-regulate secreted proteins at late to stationary growth phases *in vitro* (Camara et al., 2002; Fischetti, 2006).

6.1.3.4 Intercellular polysaccharide adhesin (*ica*) of *S. aureus*

Over 90% of clinical *S. aureus* strains possess capsular polysaccharides. Capsule production is believed to decrease phagocytosis *in vitro*, and has been shown to enhance *S. aureus* virulence in a mouse bacteriaemia model (Harris et al., 2002).

Biofilm formation can be described as a two step process that first requires adhesion of bacteria to a surface followed by cell-cell adhesion, forming multiple layers of the biofilm. This process in *S. aureus* is associated with the polysaccharide intercellular adhesin (PIA), which is composed of linear β-1,

6-linked glucosaminylglycans. The *ica* locus is thought to mediate cell-cell adhesion and PIA production (Cramton et al., 1999). The locus itself is comprised of four intercellular adhesion genes (*icaA, icaB, icaC* and *icaD*) and one regulator gene (*icaR*) which appears to function as a repressor (Cafiso et al., 2004; Conlon et al., 2002a; Gotz, 2002; Mack et al., 1996; Ziebuhr et al., 1999). *IcaA* and *icaD* play a significant role in *S. aureus* and *S. epidermidis* biofilm formation (Yazdani et al., 2006). The product of *icaA* is a N-acetylglucosamine transferase, *icaC* and *icaD* encode membrane proteins and *icaB* encodes a signal peptide that is secreted into the medium (Fig. 6.2) (Gelosia et al., 2001).

6.1.4 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Previous studies have examined gene expression in bacterial biofilms including those produced by *S. aureus* and *P. aeruginosa* (Arciola et al., 2001; Conlon et al., 2002a; Cramton et al., 1999; Gelosia et al., 2001; Mah et al., 2003; Sakoulas et al., 2002). For example, Cafiso *et al.* (2004) used this approach to determine the expression of the *icaADBC* operon in *S. epidermidis* strains isolated from catheter related and nosocomial infections and correlated expression to biofilm production. Mah *et al.* (2003) used RT-PCR to identify expression of the *ndvB* locus which is required for the synthesis of periplasmic glucans in *P. aeruginosa*. RT-PCR is a sensitive method but is at best only semi-quantitative. To overcome this limitation a quantitative real-time PCR can be employed which allows monitoring the formation of the product in real time. The monitoring of product is accomplished using various types of fluorescent dyes.
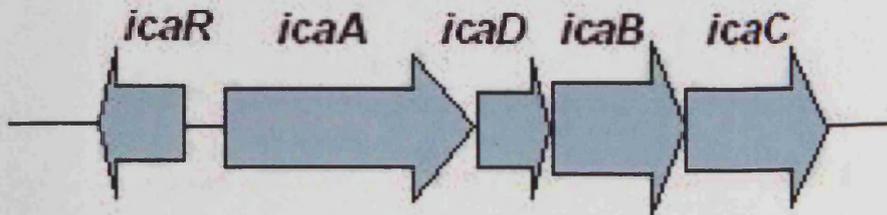
Fig. 6.2 Schematic of the *ica* locus.
The operon is composed of the *icaR* (regulator gene) and *icaADBC* (biosynthesis) genes. The *icaR* gene is transcribed divergently from the other four genes and the *icaA, B, C* and *D* genes appear to be translated from a single transcript.

6.1.5 Microarray study

An 'array' refers to the regular arrangement of oligonucleotide or cDNA representations of genes on a solid support such as nylon membranes and plastic or glass microscope slides (Whatling et al., 2003). Many complete genomic sequences of microorganisms are now available. The analysis of bacterial genomes has already contributed to our understanding of the molecular evolution and diversity of human pathogens including *Campylobacter jejuni*, *Vibrio cholerae*, *Streptococcus pyogenes*, *Helicobacter pylori*, *Mycobacterium bovis* and *S. aureus*. Such information has been invaluable in the development of microbial microarray techniques. Microarrays are now increasingly being used for bacterial genome analysis and the approach has proven to be of great value in helping elucidate the genomic diversity and evolutionary relationships within species (Fitzgerald et al., 2001; Saunders et al., 2004a).

However, gene expression analysis is the most common application of DNA/oligonucleotide microarrays. In this technique, RNA isolated from two samples (control and test sample) is fluorescently labelled, generally with cyanine 3 (CY3 green) and with cyanine 5 (CY5 red) before being hybridised to a microarray consisting of large numbers of oligonucleotides orderly arranged on the surface of a glass microscope slide (Fig. 6.3). These two cyanine dyes have different absorption and emission spectra. After hybridisation, a high resolution scanner records the signal. Green or red signals indicate that only one of the targets has hybridised to the probe, and yellow shows that both control and test sample have hybridised (Ljperen and Saunders, 2004).

Microarrays represent a relatively recent method for analysing bacterial genotypes. The advantages of microarrays lie in their flexibility, the versatility in the use of probes to test sample material and the fact that probes can be used to detect DNA sequences linked to specific phenotypic characteristics (Ljperen and Saunders, 2004).
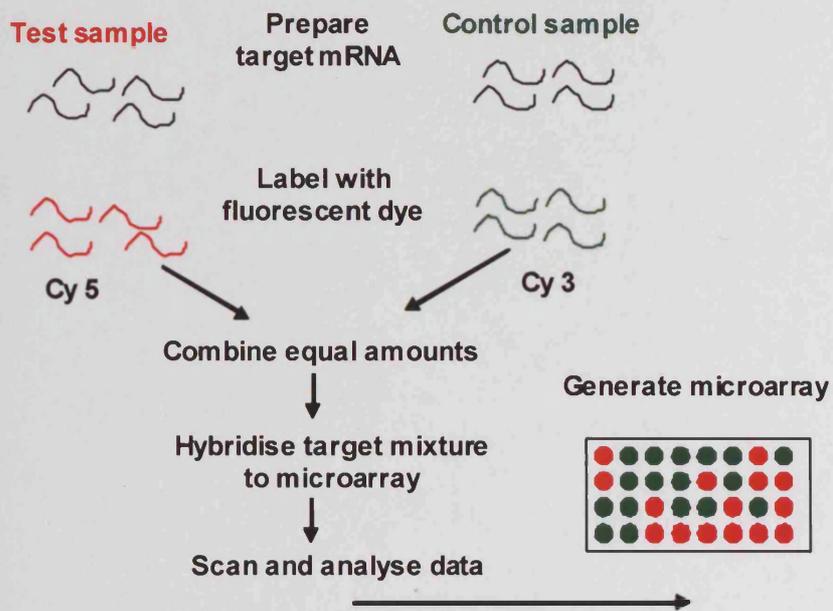
Fig. 6.3 Schematic procedure involved in microarray analysis.

Microarray studies have been used to identify global gene expression profiles of biofilms (Lazazzera, 2005). Whiteley *et al.* (2001) performed the first DNA microarray study to analyse gene expression in *P. aeruginosa* planktonic and biofilm cells. This initial microarray showed that approximately 1% of genes were differentially expressed between biofilm and planktonic growth phases and that *rpoS* gene expression was important in both biofilm formation and resistance to the antibiotic tobramycin (Whiteley et al., 2001). Resch *et al.* (2005) carried out a microarray analysis to determine which genes were up-regulated in *S. aureus* biofilms. Their data reported up-regulation of the *ica* genes in early biofilms (6-8 h of growth) compared to planktonic cells, suggesting that *ica* up-regulation was not needed after attachment and biofilm formation had begun (Resch et al., 2005). Resch *et al.* (2005) found that a range of virulence factors (*e.g.* proteases) and toxins were up-regulated in planktonic cells, which could be useful to cause acute infection and to invade cells. It has been suggested that toxins and proteases are not important for biofilm persistence once established (Resch et al., 2005).

Printed arrays can be used for either Comparative Genome Hybridisation (CGH) or expression profiling work. The *S. aureus* microarray used in this study consists of 720 oligonucleotide probes representing the virulence and housekeeping genes of *S. aureus*. Data derived from the array will allow comparative analysis of gene expression by isolates from *S. aureus* biofilms generated on the CDFF and from planktonic *S. aureus* in exponential and stationary phase.

6.2 AIMS

The aims of this chapter were:

To use both RT-PCR and microarray analysis to examine the *in vitro* biofilm gene expression of two chronic wound bacterial species, namely *S. aureus* and *P. aeruginosa*. Biofilms were generated for this study using the Constant Depth Film Fermenter as described in Chapter 4.

6.3 Methods

6.3.1 Processing of CDFF biofilms

Biofilms were prepared using the CDFF as described previously (Chapter 4, section 4.3.12). CDFF biofilms for RT-PCR were recovered and stored in a 2 ml microtube with 1.5 ml RNAlater® (Ambion Europe Ltd, Huntington, UK) solution before RNA extraction (section 6.3.3) and were maintained at -80°C until required.

6.3.2 Preparation of bacteria for planktonic growth

In addition to growth in a biofilm state (section 6.3.1), gene expression was also assessed for planktonic strains of *P. aeruginosa* D40 *and S. aureus* D76 cultured in BHI medium at 37°C.

As described previously (Chapter 4, section 4.3.12.1), overnight cultures were initially prepared to generate inocula for planktonic growth experiments. This overnight culture was diluted two hundred-fold in 20 ml BHI (an identical dilution factor as used for the CDFF inoculum) and 7 broths for each bacterial species were incubated with shaking at 37°C for 7 d. Each day, the cells were centrifuged and the supernatant removed and new medium was added. At each 24 h time point, the cells from one of the broths were harvested by centrifugation and stored in a 2 ml microtube with 1.5 ml RNAlater® (Ambion, Europe Ltd, Huntington, UK) solution prior to RNA extraction. The pelleted cells in RNAlater were stored at -80 °C until processed.

6.3.3 RNA extraction

RNA was extracted using two different methods, employing either a RiboPure Bacteria Kit (Ambion Ltd., Warrington, UK) or a Qiagen RNeasy Kit (Qiagen, West Sussex, UK). RNA samples were kept on ice throughout the RNA extraction whenever possible. For the RiboPure Bacteria Kit, biofilm and planktonic cells from the RNAlater were collected by centrifugation for 5 min (16,000 *g*). The supernatant was decanted and 350 µl RNAwiz (Ambion, RiboPure Bacteria Kit) added to the bacteria which were resuspended by

vortexing vigorously for 15 s. The bacteria were transferred to a tube containing 250 μl of sterile zirconia beads and then homogenised for 2-5 min using a Mini-BeadBeater-8 (Stratech Scientific Ltd., Soham, UK). After disruption, cells were centrifuged for 5 min at 4°C at 16,000 g and the bacterial lysate transferred to a fresh 1.5 ml tube. The lysate volume was estimated (200 - 250 μl) and 0.2x volumes of chloroform (Sigma, Poole, UK) added and shaken vigorously for 30 s prior to incubation for 10 min at room temperature. After centrifugation (5 min, 4°C, 16,000 g) the aqueous phase containing the partially purified RNA (150 - 200 μl) was transferred to a fresh 1.5 ml eppendorf tube and 0.5x volumes of absolute ethanol added. The lysate/ethanol mixture was transferred to a filter cartridge, which was placed into a 2 ml collection tube, and centrifuged for 1 min. The flow-through was discarded and the RNA washed according to the manufacturer's recommended protocol.

For the Qiagen RNeasy Kit, 200 μl of biofilm cells or planktonic cells from the RNAlater were collected by centrifugation for 5 min at 4°C (16,000 g). The supernatant was decanted and the cells re-suspended in 200 μl lysis buffer, containing 2x TE buffer, 1.2% Triton X-100, 0.3 mg ml^{-1} lysozyme, 0.03 mg ml^{-1} lysostaphin and nuclease free water. The mixture was incubated at 37°C for 45 min. To the lysate, 350 μl RLT extraction medium (Qiagen, Crawley, UK) was added and mixed thoroughly by vortexing vigorously. The RNeasy Mini Kit (Qiagen) was used to complete total RNA extraction according to the manufacturer's recommended protocol. Potential DNA contamination of all samples was then removed using RNase-Free DNase I (Qiagen) treatment following the manufacturer's instructions.

6.3.4 cDNA synthesis

50 ng $μl^{-1}$ of total RNA starting material or a nuclease free water control was placed into 0.2 ml thin-walled eppendorf tubes. To each sample, the following components were added: 5 μl (x10) thermophilic DNA polymerase buffer (100 mM Tris-HCl, 500 mM KCl and 1% Triton X-100), 5 μl 10 mM dNTPs, 10 μl (25 mM) MgCl, 4 μl (10 U $μl^{-1}$) RNase inhibitor, 2.5 μl (500 ng

ml^{-1}) random hexamers and 1 μl (200 U μl^{-1}) MMLV reverse transcriptase (Promega, Southampton, UK). The final volume was made up to 50 μl with nuclease free diethyl-pyrocarbonate (DEPC) treated water. Addition of nuclease free water in replacing the constituents described above provided a non-RT control. All samples were vortexed, centrifuged for 1 min at 8,000 g and incubated at 25°C for 10 min, 42°C for 60 min and 95°C for 5 min to generate cDNA. The samples were subsequently cooled to 4°C and stored at -20°C until required for PCR.

6.3.5 PCR on cDNA samples

PCR targeted a number of virulence gene families of *P. aeruginosa* and *S. aureus* including outer membrane protein genes, alginate genes, the intercellular adhesion locus (*ica*) and the accessory gene regulator (*agr*). For each gene, a specific primer pair was used to yield an amplicon of distinct size (Tables 6.2-6.3). A variety of *ica* primers were examined, which were previously described (Cafiso et al., 2004; Knobloch et al., 2002). In the case of *icaA*, two primer sets were used, *S. aureus* specific and those previously used for *S. epidermidis*. Additionally *ica* primers for *icaA, icaB, icaC, icaD* and *icaR* were designed in this study using the software program Primer3 (Rozen and Skaletsky, 2000).

The primers and reaction conditions (Table 6.4) were as previously described for *agr* (Ben Ayed et al., 2006; Shopsin et al., 2003), alginate (Edwards and Saunders, 2001) and for outer membrane proteins (Mah et al., 2003). The constitutively expressed gene encoding the 50S ribosomal protein L21 (*rplU*) was used as a control for *P. aeruginosa* (Mah et al., 2003) and the phosphate acetyltransferase (*pta*) house-keeping gene of *S. aureus* was also amplified as a RT-PCR control (Enright et al., 2000). Additional controls for each reaction included, genomic DNA, DNAse I treated RNA not treated with reverse transcriptase (to establish that genomic DNA amplification was not present) and a water template control. A typical reaction mix contained 5 μl of cDNA template, together with 1.5 mM MgCl$_2$, 2.5 U of Taq polymerase, 0.25 mM of each primer, 5x Taq buffer and 0.2 mM dNTP mix.

Table 6.2 Primers used to amplify virulence factors (*ica*, *agr* genes) of *S. aureus*.

Primer	Sequence (5'-3') Forward/Reverse	Target	Expected product size (bp)	Reference
icaAa F	TGG CTG TAT TAA GCG AAG TC	*icaA*	669	(Knobloch et al.,
icaAa R	CCT CTG TCT GGG CTT GAC C	specific		2002)
icaRa F	CCA AAT TTT TGC GAA AAG GA	*icaR*	186	In-house
icaRa R	TAC GCC TGA GGA ATT TTC TG			designed
IcaAb F	CGC ACT CAA TCA AGG CAT TA	*icaA*	240	In-house
IcaAb R	CCA GCA AGT GTC TGA CTT CG			designed
IcaDa F	ACC CAA CGC TAA AAT CAT CG	*icaD*	211	In-house
IcaDa R	GCG AAA ATG CCC ATA GTT TC			designed
IcaBa F	CAC ATA CCC ACG ATT TGC AT	*icaB*	240	In-house
IcaBa R	TCG GAG TGA CTG CTT TTT CC			designed
agr I R	GTC ACA AGT ACT ATA AGC TGC GAT	*agr*	440	(Shopsin et al., 2003)
agr II R	GTA TTA CTA ATT GAA AAG TGC CAT AGC	*agr*	572	(Shopsin et al., 2003)
agr III R	CTG TTG AAA AAG TCA ACT AAA AGC TC	*agr*	406	(Shopsin et al., 2003)
agr IV R	CGA TAA TGC CGT AT ACC CG	*agr*	588	(Shopsin et al., 2003)
pan-agr F	ATG CAC ATG GTG CAC ATG C	*agr*		(Shopsin et al., 2003)

Table 6.2 continued: Primers used to amplify virulence factors (*ica*, *agr* genes) and control genes of *S. aureus*.

Primer	Sequence (5'-3') Forward/Reverse	Target	Expected product size (bp)	Reference
pta F *pta* R	GTT AA ATC GTA TTA CCT GAA GG GAC CCT TTT GTT GAA AAG CTT AA	*pta* control	474	(Enright et al., 2000)
icaRb F *icaRb* R	TAC TGT CCT CAA TAA TCC CGA A GGT ACG ATG GTA CTA CAC TTG ATG	icaR	453	(Cafiso et al., 2004)
icaDb F *icaDb* R	CAG ACA GAG GCA ATA TCC AAC ACA AAC AAA CTC ATC CAT CCG	icaD	225	(Cafiso et al., 2004)
icaBb F *icaBb* R	ATG GCT TAA AGC ACA CGA CGC TAT CGG CAT CTG GTG TGA CAG	icaB	526	(Cafiso et al., 2004)
icaC F *icaC* R	ATC ATC GTG ACA CAC TTA CTA ACG CTC TCT TAA CAT CAT TCC GAC GCC	icaC	934	(Cafiso et al., 2004)

Table 6.3 Primers used to amplify virulence factors and control genes of *P. aeruginosa*.

Primer	Sequence (5'-3') Forward/Reverse	Target	Expected product size (bp)	Reference
cupA1 F	CAT GCG CAG TGG TAT TGG CCT TTG	*cupA1*	251	(Mah et al., 2003)
cupA1 R	GAA CAG GGT GGT GAA ATG CTC GTC			
ndvB F	CGA ACA GAT GCG CAC CGA CC	*ndvB*	650	(Mah et al., 2003)
ndvB R	CGC AGG TAG ATG GCC TGG TC			
tolA F	CAC GTT CTG ATC TTC GCC ATG CTG	*tolA*	620	(Mah et al., 2003)
tolA R	GTC GGT CGT ATC CGA AAG GAA CTC			
algD F	GCG ACC TGG ACC TGG GCT	alginate D	145	(Edwards and Saunders, 2001)
algD R	TCC TCG ATC ACG GGG ATC			
algU F	GCT CGC GGG CGT CGG C	alginate U	167	(Edwards and Saunders, 2001)
algU R	CGC AAA TCC TCG GGC AAC			
mucA F	GGA AAC TCT GTC CGC TGT GAT GGA	alginate *mucA*	141	(Edwards and Saunders, 2001)
mucA R	GGC TCG CGG TGC ATG ACG			
mucB F	GCT GCC GAC GCT TCC GAC TGG CT	alginate *mucB*	130	(Edwards and Saunders, 2001)
mucB R	CGC TGT CCA CGC GAT GCC			
rplU F	CGC AGT GAT TGT TAC CGG TG	*rplU* control	293	(Mah et al., 2003)
rplU R	AGG CCT GAA TGC CGG TGA TC			

Table 6.4 PCR conditions used for amplification of each of the primer pairs (virulence factors) in this study

Bacteria	P. aeruginosa			S. aureus			
Target gene	algD, algU, mucA, mucB	ndvB	tolA, cupA1, rplU	icaA	agr	icaA, icaB, icaD, icaR	pta
Denaturating step	97°C (1 min)	95°C (2 min)	95°C (2 min)	94°C (5 min)	94°C (3 min)	94°C (2 min)	95°C (5 min)
Cycles	35	35	35	35	30	30	30
Denaturing temp.	94°C (1 min)	95°C (30 sec)	95°C (30 sec)	94°C (30 sec)	94°C (1 min)	94°C (30 sec)	55°C (1 min)
Annealing temp.	50°C (1 min)	64°C (1 min)	56°C (1 min)	60°C (30 sec)	55°C (1 min)	55°C (30 sec)	72°C (1 min)
Extension temp.	72°C (1 min)	72°C (1 min)	72°C (1 min)	72°C (30 sec)	72°C (1 min)	72°C (1 min)	95°C 1 min)
Final extension	72°C (5 min)	72°C (10 min)	72°C (10 min)	72°C (1 min)	72°C (3 min)	72°C (5 min)	72°C (5 min)

6.3.6 Confirmation of PCR products by gel electrophoresis

PCR products (10 µl) were visualised following standard gel electrophoresis in a 0.75 % agarose gel (Sigma, UK) with ethidium bromide staining (10 µg µl^{-1}). Gel images were obtained using a Gel-Doc 2000 (Bio-Rad, Hemel-Hempstead, UK) and the average band densities for each PCR product were measured using the Discovery Series™ Quantity One$^®$ (Bio-Rad) software. Estimation of semi-quantitative values involved defining lanes and bands on the gel image following the Quantity One user guide. After defining lanes, a lane-based background subtraction was performed with bands identified using the Detect Bands command and then the values of a standard (a quantitative MassRuler DNA Ladder; low range, Fermentas, York, UK) being entered. The quantity of PCR product in the experimental bands was determined using this standard.

To obtain additional confirmation of the PCR product size and sequence, PCR products were cleaned using the QIAquick PCR Purification Kit (Qiagen) and sequenced as described previously in Chapter 4 (section 4.3.2).

6.3.7 Microarray study for S. aureus gene expression

The microarray study was done in collaboration with Dr Nick Saunders at the Health Protection Agency in Colindale, London, UK. The keywords used for virulence searches in the original design of this array were toxin, leukocidin, coagulase, adhesion, protease, nuclease, lipoprotein, lipase, capsular polysaccharide, binding regulatory peptidase, staphylococcal cassette chromosome (SCC) and iron (Saunders et al., 2004a). This original array (Saunders et al., 2004a) has since been adapted to accommodate an additional 337 S. aureus virulence associated genes whose sequences have been obtained. A further 30 E. coli gene sequences were also added to the array as negative controls. Therefore the revised array comprised of probes for 750 genes in total (Appendix III). The microarray described here was originally used to provide an insight into the pathogenicity of the S. aureus strains. In this present study this microarray was used in the comparative

analysis of virulence gene expression between S. aureus biofilms and S. aureus planktonic cultures (exponential and stationary growth phases). RNA was extracted from the S. aureus biofilms and planktonic growth as described in section 6.3.3.

6.3.7.1 CDFF biofilm samples for microarray study

Staphylococcus aureus biofilms were prepared using the CDFF as described previously (Chapter 4, section 4.3.12). The only difference was that the biofilms were recovered only after 7 d incubation period instead of every 24 h for 7 d.

6.3.7.2 Preparation of planktonic growth for microarray analysis

In addition to the biofilm study, gene expression for *S. aureus'* (D76) was also assessed for planktonic cultures. The planktonic samples were harvested at various points along the growth curve, namely in exponential phase (3 h) and in stationary phase (10 h). Overnight cultures were used to set up planktonic exponential and planktonic stationary phase and diluted 1:500. An aliquot (1 ml) was added to wells of a 24 microtitre well plate and placed in the plate reader (Fluostar Optima, BMG Labtech, UK). The absorbance was measured at 600 nm for 48h (at 37°C, with agitation).

6.3.7.3 DNA extraction for comparative genome hybridisation

DNA was extracted from an overnight culture in BHI broth of each pure bacterial isolate using a Puregene® DNA isolation kit (Gentra Systems, Minneapolis, USA) by following the "Gram-positive extraction" protocol (http://www.gentra.com/technical assistance/protocols.asp).

6.3.7.3.1 Indirect labelling of DNA for comparative genome hybridisation

DNA (1 µg) was mixed with 2 µl of random nonamers (Invitrogen, Paisley, UK) and the volume was adjusted with water to 16 µl in total. The mixture was heated to 95°C for 30 s in a PCR thermal cycler then cooled on ice and 4 µl of the following master mix added: 2 µl exo-Klenow buffer (10x; New England BioLabs, Ipswich, UK) 1 µl dNTP mix (20x 1:1 aa-dUTP mix;

Invitrogen) 1 µl exo-Klenow (5 units). The preparation was incubated at room temperature for 30 min followed by subsequent incubation at 37°C for 60 min. For the cleanup step, the 'Minelute Reaction Cleanup' (Qiagen) kit was used and the manufacturer's instructions followed before proceeding to hybridisation (see section 6.3.7.7).

6.3.7.4 RNA extraction

RNA from *S. aureus* biofilms and planktonic growth was extracted using the Qiagen RNeasy Kit protocol and the maufacturer's recommended protocol (6.3.3).

6.3.7.4.1 Aminoallyl cDNA synthesis protocol for total RNA extraction

For all steps, the ChipShot indirect labelling kit was used (Promega, Southampton, UK).

For each indirect synthesis reaction, the following mix was prepared, 1 µg of total RNA, 1 µl of random primers (3 µg µl^{-1}), and nuclease free water to a volume of 20 µl. The RNA/primer solution was incubated at 70°C for 10 min and then placed on ice. Twenty µl of extension mix containing 8 µl ChipShot Reverse Transcriptase (5x) reaction buffer, 4.8 µl magnesium chloride (25 mM), 4 µl aminoallyl-dNTP mix and 3.2 µl ChipShot Reverse Transcriptase was added to the RNA/primer solution, vortexed, spun briefly and incubated at 22-25°C for 10 min, followed by incubation at 42°C for 2 h. To each cDNA synthesis reaction 1 µl RNase H and 0.35 µl RNase solution was added, mixed gently and incubated at 37°C in a thermal cycler for 15 min.

6. 3.7.4.2 Aminoallyl cDNA purification

To 40 µl of the synthesised cDNA, 4 µl sodium acetate (3M; pH 5.2) and 225 µl of binding solution (Promega Kit, proprietary contents) was added and vortexed gently for 5-10 min. The solution was applied to a ChipShot membrane on a spin column, which was placed into a collection tube. The column was incubated for 5 min at RT, then spun down at 10,000 *g* for 1 min and the flow-through discarded. The column was washed 3 times with 500 µl

80% ethanol, centrifuged at 10,000 *g* for 1 min. To remove traces of ethanol, the column was centrifuged again at 10,000 *g* for 1 min. After the washing steps, the column was placed in a clean collection tube and purified cDNA eluted with 65 μl of 100 mM sodium bicarbonate (pH 9.0). The column was incubated at RT for 1 min and centrifuged at 10,000 *g* for 1 min. Sixty μl of cDNA was recovered after centrifugation. The cDNA was then labelled and purified for microarray analysis as described below.

6. 3.7.4.3 Conjugation of CyDye NHS Ester to Aminoallyl cDNA

To prevent photo bleaching, the reactive dyes were protected from light at all times. To each dry aliquot of CyDye NHS ester (Amersham, UK) 5 μl of dimethyl sulphoxide (DMSO) was added and mixed gently to ensure that the CyDye NHS ester was thoroughly resuspended. Two μl of CyDye solution was added to each eluted 60 μl aminoallyl cDNA, vortexed gently and incubated at 23°C in a thermal cycler for 1 h. Planktonic samples were stained with CY5 and biofilm samples were stained with CY3 dyes.

6. 3.7.4.4 Purifying CyDye labelled cDNA

To the 60 μl of the synthesised and labelled cDNA, 8 μl of sodium acetate (3M, pH 5.2) and 440 μl of binding solution (ChipShot indirect labelling kit, Promega) was added. The mixture was vortexed gently for 5-10 s and applied to a ChipShot membrane column (ChipShot indirect labelling kit, Promega) which was placed into a collection tube. The column was incubated at room temperature for 5 min and then centrifuged at 10,000 *g* for 1 min. The flow-through was discarded and the column was washed (x3) with 500 μl 80% v/v ethanol, centrifuged again at 10,000 *g* for 1 min and followed by a final centrifuge step to remove traces of ethanol. The column was placed in a clean collection tube and 30 μl elution buffer (Promega Kit, proprietary contents) was added, incubated at RT for 1 min and centrifuged at 10,000 *g* for 1 min. The elution step was repeated with another 30 μl elution buffer as described above. Eluted cDNA was stored in a light proof container at 4°C until required for the microarray study.

6.3.7.5 Printing

Slides were printed using a Genetix Qarray Mini platform. The oligonucleotides probes were printed in replicates of four, arranged in a random pattern using Telechem Arraylt Stealth pins (Genetix Limited, Hampshire, UK).

6.3.7.6 Slide processing

Printed microarray slides were placed in a chamber at 42°C with 50-75% relative humidity provided by a saturated solution of NaCl for 16-18 h. Excess spotting solution was washed off with 0.2% SDS by agitation in a slide washer for 2 min at 20-25°C. The slides were then washed, with shaking, three times in sterile distilled water for 1 min each wash. The microarrays were then incubated for 20 min at 50°C in distilled water, dried by centrifugation (10,000 g for 5 min) and stored in the dark at room temperature (Saunders et al., 2004a).

6.3.7.7 Hybridisation

Hybridisation was performed using the Lucidea SlidePro Hybridisation Instrument (GE Healthcare, Chalfont St Giles, UK). The software was programmed to perform a series of washes to the hybridisation chambers and their respective tubing on dummy slides before inserting the arrays and initiating the pre-hybridisation process. The hybridisation mix was then injected and hybridisation allowed to take place for up to 7 h. Any unbound probe mix was washed away with isopropanol and the arrays were air dried ready for analysis (Saunders et al., 2004a).

6.3.7.8 Scanning and Data analysis

The arrays were scanned using the Gentix aQuire Scanner. Fluorescent signals for the differentially labelled samples were detected at wavelengths of 532 nm for Cy3 and 639 nm for Cy5. The images were analysed using QScan software (Genetix). The software enables superimposing of two images, subtraction of background and calculation of the ratio of the two colour signals for comparative analysis.

6.4 Results

For RNA extraction, two different methods were employed, the RiboPure Bacteria Kit and the Qiagen RNeasy Kit. Results obtained with the Qiagen RNeasy Kit were more consistent and the RNA yield was generally higher than obtained with the RiboPure Bacteria Kit. Whenever possible the Qiagen RNeasy Kit was used to generate RNA.

6.4.1 RT-PCR

The housekeeping genes *rplU* and *pta* were successfully amplified from *P. aeruginosa* and *S. aureus,* respectively, indicating that gene expression could be routinely and consistently detected (Fig. 6.4-6.7).

6.4.2 Expression of genes encoding outer membrane surface proteins of *P. aeruginosa*

Messenger RNA from the *tolA* gene was detected from biofilm samples after day 3, whilst *cupA1* was expressed from day 1 to day 6 and not expressed on day 7. In contrast, no expression of *ndvB* was observed in any of the biofilm samples over the 7 d of the experiment.

Inconsistent expression of the outer membrane proteins was observed in the planktonic samples. *TolA* was only expressed on day 1 and day 3 and was not expressed on any of days 2, 4, 5, 6 and 7. The *cupA1* gene was only expressed on day 5, day 6 and day 7. Again no expression of *ndvB* was observed (Table 6.5).

6.4.3 Expression of alginate genes by *P. aeruginosa*

RT-PCR was performed on biofilm and planktonic samples from day 1 to day 7 of incubation. The target genes *algD, mucA* and *mucB* were expressed in all biofilm samples throughout the 7 d analysis. *AlgU* expression in biofilm samples was only detected after day 3. In comparison, analysis of planktonic cultures also demonstrated expression of these target genes, although for each gene, expression was inconsistent over the 7 d study period (Table 6.5; Fig. 6.4-6.5).

Table 6.5 Detection of gene families associated with *P. aeruginosa* biofilm formation.

	Samples	*algD*	*algU*	*mucA*	*mucB*	*tolA1*	*cupA1*	*ndvB*
Day 1	Biofilm	+	-	+	+	+	+	-
	Planktonic	+	-	+	+	+	-	-
Day 2	Biofilm	+	-	+	+	-	+	-
	Planktonic	-	-	-	-	-	-	-
Day 3	Biofilm	+	+	+	+	+	+	-
	Planktonic	+	+	+	+	+	-	-
Day 4	Biofilm	+	+	+	+	+	+	-
	Planktonic	+	+	+	+	-	-	-
Day 5	Biofilm	+	+	+	+	+	+	-
	Planktonic	-	-	-	-	-	+	-
Day 6	Biofilm	+	+	+	+	+	+	-
	Planktonic	+	+	+	+	-	+	-
Day 7	Biofilm	+	+	+	+	+	+	-
	Planktonic	+	+	+	+	-	+	-

For all RT-PCRs control gene expression (*rplU*) was routinely detected. +, detected; -, not detected; highlighted in pink are samples where differences in gene expression from planktonic mode of growth were seen.
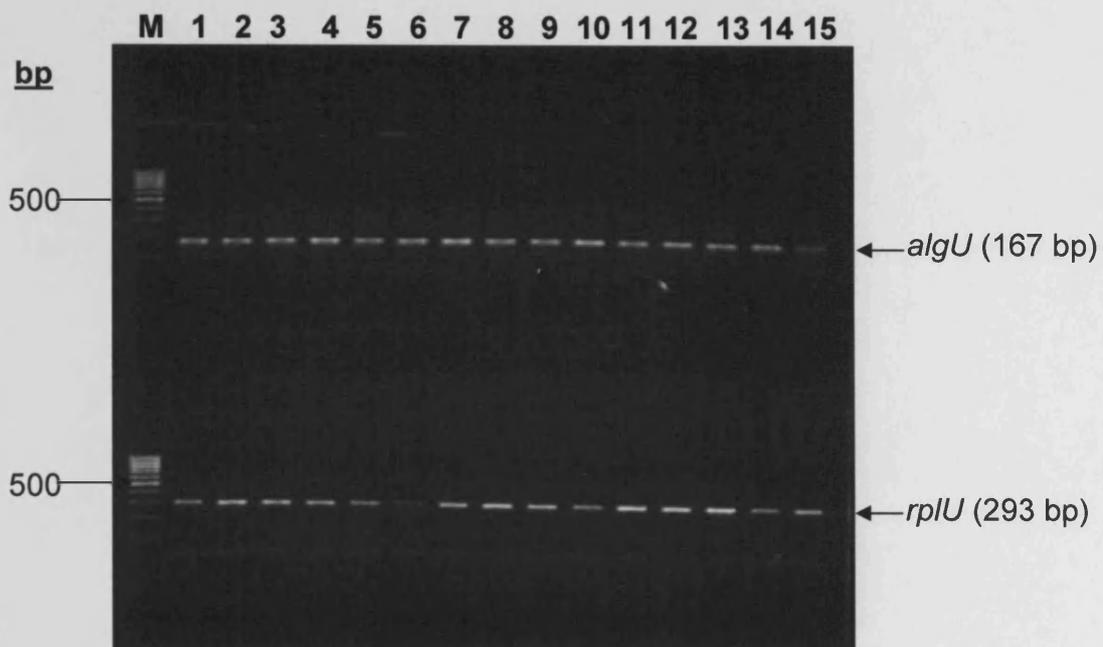
Fig. 6.4 Agarose gel showing typical RT-PCR products during RHE infection of *P. aeruginosa* housekeeping (*rplU*) and virulence (*algU*) genes. Lanes: M, low range MassRuler DNA ladder (Fermentas, UK); 1-3, biofilm samples day 3; 4, planktonic sample day 3; 5-6, biofilm samples day 4; 7, planktonic sample day 4; 8-9, biofilm samples day 5; 10, planktonic sample day 5; 11-13, biofilm samples day 6, 14, planktonic sample day 6; 15, biofilm sample day 7
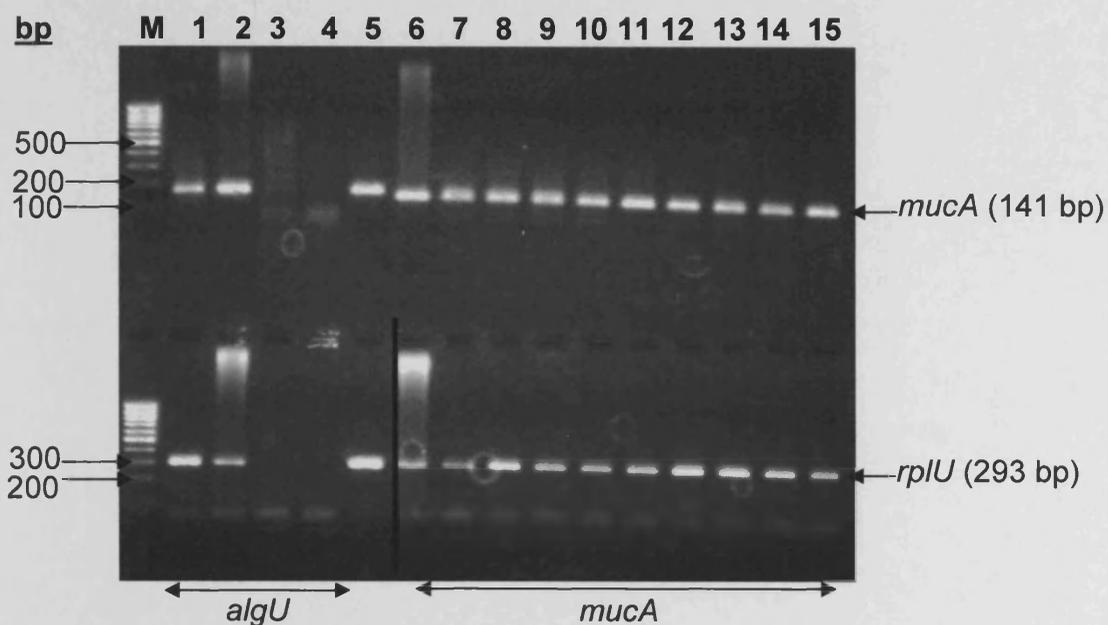
Fig. 6.5 Agarose gel showing typical RT-PCR products during RHE infection of *P. aeruginosa* housekeeping (*rplU*) and virulence (*algU, mucA*) genes.
Lanes: M, low range MassRuler DNA ladder (Fermentas, UK); 1, biofilm sample day 7; 2, planktonic sample day 7; 3, H$_2$O; 4, pure RNA; 5, genomic DNA; 6-8, biofilm samples day 1; 9, planktonic sample day 1; 10, biofilm sample day 2; 11-13, biofilm samples day 3, 14, planktonic sample day 3; 15, biofilm sample day 4

6.4.4 Expression of accessory gene regulator (agr) in S. aureus

All biofilm and planktonic samples expressed the agr gene for all 7 days (Table 6.6).

6.4.5 Expression of the intercellular adhesion (ica) genes in S. aureus

RT-PCR using three sets of primers was undertaken. RT-PCR products were generated using icaAa primers (Knobloch et al., 2002) targeting the entire ica locus of S. aureus, in biofilm samples from day 3 to day 7 (Fig. 6.6-6.7). However, no expression of the icaAa gene was evident in any of the planktonic samples. In contrast, RT-PCR products were not detected using alternative primers (icaBb, icaCb, icaDb and icaRb) specific to the ica operon of S. epidermidis (Cafiso et al., 2004).

Using a third set of 'in-house' prepared primers, S. aureus ica genes were found to generate RT-PCR products for all biofilm samples from 2 d onwards, with icaDa expressed only in biofilm samples from day 3. Using these primers on planktonic samples, icaAb expression was evident on days 4-6, icaBa expression occurred on days 6 and 7 and icaDa and icaRa were expressed on days 3-6 (Table 6.6).

6.4.6 Semi-quantitative comparison of gene expression using RT-PCR

Semi-quantitative analysis using RT-PCR for all the target genes indicated varying quantities of mRNA for the expressing strains.

The majority of the biofilm specific genes in P. aeruginosa were upregulated in the biofilm state (27 out of 42). However, algU, mucA and mucB appeared to be down regulated in the biofilm state from day 5 onwards (Table 6.7). Between the two growth phases, there appeared to be greater up regulation of ica gene expression in the biofilm state, with only three samples demonstrating an apparent down regulation compared to the planktonic state. The majority of agr genes were upregulated in the biofilm state, 6 out of 7 (Table 6.8).

Table 6.6 Detection of gene families associated with *S. aureus* biofilm formation.

	Samples	*icaAa*	*icaAb*	*icaBa*	*icaDa*	*icaRa*	*agr*
Day 1	Biofilm	-	-	-	-	-	+
	Planktonic	-	-	-	-	-	+
Day 2	Biofilm	-	+	+	-	+	+
	Planktonic	-	(pink)	(pink)	-	(pink)	+
Day 3	Biofilm	(pink) +	+	+	+	+	+
	Planktonic	-	(pink)	(pink)	+	+	+
Day 4	Biofilm	(pink) +	+	+	+	+	+
	Planktonic	-	+	(pink)	+	+	+
Day 5	Biofilm	(pink) +	+	+	+	+	+
	Planktonic	-	+	(pink)	+	+	+
Day 6	Biofilm	(pink) +	+	+	+	+	+
	Planktonic	-	+	+	+	+	+
Day 7	Biofilm	(pink) +	+	+	+	+	+
	Planktonic	-	(pink)	+	(pink)	(pink)	+

For all RT-PCRs control gene expression (*pta*) was routinely detected. +, detected; -, not detected; highlighted in pink are samples where differences in gene expression from planktonic mode of growth were seen.

Fig. 6.6 Agarose gel showing typical RT-PCR products during RHE infection of *S.aureus* housekeeping (pta) and virulence (icaA) genes.
Lanes: M, low range MassRuler DNA ladder (Fermentas, UK); 1-3, biofilm samples day 3; 4-6, biofilm samples day 4; 7-9, biofilm samples day 5; 10, biofilm sample day 6; 11-12, biofilm samples day 7, 13, H₂O; 14, pure RNA; 15, genomic DNA
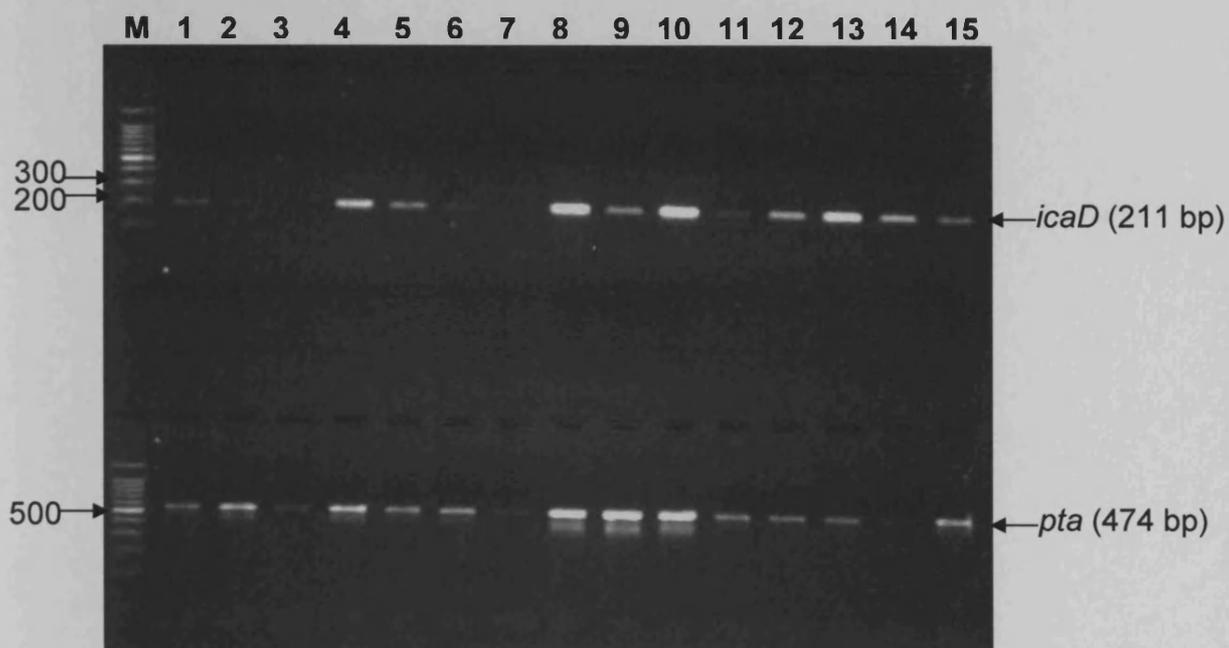
Fig. 6.7 Agarose gel showing typical RT-PCR products during RHE infection of *S. aureus* housekeeping (*pta*) and virulence (*icaD*) genes.
Lanes: M, low range MassRuler DNA ladder (Fermentas, UK); 1-2, biofilm samples day 3; 3, planktonic sample day 3; 4-6, biofilm samples day 4; 7, planktonic sample day 4; 8-10, biofilm samples day 5; 11, planktonic sample day 5; 12-13, biofilm samples day 6; 14, planktonic sample day 6; 15, biofilm sample day 7.

Table 6.7 Detection of gene families associated with pathogenesis of *P. aeruginosa* showing semi-quantification of RT-PCR products produced.

	Samples	*algD*	*algU*	*mucA*	*mucB*	*tolA1*	*cupA1*	*ndvB*
Day 1	Biofilm	106	-	119	139	-	133	-
	Planktonic	74	-	110	86	99	-	-
Day 2	Biofilm	79	-	111	92	-	202	-
	Planktonic	-	-	-	-	-	-	-
Day 3	Biofilm	80	94	110	89	64	192	-
	Planktonic	61	96	95	85	53	-	-
Day 4	Biofilm	80	147	145	116	155	179	-
	Planktonic	62	92	92	75	-	-	-
Day 5	Biofilm	71	83	92	77	102	214	-
	Planktonic	70	106	108	98	-	-	-
Day 6	Biofilm	68	84	97	75	54	168	-
	Planktonic	67	96	112	106	-	134	-
Day 7	Biofilm	76	86	85	87	76	-	-
	Planktonic	69	125	141	101	-	164	-

For all RT-PCRs control gene expression was routinely detected. The numbers represent the relative quantity (as a percentage) of PCR amplicon compared with the constitutively expressed gene product *rplU*. Highlighted in pink indicates samples where up-regulation in gene expression occurred in the biofilm state. Highlighted in yellow indicates samples where down-regulation in gene expression occurred in the biofilm state.

Table 6.8   Detection of gene families associated with *S.aureus* biofilms showing semi-quantification of RT-PCR products produced.

	Samples	*icaAa*	*icaAb*	*icaBa*	*icaDa*	*icaRa*	*agr*
Day 1	Biofilm	-	-	-	-	68	125
	Planktonic	-	-	-	-	-	80
Day 2	Biofilm	-	47	43	-	105	157
	Planktonic	-	-	-	-	-	80
Day 3	Biofilm	75	88	81	170	107	85
	Planktonic	-	-	-	105	55	78
Day 4	Biofilm	94	88	97	134	146	74
	Planktonic	-	90	-	102	100	113
Day 5	Biofilm	80	98	103	243	58	190
	Planktonic	-	75	-	117	72	112
Day 6	Biofilm	100	218	279	76	87	121
	Planktonic	-	144	169	62	103	95
Day 7	Biofilm	96	60	82	127	57	109
	Planktonic	-	-	61	-	-	92

For all RT-PCRs control gene expression was routinely detected. The numbers represent the relative quantity (as a percentage) of the PCR amplicon compared with the constitutively expressed gene product *pta*. Highlighted in pink indicates samples where up-regulation in gene expression occurred in the biofilm state. Highlighted in yellow indicates where down-regulation in gene expression occurred in the biofilm state.

6.4.7 Microarray study for *S. aureus* gene expression

6.4.7.1 Comparative genome hybridisation

In comparative genome hybridisation experiments, two genomic DNA samples one from wound isolate *S. aureus* D76 and the other from *S. aureus* MW2 (Baba et al., 2002) were simultaneously hybridised to the microarray and detected using different fluorochromes. In this study the presence and absence of the genes on the array for the genome of the test strains was confirmed. Hybridisation with the *S. aureus* D76 strain was found to occur with signals evident for 486 genes and with *S. aureus* MW2, 550 genes were present. The majority of these genes present in *S. aureus* D76 were hypothetical proteins related to the ATP-binding protein, exotoxins and capsular polysaccharide synthesis enzymes. The intercellular adhesion proteins icaA, icaB, icaD and the regulator icaR were also present as well as the staphylococcal accessory regulator A (*sarA*), which is implicated in biofilm formation (Appendix IV). Virulence factors such as proteases, zinc metalloproteinase, thermonuclease, adhesins, alpha-, beta-, gamma-haemolysin were also present. It seems that the D76 strain carries drug resistance in various forms including a hypothetical protein, which is similar to tetracycline resistance proteins as well as multidrug resistance proteins. Interestingly, phage related proteins, such as staphylococcal phage phi11, phage integrase family, hypothetical protein which is similar to *Enterococcus faecalis* plasmid pPD1 and a lytic regulatory protein truncated with Tn554 were also present in the *S. aureus* D76 strain which could promote gene transfer (Appendix IV).

6.4.7.2 *Staphylococcus aureus* gene expression as determined using the microarray

Comparative gene expression between *S. aureus* D76 biofilm samples after 7 d of incubation in the CDFF was compared against both stationary and exponential phase planktonic growth. Microarray analysis revealed that both growth phases exhibited common expression of the majority of genes, although some differences were found. Figure 6.8 (biofilm against

exponential phase) and Fig. 6.9 (biofilm against stationary phase) illustrate the up-regulation of genes between the two growth phases. The genes which are distributed around the line at 1 are non changeable. The genes above this line are up-regulated in the biofilm phase and the genes beneath the line are up-regulated in the planktonic phase (Appendix III, for detailed gene names).

Figure 6.10 shows expression of genes of interest in biofilm phase against exponential planktonic phase. For example, the regulatory genes such as the accessory gene regulator (agrC1 – group1) and the sarA were more highly expressed in the biofilm state than in the exponential planktonic phase as well as few hypothetical proteins such as SA1999 (hypothetical protein, similar to the regulatory protein SIR2 family), MW0596 (hypothetical protein, similar to iron dependent repressor) and MW2499 (hypothetical protein, similar to short chain oxireductase). However, several hypothetical genes seemed to be up-regulated in the exponential planktonic growth phase compared to biofilm growth mode such as MW0595 (hypothetical protein, similar to the ABC transporter ATP-binding protein), MW0339 (hypothetical protein, similar to GTP-binding protein), MW0283 (hypothetical protein, similar to branched chain amino acid uptake carrier), MW0384 (Exotoxin 18, Genomic island nu Sa alpha 2) and MW2217 (secretory antigen precursor).

Figure 6.11 shows expression of genes of interest in the biofilm phase against the stationary planktonic phase. Genes which are up-regulated in the biofilm phase compared to the stationary planktonic phase included MW1834 (hypothetical protein, similar to ferritin), MW0396 (hypothetical protein), MW1041 (hypothetical protein, similar to fibrinogen binding protein), SA0857 (hypothetical protein, similar to negative regulator of genetic competence mecA), SAV0151 Cap8c (capsular polysaccharide synthesis enzyme Cap8c), SA1174 (SOS regulatory LexA protein) and MW2129 hysA (hyaluronate lysate precursor).

In comparison hypothetical proteins such as MW0757, MW1744, MW0579 (similar to esterase/lipase), MW1508 (similar to ABC transporter ATP-binding protein) and SA2272 were up-regulated in the stationary planktonic growth phase.
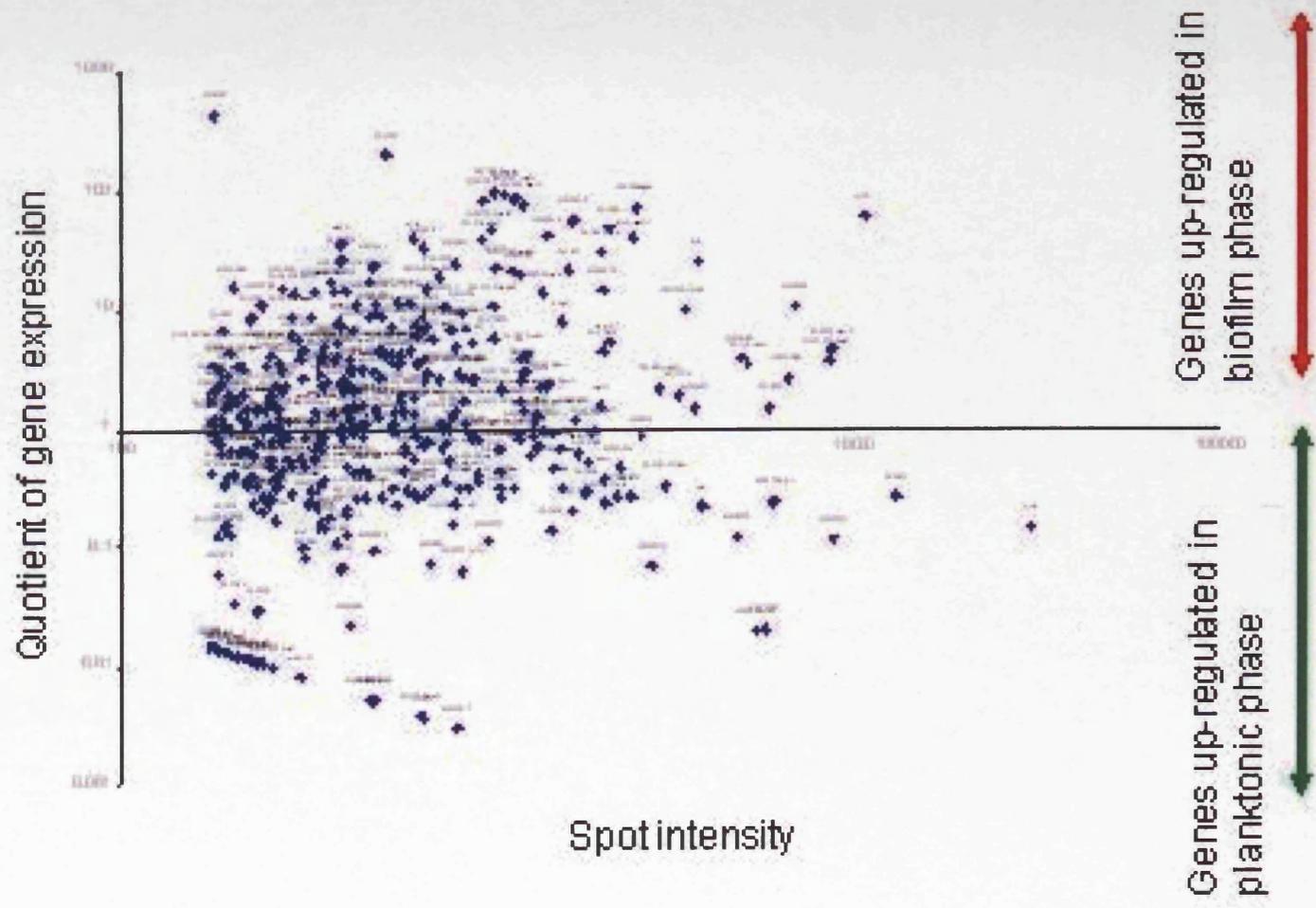
Fig. 6.8 Expression of genes in biofilm against exponential planktonic phase of *S. aureus* D76
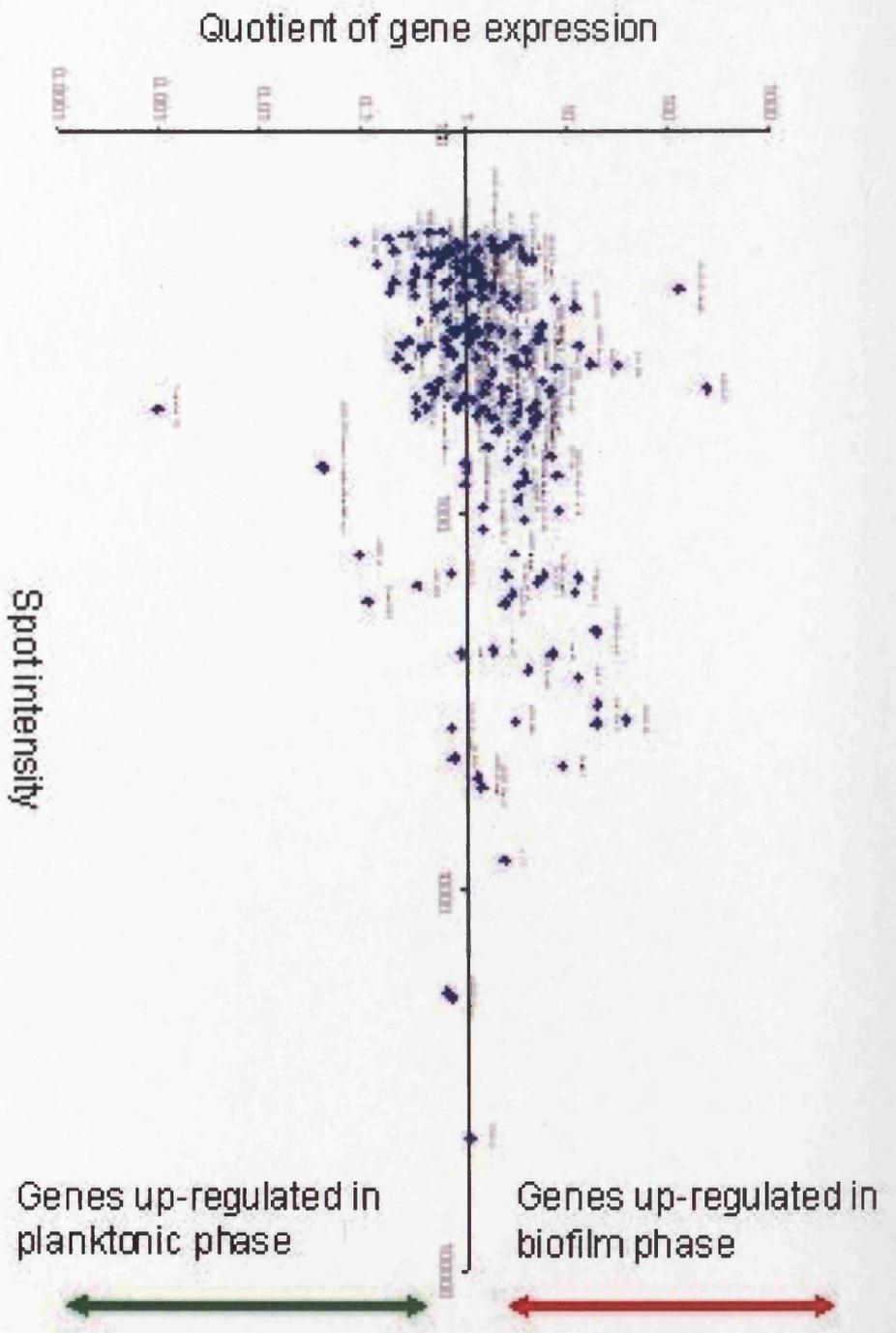
Fig. 6.9 Expression of genes in biofilms against stationary plankonic phase of S. *aureus* D76
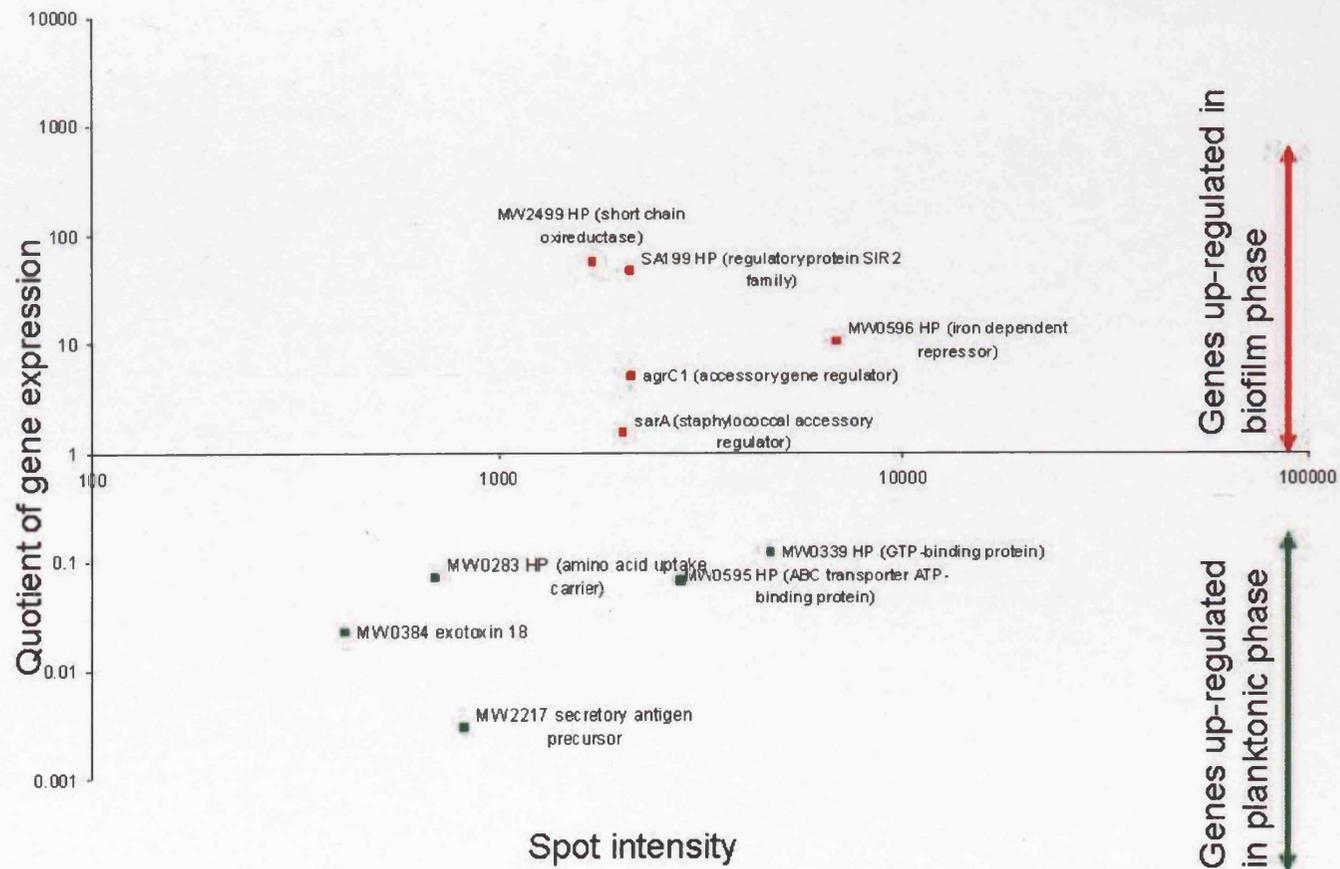
Fig. 6.10 Expression of genes of interest in biofilm against exponential growth phase of *S. aureus* D76.
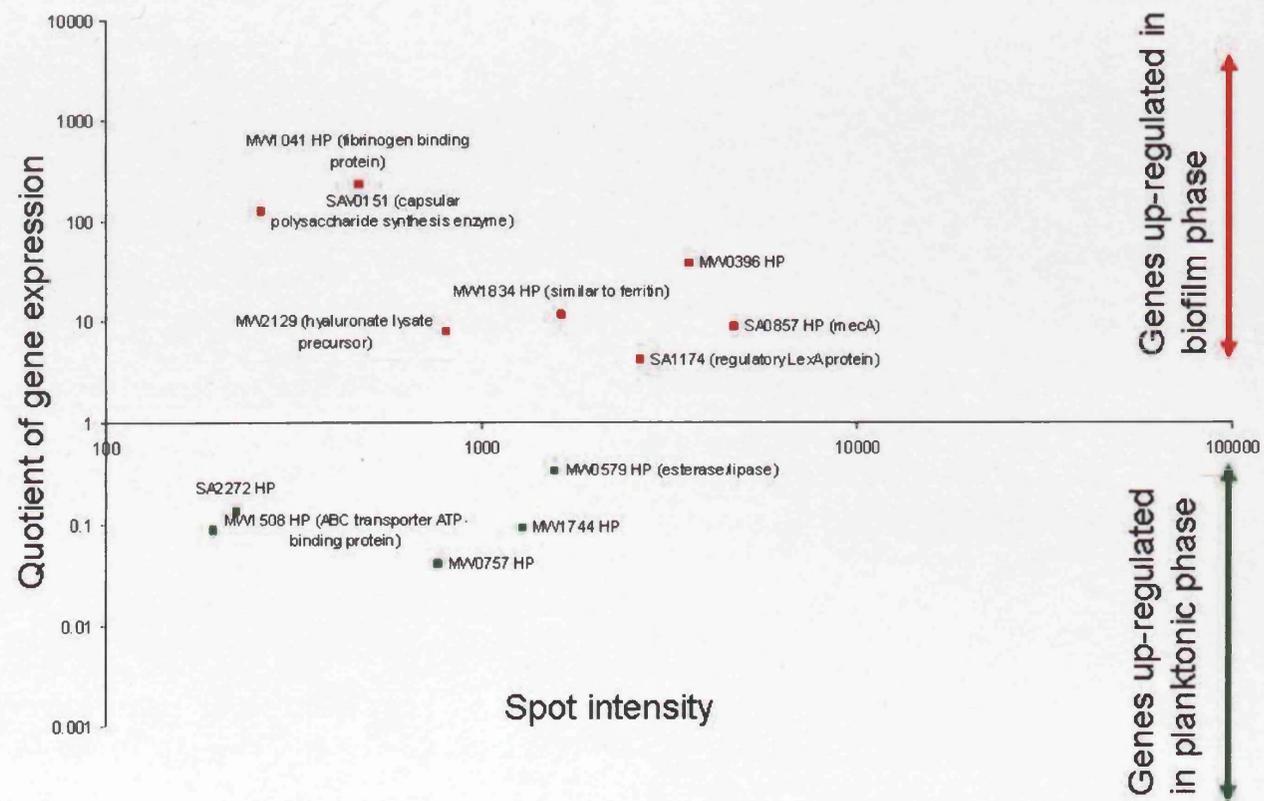
Fig. 6.11 Expression of genes of interest in biofilm against stationaryl growth phase of *S. aureus* D76.

6.5 Discussion

In the past, research has largely focused on the *in vitro* study of organisms grown in liquid media. However, in recent years it has been recognized that in their natural settings bacterial cells are most often found in close association with surfaces and interfaces, in the form of aggregates referred to as biofilms (Branda et al., 2005). It has been suggested that adherent and biofilm bacteria behave differently (Literature Review, section 1.1.1, Table 1.1) and hence may express a different pattern of genes compared with their planktonic counterparts (Becker et al., 2001; Beloin and Ghigo, 2005). Whiteley *et al.* (2001) showed that gene expression in *P. aeruginosa* biofilm cells was remarkably similar to their planktonic counterparts under similar environment conditions but that there were a small number of significant differences. Such differences in gene expression could perhaps explain important phenotypic traits of biofilms including the high antibiotic resistance of biofilm cells, or even aid in the development of rapid screens for agents that block biofilm maintenance (Whiteley et al., 2001).

The present study focused on comparing specific gene expression for both *P. aeruginosa* and *S. aureus* when cultured in biofilms and in planktonic states. The significance of this work was that the detection, albeit *in vitro*, of an up-regulated biofilm gene product could be important if also manifested in a chronic wound biofilm state. Differential expression between the two growth states could theoretically provide evidence of virulence mechanisms or strategies promoting the survival of the bacteria in biofilms. This knowledge could in the future be exploited in the development of management and treatment regimes (Beloin and Ghigo, 2005).

The selected bacterial species were studied as these organisms have previously been shown to be frequently encountered in chronic wounds (Davies et al., 2007; Davies et al., 2004) and in chronic infections (Parsek and Singh, 2003). In addition, previous chapters reported in this thesis (Chapter 4 and Chapter 5) have shown that these organisms predominated over other bacterial species in biofilm models.

The specific aims of this Chapter were to examine the expression of previously proposed 'biofilm specific genes' and to compare their expression in planktonic and biofilm growth phases. It addition exploratory studies were also performed using a microarray analysis.

Bacteria growing in biofilms possess characteristics distinct from their planktonic counterparts, e.g. biofilm bacteria are more resistant to antimicrobial treatments (Whiteley et al., 2001). The outer membrane surface proteins are of particular interest in P. aeruginosa due to their involvement in the transport of antibiotics (Stover et al., 2000). Mah et al. (2003) proposed a mechanism of biofilm-specific antibiotic resistance wherein periplasmic glucans bind to, and sequester antibiotics. It has been suggested that the gene, ndvB is required for the synthesis of periplasmic glucans (Mah et al., 2003). However, in this study ndvB was not found to be expressed in P. aeruginosa biofilms. This contrasts with the results of Mah et al. (2003) who predicted and showed that ndvB was exclusively expressed in biofilm grown cells using a microtitre plate assay for biofilm formation. Although Mah et al. (2003) data supports an involvement for ndvB in biofilm antibiotic resistance, it may be that some strains of P. aeruginosa have compensatory mechanisms for ndvB mutations or because of strain variations.

Previous studies have examined how alginate overproduction influences P. aeruginosa biofilm development and function, using a non-mucoid P. aeruginosa wild type and a mucoid alginate overproducing mutant strain (Hentzer et al., 2001). These studies showed that in cystic fibrosis (CF) patients, conversion to the mucoid phenotype resulted in an altered biofilm structure and increased antimicrobial resistance. RT-PCR of alginate gene expression in P. aeruginosa in the present study did not show a direct correlation with biofilm formation. The majority of planktonic samples were also found to express algD, algU, mucA and mucB. These data might suggest that some bacteria in the broth cultures had biofilm phenotypes or that expression of these genes is not an exclusive property of biofilms. In

fact Lazazzera *et al.* (2005) suggests that subsets of genes expressed in biofilms are also expressed under different planktonic conditions.

The expression of *S. aureus* virulence factors is controlled by the quorum dependent accessory gene regulator (*agr*) and can be classified into 4 (I-IV) specific *agr* groups (Shopsin et al., 2003; Yarwood et al., 2002). Group I and II have been linked with enterotoxin mediated and invasive disease, while groups III and IV have been associated with non-invasive and exfoliating-producing disease (Jarraud et al., 2002). The *S. aureus* wound isolate D76 used in this study expressed *agr* I in all biofilm and planktonic samples. It may therefore be possible that this *S. aureus* strain plays a role in impaired healing by the production of this toxin which then attacks the patient epithelial cells.

Studies have shown that PIA, a homoglycan consisting of β-1,6-linked N-acetylglucosamine which is encoded by the *ica* operon, is required for biofilm formation (Conlon et al., 2002b; Mack et al., 1996). Mack *et al* (1999; 1996) suggested that PIA mediates intercellular adhesion and that bacteria have no direct contact to the surface following the accumulative phase but remain in the biofilm via mechanisms mediating cell-to-cell adhesion (Rohde et al., 2005). *Staphylococcus epidermidis*, a commensal of human skin and mucous membranes, is also a predominant cause of foreign-body-associated infections. PIA is a significant virulence factor and essential for *S. epidermidis* and *S. aureus* cell accumulation (Knobloch et al., 2004). However, investigations into the relationship between the *ica* genotype and biofilm formation of *S. aureus* has in the past led to contradictory results by different investigators (Knobloch et al., 2002). Cramton *et al.* (1999) characterised *S. aureus* isolates that were *ica* positive and showed that deletion of the *ica* genes eliminated the ability to produce polysaccharide intercellular adhesion (PIA) and form a biofilm *in vitro*. In contrast, Yazdani *et al.* (2006) evaluated the biofilm forming capacity and the presence of *icaAD* (associated with slime production) among *S. aureus* isolated from wound infections. All fifty strains identified, were found to carry the *icaAD* genes,

with only 26 of the strains found to produce a biofilm *in vitro*. Hence, this study indicated that whilst there was a high prevalence of the *icaAD* genes amongst the *S. aureus* isolates, their presence was not always associated with biofilm formation *in vitro* (Yazdani et al., 2006). Despite the importance of *ica* genes, not much is known about the regulation of the *ica* locus itself (Jefferson et al., 2003). Numerous recent publications report on factors influencing PIA or biofilm production *in vitro*. Such factors include the level of glucose and other sugars in the culture medium, extent of osmolarity and anaerobiosis, low levels of ethanol, and iron restriction (Cramton et al., 2001; Fluckiger et al., 2005; Jefferson et al., 2003; Rachid et al., 2000a; Rachid et al., 2000b).

The observation that biofilms are not wholly dependent upon expression of the *ica* operon is also supported by the findings of Muller *et al.* (1993) who found that a few *S. epidermidis* strains were able to form a biofilm with no apparent production of polysaccharide (Cramton et al., 2001). Importantly, the majority of clinical *S. aureus* strains contain the *ica* operon (Arciola et al., 2001; Cramton et al., 1999; Fitzpatrick et al., 2005; Rohde et al., 2005), and expression of the *ica* operon and biofilm production is controlled under *in vitro* conditions (Cramton et al., 2001; Fitzpatrick et al., 2005; O'Gara, 2007).

In the present investigation, RT-PCR detection of expressed genes of the *S. aureus ica* operon used primers previously validated by Knobloch *et al.* (2002), primers designed by Cafiso *et al.* (2004) and also 'in-house' designed primers. The failure of the Cafiso *et al.* (2004) primers to yield PCR products with any of the specimens likely relates to sequence variations between *S. epidermidis* and *S. aureus*, as the former species was used as the basis of the primer design by Cafiso *et al.* (2004).

It was evident from the results (Table 6.6) that *ica* genes were expressed in both biofilm and planktonic samples. As mentioned previously for *P. aeruginosa* strains, a biofilm occurrence within the planktonic samples could be envisaged due to the extended incubation time of the culture (7 days)

even though the broth was replenished at 24 h intervals. A possible improvement could be to generate continuous planktonic growth within a dedicated fermenter (*e.g.* a chemostat).

After 48 h, all biofilm samples were found to express *icaA, icaB* and *icaR* whilst *icaD* was expressed only after 72h. This data suggests that using the CDFF for biofilm formation, 48 h incubation was necessary to get an established biofilm of *S. aureus*.

Semi-quantification of the RT-PCR products indicated that for the majority of targeted genes, expression was up-regulated in the biofilm samples compared to the planktonic ones. The up-regulation of the *ica* genes in the biofilm mode of growth is consistent with previous work (Resch et al., 2005). The data therefore suggests that whilst there is differential gene expression between biofilm and planktonic cultures, expression does vary with other factors including culture age and for the studied genes, expression did not appear exclusive to a particular mode of growth. Previous studies also highlight that environmental signals such as temperature, osmolarity, pH, iron and oxygen play a role in biofilm development and therefore alter gene expression (Costerton et al., 1995). It also highlights the uniqueness of biofilms and that a universal biofilm gene-expression pattern still remains a wide-open area of investigation (Beloin and Ghigo, 2005).

The detection of biofilm specific genes in this present study seemed to be more complex than expected and it also appeared to be very difficult to maintain for 7 d planktonic cultures. Further studies are still required to fully elucidate the genetics of bacterial growth (Waite et al., 2005). Considering these contrasting results, the findings could be indicative that a wide range of genes influence biofilm development under specific conditions. More work remains to be done, *e.g.* the discovery of other genes which are critical for biofilm development. Improved understanding of which genes and proteins are differentially expressed under biofilm and planktonic growth conditions and how the morphology and physiology of biofilm cells differs from those of

planktonic cells is needed to understand the high persistence and antimicrobial resistance of biofilm cells (Resch et al., 2005). It is also important to highlight how widespread biofilms are with more than 99.9% of bacteria found to grow in biofilms (Donlan and Costerton, 2002) and that biofilm research encompasses studies of multispecies communities (Lewis, 2001).

In this present study, exploratory experiments using the microarray technique at the Health Protection Agency in Colindale were employed. The microarray used in this study was designed to look for potential virulence associations (section 6.3.8.7) and not only for biofilm-specific genes. Therefore a direct comparison of the microarray data with the RT-PCR results was difficult. Of the genes targeted by RT-PCR, only *agr* and *ica* genes were housed on the microarray. Comparative genome hybridisation was first used to confirm if the genes on the array were present or absent within the *S. aureus* D76 genome. The 486 genes present included the ica proteins, the *sarA* gene, virulence factors, phage related proteins which are involved with gene transfer events and multidrug resistance proteins.

The microarray expression work showed that several regulatory genes including *agrC1* (accessory gene regulator, group 1) and SA0573 sarA (staphylococcal accessory regulator A) had a higher expression in biofilm samples compared with planktonic exponential samples. Significantly, *agr* expression was also confirmed previously by RT-PCR in all biofilm and planktonic samples. By RT-PCR the majority of *agr* genes were up-regulated in the biofilm state, 6 out of 7. It is important to note that a network of regulatory genes including *agr* and *sar* operons control the expression of *S. aureus* virulence factors (Saunders et al., 2004b). The microarray data provided detailed information concerning gene carriage and increase or decrease in the level of gene between planktonic and biofilm mode of growth in the *S. aureus* D76 wound isolate. In this microarray study there was no evidence for the expression of the *ica* genes. This correlates with previous findings reported by Resch *et al* (2005) who suggested that these genes

were not needed after initial attachment and start of biofilm formation as the biofilm samples used for this study were 7 d old. Transcriptomic analysis opens the door for future work and provides a powerful tool to assess differential gene expression patterns under biofilm and planktonic growth conditions (Resch et al., 2005) as well as in other systems. However, more work still needs to be done to fully understand the genetics of bacterial biofilm growth (Waite et al., 2005).

In future studies, it would be interesting to examine gene expression from a mixed biofilm population and compare it with a mixed planktonic population. In this present study, mixed biofilms prepared in the CDFF and mixed planktonic preparations were generated but due to time constraints, the RT-PCR process was not pursued with these populations. It would certainly be valuable to extend the current study to apply a real-time PCR to detect, identify and quantify the relevant genes of interest over time between the planktonic and biofilm modes of growth to confirm the apparent up-regulation of the genes identified in this study. The real-time technique would allow more accurate monitoring of gene expression over time.

In general, microarray technology whilst providing a significant amount of information does suffer from the disadvantage of relating the findings to the role of the gene products, simply due to our currently limited knowledge concerning the hypothetical proteins and their relevant role in biofilm formation. Therefore, it would be helpful to actually identify first biofilm related genes and then to monitor the expression in samples. So far neither the microarray nor the RT-PCR study provided any direct indications related to genes associated with chronic wound biofilms. In future, and accompanying the development of these techniques it would be interesting to look at bacterial gene expression within actual clinical wound samples, and maybe compare specimens from both the normally healing phenotype and those that fail to heal.

6.6 Summary

In summary, this present study showed:

1. That the majority of the examined *P. aeruginosa* and *S. aureus* genes previously associated with biofilm formation were expressed in biofilm samples. Some of these genes were also expressed in planktonic samples suggesting that there might be a biofilm phenotype present or that the expression of the genes was not exclusive to specific growth modes.

2. Semi-quantification of the RT-PCR products from these genes did indicate that the majority were up-regulated in the biofilm samples compared to planktonic ones.

3. Altered gene expression between biofilm and planktonic samples was also apparent from exploratory microarray studies of *S. aureus*. In particular, confirmation of up-regulation of *agrC1, sarA* and the capsular polysaccharide synthesis enzyme Cap8c in biofilm grown cells was evident using the array method.

General Discussion

The significance of biofilm related infections is becoming increasingly evident and it has been suggested that over 65% of all hospital infections have their origin in biofilms (Costerton et al., 1999; Donlan, 2001; Donlan and Costerton, 2002; Douglas, 2003; Ramage et al., 2006). As a consequence, it is not surprising that much research in recent years has been focussed at improving our understanding of biofilms and the options available to combat them. The debate surrounding what a biofilm actually is remains an ongoing one. It is always difficult to define a biofilm and to compare biofilms with other studies due to the lack of standardised systems and protocols (Parsek and Singh, 2003). In this study, a biofilm was classed as being formed once the microorganisms (in this work *C. albicans* or chronic wound bacteria) were attached to a surface and produced multilayered structures.

Biofilms are ubiquitous throughout natural, industrial and clinical environments (Parsek and Singh, 2003). A distinguishing feature of these biofilms is the presence of aggregated microcolonies of cells that are attached to a surface (Hall-Stoodley et al., 2004). In the clinical environment, biofilms provide a reservoir of potentially pathogenic organisms that are often resistant to standard antibiotic concentrations. Elevated antibiotic resistance might be affected by phenotypic changes in the organisms which make up a biofilm. These changes include slower growth rate and expression of new multi-drug-resistant pumps (Anwar et al., 1992; Hoyle and Costerton, 1991; O'Toole et al., 2000). Another clinical problem of biofilms is that they can cause device failure when adhered to biomaterials used in medical devices (Lewis, 2001; Mah and O'Toole, 2001). Indeed, once a biofilm is established, the removal of bacteria by host defence mechanisms is also very difficult, and in the case of medical devices, often the only treatment option is the removal and replacement of the medical device.

Candida albicans is a commensal fungus of humans, commonly found in the oral cavity, gastrointestinal tract, female genital tract and occasionally on the skin (Hube and Naglik, 2001; Watts et al., 1998). Although considered as a commensal of healthy individuals, this fungus frequently causes superficial

infections when the normal microbial flora is unbalanced or there is a debilitation in the hosts' defence mechanisms. Hence, in immunocompromised patients, *C. albicans* may invade the tissue, penetrate the blood vessels and cause life threatening systemic infections (Felk et al., 2002).

In this study, it was shown that *C. albicans* strains isolated from different oral conditions were capable of forming biofilms and invading a reconstituted human oral epithelium (RHE) model. Using CLSM a heterogeneous invasion pattern of the tissue was evident with the strains of *C. albicans,* with the surface mainly colonised by the yeast form and a complex network of filaments occurring within the tissue. These filamentous hyphae were seen to physically penetrate the cells and exhibited budding within the tissue. Hyphae are indeed considered to be the principle invasive forms of *C. albicans* and are frequently identified in infected tissue (Felk et al., 2002). At the time of writing no other CLSM investigations regarding *C. albicans* biofilm formation ability and invasion in an *in vitro* model have been reported. In this present study, the ability of *C. albicans* to invade the RHE appeared to correlate with the isolates' capacity to exhibit a yeast/hyphal transition and in all but one case, isolates categorised as the 'high invaders' revealed a predominance of the hyphal morphology within the tissue. It was also evident that four of the five strains identified as high invaders had previously been isolated from chronic hyperplastic candidosis conditions. This could suggest that there is a particular strain type associated with this form of oral candidosis. It was clear from the pattern of RHE infection that *C. albicans* is heterogenous with respect to tissue invasion by strains, and whilst the host condition obviously plays an important role in the occurrence of infection, this study suggests that inherent strain virulence could also contribute.

Destruction of host tissues by *Candida* may be facilitated by the release of hydrolytic enzymes into the environment. The most widely studied hydrolytic enzymes of *C. albicans* are the secreted aspartyl proteinases (*SAPs*) and phospholipases (*PLs*). At present, 10 different genes have been identified

that encode for candidal *SAPs* (Hube, 1996; Hube et al., 1997; Sanglard et al., 1997). The exact role of these enzymes in virulence remains unclear, however their ability to degrade host extracellular matrix proteins, cell membranes, host surface molecules and their involvement in host tissue invasion are obvious pathogenic factors (Naglik et al., 2003). The production of phospholipases could contribute to host cell membrane damage which could promote cell lysis or expose receptors to facilitate adherence (Ghannoum, 2000; Ibrahim et al., 1995).

In addition to hydrolytic enzymes, adherence factors of *C. albicans* are also regarded as being important in its virulence. Recently, a gene family collectively referred to as 'agglutinin-like-sequences' (*ALS*) genes have been studied with respect to their role in promoting *Candida* infection. Eight *Candida ALS* genes encoding large glycoproteins have been shown to promote candidal adhesion to host surfaces (Guar and Klotz, 1997; Hoyer et al., 2001).

In this present study, the expression of these ALS genes together with those encoding for hydrolytic enzymes were analysed to determine whether expression correlated with biofilm formation and pattern of RHE infection. As reported in Chapter 3, *C. albicans* strains that had previously been categorised as 'high invaders' (Chapter 2) were also found to be consistent producers of *SAPs* 4-6 despite the level of expression of these genes being strain dependent. *SAP* genes 4-6 are believed to be important in controlling yeast to hyphal transition (Naglik et al. 2003) and therefore might have had an indirect link with invasion through promoting hyphal development. Whilst there was a largely consistent expression pattern observed between the planktonic and the biofilm growth phases of the *Candida* strains examined, there were some notable differences. These included up-regulation of certain *SAPs* and *ALS* expression in the presence of the tissue and up-regulation of *PLs* expression in the planktonic state. The findings would appear to imply that culture environment was an important factor in inducing expression of these genes. The fact that phospholipase genes were up-

regulated in the absence of RHE could indicate that expression results from a 'starvation trigger' that may not be present when the tissue is available as a contributing source of additional nutrients. The up-regulation of both *ALS* and *SAP* genes in environments containing the tissue could, in contrast, relate to adhesion based triggers. Further work is required to investigate and validate these hypotheses.

An additional area of work using this RHE model could be in the investigation of non-*C. albicans Candida* (NCAC) species. Several studies have shown an increase in the incidence of candidaemia and a shift from the involvement of NCAC species, namely *Candida parapsilosis, C. tropicalis, C. guilliermondii, C. glabrata, C. krusei, C. lusitaniae, C. kefyr* and *C. dubliniensis* (Bassetti et al., 2006; Nucci and Colombo, 2007; Pfaller and Diekema, 2002; Weinberger et al., 2005). *C. tropicalis* has emerged as the second most frequent species of all candidemias after *C. albicans* (Nucci and Colombo, 2007; Weinberger et al., 2005). Other NCAC species frequently isolated from blood include *C. glabrata* and *C. parapsilosis* (Bassetti et al., 2006). It has been observed that the prevalence of NCAC species from the oral cavities of patients with advanced cancer is also increasing. An explanation for this could be the increased use of antifungal drugs (*e.g.* fluconazole) or the advancements in methods for identifying the recovered yeast species (Bassetti et al., 2006; Davies et al., 2002). This recent recognition of the importance of NCAC species in human infection would support studies examining the biofilms produced by these species and assessing their roles in infection. Therefore, analysis of clinical NCAC species biofilms within the RHE model could aid in clarifying their role in both candidemia and oral candidosis.

With regards to chronic wound biofilms (produced by *P. aeruginosa, S. aureus, M. luteus* and *S. oralis)* differences in biofilm formation were again evident between the species examined. Several *in vitro* models were used to study these biofilms, including a microtitre plate assay, the constant depth film fermenter (CDFF) and the RHE system. Chronic wound bacteria were able to form biofilms (both individually and in mixed culture) in all these

models, although in the case of the RHE, invasion only occurred once the integrity of the epithelium barrier was disrupted. It is also important to highlight that biofilm formation in the CDFF was observed over a period of 7 d, in contrast to the formation on the RHE, which was only observed between 24-28 h. It may be that greater levels of biofilm might have occurred on the RHE if a prolonged incubation time had been utilised, and again this is an area for potential future development.

To assess whether synergism or antagonism occurred between the studied bacterial species in the microtitre plate assay, different combinations of bacteria in mixed biofilms were analysed. In these mixed species biofilms, both *P. aeruginosa* and *S. aureus* appeared to antagonise the biofilm forming ability of *S. oralis* and *M. luteus*. In the case of the CDFF, all four bacterial species were added simultaneously and *M. luteus* appeared to be 'out-competed' in these situations by the other organisms present and *S. oralis* was often at the limits of detection. *Micrococcus luteus* is not generally considered to be a human pathogen, whereas both *P. aeruginosa* and *S. aureus* are. It is tempting to speculate that in chronic wound situation the introduction of either *P. aeruginosa* or *S. aureus* to the environment may cause replacement of harmless skin commensals such as *M. luteus,* thus causing an ecological shift to the infected state. Such a situation has indeed previously been postulated to occur in plaque mediated diseases (Marsh, 2003). To confirm this hypothesis, there is a need for more in depth cultural analysis of the chronic wound environment, and identification of the bacterial species involved. Potentially, if this situation did occur, then there may be a possible role for probiotic therapy in chronic wounds that could reverse the microbial ecology to a healthy state. The CDFF would provide an ideal system in which to model such interactions.

In this present study, an attempt was made to add anaerobic bacteria simultaneously with aerobic bacteria to form a 'wound' biofilm consortium in the CDFF. However, the anaerobic species were not subsequently detected when the mixed species biofilm was cultured and therefore these organisms

were not included in further studies. The reason for this 'loss' of the anaerobic bacteria could be that the CDFF did not provide a truly anaerobic environment, as anaerobic gas was not infused into the fermenter. Alternatively, it may have been that the anaerobic species needed a richer growth medium than that provided by the BHI broth used. It is known from previous research that it is sometimes difficult to maintain anaerobic culture within such environments. Denaturing Gradient Gel Electrophoresis (DGGE) is a technique that could be used on DNA recovered from the biofilms to confirm the presence of the anaerobic organisms as low number constituents within the biofilm. DGGE is a more sensitive technique than traditional culture techniques, whereby total population DNA is amplified by PCR, enabling detection of organisms even when present in very low numbers (Spratt, 2004) .

To test the statement that biofilms are inherently more resistant to antimicrobial and bactericidal agents, povidone-iodine (PVP1) was used. PVP1 is a bacteriostatic and antimicrobial agent which is commonly used as a topical agent in the treatment of wound infections (Mertz et al., 1999). Initial experiments with PVP1 showed that planktonic organisms were susceptible to 1% PVP1 which is the concentration that is believed to be available to the wound surface from PVP1 integrated wound dressings such as Inadine (Johnson & Johnson). Against CDFF generated biofilms however, 1% PVP1 had little long term effect on the number of viable cells. It was interesting to see that after live/dead staining of PVP1 biofilms, a majority of 'dead' cells was evident. 'Persister cells' may be in existence in the established biofilm, which, whilst dormant are less susceptible to antimicrobial activity but are able to effect re-colonisation of the biofilm after treatment is stopped. Another possible explanation for this staining pattern could be that PVP1 did not actually kill the bacteria and only damaged the cell membrane which is reflected as appearing 'dead' by the live/dead stain. However a reduction in viable cell numbers was observed following culture, which lends more support to the 'persister' cell theory. It would have been interesting to test other antimicrobial agents on CDFF generated biofilms.

For example, Rasmussen and Givskov (2006) have suggested that quorum sensing inhibitors (QSI) may have value as potentially new antimicrobial agents to eradicate *in vitro* biofilms and such molecules could readily be tested in the future using the CDFF model. Garlic extract, carrots, chilli, water lily, and patulin and penicillic acid (both compounds produced by some members of the fungal genus *Penicillium*) have all been found to inhibit QS and could be tested in this way (Ramsey and Wozniak, 2005; Rasmussen et al., 2005; Rasmussen and Givskov, 2006).

Whilst much is known of the physiology of organisms in biofilms little is known about biofilm architecture. An important aim of this study was to examine the spatial distribution and structure of chronic wound bacterial biofilms in both *in vitro* models and in actual wound tissue. Chronic wounds are known to be colonised by a diverse microflora, including anaerobic bacteria, and it has been speculated that bacteria exist in these highly persistent biofilm communities (James et al., 2007). Fluorescent *in situ* hybridisation (FISH) with peptide nucleic acid probes (PNA) was used to assess biofilm architecture. The data showed that in bacterial biofilms established in the CDFF, the organisms grew as aggregates and in species specific zones, suggesting that the bacterial species did not integrate well with one another under these conditions. *Pseudomonas aeruginosa* was the predominant constituent of these CDFF generated biofilms as demonstrated by FISH and indeed previously by viable count estimation. The distribution of mixed organisms confirmed the previous cultural findings, suggesting that species antagonism occurred within the community. Bjarnsholt *et al.* (2008) analysed sections from chronic wounds by PNA FISH using a *P. aeruginosa* specific (Texas red labelled) and a universal bacterial (FITC labelled) probe and also found distinct microcolonies of *P. aeruginosa* and cocci shaped bacteria when viewed by CLSM. Another study using a porcine model, inoculated partial thickness wounds with a *S. aureus* wound isolate strain and showed aggregates of microcolonies of bacteria embedded within and attached to the wound bed (Davis et al., 2008). James *et al.* (2007) also observed Gram-positive cocci in large aggregates in all acute and chronic

wounds. Unfortunately in this study, the presence of any EPS matrix generated by the chronic wound bacterial biofilms was not established.

Unlike the findings seen with *C. albicans*, none of the bacterial species were able to invade the tissue model and these bacterial biofilms were found to be limited using an undamaged RHE surface. Subsequent analysis of chronic wound biopsies showed the presence of bacteria, but it was difficult to confirm whether an actual biofilm was present due to the condition of the archived biopsy tissue analysed. These samples were from chronic wounds which clinically were not regarded as infected. Certainly, in the case of clinically infected wounds, biofilms are evident on the surface of many patient wounds particularly when *P. aeruginosa* is involved, as it has a characteristic green colouration and strong odour.

Bacteria possess adhesins which are thought to be involved in biofilm formation. For example, Love *et al* (1997) showed that antigen I/II polypeptides are involved in the attachment of oral streptococci to collagen and that their activity may be crucial for penetration of human root dentinal tubules and infection of the tooth root canal system and pulp. *Pseudomonas aeruginosa* also promotes attachment and biofilm formation through its twitching and flagella mediated motility. Furthermore, some bacteria have the ability to actually invade the tissues, as evident with spirochetes, *Salmonella, Escherichia, Shigella* and *Yersinia* spp. (Barocchi et al., 2002). There are, however, no specific bacterial morphological forms that actually promote epithelium invasion as seen with the hyphae of *C. albicans*.

Prokaryotic (bacteria) as well as eukaryotic organisms (fungi) produce destructive enzymes, such as proteinases, phospholipases and DNases, which damage the epithelial cells and tissues, and indirectly promote an inflammatory response (Percival and Rogers, 2005). In addition, recent studies have also identified numerous bacterial genes or factors as being 'essential' or 'required' for biofilm formation (Caiazza and O'Toole, 2003; Cramton et al., 1999; Hall-Stoodley et al., 2004; Valle et al., 2003). These

genes regulate or express surface-adhesion proteins, appendages such as pili, flagella and the EPS matrix (Hall-Stoodley et al., 2004). Bacterial surface proteins contribute significantly to adhesion and include the autolysin AtlE of *Staphylococcus epidermidis*, the biofilm associated protein (Bap) and the accumulation associated protein (AAP) (Hall-Stoodley et al., 2004).

Staphylococcus aureus and *P. aeruginosa* biofilms generated in the CDFF were used to detect biofilm and virulence related genes by RT-PCR. *Staphylococcus aureus* gene expression was further analysed by microarray technology at the Health Protection Agency in Colindale, London, UK. The *in vitro* study showed that the majority of the examined genes, previously associated with biofilm formation by *S. aureus* and *P. aeruginosa* were expressed in biofilm samples. Some of these genes were also expressed in planktonic samples, suggesting that there might be some organisms with a biofilm phenotype present, or that the expression of the genes was not exclusive to the biofilm mode of growth. Furthermore, semi-quantification of the RT-PCR products from these genes indicated that the majority were 'up regulated' in the biofilm samples compared to the planktonic ones. Altered gene expression for *S. aureus* between biofilm and planktonic samples was also apparent from the exploratory microarray data. The expression of *S. aureus* virulence associated factors is controlled by a network of regulatory genes including *agr* and the *sar* operon (Saunders et al., 2004). Confirmation of up-regulation of *agr* and *sarA* was evident using the microarray method in this present study. Beenken *et al.* (2004) similarly investigated differential gene expression in a mature *S. aureus* biofilm and revealed that genes which were part of the *sarA* regulon were also differentially expressed in biofilms.

Following study of these chronic wound isolates, several areas for further investigation have been identified. Additional molecular analysis *e.g.* quantitative real time PCR (qRT-PCR) for gene quantification would prove valuable in elucidation the role of individual genes in a biofilm state. qRT-PCR would provide more accurate quantitative data on specific gene

expression compared with the semi-quantitative RT-PCR used here. qRT-PCR allows quantification of the gene product and could also be used to validate the microarray data. Biofilm related gene expression could be further assessed with mixed species biofilm samples and with biofilm samples that have been treated with PVP1 or other antimicrobial agents.

FISH and CLSM analysis on fresh or well preserved clinical biopsies (CHC and chronic wounds) could be used to highlight the location of causative organisms within the tissue. This could actually aid in the determination of which species act as primary colonisers of the infection and which organisms actually invade the tissue and cause harm. The FISH technique could facilitate estimation of the relative proportions of organisms within the biopsy and could be valuable in assessing biofilm management strategies or evaluating the effectiveness of antimicrobials against the biofilm consortium *in vivo*. The application of such tests in the clinical setting in the not too distant future seems highly probable. It is still not clear to what extent microorganisms in a wound are surface colonisers or how deep they penetrate the wound tissue. This could affect the outcome for the patient and the strategy for treatment. For instance, whether prescription of systemic antibiotics is necessary or whether antimicrobial wound dressings are sufficient.

Succession studies for biofilm formation represents an area that appears to be neglected by researchers and warrants future investigation. Even in this present study, all species were added simultaneously to the CDFF. Without time constraints, it would have been interesting to add the species of interest in a successive manner, *e.g.* i) first add suspect commensal species and then the pathogenic organisms or, ii) aerobic organisms and then in a mature biofilm add anaerobic ones. This could give a better understanding of how biofilms form *in vivo*.

The characterisation of biofilms as shown in this study is extremely complex. The RHE used is by far, the closest model to the *in vivo* situation for studying

biofilm formation. Only with further knowledge can our understanding of 'biofilms' be improved. Such research is warranted, as it may facilitate the development of new diagnostic tools and techniques which could be applied to any biofilm related infections.

Biofilms are currently a subject of intense research interest and a major public health issue in the emergence of antimicrobial resistance. This study attempted to characterise biofilms associated with infections and their feature that promote virulence and resistance to antimicrobial agents. In conclusion, principal findings of this study showed

- that strain and species variation in terms of biofilm formation, infection type and invasion of the RHE tissue was evident, particularly for *Candida albicans*.

- Bacterial interaction studies highlighted that *P. aeruginosa* was the predominant organism in mixed species biofilms using *in vitro* studies which could have importance when extrapolated to the clinical situation.

- The limited long term effect of PVP1 on biofilms has potential significance in how chronic wounds are therapeutically managed.

- However, it is still difficult to determine, whether the presence of either *S. aureus* or *P. aeruginosa* in chronic wounds reflects microbial infection or colonisation. If the former, then the occurrence of these organisms might be valuable indicators of the likely occurrence of a non-healing wound phenotype.

- Moreover, the appreciation that biofilms are involved in chronic wounds reinforces the need for continued study of this subject with particular emphasis directed at the involvement of both *S. aureus* and *P. aeruginosa*.

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Appendix I. Bacterial media

Constituents of BM medium (McKee et al., 1985)

BM medium	g l^{-1}
Proteose peptone (Oxoid, Unipath Ltd. Basingstoke, UK)	10
Trypticase peptone (Becton-Dickson UK Ltd, Oxford, UK	5
Yeast extract (Oxoid, UK)	5
potassium chloride (Sigma, Poole)	2.5
Glucose (BDH, WWR International, Lutterworth, UK)	10
Cysteine HCl	0.5
Haemin (Sigma, Poole)	0.005
Vitamin K1	0.001
Glucose	10
H$_2$O	1000 ml

Two other reagents, 0.5 gl^{-1} cysteine hydrochloride (BDH, WWR International, Lutterworth, UK) and 0.001 gl^{-1} vitamin K (Sigma, Poole), were each filter sterilised separately, and added aseptically to the autoclaved BM media after cooling.

Constituents of simulated wound fluid (Addison et al., 2004)

Simulated wound fluid	g l^{-1}
calcium-chloride	1.5
Sodium-chloride	12
Tris-HCl	5
Bovine Serum Albumin (BSA), pH 7.5	20
H$_2$O	1000 ml

The medium was modified by adding 2 gl^{-1} glucose to provide an additional carbon source and sterilised by autoclaving.

Appendix II.

Sequences of
Streptococcus oralis (B52),
Staphylococcus aureus (D76),
Pseudomonas aeruginosa (D40),
Micrococcus luteus (B81)
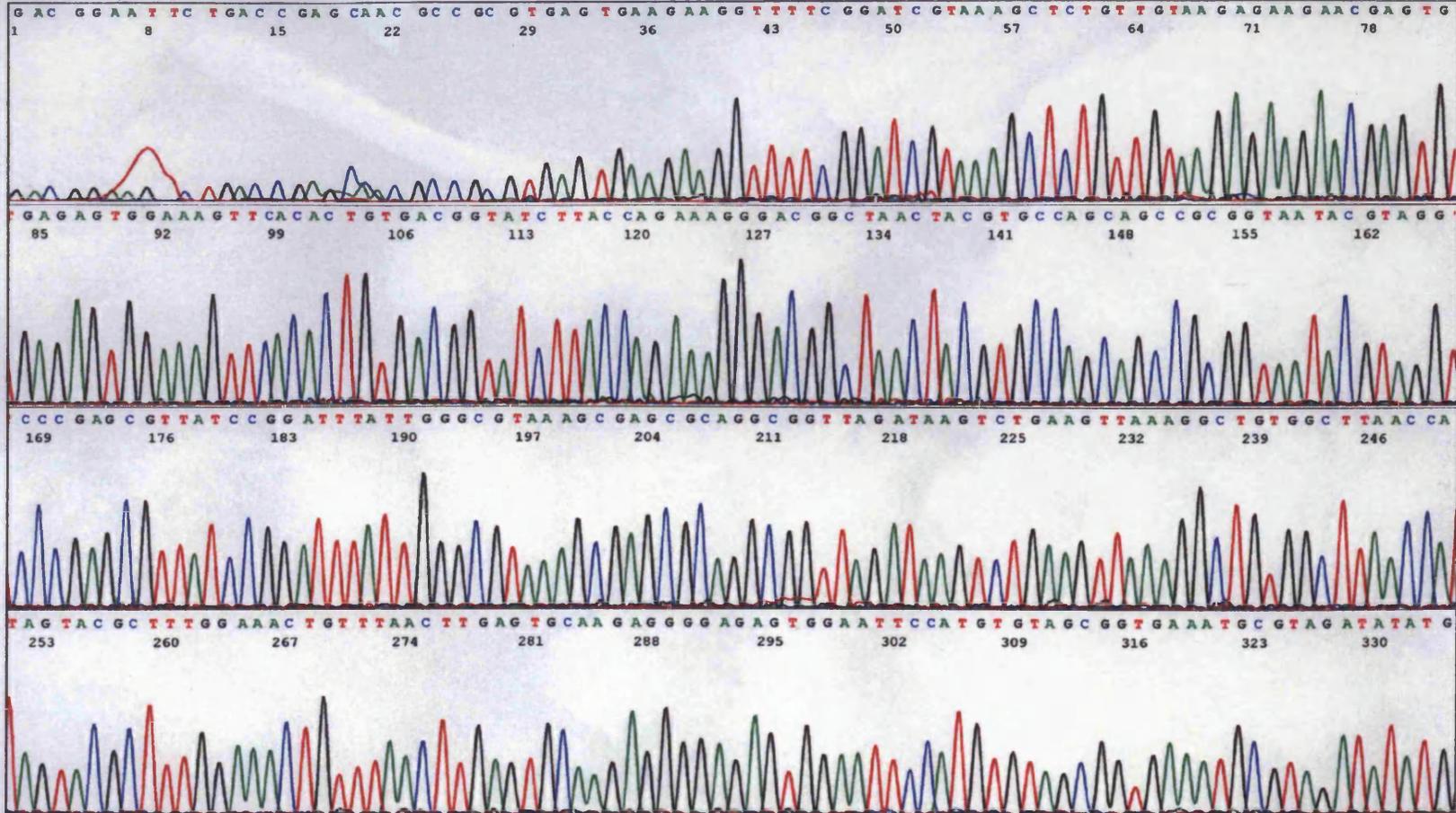
Sequence of *Streptococcus oralis* (B52)

Sequence of *Streptococcus oralis* (B52) continued

Sequence of *Staphylococcus aureus* (D76)

Sequence of *Staphylococcus aureus* (D76) continued

C G G T G A A A T G C G C A G A G A T A T G G A G G A A C A C C A G T G G C G A A G G C G A C T T T C T G G T C T G T A A C T G A C G C T G A T G T G C G A A A G C G
337   344   351   358   365   372   379   386   393   400   407   414

T G G G G A T C A A A C A G G A T T A G A T A C C C T G G T A G T C C A C G C C G T A A A C G A T G A G T G C T A A G T G T T A G G G G G T T T C C G C C C C T T A G
421   428   435   442   449   456   463   470   477   484   491   498

T G C T G C A G C T A A C G C A T T A A G C A C T C C G C C T G G G G A G T A C G A C C G C A A G G T T G A A A C T C A A A G G A A T T G A C G G G G A C C G C A
505   512   519   526   533   540   547   554   561   568   575   582

C A A G C G G T G G A G C A T G T G G T T T A A T T C G A A G C A A C G C G A A G A A C C T T A C C A A A T C T T G A C A T C C T T T G A C A A C T C T A G A G A T
589   596   603   610   617   624   631   638   645   652   659

Sequence of *Pseudomonas aeruginosa* (D40)

Sequence of *Pseudomonas aeruginosa* (D40) continued

Sequence of *Micrococcus luteus* (B81)

Sequence of *Micrococcus luteus* (B81) continued
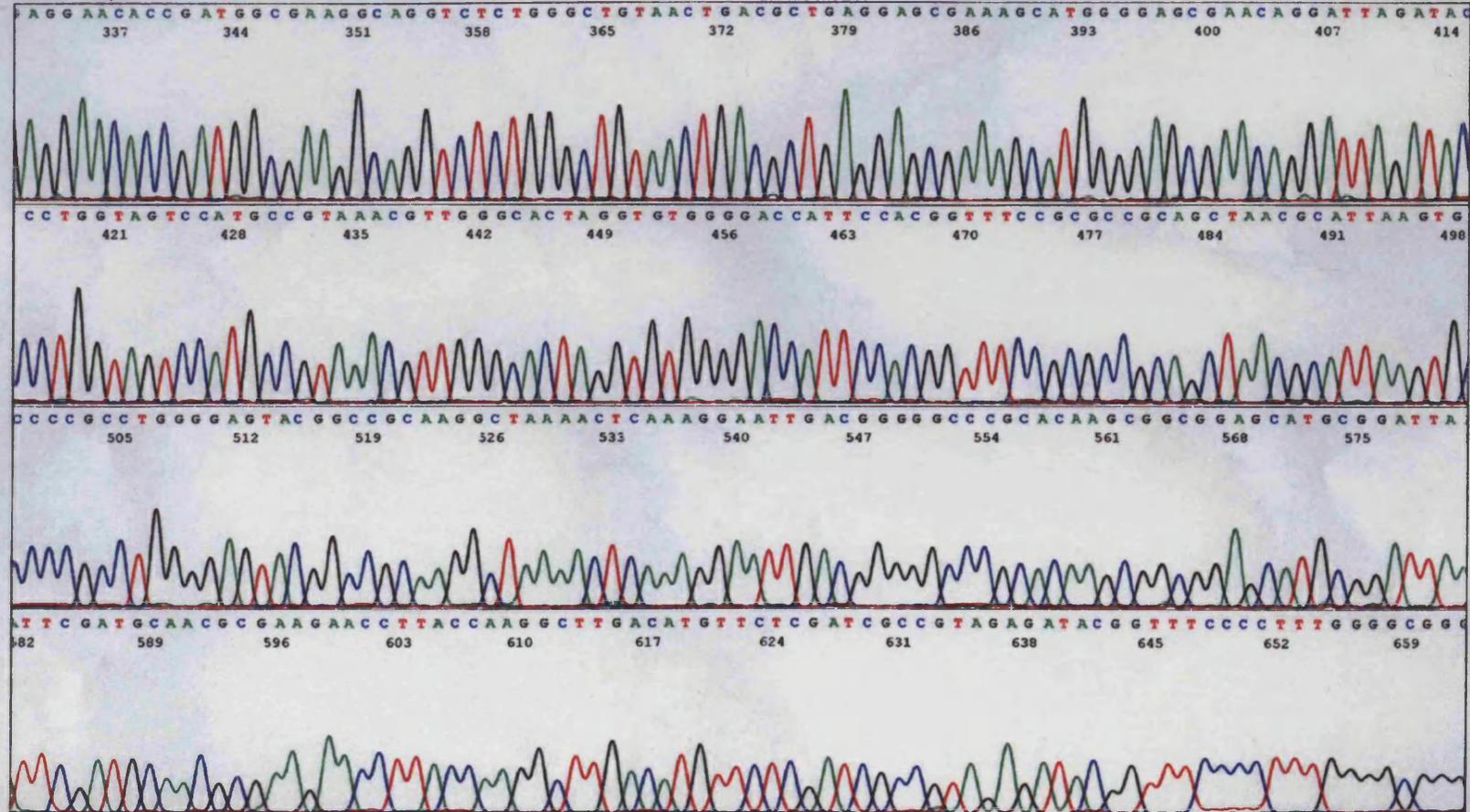
Appendix III. Microarray gene list

	ORF	Gene	Product
agrC1	af210055	agrC	group 1 accessory gene regulator
agrC2	af001782	agrC	group 2 accessory gene regulator
agrC3	af001783	agrC	group 3 accessory gene regulator
agrC4	af288215	agrC	group 4 accessory gene regulator
bbp	O24	bbp	sdrE homolog (bone sialoprotein binding protein)
clfA	MW0764	clfA	fibrinogen-binding protein
clfB	MW2551	clfB	clumping factor B
coa	MW0206	coa	staphylcoagulase precursor
comGC	MW1494	comGC	exogenous DNA-binding protein
E16-0102c	E16-0102c	E16 102c	TCRTETB/TCRTETA
E16-0463	E16-0463	E16 463	BACTRLTOXIN
E16-0467	E16-0467	E16 467	TOXICSSTOXIN/BACTRLTOXIN
E16-0469	E16-0469	E16 469	TOXICSSTOXIN
E16-0475	E16-0475	E16 475	TOXICSSTOXIN
E16-0776	E16-0776	E16 776	SECYTRNLCASE
E161084c	E16-1084c	E16 1084c	V8PROTEASE
E161208c	E16-1208c	E16 1208c	BICOMPNTOXIN
E16-1212c	E16-1212c	E16 1212c	TOXICSSTOXIN
E16-1213c	E16-1213c	E16 1213c	TOXICSSTOXIN
E16-1949	E16-1949	E16 1949	BLACTAMASEA
E16-2023c	E16-2023c	E16 2023c	V8PROTEASE
E16-2024c	E16-2024c	E16 2024c	V8PROTEASE
E16-2025c	E16-2025c	E16 2025c	V8PROTEASE
E162027c	E16-2027c	E16 2027c	V8PROTEASE
E162040c	E16-2040c	E16 2040c	BACTRLTOXIN
E16-2041c	E16-2041c	E16 2041c	BACTRLTOXIN
E16-2042c	E16-2042c	E16 2042c	BACTRLTOXIN
E162902c	E16-2902c	E16 2902c	THERMOLYSIN
E16-2979c	E16-2979c	E16 2979c	SUBTILISIN
E16465	E16-0465	E16 465	TOXICSSTOXIN/BACTRLTOXIN
E16472	E16-0472	E16 472	TOXICSSTOXIN/BACTRLTOXIN
ebhA	SA1267	ebhA	similar to streptococcal adhesin emb
ebhB	SA1268	ebhB	similar to streptococcal adhesin emb
efb	strain 4074	efb	fibrinogen-binding protein
epbS	MW1369 bacteriophage	epbS	elastin binding protein
eta	phi ETA	eta	Staphylococcus aureus exfoliative toxin A
etb	etb plasmid	etb	Staphylococcus aureus exfoliative toxin B
fhuA	MW0609	fhuA	ferrichrome transport ATP-binding protein
fhuB	MW0610	fhuB	ferrichrome transport permease
fhuG	MW0611	fhuG	ferrichrome transport permease
fnbB	MW2420	fnbB	fibronectin-binding protein homolog
geh	MW0297	geh	glycerol ester hydrolase
gi|46019590	agr		type 2 7-11 27
gi|46019594	agr		type 2
gi|46019594	agr		type 2 7-11
gi|46019602	agr		type 3 subst specific 4 5
gi|46019606	agr		type 3 subst specific 4
gi|46019610	agr		type 3 subst specific 3-6 26
gi|78172207	agr		type 4 subset specifi 2 14 24
gi|78172207	agr		type 4 specific
gi|78172212	agr		type 1 subset specific 1 12 25

gi|78172212	agr		type 1 subset specific 1 12 16 17 20 25
gmk	MW1092	gmk	guanylate kinase
hla	MW1044	hla	alpha toxin; alpha haemolysin
hlb	MW1881	hlb	beta haemolysin
hlgB	MW2344	hlgB	gamma-hemolysin component B
hlgC	MW2343	hlgC	gamma-hemolysin component C
hlgII	MW2342	hlgA	gamma haemolysin chain II
hu	MW1362	hu	DNA-binding protein II
icaA	MW2586	icaA	intercellular adhesion protein A
lepA	MW1536	lepA	GTP-binding protein
lgt	MW0723	lgt	prolipoprotein diacylglyceryl transferase
lip	MW2590	lip	triacylglycerol lipase precursor
lsp	MW1079	lsp	lipoprotein signal peptidase
modA	MW2197	modA	probable molybdate-binding protein
modC	MW2195	modC	molybdenum transport ATP-binding protein
msmX	MW0189	msmX	multiple sugar-binding transport ATP-binding protein
mupR	j2870	mupR	isoleucyl tRNA synthetase
MW0031	MW0031	mecA	penicillin binding protein 2 prime
MW0040	MW0040	MW0040	hypothetical protein
MW0042	hypothetical protein		hypothetical protein
MW0043	hypothetical protein		hypothetical protein
MW0045	hypothetical protein		hypothetical protein
MW0047	hypothetical protein		hypothetical protein
MW0051	MW0051	seh	staph enterotoxin h
MW0052	MW0052	MW0052	truncated hypothetical protein, similar to enterotoxin SEO
MW0053	hypothetical protein		
MW0062	MW0062	MW0062	hypothetical protein similar to macrolide-efflux determinant
MW0063	MW0063		portal protein
MW0064	MW0064		hypothetical protein, similar to transcriptional regulator (LysR family)
MW0065	MW0065		terminase large subunit
MW0066	MW0066	MW0066	hypothetical protein, similar to transcriptional regulator
MW0074	hypothetical protein		
MW0077	MW0077	MW0077	hypothetical protein, similar to transcription regulator AraC/XylS family
MW0079	MW0079	MW0079	hypothetical protein similar to transmembrane efflux pump protein
MW0085	MW0085	sarH1	staphylococcal accessory regulator A homologue
MW0092	MW0092	MW0092	hypothetical protein similar to multi-drug resistance efflux pump
MW0105	MW0105	MW0105	hypothetical protein, similar to capsular polysaccharide synthesis protein 14H
MW0108	MW0108	MW0108	hypothetical protein
MW0111	MW0111	MW0111	hypothetical protein similar to tetracyclin resistance protein
MW0120	MW0120		truncated replication initiator protein
MW0147	MW0147	MW0147	hypothetical protein similar to membrane lipoprotein SrpL
MW0161	MW0161	MW0161	hypothetical protein, similar to isochorismatase
MW0167	MW0167	MW0167	hypothetical protein, similar to transcription regulator
MW0171	MW0171		hypothetical protein
MW0172	MW0172	MW0172	hypothetical protein, similar to ABC transporter ATP-binding protein
MW0178	MW0178	MW0178	hypothetical protein, similar to ABC transporter ATP-binding protein
MW0182	MW0182	oppB	truncated hypothetical protein, similar to oligopeptide ABC transporter permease
MW0190	MW0190	MW0190	hypothetical protein, similar to maltose/maltodextrin-binding protein
MW0198	MW0198	MW0198	hypothetical protein, similar to two-component response

			regulator
MW0200	MW0200	MW0200	hypothetical protein similar to periplasmic-iron-binding protein BitC
MW0213	MW0213	MW0213	hypothetical protein, similar to nickel ABC transporter nickel-binding protein
MW0240	MW0240	MW0240	hypothetical protein, similar to transcription regulator GntR family
MW0250	MW0250	MW0250	hypothetical protein, similar to proton antiporter efflux pump
MW0263	MW0263	MW0263	conserved hypothetical protein, similar to diarrheal toxin incomplete ORF
MW0283	MW0283	MW0283	hypothetical protein, similar to branched-chain amino acid uptake carrier
MW0286	MW0286	MW0286	hypothetical protein, similar to ABC transporter ATP-binding protein
MW0310	MW0310	MW0310	hypothetical protein, similar to transcription regulator
MW0311	MW0311	MW0311	conserved hypothetical protein
MW0323	MW0323	MW0323	conserved hypothetical protein
MW0327	MW0327	MW0327	hypothetical protein, similar to ABC transporter ATP-binding protein
MW0339	MW0339	MW0339	hypothetical protein, similar to GTP-binding protein
MW0345	MW0345	MW0345	hypothetical protein, similar to exotoxin 2
MW0355	MW0355		hypothetical protein
MW0368	MW0368		hypothetical protein, similar to AbiD1 [Genomic island nu Sa alpha2
MW0372	MW0372		GenomicislandnuSaalpha2
MW0374	MW0374		GenomicislandnuSaalpha2
MW0376	MW0376	MW0376	hypothetical protein
MW0378	MW0378		GenomicislandnuSaalpha2
MW0379	MW0379		GenomicislandnuSaalpha2
MW0381	MW0381	MW0381	conserved hypothetical protein MW0381
MW0382	MW0382	set16	exotoxin 16
MW0383	MW0383	set17	exotoxin 17
MW0384	MW0384	set18	exotoxin 18
MW0385	MW0385	set19	exotoxin 19
MW0386	MW0386	set20	exotoxin 20
MW0387	MW0387	set21	exotoxin 21
MW0388	MW0388	set22	exotoxin 22
MW0389	MW0389	set23	exotoxin 23
MW0390	MW0390	set24	exotoxin 24
MW0391	MW0391	set25	exotoxin 25
MW0394	MW0394	set26	exotoxin 26
MW0395	MW0395	MW0395	hypothetical protein
MW0396	MW0396	MW0396	hypothetical protein
MW0397	MW0397	lpl10	hypothetical protein
MW0398	MW0398	lpl11	hypothetical protein
MW0399	MW0399	lpl12	hypothetical protein
MW0400	MW0400	lpl13	hypothetical protein
MW0401	MW0401	lpl14	hypothetical protein
MW0402	MW0402	MW0402	hypothetical protein
MW0416	MW0416	MW0416	hypothetical protein, similar to ABC transporter ATP-binding protein
MW0418	MW0418	MW0418	hypothetical protein similar to lactococcal lipoprotein
MW0513	MW0513	MW0513	hypothetical protein, similar to Cu binding protein (Mn oxidation)
MW0531	MW0531	yqiL	acetyl-CoA c-acetyltransferase
MW0543	MW0543	pta	phosphotransacetylase
MW0552	MW0552		major tail protein
MW0553	MW0553		conserved hypothetical protein
MW0554	MW0554		putative primase

MW0558	MW0558		conserved hypothetical protein
MW0559	MW0559		conserved hypothetical protein
MW0560	MW0560		hypothetical protein
MW0573	MW0573	MW0573	hypothetical protein similar to iron-binding protein
MW0574	MW0574	MW0574	hypothetical protein similar to iron(III) ABC transporter permease protein
MW0579	MW0579	MW0579	hypothetical protein similar to esterase/lipase
MW0593	MW0593	MW0593	lipoprotein, Streptococcal adhesin PsaA homologue
MW0594	MW0594	MW0594	conserved hypothetical protein
MW0595	MW0595	MW0595	hypothetical protein, similar to ABC transporter ATP-binding protein
MW0596	MW0596	MW0596	hypothetical protein similar to iron dependent repressor
MW0617	MW0617	MW0617	hypothetical protein similar to lipase LipA
MW0623	MW0623	vraF	ABC transporter ATP-binding protein
MW0651	MW0651	MW0651	hypothetical protein
MW0681	MW0681	MW0681	hypothetical protein, similar to anion-binding protein
MW0682	MW0682	MW0682	hypothetical protein, similar to ABC transporter ATP-binding protein
MW0684	MW0684	MW0684	hypothetical protein, similar to choline transport ATP-binding protein
MW0695	MW0695	MW0695	hypothetical protein similar to ferrichrome ABC transporter permease
MW0696	MW0696	MW0696	hypothetical protein similar to ferrichrome ABC transporter permease
MW0697	MW0697	MW0697	hypothetical protein similar to ferrichrome ABC transporter ATP-binding protein
MW0698	MW0698	MW0698	lipoprotein, similar to ferrichrome ABC transporter
MW0736	MW0736	tpi	triosephosphate isomerase
MW0746	MW0746		hypothetical protein (Genomic island)
MW0751	MW0751		hypothetical protein (Genomic island)
MW0754	MW0754		hypothetical protein (Genomic island)
MW0755	MW0755		hypothetical protein (Genomic island)
MW0756	MW0756		hypothetical protein (Genomic island)
MW0757	MW0757		hypothetical protein (Genomic island)
MW0758	MW0758	ear	Ear (Genomic island)
MW0760	MW0760	sel2	extracellular enterotoxin I
MW0765	MW0765	MW0765	truncated secreted von Willebrand factor-binding protein
MW0766	MW0766	MW0766	truncated secreted von Willebrand factor-binding protein
MW0767	MW0767	ssp	extracellular ECM and plasma binding protein
MW0790	MW0790	MW0790	ABC transporter ATP-binding protein homologue
MW0795	MW0795	MW0795	ABC transporter ATP-binding protein homologue
MW0870	MW0870	oppD	oligopeptide transport system ATP-binding protein OppD homologue
MW0871	MW0871	oppF	oligopeptide transport ATP-binding protein
MW0872	MW0872	MW0872	hypothetical protein, similar to peptide binding protein OppA
MW0873	MW0873	MW0873	hypothetical protein, similar to oligopeptide ABC transporter oligopeptide-binding protein
MW0874	MW0874	MW0874	oligopeptide ABC transporter ATP-binding protein homologue
MW0875	MW0875	appF	oligopeptide transport system ATP-binding protein AppF homologue
MW0897	MW0897	MW0897	hypothetical protein, similar to multidrug resistance protein-related protein
MW0919	MW0919		hypothetical protein, similar to UDP-glucose:polyglycerol phosphate glucosyltransferase
MW0921	MW0921	MW0921	hypothetical protein similar to ferrichrome ABC transporter
MW0962	MW0962	MW0962	hypothetical protein
MW0982	MW0982	potA	spermidine/putrescine ABC transporter, ATP-binding protein homolog
MW0985	MW0985	MW0985	spermidine/putrescine-binding protein precursor homolog
MW0986	MW0986	MW0986	conserved hypothetical protein
MW1015	MW1015	MW1015	hypothetical protein similar to ferrichrome ABC transporter

MW1016	MW1016	MW1016	hypothetical protein similar to ferrichrome ABC transporter
MW1040	MW1040	MW1040	fibrinogen-binding protein
MW1041	MW1041	MW1041	hypothetical protein, similar to fibrinogen-binding protein
MW1047	MW1047	MW1047	hypothetical protein, similar to exotoxin 1
MW1048	MW1048	MW1048	hypothetical protein, similar to exotoxin 4
MW1049	MW1049	MW1049	hypothetical protein, similar to exotoxin 3
MW1091	MW1091	MW1091	hypothetical protein, similar to fibrinogen binding protein
MW1160	MW1160	MW1160	hypothetical protein, similar to transcription regulator GntR family
MW1163	MW1163	MW1163	hypothetical protein, similar to 3-oxoacyl- acyl-carrier protein reductase homolog ymfl
MW1182	MW1182	glpF	glycerol uptake facilitator
MW1185	MW1185	MW1185	hypothetical protein similar to lysophospholipase
MW1189	MW1189	MW1189	hypothetical protein, similar to GTP-binding protein proteinase modulator homolog ynbA
MW1197	MW1197		hypothetical protein
MW1206	MW1206	MW1206	ABC transporter (ATP-binding protein) homolog
MW1261	MW1261	femA	factor essential for expression of methicillin resistance
MW1262	MW1262	femB	FemB protein
MW1280	MW1280	MW1280	ABC transporter (ATP-binding protein) homolog
MW1287	MW1287	MW1287	hypothetical protein, similar to alanine racemase
MW1364	MW1364	MW1364	hypothetical protein, similar to GTP binding protein
MW1378	MW1378	lukF	Panton-Valentine leukocidin chain F precursor
MW1379	MW1379	lukS	Panton-Valentine leukocidin chain S precursor
MW1386	MW1386		conserved hypothetical protein (Bacterioophage)
MW1393	MW1393		conserved hypothetical protein (Bacterioophage)
MW1400	MW1400		hypothetical protein (Bacterioophage)
MW1401	MW1401		hypothetical protein (Bacterioophage)
MW1405	MW1405		hypothetical protein (Bacterioophage)
MW1408	MW1408		hypothetical protein (Bacterioophage)
MW1493	MW1493	MW1493	hypothetical protein, similar to competence protein
MW1507	MW1507	MW1507	hypothetical protein, similar to ABC transporter
MW1508	MW1508	MW1508	hypothetical protein, similar to ABC transporter ATP-binding protein
MW1519	MW1519	bex	GTP-binding protain Era homolog
MW1532	MW1532	dnaK	DnaK protein
MW1547	MW1547	aroE	shikimate dehydrogenease
MW1552	MW1552	MW1552	enterotoxin homolog
MW1572	MW1572	MW1572	iron-sulfur cofactor synthesis protein homolog
MW1659	MW1659	MW1659	hypothetical protein similar to iron-sulfur cofactor synthesis protein nifZ
MW1742	MW1742		hypothetical protein
MW1744	MW1744		hypothetical protein
MW1748	MW1748		hypothetical protein
MW1749	MW1749		hypothetical protein
MW1752	MW1752	splF	serine protease
MW1753	MW1753	splC	serine protease
MW1754	MW1754	splB	serine protease
MW1755	MW1755	splA	serine protease
MW1757	MW1757		hypothetical protein, similar to Ear protein (Genomic island)
MW1758	MW1758	bsaG	hypothetical protein, similar to EpiG
MW1759	MW1759	bsaE	hypothetical protein, similar tpo EpiE
MW1760	MW1760	bsaF	hypothetical protein, similar to EpiF
MW1761	MW1761	MW1761	hypothetical protein, similar to EPIDERMIN LEADER PEPTIDE PROCESSING SERINE PROTEASE EPIP PRECURSOR [Genomic island nu Sa beta2]
MW1762	MW1762	bsaD	hypothetical protein, similar to lantibiotic epidermin

			biosynthesis protein EpiD
MW1763	MW1763	bsaC	hypothetical protein, similar to lantibiotic epidermin biosynthesis protein EpiC
MW1764	MW1764	bsaB	hypothetical protein, similar to lantibiotic epidermin biosynthesis protein EpiB
MW1765	MW1765	bsaA2	hypothetical protein, similar to gallidermin precursor
MW1766	MW1766	bsaA1	hypothetical protein, similar to gallidermin precursor
MW1767	MW1767	LukD	leukotoxin
MW1768	MW1768	LukE	leukotoxin
MW1769	MW1769 [Genomic island nu Sa beta2]		hypothetical protein (Genomic island)
MW1798	MW1798	MW1798	glutamate ABC transporter ATP-binding protein
MW1806	MW1806	MW1806	ABC transporter (ATP-binding protein) homolog
MW1811	MW1811	MW1811	hypothetical protein, similar to teichoic acid translocation ATP-binding protein tagH
MW1814	MW1814	sgtB	hypothetical protein, similar to penicillin-binding protein 1A/1B
MW1828	MW1828	map	methionyl aminopeptidase map
MW1834	MW1834	MW1834	hypothetical protein, similar to ferritin
MW1872	MW1872	MW1872	hypothetical protein, similar to ABC transporter, ATP-binding protein
MW1874	MW1874	MW1874	hypothetical protein, similar to ABC transporter, ATP-binding protein
MW1882	MW1882	MW1882	hypothetical protein
MW1885	MW1885	sak	staphylokinase precursor
MW1889	MW1889	sea	staph enterotoxin a
MW1896	MW1896	MW1896	hypothetical protein
MW1897	MW1897	MW1897	hypothetical protein
MW1898	MW1898	MW1898	hypothetical protein
MW1899	MW1899	MW1899	hypothetical protein
MW1900	MW1900	MW1900	hypothetical protein
MW1901	MW1901	MW1901	hypothetical protein
MW1902	MW1902	MW1902	hypothetical protein
MW1903	MW1903	MW1903	hypothetical protein
MW1904	MW1904	MW1904	hypothetical protein
MW1905	MW1905	MW1905	hypothetical protein
MW1906	MW1906	MW1906	portal protein
MW1907	MW1907	MW1907	hypothetical protein
MW1908	MW1908	MW1908	hypothetical protein
MW1909	MW1909	MW1909	hypothetical protein
MW1910	MW1910	MW1910	hypothetical protein
MW1911	MW1911	MW1911	hypothetical protein
MW1912	MW1912	MW1912	hypothetical protein
MW1913	MW1913	MW1913	hypothetical protein
MW1914	MW1914	MW1914	hypothetical protein
MW1915	MW1915	MW1915	hypothetical protein
MW1916	MW1916	MW1916	hypothetical protein
MW1917	MW1917	MW1917	hypothetical protein
MW1921	MW1921	MW1921	single-strand DNA-binding protein
MW1926	MW1926	MW1926	hypothetical protein
MW1929	MW1929	MW1929	hypothetical protein
MW1933	MW1933	MW1933	hypothetical protein
MW1935	MW1935	MW1935	hypothetical protein
MW1937	MW1937	seg	staph enterotoxin g
MW1938	MW1938	sek2	staph enterotoxin k
MW1939	MW1939	int	integrase
MW1941	MW1941	MW1941	hypothetical protein, similar to synergohymenotropic toxin precursor - Staphylococcus intermedius
MW1942	MW1942	MW1942	hypothetical protein similar to leukocidin chain lukM precursor

MW1988	MW1988	sigB	sigma factor B
MW1991	MW1991	rsbU	sigmaB regulation protein RsbU
MW1999	MW1999	kdpC	probable potassium-transporting ATPase C chain
MW2000	MW2000	KdpB	probable potassium-transporting ATPase B chain
MW2002	MW2002	kdpD	sensor protein KdpD
MW2013	MW2013	MW2013	lipoprotein precursor
MW2021	MW2021	MW2021	hypothetical protein, similar to single strand DNA binding protein
MW2079	MW2079	MW2079	hypothetical protein, similar to ABC transporter (ATP-binding protein)
MW2093	MW2093	MW2093	hypothetical protein, similar to multidrug resistance protein
MW2095	MW2095	MW2095	hypothetical protein, similar to multidrug transporter
MW2101	MW2101	MW2101	hypothetical protein similar to ferrichrome ABC transporter (permease)
MW2102	MW2102	MW2102	hypothetical protein similar to ferrichrome ABC transporter (permease)
MW2103	MW2103	MW2103	hypothetical protein similar to ferrichrome ABC transporter (binding prote)
MW2125	MW2125		hypothetical protein, similar to YnaE
MW2129	MW2129	hysA	hyaluronate lyase precursor
MW2140	MW2140	MW2140	hypothetical protein, similar to ABC transporter (ATP-binding protein)
MW2141	MW2141	MW2141	(ATP-binding protein) hypothetical protein, similar to ABC transporter
MW2179	MW2179	MW2179	hypothetical protein, similar to acriflavin resistance protein
MW2202	MW2202	MW2202	hypothetical protein similar to ferrichrome ABC transporter fhuD precursor
MW2217	MW2217	MW2217	secretory antigen precursor SsaA homolog
MW2259	MW2259	MW2259	conserved hypothetical protein
MW2261	MW2261	MW2261	ATP-binding protein) hypothetical protein, similar to ABC transporter
MW2273	MW2273	MW2273	hypothetical protein, similar to multidrug resistance protein
MW2280	MW2280	MW2280	hypothetical protein, similar to ABC transporter, ATP-binding protein
MW2292	MW2292		hypothetical protein, similar to export protein
MW2328	MW2328	MW2328	hypothetical protein similar to Zn-binding lipoprotein adcA
MW2336	MW2336	MW2336	hypothetical protein, similar to ABC transporter, periplasmic amino acid-binding protein
MW2337	MW2337	MW2337	hypothetical protein, similar to multidrug resistance protein
MW2351	MW2351	MW2351	hypothetical protein, similar to ABC transporter, ATP-binding protein
MW2352	MW2352	MW2352	hypothetical protein similar to lipoprotein inner membrane ABC-transporter
MW2368	MW2368	MW2368	hypothetical protein, similar to integral membrane efflux protein
MW2376	MW2376	MW2376	hypothetical protein, similar to chloramphenicol resistance protein
MW2378	MW2378	MW2378	hypothetical protein, similar to ABC transporter (ATP-binding protein)
MW2396	MW2396	MW2396	hypothetical protein, similar to glucose 1-dehydrogenase
MW2403	MW2403	MW2403	hypothetical protein, similar to oxidoreductase
MW2409	MW2409		conserved hypothetical protein
MW2421	MW2421	fnb	fibronectin-binding protein homolog (for fnbA?)
MW2431	MW2431	MW2431	hypothetical protein, similar to glucarate transporter
MW2437	MW2437	MW2437	conserved hypothetical protein
MW2446	MW2446	MW2446	hypothetical protein, similar to ABC transporter (ATP-binding protein)
MW2447	MW2447	MW2447	conserved hypothetical protein
MW2448	MW2448	srtA	sortase
MW2471	MW2471	MW2471	ferrous iron transport protein B homolog
MW2479	MW2479	MW2479	hypothetical protein, similar to mercuric ion-binding protein
MW2498	MW2498	MW2498	hypothetical protein, similar to transcription regulator acrR
MW2499	MW2499	MW2499	hypothetical protein, similar to short chain oxidoreductase

MW2503	MW2503	MW2503	hypothetical protein similar to ferrous iron transporter protein B
MW2534	MW2534		hypothetical protein
MW2543	MW2543	MW2543	hypothetical protein, similar to ABC transporter (ATP-binding protein)
MW2553	MW2553	arc	carbamate kinase
MW2557	MW2557	MW2557	hypothetical protein, similar to arginine repressor
MW2558	MW2558	aur	zinc metalloproteinase aureolysin
MW2565	MW2565	MW2565	hypothetical protein, similar to autolysin precursor
MW2566	MW2566	MW2566	hypothetical protein, similar to N-Carbamoylsarcosine Amidohydrolase
MW2574	MW2574	MW2574	hypothetical protein, similar to preprotein translocase secY
MW2612	MW2612	cna	collagen adhesin precursor
MW2627	MW2627	MW2627	hypothetical protein, similar to DNA-binding protein SpoOJ-like homolog
MWrRNA02	MWrRNA02	MWrRNA02	23S ribosomal RNA (RNA02)
MWrRNA05	MWrRNA05	MWrRNA05	16S ribosomal RNA (RNA05)
nuc	MW1211	nuc	thermonuclease
obg	MW1594	obg	SpoOB-associated GTP-binding protein
oppF		oppF	
pbp3	MW1504	pbp3	penicillin-binding protein 3
pbpA	MW1064	pbpA	penicillin-binding protein 1
pstB	MW1274	pstB	phosphate ABC transporter ATP-binding protein
rlp	MW0184	rlp	RGD-containing lipoprotein
RNAIII	MW1959	RNAIII	from agr (hld)
rot	MW1705	rot	repressor of toxins
SA0022	SA0022		hypothetical protein, similar to 5'-nucleotidase
SA0024	SA0024		hypothetical protein
SA0040	SA0040	mecI	methicillin resistance regulatory protein
SA0041	SA0041	xylR	xylose repressor homologue
SA0047	SA0047		hypothetical protein in transposon Tn554
SA0054	SA0054		hypothetical protein
SA0059	SA0059		hypothetical protein
SA0061	SA0061		hypothetical protein
SA0066	SA0066	kdpE	hypothetical protein, similar to kdp operon transcriptional regulatory protein
SA0072	SA0072		hypothetical protein
SA0073	SA0073		hypothetical protein
SA0074	SA0074		hypothetical protein
SA0076	SA0076		hypothetical protein
SA0077	SA0077		hypothetical protein, similar to serine/threonine protein kinase
SA0102	SA0102		67 kDa Myosin-crossreactive streptococcal antigen homologue
SA0103	SA0103		conserved hypothetical protein
SA0104	SA0104	SA0104	hypothetical protein, similar to transcription regulator GntR family
SA0141	SA0141		hypothetical protein
SA0142	SA0142		hypothetical protein, similar to DNA-binding protein
SA0190	SA0190		hypothetical protein
SA0191	SA0191		conserved hypothetical protein
SA0192	SA0192		hypothetical protein, similar to ABC transporter ATP-binding protein
SA0193	SA0193		hypothetical protein, similar to Enterococcus faecalis plasmid pPD1 bacI
SA0195	SA0195		hypothetical protein
SA0196	SA0196		conserved hypothetical protein
SA0197	SA0197		hypothetical protein, similar to ABC transporter ATP-binding protein
SA0211	SA0211	SA0211	acetyl-CoA acetyltransferase

SA0251	SA0251	lytR	two-component response regulator
SA0276	SA0276		conserved hypothetical protein, similar to diarrheal toxin
SA0294	SA0294	SA0294	hypothetical protein, similar to branched-chain amino acid uptake carrier
SA0298	SA0298	SA0298	hypothetical protein, similar to regulatory protein PfoR
SA0377	SA0377 [Pathogenicity island SaPIn2]		hypothetical protein
SA0378	SA0378 [Pathogenicity island SaPIn2]		hypothetical protein
SA0396	SA0396	lpl1	hypothetical protein
SA0397	SA0397	lpl2	hypothetical protein
SA0398	SA0398	lpl3	hypothetical protein
SA0400	SA0400	lpl4	hypothetical protein
SA0401	SA0401	lpl5	hypothetical protein
SA0404	SA0404	lpl8	hypothetical protein
SA0405	SA0405	lpl9	hypothetical protein
SA0573	SA0573	sarA	staphylococcal accessory regulator A
SA0598	SA0598	pbp4	
SA0610	SA0610		hypothetical protein, similar to lipase LipA
SA0614	SA0614	SA0614	hypothetical protein, similar to two-component response regulator
SA0622	SA0622	SA0622	hypothetical protein, similar to AraC/XylS family transcriptional regulator
SA0627	SA0627	SA0627	hypothetical protein, similar to LysR family transcriptional regulator
SA0641	SA0641	SA0641	conserved hypothetical protein, similar to transcriptional regulator
SA0661	SA0661	saeR	response regulator
SA0675	SA0675		hypothetical protein, similar to ABC transporter ATP-binding protein
SA0702	SA0702	llm	lipophilic protein affecting bacterial lysis rate and methicillin resistance level
SA0704	SA0704		conserved hypothetical protein
SA0726	SA0726	gapR	glycolytic operon regulator
SA0745	SA0745		hypothetical protein, similar to extracellular matrix and plasma binding
SA0836	SA0836	SA0836	hypothetical protein, similar to transcription regulator LysR family
SA0857	SA0857	SA0857	hypothetical protein, similar to negative regulator of genetic competence MecA
SA0904	SA0904	SA0904	hypothetical protein, probable ATL autolysin transcription regulator
SA0907	SA0907 COL	seb	staph enterotoxin b
SA1010	SA1010		hypothetical protein, similar to exotoxin 4
SA1013	SA1013	SA1013	hypothetical protein, similar to carbamate kinase
SA1139	SA1139	glgP	glycerol uptake operon antiterminator regulatory protein
SA1159	SA1159	SA1159	hypothetical protein, similar to two-component response regulator
SA1174	SA1174	lexA	SOS regulatory LexA protein
SA1195	SA1195	SA1195	SA1217 hypothetical protein, similar to negative regulator PhoU
SA1208	SA1208		hypothetical protein
SA1217	SA1217	SA1217	SA1217 hypothetical protein, similar to negative regulator PhoU
SA1247	SA1247	SA1247	truncated (putative response regulator ArlR (truncated-arlR)
SA1248	SA1248	SA1248	truncated (putative response regulator ArlR [S (truncated-arlR)
SA1320	SA1320		hypothetical lipoprotein
SA1329	SA1329	SA1329	ferric uptake regulator homolog
SA1337	SA1337	SA1337	transcription regulator AraC/XylS family homolog
SA1341	SA1341		hypothetical protein, similar to export protein SpcT protein
SA1383	SA1383	SA1383	ferric uptake regulator homolog
SA1516	SA1516	phoP	alkaline phosphatase synthesis transcriptional regulatory protein

SA1539	SA1539	SA1539	hypothetical protein, similar to septation ring formation regulator
SA1552	SA1552		hypothetical protein
SA1557	SA1557	ccpA	catabolite control protein A
SA1580	SA1580		multidrug resistance protein homolog
SA1584	SA1584	SA1584	lysophospholipase homolog
SA1621	SA1621	SA1621	hypothetical protein
SA1628	SA1628	splD	serine protease
SA1632	SA1632	SA1632	hypothetical protein
SA1633	SA1633	SA1633	probable beta-lactamase
SA1635	SA1635 [Pathogenicity island SaPIn3]		hypothetical protein
SA1636	SA1636 [Pathogenicity island SaPIn3]		hypothetical protein
SA1640	SA1640 [Pathogenicity island SaPIn3]		conserved hypothetical protein
SA1641	SA1641 [Pathogenicity island SaPIn3]		hypothetical protein
SA1643	SA1643	sen	staph enterotoxin n
SA1644	SA1644	yent2	enterotoxin
SA1646	SA1646	sei	staph enterotoxin i
SA1647	SA1647	sem	staph enterotoxin m
SA1649	SA1649	SA1649	conserved hypothetical protein
SA1666	SA1666	SA1666	two-component response regulator homolog
SA1676	SA1676	SA1676	hypothetical protein, similar to regulatory protein (pfoS/R)
SA1678	SA1678	SA1678	transcription regulator Fur family homolog
SA1700	SA1700	vraR	two-component response regulator
SA1748	SA1748	SA1748	hypothetical protein, similar to transcription regulator, GntR family
SA1766	SA1766 [Bacteriophage phiN315]		hypothetical protein
SA1768	SA1768	SA1768	hypothetical protein
SA1769	SA1769	SA1769	hypothetical protein
SA1770	SA1770	SA1770	hypothetical protein
SA1771	SA1771	SA1771	hypothetical protein
SA1772	SA1772	SA1772	hypothetical protein
SA1773	SA1773	SA1773	hypothetical protein
SA1774	SA1774	SA1774	hypothetical protein
SA1775	SA1775	SA1775	hypothetical protein, similar to scaffolding protein
SA1776	SA1776	SA1776	hypothetical protein
SA1777	SA1777	SA1777	hypothetical protein
SA1778	SA1778	SA1778	hypothetical protein
SA1791]	SA1791		hypothetical protein [Bacteriophage phiN315]
SA1794	SA1794		hypothetical protein [Bacteriophage phiN315]
SA1795	SA1795		hypothetical protein [Bacteriophage phiN315]
SA1797	SA1797	SA1797	hypothetical protein
SA1800	SA1800	SA1800	hypothetical protein
SA1804	SA1804	SA1804	hypothetical transcriptional regulator
SA1805	SA1805	SA1805	repressor homolog
SA1806	SA1806]		probable ATP-dependent helicase [Bacteriophage phiN315
SA1808	SA1808	SA1808	probable ss-1,3-N-acetylglucosaminyltransferase
SA1809	SA1809	SA1809	hypothetical protein
SA1819	SA1819	tsst-1	toxic shock syndrome toxin-1
SA1822	SA1822 [Pathogenicity island SaPIn1]		hypothetical protein
SA1826	SA1826 [Pathogenicity island SaPIn1]		hypothetical protein
SA1828	SA1828 [Pathogenicity island SaPIn1]		hypothetical protein
SA1829	SA1829 [Pathogenicity island SaPIn1]		hypothetical protein
SA1833	SA1833	SA1833	hypothetical protein, similar to transcription regulator
SA1834	SA1834 [Pathogenicity island SaPIn1]		hypothetical protein

SA1883	SA1883	kdpE	KDP operon transcriptional regulatory protein KdpE
SA1888	SA1888	SA1888	hypothetical protein, similar to rod shape determining protein RodA
SA1949	SA1949	SA1949	lytic regulatory protein truncated with Tn554 (truncated-SA)
SA1953	SA1953	tnpC	transposition regulatory protein
SA1954	SA1954	tnpB	transposition regulatory protein
SA1955	SA1955	tnpA	transposition regulatory protein
SA1956	SA1956	SA1956	lytic regulatory protein truncated with Tn554
SA1970	SA1970		hypothetical protein, similar to multidrug resistance protein
SA1999	SA1999	SA1999	hypothetical protein, similar to regulatory protein, SIR2 family
SA2002	SA2002	SA2002	hypothetical protein, similar to transcription regulator MerR family
SA2004	SA2004		conserved hypothetical protein
SA2005	SA2005		conserved hypothetical protein
SA2010	SA2010		hypothetical protein, similar to RNA-directed DNA polymerase from retron EC86
SA2011	SA2011		hypothetical protein
SA2012	SA2012		hypothetical protein
SA2060	SA2060	SA2060	hypothetical protein, similar to transcription regulator MarR family
SA2063	SA2063	moaA	molybdenum cofactor biosynthesis protein A
SA2092	SA2092	SA2092	hypothetical protein, similar to transcription regulator
SA2108	SA2108	SA2108	hypothetical protein, similar to transcription regulator, RpiR family
SA2115	SA2115	SA2115	hypothetical protein, similar to transcriptional regulator
SA2119	SA2119	SA2119	hypothetical protein, similar to dehydrogenase
SA2123	SA2123	SA2123	hypothetical protein, similar to transcription regulator LysR family
SA2124	SA2124	fosB	fosfomycin resistance protein fofB
SA2144	SA2144	SA2144	hypothetical protein, similar to transcriptional regulator (TetR/AcrR family)
SA2147	SA2147	tcaR	TcaR transcription regulator
SA2151	SA2151	SA2151	hypothetical protein, similar to two component response regulator
SA2165	SA2165	SA2165	hypothetical protein, similar to transcriptional regulator tetR-family
SA2169	SA2169	SA2169	hypothetical protein, similar to transcription regulatory protein
SA2174	SA2174	SA2174	hypothetical protein, similar to transcriptional regulator
SA2178	SA2178	SA2178	hypothetical protein, similar to transcriptional regulator
SA2259	SA2259		conserved hypothetical protein
SA2272	SA2272		hypothetical protein
SA2286	SA2286	sarH3	staphylococcal accessory regulator A homolog
SA2296	SA2296	SA2296	hypothetical protein, similar to transcriptional regulator, MerR family
SA2308	SA2308	SA2308	hypothetical protein, similar to transcription regulator MarR family
SA2314	SA2314		hypothetical protein, similar to ABC transporter (ATP-binding protein)
SA2320	SA2320	SA2320	hypothetical protein, similar to regulatory protein pfoR
SA2330	SA2330	SA2330	hypothetical protein, similar to transcription regulator
SA2340	SA2340	SA2340	hypothetical protein, similar to transcriptional regulator tetR-family
SA2357	SA2357	SA2357	hypothetical protein, similar to regulatory protein (pfoS/R)
SA2379	SA2379	SA2379	hypothetical protein, similar to transcriptional regulator tetR-family
SA2382	SA2382	SA2382	truncated hypothetical protein, similar to glutamyl-endopeptidase
SA2389	SA2389	SA2389	truncated hypothetical protein, similar to metalloproteinase mpr precursor
SA2418	SA2418	SA2418	hypothetical protein, similar to two-component response regulator
SA2421	SA2421	SA2421	hypothetical protein, similar to transcriptional regulator
SA2424	SA2424	SA2424	hypothetical protein, similar to transcription regulator Crp/Fnr

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family protein

SA2458icaR	SA2458	icaR	ica operon transcriptional regulator IcaR
SA2472	SA2472	SA2472	hypothetical protein, similar to ATP phosphoribosyltransferase regulatory subunit
SA2483	SA2483		hypothetical protein
SA2500gidA	SA2500	gidA	glucose inhibited division protein A
saeR	MW0668	saeR	response regulator
sarR	MW2213	sarR	staphylococcal accessory regulator A homolog
SAV0149	SAV0149	capA	capsular polysaccharide synthesis enzyme Cap5A
SAV0150	SAV0150	capB	capsular polysaccharide synthesis enzyme Cap5B
SAV0151	SAV0151	capC	capsular polysaccharide synthesis enzyme Cap8C
SAV0153	SAV0153	capE	capsular polysaccharide synthesis enzyme Cap8E
SAV0154	SAV0154	capF	capsular polysaccharide synthesis enzyme Cap5F
SAV0155	SAV0155	capG	capsular polysaccharide synthesis enzyme Cap5G
SAV0156	SAV0156	capH	capsular polysaccharide synthesis enzyme O-acetyl transferase Cap5H
SAV0157	SAV0157	capI	capsular polysaccharide synthesis enzyme Cap5I
SAV0158	SAV0158	capJ	capsular polysaccharide synthesis enzyme Cap5J
SAV0159	SAV0159	capK	capsular polysaccharide synthesis enzyme Cap5K
SAV0160	SAV0160	capL	capsular polysaccharide synthesis enzyme Cap5L
SAV0161	SAV0161	capM	capsular polysaccharide synthesis enzyme Cap5M
SAV0162	SAV0162	capN	capsular polysaccharide synthesis enzyme Cap5N
SAV0163	SAV0163	capO	capsular polysaccharide synthesis enzyme Cap8O
SAV0164	SAV0164	capP	capsular polysaccharide synthesis enzyme Cap5P
SAV0195	SAV0195	hsdR	probable type I restriction enzyme restriction chain
SAV0398	SAV0398	tetM	tetracycline resistance protein
SAV0416	SAV0416	SAV0416	hypothetical protein
SAV0417	SAV0417	SAV0417	hypothetical protein
SAV0418	SAV0418	SAV0418	probable transposase
SAV0422	SAV0422	set6	exotoxin 6
SAV0423	SAV0423	set7	exotoxin 7
SAV0424	SAV0424	set8	exotoxin 8
SAV0425	SAV0425	set10	exotoxin10
SAV0426	SAV0426	set11	exotoxin11
SAV0427	SAV0427	set12	exotoxin 12
SAV0428	SAV0428	set13	exotoxin 13
SAV0429	SAV0429	set14	exotoxin 14
SAV0431	SAV0431	hsdM	probable type I site-specific deoxyribonuclease LldI chain
SAV0432	SAV0432	hsdS	probable restriction modification system specificity subunit c
SAV0433	SAV0433	set15	exotoxin 15
SAV0993	SAV0993	appF	oligopeptide transport system ATP-binding protein AppF homologue
SAV1113	SAV1113	SAV1113	conserved hypothetical protein
SAV1119	SAV1119	vacA	putative vacuolating cytotoxin paralog
SAV1622	SAV1622	thdF	possible thiophene and furan oxidation protein
SAV1827	SAV1827	yent1	enterotoxin
SAV1830	SAV1830	seo	enterotoxin
SAV1948	SAV1948	sep	enterotoxin P
SAV2009	SAV2009	sec3	enterotoxin typeC3
SAV2352	SAV2352	SAV2352	hypothetical protein
SAV2499	SAV2499	sarH2	staphylococcal accessory regulator A homolog
SAV2662	SAV2662	SAV2662	capsular polysaccharide biosynthesis
SAV2663	SAV2663	SAV2663	capsular polysaccharide biosynthesis
SAV2664	SAV2664	SAV2664	capsular polysaccharide biosynthesis
SAV2667	SAV2667	icaD	intercellular adhesion protein D

SAV2668	SAV2668	icaB	intercellular adhesion protein B
SAV2669	SAV2669	icaC	intercellular adhesion protein C
sbi	MW2341	sbi	IgG binding protein
sdhB	MW1032	sdhB	succinate dehydrogenase iron-sulfur protein subunit (MW2)
sdrC	MW0516	sdrC	Ser-Asp rich fibrinogen-binding bone sialoprotein-binding protein
sdrD	MW0517	sdrD	Ser-Asp rich fibrinogen-binding bone sialoprotein-binding protein
sdrE	MW0518	sdrE	Ser-Asp rich fibrinogen-binding bone sialoprotein-binding protein
sec4	MW0759	sec4	staph enterotoxin c
sed	Plasmid pIB485	sed	staph enterotoxin d
see	FRI918	see	staph enterotoxin e
sej	Plasmid pIB485	sej	staph enterotoxin j
sirA	MW0088	sirA	lipoprotein
sirB	MW0087	sirB	lipoprotein
sirC	MW0086	sirC	lipoprotein
spa	MW0084	spa	IgG binding protein A
ssb	MW0342	ssb	single-strand DNA-binding protein of phage phi Sa 2mw
sspA	MW0932	sspA	serine protease V8 protease
sspB	MW0931	sspB	cysteine protease precursor
sspC	MW0930	sspC	cysteine protease
ssrP	MW0743	ssrP	ssrA-binding protein
tagH	MW0599	tagH	teichoic acid translocation ATP-binding protein
taxon:1280	taxon:1280	chp	chemotaxis inhibitory protein
vga	MW1971	vga	hypothetical ABC transporter ATP-binding protein
Type I-F	Type I-F		0.048
Type I-R	Type I-R		
Type II-F	Type II-F		0.032
Type II-R	Type II-R		
Type III-F	Type III-F		0.04
Type III-R	Type III-R		
Type IVa-F	Type IVa-F		0.104
Type IVa-R	Type IVa-R		
Type IVb-F	Type IVb-F		0.092
Type IVb-R	Type IVb-R		
Type IVc-F	Type IVc-F		0.078
Type IVc-R	Type IVc-R		
Type IVd-F5	Type IVd-F5		0.28
Type IVd-R6	Type IVd-R6		
Type V-F	Type V-F		0.06
Type V-R	Type V-R		
MecA147-F	MecA147-F		0.046
MecA147-R	MecA147-R		
mecI-F	mecI-F		0.08
mecI-R	mecI-R		
IS1272-F	IS1272-F		0.08
mecR1-R	mecR1-R		
ccrAB-ß2	ccrAB-ß2		0.08
c̃crAB-2	c̃crAB-2		0.08
c̃crAB-3	c̃crAB-3		0.08
c̃crAB-4	c̃crAB-4		0.08
ccrC-F	ccrC-F		0.08
ccrC-R	ccrC-R		
MW0251	SAV1661/SA1486/MW1605	family A24A unassigned peptidases	

MW1017	SAV0400	family C40 unassigned peptidases (SAV0400 protein)	
MW1390	SAV1909/MW1850/SA1725	staphopain A	stp
MW1482	SAV0913	D-alanyl-glycyl peptidase (staphylococcal phage phi11) (LytA protein)	lytA
MW1501	SAV1247/SA1090	D-alanyl-glycyl peptidase (staphylococcal phage phi11) (LytN protein)	lytN
MW1895	SAV0909	family C51 unassigned peptidases (SAV0909 protein)	
MW1936	SAV2690	pyroglutamyl-peptidase I (prokaryote) (SAV2690 protein)	pcp
SA0509	MW1017	sortase B	srtB
SA0668	SAV1384/MW1272	oligopeptidase F	
SA0879	SAV1000	oligopeptidase F	
SA1360	SAV0511	FtsH-2 peptidase	ftsH
SA1814	SAV1279/MW1162	subfamily M16C unassigned peptidases (SAV1279 protein)	
	SAV0942	family M17 unassigned peptidases (SAV0942 protein)	ampA
SAV0012	SAV1708/MW1651	Mername-AA019 peptidase	
SAV0102			
SAV0183	SAV1529	subfamily M24B non-peptidase homologues (SAV1529 protein)	
SAV0209	MW1482	subfamily M24B unassigned peptidases (MW1482 protein)	
	SA1360/SAV1530	subfamily M24B unassigned peptidases (SA1360 protein)	
SAV0212	SA1814/MW1943	subfamily M20A unassigned peptidases (SAV2006 protein)	
SAV0276	SAV1751/MW1694	subfamily M20A unassigned peptidases (SAV1751 protein)	
SAV0400	SAV0743/SA0698	peptidase T	pepT
SAV0457	SAV1512/MW1465	subfamily M20B unassigned peptidases (SAV1512 protein)	
SAV0511	SAV2133/SA1935	subfamily M20D non-peptidase homologues (HmrA protein)	hmrA
SAV0520	SAV0549/MW0504	family M20D unassigned peptidases (SAV0549 protein)	
SAV0526	SAV1398/MW1286	family M20D unassigned peptidases (hippurate hydrolase)	hipO
SAV0549	SAV2329/SA2120/MW2250	family M20D unassigned peptidases (SAV2329 protein)	
SAV0612	SAV0102/SA0098/MW0078	family M20D unassigned peptidases (SAV0102 protein)	
SAV0743	SAV1366/MW1253	family M42 unassigned peptidases (SAV1366 protein)	
SAV0768	SAV2455/SA2244/MW2379	family M42 unassigned peptidases (SAV2455 protein)	
SAV0909	SAV1745/SA1566/MW1688	family M42 unassigned peptidases (SAV1745 protein)	
SAV0913	SAV1201	dihydro-orotase	pyrC
SAV0942	SAV2051/MW1975	family M22 non-peptidase homologues (SAV2051 protein)	
SAV0965	SAV2049/SA1854/MW1973	family M22 unassigned peptidases (SAV2049 protein)	
SAV1000	SAV1262/MW1145	RseP peptidase	
SAV1044	SAV0276/SA0265	lysostaphin (peptidoglycan hydrolase)	lytM
SAV1057			
SAV1068	MW1895	subfamily M23B unassigned peptidases (MW1895 protein)	

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SAV1070	MW1390	subfamily M23B unassigned peptidases (MW1390 protein)
SAV1201	SAV0212/SA0205/MW0188	subfamily M23B unassigned peptidases (SAV0212 protein)
SAV1247	SAV1879	aminopeptidase S (*Staphylococcus aureus*) (aminopeptidase ampS) ampS
SAV1253	SAV1811/SA1629	SplC g.p. (*Staphylococcus aureus*) (m-type 2) splC
SAV1262	SAV1813/SA1631	subfamily S1B unassigned peptidases (exoprotein A) splA
SAV1279	SAV1810/SA1628	subfamily S1B unassigned peptidases (serine protease splf) splD/splF
SAV1366	SAV1728	subfamily S1C unassigned peptidases (SAV1728 protein)
SAV1384	SA0879/MW0903	subfamily S1C unassigned peptidases (SA0879 protein) htrA
SAV1398	SAV2596/SA2389	family S1 unassigned peptidases (SAV2596 protein)
SAV1420	SAV1070	amidophosphoribosyltransferase precursor purF
SAV1512	SAV2154	glucosamine-fructose-6-phosphate aminotransferase glmS
SAV1529	MW0251/SA0264	family C59 non-peptidase homologues
SAV1612	SAV1253	CodW component of CodWX peptidase clpQ
SAV1613	SAV0209/MW0185	gamma-glutamyltransferase 1 (bacterial)
SAV1661	SA0668	family C26 unassigned peptidases
SAV1708	SAV2675/SA2467	family C56 non-peptidase homologues (imidazole glycerol phosphate synthase subunit HisH) hisH
SAV1728	SAV0520/SA0478/MW0475	family C56 non-peptidase homologues
SAV1745	SAV1068/MW0951	family C56 non-peptidase homologues (phosphoribosylformylglycinamidine synthase I) purQ
SAV1751	SAV1875	family C56 non-peptidase homologues (orf 1 protein)
SAV1765	SA0509/MW0506	family C56 unassigned peptidases (SA0509 protein) HCHA
SAV1793	SAV2451/MW2375	subfamily S9C non-peptidase homologues (SAV2451 protein)
SAV1810	SAV1793/SA1611/MW1731	subfamily S9C unassigned peptidases
SAV1811	SAV0612/SA0569/MW0576	family S33 non-peptidase homologues
SAV1813	SAV1765	family S33 non-peptidase homologues
SAV1875	SAV0012/SA0011/MW0012	family S33 unassigned peptidases
SAV1879	SAV0457/SA0416	family S33 unassigned peptidases

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SAV1909	SAV1044/SA0897/MW0928	family S33 unassigned peptidases	
SAV1964	SAV2441/MW2365	family S12 unassigned peptidases (SAV2441 protein)	flp
SAV1998	SAV1057	family S12 unassigned peptidases (Fmt protein)	fmt
SAV2049	SAV1998	family S24 unassigned peptidases (SAV1998 protein)	
SAV2051	MW1936	family S24 unassigned peptidases	
SAV2133	SAV0965	signal peptidase SpsB (*Staphylococcus aureus*) (SpsB protein)	spsB
SAV2154	SAV0526/SA0484/MW0481	family S16 non-peptidase homologues	radA
SAV2329	SAV0768	peptidase Clp (type 1)	clpP
SAV2441	SAV1964	family S14 non-peptidase homologues (SAV1964 protein)	
SAV2451	SAV1420	subfamily S41A unassigned peptidases (SAV1420 protein)	ctpA
SAV2455	MW1501/SAV1549/SA1379	family S54 unassigned peptidases (SAV1549 protein)	
SAV2596	SAV0183/SA0177/MW0157	ornithine acetyltransferase precursor	argJ
SAV2675	SAV1613/MW1563	family U32 unassigned peptidases (SAV1613 protein)	
SAV2690	SAV1612/MW1562	family U32 unassigned peptidases (SAV1612 protein)	

Appendix IV. Genes present by *S. aureus* D76 as analysed by comparative genome hybridisation.

Gene ID	Name	Function
Bbp	bbp	sdrE homolog (bone sialoprotein binding protein)
clfa	Clfa	fibrinogen-binding protein
clfb	clfb	clumping factor B
Coa	Coa	staphylcoagulase precursor
comGC	comGC	exogenous DNA-binding protein
E161084c	E161084c	V8PROTEASE
E16-1949	E16-1949	BLACTAMASEA
E16-2023c	E16-2023c	V8PROTEASE
E162040c	E162040c	BACTRLTOXIN
E16-2042c	E16-2042c	BACTRLTOXIN
E162902c	E162902c	THERMOLYSIN
ebhA	ebhA	similar to streptococcal adhesin emb
ebhB	ebhB	similar to streptococcal adhesin emb
efb	efb	fibrinogen-binding protein
fhuA	fhuA	ferrichrome transport ATP-binding protein
fhuB	fhuB	ferrichrome transport permease
fnbB	fnbB	fibronectin-binding protein homolog
geh	geh	glycerol ester hydrolase
gmk	gmk	guanylate kinase
hla	hla	alpha toxin; alpha haemolysin
hlb	hlb	beta haemolysin
hlgB	hlgB	gamma-hemolysin component B
hlgC	hlgC	gamma-hemolysin component C
hlgII	hlgII	gamma haemolysin chain II
hu	hu	DNA-binding protein II
icaA	icaA	intercellular adhesion protein A
lepA	lepA	GTP-binding protein
lip	lip	triacylglycerol lipase precursor
lsp	lsp	lipoprotein signal peptidase
modA	modA	probable molybdate-binding protein

Gene ID	Name	Function
modC	modC	molybdenum transport ATP-binding protein
msmX	msmX	multiple sugar-binding transport ATP-binding protein
MW0053	MW0053	hypothetical protein
MW0074	MW0074	hypothetical protein
MW0077	MW0077	hypothetical protein, similar to transcription regulator AraC/XylS family
MW0079	MW0079	hypothetical protein similar to transmembrane efflux pump protein
MW0085	MW0085	staphylococcal accessory regulator A homologue
MW0092	MW0092	hypothetical protein similar to multi-drug resistance efflux pump
MW0105	MW0105	hypothetical protein, similar to capsular polysaccharide synthesis protein 14H
MW0108	MW0108	hypothetical protein
MW0111	MW0111	hypothetical protein similar to tetracyclin resistance protein
MW0120	MW0120	truncated replication initiator protein
MW0147	MW0147	hypothetical protein similar to membrane lipoprotein SrpL
MW0161	MW0161	hypothetical protein, similar to isochorismatase
MW0167	MW0167	hypothetical protein, similar to transcription regulator
MW0171	MW0171	hypothetical protein
MW0172	MW0172	hypothetical protein, similar to ABC transporter ATP-binding protein
MW0178	MW0178	hypothetical protein, similar to ABC transporter ATP-binding protein
MW0182	MW0182	truncated hypothetical protein, similar to oligopeptide ABC transporter permease
MW0190	MW0190	hypothetical protein, similar to maltose/maltodextrin-binding protein
MW0198	MW0198	hypothetical protein, similar to two-component response regulator
MW0200	MW0200	hypothetical protein similar to periplasmic-iron-binding protein BitC
MW0213	MW0213	hypothetical protein, similar to nickel ABC transporter nickel-binding protein
MW0240	MW0240	hypothetical protein, similar to transcription regulator GntR family
MW0250	MW0250	hypothetical protein, similar to proton antiporter efflux pump
MW0251	MW0251	family A24A unassigned peptidases
MW0263	MW0263	conserved hypothetical protein, similar to diarrheal toxin incomplete ORF
MW0283	MW0283	hypothetical protein, similar to branched-chain amino acid uptake carrier
MW0286	MW0286	hypothetical protein, similar to ABC transporter ATP-binding protein
MW0310	MW0310	hypothetical protein, similar to transcription regulator
MW0311	MW0311	conserved hypothetical protein
MW0323	MW0323	conserved hypothetical protein
MW0327	MW0327	hypothetical protein, similar to ABC transporter ATP-binding protein
MW0339	MW0339	hypothetical protein, similar to GTP-binding protein

Gene ID	Name	Function
MW0345	MW0345	hypothetical protein, similar to exotoxin 2
MW0355	MW0355	hypothetical protein
MW0368	MW0368	hypothetical protein, similar to AbiD1 [Genomic island nu Sa alpha2]
MW0372	MW0372	GenomicislandnuSaalpha2
MW0381	MW0381	conserved hypothetical protein MW0381 [Genomic island nu Sa alpha2]
MW0382	MW0382	exotoxin 16
MW0383	MW0383	exotoxin 17
MW0384	MW0384	exotoxin 18
MW0385	MW0385	exotoxin 19
MW0386	MW0386	exotoxin 20
MW0388	MW0388	exotoxin 22
MW0389	MW0389	exotoxin 23
MW0390	MW0390	exotoxin 24
MW0391	MW0391	exotoxin 25
MW0396	MW0396	hypothetical protein
MW0400:lp13	MW0400:lp13	hypothetical protein
MW0416	MW0416	hypothetical protein, similar to ABC transporter ATP-binding protein
MW0418	MW0418	hypothetical protein similar to lactococcal lipoprotein
MW0513	MW0513	hypothetical protein, similar to Cu binding protein (Mn oxidation)
MW0531:yqiL	MW0531:yqiL	acetyl-CoA c-acetyltransferase
MW0543	MW0543	phosphotransacetylase
MW0553	MW0553	conserved hypothetical protein
MW0554	MW0554	putative primase
MW0558	MW0558	conserved hypothetical protein
MW0559	MW0559	conserved hypothetical protein
MW0560	MW0560	hypothetical protein
MW0573	MW0573	hypothetical protein similar to iron-binding protein
MW0574	MW0574	hypothetical protein similar to iron(III) ABC transporter permease protein
MW0579	MW0579	hypothetical protein similar to esterase/lipase
MW0593	MW0593	lipoprotein, Streptococcal adhesin PsaA homologue
MW0595	MW0595	hypothetical protein, similar to ABC transporter ATP-binding protein
MW0596	MW0596	hypothetical protein similar to iron dependent repressor
MW0617	MW0617	hypothetical protein similar to lipase LipA
MW0623:vraF	MW0623:vraF	ABC transporter ATP-binding protein

Gene ID	Name	Function
MW0651	MW0651	hypothetical protein
MW0681	MW0681	hypothetical protein, similar to anion-binding protein
MW0682	MW0682	hypothetical protein, similar to ABC transporter ATP-binding protein
MW0695	MW0695	hypothetical protein similar to ferrichrome ABC transporter permease
MW0696	MW0696	hypothetical protein similar to ferrichrome ABC transporter permease
MW0697	MW0697	hypothetical protein similar to ferrichrome ABC transporter ATP-binding protein
MW0698	MW0698	lipoprotein, similar to ferrichrome ABC transporter
MW0736:tpi	MW0736:tpi	triosephosphate isomerase
MW0746	MW0746	hypothetical protein [GenomicislandnuSaalpha3mw]
MW0751	MW0751	hypothetical protein [GenomicislandnuSaalpha3mw]
MW0754	MW0754	hypothetical protein [GenomicislandnuSaalpha3mw]
MW0755	MW0755	hypothetical protein [GenomicislandnuSaalpha3mw]
MW0756	MW0756	hypothetical protein [GenomicislandnuSaalpha3mw]
MW0765	MW0765	truncated secreted von Willebrand factor-binding protein
MW0766	MW0766	truncated secreted von Willebrand factor-binding protein
MW0767:ssp	MW0767:ssp	extracellular ECM and plasma binding protein
MW0790	MW0790	ABC transporter ATP-binding protein homologue
MW0795	MW0795	ABC transporter ATP-binding protein homologue
MW0871:OppF	MW0871:OppF	oligopeptide transport ATP-binding protein
MW0872	MW0872	hypothetical protein, similar to peptide binding protein OppA
MW0873	MW0873	hypothetical protein, similar to oligopeptide ABC transporter oligopeptide-binding protein
MW0874	MW0874	oligopeptide ABC transporter ATP-binding protein homologue
MW0875:AppF	MW0875:AppF	oligopeptide transport system ATP-binding protein AppF homologue
MW0897	MW0897	hypothetical protein, similar to multidrug resistance protein-related protein
MW0921	MW0921	hypothetical protein similar to ferrichrome ABC transporter
MW0962	MW0962	hypothetical protein
MW0982	MW0982	spermidine/putrescine ABC transporter, ATP-binding protein homolog
MW0985	MW0985	spermidine/putrescine-binding protein precursor homolog
MW0986	MW0986	conserved hypothetical protein
MW1015	MW1015	hypothetical protein similar to ferrichrome ABC transporter
MW1016	MW1016	hypothetical protein similar to ferrichrome ABC transporter
MW1017	MW1017	family C40 unassigned peptidases (SAV0400 protein)
MW1040	MW1040	fibrinogen-binding protein
MW1041	MW1041	hypothetical protein, similar to fibrinogen-binding protein

Gene ID	Name	Function
MW1047	MW1047	hypothetical protein, similar to exotoxin 1
MW1048	MW1048	hypothetical protein, similar to exotoxin 4
MW1091	MW1091	hypothetical protein, similar to fibrinogen binding protein
MW1160	MW1160	hypothetical protein, similar to transcription regulator GntR family
MW1163	MW1163	hypothetical protein, similar to 3-oxoacyl- acyl-carrier protein reductase homolog ymfI
MW1182:glpF	MW1182:glpF	glycerol uptake facilitator
MW1185	MW1185	hypothetical protein similar to lysophospholipase
MW1189	MW1189	hypothetical protein, similar to GTP-binding protein proteinase modulator homolog ynbA
MW1206	MW1206	ABC transporter (ATP-binding protein) homolog
MW1261:femA	MW1261:femA	factor essential for expression of methicillin resistance
MW1262:femB	MW1262:femB	FemB protein
MW1280	MW1280	ABC transporter (ATP-binding protein) homolog
MW1287	MW1287	hypothetical protein, similar to alanine racemase
MW1364	MW1364	hypothetical protein, similar to GTP binding protein
MW1386	MW1386	conserved hypothetical protein [BacteriophagephiSa2mw]
MW1390:stp	MW1390:stp	stp staphopain A
MW1393	MW1393	conserved hypothetical protein [BacteriophagephiSa2mw]
MW1400	MW1400	hypothetical protein [BacteriophagephiSa2mw]
MW1401	MW1401	hypothetical protein [BacteriophagephiSa2mw]
MW1405	MW1405	hypothetical protein [BacteriophagephiSa2mw]
MW1408	MW1408	hypothetical protein [BacteriophagephiSa2mw]
MW1482: lytA	MW1482: lytA	D-alanyl-glycyl peptidase (staphylococcal phage phi11) (LytA protein)
MW1493	MW1493	hypothetical protein, similar to competence protein
MW1501:lytN	MW1501:lytN	D-alanyl-glycyl peptidase (staphylococcal phage phi11) (LytN protein)
MW1508	MW1508	hypothetical protein, similar to ABC transporter ATP-binding protein
MW1519:bex	MW1519:bex	GTP-binding protain Era homolog
MW1532:dnaK	MW1532:dnaK	DnaK protein
MW1547:aroE	MW1547:aroE	shikimate dehydrogenease
MW1552	MW1552	enterotoxin homolog
MW1572	MW1572	iron-sulfur cofactor synthesis protein homolog
MW1659	MW1659	hypothetical protein similar to iron-sulfur cofactor synthesis protein nifZ
MW1752:splF	MW1752:splF	serine protease
MW1753:splC	MW1753:splC	serine protease
MW1754:splB	MW1754:splB	serine protease

Gene ID	Name	Function
MW1755:splA	MW1755:splA	serine protease
MW1757	MW1757	hypothetical protein, similar to Ear protein (GenomicislandnuSabeta2)
MW1758:bsaG	MW1758:bsaG	hypothetical protein, similar to EpiG (Genomic island nu Sa beta2)
MW1759:bsaE	MW1759:bsaE	hypothetical protein, similar tpo EpiE
MW1760:bsaF	MW1760:bsaF	hypothetical protein, similar to EpiF (Genomic island nu Sa beta2)
MW1761	MW1761	hypothetical protein, similar to EPIDERMIN LEADER PEPTIDE PROCESSING SERINE PROTEASE EPIP PRECURSOR [Genomic island nu Sa beta2]
MW1762:bsaD	MW1762:bsaD	hypothetical protein, similar to lantibiotic epidermin biosynthesis protein EpiD
MW1763:bsaC	MW1763:bsaC	hypothetical protein, similar to lantibiotic epidermin biosynthesis protein EpiC
MW1764:bsaB	MW1764:bsaB	hypothetical protein, similar to lantibiotic epidermin biosynthesis protein EpiB
MW1765	MW1765	hypothetical protein, similar to gallidermin precursor
MW1766:bsaA1	MW1766:bsaA1	hypothetical protein, similar to gallidermin precursor
MW1767:LukD	MW1767:LukD	leukotoxin
MW1768:LukE	MW1768:LukE	leukotoxin
MW1769	MW1769	hypothetical protein [GenomicislandnuSabeta2]
MW1798	MW1798	glutamate ABC transporter ATP-binding protein
MW1806	MW1806	ABC transporter (ATP-binding protein) homolog
MW1811	MW1811	hypothetical protein, similar to teichoic acid translocation ATP-binding protein tagH
MW1814:sgtB	MW1814:sgtB	hypothetical protein, similar to penicillin-binding protein 1A/1B
MW1828:map	MW1828:map	methionyl aminopeptidase map
MW1834	MW1834	hypothetical protein, similar to ferritin
MW1872	MW1872	hypothetical protein, similar to ABC transporter, ATP-binding pro
MW1874	MW1874	hypothetical protein, similar to ABC transporter, ATP-binding protein
MW1882	MW1882	hypothetical protein
MW1885:sak	MW1885:sak	staphylokinase precursor
MW1895	MW1895	subfamily M23B unassigned peptidases (MW1895 protein)
MW1896	MW1896	hypothetical protein
MW1897	MW1897	hypothetical protein
MW1898	MW1898	hypothetical protein
MW1899	MW1899	hypothetical protein
MW1900	MW1900	hypothetical protein
MW1901	MW1901	hypothetical protein
MW1902	MW1902	hypothetical protein
MW1903	MW1903	hypothetical protein

Gene ID	Name	Function
MW1904	MW1904	hypothetical protein
MW1905	MW1905	hypothetical protein
MW1906	MW1906	portal protein (phage portal protein)
MW1907	MW1907	hypothetical protein
MW1908	MW1908	hypothetical protein
MW1909	MW1909	hypothetical protein
MW1910	MW1910	hypothetical protein
MW1911	MW1911	hypothetical protein
MW1912	MW1912	hypothetical protein
MW1913	MW1913	hypothetical protein
MW1915	MW1915	hypothetical protein
MW1916	MW1916	hypothetical protein
MW1917	MW1917	hypothetical protein
MW1926	MW1926	hypothetical protein
MW1929	MW1929	hypothetical protein
MW1936: pcp	MW1936: pcp	pcp pyroglutamyl-peptidase I (prokaryote) (SAV2690 protein)
MW1939:int	MW1939:int	integrase (phage integrase family)
MW1941	MW1941	hypothetical protein, similar to synergohymenotropic toxin precursor - *Staphylococcus intermedius*
MW1942	MW1942	hypothetical protein similar to leukocidin chain lukM precursor
MW1988:sigB	MW1988:sigB	sigma factor B
MW1991:rsbU	MW1991:rsbU	sigmaB regulation protein RsbU
MW1999:kdpC	MW1999:kdpC	probable potassium-transporting ATPase C chain
MW2000:KdpB	MW2000:KdpB	probable potassium-transporting ATPase B chain
MW2002:KdpD	MW2002:KdpD	sensor protein KdpD (sensor histidine kinase, OmpR family)
MW2013	MW2013	lipoprotein precursor
MW2021	MW2021	hypothetical protein, similar to single strand DNA binding protein
MW2079	MW2079	hypothetical protein, similar to ABC transporter (ATP-binding protein)
MW2093	MW2093	hypothetical protein, similar to multidrug resistance protein
MW2095	MW2095	hypothetical protein, similar to multidrug transporter
MW2101	MW2101	hypothetical protein similar to ferrichrome ABC transporter (permease)
MW2102	MW2102	hypothetical protein similar to ferrichrome ABC transporter (permease)
MW2103	MW2103	hypothetical protein similar to ferrichrome ABC transporter (binding prote)
MW2129:hysA	MW2129:hysA	hyaluronate lyase precursor

Gene ID	Name	Function
MW2140	MW2140	hypothetical protein, similar to ABC transporter (ATP-binding protein)
MW2141	MW2141	(ATP-binding protein) hypothetical protein, similar to ABC transporter
MW2179	MW2179	hypothetical protein, similar to acriflavin resistance protein
MW2202	MW2202	hypothetical protein similar to ferrichrome ABC transporter fhuD precursor
MW2217	MW2217	secretory antigen precursor SsaA homolog
MW2259	MW2259	conserved hypothetical protein
MW2261	MW2261	ATP-binding protein, hypothetical protein, similar to ABC transporter
MW2273	MW2273	hypothetical protein, similar to multidrug resistance protein
MW2280	MW2280	hypothetical protein, similar to ABC transporter, ATP-binding protein
MW2328	MW2328	hypothetical protein similar to Zn-binding lipoprotein adcA
MW2336	MW2336	hypothetical protein, similar to ABC transporter, periplasmic amino acid-binding protein
MW2337	MW2337	hypothetical protein, similar to multidrug resistance protein
MW2351	MW2351	hypothetical protein, similar to ABC transporter, ATP-binding protein
MW2352	MW2352	hypothetical protein similar to lipoprotein inner membrane ABC-transporter
MW2368	MW2368	hypothetical protein, similar to integral membrane efflux protein
MW2376	MW2376	hypothetical protein, similar to chloramphenicol resistance protein
MW2378	MW2378	hypothetical protein, similar to ABC transporter (ATP-binding protein)
MW2396	MW2396	hypothetical protein, similar to glucose 1-dehydrogenase
MW2403	MW2403	hypothetical protein, similar to oxidoreductase
MW2421:fnb	MW2421:fnb	fibronectin-binding protein homolog (for fnbA?)
MW2431	MW2431	hypothetical protein, similar to glucarate transporter
MW2437	MW2437	conserved hypothetical protein
MW2446	MW2446	hypothetical protein, similar to ABC transporter (ATP-binding protein)
MW2447	MW2447	conserved hypothetical protein
MW2448:srtA	MW2448:srtA	sortase
MW2471	MW2471	ferrous iron transport protein B homolog
MW2479	MW2479	hypothetical protein, similar to mercuric ion-binding protein
MW2498	MW2498	hypothetical protein, similar to transcription regulator acrR
MW2499	MW2499	hypothetical protein, similar to short chain oxidoreductase
MW2503	MW2503	hypothetical protein similar to ferrous iron transporter protein B
MW2534	MW2534	hypothetical protein
MW2543	MW2543	hypothetical protein, similar to ABC transporter (ATP-binding protein)
MW2553:arc	MW2553:arc	carbamate kinase
MW2557	MW2557	hypothetical protein, similar to arginine repressor

Gene ID	Name	Function
MW2558:aur	MW2558:aur	zinc metalloproteinase aureolysin
MW2566	MW2566	hypothetical protein, similar to N-Carbamoylsarcosine Amidohydrolase
MW2574	MW2574	hypothetical protein, similar to preprotein translocase secY
MWrRNA02	MWrRNA02	23S ribosomal RNA (RNA02)
MWrRNA05	MWrRNA05	16S ribosomal RNA (RNA05)
nuc	nuc	thermonuclease
obg	obg	SpoOB-associated GTP-binding protein
pbp3	pbp3	penicillin-binding protein 3
pbpA	pbpA	penicillin-binding protein 1
pstB	pstB	phosphate ABC transporter ATP-binding protein
rlp	rlp	RGD-containing lipoprotein
rot	rot	repressor of toxins
SA0074	SA0074	hypothetical protein
SA0102	SA0102	67 kDa Myosin-crossreactive streptococcal antigen homologue
SA0103	SA0103	conserved hypothetical protein
SA0104	SA0104	hypothetical protein, similar to transcription regulator GntR family
SA0190	SA0190	hypothetical protein
SA0191	SA0191	conserved hypothetical protein
SA0192	SA0192	hypothetical protein, similar to ABC transporter ATP-binding protein
SA0193	SA0193	hypothetical protein, similar to Enterococcus faecalis plasmid pPD1 bacl
SA0195	SA0195	hypothetical protein
SA0196	SA0196	conserved hypothetical protein
SA0197	SA0197	hypothetical protein, similar to ABC transporter ATP-binding protein
SA0211	SA0211	acetyl-CoA acetyltransferase
SA0251:lytR	SA0251:lytR	two-component response regulator
SA0276	SA0276	conserved hypothetical protein, similar to diarrheal toxin
SA0294	SA0294	hypothetical protein, similar to branched-chain amino acid uptake carrier
SA0298	SA0298	hypothetical protein, similar to regulatory protein PfoR
SA0509	SA0509:srtB	srtB sortase B
SA0573:sarA	SA0573:sarA	staphylococcal accessory regulator A
SA0598:pbp4	SA0598:pbp4	
SA0610	SA0610	hypothetical protein, similar to lipase LipA
SA0614	SA0614	hypothetical protein, similar to two-component response regulator
SA0622	SA0622	hypothetical protein, similar to AraC/XylS family transcriptional regulator

Gene ID	Name	Function
SA0627	SA0627	hypothetical protein, similar to LysR family transcriptional regulator
SA0641	SA0641	conserved hypothetical protein, similar to transcriptional regulator
SA0661:saeR	SA0661:saeR	response regulator
SA0668	SA0668	oligopeptidase F
SA0675	SA0675	hypothetical protein, similar to ABC transporter ATP-binding protein
SA0675	SA0675	hypothetical protein, similar to ABC transporter ATP-binding protein
SA0702:llm	SA0702:llm	lipophilic protein affecting bacterial lysis rate and methicillin resistance level
SA0704	SA0704	conserved hypothetical protein
SA0726:gapR	SA0726:gapR	glycolytic operon regulator
SA0745	SA0745	hypothetical protein, similar to extracellular matrix and plasma binding
SA0836	SA0836	hypothetical protein, similar to transcription regulator LysR family
SA0857	SA0857	hypothetical protein, similar to negative regulator of genetic competence MecA
SA0879	SA0879	oligopeptidase F
SA0904	SA0904	hypothetical protein, probable ATL autolysin transcription regulator
SA1010	SA1010	hypothetical protein, similar to exotoxin 4
SA1013	SA1013	hypothetical protein, similar to carbamate kinase
SA1139:glgP	SA1139:glgP	glycerol uptake operon antiterminator regulatory protein
SA1159	SA1159	hypothetical protein, similar to two-component response regulator
SA1174:LexA	SA1174:LexA	SOS regulatory LexA protein
SA1195	SA1195	SA1217 hypothetical protein, similar to negative regulator PhoU
SA1208	SA1208	hypothetical protein
SA1217	SA1217	SA1217 hypothetical protein, similar to negative regulator PhoU
SA1247	SA1247	truncated (putative response regulator ArlR (truncated-arlR)
SA1248	SA1248	truncated (putative response regulator ArlR [S (truncated-arlR)
SA1329	SA1329	ferric uptake regulator homolog
SA1337	SA1337	transcription regulator AraC/XylS family homolog
SA1341	SA1341	hypothetical protein, similar to export protein SpcT protein
SA1360:ftsH	SA1360:ftsH	ftsH FtsH-2 peptidase
SA1383	SA1383	ferric uptake regulator homolog
SA1516:phoP	SA1516:phoP	alkaline phosphatase synthesis transcriptional regulatory protein
SA1539	SA1539	hypothetical protein, similar to septation ring formation regulator
SA1552	SA1552	hypothetical protein
SA1580	SA1580	multidrug resistance protein homolog
SA1584	SA1584	lysophospholipase homolog

Gene ID	Name	Function
SA1621	SA1621	hypothetical protein
SA1628:splD	SA1628:splD	serine protease
SA1632	SA1632	hypothetical protein
SA1640	SA1640	hypothetical protein (PathogenicityislandSaPIn3)
SA1643:sen	SA1643:sen	staph enterotoxin n
SA1644:yent2	SA1644:yent2	enterotoxin
SA1646:sei	SA1646:sei	staph enterotoxin i
SA1647:sem	SA1647:sem	staph enterotoxin m
SA1649	SA1649	conserved hypothetical protein
SA1666	SA1666	two-component response regulator homolog
SA1676	SA1676	hypothetical protein, similar to regulatory protein (pfoS/R)
SA1678	SA1678	transcription regulator Fur family homolog
SA1814	SA1814	subfamily M16C unassigned peptidases (SAV1279 protein)
SA1883:KdpE	SA1883:KdpE	KDP operon transcriptional regulatory protein KdpE
SA1888RodA	SA1888RodA	hypothetical protein, similar to rod shape determining protein RodA
SA1949	SA1949	lytic regulatory protein truncated with Tn554 (truncated-SA)
SA1970	SA1970	hypothetical protein, similar to multidrug resistance protein
SA1999	SA1999	hypothetical protein, similar to regulatory protein, SIR2 family
SA2002	SA2002	hypothetical protein, similar to transcription regulator MerR family
SA2004	SA2004	conserved hypothetical protein
SA2005	SA2005	conserved hypothetical protein
SA2060	SA2060	hypothetical protein, similar to transcription regulator MarR family
SA2063:moaA	SA2063:moaA	molybdenum cofactor biosynthesis protein A
SA2092	SA2092	hypothetical protein, similar to transcription regulator
SA2108	SA2108	hypothetical protein, similar to transcription regulator, RpiR family
SA2115	SA2115	hypothetical protein, similar to transcriptional regulator
SA2119	SA2119	hypothetical protein, similar to dehydrogenase
SA2123	SA2123	hypothetical protein, similar to transcription regulator LysR family
SA2124:fosB	SA2124:fosB	fosfomycin resistance protein fofB
SA2144	SA2144	hypothetical protein, similar to transcriptional regulator (TetR/AcrR family)
SA2147:tcaR	SA2147:tcaR	TcaR transcription regulator
SA2151	SA2151	hypothetical protein, similar to two component response regulator
SA2165	SA2165	hypothetical protein, similar to transcriptional regulator tetR-family
SA2169	SA2169	hypothetical protein, similar to transcription regulatory protein

Gene ID	Name	Function
SA2174	SA2174	hypothetical protein, similar to transcriptional regulator
SA2178	SA2178	hypothetical protein, similar to transcriptional regulator
SA2308	SA2308	hypothetical protein, similar to transcription regulator MarR family
SA2314	SA2314	hypothetical protein, similar to ABC transporter (ATP-binding protein)
SA2330	SA2330	hypothetical protein, similar to transcription regulator
SA2357	SA2357	hypothetical protein, similar to regulatory protein (pfoS/R)
SA2379	SA2379	hypothetical protein, similar to transcriptional regulator tetR-family
SA2418	SA2418	hypothetical protein, similar to two-component response regulator
SA2421	SA2421	hypothetical protein, similar to transcriptional regulator
SA2424	SA2424	hypothetical protein, similar to transcription regulator Crp/Fnr family protein
SA2458:IcaR	SA2458:IcaR	ica operon transcriptional regulator IcaR
SA2472	SA2472	hypothetical protein, similar to ATP phosphoribosyltransferase regulatory subunit
SA2483	SA2483	hypothetical protein
SA2500:gidA	SA2500:gidA	glucose inhibited division protein A
saeR	saeR	response regulator
sarR	sarR	staphylococcal accessory regulator A homolog
SAV0012	SAV0012	ampA family M17 unassigned peptidases (SAV0942 protein)
SAV0102	SAV0102	Mername-AA019 peptidase
SAV0149:Cap5A	SAV0149:Cap5A	capsular polysaccharide synthesis enzyme Cap5A
SAV0153Cap8E	SAV0153Cap8E	capsular polysaccharide synthesis enzyme Cap8E
SAV0154Cap5F	SAV0154Cap5F	capsular polysaccharide synthesis enzyme Cap5F
SAV0155:Cap5G	SAV0155:Cap5G	capsular polysaccharide synthesis enzyme Cap5G
SAV0156Cap5H	SAV0156Cap5H	capsular polysaccharide synthesis enzyme O-acetyl transferase Cap5H
SAV0157Cap5I	SAV0157Cap5I	capsular polysaccharide synthesis enzyme Cap5I
SAV0158:Cap5J	SAV0158:Cap5J	capsular polysaccharide synthesis enzyme Cap5J
SAV0159:Cap5K	SAV0159:Cap5K	capsular polysaccharide synthesis enzyme Cap5K
SAV0161Cap5M	SAV0161Cap5M	capsular polysaccharide synthesis enzyme Cap5M
SAV0162:Cap5N	SAV0162:Cap5N	capsular polysaccharide synthesis enzyme Cap5N
SAV0163:Cap8O	SAV0163:Cap8O	capsular polysaccharide synthesis enzyme Cap8O
SAV0183	SAV0183	subfamily M24B non-peptidase homologues (SAV1529 protein)
SAV0195:hsdR	SAV0195:hsdR	probable type I restriction enzyme restriction chain
SAV0212	SAV0212	subfamily M24B unassigned peptidases (SA1360 protein)
SAV0276	SAV0276	subfamily M20A unassigned peptidases (SAV2006 protein)
SAV0398:tetM	SAV0398:tetM	tetracycline resistance protein

Gene ID	Name	Function
SAV0422:set6	SAV0422:set6	exotoxin 6
SAV0424:set8	SAV0424:set8	exotoxin 8
SAV0425:set10	SAV0425:set10	exotoxin10
SAV0426:set11	SAV0426:set11	exotoxin11
SAV0428:set13	SAV0428:set13	exotoxin 13
SAV0429:set14	SAV0429:set14	exotoxin 14
SAV0431hsdM	SAV0431hsdM	probable type I site-specific deoxyribonuclease LIdI chain
SAV0433	SAV0433	exotoxin 15
SAV0457:pepT	SAV0457:pepT	peptidase T
SAV0511	SAV0511	subfamily M20B unassigned peptidases (SAV1512 protein)
SAV0520:hmrA	SAV0520:hmrA	subfamily M20D non-peptidase homologues (HmrA protein)
SAV0526	SAV0526	family M20D unassigned peptidases (SAV0549 protein)
SAV0549:hipO	SAV0549:hipO	family M20D unassigned peptidases (hippurate hydrolase)
SAV0612	SAV0612	family M20D unassigned peptidases (SAV2329 protein)
SAV0743	SAV0743	family M20D unassigned peptidases (SAV0102 protein)
SAV0768	SAV0768	family M42 unassigned peptidases (SAV1366 protein)
SAV0942:pyrC	SAV0942:pyrC	dihydro-orotase
SAV0965	SAV0965	family M22 non-peptidase homologues (SAV2051 protein)
SAV0993:appF	SAV0993:appF	oligopeptide transport system ATP-binding protein AppF homologue
SAV1000	SAV1000	family M22 unassigned peptidases (SAV2049 protein)
SAV1044	SAV1044	RseP peptidase
SAV1057:lytM	SAV1057:lytM	lysostaphin (peptidoglycan hydrolase)
SAV1068	SAV1068	subfamily M23B unassigned peptidases (MW1895 protein)
SAV1070	SAV1070	subfamily M23B unassigned peptidases (MW1390 protein)
SAV1113	SAV1113	conserved hypothetical protein
SAV1119:vacA	SAV1119:vacA	putative vacuolating cytotoxin paralog
SAV1201	SAV1201	subfamily M23B unassigned peptidases (SAV0212 protein)
SAV1253	SAV1253	SpIC g.p. (Staphylococcus aureus) (m-type 2)
SAV1279:splD/splF	SAV1279	subfamily S1B unassigned peptidases (serine protease splf)
SAV1366	SAV1366	subfamily S1C unassigned peptidases (SAV1728 protein)
SAV1384:htrA	SAV1384:htrA	subfamily S1C unassigned peptidases (SA0879 protein)
SAV1420:purF	SAV1420:purF	amidophosphoribosyltransferase precursor
SAV1512:glmS	SAV1512:glmS	glucosamine-fructose-6-phosphate aminotransferase
SAV1529	SAV1529	family C59 non-peptidase homologues

Gene ID	Name	Function
SAV1613	SAV1613	gamma-glutamyltransferase 1 (bacterial)
SAV1622:thdF	SAV1622:thdF	possible thiophene and furan oxidation protein
SAV1661	SAV1661	amily C26 unassigned peptidases
SAV1708:hisH	SAV1708:hisH	family C56 non-peptidase homologues (imidazole glycerol phosphate synthase subunit HisH)
SAV1745:purQ	SAV1745:purQ	family C56 non-peptidase homologues (phosphoribosylformylglycinamidine synthase I)
SAV1751	SAV1751	family C56 non-peptidase homologues (orf 1 protein)
SAV1765:HCHA	SAV1765:HCHA	family C56 unassigned peptidases (SA0509 protein)
SAV1793	SAV1793	subfamily S9C non-peptidase homologues (SAV2451 protein)
SAV1810	SAV1810	subfamily S9C unassigned peptidases
SAV1811	SAV1811	family S33 non-peptidase homologues
SAV1813	SAV1813	family S33 non-peptidase homologues
SAV1827:yent1	SAV1827:yent1	enterotoxin
SAV1830:seo	SAV1830:seo	enterotoxin
SAV1875	SAV1875	family S33 unassigned peptidases
SAV1879	SAV1879	family S33 unassigned peptidases
SAV1909	SAV1909	family S33 unassigned peptidases
SAV1998:fmt	SAV1998:fmt	family S12 unassigned peptidases (Fmt protein)
SAV2049	SAV2049	family S24 unassigned peptidases (SAV1998 protein)
SAV2051	SAV2051	family S24 unassigned peptidases
SAV2133:spsB	SAV2133:spsB	signal peptidase SpsB (Staphylococcus aureus) (SpsB protein)
SAV2154:radA	SAV2154:radA	family S16 non-peptidase homologues
SAV2329:clpP	SAV2329:clpP	peptidase Clp (type 1)
SAV2352	SAV2352	SAV2352 hypothetical protein
SAV2451:ctpA	SAV2451:ctpA	subfamily S41A unassigned peptidases (SAV1420 protein)
SAV2455	SAV2455	family S54 unassigned peptidases (SAV1549 protein)
SAV2662	SAV2662	SAV2662 capsular polysaccharide biosynthesis
SAV2663	SAV2663	SAV2663 capsular polysaccharide biosynthesis
SAV2664	SAV2664	SAV2664 capsular polysaccharide biosynthesis
SAV2667:icaD	SAV2667:icaD	intercellular adhesion protein D
SAV2668:icaB	SAV2668:icaB	intercellular adhesion protein B
SAV2675	SAV2675	family U32 unassigned peptidases (SAV1613 protein)
SAV2690	SAV2690	family U32 unassigned peptidases (SAV1612 protein)
SAV2690	SAV2690	family U32 unassigned peptidases (SAV1612 protein)
sbi	sbi	IgG binding protein

Gene ID	Name	Function
sdhB	sdhB	succinate dehydrogenase iron-sulfur protein subunit (MW2)
sdrC	sdrC	Ser-Asp rich fibrinogen-binding bone sialoprotein-binding protein
sdrD	sdrD	Ser-Asp rich fibrinogen-binding bone sialoprotein-binding protein
sdrE	sdrE	Ser-Asp rich fibrinogen-binding bone sialoprotein-binding protein
sirA	sirA	lipoprotein
sirB	sirB	lipoprotein
sirC	sirC	lipoprotein
spa	spa	IgG binding protein A
splE:S.hyicus	splE:S.hyicus	
ssb	ssb	single-strand DNA-binding protein of phage phi Sa 2mw
sspA	sspA	serine protease V8 protease
sspB	sspB	cysteine protease precursor
sspC	sspC	cysteine protease
tagH	tagH	teichoic acid translocation ATP-binding protein
taxon:1280 chp	taxon:1280 chp	chemotaxis inhibitory protein
vga	vga	hypothetical ABC transporter ATP-binding protein