Characterisation of Staphylococcal and *Candida* Populations from Healthy and Diseased Oral and Subgingival Sites Reveals a High Prevalence and Diversity of the Arginine Catabolic Mobile Element ACME in *Staphylococcus epidermidis*

A thesis submitted to the University of Dublin in fulfilment of the requirements for the degree of Doctor of Philosophy by

Aoife M. O'Connor

September 2017

Microbiology Research Unit
Division of Oral Biosciences
Dublin Dental University Hospital
University of Dublin
Trinity College

Declaration

I hereby declare that this thesis has not previously been submitted for a degree at this or any other university, and that it represents my own unaided work, except where duly acknowledged in the text. I agree to deposit this thesis in the University's open access institutional repository or allow the library to do so on my behalf, subject to Irish Copyright Legislation and Trinity College Library conditions of use and acknowledgement.

_____

Aoife M. O'Connor, B.A. (Mod) Microbiology

This thesis is dedicated to the loving memory of my granny Kathleen O'Connor, a strong and compassionate woman, who always showed a keen interest in my education.

Summary

The oral cavity harbours a diverse microbiological population, that exists mainly as plaque biofilm, the accumulation of which is associated with oral diseases such as periodontal disease and peri-implantitis. Traditionally, staphylococci were considered transient members of the oral flora, and not thought to contribute to plaque-associated diseases. In contrast, *Candida* species, particularly *Candida albicans*, are well-recognised oral commensals and opportunistic pathogens. Detailed investigations of oral staphylococcal and *Candida* populations in healthy individuals with and without dental implants and in patients with periodontal disease are currently lacking.

The aim of this study was to determine the prevalence and abundance of staphylococcal and *Candida* species in periodontal pockets, subgingival sites and oral rinses from patients with periodontal disease (n=20), healthy patients with dental implants (n=31) and orally healthy participants without implants (n=64). Participants were subjected to oral rinse, nasal, periodontal pocket and subgingival site sampling. Staphylococci were recovered on selective agar media and definitively identified by Matrix Assisted Laser Desorption Ionization Time-of-Flight analysis. *Candida* species were recovered on CHROMagar Candida™ medium (CHROMagar, France) and definitively identified by polymerase chain reaction (PCR) using species-specific primers.

Staphylococci were prevalent in all three participant groups and *Staphylococcus epidermidis* predominated in all oral sites investigated. This species was most prevalent in oral rinses (18/20, 90%), periodontal pockets (6/20, 30%) and subgingival sites (4/20, 20%) of patients with periodontal disease, but was also highly prevalent in healthy patients with implants [oral rinses (25/31, 80.6%) and subgingival sites (5/31, 16.1%)] and in orally healthy participants [oral rinses (43/64, 67.2%) and subgingival sites (5/64, 7.8%)]. The average cell density of *S. epidermidis* was also significantly higher in oral rinses from patients with periodontal disease [82.4 ± 218.9 colony forming units (CFU)/ml] compared to orally healthy participants [20.4 ± 14.8 CFU/ml] (p = 0.0153). In contrast, *Staphylococcus aureus* was much less prevalent [patients with periodontal disease: oral rinses (5/20, 25%), periodontal pockets (0/20, 0%) and subgingival sites (0/20, 0%); healthy patients with implants: oral rinses (15/31, 48.4%), subgingival sites (4/31, 12.9%); orally healthy participants: oral rinses (19/64, 29.7%), subgingival sites (5/64, 7.8%)].

Candida albicans predominated in all participant groups [patients with periodontal disease: oral rinses (11/20, 55%), periodontal pockets (4/20, 20%) and subgingival sites (2/20, 10%); healthy patients with implants: oral rinses (14/31, 45.2%) and subgingival sites (7/31, 22.6%); orally healthy participants: oral rinses (17/64, 26.6%) and subgingival sites (3/64, 4.7%)]. The average *C. albicans* cell density was also significantly higher (p = <0.05) in oral rinses of patients with periodontal disease (215.2 ± 487.8 CFU/ml) than in the other participant groups (34.7 ± 70.6 CFU/ml and 7.8 ± 25.9 CFU/ml).

In total 227 *S. epidermidis* and 78 *S. aureus* oral-nasal isolates were screened using the *S. aureus* Genotyping Kit 2.0 microarray system (Alere, Germany) and 24 *C. albicans* isolates underwent ABC genotyping and multilocus sequence typing (MLST) using the current consensus

MLST scheme for *C. albicans* (www.pubmlst.org/calbicans) to identify genomic markers or clonal lineages that might be associated with a participant group, anatomical sites or oral disease state(s).

A diverse population of *C. albicans* isolates was identified among the three groups of participants by MLST. No clonal lineages were particularly associated with oral health status or anatomical site. Diverse populations of *S. aureus* and *S. epidermidis* were also detected in all three participant groups by microarray profiling. Genes encoding resistance to antimicrobial agents including macrolides, tetracycline, and methicillin were more prevalent in *S. epidermidis*, whereas genes encoding virulence factors were typically more prevalent in *S. aureus*.

Interestingly, microarray profiling revealed that the *arc* genes carried by the arginine catabolic mobile element (ACME) were highly prevalent in oral *S. epidermidis*. The prevalence of each of three previously described ACME types (I: *arc* and *opp3* genes, II: *arc* genes only, and III: *opp3* genes only) was investigated in 143 *S. epidermidis* isolates by multiplex PCR using ACME-*arc*- and ACME-*opp3*-specific primers. A total of 85/143 (59.4%) isolates harboured ACME, of which 60/85 (70.6%) harboured ACME II, 16/85 (18.8%) harboured ACME I and 9/85 (10.6%) harboured ACME III. ACME was significantly (p = 0.016) more prevalent among isolates from periodontal pockets (7/9, 83%) compared to subgingival sites of healthy participants (3/5, 60%).

The genomic diversity of 25 ACMEs from *S. epidermidis* isolates selected as representative of participant groups and oral sites [type I (n=2), type II (n=20) and type III (n=3)] was investigated by whole genome sequencing (WGS). This was the first detailed investigation of the structural organisation of ACME type III to date. Surprisingly, all three ACME III-positive isolates belonged to the extremely rare *S. epidermidis* sequence type (ST) ST329, suggesting that this ST may represent an ancestral strain of historic ACME rearrangements that retains ACME III as a remnant. The ACMEs characterised were frequently components of composite genetic elements, often co-located with staphylococcal cassette chromosome (SCC)-associated genes. Based on the size of the elements and presence or absence of SCC-associated genes, highly diverse composite elements were identified in 16/25 isolates in association with ACME types I-III. Typically *copA* is located at the 3' end of ACME, and the *ars* operon located downstream of ACME, however, these were internalised within ACME composites containing ACME I (n=2) and III (n=3) and directly into ACME II (n=2) highlighting the genomic plasticity of these elements.

The ACME-*arc* operon encodes an arginine deaminase pathway thought to enable staphylococcal persistence in nutrient and oxygen poor environments by metabolism of L-arginine for energy and pH regulation. Based on the high prevalence of ACME-*arc* and the low prevalence of ACME III lacking ACME-*arc* in periodontal pockets and subgingival sites, it is likely that this operon facilitates the adaptation of *S. epidermidis* to the semi-anaerobic environment of these sites.

The present study revealed a significant enrichment of *S. epidermidis* harbouring a diverse range of ACMEs in subgingival sites and periodontal pockets of patients with periodontal disease, particularly ACME types encoding the *arc*-genes, suggesting that these genes confer a survival advantage on *S. epidermidis* in these diseased semi-anaerobic sites.

Table of Contents

Acknowledgements

Firstly, I would like to express my sincere gratitude to my supervisor Professor David Coleman, for his continuous support, patience and immense knowledge throughout the past four years. Thank you for giving me the opportunity to undertake a Ph.D. research project. I would equally like to acknowledge and thank my supervisor Dr. Brenda McManus for all her encouragement, patience, and advice. Thank you for always being available to help me with all aspects of this project, I have learned so much working with you over the past four years.

I wish to thank Dr. Gràinne Brennan and all the staff in the National MRSA Reference Laboratory who were always so helpful, and in particular, I would like to thank Tanya Flemming for setting up countless MALDI-TOF runs. Thank you to the Moyne Institute preparation room for suppling culture media to the laboratory.

I would like to acknowledge the funding for this project provided by the Microbiology Unit, Division of Oral Bioscience, Dublin Dental University Hospital. Thank you for the chance to present my research internationally, to undertake training course both in Ireland and abroad, and for providing anything required for my research. Thank you to Dr. Mary O'Donnell for managing the Microbial Research Unit so efficiently, and for acting as gatekeeper on this project. I am grateful to Dr. Michael O'Sullivan and Dr. Ioannis Polyzois for providing me with the clinical samples that were investigated in the present study, and to Dr. Peter Kinnevey for helping me with the whole genome data analysis involved in this project. Thank you to Dr. Keira Malone for sharing additional control isolates.

I'd like to extended my deepest thanks to all staff and students in the Microbiology Research Unit, both past and present who made me feel so welcome from day one. I was so fortunate to work with, and become close friends with such a wonderful, hilarious, and supportive group of people. Dr. Peter Flannagan, what can I say? You have made laugh, you have made me cry, your friendship knows no boundaries (or iLines), thank you for the decades of entertainment you squashed into the past four years. Thank you to Dr. Maria Boyle and Dr. Anna Shore for all the advice and

encouragement during my project. I'd like to thank Megan Earls for teaching me how to set up whole genome sequencing runs, and for knowing when drinks are required. To Dr. Emily Deasy and Elaine Malone, thanks for always being available for a chat. Lastly, a special thank you to the one and only Dr. Alexandros Lazaris. Your nuggets of 'wisdom' and honesty are truly refreshing, thank you for your friendship, the last four years wouldn't have been the same without you.

To the Foxpack (Louise, Grainne, Doireann, and Alison), Fiona, and Sorcha, thank you for the many dinners, trips, and parties over the past four years, and for always showing interest in my research. I am so grateful for all your friendship.

I'd like to thank my sisters; Tara and Niamh for all their support and for always saying what they are thinking, could I be anymore grateful! To Amber, the best dog a girl could ask for. A special thank you to Dr. Paul Barry, for always believing in me. Thank you for your love and endless support, here's where the fun begins!

Finally, I express my profound gratitude to my parents James and Martina; for providing me with unfailing support and continuous encouragement throughout my years of study. This accomplishment would not have been possible without them. Thank you.

Index of Tables

Page number refers to the text page preceding the table

Index of Figures

Page number refers to the text page preceding the figure

Abbreviations

aa	Amino acid
ACME	Arginine catabolic mobile element
AIDS	Acquired immunodeficiency syndrome
agr	Accessory gene regulator
ATP	Adenosine triphosphate
BHIA	Brain Heart Infusion Agar
BLAST	Basic local alignment search tool
bp	Base pair
BSI	Blood stream infection
BURST	Based upon related sequence types
ca.	Approximately
CA	Community associated
CA-MRSA	Community associated methicillin resistant *Staphylococcus aureus*
CBA	Columbia blood agar
CC	Clonal complex
CDC	Centers for disease control and prevention
CFU	Colony forming units
CFU/ml	Colony forming units per milliliter
CHIP	Chemotaxis inhibitory protein
CoNS	Coagulase negative staphylococci
CoPS	Coagulase positive staphylococci
DLV	Double locus variant

DDUH	Dublin Dental University Hospital
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphate
DST	Diploid sequence type
e.g.	*Exempli graita*; for example
EARS-net	Eruopean Antimicrobial resistance surveillance network
EDTA	Ethylenediaminetetraacetic acid
et. al.	*Et alia*; and others
etc.	*Et cetera;* and the rest
EUCAST	European committee on antimicrobial susceptibility testing
g	Gravitational force
h	Hours
HA	Hospital acquired
HA-MRSA	Hospital acquired methicillin resistant *Staphylococcal aureus*
HCL	Hydrochloric acid
HGAP	Hierarchical Genome Asssembly Process
HIV	Human immunodeficiency virus
HRP	Horseradish pyruvate
i.e.	*Id est*; that is
IEC	Immune evasion complex
IgG	Immunoglobulin G
IS	Insertion sequence
in vitro	Within the glass; performing a given procedure in a controlled

	environment outside of a living organism.
kb	Kilobase
L	Litre
LA	Livestock associated
M	Molar
mg	Milligram
MGE	Mobile genetic element
min	Minute
ml	Millilitre
MLST	Multi locus sequence type
mM	Milli molar
MRSA	Methicillin resistant *Staphylococcus aureus*
MRSE	Methicillin resistant *Staphylococcus epidermidis*
MSA	Manitol salt agar
MS-CoNS	Methicillin susceptible coagulase negative staphylococci
MSCRAMMs	Microbial surface components recognizing adhesive matrix molecules
MSSA	Methicillin susceptible *Staphylococcus aureus*
MSSE	Methicillin susceptible *Staphylococcus epidermidis*
N/A	Not applicable
NGS	Next generation sequencing
NMRSARL	National Methicillin Resistant *Staphylococcus aureus* Reference Laboratory

ORF	Open reading frame
PBP	Penicillin binding protein
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
pH	potential hydrogen
PVL	Panton-Valentine Leukocidin
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
rpm	Revolutions per minute
RT-PCR	Real-time polymerase chain reaction
s	Seconds
SaPI	Staphylococcal Pathogenicity Island
SCC	Staphylococcal chromosomal cassette
SCC*mec*	Staphylococcal chromosomal cassette *mec*
SCIN	Staphylococcal complement inhibitor
SMRT	Single molecule real time
SNV	Single nucleotide variant
SSTI	Skin and Soft Tissue Infection
ST	Sequence Type
TBE	Tris/Borate/EDTA
TE	Tris/EDTA
Tris	Tris (hydroxymethyl) aminoethane

TSA	Trypticase soy agar
UK	United Kingdom
UPGMA	Unweighted pair group method according to arithmetic averages
USA	United States of America
w/v	Weight/volume
wgMLST	Whole genome sequencing multi-locus sequence typing
WGS	Whole genome sequencing
WHO	World Health Organisation
°C	Degrees celcius
µg	Microgram
µl	Microliter
%	Percentage
≥	Greater than or equal to
≤	Less than or equal to
>	Greater than
<	Less than
Δ	Deletion (of part of a gene)
n	Number
α	Alpha
β	Beta
γ	Gamma
′	Prime

Publications

Some of the original work presented in this thesis has been published in a refereed international publication as listed below. Offprints of the proofs of the manuscripts are included at the end of the thesis (Appendix 1).

1. **McManus, B.A., O'Connor, A.M., Kinnevey, P.M., O'Sullivan, M., Polyzois, I., Coleman, D.C. (2017).** First detailed genetic characterization of the structural organization of type III arginine catabolic mobile elements (ACMEs) harboured by *Staphylococcus epidermidis* using whole genome sequencing. *Antimicrob. Agents Chemother*. DOI:10.1128/AAC.01216-17.

Chapter 1

General Introduction

1.1 The oral cavity and its microenvironments

The oral cavity consists of a wide variety of heterogeneous microenvironments composed of hard and soft tissue types as well as different shedding and non-shedding surfaces such as teeth, mucosa and the tongue (1, 2). Each of these distinct tissue types and surfaces give rise to numerous intraoral niches, which harbour distinct microbial communities. For example, each of the tongue's different surfaces (e.g. dorsum, lateral border) are anatomically different to each other and harbour distinct microbial communities (1).

Oral microbial communities commonly exist as biofilms; highly structured and organised communities of microorganisms encased within a matrix of secreted extracellular polymer substances (3). The complex biofilms that form on the hard non-shedding surface of teeth are commonly known as dental plaque. *Streptococcus*, *Actinomyces*, *Porphyromonas* species and spirochetes are key pathogens in dental plaque. Saliva coats the teeth and provides the microbial residents of plaque with a constant source of peptides and proteins (4).

The formation and build-up of dental plaque is associated with oral diseases such as gingivitis, caries, halitosis and periodontal diseases due to the metabolites produced by the microbial residents of plaque biofilm. For example, the metabolic products formed by dental plaque microbial communities in subgingival plaque can induce host immune responses resulting in gingival inflammation and bleeding (gingivitis) (4). Microbes residing in supragingival plaque can metabolise carbohydrates present in saliva to organic acids such as lactic and acetic acid and prolonged exposure of the tooth to the acidic biofilm can result in dental caries (4). Good oral hygiene practices including regular teeth brushing, flossing, and periodic mechanical descaling are the most effective methods of controlling dental plaque in the oral cavity.

Minimising oral dental plaque levels in the oral cavity is important as oral health plays an important role in the overall systemic health. Systemic diseases currently associated with poor oral hygiene include cardiovascular diseases, atherosclerosis, rheumatoid arthritis and aspiration pneumonia (5–7). For example aspiration pneumonia is mediated by pathogens associated with dental plaque and is most likely to occur in high-risk groups such as immunocompromised individuals and in the elderly (7, 8). The periopathogens *Porphyrmonas gingivalis* and *Fusobacterium nucleatum* have been detected in atherosclerotic plaque and associated with cardiovascular disease, respectively (5, 9).

1.2 Periodontal disease

The periodontium exists as a support structure to the teeth and is composed of the gingival crevice, periodontal ligament, root cementum and alveolar bone (10). Periodontal disease is an inflammatory disease that is preceded by gingivitis, a less invasive reversible form of periodontal disease that causes expansion of the gingival margin and gingival swelling and bleeding (11). Visible dental plaque will start to develop on teeth 24 h following oral hygiene practices and an absence of oral hygiene practices for 10-21 days results in gingivitis. However, gingivitis can be reversed within one week if effective oral hygiene practices are reintroduced (12). If left untreated, the eventual detachment of the gingiva from the tooth results in the formation of a periodontal pocket (10). Periodontal pockets allow the build-up of dental plaque deep within the periodontal tissue, eventually leading to loss of supporting tissue, periodontal ligaments and the reabsorption of the alveolar bone around the tooth (Fig. 1.1) (7, 10). *Porphyromonas gingivalis*, *Prevotella intermedia*, *Treponema denticola* and *Tannerella forsythia* were previously thought to be the causative pathogens of periodontal disease but research on staphylococcal and *Candida* populations in association with this disease are sparse. However, it has recently been suggested that the development of periodontal disease can be attributed to disruption of polymicrobial synergy between the resident microbes of dental plaque due to increased microbial diversity (7, 13, 14). This dysbiosis triggers the expression of virulence factors by resident pathogenic bacteria leading to the secretion of toxins which cause gum irritation (6, 7). The consequent host inflammatory response leads to an influx of innate immune cells such as neutrophils causing further destruction of the periodontal tissue (7).

Predisposing factors for the development of periodontal disease include immunosuppression, poor oral hygiene, and smoking (15). One of the biggest risk factors for periodontal disease is low socioeconomic status; this population usually cannot afford dental treatments and places a low importance on oral hygiene (11, 16). The current classifications for periodontal disease were introduced in 1999 (17). Periodontal disease can be classified as chronic or aggressive. Chronic periodontal disease is a slowly progressing disease that is plaque related and can present in a localised or generalised form and leads to loss of attachment and bone loss (11, 18). Aggressive periodontal disease is not plaque associated and involves the swift loss of attachment and bone in patients typically under the age of 30 years and can usually be traced through families (13, 18).

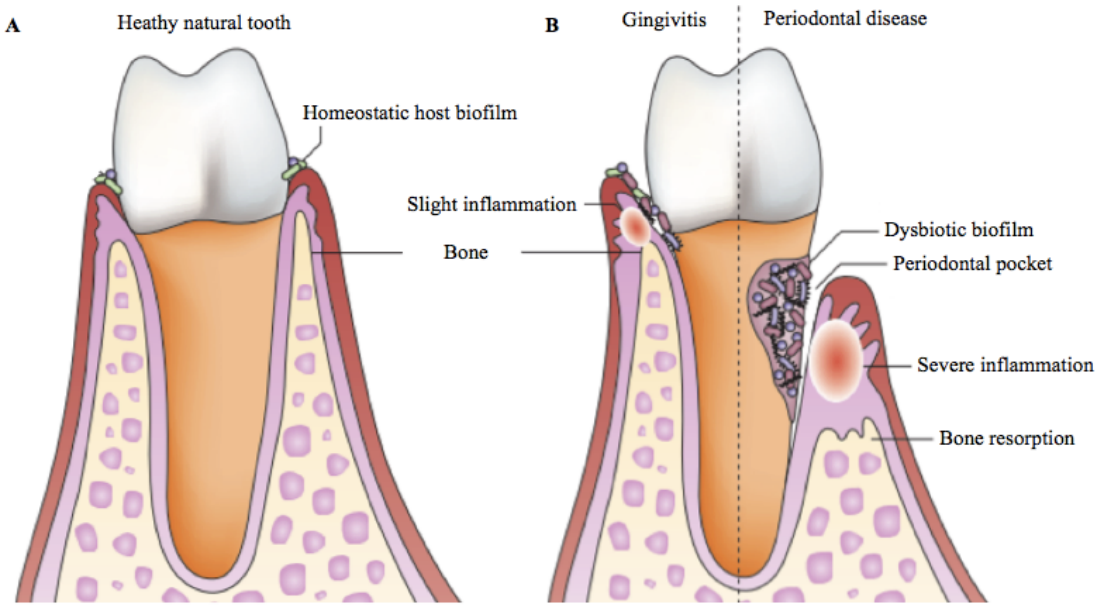


Figure 1.1 Progression from periodontal health to gingivitis and eventually periodontitis or periodontal disease. A) In periodontal health, symbiotic biofilm develops along the gingival margin and does not activate any immune response from the host. B) During gingivitis the size of the gingival margin increases, homoeostasis of the biofilm is disrupted by increased microbial diversity and localised inflammation occurs. The development of a periodontal pocket indicates the progression of gingivitis to periodontal disease. Figure adapted from Hajishengallis *et al.*, (7).

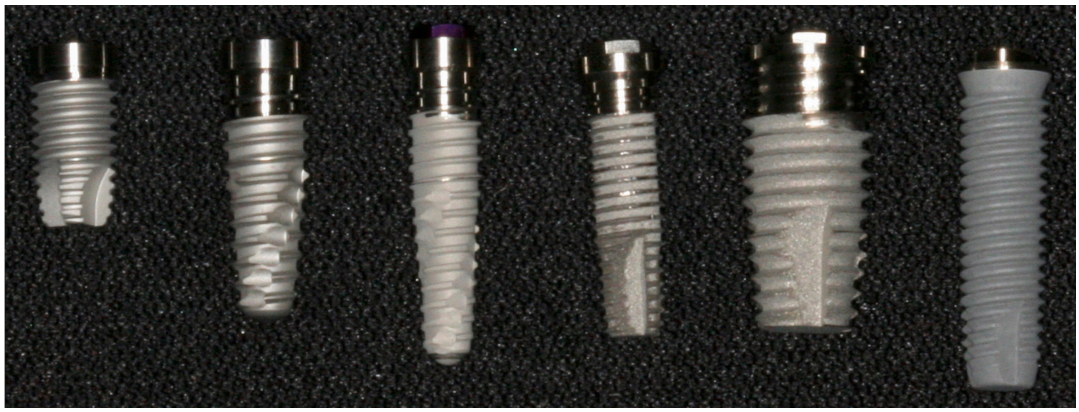
The diagnosis of periodontal disease is based on signs of inflammation, probing depths, bleeding on probing and periodontal ligament attachment loss (clinical attachment loss >3 mm) (17, 18). The diagnoses of periodontal disease is subjective leading to difficulties calculating the global prevalence of this disease (16, 18). It is estimated that 50-90% of adults worldwide have gingivitis and 80% of adults worldwide will have a minimum one tooth affected by periodontal disease at some stage during their lifetime (6, 11).

1.3 Peri-implantitis

Dental implants are indwelling titanium medical devices that are inserted into the patients mandibular or maxillary bone and by a process known as osseointegration, integrates into the jawbone to form a structural and functional connection (Fig. 1.2). This process anchors the prosthetic restoration to the jaw where there has been tooth loss due to trauma, periodontal disease or other diseases (19, 20). Healthy dental implants have homoeostatic dental plaque at their gingival crevice and are fully integrated into bone. There are no periodontal ligaments surrounding an implant and therefore the gingival crevice is deeper around an implant than around a natural tooth. Healthy implants generally have a probing depth of 3 mm (20, 21). Implant failure can occur in the early stages of placement due to osseointegration failure, or in later stages of placement most commonly due to dysbiosis of the implant biofilm and microbes causing infection in periodontal tissue (20).

Peri-implant mucositis, a less severe, reversible disease characterised by localised inflammation in the soft periodontal tissue surrounding the failing implant always precedes peri-implantitis (20, 22). The progression from peri-implant mucositis to peri-implant disease is slow and can be difficult to diagnose (23). Further inflammation leading to the destruction of the alveolar bone can indicate the progression of the disease to peri-implantitis. Bone loss can also occur due to improper implant placement and due to surgical trauma; the diagnosis of peri-implantitis must show that these are not the causes of bone loss (21, 23). The destruction of the alveolar bone may potentially lead to mobility of the dental implant, known as implant failure (19, 20). Similar to periodontal disease biofilm, *P. gingivalis*, *T. denticola*, *Fusobacterium nucleatum*, *P. intermedia* and *Aggregatibacter actinomycetemcomitans*

A



B

Figure 1.2 Photographs of examples of distinct titanium dental implants. A) Six examples of titanium dental implants which are used to replace teeth lost through trauma or oral disease. B) A titanium dental implant with the crown attached. Photographs reproduced with kind permission from Dr. I. Polyzois, Division of Restorative Dentistry and Periodontology, DDUH.

are also associated with implant disease, however *Staphylococcus aureus*, *Staphylococcus epidermidis*, coliforms, *Pseudomonas* spp. and *Candida* spp. have also been isolated from peri-implantitis biofilms (20, 24).

Due to the slow progression from peri-implant mucositis to peri-implantitis, early diagnoses and treatment is possible. Treatment of peri-implant mucositis usually involves mechanical debridement of the dental plaque and regular maintenance of the implant until reduced sign of inflammation are achieved (21, 22).

Whilst the predisposing factors for the development of periodontal disease are comparable to those of peri-implantitis, smoking is also a risk factor for the development of peri-implantitis (25). A patient with a previous history of periodontal disease and tooth loss due to periodontal disease prior to implant placement is considered severely at risk for the future development of peri-implantitis (25, 26).

1.4 Staphylococcal species

Staphylococci are Gram-positive bacterial species, some of which are commonly associated with the normal colonising microbial flora of human and animal skin and mucous membranes. Many of these species are commensal organisms and opportunistic pathogens, and are frequently associated with chronic and acute infections such as superficial skin and soft tissue infections (SSTIs) and prosthetic joint infections. Staphylococcal species can be divided into two distinct groups based on their ability or inability to produce the enzyme coagulase, which is a prothrombin activator that converts fibrinogen into fibrin. Coagulase-positive staphylococcal (CoPS) species such as *S. aureus* and *Staphylococcus pseudintermedius* produce this enzyme, whereas coagulase-negative staphylococci (CoNS) such as *S. epidermidis* and *Staphylococcus haemolyticus* do not. To date, 47 distinct staphylococcal species have been described, the majority of which are CoNS (38/47) (27). By far, the most predominant and pathogenic CoPS in humans is *S. aureus*. The most predominant CoNS are typically commensals such as *S. epidermidis*, *S. haemolyticus*, and *Staphylococcus hominis* that can cause opportunistic clinical infections in humans (27).

1.4.1 Identification of staphylococcal species

Previously, selective and differential agars such as mannitol salt agar (MSA) and Baird Parker were used to select for staphylococcal species and to differentiate *S. aureus* from CoNS. Mannitol salt agar contains a high salt concentration, mannitol and phenol red, *S.*

aureus metabolises mannitol, altering the pH and causing a colour change in the agar surrounding the colony from red to yellow. Coagulase negative staphylococci can also grow on MSA as pale pink colonies that do not cause a colour change. Baird parker agar is a selective agar for CoPS that contains lithium chloride and potassium tellurite, *S. aureus* produce black colonies with a halo caused by tellurite reduction (28).

More recently chromogenic media is used for the presumptive identification of *S. aureus* and CoNS. The most widely used medium is Sa*Select*™, this medium is selective for staphylococcal species, *S. aureus* along with CoNS such as *S. epidermidis*, *S. haemolyticus*, and *Staphylococcus saprophyticus* can be presumptively identified based on colony colour and morphology on this medium (29).

1.4.2 Genetic transfer among staphylococci

Both CoPS and CoNS species can harbour a diverse range of antimicrobial resistance genes and virulence factor genes encoded on mobile genetic elements (MGEs) and frequently these MGEs are exchanged between different species (30). Mobile genetic elements can exist outside of the bacterial chromosome as plasmids, or integrate into genomic DNA such as insertion sequences, transposons, chromosomal cassettes, lysogenic bacteriophages and pathogenicity islands (31). Based on accumulating evidence it has been postulated that CoNS possibly act as a reservoir of MGEs that can transmit useful genetic material such as genes encoding antimicrobial resistance into *S. aureus,* enhancing the ability of this species to invade, cause tissue damage, and persist during infection of human and animal hosts (32, 33).

1.4.2.1 Methicillin-resistance and the staphylococcal chromosomal cassette (SCC)

Staphylococcal chromosomal cassettes (SCC), particularly those encoding the methicillin resistance gene *mecA* (SCC*mec*), are among the best characterised staphylococcal MGEs to date. These are large elements ranging from 20 to 67 kb in size that integrate into the chromosomal *orfX* locus in staphylococci and often harbour genes encoding antimicrobial resistance and virulence factors (34–36). These elements also encode regulatory genes and chromosome cassette recombinases (*ccr*) gene complexes, the latter of which regulate the insertion and excision of the MGE (37–39) into the bacterial chromosome.

Staphylococcus aureus strains that harbour SCC*mec* elements are referred to as methicillin-resistant *S. aureus* (MRSA) and are a major cause of nosocomial infections

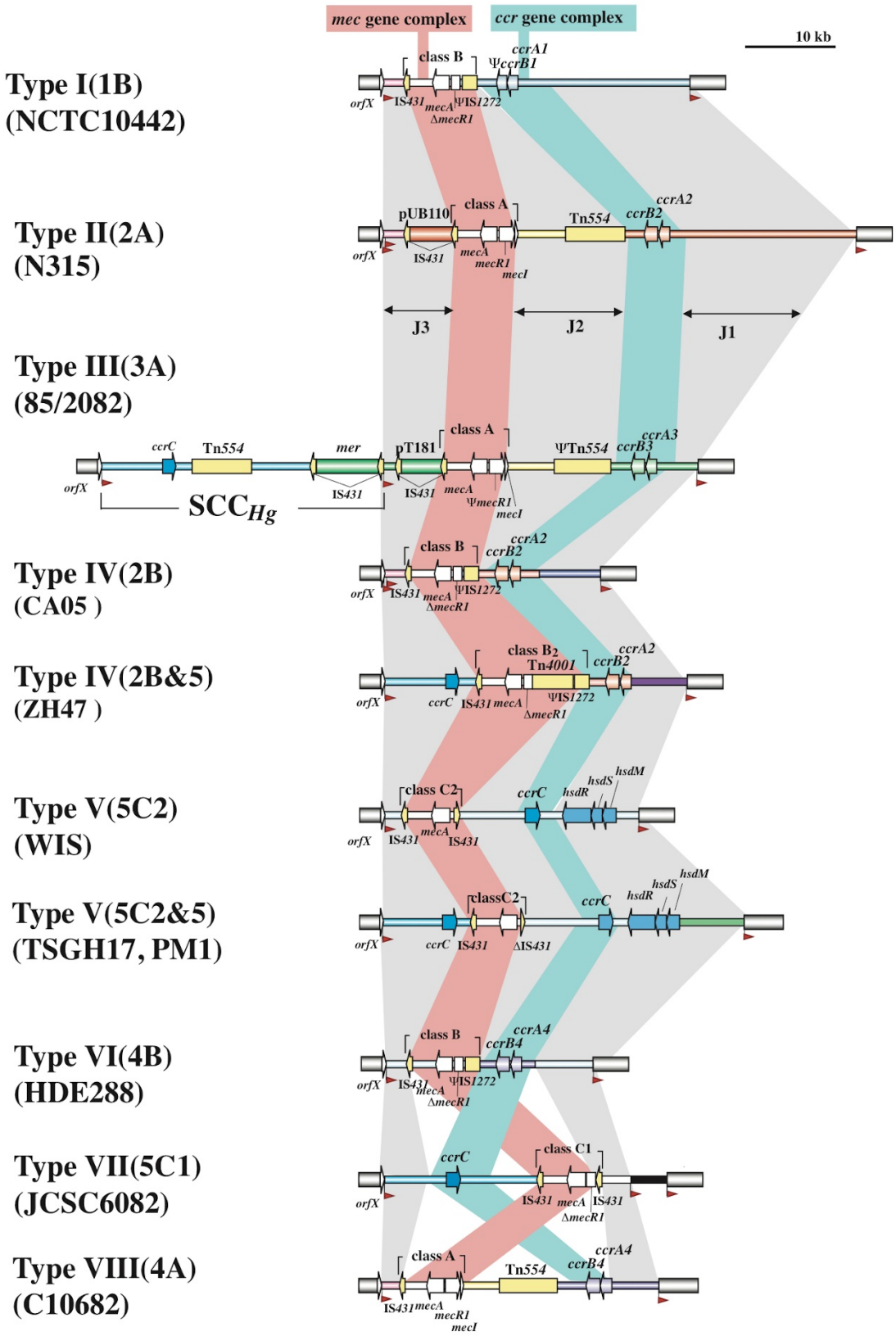
worldwide. To date 12 different SCC*mec* types (I-XII) have been described in MRSA, with each type harbouring a different combination of the *mec* gene and *ccr* gene complexes (34–36, 40) (Fig. 1.3). The *mec* gene complex is composed of varying combinations of the *mec* gene (either *mecA* or *mecC*) and the *mec* regulatory genes (*mecI*, *mecRI*, and *mecR2*). Currently there are five classes (A-E) of the *mec* gene complex that have been detected in MRSA. The *mec* gene classes A-D all contain the *mecA* and various regulatory genes, whereas class E contains the recently described *mecC* gene and its accompanying regulatory genes (35). The *ccr* gene complex is composed of varying combinations of *ccr* genes: *ccrA*, *ccrB*, *ccrC* and *ccrAA* (37, 41). To date, eight different types of *ccr* genes complexes have been described in MRSA (42). The joining (J) regions are the genomic regions surrounding the *ccr* and *mec* gene complexes that can harbour MGEs containing additional antimicrobial resistance determinants (27, 43). Based on genomic differences such as the presence or absence of genes or non-coding sections in the J-region, SCC*mec* elements can be further subtyped. (42).

Several distinct SCC*mec* types that have been identified in MRSA have also been identified in *S. epidermidis* and other CoNS in addition to many SCC*mec* types yet to be identified in MRSA (27, 44–46). This has led to the hypothesis that SCC*mec* elements originated in CoNS (44, 47).

Pseudo-SCC*mec* elements which encode *mecA* but lack any *ccr* gene complexes, have been detected in MRSA and some CoNS such as *S. hominis* (27, 48). Other SCC elements lacking *mecA* have also been characterised that encode antibiotic resistance determinants such as fusidic acid resistance, or encode resistance to metalloids and transition metals such as cadmium or mercury which have bactericidal properties (27, 49–51).

1.4.3 *Staphylococcus aureus*

Staphylococcus aureus is a commensal coloniser of the squamous epithelium of the anterior nares in humans. Approximately 20% of the human population is persistently colonised, while 60% of the population are transient carriers (52). This species is an invasive global pathogen capable of causing superficial skin and soft tissue infections (SSTIs) as well as aggressive life threatening infections such as septicaemia, toxic shock syndrome, and pneumonia (52). Additionally, *S. aureus* is a major healthcare


mec gene complex
ccr gene complex
10 kb
Type I(1B)
(NCTC10442)
class B
ccrA1
ΨccrB1
orfX
IS431
mecA
ΨIS1272
ΔmecR1
Type II(2A)
(N315)
pUB110
class A
Tn554
ccrA2
ccrB2
orfX
IS431
mecA
mecR1
mecI
J3
J2
J1
Type III(3A)
(85/2082)
ccrC
Tn554
mer
pT181
class A
ΨTn554
ccrA3
ccrB3
orfX
IS431
IS431
mecA
ΨmecR1
mecI
SCCHg
Type IV(2B)
(CA05)
class B
ccrA2
ccrB2
orfX
IS431
mecA
ΨIS1272
ΔmecR1
Type IV(2B&5)
(ZH47)
class B2
Tn4001
ccrA2
ccrB2
orfX
ccrC
IS431
mecA
ΨIS1272
ΔmecR1
Type V(5C2)
(WIS)
class C2
ccrC
hsdR
hsdS
hsdM
orfX
IS431
mecA
IS431
Type V(5C2&5)
(TSGH17, PM1)
classC2
ccrC
hsdR
hsdS
hsdM
orfX
ccrC
IS431
ΔIS431
Type VI(4B)
(HDE288)
class B
ccrA4
ccrB4
orfX
IS431
mecA
ΨIS1272
ΔmecR1
Type VII(5C1)
(JCSC6082)
ccrC
class C1
orfX
IS431
mecA
IS431
ΔmecR1
Type VIII(4A)
(C10682)
class A
Tn554
ccrA4
ccrB4
IS431
mecA
mecR1
mecI

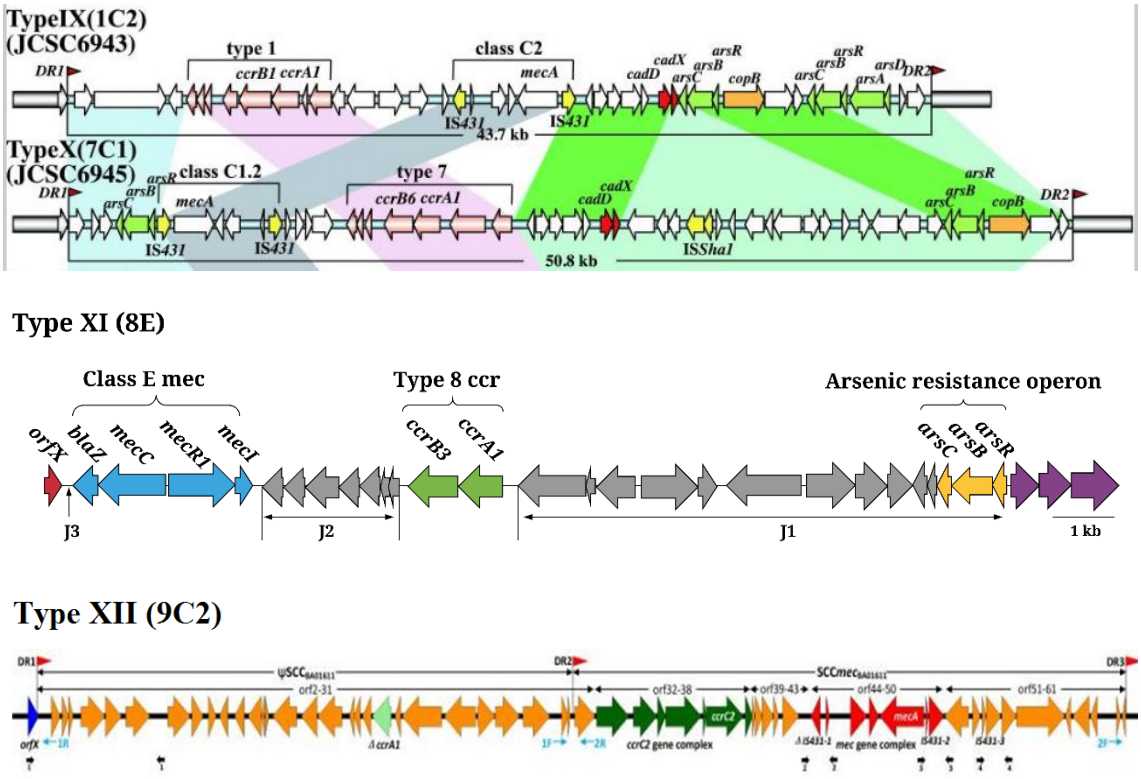


Figure 1.3 Diagram showing the genetic organisation of SCC*mec* types I-XII. SCC*mec* I, NCTC10442 (GenBank accession number AB033763); SCC*mec* type II, N315 (D86934); SCC*mec* type III*mer,* 85/2082 (AB037681); SCC*mec* type IV, CA05 (AB0633172) and Zh47 (AM292304); SCC*mec* type V, CA05 (AB063172); SCC*mec* type VI HDE288 (AF411935); SCC*mec* type VII, JCSC6082 (AB373032); SCC*mec* type VIII C10682, (C10682); SCC*mec* type IX, JCSC6943, (AB505630); SCC*mec* type X, JCSC6945 (AB478780); SCC*mec* type XI, M10/0061 (FR823292.1), and SCC*mec* type XII, BA01611 (KR187111). The alphabetic and integer designations in parenthesis following the SCC*mec* type specify the *mec* and *ccr* complex types for each SCC*mec* type i.e. 1B after SCC*mec* type I refers to *ccrAB1* and class B *mec*. Modified from IWG-SCC 2009 (SCC*mec* I – IX), Li *et al.,* 2011 (SCC*mec* X and XI) Shore *et al*., 2011 (SCC*mec* XI) and Wu *et al*., 2015 (SCC*mec* XII) (35, 36, 40, 42).

associated pathogen, and is known to be a significant cause of wound and device related infections (53). These infections can prove difficult to treat due to the emergence of antibiotic resistant strains of this species such as MRSA, a major cause of nosocomial infections worldwide (54, 55).

Compared to other staphylococci, the greatest impact of SCC*mec* has been observed in MRSA, although the prevalence and diversity of SCC*mec* is higher among the CoNS population.

Historically, three main lineages of MRSA evolved independently in hospital, community and agricultural settings; these were typically separated according to SCC*mec* types present and isolate origin. The typical features of healthcare-associated MRSA (HCA-MRSA) were the presence of SCC*mec* I, II or III and the acquisition of these strains in a healthcare environment, especially hospitals (41). Community-associated MRSA (CA-MRSA) emerged independently of HA-MRSA, and were considered more genetically diverse than HCA-MRSA populations, and tended to harbour smaller SCC*mec* elements such as SCC*mec* types IV and V (56). Community-associated MRSA emerged as a significant cause of infections in the community and was predominantly associated with SSTIs, however it has also been associated with life threatening infections (57–61). Most recently, MRSA has emerged in the agricultural sector and has been associated with infection in livestock (LA-MRSA). Livestock-associated MRSA clones are genetically distinct from HCA-MRSA and CA-MRSA as they do not harbour genes associated with the immune evasion cluster (IEC) which are required by MRSA to successfully infect a human host (62). Heathcare-assocaited MRSA and CA-MRSA typically harbour lysogenic prophages encoding the IEC integrated into the chromosomal beta-toxin gene *hlb*, a characteristic feature of human strains. As the global population of MRSA continues to evolve, the genotypic features associated with each population become less distinct, as evidenced in recent reports of MRSA outbreaks within hospitals caused by strains originally considered to be CA-MRSA (63–65).

1.4.3.1 Adhesion, immune evasion and virulence

Staphylococcus aureus has the ability to express a diverse range of virulence factors, including a wide range of proteins such as superantigens (SAGs) and staphylococcal superantigen-like proteins (SSL), which enhance its pathogenicity by disrupting and/or circumventing the hosts immune response. To date 23 distinct SAGs such as toxic

shock syndrome toxin (TSST-1), staphylococcal enterotoxins (SEs) and SE-like superantigens have been identified in *S. aureus* (66). These are small exoproteins that can bind to both MHC class II molecules and T-cell receptors, which can lead to a large cytokine release which causes toxic shock (66). Immune modulating proteins, such as the chemotaxis inhibitory protein of staphylococci (CHIPs) or the staphylococcus complement inhibitor (SCIN) target elements of the hosts innate immune response preventing neutrophil migration to the infection site and preventing opsonophagocytosis (67). Opsonophagocytosis is the immune process whereby an opsonin such as an antibody is bound to a pathogen marking if for phagocytosis by immune cells such as neutrophils. Panton-Valentine leucocidin (PVL) is a bi-component leukocidal toxin harboured primarily by CA-MRSA strains that can form pores in leukocytes (68).

Surface proteins expressed by *S. aureus* enable it to anchor to host tissues consisting of extracellular matrix proteins such as collagen, fibronectin, and fibrinogen. These *S. aureus* surface proteins are known as microbial surface components recognising adhesive matrix molecules (MSCRAMMs) (69). Some of the most well characterised MSCRAMMs are proteins FnbA and FnbB which bind to fibronectin, ClfA and ClfB which bind to fibrinogen, and Cna which is a collagen-binding protein (69, 70). These molecules can also enable *S. aureus* to evade the host's immune response, causing further tissue damage or toxic shock (69).

1.4.3.2 Antimicrobial Resistance

Although *S. aureus* is naturally susceptible to antibiotics, it can acquire genes encoding antimicrobial resistance on MGEs. Some of the most clinically relevant antimicrobial genes encoded on MGEs detected in *S. aureus* are *ileS2*, *fusB*/*C*, and *vanA* encoding resistance to murpirocin, fusidic acid, and glycopeptides, respectively. Due to the common antimicrobial target site of several agents, some genes can confer resistance to multiple antimicrobial classes, such as *cfr*. By encoding a protein that changes the accessibility of the linezolid binding site in the 23S rRNA gene, the *cfr* gene confers resistance to all antimicrobials (phenicols, lincosamides, oxazolidinones, pleuromotilins, and streptogramin A compounds) that target this site giving rise to an antimicrobial resistance pattern known as the $PhLOPS_A$ phenotype (71, 72).

1.4.4 *Staphylococcus epidermidis*

Staphylococcus epidermidis is a commensal organism of the skin and nares, and like all CoNS, it is less virulent than *S. aureus*. However, based on its classification as a commensal organism, *S. epidermidis* was often regarded as a contaminant when isolated from clinical infections (73). More recently *S. epidermidis* has been associated with nosocomial infections, particularly with foreign body related infections such as catheter-related septicaemia, or prosthetic joint infections (27, 74, 75).

1.4.4.1 Adhesion, immune evasion and virulence

The primary method by which *S. epidermidis* causes infection is by producing MSCRAMMs both for surface adherence and intercellular adhesion that are involved in the formation of an extracellular polysaccharide matrix also referred to as biofilm (76). This opportunistic pathogen is adept at building biofilms *in vivo*, which offer protection from antibiotics and the host's immune response, while also being difficult to eradicate once established around an infected prosthesis (75, 77). *Staphylococcus epidermidis* can readily colonise metals such as titanium, which is a lightweight metal from which many prosthetic joint components are made, along with the majority of commercially available dental implants (78, 79). The MSCRAMMs known as serine-aspartate repeat (Sdr) proteins are expressed by *S. epidermidis* and play a critical role in the adherence of this species to fibrinogen-coated surfaces and binding to collagen I (80). The SesC protein is usually detected in biofilm-associated *S. epidermidis* isolates and is comparable to clumping factor A protein in *S. aureus*. Progression from the adherence phase of biofilm formation to the accumulation phase involves increased intercellular adhesion. The most well characterised protein involved in this stage progression is polysaccharide intercellular adhesin (PIA) which is encoded by *icaA/D/B/C* and is critical for the formation of the extracellular matrix (76, 80, 81).

The mobile virulence factor known as the arginine catabolic mobile element (ACME) is frequently detected in *S. epidermidis* and is thought to enable staphylococcal species to survive in adverse environments. It is thought that ACME was assembled in *S. epidermidis* prior to its transfer into the CA-MRSA strain USA300 (82). There are three distinct ACME types defined based on the presence of both the *arc* and *opp3* operons (type I), *arc* operon only (type II), and the *opp3* operon only (III) (38, 54). The *arc* operon is involved in the metabolism of L-arginine resulting in the metabolites ornithine, ammonia, ATP, and CO_2 (83, 84). These metabolites provide an energy

source, and regulate the internal pH of bacteria in adverse environments. The precise role of *opp3* is unknown, it encodes an oligopeptide permease ABC transporter, and it is hypothesised it could play a role in nutrient uptake. To date ACME has been detected in *S. aureus*, *S. epidermidis* and *S. haemolyticus* (54, 85).

1.4.4.2 Antimicrobial resistance

Due to the diverse range of antibiotic resistance genes harboured by CoNS such as *S. epidermidis,* together with the propensity of these species to form biofilms, treating infections caused by CoNS can be difficult.

As mentioned previously the prevalence and diversity of SCC and SCC*mec* elements among CoNS is much greater compared to *S. aureus*. Species such as *S. epidermidis* and *S. haemolyticus* are thought to act as reservoirs for these MGEs, and CoNS often harbour non-typable SCC*mec* elements encoding novel *mec* and *ccr* gene complexes (45, 46, 86, 87). A higher prevalence of oxacillin resistance has been detected in clinical *S. epidermidis* and *S. haemolyticus* isolates (>80%) compared to other clinically important CoNS such as *S. lugdunensis* (7.9%) (27). Resistance to glycopeptides such as vancomycin was detected in *S. epidermidis* and *S. haemolyticus* prior to the first detection of reduced susceptibility in *S. aureus*, however the prevalence of glycopeptide resistance CoNS remains low despite the widespread use of vancomycin (27, 88). To date several distinct *cfr*-plasmids and multiple chromosomal *cfr* segments have been detected in CoNS (such as *S. sciuri*, *S. simulans*, and *S. warneri*) recovered from animals (89). The *cfr* gene can mediate $PhLOPS_A$ resistance and has also been detected in clinical CoNS such as *S. epidermidis* and *S. haemolyticus*, however, the overall prevalence of linezolid resistance is low among CoNS (90).

1.5 *Candida* species

1.5.1 *Candida* infection

Incidences of fungal related infections have increased in recent decades and are commonly associated with immunocompromised, elderly, pregnant or diabetic individuals (91–93). However, almost all of these mycotic infections are associated with *Candida*, *Aspergillus*, or *Cryptococcus* species (91). Recently, *Candida* species have been reported to be in the top five aetiological agents of nosocomial bloodstream infections (BSIs) in the United States (91, 94).

Most *Candida* species are relatively harmless eukaryotic yeasts that are members of the Ascomycota phylum, and have been recovered from both mammalian and environmental sources. *Candida* species are commonly identified as part of the healthy microflora on mucosal surfaces including the oral cavity, gastrointestinal tract, and vaginal tract (92, 93). Certain *Candida* species are opportunistic pathogens that can cause infection when the hosts immunity is compromised, and to date over 17 different species have been associated with disease in humans (91, 92). There are many factors associated with increased risk of invasive *Candida* infections for example the use of broad-spectrum antibiotics, damage to the skin barrier associated with indwelling medical devices such as vascular catheters, the use of prosthetic devices such as dentures or dental implants, and burns (91–93). Individuals with underlying conditions such as cystic fibrosis, diabetes, or human immune deficiency virus (HIV) infection are also prone to *Candida* related infections (95–97).

Candida infections in humans are typically attributed to *Candida albicans*, *Candida glabrata*, *Candida parapsilosis*, *Candida krusei*, and *Candida dubliniensis* (91–93). Despite increasing rates of infection caused by non-*C. albicans Candida* species, *C. albicans* remains the most prevalent species and the predominant cause of systemic and oral candidiasis, as well as nosocomial *Candida* infections (91–93, 98). Interestingly, the overall prevalence of *C. albicans* infections has gradually declined over recent decades while cases of non-*C. albicans Candida* infections have increased (99). A major contributory factor to this change has been the increased use of the azole antifungal drug fluconazole, to which species such as *C. krusei*, amongst others, are inherently resistant (99). Another contributory factor is possibly the increase in life expectancy, as species like *C. glabrata* are more commonly associated with infections in the elderly. *Candida glabrata* and *C. tropicalis* are more commonly associated with patients with solid organ or haematological malignancies, whereas *C. krusei* is more common in patients undergoing fluconazole prophylaxis, or in haematopoietic stem cell recipients (99).

1.5.2 Identification of *Candida* species

Chromogenic agar media are commonly used for the presumptive identification of medically important *Candida* species. Currently the most widely utilised medium for this purpose is CHROMagar Candida™ medium (100). This medium is selective for

Candida species and several distinct *Candida* species can be presumptively identified based on colony colour and morphology developed on this medium (Fig. 1.4).

Prior to the identification of *C. dubliniensis*, the ability to produce germ-tubes in serum was considered a definitive characteristic of *C. albicans* and was utilised to differentiate this dimorphic *Candida* species from other *Candida* species. However, this protocol has been adapted since the discovery of *C. dubliniensis*, a species that is very closely related to *C. albicans* and can also form germ-tubes in serum. *Candida albicans* can form germ-tubes following incubation in medium containing *N*-acetylglucosamine, whereas *C. dubliniensis* cannot (101), which enabled phenotypic differentiation between these species.

Both *C. albicans* and *C. dubliniensis* can both produce chlamydospores on Rice Agar Tween medium (RAT agar), differentiating them from other medically relevant *Candida* species. Chlamydospores are spore-like structures that usually are produced at the termini of pseudohyphae. *Candida albicans* typically produces a single chlamydospore at the end of a pseudohyphae when grown on RAT agar, whereas *C. dubliniensis* can produce multiple chlamydospores at pseudohyphal termini when grown on the same medium (102). However, not all *C. dubliniensis* isolates show this multiple chlamydospore characteristic (103).

Genotypic identifications methods such as polymerase chain reaction (PCR) amplification and DNA sequence analysis of intergenic spacer regions of the ribosomal (rDNA) have proven very successful for definitive identification of *Candida* species. (104). Unlike most medically relevant *Candida* species which can be presumptively identified based on colony morphology on chromogenic media, genotypic analysis is one of the most rapid methods for definitive identification and differentiation of the closely related *C. albicans* and *C. dubliniensis*, which share over 90 % DNA sequence homology (105). Currently one of the most rapid and definitive methods to discriminate between these two species is PCR amplification using oligonucleotide primers that target divergent DNA sequence between the two species (106).

1.6 Microbial Typing Systems

Molecular typing schemes have proved essential in the epidemiological and global population structural analysis of microbial pathogens. Such analyses are fundamental in facilitating the understanding of the dynamics of infectious microorganisms within

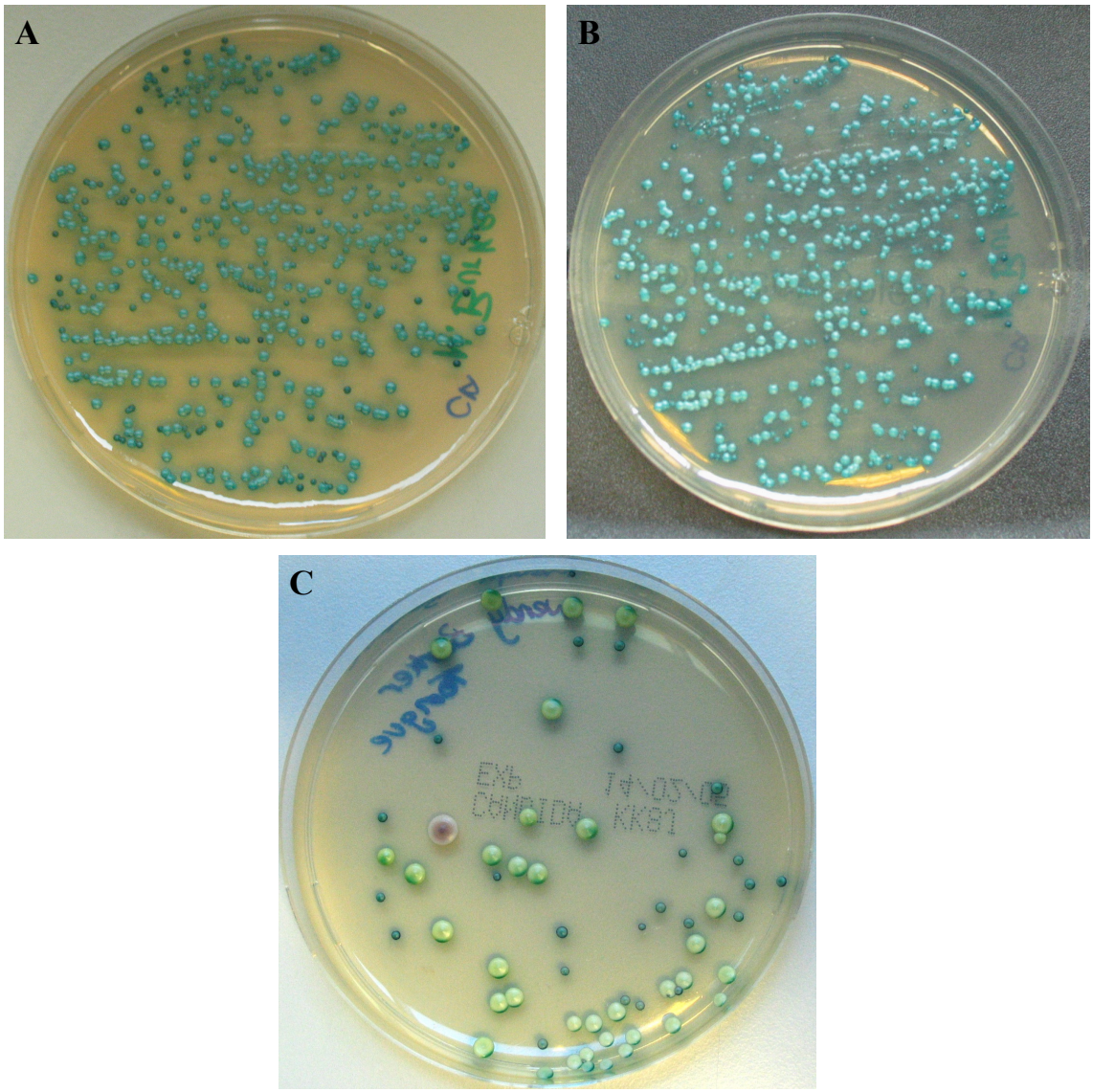


Figure 1.4 Photographs showing *Candida* species growing on CHROMagar Candida™ agar following 48 h incubation at 37°C. Panels A and B show plates with *C. albicans* (larger light blue/green colonies) and *C. dubliniensis* (smaller dark blue/green colonies) colonies. Panel C show a plate containing *C. albicans* and *C. dubliniensis* colonies and one *C. glabrata* colony (dark pink with a pale pink edge) among

human, animal and plant populations. Investigations into microbial populations can also provide information on the complexities between commensal and infectious organisms, possible origins of infections, facilitate the tracking of the emergence of antimicrobial/antifungal resistance with microbial populations, and finally provide detailed information on the genetic relatedness between isolates within the same species (93). Systems developed to investigate microbial populations should be able to (i) accurately discriminate between isolates belonging to the same species that are highly related but non-identical; (ii) recognise identical strains among a collection of isolates and generate reproducible data; (iii) be unaffected by high-frequency genome reorganisation and evolutionary pressures so that genetic changes are reasonable stable over time and reflect evolutionary change only by mutating with a medium frequency; (iv) determine the genetic distances between isolates that vary in genetic relatedness; (v) be amenable to computer-based analysis to enable data analysis, normalisation and storage, and (vi) be directly comparable between different laboratories worldwide (93).

1.6.1 Molecular typing of staphylococci

Recently phenotypic testing of staphylococcal species such as antibiotic susceptibility and biochemical testing are used to initially type isolates, which are then investigated by genotypic methods such as pulsed-field gel electrophoresis (PFGE), *spa* typing, multilocus sequence typing (MLST) and SCC*mec* typing. Pulsed field gel electrophoresis typing method is based on isolate profiling by comparing the size of chromosomal fragments generated by digestion with restriction endonucleases. The large DNA fragments are separated by switching the direction of the electrical field during electrophoresis, resulting in distinct PFGE patterns (107). The interpretation of the PFGE pattern can be subjective, reproducibility of patterns can vary between laboratories, considerable technical expertise is required, and it is time-consuming, this technique has been largely been succeeded by molecular techniques. Next generation sequencing (NGS) is increasingly being used to type isolates because of the increased accuracy and information it can provide.

1.6.1.1 Multilocus sequence typing

Multilocus sequence typing is an established technique used for typing isolates within a species, and the data generated can be used to group closely related isolates into

sequence types (STs) and clonal complexes (CCs). This technique involves the amplification of seven highly conserved genes (often referred to as housekeeping genes) by PCR and sequencing of the PCR product (108). Sequence variations at each gene are identified as distinct alleles and assigned a specific integer, with the combination of the seven integers yielding an allelic profile. The allelic profile is used to assign a ST to the isolate using the international MLST database for the specific species. The STs can be assigned to distinct CCs based on their genetic relationship using the based upon related sequence types (BURST) algorithm, and this software can also deduce the probable founding genotype for each CC (109). Additionally, computer based analysis of sequence data or allelic profiles generated from MLST analysis can be used to create phylogenetic trees based on unweighted pair group method with arithmetic advantages (UPGMA) or neighbouring joining methods to visually display the genetic relatedness of the isolates being investigated (93).

MLST is used worldwide, the results are unambiguous as they are based on sequences, and is supported by an international nomenclature that enables consistent defining of isolates based on the ST or CC they belong to. MLST schemes have been developed for many bacterial and fungal species such as *S. aureus*, *S. epidermidis*, and *C. albicans* (110–112). Limitations of this typing method are that it only compares a small section of the core genome, is laborious, and expensive.

1.6.1.2 Staphylococcus aureus *protein A (spa) typing*

Sequencing of the polymorphic X region of the protein A gene (*spa*) has been utilised as an effective typing technique suitable for both outbreak and long term investigations (113). The X region is highly diverse, variations in this region are caused by duplication and deletion of repetitive units, as well as point mutations within the repeat units (113). A *spa* type number is assigned to an isolate based on the sequence uploaded to a web-based database (http://spa.ridom.de/). This typing method is rapid and is supported by international nomenclature enabling inter-laboratory comparisons, and can detect greater population diversity compared to MLST (113).

1.6.1.3 Staphylococcal cassette chromosome mec *typing*

The SCC*mec* type can be defined by the characterisation of the *ccr* and *mec* complex types detected in MRSA isolates. Further SCC*mec* subtypes can be identified based on structural differences in the J regions. Many different SCC*mec* typing schemes have

been developed for MRSA, and typically involve numerous multiplex PCRs (114, 115). However, none of the current SCC*mec* PCR based typing schemes detect all SCC*mec* types and subtypes identified in MRSA to date. Combining SCC*mec* typing with MLST and *spa* type enables greater discrimination between MRSA isolates that belong to the same clone. A consensus international SCC*mec* typing nomenclature based on *mec* and *ccr* gene types has been established to streamline the naming of newly identified SCC*mec* types, subtypes and variants (42). To date, no SCC*mec* typing scheme has been developed for CoNS; SCC*mec* elements detected in CoNS tend to be typed based on the MRSA tying scheme (44). However, due to the higher variation in SCC*mec* elements harboured by CoNS, many of the elements detected are non-typeable SCC*mec* variants.

1.6.1.4 DNA microarray profiling

The availability of the whole genome sequence of *S. aureus* lead to the development of the high-throughput DNA microarray platform, the *S. aureus* Genotyping Kit 2.0 (Alere, Jena, Germany). This microarray platform can be utilised to rapidly screen large numbers of *S. aureus* isolates for the presence of important virulence factor and antimicrobial resistance genes and typing markers. This technology can provide detailed genetic information and accurately assign isolates to an ST and/or CC, and identify SCC*mec* types (116). The *S. aureus* Genotyping Kit 2.0 contains oligonucleotide probes specific for 336 *S. aureus* gene sequences and alleles, these include antimicrobial resistance such as those encoding resistance to macrolides, tetracycline or beta-lactams and virulence factor genes such as MSCRAMMs, biofilm associated genes and ACME.

The *S. aureus* DNA microarray can also be used to investigate the prevalence of antimicrobial resistance gene, SCC*mec* types and some virulence factors such as ACME in *S. epidermidis* as both these species can harbour the same genes (46).

1.6.1.5 Whole genome sequencing (WGS)

Over the past decade, the availability of NGS technologies has increased dramatically, and greatly reduced the cost of WGS. The use of WGS as a typing tool has proven successful for the tracking of local outbreaks in hospitals, primarily due to the higher discrimination power between isolates, and for longterm population studies. Whole-genome MLST (wgMLST) is a highly discriminatory typing method that has been developed for *S. aureus*, which examines 1500-2500 genome-wide loci for sequence

variation, in contrast to conventional MLST scheme based on seven loci (65). Core-genome MLST (cgMLST) excludes loci located on the accessory genome and also offers a much higher discriminatory power than conventional MLST.

1.6.2 Molecular typing of *Candida*

1.6.2.1 DNA fingerprinting using complex species-specific probes

Prior to the advent of direct sequencing methods and MLST, the most successful DNA-based typing technique for *Candida* isolates incorporated Southern hybridisation of restriction endonucleases-digested chromosomal DNA to a complex species-specific DNA fingerprinting probes that identified repetitive DNA sequences dispersed throughout the species genome (117, 118). This method has been used extensively due to its high discriminatory power, however the methodology is technically demanding and inter-laboratory comparisons are difficult (93). During the last two decades, the increased availability of sequencing technologies enabled the development of typing schemes based on direct DNA sequence comparisons.

1.6.2.2 ABC Genotyping

Candida albicans ABC genotyping can be used to separate *C. albicans* isolates into three distinct genotypes based on the presence, absence or heterozygous presence of a transposable intron in the 25S rDNA gene (93, 119). ABC genotyping involves the PCR amplification of the internal transcribed spacer region of the 25S rDNA gene, and PCR product separation by agarose gel electrophoresis (119). *Candida albicans* genotypes A and B are identified by a single 450 bp or 840 bp product, respectively, whereas genotype C is identified by two products 450 bp and 840 bp (119). This typing technique is quick, cheap, and the results are easily interrupted, however ABC genotyping cannot detect the overall genetic diversity of the *C. albicans* population and is typically use in conjunction with other more specific typing methods to investigate *C. albicans* populations (93).

1.6.2.3 Multilocus sequencing typing (MLST) of diploid species

Candida albicans MLST involves the PCR amplification and DNA sequence analysis of seven housekeeping genes (*AAT1a*, *ACC1*, *ADP1*, *MPI1b*, *SYA1*, *VPS13*, *ZWF1b*) that are under stabilising selection pressure (120, 121). Due to the diploid nature of *C.*

albicans and most other *Candida* species, an increased potential for sequence variation exists due to heterozygous nucleotide sites, which provide additional genotypes. For this reason, for the majority of diploid organisms the ST is referred to as a diploid sequence type (DST). Using MLST for *Candida* population analysis has been highly successful due to its high reproducibility, the speed to which it can be carried out and it has a good discriminatory power. A major advantage of MLST, particularly for analysis of *C. albicans* populations, is that the data generated is directly comparable between different laboratories worldwide and usually made available online via the curated *C. albicans* MLST database (https://pubmlst.org/calbicans/).

1.6.2.4 DNA microarray technology

More recently DNA microarray technology has been combined with MLST to develop an array for high-throughput SNP detection in *C. albicans* (122). This array detects SNPs previously detected by MLST, in conjunction with other SNPs on chromosomes that are not included in the MLST scheme (122). To date this MLST-biased SNP microarray has not be applied to large numbers of *C. albicans* isolates for population analysis.

1.6.2.5 Whole genome sequence based typing systems

As WGS technologies develop and become more cost efficient, it is likely a whole genome-based MLST scheme will be established for *Candida* species, revolutionising epidemiological studies of *Candida* species, similar to what has already been developed for some bacterial species. The development of a wgMLST scheme for *Candida* species will be more difficult to create compared to a bacterial wgMLST schemes, due to the larger size of the *Candida* genomes, the presence of multiple chromosomes, and additionally, the diploid nature of the majority of these species which means heterozygous states need to be accommodated for in genomic analyses and associated software packages.

1.7 Project aims

Understanding the polymicrobial relationships between oral microbes in dental plaque is important for enhancing our understanding of oral biofilm diseases. Currently, staphylococcal species are not considered oral commensals, but rather are considered as transient members of the oral flora. These species are of particular interest due to the

extensive range of antimicrobial resistance and virulence factor genes they can harbour that can enable them to persist in biofilm and cause persistent infection. To date, a comprehensive molecular characterisation of such species recovered from periodontal pockets, subgingival sites, and oral rinse samples of individuals during states of oral health and disease is lacking. Furthermore, the co-isolation of staphylococci and *Candida*, an important oral commensal and opportunistic yeast pathogen, in the oral cavities of healthy patients and those with plaque-related disease has not yet been determined.

The aims of the present study are:

- To determine if there is a significant difference in the prevalence and/or abundance of staphylococcal and *Candida* species in the oral rinse samples, periodontal pockets and subgingival sites of patients with periodontal disease, healthy patients with implants, and orally healthy participants.
- To undertake a comparative molecular characterisation and typing of *S. aureus* and *S. epidermidis* isolates recovered from the three distinct anatomical sites investigated of each participant group using DNA microarray technology. This technology was utilised to identify genes encoding antimicrobial resistance or virulence factors that may be particularly associated with distinct environments such as healthy or diseased subgingival sites and periodontal pockets.
- The molecular characterisation of recovered staphylococci indicated a high prevalence of the virulence factor ACME among *S. epidermidis.* The prevalence and types of ACME was further determined by PCR and WGS to investigate the genetic diversity of ACME and its association with particular environments such as periodontal pockets, subgingival sites and oral rinses samples in the participant groups sampled.
- A selection of *C. albicans* isolates were characterised using MLST to determine if there was any CC or DST enrichment associated with oral health status that could potentially serve as a marker for disease.

Chapter 2

General Materials and Methods

2.1 General microbiology methods

2.1.1 Culture and storage

Staphylococcal species were routinely cultured on MSA, Sa*Select*™ (Bio-Rad, Hercules, CA, USA) or trypticase soy agar (TSA), at 37°C for 48 h (both supplied by Oxoid Ltd., Hampshire, UK) or on Columbia blood agar (CBA) (Fannin Ltd., Dublin, Ireland) for DNA extraction prior to DNA microarray analysis.

Candida species were routinely cultured on CHROMagar Candida™ medium (CHROMagar, Paris, France), or on yeast extract peptone dextrose agar (YEPD) (Oxoid Ltd.) at 37°C for 48 h. For liquid culture, *Candida* species were grown at 37°C in YEPD broth in an orbital incubator (Gallenkamp, Leicester, UK) set at 200 RPM for 24 h.

Staphylococcal and *Candida* species were maintained in Microbank cryogenic vials (Pro-lab Diagnostics, Cheshire, UK) at -80°C. Isolates were reactivated by removing a single bead from the vial using sterile tweezers and using this bead to inoculate an appropriate agar plate followed by incubation in a static incubator (Gallenkamp) for 48 h at 37°C.

2.1.2 Chemicals, enzymes, and oligonucleotides

All chemicals used, unless otherwise specified, were of analytical grade or molecular biology grade and were purchased from Sigma-Aldrich Ltd. (Co. Wicklow, Ireland). Enzymes and other reagents for molecular biology procedures such as dNTPS, 5X green GoTaq® flexi buffer, DNA loading dye, 1 kb and 100 bp DNA molecular size reference markers were purchased from the Promega Corporation (Madison, WI, USA). Lysozyme and lysostaphin were purchased from Sigma-Aldrich Ltd. GoTaq® DNA polymerase (Promega) was used in all polymerase chain reactions (PCRs). All custom synthesised oligonucleotides were purchased from Sigma-Aldrich Ltd. at a concentration of 100 µM and diluted to a working concentration of 10 µM using molecular biology reagent water (Sigma-Aldrich Ltd.) and stored at -20°C. GelRed™ (Biotium, Fremont, CA, USA) was used to fluorescently stain DNA for agarose gel electrophoresis.

Purification of PCR products was carried out using the GenElute™ PCR Clean-Up Kit (Sigma-Aldrich Ltd.) according to the manufacturer's instructions. When more than 10 PCR amplimers required purification, these were processed simultaneously

using the QIAquick 96-well PCR purification kit (Qiagen, West Sussex, UK) according to the manufacturer's instructions.

2.1.3 Buffers and solutions

A 5 × concentration of TBE buffer was prepared using 0.45 M Trizma base, 0.45 M boric acid, 0.01 M EDTA, pH 8. This was diluted to 0.5 × in purified water (Milli-Q® Direct 8 water purification system, Millipore Ireland, Cork, Ireland) for use as the buffer solution for agarose gel electrophoresis.

For extraction of nucleic acid from *Candida* isolates a cell lysis buffer consisting of 2% (v/v) Triton X-100, 1 mM EDTA, 1% (w/v) sodium dodecyl sulphate (SDS), 100 mM NaCl, and 10 mM Tris-HCl, pH 8 was prepared and stored at 4°C.

The solution phenol:chloroform:isoamyl alcohol (24:24:1) was prepared using 24 ml of liquefied phenol washed in Tris-buffer (Sigma-Aldrich Ltd.), 24 ml of chloroform, and 1 ml of isoamyl alcohol. This solution was stored in the dark at 4°C.

Phosphate buffered saline (PBS) tablets (Oxoid Ltd.) were dissolved in purified water and sterilised by autoclaving in a Tomy SX-500E autoclave (Tomy Kogyo Co., Ltd., Tokyo, Japan) according to manufacturer's instructions.

Agarose was prepared for gel electrophoresis at concentrations of 1 or 2 % (w/v) by dissolution of agarose powder (Sigma-Aldrich) in TBE buffer, GelRed™ was added to the agarose gel at a final concentration of 1% (v/v). Agarose gel electrophoresis was carried out using Consort (B-2300 Turnhout, Belgium) power pack model EV222 set at 102 V and 80 mA, Galileo bioscience (Cambridge, MA, USA) gel boxes and well-spacing combs (Thermo Scientific, Waltham, MA, USA). Agarose gel electrophoresis was carried out for 2-3 h.

Following electrophoresis, gels were visualised using ultraviolet light at a wavelength of 312 nm in an Alpha Innotech transilluminator (Protein Simple, San Jose, CA, USA) model AVT26U and the AlphaImager mini software (Protein Simple). Images were printed using a Mitsubishi (Sant del Vallés, Barcelona, Spain) printer model P93DW.

2.1.4 Nucleic acid extraction

2.1.4.1 Bacterial DNA extraction

Bacterial isolates were lawned on TSA and incubated at 37°C for 24 h. Following incubation, the cells were collected from a 3 cm^2 area using a sterile loop (Greiner Bio-One, Gloucestershire, UK) and suspended in a 1.5 ml Eppendorf tube (Eppendorf, Hamburg, Germany) containing 250 µl lysis buffer (0.05 mg lysostaphin, 0.02 g lysozyme in 1 ml TE buffer [Tris-EDTA, 10 mM Tris-HCL and 1 mM ETDA, pH 8]) to disrupt cell wall structures. Bacterial cell lysis was carried out at 37°C for 2 h with shaking at 200 rpm in an orbital incubator (Gallenkamp). Following cell lysis, 25 µl of proteinase K (20 µg/ml) and 200 µl of buffer AL (both supplied in the Qiagen DNeasy® Blood and Tissue kit) were added to the lysate which was subsequently incubated for 30 min at 70°C to degrade cellular proteins. DNA was isolated from the cell lysate using DNeasy® mini spin columns and collection tubes supplied in the Qiagen DNeasy® Blood and Tissue kit according to the manufacturer's instructions. Purified DNA was eluted in 50 µl of molecular biology reagent water (Sigma-Aldrich Ltd.) and stored at -20°C.

2.1.4.2 DNA extraction for DNA microarray analysis

Single colonies of staphylococcal isolates for DNA microarray analysis were lawned on CBA plates and incubated at 37°C for 18 h. Cells from a 3 cm^2 area were collected and suspended in 200 µl lysis buffer A1 containing lysis enhancer A2, (both supplied with the *S. aureus* Genotyping kit 2.0 (Alere Technologies GmbH, Jena, Germany)). The cell suspension was vortexed and cell lysis was carried out in a Cliften NE5-28 shaking waterbath (Nickel Electro Ltd., North Somerset, UK) at 37°C for 60 min at 250 rpm. Following cell lysis, protein degradation of lysed cells and DNA purification was carried out using the Qiagen DNeasy® Blood and Tissue kit as previously described in section 2.1.4.1.

2.1.4.3 Candida *DNA extraction*

Prior to isolation of DNA, *Candida* isolates were grown in 2 ml YPD broth for 18 h at 37°C in an orbital incubator (Gallenkamp) at 200 rpm. *Candida* cells were harvested from 1.5 ml of the 18 h culture by centrifugation at 14,000 × *g*. The supernatant was discarded and the remaining pellet was resuspended in 200 µl cell lysis buffer (see section 2.2.1) by vortexing. The resultant suspension was transferred to a 2 ml screw

capped tube (Starstedt AG, Nümbrecht, Germany) containing 0.3 g of 425-600 µm acid-washed glass beads (Sigma-Aldrich Ltd.). A 200 µl aliquot of phenol:chloroform:isoamyl alcohol (24:24:1) was then added to the tube. Cell disruption was carried out in a Mini-Beadbeater (Biospec Products, Bartlesville, OK, USA) for 20 sec at 3250 oscillations per minute. Following disruption, extracted DNA was collected by centrifugation at 14,000 × *g* for 10 min. The aqueous phase was removed and extracted with an equal volume of chloroform:isolamyl alcohol (24:1) twice. The nucleic acids were precipitated by adding 400 µl ice-cold 70% (v/v) ethanol stored at -20ºC and 20 µl 3 M sodium acetate. Precipitated DNA was collected by centrifugation at 14,000 × *g* for 10 min. The purified DNA pellet was washed in 70% (v/v) ethanol, dried and resuspended in 50 µl of molecular biology reagent water (Sigma-Aldrich Ltd.) and stored at -20°C.

2.1.5 Determination of concentrations and quality of extracted DNA and DNA sequencing reactions

Staphylococcal DNA contained in 1.5 ml Eppendorf tubes was concentrated by evaporation in a QBD2 dry heating block (Grant, Cambridgeshire, UK) at 70°C for 30 min with the lids of the tubes open.

The DNA concentration of staphylococcal and *Candida* samples was measured using a Nanodrop 2000c spectrophotometer (Thermo Scientific) and quality checked using the OD 260:280 ratio which can indicate the presence of proteins and other contaminates in the DNA sample. For general use in PCR, each DNA sample was normalised to 60 ng/µl using ultrapure water (Sigma-Aldrich Ltd.). For extracted DNA samples being subjected to DNA microarray analysis, DNA samples were normalised to concentrations between 0.5-1.5 µg/µl.

Sequencing reactions were performed commercially by Source Bioscience (Source Bioscience, Co. Waterford, Republic of Ireland) using the Sanger sequencing method with an ABI 3730*xl* Sanger DNA analyser and an ABI BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems, Carlsbad, CA, USA).

2.2 Participant recruitment and sample collection

2.2.1 Study group

Ethical approval for this study was granted by the Faculty of Health Sciences Ethics Committee of Trinity College in February 2014 (Appendix 1). Participants were recruited from February 2014 until October 2016. The study group consisted of 31 healthy patients with dental implants, 20 patients with periodontal disease, and 64 orally healthy participants. All participants were over 18 years of age and provided informed, written consent prior to participation (Appendix 1). Participants with periodontitis included in this study met the following criteria: they were over 18 years of age; had a minimum of one periodontal site with a probing depth of greater than 6 mm, bleeding on probing (BOP) and had provided informed consent. Participants were excluded from participation if they met any of the following criteria: pregnancy or lactation, diabetes or asthma, steroid treatment during the previous year, or antibiotic or antifungal treatment in the previous two months. Patient recruitment was carried out by sending an information leaflet regarding the study to suitable participants one week prior to scheduled dental appointments at the Dublin Dental University Hospital (DDUH). Clinical sampling of recruited participants was undertaken during these routine dental appointments. Dental students were recruited as orally healthy controls by using informative posters advertising the study in DDUH.

2.2.2 Clinical sample collection

Qualified Dentists at the DDUH carried out all clinical sampling of volunteer participants. A minimum of three subgingival sites were sampled from each patient and three sites were sampled from each orally healthy participant. The subgingival sites sampled were periodontal pockets, healthy natural tooth sites, diseased implants and healthy implants according to the oral status of each participant. Each subgingival site or periodontal pocket was sampled by inserting a PerioPaper™ gingival fluid collection strip (Oraflow, NY, USA) into the subgingival crevice surrounding natural teeth or healthy oral implants or periodontal pocket for 30 s. Following sampling, the collection strips were placed into sterile 2 ml screw capped tubes (Starstedt AG) containing 1 ml nutrient broth (NB) (Oxoid Ltd.). A minimum of three subgingival sites were sampled from each patient and three from each orally healthy student participant. The

subgingival sites sampled were periodontal pockets, healthy natural tooth sites, diseased implants and healthy implants according to the oral status of each participant.

Oral rinse samples were collected by providing each participant with a sterile 100 ml plastic container (Starstedt AG) containing 25 ml sterile PBS, and instructing the participant to rinse this PBS around their oral cavity for 30 s before returning the washings to the same container.

The anterior nares of each participant were sampled using nitrogen-gassed VI-packed sterile transport swabs (Starstedt AG). Participants also completed a questionnaire comprising of medically relevant details including sex, age, tobacco use, and current oral health such as gingival redness and/or bleeding on probing (Appendix 2).

All samples were immediately transported to the microbiology laboratory and stored at 4^{o}C until processing which was completed within 4 h of collection as described in Chapter 3, Section 3.1.2.

Chapter 3

Prevalence and Abundance of Staphylococcal and *Candida* Species Recovered from Distinct Anatomical Sites in the Oral Cavities of Patients With Periodontal Disease, Healthy Patients With Implants, and Orally Healthy Participants

3.1 Introduction

3.1.1 Staphylococcal species in the oral cavity

Staphylococcal species such as *S. aureus* and *S. epidermidis,* amongst others, are well known colonisers of human and mammalian skin and mucous membranes. However, *S. aureus* is also a significant pathogen capable of causing superficial skin and soft tissue infections as well as life-threatening systemic infections such as septicaemia in humans (54). *Staphylococcus epidermidis* is significantly less pathogenic than *S. aureus* but has been previously linked with persistent infections associated with indwelling medical devices such as urinary and vascular catheters (75). Staphylococcal species produce adhesion proteins such as capsular polysaccharide adhesion (PSA) that are involved in the formation of an extracellular polysaccharide matrix in biofilms (76). Biofilm formation enhances the survival of both these species by providing protection against disinfectants, antibiotics and immune system factors as well as providing a suitable environment for horizontal gene transfer. *Staphylococcus aureus* and *S. epidermidis* are often associated with biofilm formation *in vivo* on titanium alloy-surfaced implants such as prosthetic joint replacements (76).

Interestingly, staphylococcal species were not previously considered a part of the normal oral flora and when they were recovered from the oral cavity they were considered transient members (123). More recently researchers have investigated the prevalence of staphylococcal species in the oral cavity during oral health, periodontal disease, and peri-implantitis by sampling the oral cavity in general and more specifically, dental plaque recovered from periodontal pockets and subgingival sites (24, 124–128). *Staphylococcus aureus* has been associated with oral diseases such as osteomyelitis of the jaw and periodontal disease (129–131) and, in combination with *Candida* species, has also been associated with angular cheilitis (129). Previously *S. aureus* was associated with peri-implantitis based on the application of DNA:DNA checkerboard technology; however, this technology lacks discrimination in the differentiation of *S. aureus* from *S. epidermidis* (78, 79). The DNA:DNA checkerboard technology approach involves the hybridisation of DNA extracted from clinical samples to probes generated from total cellular DNA of reference organisms. As *S. aureus* and *S. epidermidis* and other CoNS species can share antimicrobial agent resistance genes, virulence-associated genes, as well as other mobile genetic elements in common, considerable opportunities exist for cross-hybridisation, resulting in-false positive

identification of *S. aureus* (132). Furthermore, as the method is solely based on DNA, hybridisation does not distinguish between viable and non-viable bacterial organisms (132).

Overall the prevalence of staphylococcal species recovered from the oral cavity across the different studies varies, possibly due to the range of different isolation and species identification techniques used. For example, one study reported that the prevalence of *S. aureus* in periodontal pockets was 29.2%, however this investigation used DNA:DNA checkerboard technology to identify the staphylococcal species present (130), while another study specifically investigated the prevalence of *S. aureus* in periodontal pockets (68.2%) using quantitative real-time PCR (q-PCR) and did not investigate the prevalence of CoNS (24).

Recent studies reported that *S. epidermidis* and *S. aureus* are the most frequently recovered staphylococcal species from anatomical sites in the oral cavity. However, the presence of other species including *S. warneri*, *S. hominis*, *S. haemolyticus*, and *S. saprophyticus* has also been reported (123–125, 128). Previous studies of patients with periodontal disease reported staphylococcal species oral prevalence rates ranging from 61.36% to 84% (125, 133). However, another study reported a 94% prevalence of staphylococci in orally healthy participants (134). These studies used oral rinse techniques to sample the oral cavity in general, cultured staphylococci on selective media such as MSA, and identified staphylococcal species based on coagulase tests or biochemical tests such as the commercially available API Staph system. The reported prevalence of staphylococcal species in oral rinse samples from patients with peri-implantitis was 72.3%, using both culture based and 16S rDNA PCR identification techniques (132). The predominant staphylococcal species identified in the oral rinse samples of patients with peri-implantitis were *S. epidermidis* (47.4%) followed by *S. aureus* (21.1%) (132). The prevalence of staphylococcal species in periodontal pockets sampled in previous studies ranged from 50 – 86.7% (123, 124, 128). Two of these studies reported that *S. epidermidis* was the most prevalent species (ranging from 45-80%) (123, 124), whereas a the third study reported *S. auricularis* (31.4%) followed by *S. epidermidis* (21.4%) as the most predominant species (128). Periodontal pockets examined in these studies were sampled using paper points, staphylococcal species were cultured on selective media such as MSA, and identified based on coagulase tests or the API Staph system. Another previous study reported the prevalence of staphylococcal species in peri-implant pockets to be 44.8%; *S. epidermidis* and *S. aureus* were detected

in 31% and 10.34% of these pockets, respectively (132). The prevalence of *S. epidermidis* in periodontal pockets suggests this species could possibly be contributing to periodontal disease and/or biofilm formation in this distinct anatomical site. Periodontal pockets are predominantly anaerobic and it is perhaps unexpected or unusual to recover staphylococcal species from such an environment. It is critical that supragingival biofilm and saliva are removed from the periodontal pocket site prior to sampling to avoid cross contamination of the periodontal pocket sample. Unfortunately, studies investigating the prevalence of staphylococcal species in distinct oral anatomical sites rarely report relative abundance data and therefore it is difficult to define the difference between staphylococcal carriage and infection in these sites.

3.1.2 *Candida* in the oral cavity

Candida albicans is the most prevalent *Candida* species in the oral cavity, and is present in the oral cavities of 30-45% of healthy adults (98, 135). *Candida albicans* is also the most prevalent *Candida* species in the oral cavities of individuals with oral disease such as periodontal disease (47.6%) and oral leukoplakia (70.5%) or in patients with underlying diseases such as acquired immunodeficiency syndrome (AIDS) (85%), diabetes (60%) and autoimmune polyendocrinopathy-candidiasis- ectodermal dystrophy (APECED) (75%) (95, 96, 136–139). *Candida albicans* is known to play a direct role in the chronic infection of oral leukoplakia (*Candida* leukoplakia). Persistent *Candida* infection such as occurs in patients with APECED or oral leukoplakia have been associated with increased risk of oral carcinoma development due to the oxidation of ethanol or fermentation of glucose by *Candida* species leading to the production of the highly carcinogenic acetaldehyde (138, 140). Other *Candida* species such as *C. glabrata* and *C. dubliniensis* have previously been associated with immunocompromised individuals such as HIV-infected and AIDS patients and patients undergoing organ transplantations (141, 142).

Patients with gingivitis who use dental devices such as orthodontic appliances have a significantly higher prevalence of *C. albicans* associated with subgingival sites (143). *Candida albicans* was recovered from subgingival sites of 17% of patients with gingivitis using orthodontic devices compared to 1.9% of patients with gingivitis but without orthodontic devices (143). Two separate previous studies have reported a similar prevalence of *C. albicans* recovered from the oral cavities of patients with

periodontal disease (10, 139). *Candida albicans* was detected in periodontal pockets of 10/21 (47.6%) patients with periodontal disease (10) and 8/17 (47%) of patients with severe periodontal disease compared to 3/20 (15%) subgingival sites in orally healthy adults (139). In both studies *C. albicans* was recovered in association with other *Candida* species such as *C. dubliniensis* and *C. glabrata*, and a greater abundance of *Candida* species was associated with instances of disease (10, 139). Very few studies have specifically investigated the prevalence of *Candida* species in subgingival sites or periodontal pockets or compared the *Candida* prevalence in these sites across multiple patient groups. However, a higher prevalence of *Candida* recovered from periodontal pockets compared to subgingival sites has been reported previously, (10, 139, 143) suggesting that *Candida* may play a role in the progression of periodontal disease.

The predominance of *Candida* species and *S. epidermidis* in periodontal pockets suggests that these species can survive and possibly contribute to biofilm formation in this distinct anatomical site.

3.1.3 Species identification using MALDI-TOF

Chromogenic media such as Sa*Select*™ (Bio-Rad) and CHROMagar Candida™ medium (CHROMagar, Paris, France) contain chromogenic substrates that enable the presumptive identification of staphylococcal and *Candida* species, respectively, based on colony colour patterns and morphology (29, 100, 144).

Matrix Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) technology is based on the comparison of ribosomal protein ion spectra. Each species yields a distinctive MALDI-TOF spectrum, which can be compared to a database containing a library of spectra representing many different microbial species. Briefly, a microbial culture is irradiated in a matrix by a laser, ribosomal protein ions are accelerated through a vacuum tunnel where they are separated by size and detected by a detector at the opposite end of the vacuum tunnel as spectra (Fig. 3.1) (145). The spectrum obtained is compared to an extensive spectra database and the identification is given a quality score (145). This technology has significantly reduced the time, expense and labour required for bacterial and fungal species identification in the clinical laboratory.

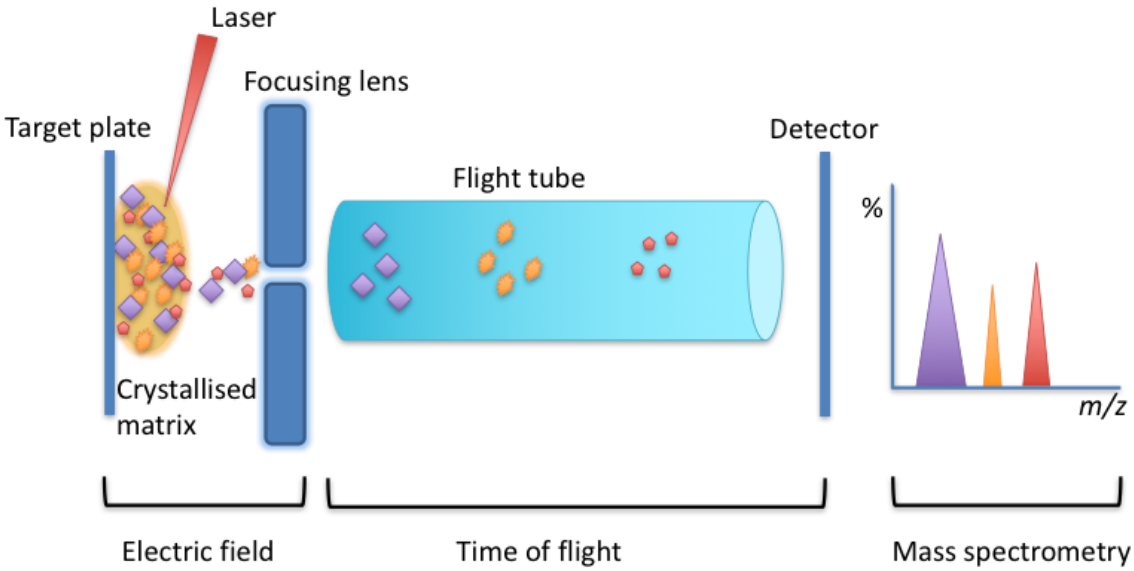


Figure 3.1 Schematic diagram of microbial identification by matrix assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF MS). MALDI-TOF MS has emerged as a powerful tool for microbial identification whereby microorganisms are identified using either intact cells or cell extracts. The colours purple, orange and red represent three different ribosomal proteins embedded in the crystallised matrix which is represented as the faint orange background on the target plate. Desorption of the ribosomal proteins occurs when the laser hits the matrix in the electric field and the ribosomal ions are accelerated into the flight tube. Separation of the ions occurs in the flight tube and the divergence in time of flight of the ions is detected in the detector and converted into spectra that can be compared with profiles for a wide range of reference microbial species stored in a database. Figure adapted from J. Lavigne *et al.* (145).

3.1.4 Objectives

The aim of this part of the present study was to determine the prevalence and abundance of all staphylococcal and *Candida* species recovered from oral rinse samples, periodontal pockets and subgingival sites of three distinct participant groups including patients with periodontal disease, healthy patients with oral implants and orally healthy participants. Staphylococcal and *Candida* species were identified using chromogenic media, MALDI-TOF or PCR. More specifically, this study aimed to:

- Determine if there is significant difference in the prevalence and abundance of staphylococcal and *Candida* species in the oral rinse samples of patients with periodontal disease and healthy patients with implants in comparison to orally healthy participants.
- Determine if there is a significant difference in the prevalence and abundance of the same species in the periodontal pockets and subgingival sites of the two previously mentioned patient groups in comparison to orally healthy participants.
- Investigate if participants in this study harboured the same staphylococcal species in their nasal and oral cavities.

3.2 Materials and methods

3.2.1 Clinical sample collection

Qualified Dentists at the DDUH carried out all clinical sampling of volunteer participants as described previously (Chapter 2, Section 2.2).

3.2.2 Isolation of staphylococci and *Candida* from clinical samples

Oral rinse samples were recovered from study participants as described in Chapter 2, Section 2.2.2. Collected oral rinse samples were vortexed in their collection containers at maximum speed using a Heidolph Reax vortex (Heidolph Instruments GmbH & Co., Schwabach, Germany) for 30 s, after which time 1 ml aliquots were transferred into separate sterile 1.5 ml Eppendorf microfuge tubes. These were then centrifuged at 20,000 × *g* for 1 min using an Eppendorf 5417C bench top centrifuge (Eppendorf, Hamburg, Germany). Following centrifugation, the supernatant from each sample was removed and discarded and the pellet resuspended in 300 µl of NB solution. Aliquots (100 µl) of the resulting suspension were then plated on MSA and Sa*Select*™ agar media to recover staphylococcal species and onto CHROMagar™ Candida agar plates to recover *Candida* species. To allow for greater colour differentiation between species on selective media and in particular between *S. aureus* and *S. epidermidis* isolates on Sa*Select*™, all plates were incubated for 48 h at 37°C.

The nares of participants were sampled to investigate if staphylococcal trafficking was occurring between the nares and oral cavity. Swabs used to sample the anterior nares were used to lawn the entire surface of MSA and Sa*Select*™ plates while rotating the swab. All plates were incubated for 48 h at 37°C.

PerioPaper samples were vortexed in their sample containers with 1 ml NB at maximum speed for 1 min. Immediately afterwards, 50 µl aliquots of the resulting suspension were then plated onto separate MSA, Sa*Select*™ and CHROMagar™ Candida plates to recover staphylococcal and *Candida* species, respectively. All plates were incubated for 48 h at 37°C.

Single colony purification of morphologically distinct staphylococcal or *Candida* colonies from primary isolation plates was carried out by subculture onto TSA or YEPD agar, respectively, followed by incubation at 37°C for 48 h. All isolates were preserved on Microbank™ cryostorage beads at -80°C for long-term storage prior to

further analysis. Isolates recovered from patients with periodontal disease and healthy patients with implants were assigned a name beginning with the letter P and I, respectively followed by a numerical value to indicate distinct patients. Isolates recovered from orally healthy participants were assigned a name beginning with the letter C and a three-digit numerical value to indicate distinct participants.

3.2.3 Quantitative species identification on differential and selective media

3.2.3.1 Presumptive identification and quantitation of staphylococci

Following incubation, the number of each distinct colony type recovered from oral rinse and PerioPaper™ samples was recorded as relative abundance in colony forming units per ml (CFU/ml). Each colony type recovered from nasal swabs was stored as a representative of the nasal staphylococcal population however relative abundance data was not recorded for these samples. Colonies that were pink to orange in colour on Sa*Select* after 48 h incubation were presumptively identified as *S. aureus*, whereas distinctive pale pink colonies that were smaller in size than *S. aureus* were presumptively identified as *S. epidermidis* (Fig. 3.2) (29). Staphylococcal species such as *S. saprophyticus* (blue/green colour), *S. lugdunensis* (pale-yellow), *S. haemolyticus* (white-yellow), *S. warneri* (white-yellow) and *S. sciuri* (dark-yellow) were also presumptively identified on Sa*Select*™ after 48 h (29). Colonies on MSA that turned the surrounding agar from red to yellow were presumptively identified as *S. aureus*.

3.2.3.2 Presumptive identification and quantitation of Candida *species*

Following incubation, the number of each distinct colony type recovered from oral rinse and PerioPaper™ samples on CHROMagar Candida™ was recorded and calculated as abundance in colony forming units per ml (CFU/ml). Distinct green colonies of a moderate size were presumptively identified as *C. albicans* (Fig. 3.3), small dark green-blue colonies were presumptively identified as *C. dubliniensis* (100). Less frequently recovered species such as *C. glabrata* (deep pink colonies with a light pink edge) were recovered and presumptively identified based on colony colour and morphology (100).

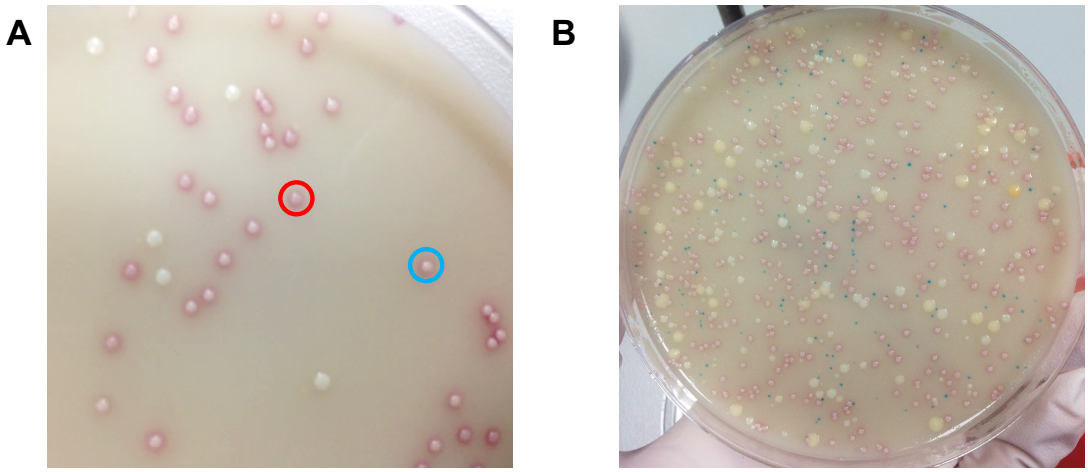


Figure 3.2 Photographs showing the appearance of staphylococcal species frequently recovered from clinical samples on chromogenic Sa*Select*™ agar after 48 h incubation. The blue circle in panel A highlights a presumptive *S. epidermidis* colony based on its pale pink colour. The red circle in panel A highlights a pink colony presumptively identified as *S. aureus*. Panel B shows an example of the range of staphylococci that can be presumptively distinguished on an Sa*Select* plate after 48 h incubation at 37°C. *Staphylococcus epidermidis* (pale pink colonies), *S. saprophyticus* (blue/green colonies), *S. lugdunensis* (pale yellow colonies) and *S. haemolyticus* or *S. warneri* (white-yellow colonies).

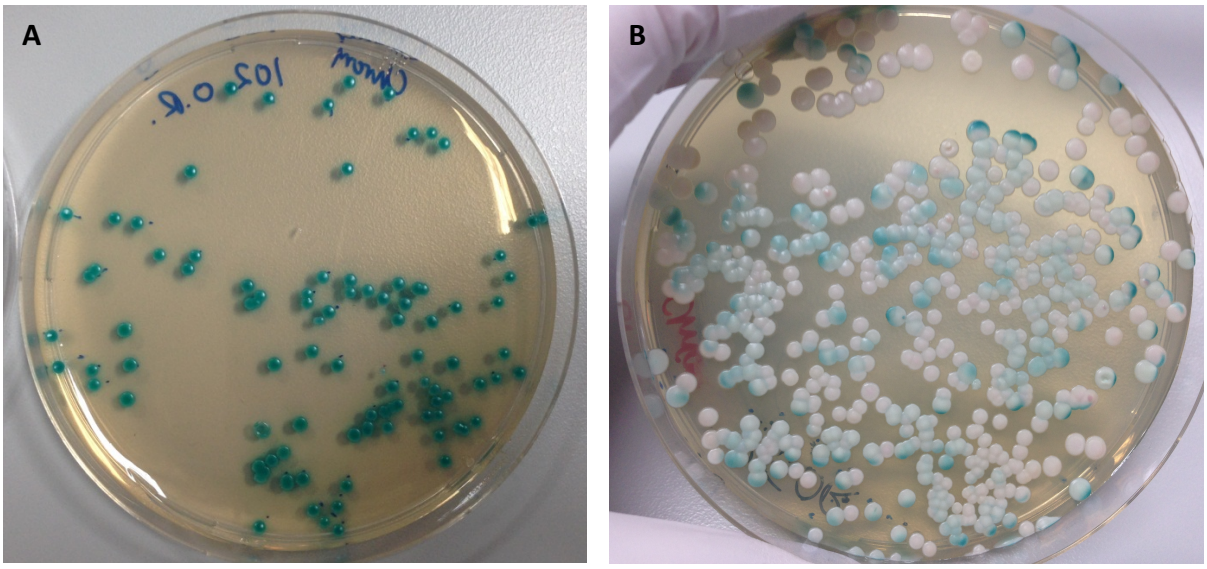


Figure 3.3 Photographs showing *Candida* species recovered from clinical samples on chromogenic CHROMagar Candida™ agar following 48 h incubation. Panel A shows *C. albicans* colonies recovered from an oral rinse sample of a healthy student grown on CHROMagar at 37°C for 48 h. Colony colours formed by *Candida albicans* on CHROMagar™ vary from light green to dark green depending on the strain. Panel B shows a mix of *C. albicans* (light green) and *C. glabrata* (pale pink) recovered from an oral rinse sample of a healthy patient with implants.

3.2.4 Definitive identification of staphylococcal species using matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF)

MALDI-TOF technology was used to definitively identify staphylococcal isolates recovered from study participants. Bacterial isolates were cultured on CBA at 37°C for 48 h prior to identification by MALDI-TOF which was performed using the VITEK® MS system (bioMérieux, Marcy L'Etoile, France) according to the manufacturer's instructions. Species identification was based on confidence values issued by the VITEK® MS system and only confidence values ≥ 99.9% were accepted for identification. Any isolate that yielded low confidence values or no identification was re-tested.

3.2.5 Definitive identification of *Candida* species using species-specific PCR

Definitive identification of *C. albicans* and *C. dubliniensis* isolates was carried out by multiplex PCR as described previously by Donnelly *et al.* (106). DNA extracted from *Candida* isolates (Chapter 2, Section 2.1.4.3) was normalised to a concentration of 60 ng/µl and a 1µl aliquot containing 60 ng DNA was used for amplification in 50 µl reaction volumes consisting of 10 µM of each primer pair caACT, DUB and RNA (Table 3.1), 1.5 mM $MgCl_2$, 5X green GoTaq flexi buffer, sterile water, 200 µM dNTPs and 2.5 U of GoTaq DNA polymerase. The caACT and DUB primers amplify specific regions of the *ACT1* gene and *ACT1* intron, respectively. Pan-fungal primers that amplify a conserved region of the gene encoding rRNA were used as a positive PCR control (Table 3.1) (106, 146). The cycling conditions consisted of an initial denaturation step at 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 3 min, and ending with final elongation step at 72°C for 10 min. Agarose gel electrophoresis was carried out as described in Chapter 2, Section 2.1.3.

All *Candida* isolates yielded an amplimer approximately 1768 bp in size corresponding to the pan-fungal primers, whereas *C. albicans* and *C. dubliniensis* isolates yielded a second amplimer of either 614 bp or 288 bp in size, respectively.

3.2.6 Statistical analysis

Statistical analyses (Fisher's exact tests and student *t*-tests) to compare species prevalence and abundance between the three distinct participant groups was carried out using GraphpadQuickCalcs, GraphPad Software

Table 3.1 Oligonucleotide primers used for the analysis of *Candida* species

Primer pairs	Expected amplicon size	Primer sequence (5'→ 3')	Primer target	GenBank accession number	Amplicon coordinates[a]	Reference
caACT[b] F	1768 bp	CGGAATTCAATGGACGGTGGTATGTT	*ACT1* gene	X16377	-1 → +17	(106)
caACT[b] R		CGGAATTCAATGGATGGACCAGATTCGTCG			+1746 → +1767	
DUB[c] F	288 bp	GTATTTGTCGTTCCCCTTTC	*ACT1* intron	AJ236897	+ 251 → +270	(106)
DUB[c] R		GTGTTGTGTGCACTAACGTC			+ 519 → + 538	
RNA[d] F	614 bp	GCATATCAATAAGCGGAGGAAAAG	Large ribosomal	X83718	+40 → + 63	(146)
RNA[d] R		CGTCTTGAAACACGGACC	subunit gene		+638→ +655	

[a] Amplicon coordinates are shown in the 5' to 3' direction, the first base of the start codon ATG being +1.

[b] caACT primers specifically amplify a coding region if the *ACT1* gene in *C. albicans*.

[c] DUB primers specifically amplify a non-coding intron region of the *ACT1* intron in *C. dublinensis*

[d] RNA primers specifically amplify a region in the D1-D2 region of the large ribosomal subunit gene in fungal species.

Abbreviations: bp, base pair.

(http://www.graphpad.com/QuickCalcs/). A p value < 0.05 was deemed statistically significant.

3.3 Results

3.3.1 Prevalence of staphylococcal and *Candida* species among participants investigated.

The prevalence of staphylococcal and *Candida* species recovered from oral rinse samples, nares, periodontal pockets, and subgingival sites of the three distinct participant groups investigated is outlined in Table 3.2. The average age range of patients with periodontal disease was 45-54 years, the average age ranges of healthy patients with implants and orally healthy participants was slightly lower at 34-44 years and 18-24 years, respectively. Of patients with periodontal disease 6/20 (30%) were male, and 14/20 (70%) were female, of healthy patients with implants 12/31 (38.7%) were male, and 19/31 (61.2%) were female, and finally of orally healthy participants 27/64 (42.1%) were male and 37/64 (57.8%) were female. Among patients with periodontal disease, healthy patients with implants and orally healthy participants 5/20 (25%), 5/31 (16.1%), and 2/64 (3.1%) were smokers.

3.3.1.1 Prevalence of staphylococcal and Candida *species among patients with periodontal disease.*

Staphylococcal species were recovered from the oral rinse samples of 20/20 (100%) of patients with periodontal disease investigated, 18/20 of which also yielded staphylococci from their nasal cavities (Table 3.2). Staphylococcal species were also recovered from subgingival sites and periodontal pockets of five (20%) and nine (45%) patients with periodontal disease, respectively.

The coagulase negative species *S. epidermidis* was the predominant staphylococcal species isolated from patients with periodontal disease, and was recovered from 18/20 (90%) oral rinse samples and 17/20 (85%) nasal samples (Table 3.2). *Staphylococcus epidermidis* was recovered from both the nasal and oral cavities of 15 patients with periodontal disease. *Staphylococcus epidermidis* was recovered from the subgingival sites and periodontal pockets of 4/20 (20%) and 6/20 (30%) of patients with periodontal disease, respectively.

Staphylococcus aureus was the second most prevalent staphylococcal species identified among samples taken from patients with periodontal disease, and was detected in oral rinse samples of 5/20 (25%) and in nares samples of 5/20 (25%) patients (Table 3.2). This species was recovered from both the nasal swab and oral rinse

Table 3.2 Prevalence of *S. epidermidis*, *S. aureus* and *Candida* species detected among the study participants investigated

Staphylococcal and/or *Candida* species identified	Number of individuals from whom species were recovered (%)			
	Oral rinse (%)	Nasal swab (%)	Perio Paper™ strips[a] (%)	
Patients with periodontal disease (*n*=20)[b]			**Subgingival sites**	**Periodontal pockets**
Staphylococcal species	20/20 (100)	18/20 (90)	5/20 (25)	9/20 (45)
S. epidermidis	18/20 (90)	17/20 (85)	4/20 (20)	6/20 (30)
S. aureus	5/20 (25)	5/20 (25)	0/20 (0)	0/20 (0)
Other staphylococcal species[b]	9/20 (45)	11/20 (55)	3/20 (15)	4/20 (20)
Candida species	11/20 (55)	N/A	3/20 (15)	4/20 (20)
C. albicans	11/20 (55)	N/A	2/20 (10)	4/20 (20)
Other *Candida* species[b]	1/20 (5)	N/A	1/20 (5)	1/20 (5)
Healthy patients with implants (*n*=31)[b]				
Staphylococcal species	30/31(96.8)	30/31 (96.8)	11/31 (35.5)	N/A
S. epidermidis	25/31 (80.6)	24/31 (77.4)	5/31 (16.1)	N/A
S. aureus	15/31 (48.4)	14/31 (45.2)	4/31 (12.9)	N/A
Other staphylococcal species[b]	16/31 (51.6)	14/31 (45.2)	4/31 (12.9)	N/A
Candida species	19/31 (61.3)	N/A	8/31 (25.8)	N/A
C. albicans	14/31 (45.2)	N/A	7/31 (22.6)	N/A
Other *Candida* species[b]	6/31 (19.4)	N/A	2/31 (6.5)	N/A
Orally healthy participants (*n*=64)[b]				
Staphylococcal species	54/64 (84.4)	61/64 (95.3)	11/64 (17.2)	N/A
S. epidermidis	43/64 (67.2)	55/64 (85.9)	5/64 (7.8)	N/A
S. aureus	19/64 (29.7)	12/64 (18.75)	5/64 (7.8)	N/A
Other staphylococcal species[b]	13/64 (20.3)	22/64 (34.4)	2/64 (3.1)	N/A
Candida species	24/64 (37.5)	N/A	3/64 (4.7)	N/A
C. albicans	17/64 (26.6)	N/A	3/64 (4.68)	N/A
Other *Candida* species[b]	9/64 (14.1)	N/A	0/64 (0)	N/A

[a] Perio Paper™ Strips were used to sample healthy subgingival sites in healthy participants, in healthy patients with implants and both healthy subgingival sites and periodontal pockets in patients with periodontal disease.

[b] Other staphylococcal species identified included *S. hominis*, *S. haemolyticus*, *S. warneri*, *S. pasteuri*, *S. saprophyticus*, *S. capitis*, *S. vitulinus*, *S. lugdunensis*. Other *Candida* species identified included *C. dubliniensis* and *C. glabrata*.

Abbreviations: NA, not applicable.

samples of two distinct patients. Although *S. aureus* was the second most prevalent staphylococcal species recovered by oral rinse sampling, it was not recovered from any subgingival site or periodontal pocket samples (Table 3.2).

Other staphylococcal species recovered from the oral cavity (9/20, 45%) and nares (11/20, 55%) included *S. haemolyticus*, *S. warneri, S, pasteuri*, and *S. saprophyticus* and these species were always co-isolated with *S. epidermidis* (Table 3.2). *Staphylococcus hominis*, *S. equorum*, *S. warneri*, and *S. pasteuri* were recovered from subgingival sites and periodontal pockets. Patient P15 did not yield *S. epidermidis* from their oral rinse or subgingival site samples, however samples from these sites yielded *S. equorum*. Patients P13 yielded *S. hominis* from a subgingival site and P18 yielded *S. warneri* from periodontal pockets and interestingly, these species were not recovered from these patients' oral rinse samples. Patients P22 harboured a diverse range of staphylococcal species; *S. epidermidis*, *S. pasteuri* and *S. capitis* were recovered from an oral rinse sample, *S. warneri* and *S. pastueri* were recovered from periodontal pockets, and finally, *S. epidermidis* and *S. pastueri* were recovered from subgingival sites.

Candida species were recovered from the oral rinse sample of 11/20 (55%), the subgingival sites of 3/20 (15%) and the periodontal pockets of 4/20 (20%) patients with periodontal disease investigated (Table 3.2). All five patients from whom *Candida* species were recovered from subgingival sites and/or periodontal pockets also yielded the same *Candida* species from their oral rinse sample.

The most common *Candida* species identified was *C. albicans*, which was detected in the oral rinse sample of 11/20 (55%), the subgingival sites of 2/20 (10%) and periodontal pockets of 2/20 (10%) patients with periodontal disease (Table 3.2). *Candida albicans* was recovered from the oral rinse sample and a periodontal pocket but not from subgingival sites of Patient P13. In contrast, Patient P14 yielded *C. albicans* from the oral cavity and subgingival sites but not from periodontal pockets. *Candida dubliniensis* was recovered from the oral rinse sample, subgingival sites and periodontal pockets of one patient (Patient P9).

3.3.1.2 Prevalence of staphylococcal and Candida *species among healthy patients with implants.*

Staphylococcal species were recovered from the oral rinse sample and nasal cavity of 30/31 (96.8%) and 30/31 (96.8%) healthy patients with implants, respectively (Table

3.2). Staphylococcal species were not recovered from the nares of Patient I4 or the oral rinse sample of Patient I19. Staphylococci were also identified in subgingival sites of healthy implants or natural teeth of 11/31 (35.5%) participants in this group (Table 3.2).

Similar to the patients with periodontal disease, the most prevalent staphylococcal species recovered from the oral rinse samples (25/31, 80.6%) and nares (24/31, 77.4%) of healthy patients with implants was *S. epidermidis* (Table 3.2). *Staphylococcus epidermidis* was recovered from both oral rinse samples and nares samples of 19 patients. This species was also recovered from subgingival sites in five (5/31, 16.1%) patients (Table 3.2), and all five of these patients also harboured *S. epidermidis* in their oral rinse samples.

The second most prevalent staphylococcal species recovered from oral rinse and nasal samples was *S. aureus*, which was recovered from 15/31 (48.4%) and 14/31 (45.2%) of samples, respectively (Table 3.2). This species was recovered from both oral rinse and nasal samples of 12 distinct patients. *Staphylococcus aureus* was almost twice as prevalent in oral rinse samples from healthy patients with implants than the corresponding samples from patients with periodontal disease although the difference was not statistically significant ($p = 0.1428$). This species was also recovered from the subgingival sites of 4/31 (12.9%) of healthy patients with implants and was co-isolated from a subgingival site with *S. epidermidis* in 1/4 (25%) of these patients. Three of these patients (Patients I3, I15 and I28) yielded *S. aureus* from their oral rinse and nares samples also. The remaining patient (Patient I33) yielded *S. aureus* from their oral rinse sample also.

Additional staphylococcal species including *S. warneri*, *S. pasteuri*, *S. capitis*, *S. haemolyticus*, *S. hominis*, *S. saprophyticus*, *S. lugdunensis*, and *S. cohnii* were also recovered from the oral rinse samples, nares and subgingival sites of 22/31 (71%) healthy patients with implants. Patient I30 harboured *S. aureus, S. epidermidis, S. capitis* and *S. saprophyticus* in their oral rinse sample, however only *S. capitis* and *S. saprophyticus* were recovered from this patient's subgingival sites. Another patient (Patient I26) yielded *S. aureus, S. epidermidis* and *S. capitis* from their oral rinse sample, *S. pasteuri* and *S. capitis* from subgingival sites, and *S. pasteuri*, *S. epidermidis* and *S. warneri* from the nares. Two distinct patients each yielded *S. hominis* from subgingival sites; however, this species was not recovered from oral rinse or nares samples of these patients.

Candida species were recovered from the oral rinse samples and subgingival sites of 19/31 (61.3%) and 8/31 (25.8%) of healthy patients with implants, respectively (Table 3.2). As observed in the patients with periodontal disease, *C. albicans* was the most common *Candida* species identified, detected in the oral rinse samples of 14/31 (45.2%) and subgingival sites of 7/31 (22.6%) of these patients (Table 3.2). Five patients yielded *C. albicans* from both oral rinse samples and subgingival sites.

Candida dubliniensis was recovered from the oral rinse samples of five patients (5/31, 16.1%) and the subgingival sites of two patients (2/31, 6.5%) with implants. Both patients from whom *C. dubliniensis* was detected in their subgingival sites, also harboured it in their oral rinse samples. Patient I26 yielded *C. glabrata* from their oral rinse sample.

3.3.1.3 Prevalence of staphylococcal and Candida *species among orally healthy participants.*

Staphylococcal species were recovered from the oral rinse samples of 54/64 (84.4%), the nares 61/64 (95.3%) and subgingival sites of 11/64 (17.2%) of the orally healthy participants investigated (Table 3.2).

Staphylococcus epidermidis was the most prevalent staphylococcal species identified, detected in the oral rinse samples of 43/64 (67.2%), the nares 55/64 (85.9%) and subgingival sites of 5/64 (7.8%) of orally healthy participants (Table 3.2). This species was recovered from both the oral rinse and nares samples of 33 orally healthy participants. The prevalence of *S. epidermidis* in oral rinse samples was significantly lower ($p = 0.0501$) in orally healthy participants than patients with periodontal disease. All five participants that yielded *S. epidermidis* from subgingival sites also yielded it from their oral rinse and nares samples. The prevalence of *S. epidermidis* recovered from subgingival sites was significantly lower ($p = 0.0189$) than in periodontal pockets in patients with periodontal disease.

The prevalence of *S. aureus* in the oral cavity (19/64, 29.7%) was less than half that of *S. epidermidis* (43/64, 67.2%) (Table 3.2). Twelve participants (12/64, 18.75%) harboured *S. aureus* in their nares, and five (5/63, 7.8%) participants harboured it in subgingival sites (Table 3.2). *Staphylococcus aureus* was harboured in both the nasal and oral cavities of six distinct participants in this group. Two orally healthy participants (Participants C3 and 200) yielded *S. aureus* from subgingival sites, oral rinse sample, and the nasal cavity, one participant (Participant 212) yielded *S. aureus*

from subgingival sites and their nasal cavity and one participant (Participant 323) yielded *S. aureus* from subgingival sites only. *Staphylococcus aureus* and *S. epidermidis* were both co-isolated from subgingival sites and the oral rinse sample of Participant 217.

Overall, the prevalence of *Candida* species was lowest in the orally healthy participant groups compared to the other two sample groups in each anatomical site sampled. *Candida* species were recovered from oral rinse samples of 24/64 (37.5%) and subgingival sites of 3/64 (4.7%) orally healthy participants (Table 3.2). *Candida albicans* was predominant, recovered from the oral rinse samples of 17/64 (26.6%) and subgingival sites of 3/64 (4.7%) participants (Table 3.2). The prevalence of *C. albicans* in oral rinse samples from orally healthy participants was significantly lower (p = 0.0288) than in the same samples from patients with periodontal disease (Table 3.2). The prevalence of *C. albicans* was also lower in oral rinse samples compared to healthy patients with implants, however it was not statistically significant (p = 0.1016) (Table 3.2). All three participants who harboured *C. albicans* in subgingival sites also harboured it in their oral rinse samples. *Candida dubliniensis* and *C. glabrata* were recovered by oral rinse sampling of 5/64 (7.8%) and 4/64 (6.25%) of participants, respectively.

3.3.1.4 Comparison of the prevalence of staphylococcal and Candida *species among the three groups of participants investigated.*

Staphylococcus epidermidis was the most prevalent staphylococcal species recovered from all three distinct anatomical sites sampled among the three participant groups (Table 3.2). *Staphylococcus epidermidis* predominated in oral rinse samples from all three patient groups, and this species was almost significantly more prevalent (p = 0.0501) among patients with periodontal disease (90%) compared to the orally healthy participants (67.2%). Similarly, *S. epidermidis* was also more prevalent in subgingival sites (20%) and periodontal pockets (30%) of patients with periodontal disease than in subgingival sites of healthy patients with implants (16.1%) and was significantly more prevalent (p = 0.0189) in periodontal pockets of patients with periodontal disease (30%) than in subgingival sites of orally healthy participants (7.8%).

Interestingly, the prevalence of *S. aureus* was considerably lower in oral rinse samples of patients with periodontal disease (25%) than in healthy patients with implants (48.4%) (Table 3.2), although this was not significant (p = 0.1428).

Participants from both the healthy patients with implants and orally healthy participants yielded *S. aureus* from subgingival sites (12.9% and 7.8%, respectively). In contrast, this species was not detected in the subgingival sites or periodontal pockets of patients with periodontal disease (0%) (Table 3.2).

The prevalence of *Candida* (predominantly *C. albicans*) in the oral cavity was higher among healthy patients with implants (61.3%) and patients with periodontal disease (55%) and lowest among orally healthy controls (37.5%) (Table 3.2). The difference in the prevalence of *Candida* from oral rinse samples between healthy patients with implants and orally healthy participants was significant (p = 0.047). Similarly, *Candida* species were significantly more prevalent (p = 0.0048) in subgingival sites of healthy patients with implants (25.8%) compared to the corresponding sites in orally healthy participants (4.7%) (Table 3.2). *Candida* species were also significantly more prevalent (p = 0.0432) in the subgingival sites of patients with periodontal disease (15%) than the corresponding sites of orally healthy participants (4.7%).

3.3.2 Abundance of staphylococcal and *Candida* species among participants investigated

The abundance of *S. epidermidis*, *S. aureus* and *C. albicans* recovered from oral rinse samples, periodontal pockets and subgingival sites of the three distinct participant groups examined is outlined in Tables 3.3, 3.4 and 3.5, respectively.

3.3.2.1 Abundance of S. epidermidis*,* S. aureus *and* C. albicans *among patients with periodontal disease.*

Staphylococcus epidermidis was detected in the oral rinse sample of 18/20 (90%) patients with periodontal disease and over half of these patients (13/20, 65%) yielded between 1-50 CFU/ml (Table 3.3). The remaining five samples yielded densities of >51 CFU/ml (Table 3.3) resulting in a mean *S. epidermidis* cell density of 82.35 ± 218.9 CFU/ml among oral rinse samples from this patient group (Table 3.3).

Overall, 5/38 subgingival sites examined in four patients with periodontal disease yielded *S. epidermidis*, three of these sites yielded between 1-50 CFU/ml, whereas the remaining two sites yielded between 101-500 CFU/ml, resulting in an average of 20 ± 78.4 CFU/ml (Table 3.3). Of the 35 periodontal pockets sampled, six

Table 3.3 Abundance of *S. epidermidis* recovered from different anatomical sites in patients with periodontal disease, healthy patients with implants and healthy participants

		S. epidermidis density range[a]					
Participant group (*n*)	**Sample site (*n*)**	**0 CFU/ml**	**1-50 CFU/ml**	**51-100 CFU/ml**	**101-500 CFU/ml**	**>501 CFU/ml**	**Mean cell density (CFU/ml)**
		Number (%) of samples yielding range of CFU/ml					
Periodontal disease (20)	OR (20)	2/20 (10)	13/20 (65)	2/20 (10)	2/20 (10)	1/20 (5)	82.35
	PP[b] (35)	29/35 (82.9)	3/35 (8.6)	0/35 (0)	1/35 (2.9)	2/35 (5.7)	42.6
	SG (38)	33/38 (86.8)	3/38 (7.9)	0/38 (0)	2/38 (5.3)	0/38 (0)	20
Healthy patients with implants (31)	OR (31)	6/31 (19.4)	14/31 (45.2)	5/31 (16.1)	4/31 (12.9)	2/31 (6.5)	189.3
	SG (145)	138/145 (95.2)	5/145 (3.4)	1/145 (0.7)	1/145 (0.7)	0/145 (0)	3.2
Healthy participants (64)	OR (64)	21/64 (32.8)	36/64 (56.3)	2/64 (3.1)	5/64 (7.8)	0/64 (0)	20.4
	SG (185)	179/185 (96.8)	4/185 (2.2)	0/185 (0)	2/185 (1.1)	0/185 (0)	3.6

[a] *Staphylococcus epidermidis* density counts for each oral rinse, subgingival site and periodontal pocket sample were determined based on colony counts on differential and selective media after 48 h incubation and were recorded as relative abundance in colony forming units per ml (CFU/ml).

[b] Periodontal pockets were present in patients with periodontal disease only.

Abbreviations: CFU, colony forming units; OR; oral rinse, PP; periodontal pocket; SG; subgingival site.

Table 3.4 Abundance of *S. aureus* recovered from different anatomical sites in patients with periodontal disease, healthy patients with implants and orally healthy participants

		S. aureus density range[a]					
Participant group (*n*)	**Sample site (*n*)**	**0 CFU/ml**	**1-50 CFU/ml**	**51-100 CFU/ml**	**101-500 CFU/ml**	**>501 CFU/ml**	**Mean cell density (CFU/ml)**
		Number (%) of samples yielding range of CFU/ml					
Periodontal disease (20)	OR (20)	15/20 (70)	4/20 (25)	1/20 (5)	0/20 (0)	0/20 (0)	4.4
	PP[b] (35)	35/35 (100)	0/35 (0)	0/35 (0)	0/35 (0)	0/35 (0)	0
	SG (38)	38/38 (100)	0/38 (0)	0/38 (0)	0/38 (0)	0/38 (0)	0
Healthy patients with implants (31)	OR (31)	16/31 (51.6)	9/31 (29)	1/31 (3.2)	2/31 (6.5)	3/31 (9.7)	173
	SG (145)	137/144 (94.5)	3/144 (2.1)	0/144 (0)	1/144 (0.7)	4/144 (2.8)	57.2
Healthy participants (64)	OR (64)	45/64 (70.3)	13/64 (20.3)	1/64 (1.6)	4/64 (6.3)	1/64 (1.6)	46.9
	SG (185)	179/185 (96.8)	4/185 (2.2)	1/185 (0.5)	0/186 (0)	1/186 (0.5)	28.2

[a] *Staphylococcus aureus* density counts for each oral rinse, subgingival site and periodontal pocket sample were determined based on colony counts on differential and selective media after 48 h incubation and were recorded as relative abundance in colony forming units per ml (CFU/ml).

[b] Periodontal pockets were present in patients with periodontal disease only.

Abbreviations: CFU, colony forming units; OR; oral rinse, PP; periodontal pocket, SG; subgingival site.

Table 3.5 Abundance of *C. albicans* recovered from different anatomical sites in patients with periodontal disease, healthy patients with implants and orally healthy participants

		C. albicans density counts[a]					
Participant group (*n*)	**Sample site (*n*)**	**0 CFU/ml**	**1-50 CFU/ml**	**51-100 CFU/ml**	**101-500 CFU/ml**	**>501 CFU/ml**	**Mean cell density (CFU/ml)**
		Number (%) of samples yielding range of CFU/ml					
Periodontal disease (20)	OR (20)	9/20 (45)	5/20 (25)	1/20 (5)	2/20 (10)	3/20 (15)	215.15
	PP[b] (35)	31/35 (88.6)	1/35 (2.9)	1/35 (2.9)	2/35 (5.7)	0/35 (0)	19.4
	SG (38)	34/38 (89.5)	2/38 (5.3)	1/38 (2.6)	0/38 (0)	1/38 (2.6)	119
Healthy patients with implants (31)	OR (31)	17/31 (54.8)	8/31 (25.8)	2/31 (6.5)	4/31 (12.9)	0/31 (0)	34.7
	SG (145)	136/145 (93.8)	5/145 (3.4)	0/145 (0)	1/145 (0.7)	3/145 (2.1)	88.2
Healthy participants (64)	OR (64)	47/64 (73.4)	15/64 (23.4)	0/64 (0)	2/64 (3.1)	0/64 (0)	7.8
	SG (185)	181/185 (97.8)	2/185 (1.1)	0/185 (0)	1/185 (0.5)	1/185 (0.5)	9.3

[a] *Candida albicans* density counts for each oral rinse, subgingival site and periodontal pocket sample were determined based on colony counts on differential and selective media after 48 h incubation and were recorded as relative abundance in colony forming units per ml (CFU/ml).

[b] Periodontal pockets were present in patients with periodontal disease only.

Abbreviations: CFU; colony forming units, OR; oral rinse, PP; periodontal pocket, SG; subgingival site.

pockets from six patients yielded *S. epidermidis*, three pockets yielded 1-50 CFU/ml, while the remaining three sites yielded densities >101 CFU/ml. The mean cell density of *S. epidermidis* in periodontal pockets was 42.6 ± 161.8 CFU/ml (Table 3.3).

Staphylococcus aureus was three times less prevalent than *S. epidermidis* in the oral rinse samples of these patients and was recovered from only six patients (6/20, 30%) with periodontal disease (Table 3.4). Five of these patients yielded between 1-50 CFU/ml, while the remaining patient yielded <100 CFU/ml. The mean average cell density of *S. aureus* in oral rinse samples was 4.4 ± 12.3 CFU/ml (Table 3.4). *Staphylococcus aureus* was not detected in any subgingival sites or periodontal pockets sampled in patients with periodontal disease (Table 3.4).

Candida albicans was recovered from 11/20 (55 %) oral rinse samples from patients with periodontal disease (Table 3.5). Of these 11 samples, five, one, two and three yielded *C. albicans* in the range 1-50, 51-100, 101-500 and >501 CFU/ml, respectively (Table 3.5). The average cell density recovered by oral rinse sampling was 215.15 ± 487.8 CFU/ml.

Of the 38 subgingival sites sampled, two (2/38, 5.3%) yielded *C. albicans* at cell densities between 1-50 CFU/ml, and two samples yielded between 51-100 CFU/ml (1/38, 2.6%) and >501 CFU/ml (1/38, 2.6%), respectively (Table 3.5). The mean cell density of *C. albicans* in subgingival sites was 119 ± 713.4 CFU/ml. Four (4/35) periodontal pockets sampled harboured *C. albicans*, half of these sites yielded 101-500 CFU/ml, while the remaining two sites yielded between 1-50 CFU/ml and 51-100 CFU/ml, resulting in a mean cell density of 19.4 ± 73.4 CFU/ml (Table 3.5).

3.3.2.2 Abundance of S. epidermidis*,* S. aureus *and* C. albicans *among healthy patients with implants.*

In total, a high prevalence of *S. epidermidis* 25/31 (80.6%) was observed in the oral rinse samples of healthy patients with implants and 14/31 (45.2%) of these samples yielded *S. epidermidis* in the density range 1-50 CFU/ml (Table 3.3). The remaining 11 samples yielded *S. epidermidis* in the density ranges 51-100 CFU/ml (5/31, 16.1%), 101-500 CFU/ml (4/31, 12.9%), and >501 CFU/ml (2/31, 6.5%). The average cell density of *S. epidermidis* recovered by oral rinse sampling of this patient group was 189.3 ± 546.7 CFU/ml, which was twice as high as the corresponding average cell

density recovered from patients with periodontal disease, although the difference was not significant ($p = 0.4102$) (Table 3.3).

Seven subgingival sites (7/145, 4.8%) sampled from healthy patients with implants yielded *S. epidermidis*; five of the samples yielded cell densities between 1-50 CFU/ml, whereas the remaining two samples ranged from 51-100 CFU/ml and 101-500 CFU/ml, respectively (Table 3.3). The mean average cell density recovered from subgingival sites was 3.2 ± 15.8 CFU/ml, which was significantly lower than subgingival sites in patients with periodontal disease ($p = 0.0167$).

Staphylococcus aureus was recovered from 15/31 (48.4%) oral rinse samples and nine (9/31, 29%) of these yielded cell densities between 1-50 CFU/ml (Table 3.4). One sample (3.2%) yielded 51-100 CFU/ml, two samples (6.5) yielded 101-500 CFU/ml and three samples (9.7%) yielded >500 CFU/ml, resulting in a mean average cell density from oral rinse samples of 173 ± 572.9 CFU/ml (Table 3.4).

Of the 145 subgingival samples taken from healthy patients with implants, only eight yielded *S. aureus* (Table 3.4). Four of the samples (2.8%) yielded >501 CFU/ml, three samples (2.1%) yielded 1-50 CFU/ml and one sample (0.7%) yielded 101-500 CFU/ml. The average *S. aureus* cell density recovered from subgingival sites was 57.2 ± 439.3 CFU/ml (Table 3.4).

Candida albicans was recovered from 14/31 (45.2%) of the oral rinse samples from healthy patients with dental implants, eight (8/31, 25.8%) of these yielded cell densities between 1-50 CFU/ml (Table 3.5). The remaining six samples yielded *C. albicans* cell densities between 51-100 CFU/ml (2/31, 6.55) and 1001-500 CFU/ml (4/31, 12.9%). The mean average cell density for *C. albicans* recovered by oral rinse sampling was 34.7 ± 70.6 CFU/ml (Table 3.5), six times less than the corresponding samples taken from the patients with periodontal disease ($p = 0.047$) (Table 3.5).

Nine subgingival sites sampled (9/145, 6.2%) in healthy patients with implants yielded *C. albicans*, five samples (5/145, 3.4%) yielded cell densities between 1-50 CFU/ml (Table 3.5). Three samples (3/145, 2.1%) and one sample (1/145, 0.7%) yielded *C. albicans* in the ranges >501 CFU/ml and 101-500 CFU/ml, respectively. The resulting *C. albicans* mean cell density recovered from subgingival sites was 88.2 ± 777.8 CFU/ml (Table 3.5).

3.3.2.3 Abundance of S. epidermidis*,* S. aureus *and* C. albicans *among orally healthy participants.*

The majority of oral rinse samples (36/64, 56.3%) yielded *S. epidermidis* in the range 1-50 CFU/ml (Table 3.3). Two samples (2/64, 3.1%) yielded cell densities between 51-100 CFU/ml and five samples (5/64, 7.8%) yielded cell densities of 101-500 CFU/ml. The mean average cell density for *S. epidermidis* recovered by oral rinse sampling of this participant group was 20.4 ± 40.8 CFU/ml (Table 3.3). This mean average cell density was significantly less than recovered from the corresponding samples taken from the patients with periodontal disease (p = 0.0326) and healthy patients with implants (p = 0.0153), respectively (Table 3.3).

In total, 6/185 subgingival sites examined in the 64 orally healthy participants yielded *S. epidermidis*, four of these sites (4/185, 2.2%) yielded between 1-50 CFU/ml, whereas the remaining two sites (2/185, 1.1%) yielded 101-500 CFU/ml, resulting in a average cell density of 3.6 ± 28.3 CFU/ml (Table 3.3). While a similar corresponding average cell density was recorded from healthy patients with implants (3.2 ± 15.8 CFU/ml), a significantly higher average cell density was recovered from patients with periodontal disease (20 ± 78.4 CFU/ml) (p = 0.0264) (Table 3.3).

Staphylococcus aureus was recovered from 19/64 oral rinse samples, 13 of which (20.3%) yielded *S. aureus* cell densities between 1-50 CFU/ml (Table 3.4). Of the remaining six samples, one, four and one yielded *S. aureus* in the density ranges 51-100 CFU/ml, 101-500 CFU/ml and >501 CFU/ml, respectively (Table 3.4). The mean average *S. aureus* cell density recorded from oral rinse samples was 46.9 ± 231 CFU/ml (Table 3.3). There was a 10 times greater abundance of *S. aureus* in oral rinse samples from orally healthy participants compared to patients with periodontal disease, and this species was three times less abundant than the corresponding cell densities recorded from healthy patients with implants, respectively (Table 3.4)

Six subgingival sites examined in orally healthy participants (6/185, 3.2%) yielded *S. aureus,* four of which yielded cell densities in the range 1-50 CFU/ml (Table 3.4). The cell densities recovered from the remaining two samples were 51-100 CFU/ml (1/185, 0.5%) and >501 CFU/ml (1/185, 0.5%). The mean average cell density was 28.2 ± 367.6 CFU/ml (Table 3.4).

Candida albicans was recovered by oral rinse sampling of 17 (26.6%) orally healthy participants, 15 of the samples yielded between 1-50 CFU/ml (Table 3.5). Two

samples (2/64, 3.1%) yielded cell densities in the range 101-500 CFU/ml, resulting in an overall mean average cell density from oral rinse samples of 7.8 ± 25.9 CFU/ml (Table 3.5). The corresponding average cell densities of *C. albicans* from oral rinse samples from patients with periodontal disease and patients with healthy patients with implants were significantly higher ($p = 0.00090$, $p = 0.0081$, respectively).

There was a low prevalence (4/185, 2.1%) of *C. albicans* recovered from subgingival sites in orally health participants. Two subgingival sites yielded *C. albicans* in the range 1-50 CFU/ml, while the other two sites yielded 101-500 CFU/ml and >501 CFU/ml, respectively (Table 3.5). The average *C. albicans* cell density recovered from these samples was 9.3 ± 109.6 CFU/ml (Table 3.5). The abundance of *C. albicans* was significantly higher ($p = 0.0471$) from corresponding sites in patients with periodontal disease.

3.3.2.4 Comparison of the relative abundance of S. epidermidis*,* S. aureus *and* C. albicans *recovered from the three groups of participants.*

The mean cell density of *S. epidermidis* in oral rinse samples was highest in healthy patients with implants (189.3 ± 546.7 CFU/ml) compared to patients with periodontal disease (82.35 ± 218.9 CFU/ml) and orally healthy participants (20.4 ± 40.8 CFU/ml) (Table 3.3). The abundance of *S. epidermidis* in oral rinse samples was significantly higher both in patients with periodontal disease ($p = 0.0326$) and healthy patients with implants ($p = 0.0153$) than in orally healthy participants.

The average cell density of *S. epidermidis* in subgingival sites was highest in patients with periodontal disease (20 ± 78.4 CFU/ml) compared to healthy patients with implants (3.2 ± 15.8 CFU/ml) and orally healthy participants (3.6 ± 28.3 CFU/ml) (Table 3.3). Similarly, the abundance of *S. epidermidis* was significantly higher in the subgingival sites of patients with periodontal disease than in orally healthy participants ($p = 0.0264$) and in healthy patients with implants ($p = 0.0167$). The mean average abundance of *S. epidermidis* was very significantly higher in periodontal pockets of patients with periodontal disease ($p = 0.0042$ and $p = 0.0024$), than in subgingival sites in healthy patients with implants or orally healthy participants. The mean average cell density was higher in periodontal pockets (42.6 ± 161.8 CFU/ml) than in subgingival sites (20 ± 78.4 CFU/ml) of patients with periodontal disease, although not significantly.

The highest average cell densities of *S. aureus* were recovered from the oral rinse samples and subgingival sites of healthy patients with implants (oral rinse; 173 ± 572.9 CFU/ml and subgingival sites; 57.2 ± 439.3 CFU/ml) compared to patients with periodontal disease (oral rinse sample; 4.4 ± 12.3 CFU/ml and subgingival sites; 0 ± 0 CFU/ml) and orally healthy participants (oral rinse sample; 46.9 ± 231 CFU/ml and subgingival sample; 28.2 ± 367.6 CFU/ml), however this difference was not statistically significant (Table 3.4).

The average abundance of *C. albicans* in oral rinse samples of patients with periodontal disease was significantly higher than in the corresponding samples from healthy patients with implants ($p = 0.047$) and orally healthy participants ($p = 0.0009$) (Table 3.5). Interestingly, the abundance of *C. albicans* was significantly higher ($p = 0.0081$) in oral rinse samples from healthy patients with implants compared to orally healthy participants. Patients with periodontal disease had a significantly higher ($p = 0.0471$) abundance of *C. albicans* recovered from subgingival sites compared to subgingival sites of orally healthy participants.

3.4 Discussion

Previously, *P. gingivalis*, *T. denticola*, and *T. forsythia* were considered the aetiological agents of periodontal disease due to the high relative abundance of these species detected in periodontal pockets detected using species-specific q-PCR (7, 14, 24). More recently, researchers have used microbiome analysis to reveal that the aetiology of periodontal disease is significantly more diverse than previously thought (7). The shift from periodontal health to disease typically is caused by dysbiosis in the oral microbiome. However, molecular techniques such as microbiome analysis and qPCR cannot discriminate between live and dead organisms in samples and these techniques could possibly detect the prevalence of free DNA in the periodontal pocket. To accurately detect the prevalence and relative abundance of viable microbial organisms belonging to specific microbial species, selective and differential culture techniques are required.

The combination of selective chromogenic media, specifically Sa*Select*™ and MALDI-TOF for selection and definitive identification of staphylococcal species recovered from the oral cavity was novel to the present study. In addition, the use of Sa*Select*™ enabled the visualisation of different staphylococcal species including CoNS, which allowed a greater representation of isolates to be identified by MALDI-TOF. Staphylococcal species such as *S. epidermidis*, *S. aureus*, *S. capitis*, and *S. auricularis* have previously been detected in the oral cavity using oral rinse sampling although the prevalence can vary greatly (61.36% to 94%) depending on the sampling and identification techniques used (126, 128, 133, 134). Previous studies typically isolated staphylococcal species on MSA agar, and a variety of techniques were used for identification including Gram staining, slide agglutination tests, tube coagulase tests and sugar fermentation tests and biochemical tests such as API Staph system. One previous study isolated *S. aureus* from oral rinse samples on chromogenic media (CHROMagar™ STAPH aureus) that is selective for this species only (126). The present study detected staphylococcal species including *S. epidermidis*, *S. aureus*, *S. hominis*, *S. haemolyticus*, *S. warneri*, *S. pasteuri*, and *S. saprophyticus* from 84.4 - 100% of oral rinse samples from participants in all three groups investigated (Table 3.2).

3.4.1 Prevalence and abundance of staphylococcal and *Candida* species among patients with periodontal disease

3.4.1.1 Prevalence and abundance of staphylococcal species in patients with periodontal disease

Previous studies based on selective media, Gram staining and API Staph systems reported the oral carriage of staphylococci in patients with periodontal disease to range from 61.36 - 69.5% (125, 126). In contrast, the present study recovered staphylococci from 100% of oral rinse samples from the same patient group. Currently there is no standard method for isolating and identifying staphylococcal species recovered from the oral cavity. Therefore, it is possible the variety of sampling, culture and identification techniques used by different research groups may have yielded different levels of success in recovery and identification of all staphylococcal species present, especially CoNS.

The present study revealed that 45% of patients with periodontal disease harboured staphylococci in periodontal pockets, in agreement with previous findings that detected staphylococci in 37.5 - 54% of periodontal pockets sampled (123, 125).

A previous study reported that 27.3% of patients with periodontal disease subjected to oral rinse sampling yielded *S. epidermidis* (125) in contrast to 90% (18/20) of similar patients investigated in the present study (Table 3.2). The former study used Baird-Parker agar to recover isolates; however this media does not differentiate between different staphylococcal species and therefore the presence of *S. epidermidis* could have been underestimated (125).

Staphylococcus epidermidis was more prevalent in oral rinse samples of patients with periodontal disease (18/20, 90%) compared to orally healthy participants (43/64, 67.2%), although not quite significantly (p = 0.0501). This species was however, significantly more abundant (p = 0.0153) in the oral rinse samples of patients with periodontal disease (82.35 ± 218.9 CFU/ml) than orally healthy participants (20.45 ± 40.8 CFU/ml) (Table 3.3). The capacity of *S. epidermidis* for biofilm formation and the difference in prevalence and abundance of *S. epidermidis* between patients with periodontal disease and orally healthy participants possibly correlates with differences in the prevalence of oral biofilm between these two distinct participant groups, with orally healthy participants likely have lower amounts of dental plaque.

Previous studies reported the recovery of *S. epidermidis* from 15.9 - 64.3% of periodontal pockets investigated (123–125, 128). In the present investigation, *S. epidermidis* was the most prevalent staphylococcal species identified, recovered from periodontal pockets of 30% of patients investigated. The abundance of *S. epidermidis* recovered from periodontal pocket and subgingival sites of patients with periodontal disease was significantly higher in comparison to healthy patients with implants and orally healthy participants ($p < 0.05$). This suggests that there is an overall increase in the abundance of *S. epidermidis* in the oral cavity during periodontal disease, leading to trafficking into and out of periodontal pockets and subgingival sites. In addition, the high abundance of this species in periodontal pockets potentially implicates *S. epidermidis* in biofilm formation in these pockets. However, as it is typically a commensal organism its role in disease progression is unclear. Nonetheless, *S. epidermidis* is an opportunistic pathogen that has previously been associated with infections of prosthetic hip joints and therefore a role in periodontal disease progression cannot be ruled out.

Staphylococcus aureus was previously reported as the most prevalent staphylococcal species recovered by oral rinse sampling from patients with periodontal disease (25%) (125). In the present investigation the prevalence of this species in oral rinse samples taken from patients with periodontal disease was identical, however, in contrast, *S. aureus* was not the predominant species identified (Table 3.2). *Staphylococcus aureus* was almost four times less prevalent and almost 19 times less abundant compared to *S. epidermidis* in oral rinse samples from patients with periodontal disease, possibly due to increased competition within oral biofilm from other staphylococcal species such as *S. epidermidis*.

Interestingly, previous studies that reported *S. aureus* as the predominant staphylococcal species in periodontal pockets relied on selective chromogenic media for *S. aureus* (126) or qRT-PCR (24) and reported that 13.4% and 68.2% of periodontal pockets harboured *S. aureus,* respectively. In contrast to both these studies, *S. aureus* was not recovered from any periodontal pockets or subgingival sites sampled in the present study (Table 3.2). Both of the former studies specifically investigated the prevalence of *S. aureus*, and did not report the prevalence of *S. epidermidis* or other CoNS. It is possible that the latter investigation using *S. aureus* qRT-PCR primers (targeting the *S. aureus*-specific *nuc* gene) detected *S. aureus* DNA from lysed cells, thus overestimating its prevalence (24).

3.4.1.2 Prevalence and abundance of Candida *in patients with periodontal disease*

Candida species have previously been recovered by oral rinse sampling from 42.7 - 47.6% patients with periodontal disease (10, 126) correlating with the prevalence of *Candida* in oral rinse samples of 55% of similar patients in the present study (Table 3.2).

More specifically, *C. albicans* was previously recovered from the oral rinse samples of 35.5 - 47.6% patients with periodontal disease (10, 126), also in agreement with the results of the present study (11/20, 55%). *C. albicans* was the most prevalent and abundant (average cell density 215.15 ± 487.8 CFU/ml) *Candida* species recovered from oral rinse samples of patients with periodontal disease (Tables 3.2 and 3.4) in the present study. *Candida dubliniensis* was the only other *Candida* species recovered by oral rinse sampling of these patients, detected in patient P5 who also wore a denture (Table 3.2). The presence of non-*C. albicans Candida* species is also commonly associated with denture wearers (147). The low prevalence of non-*C. albicans Candida* species is unsurprising as these are commonly associated with patients who have underlying medical conditions or who had received antibiotics or steroid treatment (141, 142) and thus would have been excluded from the present study.

Candida albicans has previously been recovered from the periodontal pockets of 30% and 19.5% of patients with periodontal disease (126, 139), correlating with the findings of the present study which recovered *C. albicans* from the periodontal pockets of 20% of patients investigated (Table 3.2). Multiple studies involving the recovery and identification of *Candida* species from clinical samples commonly utilise CHROMagar™ Candida agar and are therefore more consistent between laboratories compared to similar studies that investigated staphylococcal prevalence.

3.4.2 Prevalence of staphylococcal and *Candida* species among healthy patients with implants

3.4.2.1 Prevalence and abundance of staphylococcal species in healthy patients with implants

A previous study reported that 47.4% of oral rinse samples from patients with peri-implantitis yielded *S. epidermidis* (132). In contrast, the present study found that the prevalence of *S. epidermidis* from oral rinse sampling was considerably higher in the

oral cavities of healthy patients with implants (25/31; 80.6%) (Table 3.2). The higher prevalence rate detected in the present study very likely reflects the increased sensitivity of the recovery and identification methods used. Interestingly, the former study also reported that *S. epidermidis* was recovered from peri-implant pockets of patients with peri-implantitis (13/30, 30%) (132), which correlated with the prevalence rate detected in periodontal pockets of patients with periodontal disease (6/20, 30%) in the present study. In contrast, the prevalence of *S. epidermidis* in subgingival sites of healthy patients with implants was almost two-fold lower (5/31, 16.1%). The previously reported mean cell density of *S. epidermidis* was higher in peri-implant pockets (73 CFU/ml) than in healthy subgingival sites (55 CFU/ml) of patients with peri-implantitis, however statistical data was not available (132). In the present study, the average cell density of *S. epidermidis* in subgingival sites of healthy patients with implants was even lower (3.2 CFU/ml). *Staphylococcus epidermidis* appears to be more prevalent and abundant around diseased implants and periodontal pockets compared to healthy subgingival sites, suggesting it can readily colonise diseased tissue however it is uncertain if this opportunistic pathogen plays a role in disease progression.

A previous study that investigated the prevalence of *S. aureus* recovered from oral rinse samples from patients with peri-implantitis reported that *S. aureus* was the second most predominant species, detected in 21.1% of patients (132). In the present study, *S. aureus* was detected in the oral rinse samples of 48.4% of healthy patients with implants. It is possible that *S. aureus* is more commonly associated with healthy patients with implants due to a lack of competition with CoNS and *Candida* species in the healthy implant subgingival environment. Previous studies based on checkerboard DNA:DNA hybridisation investigated the prevalence of *S. aureus* in peri-implant pockets (40 - 70.4%) and healthy subgingival sites around implants (25 - 44%). These investigations reported an association between *S. aureus* and implants, however cross-reactivity of CoNS with the hybridization probes was not investigated (78, 79, 148). More recently, the prevalence of *S. aureus* in peri-implant pockets and subgingival sites of patients with peri-implantitis was investigated by culture on MSA agar and 16S sequence analysis for species identification and reported the species prevalence to be 3/30 (10%) and 0/30 (0%), respectively (132). *Staphylococcus aureus* was recovered from 4/31 (12.9%) of subgingival sites from healthy patients with implants in the present study. The comparatively low prevalence of *S. aureus* in both studies is not indicative of an association between *S. aureus* and peri-implantitis, however, the lower

abundance of *S. aureus* in subgingival sites of healthy patients with implants (57.2 CFU/ml) in comparison to peri-implant pockets (296 CFU/ml) (132), may suggest that *S. aureus* can perhaps adhere more readily to biofilm at peri-implantitis sites.

3.4.2.2 Prevalence and abundance of Candida *species in healthy patients with implants*

Previous studies have not investigated the prevalence of *Candida* species in association with healthy or diseased implants in the oral cavity by oral rinse sampling. In the present study, similarly to *S. epidermidis,* the prevalence of *Candida* recovered from oral rinse samples was significantly higher ($p = 0.047$) in healthy patients with implants compared to orally healthy participants (Table 3.2). This finding indicates *Candida* species are readily capable of colonising implants. These results must be interpreted with some caution as 8/31 (25.8%) of healthy patients with implants also wore a denture and these devices have previously been associated with increased prevalence of oral *Candida* carriage as they can readily adhere and form biofilms on denture surfaces (147).

Candida albicans was the predominant *Candida* species recovered from subgingival sites of healthy patients with implants (22.6%), in contrast with a previous study that reported 9% of healthy implants harboured *C. albicans* (149). Interestingly in the present study *C. albicans* was more abundant in subgingival sites (88.2 CFU/ml) compared to the oral cavity (34.7 CFU/ml) of healthy patients with implants although not significantly ($p = 0.7031$) (Table 3.4). Based on the prevalence of *C. albicans* detected in subgingival sites around healthy dental implants it is unlikely this species plays a significant role in peri-implantitis, but likely contributes to normal microbial biofilm formation on implant surface, as occurs similarly upon denture surfaces (147).

3.4.3 Prevalence of staphylococcal and *Candida* species among orally healthy participants

3.4.3.1 Prevalence and abundance of staphylococcal species in orally healthy participants

The present study which detected staphylococci in the oral rinse samples of 84.4% of participants investigated (Table 3.4), correlating with previous studies that reported a prevalence ranging from 83.9 – 94% (134, 150).

Previous research recovered *S. epidermidis* from the oral rinse samples of 41.1% of healthy participants (150), however in contrast, the current study revealed a higher prevalence of 67.2% (Table 3.2). This difference could be attributed to the different identification methods used in the previous study (150).

In the present study, staphylococcal species were recovered from the subgingival sites of 17.2% of orally healthy participants, and *S. epidermidis* was prevalent in 7.8% (Table 3.2). These findings contrast with previous studies that report the detection of *S. epidermidis* in 42.9 – 60.7% subgingival sites of healthy participants (123, 150). This discrepancy could be attributed to differences in sensitivity of sampling and culture techniques; one of the previous studies collected dental plaque, while the other lawned PerioPaper samples directly onto MSA agar (123, 150). The species identification techniques utilised in the present study (Sa*Select*™ and MALDI-TOF) are more discriminatory compared to the techniques used in the previous studies (API Staph system, or PCR amplification of the *gseA* gene encoding the serine protease GluSE) (123, 150).

According to the literature, *S. aureus* has been detected in the oral cavities of (134, 150) and in the subgingival sites of 24 - 46.4% and 3% - 33.9% of healthy participants, respectively (123, 134, 150). The results of the present study supported these previous findings (Table 3.2), as *S. aureus* was detected in the subgingival sites of (7.8%) healthy participants sampled. Despite the prevalence of *S. epidermidis* and *S. aureus* being equal in the subgingival sites of healthy participants investigated in the current study (Table 3.2), when detected, *S. aureus* was seven times more abundant than *S. epidermidis*. However, when *S. epidermidis* and *S. aureus* were recovered from the same subgingival site (as in Participant 217), *S. epidermidis* was 13 times more abundant than *S. aureus*. These results possibly indicate *S. epidermidis* is more readily able to colonise these sites and can out-compete *S. aureus* for available resources and space.

3.4.3.2 Prevalence and abundance of Candida *species in orally healthy participants*

In the present study, 37.5% of orally healthy participants yielded *Candida* species from their oral rinse sample, in agreement with previous research (10). *Candida albicans* was the predominant *Candida* species identified by oral rinse sampling (26.6%) in the present study, similar to the findings of a previous study (10).

Candida species were less prevalent and abundant in subgingival sites of orally healthy participants (4.7% prevalence, average cell density of 9.3 ± 109.6 CFU/ml) compared to periodontal pockets of patients with periodontal disease (20% prevalence, average cell density of 19.4 ± 73.4 CFU/ml) and subgingival sites of healthy patients with implants (25.8% prevalence, average cell density of 88.2 ± 777.8 CFU/ml) (Table 3.2). A possible explanation for this difference in prevalence and abundance could be the smaller physical size of subgingival sites in orally healthy participants, both the periodontal pockets and the subgingival sites around healthy implants (due to a lack of periodontal ligaments) are physically larger compared to healthy subgingival and allow for increased trafficking of oral *Candida* species into these sites (20).

3.4.4 Possible roles and interactions of staphylococcal and *Candida* species

Interestingly, *S. epidermidis* and *C. albicans* were frequently co-isolated together from oral rinse samples across the three distinct participant groups in this study, however they were co-isolated predominantly from patients with periodontal disease and healthy patients with implants (Table 3.2). Both of these species are commonly associated with biofilm formation and more specifically, oral plaque associated with periodontal disease and peri-implantitis. Based on the previous reports of microbial causes of prosthetic device failure, titanium dental implants likely act as an ideal substrate for species such as *S. epidermidis* and *C. albicans* to form biofilm upon, in the form of dental plaque. In support of this, patients with periodontal disease and healthy patients with implants harboured higher abundances of these species in their oral rinse samples compared to orally healthy controls (Tables 3.3 and 3.5). Surprisingly, *C. albicans* was more abundant in the subgingival sites (199 CFU/ml) than in the periodontal pockets (19.4 CFU/ml) in patients with periodontal disease. This may suggest that *C. albicans* is less suited to the semi-anaerobic environment of the periodontal pocket is more suited to shallower, aerobic subgingival sites. In support of this hypothesis, previous research has reported that the ability of *C. albicans* ability to form biofilm was greatly reduced under anaerobic conditions (151).

There appears to be evidence of trafficking of staphylococcal isolates between the oral cavity and the nasal cavity, especially in patients with periodontal disease as 15/18 (83.3%) patients who harboured *S. epidermidis* in their oral rinse sample also harboured it in their nares. Similar observations were also recorded in healthy patients

with implants (19/25, 76%) and orally healthy participants (33/43, 76.7%). The higher prevalence of *S. epidermidis*, *S. aureus* and *C. albicans* in oral rinse samples compared to subgingival samples or periodontal pockets across the three participant groups investigated suggests that the oral cavity is a source of contamination for subgingival and periodontal pocket sites. While these results show evidence of possible trafficking between the distinct sample sites investigated, further genetic based population analysis such as MLST is required on pairs of isolates recovered from these sites to support these findings.

In the present study, there was no co-isolation of *S. epidermidis* and *S. aureus* from periodontal pocket or subgingival sites. Previous research reported a negative association between *S. aureus* and *S. epidermidis* in the nares (52), a similar negative interaction is likely in these distinct oral anatomical sites. The ability of *S. epidermidis* to successfully form biofilm possibly enables it to out compete *S. aureus* in the different oral anatomical sites of patients with periodontal disease. The absence of *S. aureus* in periodontal pockets suggests *S. epidermidis* is better suited to this semi-anaerobic environment, possibly due to its ability to thrive in biofilm. There is likely cyclic trafficking of *S. epidermidis* into and out of periodontal pockets, subgingival sites and the oral cavity of patients with periodontal disease contributing to the over high prevalence of this species in this patient group.

Overall staphylococcal species were highly prevalent in the oral cavities across all participant groups investigated. However, species such as *S. epidermidis* were typically more prevalent and abundant in oral cavities of patients with periodontal disease compared to orally healthy participants. The frequent recovery of *S. epidermidis* and *C. albicans* from subgingival sites and periodontal pockets suggests these two species can successfully form biofilm and out-compete other staphylococcal and *Candida* species for available resources in these distinct anatomical sites.

Chapter 4

DNA Microarray Analysis of Oral *Staphylococcus aureus* and *Staphylococcus epidermidis* Isolates Recovered from Patients with Periodontal Disease, Healthy Patients with Implants and Orally Healthy Participants

4.1 Introduction

As previously described in Chapter 1, Section 1.4, staphylococcal species such as *S. aureus* and *S. epidermidis* are commensal organisms and are fundamental to the normal microbial flora of human and animal skin and mucous membranes. However, staphylococcal species have also been isolated from the oral cavity, including distinct anatomical sites within the oral cavity such as periodontal pockets and subgingival sites (24, 123–128, 150). While *S. aureus* is very well recognised and characterised cause of nosocomial infections (53, 152), less virulent CoNS species such as *S. epidermidis* and *S. lugdunensis* are not considered as significant pathogens. However, CoNS are increasingly associated with nosocomial infections, primarily in the elderly, immunocompromised, neonates, or those with foreign body related infections such as catheter- related bloodstream infections or with prosthetic joint infections (27, 74, 75). A previous study reported that almost 80% of *S. epidermidis* isolates recovered from foreign body related infections were methicillin resistant (MRSE) (77). The aetiology of CoNS infections caused by species such as *S. epidermidis* differs from *S. aureus* infections. Typically CoNS infections result from biofilm formation whereas *S. aureus* can express an abundance of different virulence factors encoded in its core genome and on MGEs, including distinct toxins, super antigens and surface proteins that can cause tissue damage, toxic shock syndrome, and enable *S. aureus* evasion of the host immune defences (69, 153).

4.1.1 Genetic transfer among staphylococcal species

The genomes of staphylococcal species such as *S. aureus* and *S. epidermidis* consists of the core and accessory genomes that account for 75% and 25% of the total genome content, respectively. The core genome encodes genes that are specific for each individual species, as well as genes that are essential for routine metabolism, growth and survival (30, 31). The accessory genome predominantly encodes non-essential genes such as antimicrobial resistance and virulence factor genes (30, 31), the majority of which are located on MGEs such as plasmids, bacteriophages, transposons and insertion sequences, pathogenicity islands and chromosomal cassettes (31). *Staphylococcus aureus* frequently exchanges DNA *via* MGEs with *S. epidermidis* (30), and it was hypothesised previously that *S. epidermidis* and other CoNS act as a reservoir

of MGEs encoding antimicrobial resistance and virulence factor genes for potential transfer to *S. aureus* (32, 86).

Staphylococcus epidermidis can harbour SCC*mec* elements that encode the methicillin resistance gene *mecA* along with its regulatory genes, and several SCC*mec* elements present in distinct MRSA linages are believed to have originated in CoNS such as *S. epidermidis* and been transferred to *S. aureus* lineages on multiple independent occasions (44, 47, 86). The virulence factor ACME, which is believed to enable staphylococcal species to survive and persist in adverse environments such as the skin, is typically located alongside SCC*mec* and SCC*mec*-like elements. The modular assembly of the ACME locus is thought to have occurred in *S. epidermidis* prior to its transfer into the CA-MRSA USA-300 clone, which is currently endemic in the USA (82).

4.1.2 *S. aureus* and *S. epidermidis* populations

Tracking of staphylococcal species during an outbreak and recording the spread of distinct strains locally and internationally is essential to accurately investigate these populations. Unfortunately, previously CoNS were not considered causative agents of infection and therefore have not been as comprehensively studied as *S. aureus*. However, in recent years a consensus MLST scheme has been developed for *S. epidermidis* and data on global isolates is maintained in an internet accessible international curated database of *S. epidermidis* STs recovered from nosocomial, carriage and environmental isolates (111, 154). Currently there is no CoNS-specific SCC*mec* typing scheme and typically SCC*mec* elements in CoNS are typed using the MRSA SCC*mec* typing scheme. However, due to the diversity of SCC*mec* and SCC elements in CoNS many such elements are non-typable with the SCC*mec* typing system developed for MRSA (44).

Multiple phenotypic and genotypic based typing methods and schemes have been developed for *S. aureus*. However phenotypic-based systems such as phage typing and antimicrobial agent resistogram typing have largely been replaced with genotypic typing methods such as PFGE, MLST, *spa* typing, SCC*mec* typing and SCC*mec* associated direct unit repeat (*dru*) typing (42, 113, 155). In recent years, whole genome sequencing (WGS) of isolates using next generation sequencing technologies has been

increasingly used for isolate typing and has enhanced sensitivity and discriminatory powers.

There are five main lineages of HCA-MRSA; CC5, CC8, CC22, CC30, CC45, and these vary in global prominence based on geographical location (59). Since 2002, ST22-MRSA-IV has been the predominant MRSA clone in Ireland (156). Compared to *S. aureus* and MRSA in particular, there has been relatively little research internationally on linages of *S. epidermidis* and their association with disease. However, more recently there have been reports of *S. epidermidis* harbouring antibiotic resistance genes being associated with hospital outbreaks (157).

A significant limitation of previous research that investigated the prevalence of staphylococcal species in distinct anatomical sites in the oral cavity such as periodontal pockets, was that staphylococcal populations recovered were not analysed further (24, 124, 125, 134). One study investigated the prevalence of MRSA in the oral cavity and periodontal pockets of patients with periodontal disease versus orally healthy controls and found that 18% (27/154) of participants harboured *S. aureus* in at least one of the sample sites, however the *mecA* gene was not detected in any of the 343 isolates analysed (158). Another study investigated antimicrobial resistance among microbial species from periodontal pockets; the authors selectively investigated samples cultured under anaerobic conditions and only recovered *S. aureus* from 1/400 participants investigated. The isolate exhibited *in vitro* resistance to amoxicillin, clindamycin, doxycycline and metronidazole (159). *Staphylococcus aureus* isolates recovered from the gingival margin of patients with periodontal disease have also been reported to harbour a greater number of virulence factors such as exotoxin encoding genes like *tst* which encode the toxins associated with toxic shock syndrome and the enterotoxin gene, *sec* (153).

Unfortunately, the prevalence of antimicrobial resistance and virulence factor genes in CoNS such as *S. epidermidis* recovered from periodontal pockets has not been investigated to date. Based on the high prevalence of *S. epidermidis* recovered from the oral cavity in the present study and the potential for this species to act as reservoir of MGEs for *S. aureus*, further detailed research into this species recovered from the oral environment is important.

4.1.3 DNA microarray profiling

Major advances in WGS technologies resulted in the publication of complete nucleotide sequences for numerous staphylococcal species, which lead to the development of DNA arrays such as the StaphyType DNA microarray platform, developed by Alere Technologies GmbH (Jena, Germany). This microarray was devised for high-throughput screening of *S. aureus* isolates in a short time period, and provides comprehensive information on antimicrobial resistance genes such as those encoding resistance to macrolides, tetracycline or beta-lactams and virulence factor genes such as toxins and MSCRAMMs harboured by isolates, along with a detailed genetic analysis that enabled SCC*mec* types to be identified and isolates to be assigned to an ST and or CC (160). Although this particular microarray was designed to screen *S. aureus* isolates, it is possible to use the array to detect genes associated with SCC*mec* such as *mecA*, *mec* regulatory genes, and *ccr* genes, antimicrobial resistance genes, and virulence factor genes such as those involved in biofilm production, harboured by CoNS (46).

4.1.4 Objectives

The aim of this part of the study was to use DNA microarray profiling to characterise a large subset of *S. epidermidis* and *S. aureus* isolates recovered from distinct anatomical sites in the oral cavity of patients with periodontal disease, healthy patients with implants and orally healthy controls.

- To determine the prevalence of antimicrobial resistance genes and virulence factor genes along with the CCs of selected *S. aureus* isolates recovered from each distinct anatomical site sampled across the three participant groups.
- To investigate the prevalence of antimicrobial resistance genes and virulence factor genes (particularly the prevalence of ACME) in *S. epidermidis* isolates recovered during the study.
- To compare isolates recovered from periodontal pockets, subgingival sites, oral rinse samples and the nasal cavity across the three different participant groups for variations in prevalence of antimicrobial and virulence factor genes.

4.2 Materials and methods

4.2.1 DNA microarray analysis

DNA microarray analysis was performed on 78 *S. aureus* and 227 *S. epidermidis* isolates using the *S. aureus* Genotyping Kit 2.0 (Alere) according to the manufacturer's instructions and as described in detail previously (116). Patients with periodontal disease, healthy patients with implants and orally healthy participants that harboured *S. aureus* and/or *S. epidermidis* in their oral rinse samples, subgingival sites, and/or periodontal pockets each had a representative isolate analysed (Table 4.1). Patients with periodontal disease and healthy patients with implants that harboured *S. aureus* and/or *S. epidermidis* in their nares also had a representative isolate analysed, whereas a selection of orally healthy participants who harboured *S. aureus* and/or *S. epidermidis* in oral rinse samples and their nares had nasal isolates analysed (Table 4.1). The *S. aureus* Genotyping Kit 2.0 contains the necessary molecular grade reagents, buffers and microarray strips to carry out microarray analysis. The microarray chips are organised into 8-well microtitre strips, and each chip contains 336 probes for genetic markers such as typing markers, virulence factor and antimicrobial resistance genes.

4.2.1.1 Biotin-dUPT labelling using linear amplification

Extracted DNA (Chapter 2, Section 2.1.4.2) was labelled with biotin-dUTP using a linear amplification reaction that used only one antisense primer per target leading to single stranded (ss) DNA products. All required primers, labelling buffer and enzymes were supplied with the *S. aureus* Genotyping kit 2.0. A master mix was prepared by combining 4.9 µl of B1 labelling buffer and 0.1 µl of B2 enzyme reagent per sample. A 5 µl aliquot of the master mix was combined with 5 µl template DNA containing 0.5-1.5 µg DNA and was placed in a Kyratec thermocycler model SC200 (Kyratec, Mansfield Australia) and subjected to the following thermocycling conditions: 96^{o}C for 5 min, followed by 45 cycles of 96^{o}C for 20 s, 50^{o}C for 20 s and 72^{o}C for 30 s.

4.2.1.2 Hybridisation of labelled PCR product to array probes.

Each well of the microarray strip was washed with 200 µl ultra-pure water (Sigma-Aldrich) prior to use. Following the washing step, 100 µl hybridisation buffer C1 (supplied with the *S. aureus* Genotyping kit 2.0), was added to each well and incubated at 55°C for 2 min with shaking 550 rpm. All incubation steps were carried out in a

Table 4.1 Summary of the numbers of isolates analysed by DNA microarray profiling according to participant group and anatomical site the isolates were recovered from

		S. aureus (*n* = 78)			*S. epidermidis* (*n* = 227)			
Participant group	***n***	**NS**	**OR**	**SG**	**NS**	**OR**	**SG**	**PP[a]**
Patients with periodontal disease	20	5	5	0	19	27	6	9
Healthy patients with implants	31	15	16	7	35	39	9	0
Orally healthy controls	64	6	19	5	30	48	5	0

[a] Periodontal pockets (PP) were detected in patients with periodontal disease only.
Abbreviations: NS, nasal swab; OR, oral rinse; SG, subgingival site

BioShake iQ thermoshaker (Q. Instruments, Jena, Germany). During this incubation step individual hybridisation mixtures consisting of 10 µl of each biotin labelled PCR product and a further 90 µl buffer C1 were prepared. Each separate hybridisation mixture was added to an individual microarray strip well and incubated at 55°C for 60 min with shaking at 550 rpm to allow hybridisation to take place.

4.2.1.3 Horseradish-peroxidase (HRP)-conjugation, staining and chip analysis

Following hybridisation, microarray strips were removed and the microarray wells were washed three times with 200 µl of wash buffer C2 (supplied with the *S. aureus* Genotyping kit 2.0). A C3/C4 mixture at a ratio of 1:100 respectively was prepared (both reagents supplied with the *S. aureus* Genotyping kit 2.0) and a 100 µl volume of the mixture was added to each microarray well and incubated for 10 min at 30°C with shaking at 550 rpm to enable binding of the bio- 16-dUTP target by the HRP-conjugate. Following incubation, the C3/C4 mixture was removed and the microarray chip was washed with a wash buffer C5 (supplied with the *S. aureus* Genotyping kit 2.0). The HRP substrate tetramethylbenzidine (labelled D1, supplied with the *S. aureus* Genotyping kit 2.0) was then used to stain the biotin-HRP conjugate by adding 100 µl aliquots to each microarray well, followed by incubation for 5 min at room temperature, after which the D1 solution was completely removed.

Analysis of each microarray chip was carried out using an ArrayMate reader with Iconoclust software version 2.0 (Alere). An image of each chip was analysed to ensure that each chip was intact and undamaged and that the staining controls were adequately stained. The staining intensity of each probe was measured automatically using the ArrayMate reader and values <0.1 were considered negative, >0.3 were considered positive and results between 0.1 and 0.3 were considered ambiguous (161).

4.2.1.4 Array data analysis

The Results Collector (Alere™) application was used to combine ArrayMate reader data files into two CSV files that were exported and analysed in the Microsoft Excel 2010 software programme (version 14.0.7177.5000). Multiple isolate result files were compiled into one Microsoft Excel document, and data were colour coded to easily distinguish between positive (red), negative (green) and ambiguous (orange) results.

4.3 Results

Isolates subjected to DNA microarray analysis were chosen as representatives of different participants and sample sites from which they were recovered (Table 4.1). In total, 78 *S. aureus*, and 227 *S. epidermidis* isolates were subjected to DNA microarray analysis.

4.3.1 Clonal lineages identified amongst *S. aureus* isolates investigated

Based on DNA microarray analysis two distinct STs and 15 distinct CCs were identified among the 78 *S. aureus* isolates investigated. The two STs detected were ST34/42 (2/78, 2.6%), and ST72 (3/78, 3.8%), and the four most prevalent CCs were CC30 (11/78, 14.1%), CC45 (10/78, 12.8%), CC398 (9/78, 11.5%), CC5 (8/78, 10.3%), the remaining CCs were each detected in seven isolates or less (Table 4.2). One isolate belonging to CC5 harboured an SCC*mec* element (Table 4.2). Isolates belonging to ST34 or ST42, and CC2250 or CC227 could not be distinguished using the DNA microarray possibly because the technology cannot discriminate between STs if single point mutations occur within the MLST genes, or possibly because the isolates ST hybridisation pattern no longer matched the parental CC pattern because the isolate is a hybrid of two distinct *S. aureus* genetic backgrounds caused by chromosomal replacements between *S. aureus* parent isolates (162).

Pairs of MSSA isolates recovered by oral rinse sampling from two distinct patients with periodontal disease were identified as belonging to the same CC (CC5). Three distinct healthy patients with implants each had *S. aureus* isolates recovered from their nares, oral rinse sample and subgingival sites identified as belonging to the same CC (CC9, CC398, CC188). Three distinct *S. aureus* isolates belonging to different STs and CCs were recovered from the nares (ST34/42 and CC15) and oral rinse sample (CC59) of a healthy patient with implants (Patient I6). Two isolates recovered from the oral rinse sample and subgingival sample of an orally healthy participant (Participant 217) were both identified as belonging to CC188. Two *S. aureus* isolates recovered from the oral rinse sample of an orally healthy participant were identified as belonging to different CCs (CC7 and CC8).

Table 4.2 Genotypic characteristics of each CC and ST identified among 78 *S. aureus* isolates recovered during the present study

CC (*n*)	Antimicrobial resistance genes[a] (*n*)	Virulence factor genes[a] (*n*)	SCC*mec* type[a] (*n*)
ST34/42 (2)	*blaZ* (2), *sdrM* (2), *fosB* (2)	*seh* (2), *lukF/S* (2), *tst* (2), *egc* (2), IEC s/c/s[b] (1), *chp* (1), *scn* (2), *sak* (2), *clfA/B* (2), *sdrC/D* (2), *fnbB/A* (2), *icaA/D/C* (2), *bbp* (2), *cap* (2)	
ST72 (3)	*blaZ* (3), *sdrM* (3), *fosB* (3)	*lukF/S* (3), *egc* (3), IEC s/c/s (3), *chp* (3), *scn* (3), *sak* (3), *clfA/B* (3), *sdrC/D* (3), *fnbB/A* (3), *icaA/D/C* (3), *bbp* (3), *cap* (3)	
CC1 (3)	*blaZ* (1), *sdrM* (3)	*seh* (3), *lukF/S* (3), *scn* (3), *sak* (3), *clfA/B* (3), *sdrC/D* (3), *fnbB/A* (3), *icaA/D/C* (3), *bbp* (3), *cap* (3)	
CC5 (8)	*blaZ* (7), *sdrM* (8), *fosB* (8), *erm*(A) (5), *mecA* (1)	*luk*F/S (8), *egc* (8), IEC s/c/s (7), *chp* (7), *scn* (8), *sak* (8), *clfA/B* (8), *sdrC/D* (8), *fnb*B/A (8), *icaA/D/C* (8), *bbp* (8), *cap* (8)	SCC*mec*-IV (1)
CC7 (2)	*blaZ* (2), *sdrM* (2)	*scn* (2), *sak* (2), *clfA/B* (2), *sdrC/D* (2), *fnbB/A* (2), *icaA/D/C* (2), *bbp* (2), *cap* (2)	
CC8 (1)	*blaZ* (1), *sdrM* (1), *fosB* (1)	*lukF/S* (1), *tst* (1) IEC s/c/s (1), *chp* (1), *scn* (1), *sak* (1), *clfA/B* (1), *sdrC/D* (1), *fnbB/A* (1), *icaA/D/C* (1), *bbp* (1), *cap* (1)	
CC9 (4)	*blaZ* (4), *sdrM* (4), *msr*(A) (1), *fosB* (4)	*lukF/S* (4), *egc* (4), IEC s/c/s (4), *chp* (4), *scn* (4), *sak* (4), *clfA/B* (4), *sdrC/D* (4), *fnbB/A* (4), *icaA/D/C* (4), *bbp* (4), *cap* (4)	
CC12 (1)	*blaZ* (1), *sdrM* (1), *fosB* (1)	*sec* (1), *lukF/S* (1), *scn* (1), *sak* (1), *clfA/B* (1), *sdrC/D* (1), *fnbB/A* (1), *icaA/D/C* (1), *bbp* (1), *cap* (1)	
CC15 (7)	*blaZ* (7), *sdrM* (7), *fosB* (7)	*lukF/S* (7), *tst* (2), *chp* (7), *scn* (7), *clfA/B* (7), *sdrC/D* (7), *fnbB/A* (7), *icaA/D/C* (7), *bbp* (7), *cap* (7)	
CC22 (6)	*blaZ* (5)	*egc* (6), IEC s/c/s (6), *chp* (6), *scn* (6), *sak* (6), *clfA/B* (6), *sdrC/D* (6), *fnbB/A* (6), *ica*A/D/C (6), *bbp* (3), *cap* (6)	

Continued overleaf

Table 4.2 continued. Genotypic characteristics of each CC and ST identified among 78 *S. aureus* isolates recovered during the present study

CC (*n*)	Antimicrobial resistance genes[a] (*n*)	Virulence factor genes[a] (*n*)	SCC*mec* type[a] (*n*)
CC30 (11)	*blaZ* (11), *sdrM* (11), *fosB* (11), *erm*(A) (2)	*lukF/S* (11), *lukF/S-PV* (3), *tst* (10) *egc* (10), IEC s/c/s (3), *chp* (3), *scn* (4), *sak* (4), *clfA/B* (11), *sdrC/D* (11), *fnbB/A* (11), *icaA/D/C* (11), *bbp* (10), *cap* (11)	
CC45 (10)	*blaZ* (10), *sdrM* (7), *erm*(A) (1)	*sec* (8), *lukF/S* (1), *egc* (10), IEC s/c/s (10), *chp* (10), *scn* (10), *sak* (10), *clfA/B* (10), *sdrC/D* (10), *fnbB/A* (10), *icaA/D/C* (10), *bbp* (10), *cap* (10)	
CC59 (2)	*sdrM* (2), *erm*(A) (1)	*lukF/S* (2), *chp* (2), *scn* (2), *clfA/B* (2), *sdrC/D* (2), *fnbB/A* (2), *icaA/D/C* (2), *bbp* (2), *cap* (2)	
CC188 (7)	*blaZ* (2), *sdrM* (7)	*lukF/S* (7), *scn* (7), *sak* (7) *clfA/B* (7), *sdrC/D* (7), *fnbB/A* (7), *icaA/D/C* (7), *bbp* (2), *cap* (7)	
CC398 (9)	*blaZ* (9), *sdrM* (9)	*lukF/S* (9), *chp* (9), *scn* (9), *clfA/B* (9), *sdrC/D* (9), *fnbB/A* (9), *icaA/D/C* (9), *bbp* (9), *cap* (9)	
CC779 (1)	*blaZ* (1), *sdrM* (1)	*lukF/S* (1), IEC s/c/s (1), *chp* (1), *scn* (1), *sak* (1), *clfA/B* (1), *sdrC/D* (1), *fnbB/A* (1), *icaA/D/C* (1), *bbp* (1), *cap* (1)	
CC2250/2277 (1)	*blaZ* (1), *fosB* (1)	*scn* (1), *sak* (1), *sdrC/D* (1), *fnbB/A* (1)	

[a] All antimicrobial resistance genes, virulence factor genes and SCC*mec* types were detected by microarray profiling using the *S. aureus* Genotyping Kit 2.0 (Alere).

[b] IEC s/c/s refers to the number of isolates that harbored all three immune evasion complex genes, *scn*, *chp*, and *sak*.

Abbreviations: CC; MLST clonal complex, ST; sequence type, SCC*mec*; staphylococcal cassette chromosome *mec*, IEC; immune evasion cluster.

4.3.2 Antimicrobial resistance and virulence factor genes harboured by *S. aureus* isolates

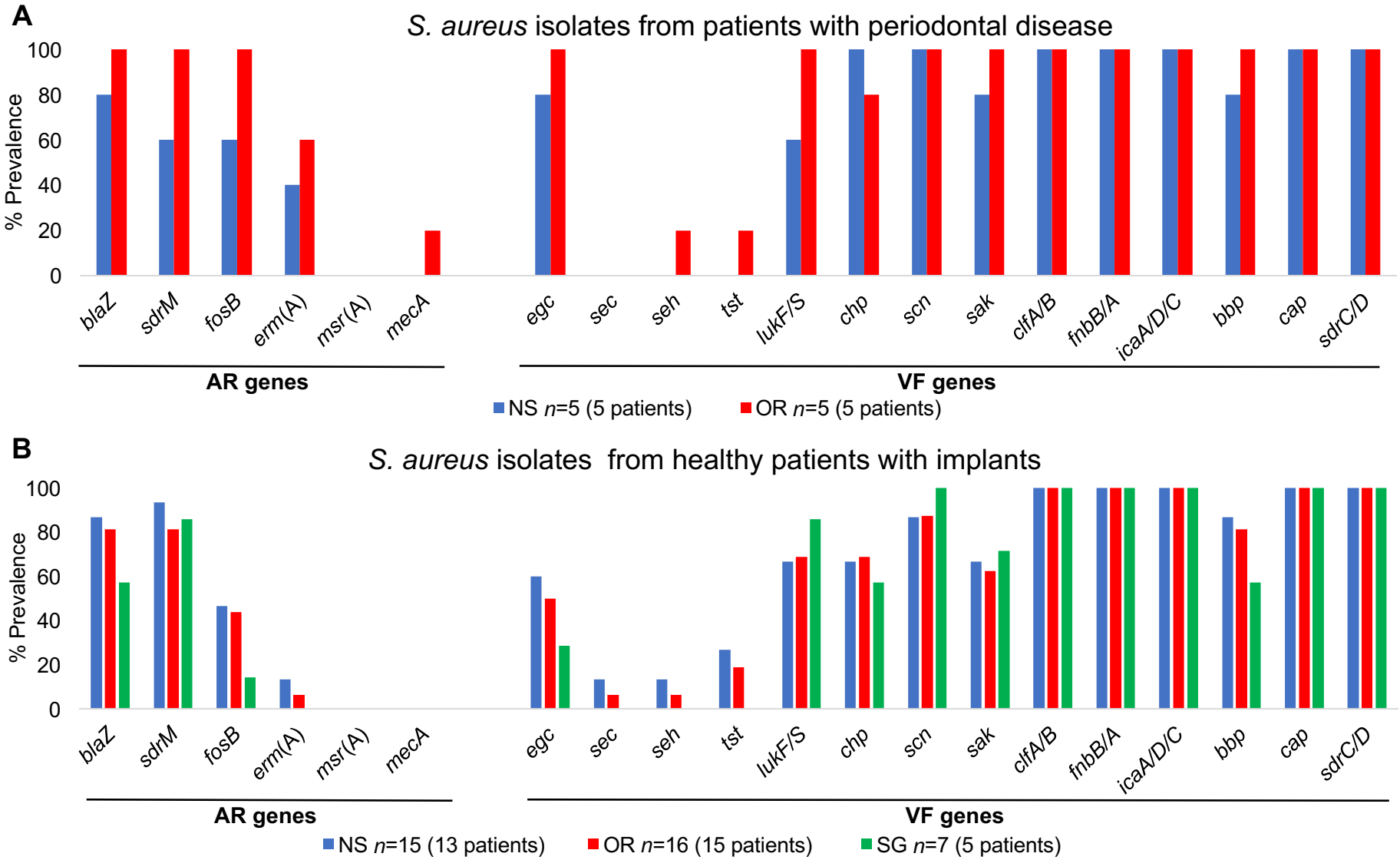
4.3.2.1 Antimicrobial resistance genes in S. aureus

Overall the *S. aureus* isolates recovered in the present study did not harbour a diverse range of antimicrobial resistance genes. Only six distinct genes encoding antimicrobial agent resistance were detected among the 78 *S. aureus* isolates subjected to DNA microarray analysis. The prevalence of the *blaZ* gene (67/78, 85.9%) encoding beta-lactam resistance and the *sdrM* gene (68/78, 87.2%) encoding an efflux pump that has been associated with low level resistance to fluoroquinolones and ethidium bromide (163) were high among *S. aureus* isolates recovered from distinct anatomical sites across all three participant groups (Fig. 4.1). The *blaZ* gene was detected in all isolates recovered from oral rinse samples (n = 5) of patients with periodontal disease and all isolates recovered from the nares (n = 6) of orally healthy participants. The lowest prevalence (4/7, 57%) was detected in isolates recovered from subgingival sites of healthy patients with implants. In contrast, 13/16 (80%) of isolates recovered from the oral cavity of the same participant groups harboured *blaZ* (Fig. 4.1B). The *sdrM* gene was detected in 100% and 86% of *S. aureus* isolates recovered from subgingival sites of orally healthy participants (5/5) and healthy patients with implants (6/7), respectively (Figs. 4.1B and 4.1C). Isolates recovered from the nares of patients with periodontal disease harboured the lowest prevalence of the *sdrM* gene (3/5, 60%).

The *fosB* gene (encoding metallothiol transferase) was detected in 100% (5/5) of *S. aureus* isolates recovered from the oral rinse samples of patients with periodontal disease (Fig. 4.1A). This gene was less commonly detected (< 60%) in *S. aureus* isolates recovered from healthy patients with implants and orally healthy controls.

The macrolide resistance gene *erm*(A) was predominantly detected in isolates recovered from the nares (2/5, 40%) and oral rinse sample (3/5, 60%) of patients with periodontal disease, and was less prevalent (< 14%) in isolates recovered from the distinct anatomical sites in the other two participant groups (Fig. 4.1).

The *msr*(A) gene encoding the macrolide efflux pump was only detected in one *S. aureus* isolate recovered from the subgingival site of an orally healthy participant


A
S. aureus isolates from patients with periodontal disease
% Prevalence
100
80
60
40
20
0
blaZ
sdrM
fosB
erm(A)
msr(A)
mecA
egc
sec
seh
tst
lukF/S
chp
scn
sak
clfA/B
fnbB/A
icaA/D/C
bbp
cap
sdrC/D
AR genes
VF genes
NS n=5 (5 patients)
OR n=5 (5 patients)
B
S. aureus isolates from healthy patients with implants
% Prevalence
100
80
60
40
20
0
blaZ
sdrM
fosB
erm(A)
msr(A)
mecA
egc
sec
seh
tst
lukF/S
chp
scn
sak
clfA/B
fnbB/A
icaA/D/C
bbp
cap
sdrC/D
AR genes
VF genes
NS n=15 (13 patients)
OR n=16 (15 patients)
SG n=7 (5 patients)

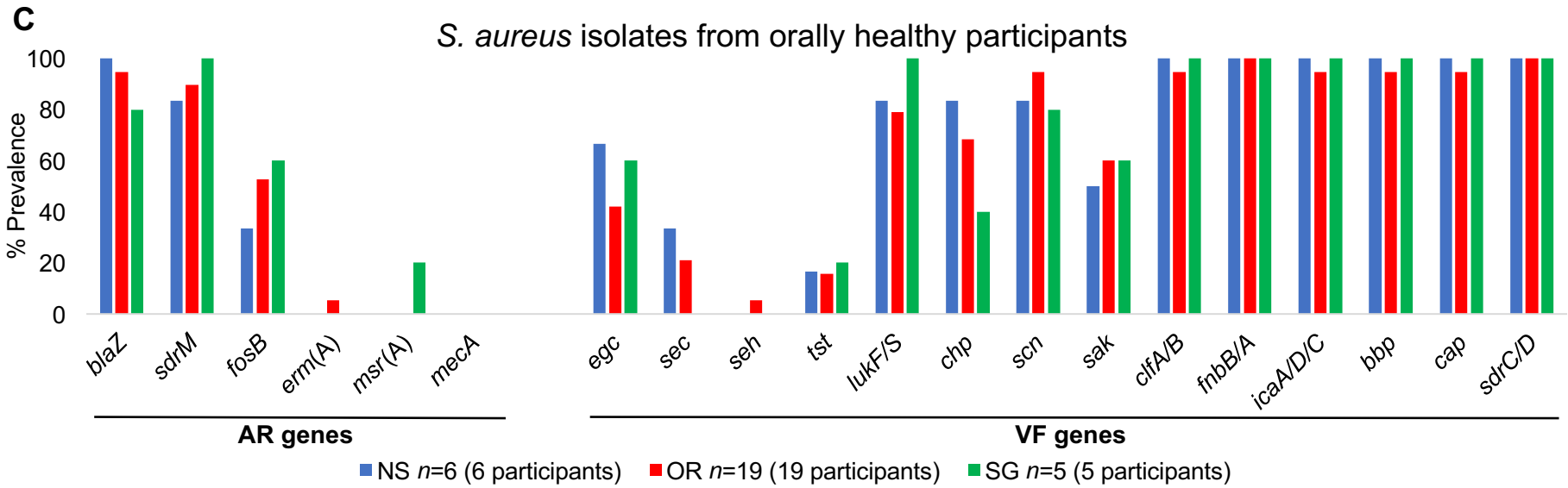


Figure 4.1 Prevalence of antimicrobial resistance (AR) and virulence factor (VF) genes detected in *S. aureus* isolates by DNA microarray profiling. A) *Staphylococcus aureus* isolates recovered from patients with periodontal disease, B) *S. aureus* isolates recovered from healthy patients with implants and C) *S. aureus* isolates recovered from orally healthy participants.

Abbreviations: NS, nasal swab; OR, oral rinse; SG, subgingival site; *egc*, enterotoxin gene cluster; *ica*, intercellular adhesion proteins.

(Fig. 4.1C) and the *mecA* gene was only detected in one isolate (CC5-MRSA-IV) recovered from the oral rinse sample of a patient with periodontal disease (Fig. 4.1A).

4.3.2.2 Virulence factor genes in S. aureus

A more diverse range of virulence factor genes were detected among the *S. aureus* isolates analysed; the prevalence of 14 virulence factor genes of interest were investigated (Fig. 4.1). The enterotoxin gene cluster *egc* was detected in *S. aureus* isolates recovered from each anatomical site investigated across the three distinct participant groups, however the highest prevalence was detected in isolates recovered from the oral rinse samples of patients with periodontal disease (5/5, 100%) and the lowest prevalence was detected among isolates recovered from subgingival sites of healthy patients with implants (2/7, 28.5%) (Fig. 4.1).

The enterotoxin C encoding gene *sec* was detected in two isolates recovered from the nares and one isolate recovered from the oral rinse sample of healthy participant with implants. Interestingly one of the isolates recovered from the nares and the isolate recovered from the oral rinse sample were from the same patient (Patient I8). The *sec* gene was also detected in two and four *S. aureus* isolates recovered from the nares and oral rinse samples of orally healthy participants, respectively (Fig. 4.1C). Pairs of isolates harbouring the *sec* gene were recovered from the nares and oral rinse samples of Participants C6 and 314.

There was a low prevalence (< 20%) of the enterotoxin encoding gene *seh* detected; *seh* was detected in 1/5 (20%) *S. aureus* isolate recovered from the oral rinse samples of patients with periodontal disease, 2/14 (14%) and 1/16 (6%) isolates recovered from nares and oral rinse samples from healthy patients with implants, respectively, and 1/19 (5.3%) of isolates recovered from an oral rinse sample of an orally healthy participant (Fig. 4.1).

The *tst* gene encoding the toxic shock syndrome toxin was detected in 13/78 (16.7%) of the *S. aureus* isolates from all three participant groups investigated (Fig. 4.1). The only isolate from a subgingival site in which the *tst* gene was detected was recovered from a healthy student.

The two leucocidin encoding genes *lukF* and *lukS* (*lukF/S*) were commonly detected (> 60%) among *S. aureus* isolates recovered from the three distinct participant groups (Fig. 4.1). All *S. aureus* isolates recovered from oral rinse samples of patients with periodontal disease and subgingival sites of orally healthy participants harboured

lukF/S. Three distinct isolates recovered from the nasal cavity, oral rinse sample and a subgingival site of an orally healthy participant (Participant C3) belonged to CC30; the nasal cavity and oral rinse isolates harboured *lukS-PV*, while the subgingival isolate harboured *lukF/S-PV*.

The immune evasion cluster (IEC) consists of a combination of five genes, the *sea* (encoding staphylococcal enterotoxin A), *sep* (encoding staphylococcal enterotoxin P), *chp* (encoding a chemotaxis inhibiting protein), *scn* (encoding a staphylococcal complement inhibitor), and *sak* (encoding staphylokinase) genes. Genes involved in the IEC gene cluster were prevalent in *S. aureus* isolates recovered from all anatomical sites sampled across the three distinct participant groups (Fig. 4.1). The predominant IEC type detected in *S. aureus* isolates recovered from healthy patients with implants (16/38, 42.1%) and orally healthy participants (14/30, 46.7%) was type B (*sak*, *chp*, and *scn*). The most prevalent IEC detected in isolates recovered from patients with periodontal disease (4/10, 40%) was type A (*sea*, *sak*, *chp*, and *scn*). Seven CC30 *S. aureus* isolates recovered from distinct sample sites in healthy patients with implants and orally healthy participants did not harbour IEC genes.

The clumping factor genes A and B (*clfA/B*) were detected in 100% (10/10) of *S. aureus* isolates recovered from each anatomical site in patients with periodontal disease (Fig. 4.1A) and healthy patients with implants (38/38) (Fig. 4.1B), and was detected in 29/30 (96.6%) of isolates recovered from orally healthy participants (Fig. 4.1C).

The *fnbA* and *fnbB* genes encoding the fibronectin-binding proteins A and B were harboured by 78/78 (100%) of *S. aureus* isolates recovered from each anatomical site sampled in patients with periodontal disease, healthy patients with implants, and orally healthy controls (Fig. 4.1).

The intercellular adhesion proteins A, C, and D, encoded by the genes *icaA*, *icaC*, and *icaD*, respectively, were harboured by 77/78 (98.7%) of *S. aureus* isolates analysed by DNA microarray in the present study (Fig. 4.1).

The bone sialoprotein-binding protein gene *bbp* was most prevalent (> 94.7%) in *S. aureus* isolates recovered from orally healthy participants and was detected in isolates recovered from the nares, oral rinse samples and subgingival sites in this group (Fig. 4.1C). The *bbp* gene was also commonly detected (> 87%) in isolates recovered from the nares and oral rinse samples of patients with periodontal disease and healthy patients with implants (Fig. 4.1).

Capsular genes were harboured by 48/48 (100%) of *S. aureus* isolates investigated that were recovered from distinct anatomical sites sampled from patients with periodontal disease and healthy patients with implants (Figs. 4.1A and 4.1B). Similarly, 29/30 (96.7%) of isolates recovered from orally healthy participants harboured capsular genes (Fig. 4.1C). Capsule type 8 was the predominant capsule type harboured by *S. aureus* isolates across the three participant groups.

4.3.3 Antimicrobial resistance and virulence factor genes harboured by *S. epidermidis* isolates

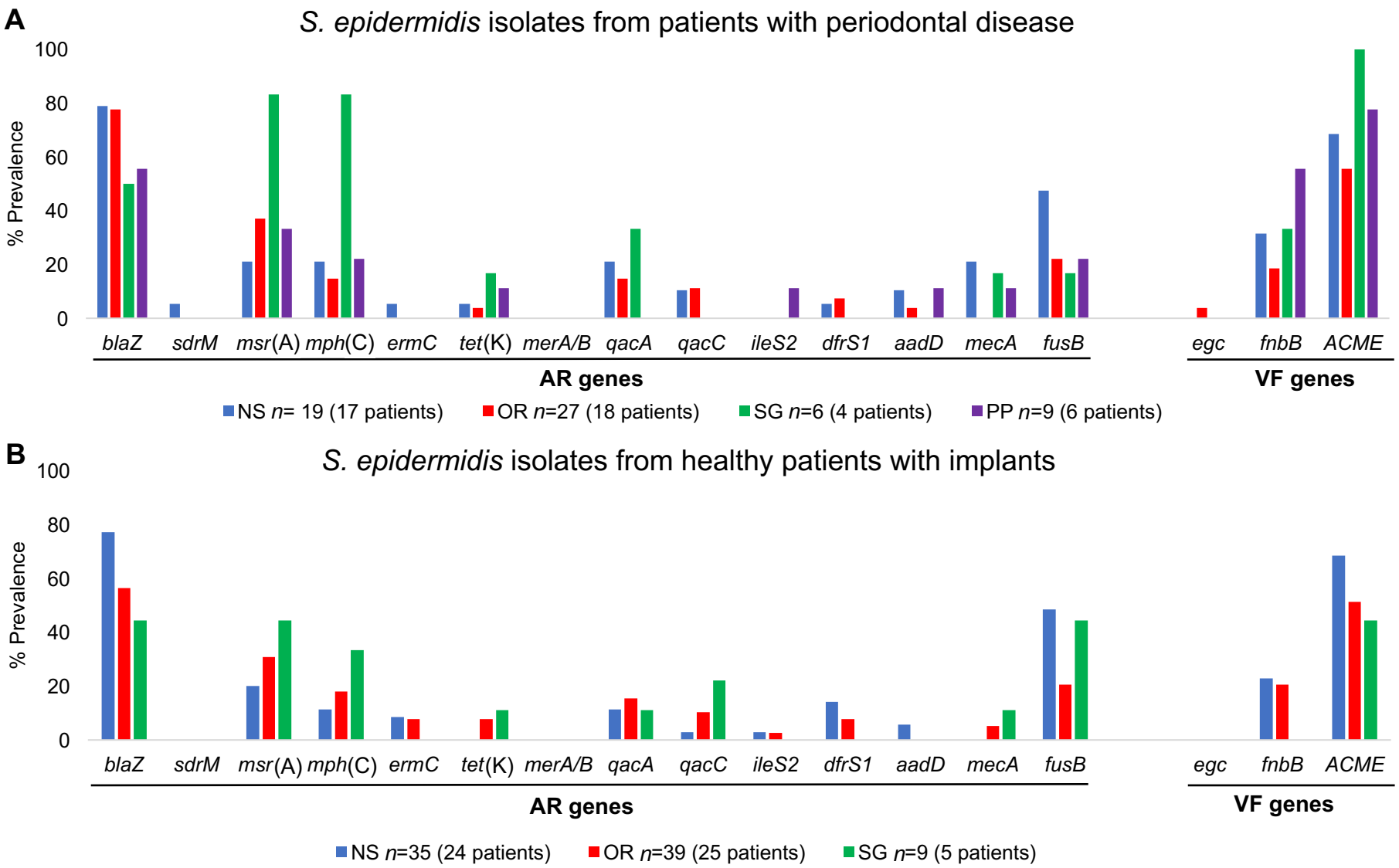
Antimicrobial and virulence factor genes were detected in *S. epidermidis* isolates recovered from the distinct anatomical sites sampled of all three participant groups. In total 61, 83 and 83 *S. epidermidis* isolates recovered from patients with periodontal disease, healthy patients with implants and orally healthy participants, respectively, were analysed.

4.3.3.1 Antimicrobial resistance genes in S. epidermidis

Similar to the *S. aureus* isolates analysed, the *blaZ* gene was the most common antimicrobial resistance gene detected among *S. epidermidis* isolates recovered from all three participant groups investigated (Fig. 4.2). In contrast to the *S. aureus* isolates, the *sdrM* gene was harboured by only one *S. epidermidis* isolate, which was recovered from the nares of a patient with periodontal disease (Fig. 4.2A).

The macrolide resistance genes *msr*(A) and *mph*(C) were detected in isolates recovered from each anatomical site sampled across the three participant groups. Interestingly, the prevalence of *msr*(A) was higher in isolates recovered from oral rinse samples (> 30.7%), periodontal pockets (33.3%) and subgingival sites (> 40%) compared to isolates recovered from the nares (< 21%) across all participant groups (Fig. 4.2). The highest prevalence of the gene *mph*(C) was in subgingival sites of patients with periodontal disease (5/6, 83.3%) (Fig. 4.2A), while the lowest was detected in isolates recovered from the nares of healthy patients with implants (4/35, 11.4%) (Fig. 4.2B).

The *erm*(C) encoding macrolide, lincosamide and streptogramin B resistance was detected in 5.3% of *S. epidermidis* isolates analysed, and was not detected in any isolates recovered from periodontal pockets or subgingival sites across the three


A
S. epidermidis isolates from patients with periodontal disease
% Prevalence
100
80
60
40
20
0
blaZ
sdrM
msr(A)
mph(C)
ermC
tet(K)
merA/B
qacA
qacC
ileS2
dfrS1
aadD
mecA
fusB
egc
fnbB
ACME
AR genes
VF genes
NS n= 19 (17 patients)
OR n=27 (18 patients)
SG n=6 (4 patients)
PP n=9 (6 patients)
B
S. epidermidis isolates from healthy patients with implants
% Prevalence
100
80
60
40
20
0
blaZ
sdrM
msr(A)
mph(C)
ermC
tet(K)
merA/B
qacA
qacC
ileS2
dfrS1
aadD
mecA
fusB
egc
fnbB
ACME
AR genes
VF genes
NS n=35 (24 patients)
OR n=39 (25 patients)
SG n=9 (5 patients)

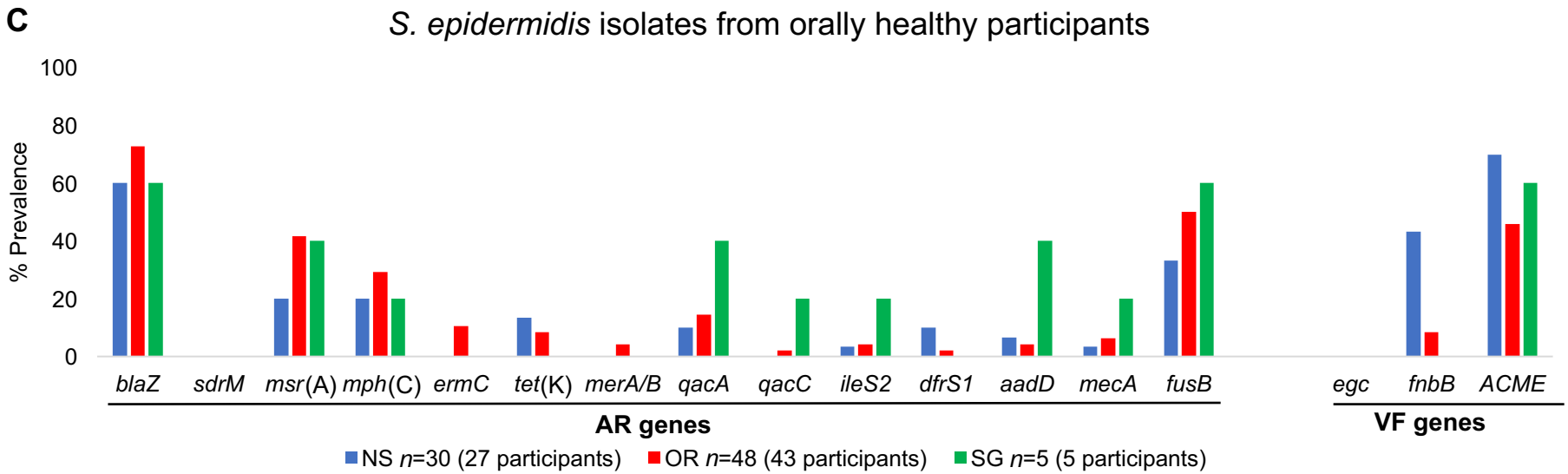


Figure 4.2 Prevalence of antimicrobial resistance (AR) and virulence factor (VF) genes detected in *S. epidermidis* isolates by DNA microarray profiling. A) *Staphylococcus epidermidis* isolates recovered from patients with periodontal disease, B) *S. epidermidis* isolates recovered from healthy patients with implants and C) *S. epidermidis* isolates recovered from orally healthy participants.

Abbreviations: NS, nasal swab; OR, oral rinse; SG, subgingival site; PP, periodontal pocket; *egc*, enterotoxin gene cluster; ACME, arginine catabolic mobile element.

participant groups (Fig. 4.2). The *tet*(K) gene encoding tetracycline resistance was detected in *S. epidermidis* isolates recovered from each of the participant groups but not necessarily from each anatomical site sampled (Fig. 4.2). Interestingly, the highest prevalence of the *tet*(K) gene was detected in subgingival sites (1/6, 16.6%) and periodontal pockets (1/9, 11.1%) of patients with periodontal disease and subgingival sites (1/9, 11.1%) of healthy patients with implants (Fig. 4.2A and 4.2B). The *tet*(K) gene was harboured by *S. epidermidis* isolates recovered from the nares, oral rinse sample, a periodontal pocket and a subgingival site of the same patient with periodontal disease (Patient P11).

The *merA/B* genes encode mercury resistance were only detected in 2/227 (0.9%) of *S. epidermidis* isolates analysed; both isolates were recovered from the oral rinse samples of two distinct orally healthy participants (Fig. 4.2C).

The *qacA* and *qacC* genes encoding resistance to quaternary ammonium compounds commonly present in antiseptics, biocdes and disinfectants were harboured by less than 14.5% of *S. epidermidis* isolates investigated (Fig. 4.2). Interestingly the highest prevalence of *qacA* and *qacC* were detected in isolates recovered from subgingival sites of orally healthy participants (2/5, 40%) and healthy patients with implants (2/9, 22.2%), although the two isolates recovered from healthy patients with implants were recovered from the same patient.

The mupirocin resistance gene *ileS2* (also known as *mupA*) was detected in *S. epidermidis* isolates recovered from the nares (1/34, 2.9%) and oral rinse (1/39, 2.6%) samples of healthy patients with implants (Fig. 4.2B), the nares (1/30, 3.3%), oral rinse samples (2/48, 4.2%), and subgingival sites (1/5, 20%) of orally healthy participants (Fig. 4.2C), however this gene was only detected in one isolate recovered from a periodontal pocket of a patients with periodontal disease (1/9, 11.1%) (Fig. 4.2A).

One of the genes encoding trimethoprim resistance (*dfrS1*) was detected only among *S. epidermidis* isolates recovered from the nares and/or oral rinse samples, and was detected in isolates recovered from all three participant groups examined, albeit infrequently (6.6%) (Fig. 4.2).

One of the genes encoding aminoglycoside resistance (*aadD*) was occasionally (5.3%) detected in isolates recovered from the distinct anatomical sites sampled in patients with periodontal disease and healthy participants with implants, and from the nares and oral rinse samples of orally healthy participants (Figs. 4.2). The *aadD* gene

was detected in 2/5 (40%) of isolates recovered from subgingival sites of orally healthy participants (Fig. 4.2C).

The *mecA* gene was harboured by 14/78 (17.9%) of *S. epidermidis* isolates investigated across each anatomical site sample and participant group (Fig. 4.2). The highest prevalence of MRSE isolates was detected in subgingival sites of patients with periodontal disease (1/6, 16.6%) and orally healthy controls (1/5, 20%). Overall the predominant *mec* gene complexes were class B (5/14, 35.7%) and class C (5/14, 35.7%) (Table 4.3). Based on the presence of class B *mec* and *ccrAB2* genes, five isolates were identified as harbouring SCC*mec* IV (Table 4.3). Another five MRSE isolates were identified as harbouring SCC*mec* V based on the presence of class C *mec* and *ccrC* genes (Table 4.3).

The gene encoding fusidic acid resistance, *fusB,* was detected in *S. epidermidis* isolates recovered from all three participant groups (37%) and from each distinct anatomical site sampled (Fig. 4.2).

4.3.3.2 Virulence factor genes in S. epidermidis

Staphylococcus epidermidis and other CoNS typically mainly harbour virulence factor genes solely associated with biofilm formation. However, three virulence factor genes were detected in the *S. epidermidis* isolates analysed in the present study. The enterotoxin gene cluster *egc* was only detected in 1/227 *S. epidermidis* isolates investigated; this isolate was recovered from the oral rinse sample of patients with periodontal disease (Fig. 4.2A). The *fnbB* gene encoding the fibronectin-binding protein B was detected in *S. epidermidis* isolates recovered from patients with periodontal disease (18/61, 29.5%), healthy patients with implants (16/83, 19.3%) and orally healthy participants (17/83, 20.4%) (Fig. 4.2). Interestingly, the only isolates recovered from subgingival sites or periodontal pockets that harboured *fnbB* were recovered from patients with periodontal disease (Fig. 4.2A). The arginine catabolic mobile element (ACME) was detected in *S. epidermidis* isolates by the presence of the *arcA*, *arcB*, *arcC* and *arcD* genes in the DNA microarray data and was identified in 135/227 (59.5%) of *S. epidermidis* isolates recovered from all three participant groups (Fig. 4.2). Interestingly, the highest prevalence of ACME was detected in *S. epidermidis* isolates recovered from subgingival sites (6/6, 100%) and periodontal pockets (7/9, 77.9%) of patients with periodontal disease (Fig. 4.2A), and the nares of orally healthy participants (21/30, 70%) (Fig. 4.2C). The prevalence of ACME in isolates recovered from

Table 4.3 SCC*mec*-associated genes detected among the 14 MRSE isolates subjected to DNA microarray profiling

Isolate	Sample site	*mec* gene complex[ab]	*ccr* genes	Presumptive SCC*mec* type	ACME-*arc*
P6NS2	NS	*mecA*, *ugpQ*, *mecR1*	*ccrC*	ND	Positive
P8NS2	NS	Class C	*ccrAA*, *ccrC*	SCC*mec* V	Positive
P8NS3	NS	Class C	*ccrAA*, *ccrC*	SCC*mec* V	Negative
P11NS1	NS	Class B	*ccrAB2*, *ccrAA*, *ccrC*	SCC*mec* IV & *ccrAA, ccrC*	Positive
P11PPHI1	SG	Class B	*ccrAB2*, *ccrAA*, *ccrC*	SCC*mec* IV & *ccrAA*, *ccrC*	Positive
P11PPP12	PP	Class B	*ccrAB2*	SCC*mec* IV	Positive
I10OR2	OR	*mecA*, Δ*mecR1*, *ugpQ*, *mecI*, *mecR1*	*ccrAB2*	ND	Negative
I29PPHI1	SG	Class C	*ccrAB2*, *ccrAA*, *ccrC*	SCC*mec* V & *ccrAB2*	Positive
I30OR1	OR	Class B	*ccrAB2*, *ccrB4*	SCC*mec* IV & *ccrB4*	Positive
C7OR1	OR	*mecA*, Δ*mecR1*, *ugpQ*, *mecI*, *mecR1*		ND	Negative
104NS2	NS	Class C	*ccrC*	SCC*mec* V	Positive
109OR2	OR	Class C	*ccrAB2*, *ccrAA*, *ccrC*	SCC*mec* V & *ccrAB2*	Negative
213OR2	OR	Class B	*ccrAB2*	SCC*mec* IV	Negative
217PP362	SG	Class D	*ccrAB2*, *ccrAA*, *ccrC*	ND	Positive

[a]The *mec* gene complexes are classified based on the presence of *mecA*, its regulatory proteins and insertion sequences. SCC*mec* types were presumptively assigned based on the *mec* and *ccr* gene complexes detected by the DNA microarray as previously described (40). Combinations of *mec* and *ccr* gene complexes not previously assigned to distinct SCC*mec* types are listed as not determined.

[b]Class A *mec* (*mecA*, *mecI*, *mecR1*, *ugpQ*, *xylR*), Class B *mec* (*mecA*, Δ*mecR1*, *ugpQ*), Class C *mec* (*mecA*, *ugpQ*), and Class D *mec* (*mecA*, Δ*mecR1*).

Abbreviation: MRSE; methicillin resistant *Staphylococcus epidermidis*, NS; nasal cavity, SG; subgingival site, PP; periodontal pocket, OR; oral cavity, ND; Not determined

subgingival sites of patients with periodontal disease was significantly higher compared to isolates recovered from subgingival sites of healthy patients with implants (p = 0.044). The lowest prevalence of ACME was detected in *S. epidermidis* isolates recovered from subgingival sites of healthy patients with implants (4/9, 44.4%) (Fig. 4.2B), followed by isolates recovered from the oral rinse sample of orally healthy participants (22/48, 45.8%) (Fig. 4.2C).

Overall 9/14 (64.3%) of MRSE isolates analysed also harboured ACME-*arc* genes. The ACME-*arc* genes were not detected in 3/3 (100%), 1/4 (25%), and 1/2 (50%) of MRSE isolates recovered from the oral rinse sample of orally healthy participants, the nares of patients with periodontal disease, and oral rinse sample of healthy patients with implants, respectively.

4.4 Discussion

The present study is the first detailed genotypic investigation into the prevalence of antimicrobial and virulence factor genes harboured by *S. aureus* (78 isolates) and *S. epidermidis* (227 isolates) isolates recovered from the nares and distinct anatomical sites in the oral cavities of patients with periodontal disease, healthy patients with implants and orally healthy participants (Table 4.1). Overall 78 *S. aureus* and 227 *S. epidermidis* isolates were screened by DNA microarray profiling.

4.4.1 Population analysis of *S. aureus* isolates recovered during the present study.

In total, 15 different clonal linages were identified among the 78 *S. aureus* isolates subjected to DNA microarray analysis in the current study (Tables 4.1 & 4.2). The predominant linages were CC30 (11/78, 14.1%), CC45 (10/78, 12.8%) and CC398 (9/78, 11.5%). Previous research revealed that 6/31 (19.4%) MSSA isolates recovered from the oral cavities of patients with peri-implantitis also belonged to CC30 (132), however, isolates belonging to CC45 or CC398 were not identified in this previous research (132). Previous studies have reported that MSSA clones are genetically diverse and don't typically group into geographical clusters, unlike MRSA strains such as ST22 MRSA-IV which has been the predominant MRSA clone in Ireland since 2002 (55, 156). The findings of the present study appear to be consistent with previous research, and show a diverse population of MSSA isolates were recovered from the participants investigated.

4.4.2 Antimicrobial resistance and virulence factor genes detected in *S. aureus* isolates

Only one of the 78 *S. aureus* isolates recovered from the oral cavities across each of the distinct participant groups was identified as MRSA (1.3%), correlating with a previous study that did not detect any MRSA in isolates recovered from the oral cavities of patients with periodontal disease and healthy participants (158).

Overall the *S. aureus* isolates analysed during the present study did not harbour many genes encoding resistance to antimicrobial agents. The genes *msr*(A) and *erm*(A) encoding macrolide resistance were detected in only one (1.3%) and nine (11.5%) of the 78 *S. aureus* isolates investigated, respectively. The prevalence of these genes in MSSA

isolates recovered from the oral cavities of similar patients has not been reported previously, however these genes have been detected in 0 – 2% and 40 – 63% of clinical MRSA isolates, respectively (161, 164). Similarly, the resistance genes *blaZ* and *fosB*, and the efflux pump gene *sdrM* were less prevalent among *S. aureus* isolates investigated in the current study (Fig. 4.1) than in MRSA isolates from previous reports (48, 160, 165). In general, MSSA isolates harbour fewer antimicrobial resistance genes than MRSA isolates, and the findings of the present study support such previous studies (166).

Staphylococcus aureus isolates recovered from each of the participant groups harboured genes encoding virulence factor and MSCRAMMs typically detected in this species (Fig. 4.1). Almost all isolates (71/78, 91.2%) harboured genes associated with the IEC, and the most prevalence type was IEC type B (*sak*, *chp*, *scn*) (33/71, 46%), which correlates with a previous study which reported 11/19 (58%) of *S. aureus* harboured this IEC type (67). Interestingly all *S. aureus* isolates (9/9, 100%) belonging to CC398 harboured IEC type C, indicating they likely belong to the human CC398 lineage rather than the animal associated CC398 lineage (62), as previous research has indicated that CC398 isolates recovered from animals are predominantly IEC negative (62). All seven IEC negative MSSA isolates belonged to CC30 and were recovered from each of the participant groups, previous studies have also detected CC30-MSSA lacking genes associated with the IEC (167).

In the present study, the *tst* gene was detected in 13/78 (16.7%) carriage *S. aureus* isolates investigated. This gene has previously been associated with CC30 MRSA (165), and 10/13 (76.9%) of the *S. aureus* isolates harbouring *tst* in the present study also belonged to CC30. The prevalence of this gene was slightly higher in the present study than in a previous report (4/6, 66.7%), which examined CC30 *S. aureus* isolates recovered from the oral cavities of patients with peri-implantitis (132). In the present study, all *tst* harbouring *S. aureus* isolates belonging to CC30 were recovered from healthy patients with implants and orally healthy controls. Based on these findings, it is likely that CC30 isolates harbouring *tst* are associated with general carriage, and are recovered from both healthy and diseased oral cavities.

Based on the prevalence of genes encoding antimicrobial resistance and virulence factors and clonal lineages present, patients with periodontal disease, healthy patients with implants and orally healthy controls harboured a diverse range of *S. aureus* isolates in their oral cavities. The low prevalence of antimicrobial resistance

genes detected suggests that these isolates were largely susceptible to antimicrobial agents.

4.4.3 Antimicrobial resistance and virulence factor genes detected in *S. epidermidis* isolates

A greater diversity of antimicrobial resistance genes was detected among *S. epidermidis* isolates compared to the *S. aureus* isolates investigated, this result is consistent previous research (27, 81, 86). The genes encoding macrolide resistance, *mph*(C), and *msr*(A) were consistently detected in 75/227 (33%) *S. epidermidis* isolates recovered from distinct anatomical sites across the three participant groups (Fig. 4.2). Interestingly, in the majority of isolates (48/50, 96%) the *mph*(C) gene was detected in association with *msr*(A), which correlates with a previous study that investigated clinical *S. epidermidis* isolates (168). This previous study reported that the prevalence of *msr*(A) in clinical *S. epidermidis* isolates was 6.4% (14/333) (168), whereas the present study detected *msr*(A) in 73/227 (32.2%) carriage isolates. In contrast, the previous study detected the *erm*(C) gene encoding macrolide/lincosamide in 134/333 (64.4%) clinical *S. epidermidis* isolates (168), whereas the present study only detected this gene in 12/227 (5.3%) carriage *S. epidermidis* isolates. However, phenotypic analysis in the previous study found that resistance to the macrolide erythromycin could be attributed to either *msr*(A) or *erm*(C), suggesting there is no association between *msr*(A) or *erm*(C) and disease or carriage *S. epidermidis* isolates.

Another interesting finding of this investigation was that, when antimicrobial resistance genes were detected in *S. epidermidis* isolates, they were more prevalent in isolates recovered from subgingival sites and periodontal pockets than in isolates recovered from oral rinse or nasal samples. Genes such as *tet*(K), *qac*(A), *ileS*(2), *msr*(A), *mph*(C), and *aadD* were more prevalent in *S. epidermidis* isolates recovered from subgingival sites and periodontal pockets of patients with periodontal disease compared to isolates recovered from oral rinse and nasal samples in the same participant groups (Fig. 4.2A). This suggests that the oral cavity and in particular, anatomical sites such as subgingival sites and periodontal pockets could possibly be acting as a reservoir for antimicrobial resistance genes. However, these results should be considered with caution due to the lower number of *S. epidermidis* isolates recovered from periodontal pockets and subgingival sites investigated compared to oral rinse isolates.

As mentioned previously, the predominant method by which *S. epidermidis* causes infection is biofilm formation, and therefore this species does not harbour many virulence factor genes associated with toxin production. The *egc* was detected in one *S. epidermidis* isolate recovered from the oral cavity of a patient with periodontal disease. *Staphylococcus epidermidis* typically do not harbour genes associated with enterotoxins, and further PCR confirmation of the presence of these genes in this isolate is required. The fibronectin gene *fnbB* is an MSCRAMM that is directly associated with biofilm formation through tissue colonisation (70). Interestingly, the only isolates recovered from periodontal pockets and subgingival sites that harboured *fnbB* were recovered from patients with periodontal disease (Fig. 4.2), possibly suggesting that *fnbB* facilitates *S. epidermidis* colonisation of this distinct anatomical site. This finding could possibly be consistent with the greater abundance of *S. epidermidis* in these diseased subgingival sites, compared to subgingival sites in healthy patients with implants and orally healthy controls. Further investigations into the prevalence of specific *S. epidermidis* virulence factor genes associated with biofilm formation in periodontal pockets is required.

The DNA microarray is not capable of identifying the clonal linages or STs among the *S. epidermidis* population. However, based on the diversity of antimicrobial resistance genes and virulence factors genes such as ACME and *fnbB* detected in isolates recovered from distinct anatomical sites across the three participant groups, it is evident the *S. epidermidis* population in these sites is diverse.

4.4.3.1 Prevalence of ACME

To date, ACME has been classified into three distinct types based on the presence of both the *arc* and *opp3* operons (type I), the *arc* operon only (type II) or the *opp3* operon only (type III). The DNA microarray can only detect the prevalence of the ACME-*arc* operon and therefore the prevalence of different ACME types cannot be identified using this technology. Furthermore, as the DNA microarray detects the *arc* genes only, the presence of ACME III is undetected using this method.

The ACME-*arc* gene cluster was not detected any of the *S. aureus* isolates investigated. In contrast 135/227 (59.5%) *S. epidermidis* isolates investigated harboured this gene cluster, suggestive of the presence of ACME types I or II. This result is consistent with previous reports on the prevalence of this element in *S. epidermidis* (154, 169, 170). Interestingly the highest prevalence of ACME-*arc* was detected in *S.*

epidermidis isolates recovered from the subgingival sites (6/6, 100%) and periodontal pockets (7/9, 77.8%) of patients with periodontal disease (Fig. 4.2). The prevalence of *S. epidermidis* harbouring ACME-*arc* was significantly higher in isolates recovered from subgingival sites of patients with periodontal disease compared to isolates recovered from subgingival sites of healthy patients with implants ($p = 0.044$). These findings correlate with a previous study that reported a higher prevalence of the ACME-*arc* in *S. epidermidis* isolates recovered from peri-implant pockets or subgingival sites compared to the oral cavity (132). The combination of these findings suggest that ACME may be a major contributing factor to the increased prevalence of *S. epidermidis* in periodontal pockets and peri-implant pockets in patients with periodontal or peri-implantitis disease.

*4.4.3.2 Prevalence of SCC*mec *types in* S. epidermidis *based on DNA microarray profiling*

In the current investigation, genes associated with the SCC*mec* element were more commonly detected in *S. epidermidis* isolates (14/227; 6.1%) than *S. aureus* isolates (1/78 isolates, 1.3%) by DNA microarray profiling. The most prevalent *mec* gene complexes detected were class B (*mecA*, Δ*mecR1*, and *ugpQ*) and class C (*mecA* and *ugpQ*), which were each detected in 5/14 (35.7%) MRSE isolates. Five *S. epidermidis* isolates presumptively harboured SCC*mec* IV elements based on the presence of class B *mec* gene complex and *ccrAB2* genes (Table 4.3), correlating with previous studies that reported SCC*mec* IV as the most prevalent SCC*mec* type among *S. epidermidis* (44, 45). Interestingly the ACME-*arc* operon was detected alongside SCC*mec*-associated genes in 9/14 of these MRSE isolates, and all four MRSE isolates recovered from periodontal pockets or subgingival sites also harboured the ACME-*arc* operon. Both SCC*mec* elements and ACME integrate at *orfX*, and are often detected alongside each other (38). However, to date there has been no definitive association between a SCC*mec* type and ACME in MRSE isolates, possibly due to the prevalence of non-typeable SCC*mec* types in MRSE isolates (169). The higher prevalence of SCC*mec* in *S. epidermidis* isolates compared to *S. aureus* isolates in the oral cavity suggests *S. epidermidis* could be acting as a reservoir for MGE such as SCC*mec* that could be transferred into MSSA in the oral cavity, however it is important to note that in the present study, evidence of horizontal gene transfer between these species was not observed.

4.4.4 Investigation into possible trafficking between oro-nasal and subgingival sites

Two or more *S. aureus* isolates recovered from separate sample sites from 18 distinct participants were analysed by DNA microarray profiling. Overall 17/18 participants harboured isolates in distinct anatomical sites which belonged to the same CC. Based on the CCs to which the *S. aureus* isolates investigated belonged, trafficking between the distinct sample sites investigated is likely. These results correlate with a previous study that identified trafficking of *S. aureus* isolates between the nares and oral cavity of four participants using pulsed-field gel electrophoresis (150).

Two or more *S. epidermidis* isolates recovered from multiple, distinct anatomical sites of 17, 20 and 25 patients with periodontal disease, healthy patients with implants and orally healthy controls were analysed by DNA microarray profiling, respectively. As identification of clonal lineages present amongst *S. epidermidis* populations cannot be determined by DNA microarray analysis, presumptive trafficking of isolates between the nares and oral cavity was inferred based on the similarity of antimicrobial resistance and virulence factor genes detected in the isolates. Based on this, presumptive trafficking of *S. epidermidis* was detected in 11/62 (17.7%) participants. Further detailed population analysis using MLST or whole genome MLST is required to definitively detect trafficking of isolates between the nares and oral cavity in these participant groups.

Using DNA microarray analysis, the genetic diversity of *S. aureus* and *S. epidermidis* isolates based on the prevalence of antimicrobial resistance and virulence factor genes was investigated, this revealed the genetic diversity of both these species between the three participant groups but also within each participant group. The detection of a diverse range of antimicrobial resistance genes in *S. epidermidis* isolates compared to *S. aureus* correlates with previous research (27, 33). Finally, the detection of a high prevalence of *S. epidermidis* harbouring ACME-*arc* in subgingival and periodontal pockets compared to *S. epidermidis* isolates recovered from oral rinse samples suggests it could provide a fitness advantage in these specific oral environments.

Chapter 5

Prevalence and Diversity of ACME among Oral and Subgingival *S. epidermidis* from Patients With Periodontal Disease and Healthy Patients With and Without Implants

5.1 Introduction

5.1.1 The arginine catabolic mobile element

The arginine catabolic mobile element (ACME) was first described as a novel MGE harboured by the *S. aureus* strain USA300 (54) and is thought to increase competitive fitness by contributing to transmission, colonisation and persistence. USA300 is an endemic strain of CA-MRSA that has been responsible for a wave of infections ranging from skin and soft tissue infections to septicaemia and necrotising pneumonia in the United States and sporadically in other countries since its emergence in the early 2000s (54, 58, 82). Since the first description, ACME has been identified in *S. epidermidis*, *S. haemolyticus* and *S. capitis* species and reportedly ranges in size from 30-34 kb (54, 85, 170). The prevalence of ACME in *S. epidermidis* isolates recovered from cases of carriage and disease has previously been reported to range between 45.8% and 67.9% (54, 154, 169, 170) and this species is considered the true origin of ACME based on in-depth phylogenetic analyses (54, 82, 83, 154, 171).

Similar to SCC*mec*, ACME integrates into the chromosomal *orfX* locus using the *attB* attachment site (54) and is flanked at the 5′ and 3′ ends by direct repeat sequences (DRs). Several previous investigations have revealed the presence of ACME integrated at *orfX* in *S. aureus* and *S. epidermidis* isolates downstream of SCC*mec*, and other SCC elements (54, 154).

5.1.2 The structure and functions of ACME

ACME is typically characterised according to the presence or absence of two operons; the *arc* operon (*arcC/B/D/A/R)* encoding an arginine deaminase pathway and the *opp3* operon (*opp3A/B/C/D/E)* encoding an oligopeptide permease ABC transporter (38, 54). To date, three distinct ACME types have been described in *S. aureus*, *S. epidermidis* and *S. haemolyticus* based on the presence of both the *arc* and *opp3* operons (type I), *arc* operon only (type II) and the *opp*3 operon only (type III) (Fig. 5.1). The ACME-*arc* has been detected in *S. haemolyticus*, however the presence or absence of the *opp3* operon was not determined and therefore the presence of ACME types I and III has not been confirmed in this species (85). In addition to the ACME *arc* and *opp*3 gene clusters, these staphylococci also harbour native chromosomal *arc*, *opp*1 and *opp*2 operons, which differ in sequence, orientation, gene order and expression levels to those contained within ACME (54, 83).

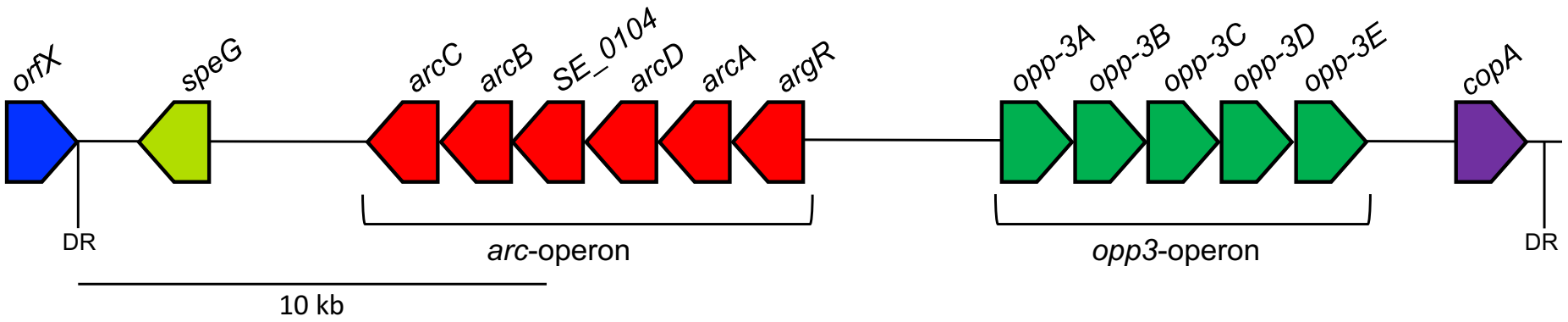


Figure 5.1 Schematic representation of the structural organisation of ACME type I. The mobile element ACME can harbour either both the *arc*-operon and *opp3*-operon (ACME type I), the *arc*-operon (ACME type II) only or the *opp3*-operon (ACME type III) only. ACME integrates at the chromosomal *orfX* locus in staphylococci and the typical location of the 5′ and 3′ direct repeat (DR) sequences that can vary in nucleotide sequence and frequency among ACME types are indicated. The *speG* gene has previously been identified alongside ACME and encodes a spermidine acetyltransferase. The *copA* gene is typically located near the 3′ end of ACME and is followed by the terminal DRs for the element. The size of ACME ranges from 30- 34 kb (54).

5.1.2.1 Genetic diversity of ACME

The prevalence and genetic diversity of ACME harboured by *S. aureus* and *S. epidermidis* has been investigated based on PCR scanning and sequencing of amplimers (38, 85, 154, 170–172). Miragaia *et al.* identified ACME subtypes based on PCR band patterns and identified a new ACME subtype (ACME-I.02) which was revealed to be highly prevalent in *S. epidermidis* isolates and that differed from ACME I-.01 (present in USA300) by 11 nucleotides (154). The *arcA* gene is reported to be highly conserved in ACME types I and II independent of the staphylococcal host species and STs (85, 169, 170). In contrast, the *opp3* operon shows higher levels of sequence diversity, in one investigation of 21 ACME type I or III MRSE isolates 11 distinct allotypes were reported (169). ACME is predominantly associated with MRSA CC8:ST8 USA300 isolates harbouring SCC*mec* IVa (100%), however ACME is highly prevalent in MRSE strains (67%), irrespective of SCC*mec* type (169, 172).

ACME types I and II recovered from different species of staphylococci such as *S. aureus*, *S. epidermidis* and *S. hamolyticus* have been characterised extensively by PCR amplification and subsequent sequencing (38, 54, 85, 154, 169), and more recently by WGS (171, 173, 174). Many of these sequences are publicly available in the GenBank database (45, 54, 173). However, to date ACME type III has been detected solely by PCR-based methods using specific primers directed towards the *arc*- and *opp3*-operons (85, 169, 170, 172). In this regard, identification of the presence of type III ACME in test isolates has been based on the detection of an *opp3* PCR amplimer and by the failure to amplify *arc* amplimers (38). To date there are no complete ACME type III sequences in the GenBank database.

5.1.2.2 The speG *gene*

Arginine-based polyamines such as spermidine and spermine play a fundamental role in cell growth, proliferation and in synthesis of nucleic acids and proteins (175). The quantity of polyamines dramatically increases in tissues undergoing rapid cell proliferation, such as during wound healing, inflammation and tumorigenesis (84, 175). Joshi *et al*. reported that exogenous polyamines are bactericidal to *S. aureus* and many Gram-positive bacteria and that staphylococci such as *S. aureus* and *S. epidermidis* lack the ability to produce polyamines *de novo* (84).

The *speG* gene has been identified alongside ACME, most notably in the USA300 lineage of MRSA (54, 82, 84) and is expressed constitutively. The gene encodes spermidine acetyltransferase (SpeG) which enhances the ability of staphylococci to colonise and persist in wounds and infection sites by mitigating the lethal effects of the polyamines produced during the healing and inflammation process (82–84). In addition, *speG* has been associated with increased biofilm formation and adhesion and decreased antibiotic susceptibility in *S. aureus* (82).

5.1.2.3 The arc-*operon*

In contrast to the native *arc* genes, the ACME-*arc* gene cluster (*arcC/B/D/A/R*) is constitutively expressed irrespective of glucose and oxygen levels in the surrounding environment. The operon encodes an arginine deaminase pathway (54, 83) responsible for the catabolism of extracellular L-arginine upon which polyamines are based, resulting in the formation of ornithine, ammonia, ATP, and CO_2 (83, 84). Ammonia production from this pathway also functions in regulation of the internal pH of staphylococci aiding strain survival in the presence of lactic acid on human skin (83).

5.1.2.4 The opp3-*operon*

The *opp3*-operon (*opp*3A/B/C/D/E) encodes an oligopeptide permease ABC transporter (38, 54). Multiple different *opp*-operons have been identified in both Gram-positive and Gram-negative bacterial species and are reported to play a role in peptide nutrient uptake, attachment to host cells, cell wall metabolism, resistance to antimicrobial peptides and chemotaxis (68, 176). A precise role for the *opp3*-operon in isolates harbouring ACME types I and III is unknown, but it may possibly play a role in pathogeneses similar to *opp*-operons in other species (86, 176).

5.1.3 Genomic sequencing technologies

5.1.3.1 First generation sequencing

More commonly known as Sanger sequencing, this method uses single stranded DNA (ssDNA), specific primers and dideoxynucleotides ddNTPs to produce short lengths (approx. 800 bp) of fluorescently labelled fragments that are electrophoresed to generate a chromatogram where each coloured peak corresponds to a different nucleotide base. Sanger sequencing is cheaper and more readily accessible than more recent sequencing

technologies, however it is unsuitable for sequencing large fragments of DNA required for WGS.

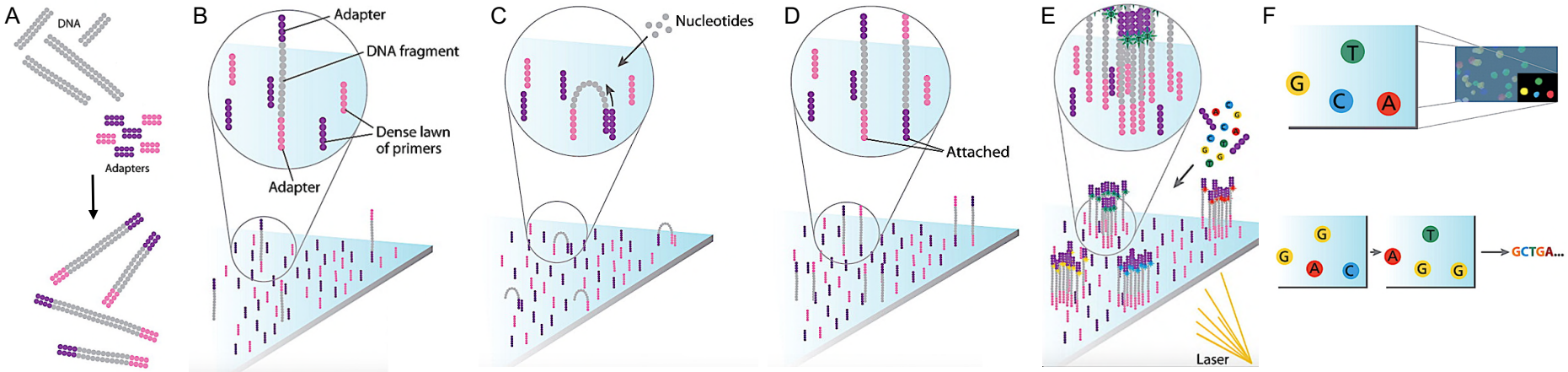
5.1.3.2 Second generation sequencing

This technology (most commonly based on Illumina-based platforms) refers to the high-throughput WGS techniques that have transformed genomic research in the last decade. The cost and time constraints of WGS based on second generation sequencing have dramatically decreased over the years, leading to an increase in the accessibility of the technology for research laboratories. Second generation sequencing platforms produce a large volume of sequence reads with extremely high levels of coverage (up to 500X) across the entire genome, ensuring the sequences are reliable and of a high quality. Despite these advances, there are still some limitations to second generation sequencing, namely the associated costs, the expertise required for computation of results and bioinformatics analysis, and its current general lack of availability for routine clinical use.

Most commercially available second generation sequencing platforms such as those from Illumina require DNA to be processed into libraries prior to sequencing (177, 178). During this step, high molecular weight sample DNA is sheared to a specific size range to yield template DNA (178, 179). Template DNA has adapters ligated to either end (Fig. 5.2) followed by a second set of motifs which contain unique primer binding sites, index reads, and flow cell adaptors.

Sequencing takes place in a flow cell which is lawned with primers that complement the adapters ligated to the template DNA. The template DNA is denatured to ssDNA and hybridised to the primers on the inside channels of the flow cell (Fig. 5.2) (177, 179). The initial ssDNA template is copied and then removed from the flow cell and the copy of the ssDNA is used to create a cluster of identical template sequences using isothermal amplification (bridge amplification) (Fig. 5.2) (179). Fluorescently labelled dNTPs are incorporated sequentially into the newly synthesised nucleotide chain to complement the template strand; a pause is required between each addition [sequencing by synthesis (SBS)] (Fig. 5.2). Each base emits a characteristic fluorescence signal that is recorded by the sequencer, which can then be used to determine the nucleotide sequence (Fig. 5.2) (177).

Second generation WGS methods such as Illumina platforms have revolutionised genome sequencing, however the requirements of DNA amplification,



Adaptors are ligated to the fragmented input template DNA

ssDNA with adaptors bind to complementary oligonucleotides on the surface of the flow cell.

- The hybridized fragment folds over and the second adapter sequence bind to its complementary oligonucleotide on the flow cell.
- Polymerase incorporates nucleotides to build a double-stranded bridge.

- The double-stranded molecule is denatured, creating two single-stranded molecules.
- The bridging process is continuously repeated. The reverse strands are washed away to create clusters of ssDNA

- Fluorescently labelled nucleotides, which are blocked at the 3'-end, compete for addition to the growing nucleotide chain.
- Nucleotides are only incorporated into the extending strand if they are complementary to the template strand.
- A laser excites each nucleotide added.

- A characteristic fluorescence, associated with the nucleotide base added to the nucleotide chain is emitted.
- The 3'-end of the strand is unblocked and the next nucleotide is incorporated into the chain.
- The sequencer records all data and sequence reads are generated.

Figure 5.2 Schematic of the Illumina Miseq sequencing technology**. (**A) Template DNA is fragmented and specialised adaptors are ligated to either end of each fragment. (B) Template DNA with adaptors binds to primers inside the flow cell channels. (C) Unlabelled nucleotides and DNA polymerase enzymes are added to the flow cell and (D) bridge amplification occurs to create millions of clusters on the flow cell. (E) The template DNA fragments in each cluster are sequenced by the sequencing by synthesis (SBS) technology. (F) Fluorescent signals are recorded and stored in the Illumina BaseSpace cloud. Figure adapted from Mardis, 2008 (177).

high probability that template strands re-hybridise rather than annealing to a new primer, and error accumulation within a cluster can impact sequence quality (179). Assembly of the short reads produced by SBS requires either a previously sequenced genome of the same species to be used as a scaffold or advanced algorithms for *de novo* assembly. For both methods, genomic structures inferred by WGS should still be confirmed by PCR amplification.

5.1.3.3 Third generation sequencing

Third generation sequencing is based on single molecule real-time (SMRT) technology, which can detect the incorporation of a single fluorescently labelled nucleotide, eliminating the requirement for library amplification (179, 180). SMRT has a higher single pass error rate caused predominantly by insertion and deletion errors, however these errors can be ameliorated using consensus sequence calls from multiple sequence reads covering each base position (179). Sequencing takes place on a SMRT cell covered with zero mode waveguide (ZMW) wells; each well contains a sequencing primer, DNA polymerase, and the template sequence (179). The extending nucleotides florescent signal is recorded in real time using powerful optical systems. On average sequence reads produced by SMRT sequencing are 10,000 bp in length, in contrast to second generation which produces average read lengths of 500 bp. SMRT sequences are primarily assembled *de novo* using the hierarchical genome-assembly process (HGAP) (181) and is usually performed commercially due to the huge costs associated with the technology and software requirements.

5.1.3.4 Fourth generation sequencing

New sequencing technologies are constantly being devised and developed. For example, fourth generation sequencing technologies such as Nanopore sequencing uses electrophoresis to sequence DNA and no DNA amplification or fluorescence labelling is required (182). This technology has been developed into a portable device that can connect to laptops and computers via USB, however high sequencing error rates must be overcome before this technology supersedes third generation sequencing.

5.1.4 Objectives

The aims of this part of the present study were:

- To determine the prevalence of each ACME type among *S. epidermidis* and *S. aureus* isolates recovered from distinct anatomical sites in the oral cavity during health and disease by multiplex PCR targeting the *arc* and *opp* operons.
- To undertake a WGS-based comparative molecular characterisation of the ACMEs harboured by a selection of *S. epidermidis* isolates.
- Utilise WGS-based technology to undertake a genomic characterisation of ACME type III for the first time.

5.2 Materials and methods

5.2.1 Detection of ACME by multiplex PCR

Staphylococcus epidermidis and *S. aureus* isolates subjected to DNA microarray analysis were also screened by multiplex PCR to detect the presence of ACME (isolates previously described in Chapter 4, Section 4.2.1). In total 197 isolates (143 *S. epidermidis* & 54 *S. aureus*) were analysed. Amplifications were performed using 30 ng of template *S. epidermidis* or *S. aureus* DNA in a 20 µl reaction volume consisting of 1 µM primers targeting the ACME-*arcA* (54) and primers targeting ACME-*opp3B* (ACME-*opp3B*_F 5′-GGATTCGCCCAAGTGATGACC-3′ and ACME-*opp3B*_R 5′-GACTGCTGGGTATGACGT-3′) genes, 1.5 mM $MgCl_2$, 5X green GoTaq flexi buffer, sterile water, 200 µM dNTPs and 2.5 U of Go Taq DNA polymerase. The *S. aureus* strain M05/0060 (USA300-ACME type I harbouring both ACME-*arc* and *opp3* genes) was used as a positive multiplex PCR control (183). The cycling conditions consisted of denaturation at 94°C for 1 min followed by 35 cycles of 30 s at 94°C, 30 s at 56°C, 45 s at 72°C, and a final elongation at 72°C for 2 min. The amplified PCR products were separated by conventional agarose gel electrophoresis in 2% (w/v) horizontal agarose gels as described in Chapter 2, Section 2.1.3.

5.2.2 Characterisation of ACME using whole genome sequencing (WGS)

In total 29 *S. epidermidis* isolates recovered from patients with periodontal disease, healthy patients with implants and orally healthy controls underwent WGS analysis (Table 5.1). Isolates were selected based on the patient group and sample site they were recovered from and the ACME type they harboured, to reflect the population of *S. epidermidis* isolates harbouring ACME detected in the present study. Trafficking of ACME between the nares and the oral cavity was investigated by the inclusion of three isolates recovered from the nares of three distinct participants who also had an isolate harbouring ACME recovered from their oral cavity undergo WGS analysis. Genomic DNA was extracted for WGS using the Qiagen DNeasy® Blood and Tissue kit as described in Chapter 2, Section 2.1.4.1. DNA concentrations were measured using a Qubit fluorometer (original model) (Thermo Scientific). Libraries were prepared using the Nextera XT library preparation reagents (Illumina, Essex, UK) and sequenced using a MiSeq desktop sequencer (Illumina).

Table 5.1 Summary of quality control metrics from each WGS run

		MiSeq quality control metrics		Read assembly quality control	
WGS run	**ACME type**	**Average %Q30[ab]**	**%PF[c]**	**N50 value[d]**	**Number of contigs[e]**
WGS run 1		79.21	54.96		
Run 1 isolates					
P11NS1	II			99354	Excluded[f]
P11OR1	II			132202	2
P11PPH21	II			73191	3
P11PPP12	II			170683	4
P14NS2	II			153789	2
P14OR1	I			83088	4
P14PPP2	II			91200	4
P14PPP3	I			93599	Excluded[f]
P8NS2	I			62476	Excluded[f]
P8OR3	II			138600	2
P9PPH12	II			102690	2
P9PPHI1	II			110857	2
P9OR1	II			123889	3
WGS run 2		82.88	90.27		
Run 2 isolates					
P19PPP1	II		6.00	163465	4
I23OR2	II		6.13	253554	3
I14OR4	II		5.02	169805	4
I12OR1	I		6.01	165364	4
I9OR1	II		5.63	178731	3
I7OR2	II		28.32	87270	Excluded[f]
201OR2	II		5.70	126373	2
200OR2	II		6.00	120908	2
33BR	II		6.83	87919	6
32BR	II		3.75	97820	2
120PPC	II		6.19	169765	4
218PP361	II		5.57	180840	1
217PP362	II		5.99	154931	4
WGS run 3		86.4	91.27		
Run 3 isolates					
P16OR1	III		7.03	NA	N/A
204OR1	III		6.89	NA	N/A
I11OR1	III		7.78	NA	N/A

[a]Values for these quality control metrics were not available for individual isolates on a WGS run.

[b]The average %Q30 value is an accuracy metric that represents the probability that the percentage of base calls is 99.9% or higher i.e. if the %Q30 value was 80, that means 80% of the base calls were accurately called 99.9% or higher.

[c]The percentage reads identified (%PF) indicates the percentage of clusters that pass the filter for the sample; the filter removes any clusters that have too high an intensity for bases other than the base called. The high intensity of bases other than the base called is most likely due to another cluster being in too close a proximity. A high %PF value is desirable.

[d]The N50 value of each isolate indicates that half the contigs of said isolate are equal to or longer in length than the N50 value.

[e]The number of contigs ACME sequences were identified on.
[f]Isolates were excluded due to poor contig lengths, or if the contig order across the ACME composite could not be confirmed by PCR.
Abbreviation: NA; not available, N/A; not applicable

5.2.3 Assemblies and bioinformatics analysis

The sequencing reads from each isolate were quality checked (Table 5.1) and then aligned to previously sequenced ACMEs and/or SCC*mec* elements in *S. epidermidis* and *S. aureus* downloaded from GenBank using Burrows-Wheeler aligner (BWA) (184). The genomic region harbouring ACME in the reference genome of *S. aureus* strain FPR3757 (GenBank accession number: CP000255.1) was used as a scaffold for alignment of reads from isolates harbouring ACME I investigated in the present study. Reads from ACME type II isolates were aligned to the genomic region harbouring ACME in the reference genome of *S. epidermidis* strain ATCC12228 (GenBank accession number: AE015929). As there was no reference ACME type III sequence available in GenBank to use as a scaffold for ACME type III reads, *de-novo* assemblies were carried out for each of the three ACME III isolates using SPAdes version 3.6 (http://bioinf.spbau.ru/en/spades). Contigs were aligned to reference ACME or SCC*mec* associated DNA sequences using BWA. Any contigs identified as harbouring SCC*mec*–related or ACME-related sequences were selected and annotated using the BioNumerics software version 7.6 annotation tool (Applied Maths, Sint-Martens-Latem, Belgium), Artemis genome browser and annotation tool (185) and BLAST software (https://blast.ncbi.nlm.nih.gov/Blast). BioNumerics and BLAST software packages were also used to predict open reading frames (ORFs). ORFs were aligned to best fit matches in GenBank and the positions of each start and stop codons were confirmed, or altered if required.

5.2.4 Contig gap closure and confirmation of ACME structure

Any gaps between ACME sequence contigs were closed by PCR using custom primers designed based on surrounding contigs followed by amplimer sequencing using Sanger sequencing (Source BioScience) (Table 5.2). Sequence data were analysed and overlapping sequences were integrated into the contig sequence using BioNumerics.

5.2.5 SMRT sequencing

Due to the lack of an appropriate ACME type III reference sequence to use as a scaffold, three *S. epidermidis* isolates (204OR1, P16OR1 and I11OR1) harbouring ACME type III were further subjected to WGS using SMRT sequencing followed by

Table 5.2 Primers used to confirm the contig order of ACME in isolates investigated

Isolate	Primer name	Primer sequence (5′ – 3′)	Amplimer size (bp)
P8OR3	P8C11F2	CGTAGATCTGATAGACTGACC	1500
	200C18iR	CTATTTTACCGTCTAAAGCG	
P9OR1	P9OR1_C31F	GAATTTATGGTTCTGTTGC	1000
	P9OR1_C20RRC	CCACAGACACTTCATCG	
	P9PPH12_C17F	GCAAAAGAGCGATAATG	2500
	200C18iR	CTATTTTACCGTCTAAAGCG	
P9PPH12	P9PPH12_C17F	GCAAAAGAGCGATAATG	2500
	200C18iR	CTATTTTACCGTCTAAAGCG	
P9PPHI1	P9HIC14F2	CATCAATTGCAAAAGAGC	1500
	200C18iR	CTATTTTACCGTCTAAAGCG	
P11OR1	P9PPH12_C17F	GCAAAAGAGCGATAATG	2500
	200C18iR	CTATTTTACCGTCTAAAGCG	
P11PPH21	P11PPH21_C22F	TCGGAGAATGATAAAGG	1500
	200C18iR	CTATTTTACCGTCTAAAGCG	
	P11PPH21_C22F	TCGGAGAATGATAAAGG	800
	P11PPH21_C48R	GATTAGGGGACGCTTTAG	
	P11PPH21C48F2	GCGTAATAATAGCGAAATG	800
	P11PPH21_C23RRC	CCATCGTATTTGAAATCG	
P11PPP12	P11PPP12C6F2	GAACTGCAGCTATTGTTTC	800
	P11PPP12C21R2	CAACAGAACCGTATTATGG	
	P11PPP12C21F2	CATTTAAACTGCCGAGTTAG	700
	P11PPP12_C27RRC	CTATAATCCTAGGGCACTG	
	P11PPP12C27F	GTTCGGTGTGCTCATATC	700
	P11PPP12C18R	CTGAATCTTCATCGATGAC	
P14NS2	P14NS2_C30F	GATCTTAAGAGACCTGCGG	1500
	P14NS2_C15R	GCAAAGAAGCAGTCTCG	
P14OR1	P14OR1_C12F	CTTTTACCCAACGACTTG	500
	P14OR1C40R	GTCACCTAATATTGCTCTAAGG	
	P14OR1_C40F	GGCATTCTTAGCTATCCAG	1500
	P14OR1_C43RRC	GATTAGGGGACGCTTTAG	
	P14OR1_C43F	GTAATAGGAGGCTGGTGTAG	4000
	P14OR1_C31RRC	CATTCTCCTCGCTTAATTC	
P14PPP2	P14PPP2_C14F	CACCATGTTCTAGGCTGC	800
	P14PPP2_C36iR	AAGTGGTACCAACAGTAG	
	P14PPP2_C36iF	CAGATCATGAAGCTAAGGCA	1000
	P14PPP2_C37RRC	GGGACGCTTTAGACGG	
	P14PPP2_C37F	GAATGGCTTGAAACGG	1500
	P14PPP2_C52R	AGTCAGCTTTACTCTCACCT	
	P14PPP2_C52F	TTGTACGATTATCAAATGTC	1100
	P14PPP2_C26R2	CACATTTCAAAGTAGGTCTAG	

Continued overleaf

Table 5.2 continued. **Primers used to confirm the contig order of ACME in isolates investigated**

Isolate	Primer name	Primer sequence (5′ – 3′)	Amplimer size (bp)
P16OR1	204-5	ATCTTTGGAACCTGGACA	5000
	204-6	CTGTTCTACTGGAGTATGTGGTC	
	204-7	TAGGTTCTCGTGCCATTG	3000
	204-8	CTCATTACGGTCGCTTAGT	
	204-9	AGATGATGAGATGGCACG	2500
	204-10	CTAAAGCCGTATCCTAAGTTG	
	P16-1F	GTCCACCTTTTTATTAATAGGG	2300
	P16-2R	GGTCTTTTAGTTGATTCAATTC	
	P16-3F	GATGGAAGTCACAGTATTCTTTG	6000
	P16-4R	CTTTTATCGCCACTGATGG	
P19PPP1	P19C6F2	CCCTTCACGTTGACC	1000
	P19C29R	CACTGCCAAAAAACATTG	
	P19C29F	GTCCTTCCTCAGTTTTACC	1500
	200C18iR	CTATTTTACCGTCTAAAGCG	
	P19C30F	GTTATTGAATGGCTTGAAAC	2000
	P19C19R	GTTGCTAATGCTAACCTTG	
I9OR1	I9C5iF	GTTGGGATGCCTCAG	1000
	200C18iR	CTATTTTACCGTCTAAAGCG	
	I9C22iF	CATGGGGCAAAGAATATAC	1500
	I9C20iR	GAGTGTATTGTCATGCGATAG	
I11OR1	I11-1 F	GGTAAATACGTAATATCGGTTG	2500
	I11-2 R	GGGTGCGAGATGAATTAC	
	I11-3 F	CCACACACTTTAGCAGAATC	3000
	I11-4 R	CTCTTATCGCCACTGATG	
	204-5	ATCTTTGGAACCTGGACA	5000
	204-6	CTGTTCTACTGGAGTATGTGGTC	
	204-7	TAGGTTCTCGTGCCATTG	3000
	204-8	CTCATTACGGTCGCTTAGT	
	204-9	AGATGATGAGATGGCACG	2500
	204-10	CTAAAGCCGTATCCTAAGTTG	
I12OR1	I12C7F	GTTCCATCGCCTACAAC	1000
	I12C21iR	GAATGTAGCTATTGTGGCG	
	I12C21iF	GGGCATTCTTAGCTATCC	1100
	200C18iR	CTATTTTACCGTCTAAAGCG	
	I12C24iF	GGGAATACTAACACAAAGGC	1500
	I12C19R	CGTAAGAAAGAGCCTAGGAC	
I14OR4	I14C6F	GGGTCTACTCTATTATTTGGG	1000
	I14C16R	CAAAAGAAAAGAGCACAGAC	
	I14C16F2	GTTATGAGGTTGGGATGC	1500
	I14C19R2	GTTCAGTGCCCTAGGATTATAG	
	I14C19F	CATTAAAGGACAAATCATTAGTG	1000
	I14C17R	CAATTTGCTTTTCTAGACCTAC	

Continued overleaf

Table 5.2 continued. **Primers used to confirm the contig order of ACME in isolates investigated**

Isolate	Primer name	Primer sequence (5′ – 3′)	Amplimer size (bp)
I23OR2	orfXF	CTTACAACGCAGCAATTAC	1300
	I23C17R	CCAGAGGTTGATTCCG	
	I23C17F	GAAAACTTGGTGGTGATG	700
	200C18iR	CTATTTTACCGTCTAAAGCG	
120PPC	120C6iF	GAGAGGCGAAGCATATC	1100
	120C17R	CATAGCGAGGATAATATTGTG	
	120C17F	GATCTGATAGACTGACCCC	900
	200C18iR	CTATTTTACCGTCTAAAGCG	
	120C20F	CTACATCTACATCAGCATGG	1400
	120C18iR	GTAGGAAGACGAGGCTG	
200OR2	200C16F	CCATCAATTGCAAAAGAG	2500
	200C18iR	CTATTTTACCGTCTAAAGCG	
201OR2	201C5F	GTATAAAATTAAACGAAAGCC	900
	201C18iR	CCTATTTTACCGTCTAAAGC	
204OR1	204-1	CCGTTAAGGATTCATAAGGC	1500
	204-2	GCAGTCCTGTTGTTACAGTTG	
	204-3	ATGCAGAAACGTTCAGAGA	4000
	204-4	CTTCTGACAGCTCTTCTATTCC	
	204-5	ATCTTTGGAACCTGGACA	5000
	204-6	CTGTTCTACTGGAGTATGTGGTC	
	204-7	TAGGTTCTCGTGCCATTG	3000
	204-8	CTCATTACGGTCGCTTAGT	
	204-9	AGATGATGAGATGGCACG	2500
	204-10	CTAAAGCCGTATCCTAAGTTG	
217PP362	orfXF2	CTTACAACGCAGCAACTATG	1600
	217C18R3	CAATTGTTGCTGTTTAGTCG	
	217C18F	GCAATAATGTAGACTGACCC	1000
	200C18iR	CTATTTTACCGTCTAAAGCG	
32BR	32C19iF	CAATTGCAAAAGAGCG	2500
	32C22R	CATACTTCCTTTAGCAAATTG	
33BR	33C3iF2	GTTATGAAGCTAGATTAATGGC	2100
	33C38R	GACACAGCCCAAGAAAG	
	33C38F	GACTGACCCCAATTAGTG	1100
	33C47R	CTAATCCTGCTAGAGATGTAATC	
	33C47F	CTCCAAAATGTCTTGCC	1000
	33C56R2	GCAATATCATTGATAAGGGG	
	33C56F	GTTAAATGACCAACAAATTTC	1100
	33C51R2	GTGCAAAGTGTCATGACTAC	
	33C51F	GGGGCAAAGAATATACG	2000
	33C28R	CTAATGTAGGACGTGGAGAC	

HGAP.3 analysis (The Genome Analysis Centre (TGAC), Norwich, UK). Genomic DNA was extracted using the Qiagen DNeasy® Blood and Tissue kit as described in Chapter 2, Section 2.1.4.1. DNA concentration and quality was checked using a Nanodrop 2000c spectrophotometer (described in Chapter 2, Section 2.1.5) to ensure it conformed to TGAC guidelines. Contigs generated from SMRT sequencing were analysed and annotated using the BioNumerics version 7.6 annotation tool (Applied Maths) and using the genome browser Artemis (185). For further confirmation of ACME III structure, the SMRT sequence generated from each isolate was also used as scaffold reference genome for alignment with the corresponding isolates previously obtained MiSeq reads.

5.2.6 Identification of direct repeats

Direct repeat sequences present in the ACME types investigated were identified by searching out previously reported DRs identified in ACME and SCC*mec* elements (38, 173) from within the WGS data using BioNumerics and Artemis software, allowing for up to six nucleotide mismatches.

5.2.7 MLST using WGS data

The sequence fragments of seven internal housekeeping genes (*arcC*, *aroE*, *gtr*, *mutS*, *pry*, *tpi* and *yqil*) (111) used for MLST analysis of *S. epidermidis* were extracted from the WGS data and STs were identified using the *S. epidermidis* MLST website (https://pubmlst.org/sepidermidis/). This typing system was previously developed for *S. epidermidis* based on PCR amplification of the seven housekeeping genes and Sanger-based sequencing of the amplimer (111).

5.2.8 Statistical analysis

To determine if the differences in the prevalence of ACME were significant between different sample sites or patient groups, two-tailed Fisher's exact tests were utilised. All calculations were carried out using GraphPad QuickCalcs (http://www.graphpad.com/quickcalcs/contingency1/). A p value < 0.05 was deemed statistically significant.

5.3 Results

5.3.1 Prevalence of ACME based on multiplex PCR

The prevalence of *S. epidermidis* and *S. aureus* recovered from the different patient and participant groups, and distinct anatomical sites has been described in Chapter 3, Section 3.3.1. Selected *S. epidermidis* and *S. aureus* isolates chosen as representative isolates recovered from individual study participants and distinct anatomical sites were screened for the presence of ACME by multiplex PCR. Overall, ACME-*arc* or *opp3* genes (or both) indicating the presence of ACME were detected in 85/143 (59.4%) *S. epidermidis* isolates and 1/54 (1.9%) *S. aureus* isolates (Table 5.3).

5.3.1.1 Prevalence of ACME among S. epidermidis *isolates*

Staphylococcus epidermidis was highly prevalent in the oral cavities of patients with periodontal disease (18/20, 90%) and ACME was detected in *S. epidermidis* isolates recovered by oral rinse sampling from 12 of these (67%) patients (Table 5.3). *Staphylococcus epidermidis* was also recovered from periodontal pockets by Perio Paper™ sampling of 6/20 (30%) patients with periodontal disease and five (5/6, 83%) of these patients yielded *S. epidermidis* with ACME. Four patients yielded pairs of *S. epidermidis* isolates that harboured the same ACME type from both oral rinse samples and periodontal pockets. Four patients with periodontal disease harboured *S. epidermidis* in subgingival sites, and ACME was detected in all subgingival *S. epidermidis* isolates from these patients (Table 5.3). Patients P4 and P11 yielded *S. epidermidis* harbouring ACME type II from oral rinse samples, periodontal pockets and subgingival sites. Patient P9 yielded *S. epidermidis* harbouring ACME II from oral rinse samples and subgingival sites.

In total, 25/31 (80.6%) orally healthy patients with dental implants yielded *S. epidermidis* from the oral cavity by oral rinse sampling, and ACME was detected in isolates from 19 (76%) of these patients (Table 5.3). In addition, *S. epidermidis* isolates were recovered from subgingival sites of five healthy patients with dental implants (16.1%) and ACME was detected in isolates harboured by four (80%) of these patients (Table 5.3). Two orally healthy patients with dental implants (Patients I1 and I29) yielded pairs of isolates harbouring different ACME types from oral rinse samples and subgingival sites, and Patient I33 yielded the same type of ACME from both of these sites. Patient I27 yielded *S. epidermidis* harbouring ACME from subgingival sites only.

Table 5.3 Prevalence of ACME types I-III harboured by *S. epidermidis* and *S. aureus* isolates from distinct anatomical sites

Patients (*n*)	Sample site	Prevalence of staphylococcal species per patient (%)	Isolates examined (*n*)[a]	Prevalence of staphylococci harbouring ACME per patient (%)	ACME types identified (*n*= patients)					
					I	II	III	I & II	I & III	II & III
				S. epidermidis						
Periodontal disease (20)	OR	18/20 (90)	27	12/18 (67)	1	8	1	0	1	1
	PP	6/20 (30)	9	5/6 (83)	0	4	0	1	0	0
	SG	4/20 (20)	6	4/4 (100)	1	3	0	0	0	0
Healthy implants (31)	OR	25/31 (80.6)	39	19/25 (76)	3	11	2	3	0	0
	SG	5/31 (16.1)	9	4/5 (80)	1	1	1	1	0	0
Orally healthy (64)	OR	43/64 (67.2)	48	22/43 (51)	3	17	1	0	0	1
	SG	5/64 (7.8)	5	3/5 (60)	0	3	0	0	0	0
				S. aureus						
Periodontal disease (20)	OR	5/20 (25)	6	0/5 (0)	0	0	0	0	0	0
	PP	0/20 (0)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	SG	0/20 (0)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Healthy implants (31)	OR	15/31 (48.4)	16	0 (0)	0	0	0	0	0	0
	SG	4/31 (12.9)	7	0/4 (0)	0	0	0	0	0	0
Orally healthy (64)	OR	20/64 (31.3)	20	1/20 (5)	0	1	0	0	0	0
	SG	5/64 (7.8)	5	0/5 (0)	0	0	0	0	0	0

[a]The presence and types of ACME among isolates investigated was determined multiplex PCR using specific primers directed towards the ACME-*arc* and/or *opp3* operons.

Abbreviations: OR; oral rinse, PP; periodontal pocket; SG; subgingival site, N/A; not applicable

Of the 64 orally healthy participants investigated, *S. epidermidis* was recovered from the oral cavity by oral rinse sampling of 43 (67.2%) and ACME was detected in isolates from 22/43 (51%) of these (Table 5.3). In addition, *S. epidermidis* isolates were recovered from the subgingival sites of 5/64 (7.8%) orally healthy participants and ACME was detected in isolates harboured by three (60%) of these patients (Table 5.3). All three participants that yielded *S. epidermidis* isolates harbouring ACME from subgingival sites also yielded isolates harbouring the same types of ACME from oral rinse samples.

Overall ACME was detected in *S. epidermidis* isolates recovered from the oral cavity by oral rinse sampling of 61.6% (53/86) of individuals across the three participant groups that harboured *S. epidermidis* in their oral rinse samples. This correlates with previous studies that investigated the prevalence of ACME in *S. epidermidis* recovered from clinical or nasal samples (154, 169, 170). The highest prevalence of ACME-positive *S. epidermidis* isolates was detected in oral rinse samples from healthy patients with implants (76%, 19/25); this prevalence was significantly higher (P = 0.016) compared to isolates recovered from oral rinse samples of orally healthy participants 51% (22/43). Furthermore, the prevalence of ACME was higher (although not significantly), among *S. epidermidis* isolates recovered from subgingival sites (100%) or periodontal pockets (83%) of patients with periodontal disease, subgingival sites of healthy patients with implants (80%) and subgingival sites of orally healthy participants (60%) compared to the isolates recovered from the oral rinse samples in these groups (51-76%) (Table 5.3).

The prevalence of *S. epidermidis* harbouring ACME was highest in isolates recovered from subgingival sites of patients with periodontal disease (4/4, 100%), followed by isolates recovered from periodontal pockets of the same patient group (5/6, 83%) (Table 5.3). The prevalence of *S. epidermidis* harbouring ACME also very high from subgingival sites of healthy patients with implants (4/5, 80%) (Table 5.3).

The prevalence of ACME was lowest in isolates recovered from subgingival sites of orally healthy participants (3/5, 60%). Patients with periodontal disease yielded significantly more (P = 0.017) isolates harbouring ACME from periodontal pockets than subgingival sites of orally healthy participants.

5.3.1.2 Prevalence of ACME among S. aureus *isolates*

Interestingly, ACME was not detected in any *S. aureus* isolates recovered from the oral cavities, periodontal pockets or subgingival sites of patients with periodontal disease or healthy patients with implants (Table 5.3).

One MSSA isolate harbouring ACME was recovered from an oral rinse sample of an orally healthy participant (1/20, 5%) (Table 5.3). However, *S. aureus* was not recovered from any subgingival sites investigated from the same participant.

5.3.1.3 Prevalence of ACME type I

ACME type I (harbouring both the ACME-*arc* and *opp3* operons) was detected in two *S. epidermidis* isolates recovered from the oral cavities of two distinct patients with periodontal disease (2/12, 16.7%), in one of these patients (P10) ACME I was detected alongside another isolate harbouring a ACME type III in the oral cavity (Table 5.3). One isolate harbouring ACME I was recovered from a periodontal pocket (Patient P14), whereas a different periodontal pocket from the same patient yielded an ACME II isolate. Patient P22 was the only patient with periodontal disease to yield a *S. epidermidis* isolate harbouring ACME I from a subgingival site.

Six healthy patients with implants (6/19, 31.6%) yielded *S. epidermidis* harbouring ACME type I from oral rinse samples (Table 5.3); in three of these patients (I2, I12, I34) ACME I was detected alongside other *S. epidermidis* isolates harbouring different ACME types (Table 5.3). Isolates harbouring ACME type I were recovered from the subgingival sites of two distinct healthy patients with implants, one of whom (Patient I29) also harboured *S. epidermidis* with a different ACME type in another subgingival site (Table 5.3). Patient I29 yielded a pair of isolates harbouring ACME type I from the oral rinse sample and a subgingival site.

In total, isolates harbouring ACME type I were recovered from the oral rinse samples of three orally healthy participants (3/22, 13.6%), but ACME type I was not detected in isolates recovered from the subgingival sites of orally healthy participants (Table 5.3).

5.3.1.4 Prevalence of ACME type II

ACME type II (harbouring the ACME-*arc* operon and lacking *opp3*) was the most prevalent type identified in isolates recovered from the oral rinse samples (n=41), periodontal pockets (n=5) and subgingival sites (n=8) of each participant group (Table

5.3). This ACME type was also detected in the only *S. aureus* isolate harbouring ACME.

ACME type II was predominant in *S. epidermidis* isolates from oral rinse samples of patients with periodontal disease (9/12, 75%) (Table 5.3), and was the only ACME type detected in isolates harboured by eight of these patients. The remaining patient (P21) harboured a pair of isolates with distinct ACME types (ACME II and III) (Table 5.3). ACME II was also detected in isolates from the periodontal pockets and subgingival sites of five and three patients in this group, respectively. The periodontal pockets of one of these patients (P14) yielded isolates with distinct ACME types (ACME I and II) (Table 5.3). Pairs of *S. epidermidis* isolates each harbouring ACME type II were recovered from periodontal pockets and subgingival sites of Patients P4 and P11, ACME type II was also detected in isolates from the oral rinse samples of these patients.

Of the 19 healthy patients with implants who harboured *S. epidermidis* isolates with ACME from oral rinse samples, 14 (14/19, 73.7%) yielded *S. epidermidis* with ACME type II, either alone or in conjunction with isolates harbouring different ACME types (Table 5.3). Of the four patients who yielded *S. epidermidis* with ACME from subgingival sites, two (2/4, 50%), harboured isolates with ACME II (Patients I29 and I33; Table 5.3) The same ACME type was detected in an isolate from the oral rinse sample of Patient I33.

Of the 22 orally healthy participants who yielded *S. epidermidis* harbouring ACME from the oral rinse samples, 18 (18/22, 81.8%) yielded isolates harbouring ACME type II, one of whom (participant 204) yielded several isolates with distinct ACME types (Table 5.3). Three of these participants yielded *S. epidermidis* with ACME from subgingival sites, all of which were identified as harbouring isolates with ACME type II (Table 5.3).

5.3.1.5 Prevalence of ACME type III

Overall ACME type III (lacking ACME-*arc* but containing *opp3*) was the least prevalent ACME type identified in the present study, harboured by 9/142 (6.3%) *S. epidermidis* isolates investigated. This ACME type was detected in *S. epidermidis* isolates recovered from the oral cavities of two distinct patients with periodontal disease (2/12, 16.7%), but was not detected in any isolates from periodontal pockets or subgingival sites (Table 5.3) of this patient group.

Two healthy patients with implants (Patients I10 and I11) (2/19, 10.5%) yielded *S. epidermidis* isolates harbouring ACME III from oral rinse samples, ACME type III was detected in one isolate recovered from a subgingival site of a different patient (I1) with healthy implants (Table 5.3).

ACME type III was detected in *S. epidermidis* isolates recovered from the oral rinse samples of two distinct orally healthy participants (2/22, 9%), and was not detected in any subgingival sites in this patient group (Table 5.3).

5.3.1.6 Participants harbouring multiple ACME types in the same sample site

Two distinct ACME types were detected in pairs of *S. epidermidis* isolates recovered from oral rinse samples of patients with periodontal disease P10 (ACME types I and III) and P21 (ACME types II and III) (Table 5.3). *Staphylococcus epidermidis* was not recovered from periodontal pockets or subgingival sites in either of these patients. Two ACME types (I and II) were detected in isolates recovered from the periodontal pocket of a patient (P14) also (Table 5.3).

ACME types I and II were detected among three separate pairs of *S. epidermidis* isolates recovered from oral rinse samples of three distinct healthy patients (I2, I12, I34) with implants. One of these patients (I34) also yielded *S. epidermidis* lacking ACME from subgingival sites. A healthy patient with implants (I29) yielded separate isolates with different ACME types I and II from distinct subgingival sites and ACME type I was also detected in a separate isolate from the oral cavity of the same patient (Table 5.3).

Two types of ACME (II and III) were detected in distinct isolates recovered from the oral rinse sample of an orally healthy participant, however *S. epidermidis* was not recovered from the subgingival sites of this participant.

5.3.2 Genetic diversity of ACME in *S. epidermidis* isolates recovered from the three participant groups

5.3.2.1 Population of S. epidermidis *investigated*

In order to investigate the genetic diversity among distinct ACME types harboured by *S. epidermidis* isolates from the oral cavity, 29 ACME-positive isolates selected as representatives of each participant group, distinct anatomical site and ACME type (Table 5.1) were subjected to WGS. Unfortunately, the contig assemblies of isolates

P8NS2, P11NS1, P14PPP3, and I7OR2 could not be confirmed by contig gap closure PCRs using many different combinations of PCR conditions and oligonucleotide primers, and therefore these four isolates were excluded from further analysis.

Based on the WGS data, the following STs were identified among the 25 isolates investigated: ST73 (5/25, 20%), ST17 (4/25, 16%), ST59 (3/25, 12%), ST153 (3/25, 12%), ST329 (3/25, 12%), ST7 (1/25, 4%), ST89 (1/25, 4%), ST130 (1/25, 4%), ST173 (1/25, 4%), ST210 (1/25, 4%), ST14 (1/25, 4%), and ST672 (1/25, 4%) (Table 5.4). Three isolates recovered from the oral rinse sample and subgingival sites of Patient P9 were identified as the same ST (ST73) (Table 5.4) and both harboured ACME type II. Two different STs were detected among the three isolates recovered from three distinct sample locations in Patient P11, all of which harboured ACME type II. Interestingly, two distinct STs were detected among the three isolates recovered from Patient P14, however ST17 was detected in the isolates recovered from the oral rinse sample (ACME I) and subgingival sites (ACME II), and an isolate harbouring ACME II identified as ST14 was only detected in an isolate recovered from the nares (Table 5.4).

5.3.2.2 Genetic diversity of the ACME arc *and* opp3 *operons*

The percentage nucleotide identity between all *arc*-operons identified in the present study, both in type I and II ACMEs ranged from 99.11 - 100% (Table 5.5). The *arc*-operons detected in isolates P9OR1, P9PPH12, P9PPHI1, P11OR1, P11PPH21, 120PPC, I9OR1, I14OR4, and 33BR all exhibited 100% nucleotide identity to each other (Table 5.5). Three of the isolates were recovered from two distinct sites in Patient P9, two isolates were recovered from two distinct isolates in Patient P11, and the remaining four isolates were each recovered from Participants 120PPC and 33BR and, Patients I9 and I14 (Table 5.4). Interestingly the range of diversity (98.73 – 100%) among the *opp3*-operons detected in ACME types I and III was slightly higher (Table 5.6), however the *opp3* operons detected in two distinct isolates harbouring ACME III (P16OR1 and I11OR1) exhibited 100% nucleotide identity. Interestingly, the ACME III *opp3*-operon identified in the third ACME III isolate from this study shared greater nucleotide similarity with the *opp3*-operons detected in ACME type III (99.98%) rather than those detected in ACME type I (98.74-98.79%) (Table 5.6).

Table 5.4 ACME-positive *Staphylococcus epidermidis* isolates subjected to WGS analysis

Participant	Isolate name	Sample site	ACME type[a]	ACME size (kb)	Allelic profile	ST[b]	Direct repeat sequences (DRs)[c]
Isolates recovered from patients with periodontal disease (*n*= 14)							
P8	P8OR3	OR	II	54.2	1-1-1-2-2-1-25	210	DR_C, F, G
P9	P9OR1	OR	II	31.1	1-5-2-6-2-1-6	73	DR_C, D
P9	P9PPH12	SG	II	31.1	1-5-2-6-2-1-6	73	DR_C, D
P9	P9PPHI1	SG	II	31.1	1-5-2-6-2-1-6	73	DR_C, D
P11	P11OR1	OR	II	31.1	1-5-2-6-2-1-6	73	DR_C, D
P11	P11PPH21	SG	II	54.1	1-5-2-6-2-1-6	73	DR_C, D
P11	P11PPP12	PP	II	31.1	2-1-1-1-2-1-1	59	DR_B, C, G, H
P14	P14NS2	Nares	II	30.7	1-1-2-1-1-1-1	14	DR_C, D
P14	P14OR1	OR	I	54.3	1-1-6-2-2-1-1	17	DR_B, C, N, O
P14	P14PPP2	PP	II	53.9	1-1-6-2-2-1-1	17	DR_A, E
P16	P16OR1	OR	III	53.9	12-29-9-8-6-5-8	329	DR_C, G, K
P19	P19PPP1	PP	II	53.4	50-58-55-4-29-10-9	672	DR_A, A, E
Isolates recovered from patients with healthy implants (*n*= 5)							
I9	I9OR1	OR	II	67.8	2-1-6-2-2-1-1	153	DR_A, B, C
I11	I11OR1	OR	III	45.1	12-29-9-8-6-5-8	329	DR_B, C, G
I12	I12OR1	OR	I	39.8	1-1-1-2-4-1-1	7	DR_B, C
I14	I14OR4	OR	II	67.8	2-1-6-2-2-1-1	153	DR_A, B, C
I23	I23OR2	OR	II	48	1-1-2-1-2-1-1	89	DR_A, C, D
Isolates recovered from orally healthy controls (*n*= 8)							
120	120PPC	SG	II	67.8	2-1-6-2-2-1-1	153	DR_A, B, C
200	200OR2	OR	II	32	1-1-6-2-2-1-1	17	DR_C, D
201	201OR2	OR	II	27	2-1-1-1-2-1-1	59	DR_C, H
204	204OR1	OR	III	65.6	12-29-9-8-6-5-8	329	DR_B, C
217	217PP362	SG	II	74.6	2-1-1-1-2-1-1	59	DR_A, C, D, G, I, L
218	218PP361	SG	II	39	1-1-1-2-1-1-1	130	DR_B, C, G
32	32BR	OR	II	32	1-6-6-1-2-1-10	173	DR_C, D
33	33BR	OR	II	48.8	1-1-6-2-2-1-1	17	DR_B, C, G, N

[a]ACME types were determined according to the presence or absence of the *arc* and/or *opp* amplicons using multiplex PCR.

[b]The ST of each isolate was determined by uploading the sequence of seven housekeeping genes to the *S. epidermidis* MLST online database (https://pubmlst.org/sepidermidis/) (111).

[c]Each direct repeat sequence represented by a letter corresponds to a specific sequence listed in Table 5.7.

Abbreviations: ACME; arginine catabolic mobile element, ST; strain type, OR; oral rinse, PP; periodontal pocket, SG; subgingival site

Table 5.5 Pairwise comparison of nucleotide identity (%) calculated between all *arc*-operons identified in ACME types I and II[a]

Isolate	P14NS2	I23OR2	201OR2	P11PPP12	200, 32[b]	ATCC12228	218PP361	P19PPP1	FPR3757	P9, P11[c]	120, I9, I14[d]	P8OR3	33BR	217PP362	P14PPP2
P14NS2	100	99.92	99.92	99.94	99.84	99.9	99.81	99.79	99.82	99.14	99.14	99.14	99.14	99.94	99.76
I23OR2	99.92	100	99.97	99.98	99.85	99.89	99.85	99.84	99.87	99.17	99.17	99.19	99.17	99.98	99.81
201OR2	99.92	99.97	100	99.98	99.82	99.85	99.82	99.81	99.84	99.14	99.14	99.16	99.14	99.98	99.77
P11PPP12	99.94	99.98	99.98	100	99.84	99.87	99.84	99.82	99.85	99.16	99.16	99.17	99.16	100	99.79
200, 32[b]	99.84	99.85	99.82	99.84	100	99.9	99.84	99.79	99.82	99.06	99.06	99.17	99.06	99.84	99.76
ATCC12228	99.9	99.89	99.85	99.87	99.9	100	99.81	99.76	99.79	99.11	99.11	99.11	99.11	99.87	99.72
218PP361	99.81	99.85	99.82	99.84	99.84	99.81	100	99.85	99.89	99.13	99.13	99.17	99.13	99.84	99.82
P19PPP1	99.79	99.84	99.81	99.82	99.79	99.76	99.85	100	99.9	99.11	99.11	99.16	99.11	99.82	99.97
FPR3757	99.82	99.87	99.84	99.85	99.82	99.79	99.89	99.9	100	99.17	99.17	99.22	99.17	99.85	99.87
P9, P11[c]	99.14	99.17	99.14	99.16	99.06	99.11	99.13	99.11	99.17	100	100	99.74	100	99.16	99.08
120, I9, I14[d]	99.14	99.17	99.14	99.16	99.06	99.11	99.13	99.11	99.17	100	100	99.74	100	99.16	99.08
P8OR3	99.14	99.19	99.16	99.17	99.17	99.11	99.17	99.16	99.22	99.74	99.74	100	99.74	99.17	99.13
33BR	99.14	99.17	99.14	99.16	99.06	99.11	99.13	99.11	99.17	100	100	99.74	100	99.16	99.08
217PP362	99.94	99.98	99.98	100	99.84	99.87	99.84	99.82	99.85	99.16	99.16	99.17	99.16	100	99.79
P14PPP2	99.76	99.81	99.77	99.79	99.76	99.72	99.82	99.97	99.87	99.08	99.08	99.13	99.08	99.79	100

[a]The *arc*-operon was defined from *arcC* to *argR* (Fig. 5.1), *arc*-operons from strains ATCC12228 (ACME type II and FPR3757 (ACME type I) were included as references.

[b]Identical ACME II *arc*-operon genomic composite sequences were detected in isolates 200OR2 and 32BR.

[c]Identical ACME II *arc*-operon genomic composite sequences were detected in isolates P9OR1, P9PPH12, P9PPHI1, P11OR1, P11PPH21.

[d]Identical ACME II *arc*-operon genomic composite sequences were detected in isolates 120PPC, I9OR1 and I14OR4.

Table 5.6 Pairwise comparison of nucleotide identity (%) calculated between the *opp3*-operons in ACME types I and III[a]

Isolate	P16OR1[b]	I11OR1	204OR1	I12OR1[c]	P14OR1[c]	FPR3757
P16OR1[b]	100	100	99.98	98.73	98.77	98.77
I11OR1	100	100	99.98	98.73	98.77	98.77
204OR1	99.98	99.98	100	98.74	98.79	98.79
I12OR1[c]	98.73	98.73	98.74	100	99.91	99.96
P14OR1[c]	98.77	98.77	98.79	99.91	100	99.96
FPR3757	98.77	98.77	98.79	99.96	99.96	100

[a] The *opp3*-operon was defined from *opp3A* to *opp3E* (Fig. 5.1), the *opp3*-operon from strain FPR3757 (ACME type I) was included as a reference.

[b] The *opp3*-operons identified in ACME type III isolates P16OR1, I11OR1 and 204OR1 are indicated in a sub-table within the dashed line.

[c] Isolates I12OR1 and P14OR1 each harboured ACME type I.

5.3.2.3 Genetic diversity of ACME I

Two *S. epidermidis* isolates harbouring ACME type I were successfully subjected to WGS (Tables 5.1 and 5.3). Both isolates harboured the complete *arc* and *opp3* operons and were divided into two ACME subtypes (ACME type I:A-B) based on the presence or absence of SCC-associated genes and the overall sizes of the elements (Fig. 5.3). Five DRs were detected among ACME type I:A (DR_B/C) and I:B (DR_N/O/B/C) (Fig. 5.3 and Table 5.7). Two nucleotide differences were observed between DR_N and DR_O, and between DR_N and DR_B (Table 5.7).

ACME types I:A and I:B were found to harbour the *copA* and *ars* operons internally upstream of the *arc* operon. In ACME type I:A these operons were located directly upstream of the internal DR_B, however in ACME type I:B, the operons were located between DR_O and DR_B (Fig. 5.3). Both these ACME types harboured the *speG* gene upstream of the *arc* operon and downstream of the *ars* and *cop* genes.

The *opp3* operon was located downstream of the *arc* operon in ACME types I:A and I:B, followed by identical 3′ end DRs (DR_C) (Fig. 5.3).

5.3.2.4 Genetic diversity of ACME II

Overall 20 *S. epidermidis* isolates harbouring ACME type II were successfully subjected to WGS to characterise the genomic structure and genetic diversity of ACME in detail (Table 5.4).

All 20 isolates harboured the entire ACME-*arc* operon (*arcC/B/D/A/R*) and lacked the *opp3* operon (Fig. 5.4, panels b-n). In each element, the ACME-*arc* operon was located upstream of ORFs encoding hypothetical proteins, sugar transporters, transposases for insertion sequence like elements and the *copA* gene encoding a copper-transporting ATPase. Each ACME was flanked by DRs at both the 5′ end directly upstream of the ACME-*arc*, and the 3′ end directly following the *cop* or *ars* genes of each element, however several ACMEs sequenced also harboured additional internal DRs. The precise location and genomic sequence of each DR varied depending on the composition of the ACME (Fig. 5.4, Table 5.7). Overall the percentage nucleotide identity between the ACME type IIs ranged from 78.56 – 100% (Table 5.8).

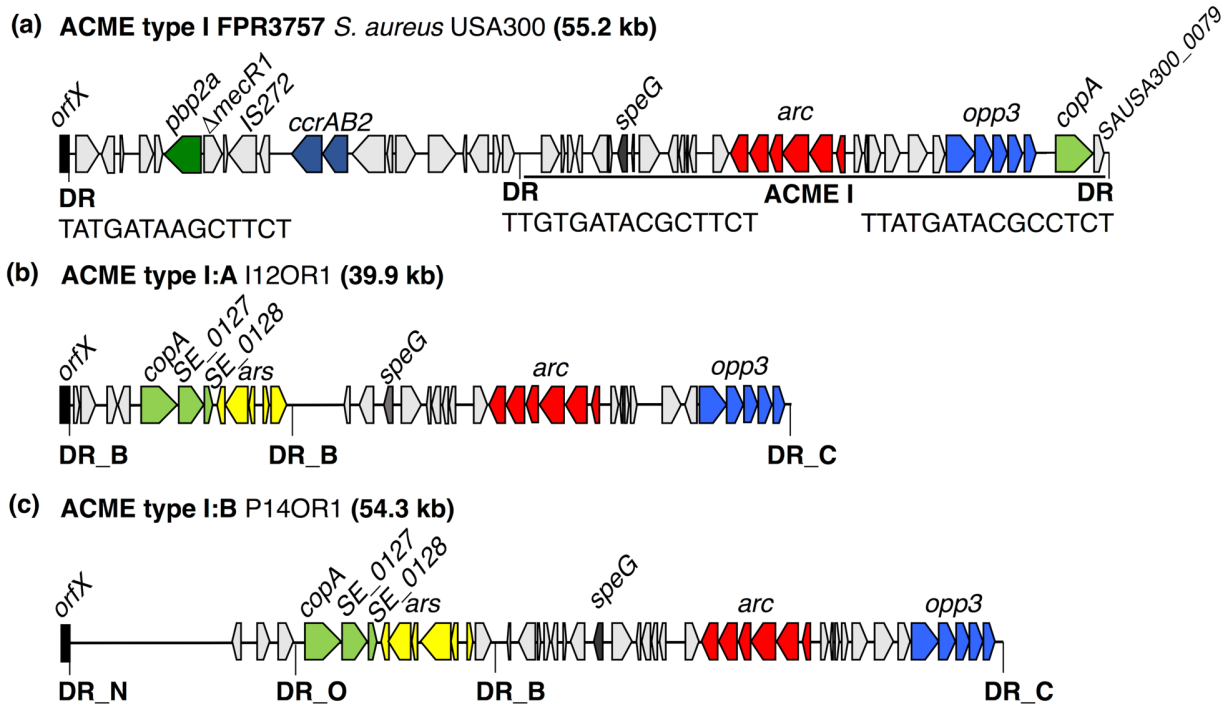
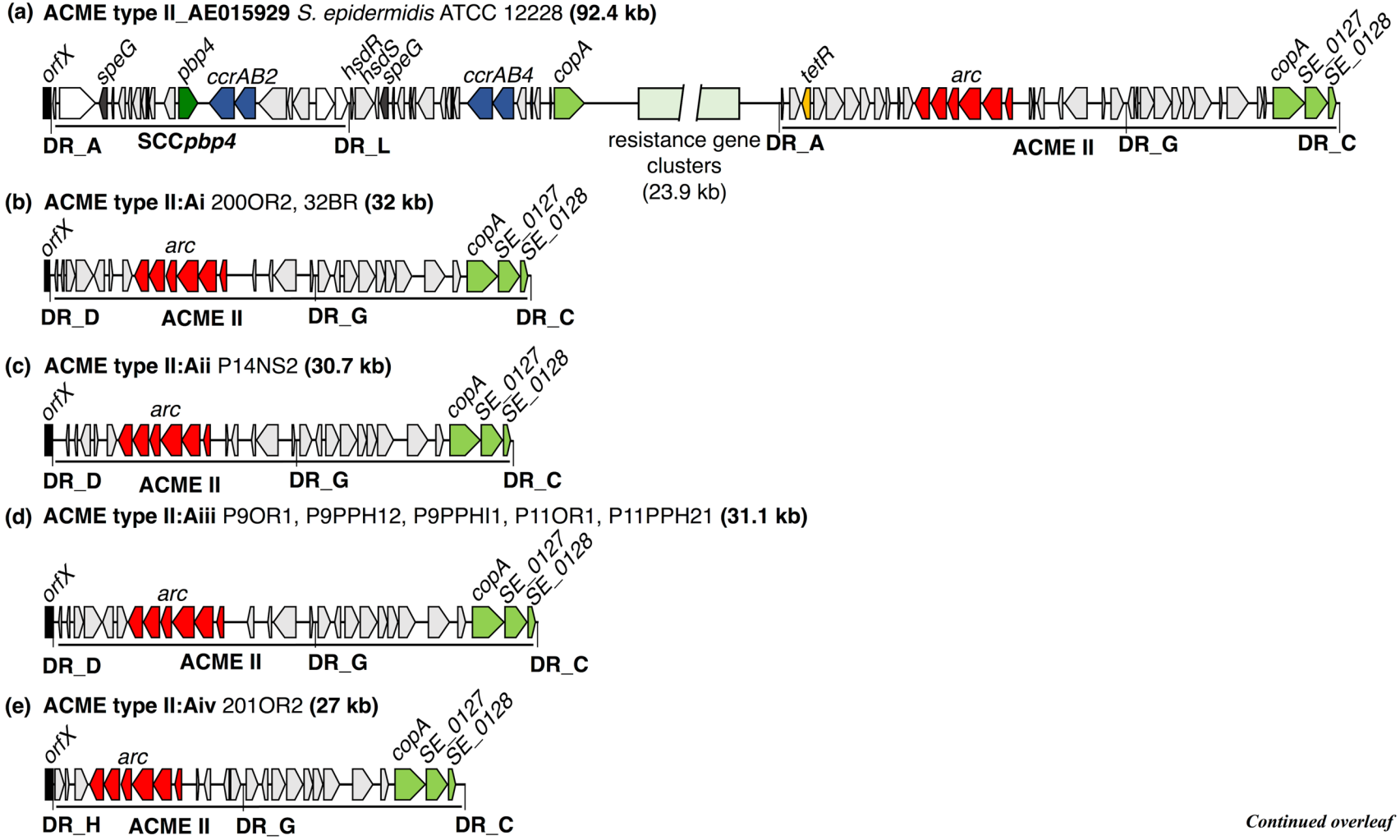


Figure 5.3 Schematic representation of ACME type I elements harboured by two distinct *S. epidermidis* isolates characterised during the present study. The ACME type I previously described in the MRSA reference USA300 strain FPR3757 (GenBank accession number CP000255) is included for comparison (a). The size of each ACME is indicated after each strain name. Each gene or group of genes of interest is differentiated by a different colour, i.e. light blue; *opp3*-operon, red; *arc*-operon, pale green; *cop*-operon, dark blue; *ccrAB* complexes, dark green; *pbp4*, dark grey; *speG*, mustard; *tetR*, light grey; genes encoding hypothetical proteins, sugar transporters, transposases and other ORFs, previously identified in ACMEs. The direction of transcription for each ORF is indicated by arrows. DRs are indicated in bold font and correspond to DR sequences listed in Table 5.7.

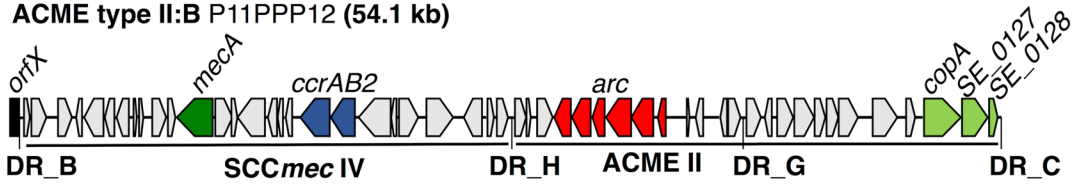
Table 5.7 Direct repeat sequences (DRs) identified among ACME types investigated in the present study

DR	Sequence
DR_A	GAAGCATATCATAAATGA
DR_B	GAAGCGTATCACAAATAA
DR_C	GAAGCGTATCGTAAGTGA
DR_D	GAAGCGTACCACAAATAA
DR_E	AATGCAAATCATAAAAGA
DR_F	GAAAGTTATCATAAGTGA
DR_G	GAAGCGTATAATAAGTAA
DR_H	GAAGCGTATCATAAGTGA
DR_I	AGAAGCGTATCACAA
DR_J	AGAGGCGTATCATAA
DR_K	GAAGCATATCATAAGTGA
DR_L	AGAAGCATATCATAAA
DR_M	GAAGGGTATCATAAATAA
DR_N	GAAGCGTATCATAAATGA
DR_O	GAAGCATATCATAAATAA

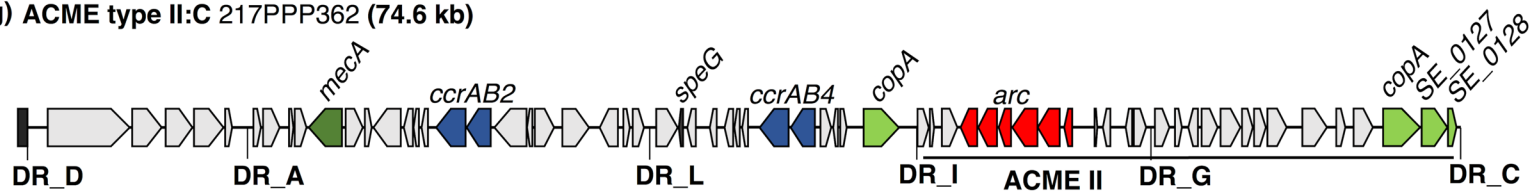


Continued overleaf

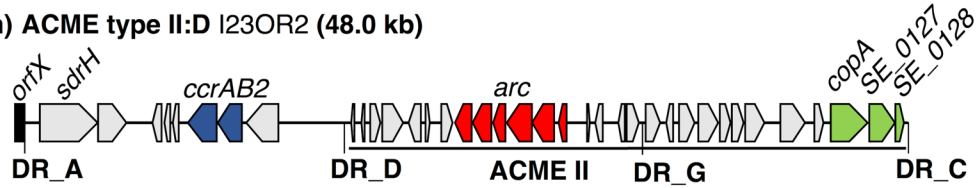
(f) ACME type II:B P11PPP12 **(54.1 kb)**



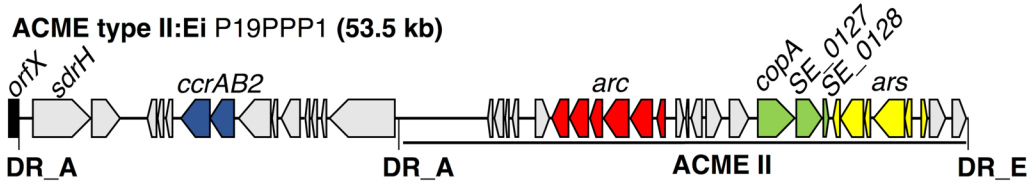
(g) ACME type II:C 217PPP362 **(74.6 kb)**



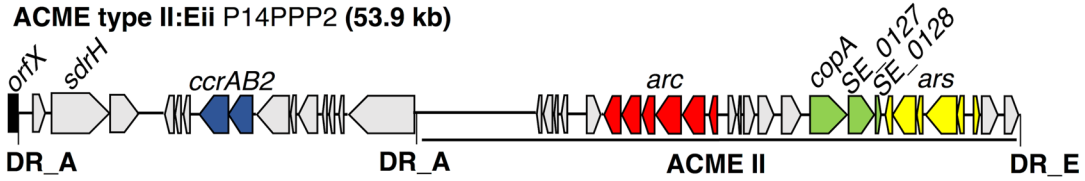
(h) ACME type II:D I23OR2 **(48.0 kb)**



(i) ACME type II:Ei P19PPP1 **(53.5 kb)**



(j) ACME type II:Eii P14PPP2 **(53.9 kb)**



Continued overleaf

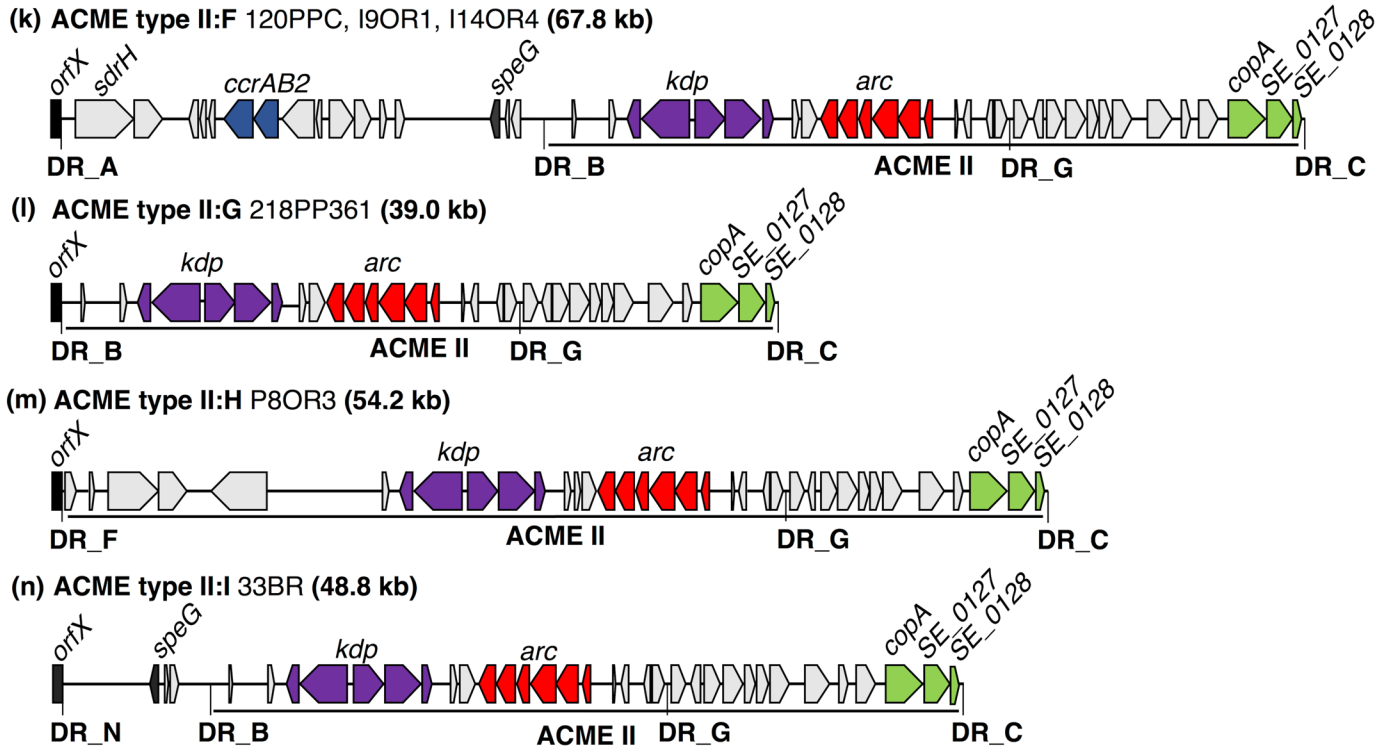


Figure 5.4 Schematic representation of ACME II and ACME II composite elements characterised in the present study. The previously described ACME type II composite element harboured by the *S. epidermidis* reference strain ATCC12228 (GenBank accession number AE015929) is included for reference. The size of each ACME is shown after the strain name. Each gene or group of genes of interest is differentiated by a different colour, i.e. red; *arc*-operon, pale green; *cop*-operon, dark blue; *ccrAB2*, light blue; *ccrAB4*, dark green; *pbp4*, dark grey; *speG*, light grey; genes encoding hypothetical proteins, sugar transporters, transposases and other ORFs, previously identified in ACMEs. The direction of transcription for each ORF is indicated by the arrow. DRs are indicated in bold font and correspond to each DR sequence listed in Table 5.7.

Table 5.8 Pairwise comparison of nucleotide identity (%) shared by all ACME type II elements investigated[a]

Isolate	200, 32[b]	P14NS2	P9, P11[c]	201OR2	P11PPP12	I23OR2	P19PPP1	120PPC	218PP361	P8OR3	33BR	217PP362	P14PPP2	ATCC2228
200, 32[b]	100	99.61	99.99	96.07	96.08	97.58	78.56	96.84	96.78	96.53	91.04	99.99	87.07	93.91
P14NS2	99.61	100	99.59	96.22	96.24	97.62	80.68	96.89	96.64	96.78	92.89	93.85	87.43	96.61
P9, P11[c]	99.99	99.59	100	95.93	95.94	97.5	79.81	96.83	96.76	96.46	92.01	91.19	83.24	93.73
201OR2	96.07	96.22	95.93	100	99.9	98.3	92.57	98.58	96.83	96.07	98.58	99.9	88.71	97.1
P11PPP12	96.08	96.24	95.94	99.9	100	98.32	92.6	98.6	96.86	96.1	98.59	100	88.77	97.28
I23OR2	97.58	97.62	97.5	98.3	98.32	100	87.32	99.45	99.17	98.03	93.96	92.97	86.3	92.42
P19PPP1	78.56	80.68	79.81	92.57	92.6	87.32	100	83.79	82.85	81.96	79.62	86.62	99.97	81.98
120, I9, I14[d]	96.84	96.89	96.83	98.58	98.6	99.45	83.79	100	99.56	96.79	100	83.62	82.11	96.51
218PP361	96.78	96.64	96.76	96.83	96.86	99.17	82.85	99.56	100	96.46	99.56	83.8	81.04	96.75
P8OR3	96.53	96.78	96.46	96.07	96.1	98.03	81.96	96.79	96.46	100	96.79	98.51	88.94	97.13
33BR	91.04	92.89	92.01	98.58	98.59	93.96	79.62	100	99.56	96.79	100	83.61	82.11	87.66
217PP362	99.99	93.85	91.19	99.99	100	92.97	86.62	83.62	83.8	98.51	83.61	100	85.55	89.4
P14PPP2	87.07	87.43	83.24	88.71	88.77	86.3	99.97	82.11	81.04	88.94	82.11	85.55	100	85.35
ATCC2228	93.91	96.61	93.73	97.1	97.28	92.42	81.98	96.51	96.75	97.13	87.66	89.5	85.35	100

[a]ACME type II nucleotide sequences were defined as regions between the DR directly upstream of the *arc*-operon and the terminal DR typically directly downstream of *copA* (Fig. 5.4 and Fig. 5.5). The corresponding sequence from the ACME II previously characterised in *S. epidermidis* strain ATCC12228 was included as a reference.

[b]Identical ACME II genomic composite sequences were detected in isolates 200OR2 and 32BR

[c]Identical ACME II genomic composite sequences were detected in isolates P9OR1, P9PPH12, P9PPHI1, P11OR1 and P11PPH21

[d]Identical ACME II genomic composite sequences were detected in isolates 120PPC, I9OR1 and I14OR4

Based on the presence or absence of SCC-associated genes and the overall size of the elements, nine structurally distinct ACME II subtypes (subtypes A-I) were identified during the current investigation (Fig. 5.4).

Four ACME type IIs were identified as subtype A as they lacked any adjacent genes previously associated with other mobile genetic elements such as SCC elements (Fig. 5.4, panels b-e). The ACME II subtype A was identified among nine different isolates investigated which were further divided into four distinct allotypes (i-iv) based on minor size and ORF variations (Fig. 5.4, panels b-e). These four ACME II:Ai-iv subtypes ranged in size from 27 kb to 32 kb (Fig. 5.4) and had identical 3′ flanking DRs (DR_C). ACME types II:Ai-iii had identical DRs (DR_D) at the 5′ end of the element (Fig. 5.4, Table 5.7), whereas the 5′ DR in ACME II:Aiv (DR_H) differed from DR_D by four nucleotides (Fig. 5.4 & Table 5.7). For the ACME II:Ai:iv subtypes, the ACME-*arc* operon was located within 6 kb of *orfX* and was located upstream of genes encoding hypothetical proteins, sugar transporters, transposases and a copper-transporting ATPase.

Eight larger ACME type IIs (subtypes B-I) ranged in size from 39 kb to 74.6 kb and were co-located with SCC-associated genes (Fig. 5.4, panels f-n). Five different DRs (DR_B, DR_D, DR_A, DR_F and DR_N) were identified at the 5′ end of these ACME elements (Fig. 5.4 and Table 5.7). ACME type II:B (Fig. 5.4, panel f) harboured both *mecA* and *ccrAB2* genes (SCC*mec* IV) followed by a DR_H (Table 5.7) upstream of the *arc* operon. Interestingly, ACME type II:B contained a second internal DR between the *arc* operon and *copA* gene (Fig. 5.4, panel f). As it lacked *speG* and contained the *mecA* gene instead of *pbp4*, the SCC-element detected alongside ACME type II:B in the present study (Fig. 5.4, panel f) differs considerably from that previously detected alongside the reference ACME type II (Fig. 5.4, panel a) in ATCC12228 (54).

The *ccrAB2* genes were identified upstream of the *arc* operon in ACME types II:D and II:E, and IIF, (Fig. 5.4, panels h - k) however these ACME types contained different internal DRs between the *ccrAB2* genes and the *arc* operon. Based on minor variations in ACME type II:E, two allotypes (i and ii) were defined within this subtype (Fig. 5.4, panels i and j, respectively). The 3′ terminal DRs for ACME types II:D and II:F were located directly after the *cop* operon, in contrast to the previously mentioned

ACME type IIs, the 3′ end DRs for ACME type II:Ei and II:Eii were located after the *ars* operon (Fig. 5.4, panels i and j).

ACME type II:F (Fig. 5.4, panel k) was the second largest element (67.8 kb) identified and harboured *ccrAB2*, *speG*, one internal DR and a *kdp* operon upstream of the *arc* operon. It was detected in three distinct isolates recovered from the oral rinse samples of two different participants and a subgingival site of a third distinct participant (Fig. 5.4, panel k).

ACME types II:G, II:H, and II:I harboured the *kdp* operon and an internal DR downstream of the *arc* operon (Fig. 5.4, panels l, m and n, respectively). The final 3′ DRs were located directly downstream of the *cop* operon in these ACME type IIs. However, the ACME type IIs were not identical due to the presence of hypothetical protein genes directly downstream of *orfX* in ACME type II:H, and the presence of *speG* in ACME type II:I (Fig 5.4, panels m and n).

In contrast to the ACME II composite element harboured by the *S. epidermidis* type strain ATCC12228, which was used as a ACME type II reference in the present study, the 20 ACME type II elements characterised within the present study were not associated with SCC*pbp4*, *ccrAB4*, internal *copA*, *tetR*, or the cadmium and mercury resistance cluster (Fig. 5.4).

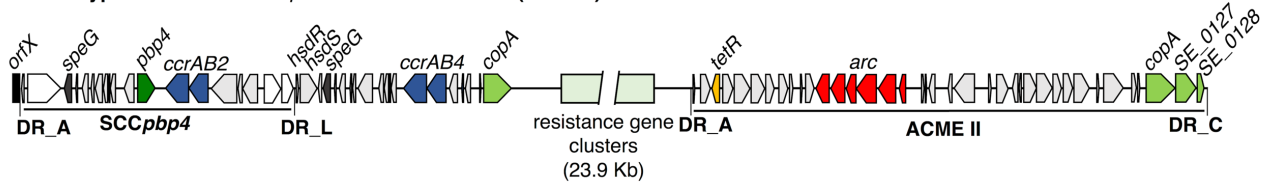
5.3.2.5 Genetic diversity of ACME III

Three ACME type IIIs were characterised by WGS in the current study (Fig. 5.5) for the first time, using a combination of SMRT- and Illumina- based sequencing platforms. Based on SMRT sequencing, the average base-pair (bp) read coverage was 265.8 bp for the three ACME III containing composite elements investigated. These composite elements varied in size from 45.1 kb to 65.6 kb, however, the ACME III was 21.3 kb in all three isolates investigated.

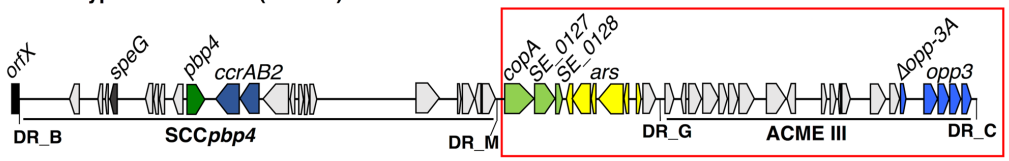
Genes previously associated with SCC elements were located adjacent to ACME III within *orfX* (Fig. 5.5). Based on the presence or absence of SCC-associated genes, three distinct ACME III subtypes (subtypes A-C) were identified (Fig 5.5, panels c-e). Genes *pbp4*, and *ccrAB2* were detected in ACME III subtypes A and B (Fig 5.5, panels c and d), and have previously been associated with ACME type II in *S. epidermidis* (Fig. 5.5, panel b). The *speG* gene was harboured in ACME type III:A only (Fig 5.5, panel c). ACME type III:B harboured two *ccr* complexes composed of a truncated *ccrB4* with *ccrA4* and *ccrAB2* (Fig 5.5, panel d).

(a) **ACME type I FPR3757** *S. aureus* USA300 **(55.2 kb)**

(b) **ACME type II AE015929** *S. epidermidis* ATCC 12228 **(92.4 kb)**



(c) **ACME type III:A** 204OR1 **(65.6 kb)**



(d) **ACME type III:B** P16OR1 **(53.9 kb)**

(e) **ACME type III:C** I11OR1 **(45.1 kb)**

Continued overleaf

Figure 5.5 Schematic representation of ACME type III composite elements characterised in the present study. The previously described ACME type I composite element harboured by the *S. aureus* reference strain FPR3757 (GenBank accession number CP000255) and ACME type II composite element harboured by the *S. epidermidis* reference strain ATCC12228 (GenBank accession number AE015929) are included for reference. The size of each ACME is shown after the strain name. Each gene or group of genes of interest is differentiated by a different colour, i.e. light blue; *opp3*-operon, red; *arc*-operon, pale green; *cop*-operon, dark blue; *ccrAB* complexes, dark green; *pbp4*, dark grey; *speG*, mustard; *tetR*, light grey; genes encoding hypothetical proteins, sugar transporters, transposases and other ORFs, previously identified in ACMEs. The direction of transcription for each ORF is indicated by the arrow. DRs are indicated in bold font and correspond to each DR sequence listed in Table 5.7. Genomic regions from *copA* to DR_C, in each ACME III exhibited >99% DNA sequence homology to each other and are enclosed in red rectangles.

Overall five different DRs were identified among the three ACME type III composites characterised. The DRs DR_B/M/G/C (Table 5.7) were detected in the ACME III subtypes A and C harboured by isolates 204OR1 and I11OR1 (Fig. 5.5, panels c and e), and DRs DR_K/G/C were detected in the ACME III subtype B harboured by isolate P16OR1 (Fig. 5.5, panel d). Each ACME III investigated started at the 5′ end by DR_G and was terminated at the 3′ end by DR_C (Table 5.7), however the DRs at the 5′ end of each ACME III composite element varied, ACME III subtypes A and C contained DR _B and this terminus, whereas ACME III subtype B contained DR_K, which differed from DR-B by only four bp (Table 5.7).

All three ACME type IIIs contained an almost identical *opp3* operon located 510 bp upstream of DR_C. Interestingly, ACME III:A contained a truncated *opp-3A* gene caused by a single nucleotide deletion at the +384 position that lead to a frameshift mutation. The nucleotide deletion was confirmed by Sanger sequencing (Fig. 5.5, panels c-e).

Surprisingly, the *copA* gene and *ars* operon were harboured internally within each ACME III composite element characterised. Typically, the *copA* gene is located at the 3′ end of ACME and the *ars* operon is characteristically located immediately downstream of ACME, an exception to this ACME organisation was detected in ACME II:E where the 3′ DR was located downstream of the *ars* operon (Fig 5.4, panels i and j). In contrast, the *copA* and *ars* genes were situated between DR_M and DR_G in ACME III subtypes A and C (Fig. 5.5, panels c and e) and in a similar location in ACME III subtype B (Fig. 5.5, panels d) despite the absence of DR_M. The *copA* gene and *ars* operon were absent downstream of ACME III in all three isolates. Interestingly, across all three ACME type III composite elements characterised, the genomic region between *copA* to DR_C exhibited >99% DNA sequence identity.

5.3.2.6 Characterisation of ACMEs recovered from distinct sample sites in the same patient

Three patients with periodontal disease (P9, P11 and P14) yielded *S. epidermidis* isolates with distinct ACME types as determined by WGS (Table 5.4).

Three isolates recovered from the oral rinse sample and two distinct subgingival sites of Patient P9 harboured ACME type II:Aiii (Fig. 5.4, panel d). However, in another case ACME types identified in isolates from the same participants were very different. Three isolates recovered from the oral rinse sample, periodontal pocket and

subgingival sites of Patient P11 harboured two genetically diverse ACME type IIs (Fig. 5.4, panels d and f). Interestingly, the isolates recovered from the oral rinse sample and subgingival site of patient P11 harboured ACME type II:Aiii, and the isolate recovered from a periodontal pocket harboured ACME type II:B (Fig. 5.4, panels d and f). Three genetically distinct ACME types were detected in the isolates recovered from the nares (ACME type II:Aii), oral rinse sample (ACME type I:B), and periodontal pocket (ACME type II:Eii) of Patient P14 (Figs. 5.3, panel c, and 5.4, panels c and j).

5.4 Discussion

5.4.1 Prevalence of ACME in *S. epidermidis* from oral rinse samples, periodontal pockets and subgingival sites

Previous research has investigated the prevalence of ACME in MRSE and MSSE isolates recovered from carriage and infection sites (154, 169, 170, 186). Overall, the reported prevalence of ACME ranged from 40% - 65.4% in MRSE isolates and was slightly higher in MSSE isolates, between 64.4% - 83% (154, 169, 170, 186). Only one study reported the prevalence of ACME types I (33.6%), II (60.9%) and III (5.5%) in MSSE carriage isolates, however none of the isolates investigated were recovered from the oral cavity (186).

Previous studies typically investigated the prevalence of ACME in *S. epidermidis* isolates recovered from multiple different sample types and/or diverse geographical areas (54, 154, 169, 170, 186). The present study is the most comprehensive investigation to date into the prevalence of *S. epidermidis* harbouring ACME recovered exclusively from distinct anatomical sites in the oral cavity of specific patient groups.

In the present study, the highest prevalence of *S. epidermidis* isolates harbouring ACME in oral rinse samples was detected in healthy patients with implants (76%, 19/25) and this prevalence rate was significantly higher (P = 0.016) than the corresponding rate in isolates recovered from oral rinse samples of orally healthy participants 51% (22/43) (Table 5.3). No significant difference was identified between the prevalence of ACME in isolates recovered from oral rinse samples of patients with periodontal disease and the other two participant groups (Table 5.3).

The present study is the first to investigate the prevalence of *S. epidermidis* harbouring ACME from periodontal pockets of patients with periodontal disease. Overall, the prevalence of ACME was highest in isolates recovered from subgingival sites in patients with periodontal disease (4/4, 100%), followed by periodontal pockets of patients with periodontal disease (5/6, 83%) and subgingival sites of healthy patients with implants (4/5, 80%). Interestingly, patients with periodontal disease yielded significantly more (P = 0.017) isolates harbouring ACME from periodontal pockets than subgingival sites of orally healthy participants (Table 5.3). The higher prevalence of ACME in *S. epidermidis* isolates recovered from periodontal pockets and subgingival

sites suggest that ACME may contribute to the fitness of *S. epidermidis* in these semi-anaerobic oral environments.

Both Miragaia *et al*. and Onishi *et al*. reported that ACME type I was the most prevalent ACME type detected in MRSE and MSSE isolates investigated (154, 170). In contrast, other studies have reported that ACME type II was the predominant ACME type (169, 186). In agreement with the latter studies, ACME type II was the most prevalent ACME type detected in *S. epidermidis* isolates investigated in the present study across the three distinct anatomical sites and distinct participant groups. The inconsistency in the reported prevalence of ACME types among different studies could be attributed to the diverse range of isolates investigated. Factors such as patient type, sample site location, use of antibiotics, and geographical location could affect the prevalence of different ACME types. The present study investigated specific anatomical sites for the carriage of *S. epidermidis* harbouring ACME in states of both health and disease. Importantly, some of these sites (i.e. periodontal pockets) are semi-anaerobic (187).

5.4.2 Genetic diversity of ACME types I, II and III

In the present study, genetically diverse ACMEs were identified in *S. epidermidis* isolates recovered from distinct sample sites in patients with periodontal disease, healthy patients with dental implants, and orally healthy participants. Utilising WGS yielded unbiased sequences of ACMEs that might not have been identified based on PCR amplification alone. For example, the internal location of *copA* downstream of the *arc* operon in ACME types 1:A and 1:B would not be expected based on the ACME type I reference strain FRP3757 (SAUSA300), and primers designed from *opp3* to *copA* based on this reference would not have produced an amplimer.

The nucleotide identity between the 13 distinct ACME type IIs, based exclusively on genomic sequence between DRs harbouring the *arc*-operon and terminal *copA* gene or *ars*-operon (Fig. 5.4), ranged from 78.56% - 99.99%. However, upon exclusion of the ACME type II identified in isolates P19PPP1 and P14PPP1, the range of nucleotide identity between each ACME type II ranges from 83.42% - 99.99%, suggesting there was considerable more divergence in P19PPP1 and P14PPP1. Isolates P19PPP1 and P14PPP2 harboured a different terminal DR (DR_E) to all the other type II elements identified (DR_C) (Table 5.7). The DR DR_C is located immediately

upstream of the *ars* operon, however DR_E is located downstream of this operon, which contributed to the lower nucleotide identity between the ACME II elements harboured by isolates P19PPP1 and P14PPP2 and the other type II elements investigated (Fig. 5.4, panels i and j, Table 5.8).

Previously ACME type III has only been characterised by PCR scanning/tiling techniques and the present study is the first to undertake a detailed structural characterisation of the element using WGS. Due to the lack of a previously published complete ACME type III sequence to use as a reference, two separate WGS techniques were utilised to comprehensively sequence three ACME type IIIs in epidemiologically unrelated *S. epidermidis* isolates and PCR was used to confirm the final structures.

Interestingly, the type III elements were divided into modular segments by DRs, as were several ACME type I and IIs, suggesting modular stepwise assembly of the ACME composite. However, across the three-distinct type III elements investigated, the region between *copA* and the terminal 3′ DRs (DR_C) was highly conserved (>97%) (Fig. 5.5). In contrast to the other ACME types investigated, the *copA* gene and SE_0128 (corresponding to SAUSA300 in FPR3757) genes were internalised within ACME III-containing composite elements (Fig. 5.5, panels c-e).

ACME type III:A harboured a truncated *opp3A* caused by a nucleotide deletion resulting in a frameshift mutation, however due to the presence of native *opp* genes in *S. epidermidis* this truncation is unlikely to have impacted the overall fitness of the isolate (Fig. 5.5, panel c). It is unlikely that the *opp3*-operon provides any major fitness advantage to *S. epidermidis* in the oral cavity, based on the low prevalence of ACME III (9/141, 6.4%) detected in the present study and the presence of a truncated *opp3A* in one ACME III. Peptide nutrient uptake, chemotaxis, and cell wall metabolism could be maintained by chromosomal *opp*-operons in the absence of the ACME-*opp3* cluster.

Several studies have investigated the nucleotide identity between *arcA* or *opp3A* genes identified in ACME elements harboured by MRSE or MSSE isolates and have reported that *arcA* is highly conserved (>99% nucleotide identity) whereas the *opp3A* gene is typically less conserved between elements (94.8-98.7% nucleotide identity) (154, 170, 186). In the present study, the percentage nucleotide identity of the *arc*-operon and *opp3*-operon shared among all ACMEs investigated (99.11 – 100% and 98.73 –100% nucleotide identity, respectively) was consistent with previous reports (Tables 5.4 and 5.5) (154, 170, 186).

Onishi *et al*. identified three ACME type I subtypes based on differences in the genomic composition between the *arcC* and *opp3E* operons (170). In the present study, the genomic sequence between *arcC* and *opp3E* in isolates I12OR1 (Fig. 5.3, panel b) and P14OR1 (Fig. 5.3, panel c) shared greater nucleotide identity with the same region in the ACME I described previously in SAUSA300 strain FPR3757 (>99.68%) (Fig. 5.3, panel a) compared to the three ACME I subtypes described previously (94.3 – 95.8%) (54, 170).

A previous study hypothesised that *S. epidermidis* harbouring SCC*mec* IV was a successfully distributed colonising MRSE strain in distinct geographical regions (170) and interestingly, SCC*mec* IV-associated genes were identified upstream of both MRSE isolates harbouring ACME types II:B and II:C (Fig. 5.4, panels f and g, respectively) investigated by WGS in the present study. The *mecA* gene is typically located upstream of ACME in MRSE strains and encodes the PBP2a protein (also known as PBP2′), which contains an adapted binding site that reduces the proteins affinity for β-lactam antibiotics (37, 154, 188). It is important to note that previous research found no association between carriage of specific ACME types and SCC*mec* elements in MRSE isolates (154, 169).

The site specific recombinases encoded by the genes *ccrA* and *ccrB* (*ccr* complex *ccrAB*) or *ccrC* play a role in the integration and excision of SCC*mec* and ACME elements at *orfX* (37, 189). Eight distinct *ccr* gene complexes have been identified to date in SCC*mec* elements consisting of *ccrA*, *ccrB*, or *ccrC* genes (42). The *ccr* gene complex is typically located upstream of ACME. Previous research identified a significant association between ACME and the *ccr* recombinase *ccrC* (169), however *ccrC* was not detected in association with ACME in any of the 25 isolates investigated by WGS in the present study. Two different studies based on PCR previously reported that the prevalence of *ccr* genes in MSSE isolates also harbouring *arcA* ranged from 36.4% - 38% (154, 170), in agreement with the findings of the present study which identified *ccr* genes in conjunction with *arcA* in 10/25 (40%) MSSE isolates investigated.

The *ccrAB2* complex was detected in ACME types II:B-II:F (Fig. 5.4, panels f-k), and ACME types III:A-III:B (Fig. 5.5, panels c and d). Interestingly both ACME types II:C and III:B harboured a second *ccr* complex, *ccrAB4* (Fig. 5.4, panel g and Fig. 5.5, panel d). However in ACME type III:B, *ccrB4* was truncated due to a nucleotide deletion leading to a frameshift mutation and a premature stop codon in the ORF. The

presence of two *ccr* complexes in ACME types II:C and III:B could possibly indicate that *ccrAB4* was previously used for a translocation, and in ACME type III:B, it is possible *ccrAB4* was interrupted upon a second translocation.

The *kdp*-operon is ubiquitous in bacteria and encodes two proteins (KdpD and KdpE) that regulate a high affinity K+ transporter known as KdpATPase (190). SCC*mec* type II harbours a *kdp*-operon downstream of the *mec* complex (42). Previous research has not investigated the prevalence of the *kdp*-operon in association with ACME in MSSE. Interestingly, four MSSE isolates harbouring distinct ACME type IIs (II:F-I) harboured the *kdp*-operon upstream of ACME but no *mec* gene (4/25, 16%) (Fig. 5.4, panels k-n). It is unknown what advantage, if any, the *kdp*-operon confers on MSSE isolates and it could possibly be a remnant of previous SCC*mec* rearrangements that occurred upstream of ACME.

The differences in genetic diversity of the *arc* and *opp3* operons, the presence of internal DRs allowing for modular rearrangements, and the detection of SCC-associated genes alongside some ACMEs investigated in the present study supports previous research that hypothesised the stepwise assembly of ACME in *S. epidermidis* (82). The prevalence of SCC-associated genes varied between each ACME type and no association between the presence of SCC-associated genes and anatomical site or participant group was identified. The acquisition of ACME by staphylococci in the oral cavity could provide a fitness advantage to staphylococci trafficking into distinct oral environments and into the nares. Based on the low prevalence of SCC*mec* elements detected (1/26, 3.8%) among isolates that were investigated by WGS, SCC*mec* likely offers no such advantage, presumably due to the absence of any antibiotic selective pressures previously associated with maintenance of SCC*mec* (191).

5.4.2.1 Population analysis of S. epidermidis *isolates investigated.*

Isolates recovered from 12 distinct patients were predominantly identified as belonging to four distinct STs (ST17, ST329, ST59 and ST153) (Table 5.4). Previously, ACME type II has been reported in isolates identified as ST17, ST59 and ST153 (154), and similarly all three isolates identified as ST59 and three isolates identified as ST153 in the present study harboured ACME type II. Four isolates identified as ST17 were recovered from three distinct participants and harboured ACME types I and II. Interestingly two of these isolates were recovered from Patient P14; one of the isolates harboured an ACME type I and the second isolate was type II (Table 5.4).

All three distinct isolates harbouring ACME III investigated in the present study were identified as the same ST type, ST329. This was surprising as to date, this ST has only been identified in 3/1068 (0.3%) allelic profiles currently listed in the MLST database (accessed 8th June 2017), and previous research from this laboratory did not identify this ST in any of the 36 *S. epidermidis* isolates investigated. It is possible ACME III could be a result of genomic rearrangements that occurred in ST329, but because it appears to confer little fitness advantage, it did not become more prevalent in the *S. epidermidis* population, but remains associated with the ST329.

5.4.3 Possible role of ACME in the oral cavity, periodontal pockets and subgingival sites

Periodontal disease is an inflammatory disease and therefore it is expected that a higher concentration of polyamines would be present in the periodontal pockets due to their association with wound healing and infection clearance. Spermidine acetyltransferase (SpeG) can mitigate the lethal effects of polyamines spermidine and spermine on staphylococci by acylation (82–84). A previous study reported environmental factors such as acidity or lack of oxygen ameliorate the bactericidal effect of polyamines on *S. aureus* (84). Similar research has not been reported on *S. epidermidis*, however, based on the similarities of polyamine toxicity to both species, a similar outcome is likely. Interestingly, only six isolates investigated by WGS in the present study harboured *speG* alongside the *arc* or *opp3* operons. This suggests SpeG does not play a role in the survival of *S. epidermidis* in the oral environment. However, it is possible the slightly acidic oral environment protects *S. epidermidis* against polyamines.

The pH of the oral cavity is typically six (192), and can vary depending on diet and the oral health status of the individual. However, it is much more difficult to determine the pH of periodontal pockets and subgingival sites because of their location in the oral cavity and the oral status of the surrounding tissue can affect pH values associated with these sites. The arginine deiminase (ADI) pathway is composed of three highly conserved enzymes encoded by the *arc*-operon, that catabolise L-arginine and can help to regulate the internal pH of the bacteria, predominantly in acidic environments (193). A by-product of the ADI pathway is ammonia synthesis, which increases the cytoplasmic pH and the environmental pH (193). Internal pH regulation

could be critical in enabling *S. epidermidis* to traffic and persist in the different environments of the oral cavity.

Staphylococci can regulate their metabolism depending on environmental factors such as oxygen levels (194). Under aerobic conditions multiple different amino acids and metabolites are consumed by staphylococci, however under anaerobic conditions the bacteria are more metabolically selective, primarily metabolising arginine, glucose, and threonine (194). The ACME-*arc* operon is expressed constitutively independent of glucose or oxygen levels. In the nutrient poor environments of periodontal pockets and subgingival sites, the catabolism of arginine would generate ATP, thus providing an energy source for the bacteria. Across all participant groups investigated in the present study, there was a greater prevalence of *S. epidermidis* harbouring ACME-*arc* recovered from periodontal pockets and subgingival sites compared to oral rinse samples. This finding supports the hypothesis that the ACME-*arc* contributes to the survival of *S. epidermidis* in the semi-anaerobic periodontal pocket and subgingival site environments.

During tissue infection, the host's arginine is synthesised into nitric oxide (NO^{-}) by the host and amplifies an inflammatory response. Thurlow *et al*. proposed that the *arc*-operon may protect NO^{-} sensitive *S. epidermidis* by competing for available host arginine (83). This would reduce the amount of arginine available for the host and possibly delay the immune response. As previously discussed, the ACME-*arc* was more prevalent in isolates recovered from periodontal pockets and subgingival sites compared to the oral rinse samples from the three distinct participant groups in the present study (Table 5.3). The catabolism of exogenous arginine by *S. epidermidis* in the periodontal pocket and subgingival site environment could dampen the hosts immune response and delay the clearance of the bacteria, providing more time for biofilm formation.

The presence of internal DRs and modular segments in multiple ACMEs investigated in the present study support the hypothesis that ACME was originally assembled in a stepwise manor. The *arc*-operon was much more prevalent (89.3%) than the *opp3*-operon (10.7%) in the *S. epidermidis* isolates harbouring ACME investigated in the present study, indicating the *arc*-operon could confer a fitness advantage, whereas the *opp3*-operon seems to be dispensable. The low prevalence of *speG* detected in association with the ACME-*arc* suggests *speG* is not required for acylation of polyamines in the oral environment. The high nucleotide identity between all the *arc*-operons identified in both ACME types I and II investigated indicate its function is

highly conserved. It is likely the ACME-*arc* plays numerous roles in enabling *S. epidermidis* to survive in the oral cavity. The catabolism of arginine by the *arc*-operon provides an energy source for the bacteria, produces ammonia to regulate external and cytosolic pH, and finally, reduces the availability of host arginine impeding the host's immune response.

Chapter 6

Population Analysis of *C. albicans*

6.1 Introduction

6.1.1 Association of *Candida* and disease

In recent decades *Candida* species such as *C. parapsilosis*, *C. glabrata*, *C. tropicalis C. krusei* and *C. dubliniensis* have emerged as causes of *Candida* infections in patients with malignancies, extremes of age, immunity deficiencies and poor responses to antifungal therapies (195, 196). Despite this, *C. albicans* remains the most prevalent *Candida* species, carried by 30-45% of normal healthy adults (98, 135). *Candida albicans* is also the most prevalent *Candida* species in the oral cavities of individuals with oral disease such as periodontal disease (47.6%) and oral leukoplakia (70.5%) or in patients with underlying diseases such as AIDS (85%), diabetes (60%) and APECED (75%) (95, 96, 136–139). Several previous studies have investigated the population structure of *C. albicans* isolates recovered from a range of distinct patient cohorts and from disparate geographical locations using DNA fingerprinting with complex, repetitive sequence-containing DNA probes and, more recently, MLST (10, 136–138, 197). The objective behind these studies was to identify any enrichment or association of particular *C. albicans* strains with disease; for example DNA fingerprinting analysis demonstrated genetic similarity and strain maintenance in *C. albicans* isolates recovered from patients with AIDS (197).

Due to the opportunistic nature of *Candida* species, underlying systemic diseases can often manifest as acute or chronic *Candida* infections, for example, chronic mucocutaneous candidiasis (CMC) is one of the main clinical symptoms and often the first manifestation of the autoimmune disorder autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) (198).

The abundance of *C. albicans* in the oral cavities of individuals infected with the HIV increases prior to candidiasis infection, indicating an impairment of the host's oral immune defences (96). The presence of oral lesions caused by candidiasis or oral hairy leukoplakia in individuals infected with HIV can indicate severe immune suppression and progression to AIDS (96, 199, 200).

Tapper-Jones *et al*. reported a higher prevalence and a significantly higher abundance of *C. albicans* in the oral cavities of patients with diabetes compared to orally healthy individuals (95), most likely associated with the decreased salivary flow and increased glucose concentration in saliva in these patients (201).

6.1.2 Epidemiological studies of *Candida* infection in patients with specific types of disease

Several previous studies have investigated if distinct populations of *C. albicans* can be associated with infections in distinct patient cohorts using MLST and ABC genotyping and several have identified associations of isolates belonging to distinct ABC genotypes or MLST clades and groups of patients with particular diseases.

ABC genotype enrichment has previously been identified in *C. albicans* isolates recovered from patients with an underlying disease and patients with orthodontic devices (138, 202). Patients with oral leukoplakia and patients with orthodontic devices had a significant enrichment of *C. albicans* isolates identified as ABC genotypes C and B, respectively, compared to orally healthy *Candida* carriers (138, 202).

Longitudinal studies based on multiple isolates recovered from individual patients with APECED, HIV or candidiasis over time have shown that unique commensal *C. albicans* strains tend to persist in the oral cavity rather than undergo strain replacement (137, 198, 203–205).

Several distinct research groups have utilised MLST and ABC genotype analysis to investigate *C. albicans* populations recovered from different patient groups and identified the occurrence of microvariation presented as loss of heterozygosity (LOH) in the DNA sequence of a proportion of *C. albicans* isolates collected from the same individual. This phenomenon is commonly observed in *C. albicans* MLST datasets (10, 136, 137, 198, 204, 205). LOH events and resulting gain of function mutations in drug resistance-associated genes *ERG11* and *TAC1* have been associated with a decrease in susceptibility to the azole class of antifungals (137, 198, 206). However to date, no studies have identified an association between LOH events and disease progression (137, 198).

6.1.3 Population structure of *C. albicans* and clade enrichment

Multiple typing methods have been devised to analyse *C. albicans* populations. Typing methods such as ABC genotyping are less discriminatory compared to the DSTs generated from MLST analysis. The MLST clades are generated based on concatenated MLST sequences, allowing the clustering of DSTs into larger closely related groups.

Standardised datasets generated from MLST analysis are typically submitted to and stored on the publically available MLST database online [3267 MLST profiles

(Database accessed on the 2nd March, 2017)]. This enables comparison between isolates collected worldwide from distinct patient groups, over different time periods, different anatomical sites, age groups, and individuals with different nationalities and ethnicities.

Large-scale studies based on the collection of isolates in this database have revealed associations between particular MLST clades and ABC genotypes, geographical origin and sample site. For example, Clades 1 and 2 consist of predominantly ABC genotype A *C. albicans* isolates recovered from the oropharynx of subjects in the UK (207). Clades 4 and 12 are associated with ABC genotype C isolates typically recovered from blood samples. However, particular clones of *C. albicans* do not appear to be associated with specific diseases; for example, it has been reported that *C. albicans* bloodstream infections are endogenous in origin, caused by the patient's own commensal organisms (204).

MLST-based investigation of the *C. albicans* population recovered from the oral cavities and periodontal pockets of patients with periodontal disease revealed an enrichment of Clade 1 *C. albicans* recovered from periodontal pockets (10). ABC genotyping analysis identified an enrichment of ABC genotype B *C. albicans* in the oral cavities of patients with fixed orthodontic devices (143). However, to date, no studies have investigated the population of isolates recovered from patients with implants or from subgingival sites by MLST analysis.

6.1.4 Objectives

The aim of this study was to comparatively characterise the population of *C. albicans* isolates recovered from patients with periodontal disease, healthy patients with dental implants and orally healthy carriers using ABC genotyping and MLST analysis.

- To determine the prevalence of specific *C. albicans* ABC genotypes and DSTs among isolates recovered from patients with periodontal disease, healthy patients with dental implants, and orally healthy carriers.
- To determine if multiple *C. albicans* isolates recovered from oral rinse and subgingival samples of the same individual were genetically identical, distinct or closely related based on MLST.
- To investigate if any enrichment of *C. albicans* clades exists among isolates recovered from the oral cavity, periodontal pockets and subgingival sites in comparison to previous studies.

6.2 Materials and methods

6.2.1 *C. albicans* isolates

Ninety-three *Candida albicans* isolates were selected out of 120 isolates as representatives of different patient groups, individual patients and the anatomical sites from which they were recovered were subjected to ABC genotype identification by PCR. A further 24 of these isolates were also subjected to MLST analysis. These 24 isolates consisted of eleven *C. albicans* isolates recovered from seven different patients with periodontitis [oral rinse samples (n = 7), a healthy subgingival site (n = 1) and periodontal pockets (n = 3)], eleven *C. albicans* isolates recovered from six different healthy patients with dental implants [oral rinse samples (n = 6), healthy subgingival sites (n = 4) and a healthy subgingival site surrounding an implant (n = 1)] and two *C. albicans* isolates recovered from the oral rinse sample and a subgingival site of one orally healthy *Candida* carrier.

6.2.2 *C. albicans* DNA extraction

Extraction of DNA from *C. albicans* isolates was performed as previously described in Chapter 2, Section 2.1.4.3.

6.2.3 ABC genotyping of *C. albicans* by PCR

Amplification of the transposable intron in the 25S rDNA was performed using 60 ng of template *C. albicans* DNA in a 50 µl reaction volume consisting of 10 µM primers CA-INT-L (5'→3' ATAAGGGAAGTCGGCAAAATAGATCCGTAA) and CA-INT-R (5'→3' CCTTGGCTGTGGTTTCGCTAGATAGTAGAT), 1.5 mM $MgCl_2$, 5X green GoTaq flexi buffer, sterile water, 200 µM dNTPs and 2.5 U of Go Taq DNA polymerase as described previously by McCullough *et al*. (119). The cycling conditions consisted of denaturation at 94ºC for 5 min followed by 30 cycles of 30 sec at 94ºC, 1 min at 58ºC, 2.5 min at 72ºC, and a final elongation at 72ºC for 10 min. The amplified PCR products were separated by conventional agarose gel electrophoresis in 2% (w/v) horizontal agarose gels as described in Chapter 2, Section 2.1.3.

6.2.4 MLST locus amplification by PCR

Twenty four *C. albicans* isolates recovered during this study were subjected to MLST analysis using the *C. albicans*-specific MLST scheme described by Bougnoux *et al.* (112). *Candida albicans* DNA was amplified in 50 µl reaction volumes consisting of 10 µM of each forward and reverse oligonucleotide primer, 1.5 mM $MgCl_2$, 5 µl of 5X green flexi buffer, 200 µM dNTPs, sterile water and 2.5 U Go Taq DNA polymerase. The oligonucleotide primers used to separately amplify the seven MLST loci are listed in Table 6.1. The cycling conditions consisted of denaturation at 94°C for 2 min followed by 30 cycles of 1 min at 94°C, 1 min 56, 1 min at 72°C, and a final elongation at 72°C for 10 min. The amplified PCR products were separated by conventional agarose gel electrophoresis in 2% (w/v) horizontal agarose gels as described in Chapter 2, Section 2.1.3. Amplified PCR products were purified using a QIAquick 96-well PCR purification kit (Qiagen) as described in Chapter 2, Section 2.1.2. Purified PCR products were normalised to a concentration of 10 ng/µl using molecular biology grade water (Sigma) prior to DNA sequencing. Sequencing reactions were performed commercially as described in Chapter 2, Section 2.1.5, using the same oligonucleotide primers used for amplification.

6.2.5 Sequence analysis

The quality of each sequence obtained was determined by manual examination of sequence chromatograms using ApE software (http://biologylabs.utah.edu/jorgensen/wayned/ape/). Sequences were trimmed to the relevant regions (Table 6.1) in SeqScape 2.6v (Applied Biosystems) based on reference allelic sequences downloaded from the *C. albicans* MLST website (https://pubmlst.org/calbicans/). Complete, trimmed allelic sequences were analysed and exported in FASTA format, from which allele numbers, allelic profiles and DSTs were assigned using the *C. albicans* MLST database (http://pubmed.org/calbicans/).

6.2.6 MLST clade identification and population structure analysis

Phylogenetic analysis was carried out on a dataset composed of the concatenated MLST sequence data from the isolates investigated in the present study as well as a selection of 41 *C. albicans* DSTs (Table 6.2) selected as representatives of 17 MLST clades previously identified in the population structure of *C. albicans* (207). These 41

Table 6.1 Loci and oligonucleotide primer sequences used for *C. albicans* MLST analysis

Allele	Amplicon size (bp)	Size of analysed MLST locus (bp)	Oligonucleotide primers used for amplification and sequencing (5' - 3')	Nucleotide coordinates for the beginning and end of each MLST locus (5' - 3')[a]	5' and 3' sequences at the beginning and end of MLST loci (5' - 3')[b]	Reference
AAT1a	478	373	F-ACTCAAGCTAGATTTTTGGC	81 - 453	5′ AATTGA	(121)
			R-CAGCAACATGATTAGCCC		3′ CGTTTC	
ACC1	519	407	F -GCAAGAGAAATTTTAATTCAATG	3251 - 3657	5′ TTTTGA	(120)
			R-TTCATCAACATCATCCAAGTG		3′ ACAAGA	
ADP1	537	443	F -GAGCCAAGTATGAATGATTTG	928 - 1360	5′ CACGTT	(120)
			R -TTGATCAACAAACCCGATAAT		3′ ATCCAA	
MPI1b	486	375	F -ACCAGAAATGGCCATTGC	454 - 828	5′ TTTAAA	(121)
			R -GCAGCCATGCATTCAATTAT		3′ GAAGCA	
SYA1	543	391	F -AGAAGAATTGTTGCTGTTACTG	2400 - 2790	5′ TAAATC	(120)
			R -GTTACCTTTACCACCAGCTTT		3′ GTATCA	
VPS13	741	403	F -TCGTTGAGAGATATTCGACTT	5151 - 5553	5′ CCTTGA	(120)
			R -ACGGATGGATCTCCAGTCC		3′ TCATGG	
ZWF1b	702	491	F -GTTTCATTTGATCCTGAAGC	903 - 1393	5′ AAAACC	(121)
			R -GCCATTGATAAGTACCTGGAT		3′ AATTAC	

[a] Nucleotide coordinates at which the locus sequence analysed for MLST begins and ends, respectively (5'-3'). Positions are indicated from the first base of the ATG start codon of each housekeeping gene being designated 1.

[b] The first (5') and last (3') six nucleotides identified at each of the seven loci in the most predominant *C. albicans* DST69 are provided for reference.

Abbreviation: bp; base pair, N/A; not applicable, F; forward primer, R; reverse primer.

Table 6.2 Representative DSTs used to assign *C. albicans* isolates to previously identified MLST clades

Clade number	Reference *C. albicans* DST[a]
1	69, 83, 104, 79
2	351, 411, 216
3	863, 726, 494
4	622, 656, 481
5	833, 347, 887
6	447, 566, 377
7	651, 761
8	602, 795
9	467, 918
10	296, 304
11	199, 204
12	307, 719
13	182, 782
14	767, 105
15	660, 517
16	690, 942
17	443, 896

[a]Concatenated MLST sequences for each DST were downloaded from the publicly available *C. albicans* MLST database (https://pubmlst.org/calbicans/). Each reference *C. albicans* DST was selected based on findings previously reported by Odds *et al*. (207).

concatenated sequences were downloaded from the *C. albicans* MLST online database (http://pubmed.org/calbicans/) and included for the purpose of assigning isolates investigated in the present study to previously defined MLST clades. A UPGMA tree was constructed using concatenated MLST sequence data from isolates from the present study and the 41 reference DSTs, a cutoff at *P* distance of 0.04 was used to assign clade numbers to isolates from the present study.

Concatenated MLST datasets resulting from isolates investigated in the present study were also compared to the concatenated MLST sequence data of 19 DSTs identified amongst isolates recovered from patients with periodontal disease and 16 DSTs recovered from orally healthy *Candida* carriers (patients attending the DDUH Accident and Emergency Department) from a previous study carried out in this laboratory (Table 6.3) (10).

The concatenated MLST sequences often contained heterozygous bases denoted by the IUPAC (International Union of Pure and Applied Chemistry) letter codes (K, M, R, S, W or Y), where each letter denotes a specific combination of two heterozygous nucleotides. As the software program BioNumerics 7.6.1 is incapable of recognising these heterozygous IPUAC codes, each site exhibiting heterozygosity was converted into two bases, one base representing each state. Corresponding sites in isolates that were homozygous were converted into two identical bases prior to phylogenetic tree construction (208).

A UPMGA dendrogram and maximum parsimony tree were constructed separately based on the concatenated MLST sequences of DSTs identified among isolates recovered during this study and the 35 DSTs identified among *C. albicans* isolates from the previous study from this laboratory using the software program BioNumerics 7.6.1 (10).

Further *C. albicans* population structure analysis was carried out using a separate algorithm based upon related sequence types (eBURST). The allelic profile and DST datasets of all *C. albicans* DSTs currently included in the *C. albicans* MLST database were used to cluster groups of related genotypes within the entire *C. albicans* population (eburst.mlst.net). Related DSTs were clustered into CCs that were identical at a minimum of six of seven loci and putative founder genotypes were predicted as the DSTs with the largest number of single locus variants (SLVs) in each CC (209).

Table 6.3 MLST datasets and ABC genotypes previously identified among *C. albicans* isolates recovered from patients with periodontal disease and healthy carriers[a]

Isolate	Alternate name[b]	DST	*AAT1a*	*ACC1*	*ADP1*	*MPI1b*	*SYA1*	*VPS13*	*ZWF1b*	CC	MLST clade	ABC genotype
Patients with periodontal disease												
RM36	RMP1dp	69	2	5	5	2	2	6	5	1	1	A
RM3	RMP2dp1	2046	2	3	2	65	2	6	5	1	1	A
RM6	RMP2dp1	2046	2	3	2	65	2	6	5	1	1	A
RM8	RMP2dp1	2046	2	3	2	65	2	6	5	1	1	A
RM15	RMP2dp2	2047	2	3	2	31	2	27	5	S	1	A
RM9	RMP2dp3	2070	2	3	2	2	2	6	20	1	1	A
RM10	RMP2dp4	73	2	3	2	2	2	6	5	1	1	A
RM14	RMP2dp5	804	2	3	2	2	2	20	5	1	1	A
RM1	RMP5dp1	2039	60	7	40	1	7	11	15	12	11	A
RM2	RMP5dp2	2040	60	7	21	1	7	114	15	12	11	A
RM4	RMP5dp3	2071	60	7	21	1	7	11	15	12	11	A
RM5	RMP5dp3	2071	60	7	21	1	7	11	15	12	11	A
RM16	RMP5or	2072	60	7	21	1	6	126	15	S	11	A
RM41	RMP7dp1	360	8	14	8	4	7	3	35	2	4	B
RM43	RMP7dp2	2073	8	7	8	4	7	3	35	2	4	B
RM49	RMP7or	2073	8	7	8	4	7	3	35	2	4	B
RM28	RMP10dp	444	2	5	5	4	2	6	5	1	1	A
RM34	RMP10dp	444	2	5	5	4	2	6	5	1	1	A
RM27	RMP10dp	444	2	5	5	4	2	6	5	1	1	A
RM25	RMP10dp	444	2	5	5	4	2	6	5	1	1	A
RM29	RMP10dp	444	2	5	5	4	2	6	5	1	1	A
RM38	RMP11or	73	2	3	2	2	2	6	5	1	1	A
RM21	RMP12dp	2038	2	2	5	2	2	242	5	1	1	A
RM23	RMP12dp	2038	2	2	5	2	2	242	5	1	1	A
RM17	RMP16dp1	2042	8	3	5	2	2	5	5	1	1	A
RM18	RMP16dp2	2043	8	3	5	2	2	6	5	1	1	A
RM26	RMP16dp3	171	8	3	6	2	2	6	5	1	1	A
RM19	RMP16or	444	2	5	5	4	2	6	5	1	1	A
RM20	RMP16or	444	2	5	5	4	2	6	5	1	1	A
RM11	RMP21or	2044	28	7	38	65	106	122	15	52	8	B
RM12	RM21dp	2045	28	7	38	2	106	122	15	52	8	B
Orally healthy *Candida* carriers												
Ca100	Ca100or	1428	2	23	5	102	2	20	20	S	1	A
Ca102	Ca102or	1660	3	5	5	2	2	76	5	1	1	A
Ca107	Ca107or	1664	4	5	6	2	2	106	5	S	1	A
Ca103	Ca103or	1661	40	24	41	21	4	76	27	S	2	A
Ca106	Ca106or	1663	4	5	4	4	139	26	4	2	2	A
Ca109	Ca109or	1666	4	2	14	4	139	41	67	S	2	A
Ca112	Ca112or	1669	36	2	6	4	49	41	4	S	2	A
Ca105	Ca105or	659	11	26	6	4	34	60	119	9	4	B
Ca104	Ca104or	1662	3	26	6	4	34	60	55	S	4	B
Ca110	Ca110or	1667	8	14	8	4	56	10	8	3	4	A
Ca111	Ca111or	1668	14	14	30	4	56	3	8	S	4	B
Ca108	Ca108or	1665	5	27	37	4	34	105	12	S	11	A
Ca114	Ca114or	1671	4	19	6	4	61	15	201	58	15	A
Ca115	Ca115or	1672	4	60	6	4	4	41	4	S	2	A
Ca116	Ca116or	1673	2	3	10	2	2	94	2	S	1	A
Ca117	Ca117or	1674	13	3	6	34	62	8	47	S	5	A

Continued overleaf

[a] Table adapted from McManus *et al*., 2012 (10).

[b] The origin of each isolate is indicated by its alternate name. Isolates named RMP were recovered from patients with periodontal disease and isolates named Ca were recovered from orally healthy *Candida* carriers. The number in each isolate name represents each individual patient or healthy carrier, and the final lower case letters indicate the anatomical site from which these isolates were recovered.

Abbreviations: DST, diploid sequence type; CC, clonal complex; OR, oral rinse; DP, periodontal pocket; S, singleton.

6.3 Results

6.3.1 ABC genotyping

In total 93 *C. albicans* isolates recovered from patients with periodontal disease, healthy patients with dental implants, and orally healthy carriers and underwent ABC genotyping analysis. Thirty two *C. albicans* isolates recovered from 11 patients with periodontal disease, 35 *C. albicans* isolates recovered from 15 patients with healthy implants, and 27 *C. albicans* isolates recovered from 17 orally healthy carriers were subjected to ABC genotyping as described previously (119). The predominant ABC genotype detected among *C. albicans* isolates recovered from 31/43 (72.1%) patients and participants and from each distinct anatomical site was genotype A (Table 6.4). Isolates identified as genotype B or C were also detected in 10/43 (23.3%) and 5/43 (11.6%) patients/participants respectively.

6.3.1.1 C. albicans *ABC genotypes detected from patients with periodontal disease*

In total, 8/11 (72.7%), 2/11 (18.2%) and 3/11 (27.3%) of patients with periodontal disease yielded ABC genotype A *C. albicans* from oral cavities, healthy subgingival sites and periodontal pockets, respectively (Table 6.4). In addition, five patients with periodontal disease yielded *C. albicans* isolates identified as genotype B [oral cavity (3/11, 27.3%), subgingival sites (1/11, 9%) and periodontal pockets (1/11, 9%)]. Interestingly, two isolates recovered from a healthy subgingival site and a separate periodontal pocket in one patient with periodontal disease were identified as distinct ABC genotypes, A and B, respectively. No ABC genotype C *C. albicans* isolates recovered from patients with periodontal disease (Table 6.4).

6.3.1.2 C. albicans *ABC genotypes detected from healthy patients with implants*

ABC genotype A was also the predominant genotype detected *C. albicans* isolates recovered from the oral cavity (10/15, 66.6%) and subgingival sites (3/15, 20%) of healthy patients with implants (Table 6.4). ABC genotype B isolates were detected in the oral cavities (4/15, 26.7%) and a single subgingival site (2/15, 13.3%) of these patients. ABC genotype C isolates were recovered from the oral cavity (1/15, 6.7%) and healthy subgingival sites (2/15, 13.3%) of patients in the same group (Table 6.4).

Table 6.4 Prevalence of *C. albicans* ABC genotypes detected among patients

Isolates from each subject group (*n*)	Number of patients[a]	ABC genotype[b] (%)		
		A	**B**	**C**
Periodontal disease				
OR (18)	11	8 (72.7)	3 (27.3)	0 (0)
SG (7)		2 (18.2)	1 (9)	0 (0)
PP (6)		3 (27.3)	1 (9)	0 (0)
Healthy implants				
OR (23)	15	10 (66.6)	4(26.7)	1 (6.7)
SG (12)		3 (20)	2 (13.3)	2 (13.3)
Orally healthy adults				
OR (23)	17	13 (76.5)	1 (5.9)	3 (17.6)
SG (4)		1 (5.9)	0 (0)	1 (5.9)

[a] Number of patients from whom *C. albicans* isolates were recovered from at a minimum of one sample site.

[b] Prevalence of *C. albicans* ABC genotypes amongst patients/participants.

Abbreviations: OR, oral rinse; PP, periodontal pocket; SG, subgingival site.

Interestingly, two isolates recovered from the same healthy subgingival site of a healthy patient with implants were identified as distinct ABC genotypes, A and C, respectively.

6.3.1.3 C. albicans *ABC genotypes detected from orally healthy carriers*

For comparison, isolates recovered from orally healthy carriers were also subjected to ABC genotype determination. ABC genotype A *C. albicans* isolates were recovered from the oral cavities of 13 orally healthy carriers (13/17, 76.5%) and a subgingival site from one of these participants (1/17, 5.9%) (Table 6.4). Three *C. albicans* isolates identified as ABC genotype C were recovered separately from the oral cavities of orally healthy carriers (Table 6.4). A further ABC genotype C isolate was recovered from the healthy subgingival site of one of these orally healthy carriers.

6.3.2 MLST analysis

Twenty four *C. albicans* isolates selected as representative isolates of each ABC genotype, anatomical sample site, patient and participant group were subjected to further analysis by MLST.

For each isolate, the presence of homozygous or heterozygous SNPs were identified within each of the seven alleles used for *C. albicans*-specific MLST. Among the 24 isolates subjected to MLST, the total number of SNPs identified for each allele were: *AAT1a*, 10; *ACC1*, 5; *ADP1*, 11; *MPIb*, 11; *SYA1*, 9; *VPS13*, 34; and *ZWF1b*, 8.

A novel *VPS13* allelic sequence was detected in a *C. albicans* isolate recovered from the healthy subgingival site of a healthy patient (Patient I4) with an implant (Table 6.5). This novel allele was designated as *VPS13* allele 292. *VPS13* allele 292 shares 99.26% identity with its closest related allele *VPS13* allele 291. There were three SNPs detected between these alleles.

Overall, 20 distinct DSTs were identified among the 24 *C. albicans* isolates subjected to MLST in this study. In total 13 novel previously undescribed DSTs were identified and designated as DSTs 3128, 3149, 3150 and 3151 – 3159, respectively (Table 6.5).

6.3.3 Population Structure analysis

A UPGMA dendrogram was constructed based on the concatenated MLST sequences identified amongst the isolates investigated in the present study and 41 DSTs chosen as

Table 6.5 Allelic profiles, DSTs, MLST clades, clonal complexes and ABC genotypes identified among *C. albicans* isolates in the present study

Patient	Isolate	Sample site	DST	*AAT1a*	*ACC1*	*ADP1*	*MPI1b*	*SYA1*	*VPS13*	*ZWF1b*	CC	MLST clade[a]	ABC genotype
Patients with periodontal disease													
P5	P5or	OR	3128	2	7	2	4	49	4	4	S	2	A
P5	P5sg	SG	3149	35	7	14	4	49	4	4	3	2	A
P8	P8or	OR	3152	60	10	21	1	34	126	12	6	ND[a]	B
P10	P10or	OR	344	13	10	15	6	7	37	15	4	3	B
P11	P11or	OR	3154	8	3	6	9	2	5	5	S	1	A
P13	P13or	OR	3155	13	10	15	4	7	160	15	4	3	B
P13	P13dp	DP	476	13	10	15	4	7	37	15	4	3	B
P14	P14or	OR	1133	2	5	5	2	2	5	5	1	1	A
P14	P14dp	DP	1133	2	5	5	2	2	5	5	1	1	A
P15	P15or	OR	79	2	5	5	9	2	6	5	1	1	A
P15	P15dp	DP	3156	2	5	5	9	2	5	5	1	1	A
Patients with healthy implants													
I4	I4or1	OR	3158	3	7	21	2	38	116	12	51	7	A
I4	I4or2	OR	3157	3	7	21	2	38	46	12	51	7	A
I4	I4sg1	SG	3159	3	7	21	2	38	292	12	51	7	A
I4	I4sg2	SG	299	4	17	21	19	27	83	22	7	12	C
I5	I5or	OR	3150	35	5	14	9	2	5	5	S	1	A
I6	I6or	OR	3151	8	14	6	4	2	10	8	2	4	C
I6	I6sg	SG	3151	8	14	6	4	2	10	8	2	4	C
I10	I10hi	HI	499	4	14	50	6	4	118	15	43	3	B
I15	I15or	OR	69	2	5	5	2	2	6	5	1	1	A
I20	I20or	OR	185	2	5	5	9	2	24	5	1	1	A
I20	I20sg	SG	185	2	5	5	9	2	24	5	1	1	A
Orally healthy volunteers													
C1	Ca1or	OR	3151	33	14	6	4	7	3	8	2	4	C
C1	Ca1sg	SG	924	8	3	6	9	2	5	5	2	4	C

[a] A UPGMA dendrogram constructed based on the concatenated MLST sequence data of all DSTs listed in this table and 41 reference DSTs from each of the 17 MLST clades was used to assign DSTs identified in the present study to previously defined clades (207) Clade numbers were not defined for DSTs that did not cluster with a reference DSTs.

Abbreviations: DST, diploid sequence type; CC, clonal complex; OR, oral rinse; SG, healthy subgingival site; DP, diseased periodontal pocket; HI, healthy implant; S, singleton; ND, not defined.

representatives of previously defined MLST clades (207). This resulted in 19/20 DSTs identified amongst the *C. albicans* isolates from the present study being assigned to previously designated clades (207). Only one DST (3152) identified in the present study could not be assigned to the previously defined clade structure.

The UPGMA dendrogram revealed that 7/20 (35%) DSTs identified in the present study belonged to Clade 1, 2/20 DSTs belonged to Clade 2, 4/20 DSTs belonged to Clade 3, 3/20 DSTs belonged to Clade 4, 3/20 DSTs belonged to Clade 7, and one DST belonged to Clade 12 (Table 6.5).

6.3.3.1 C. albicans *DSTs identified from patients with periodontal disease*

One ABC genotype B *C. albicans* isolate (DST3152) recovered from the oral cavity of a patient with periodontal disease (Patient 8) could not be assigned to a clade (Table 6.5). Of the remaining six isolates recovered from the oral cavity of patients with periodontal disease, 3/6 (50%) belonged to Clade 1 (ABC genotype A, DSTs 3154, 1133 and 79), two ABC genotype B isolates (DSTs 344 and 3155) belonged to Clade 3 (2/6, 33.3%) and one ABC genotype A isolate (DST3128) belonged to Clade 2 (1/6, 16.7%).

Two *C. albicans* isolates recovered from periodontal pockets of patients with periodontal disease [ABC genotype A (DST 1133, 3156)] were assigned to Clade 1 (2/3, 66.7%) and an ABC genotype B isolate (DST 476) was assigned to Clade 3 (1/3, 33.3%) (Table 6.1). One ABC genotype A *C. albicans* isolate (DST3149) recovered from a healthy subgingival site of a patient with periodontal disease was assigned to Clade 2 (Table 6.5).

6.3.3.2 C. albicans *DSTs identified from patients with healthy implants*

Three (3/5, 60%) ABC genotype A *C. albicans* isolates (DSTs 3150, 69 and 185) recovered from the oral cavities of three different patients with healthy implants belonged to Clade 1 (Fig. 6.1). Two (DSTs 3158 & 3157; 2/5, 40%) ABC genotype A isolates recovered from the oral cavity of patient I4 belonged to Clade 7. Two isolates (ABC genotypes C & A) recovered from the healthy subgingival sites from two patients [DSTs 3151, 185], and an ABC genotype B isolate (DST499) recovered from the healthy subgingival site surrounding an implant belonged to Clades 4, 1, 3 respectively (Table 6.5).

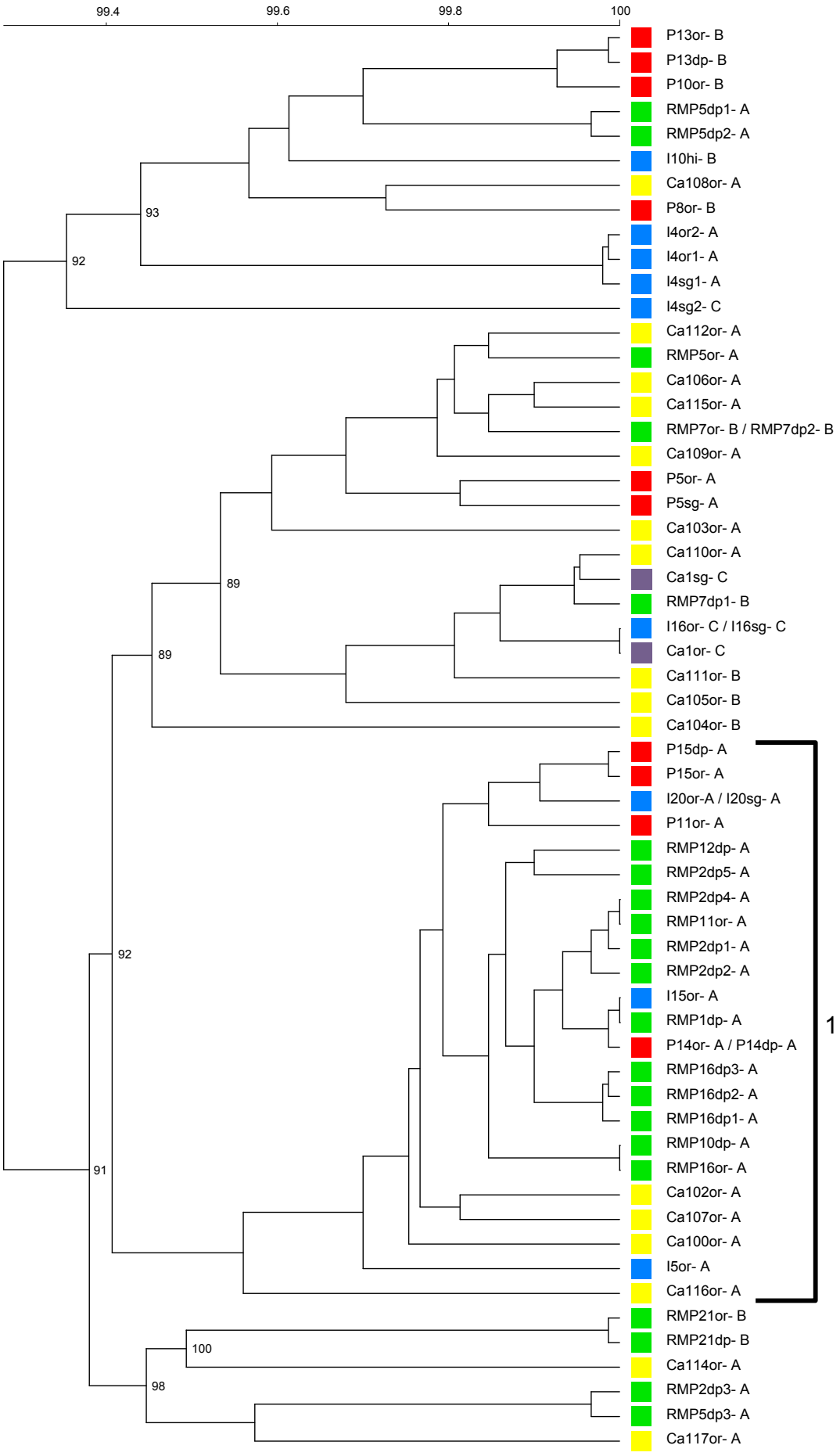

99.4
99.6
99.8
100
P13or- B
P13dp- B
P10or- B
RMP5dp1- A
RMP5dp2- A
I10hi- B
Ca108or- A
P8or- B
I4or2- A
I4or1- A
I4sg1- A
I4sg2- C
Ca112or- A
RMP5or- A
Ca106or- A
Ca115or- A
RMP7or- B / RMP7dp2- B
Ca109or- A
P5or- A
P5sg- A
Ca103or- A
Ca110or- A
Ca1sg- C
RMP7dp1- B
I16or- C / I16sg- C
Ca1or- C
Ca111or- B
Ca105or- B
Ca104or- B
P15dp- A
P15or- A
I20or-A / I20sg- A
P11or- A
RMP12dp- A
RMP2dp5- A
RMP2dp4- A
RMP11or- A
RMP2dp1- A
RMP2dp2- A
I15or- A
RMP1dp- A
P14or- A / P14dp- A
RMP16dp3- A
RMP16dp2- A
RMP16dp1- A
RMP10dp- A
RMP16or- A
Ca102or- A
Ca107or- A
Ca100or- A
I5or- A
Ca116or- A
RMP21or- B
RMP21dp- B
Ca114or- A
RMP2dp3- A
RMP5dp3- A
Ca117or- A
93
92
89
89
92
91
100
98
1

Figure 6.1. UPGMA dendrogram based on concatenated MLST sequences of *C. albicans* isolates recovered in the present study. Isolates recovered from patients with periodontal disease (P) are indicated in red, healthy patients with implants (I) are indicated in blue and two oral carriage isolates recovered from an orally healthy carrier (Ca1or and Ca1sg) are indicated in purple. A reference set of isolates previously recovered from patients with periodontal disease (RMP) and from additional orally healthy carriers were also included in this analysis, indicated in green and yellow, respectively (10). The ABC genotype identified for each isolate is indicated after each isolate name. Isolates belonging to *C. albicans* MLST Clade 1 are indicated by parenthesis. The UPGMA dendrogram was constructed based on percentage nucleotide identity. Bootstrap values indicate the percentage of times the branch arrangment occurred in 1000 randomly generated trees. Overall, isolates recovered in the present study were dispersed throughout the population structure of *C. albicans,* with no obvious clade enrichment with isolates recovered from different patient groups or anatomical sites.

6.3.3.3 C. albicans *DSTs identified from orally healthy carriers*

Two genotype C *Candida albicans* isolates (DSTs 3151 &924) recovered from the oral cavity and a healthy subgingival site of an orally healthy carrier were assigned to Clade 4 (Table 6.5; Fig. 6.1).

6.3.3.4 eBURST analysis

A population structure analysis was also carried out on the entire population of DSTs currently in the *C. albicans* MLST database (accessed 19th September 2016) using the eBurst algorithm (109) (Fig. 6.2). The most predominant CCs are expanded in Fig. 6.3, and individual DSTs identified in this study are labelled within each CC. The DSTs identified amongst isolates investigated in the present study were assigned to CCs 1, 2, 3, 4, 6, 7, 43 and 51 (Table 6.5 and Figures 6.2 & 6.3).

CC1 was identified as the largest CC in the population structure of *C. albicans* and 5/20 DSTs identified amongst 7/24 (29.1%) isolates investigated in the present study were assigned to this CC; all of these isolates were identified as ABC genotype A and also belonged to MLST Clade 1 (Table 6.5; Fig. 6.3). Singletons, defined as DSTs that did not group with any other DSTs in the MLST database, were identified in three *C. albicans* isolates recovered from the oral cavity of two patients with periodontal disease (DSTs 3128 & 3154) and one healthy patient with implants (DST 3150) (Table 6.5 & Fig. 6.2). All of these singleton isolates were identified as ABC genotype A; two of which belonged to Clade 1 and the remaining DST belonged to Clade 2 (Table 6.5).

Two *C. albicans* isolates identified as DSTs 1133 and 79 and belonging to Clade 1 were recovered from the oral cavities (2/7, 28.6%) of two different patients with periodontal disease and were assigned to CC1 (Table 6.5 & Fig. 6.3). Two isolates (DSTs 3128 & 3154) recovered from the oral cavities of two different individuals (2/7, 28.6%) were identified as singletons and they were assigned to different clades (Table 6.5 & Fig. 6.2). Three Clade 3 *C. albicans* isolates (DSTs 344, 3155 & 476) recovered from patients with periodontal disease (2/3 recovered from the oral cavities of two different patients and 1/3 recovered from a periodontal pocket) were assigned to CC4 (Table 6.5 & Fig. 6.3).

Three (3/7, 42.9%) Clade 1 *C. albicans* isolates (DSTs 69 & 185) recovered from healthy patients with implants were assigned to CC1 (Table 6.5; Fig. 6.3). Three (3/7, 42.9%) Clade 7 *C. albicans* isolates (DSTs 3157-3159) recovered from the oral cavity and subgingival site of Patient I4 were assigned to CC51 (Table 6.5; Fig. 6.2).

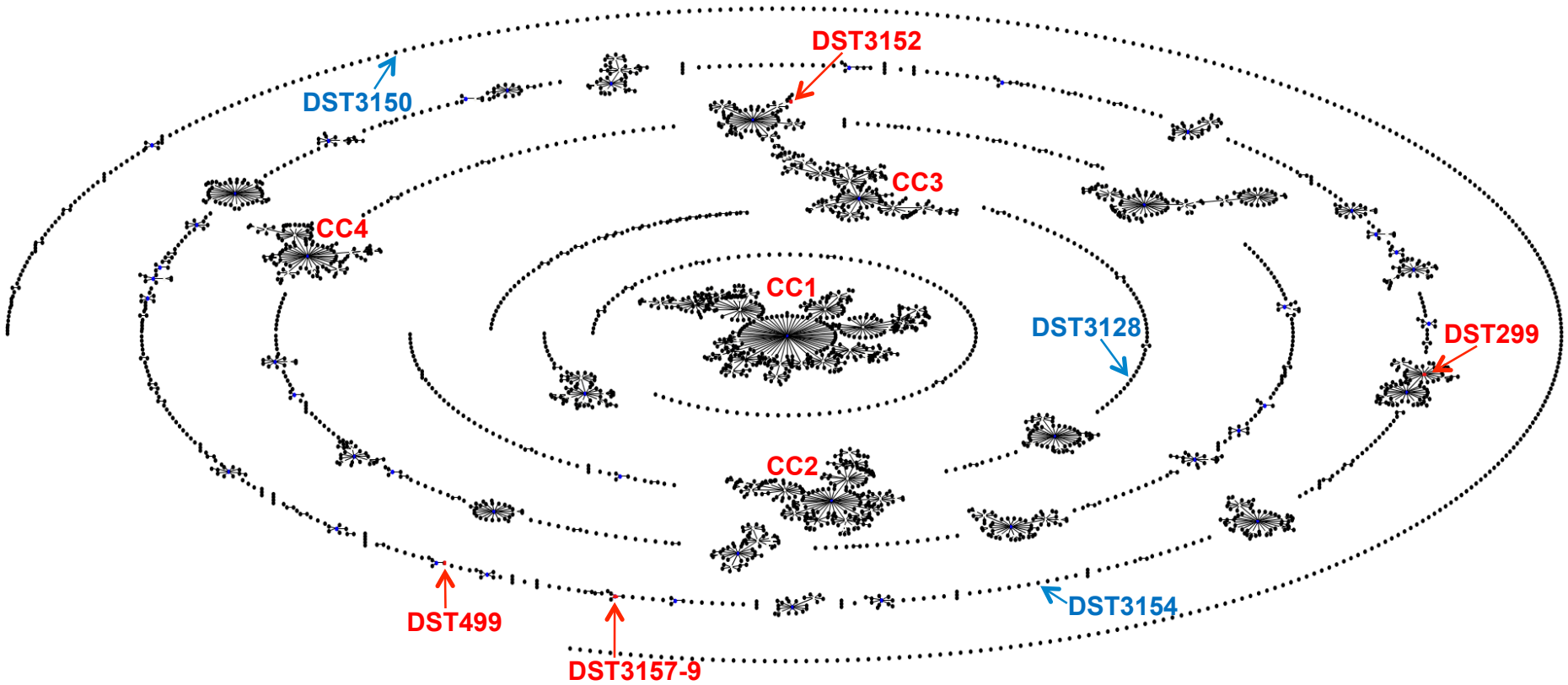


Figure 6.2 eBurst analysis of 3164 unique MLST profiles in the MLST database (http://calbicans.mlst.net/). Each black dot represents a different DST, two dots connected by a line differ by one of the seven alleles (SLV) and a dot with no line connected to it indicates a singleton. Clonal complexes 1- 4 are expanded in Figure 6.3 and DSTs identified in the present study are individually labelled in red typeface. DSTs identified in the present study as singletons are labelled in blue typeface.

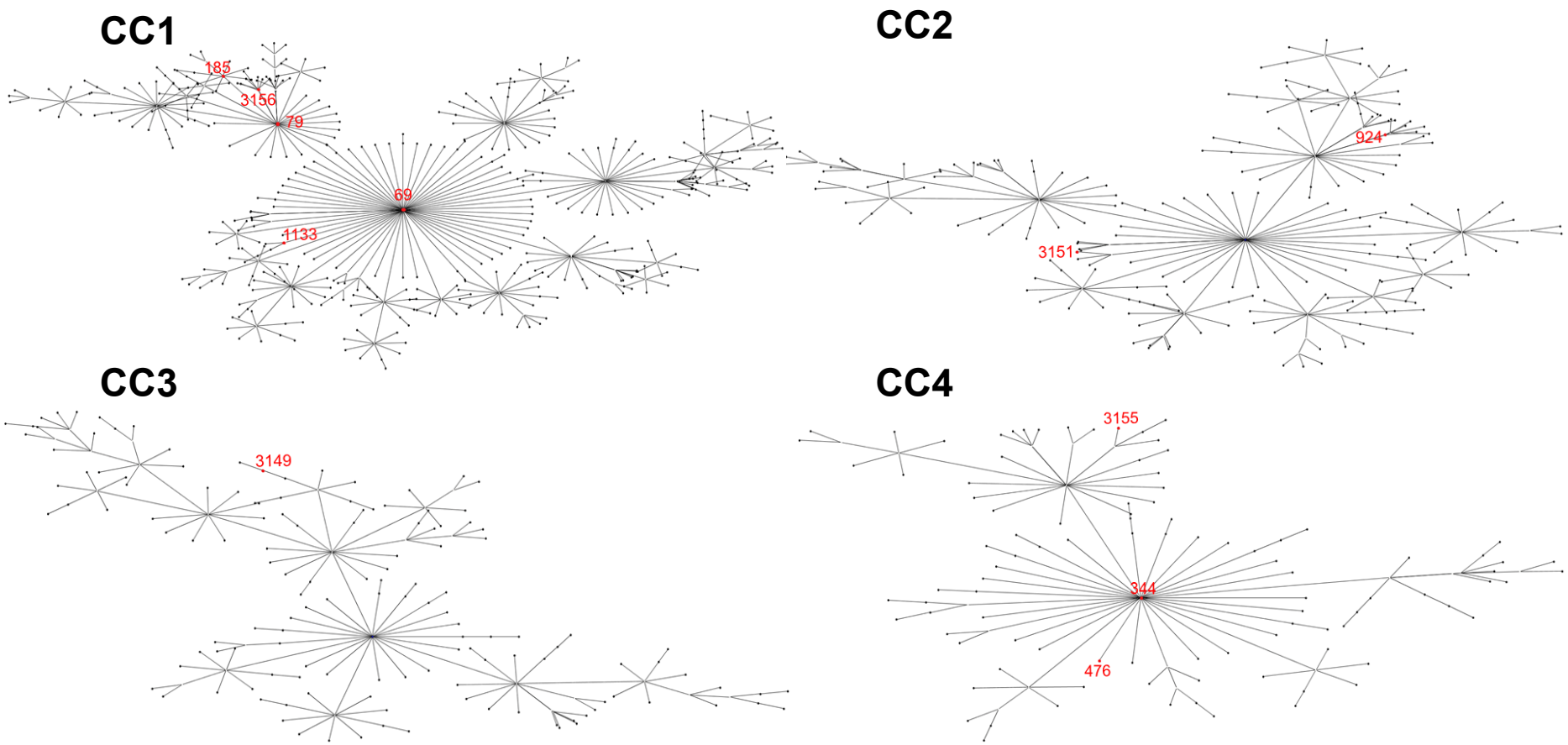


Figure 6.3 eBurst analyses of four distinct clonal complexes in the MLST database (www.calbicans.mlst.net). Each DST is represented by a dot and the line connecting two dots indicates a difference in one of the seven alleles sequenced for MLST. The length of each line is not significant. DSTs identified among isolates subjected to MLST in this study are shown in red font.

Two Clade 4 *C. albicans* isolates (DST3151) recovered from the oral cavity and a subgingival site of patient I6 and assigned to CC2 (Table 6.5; Fig. 6.3).

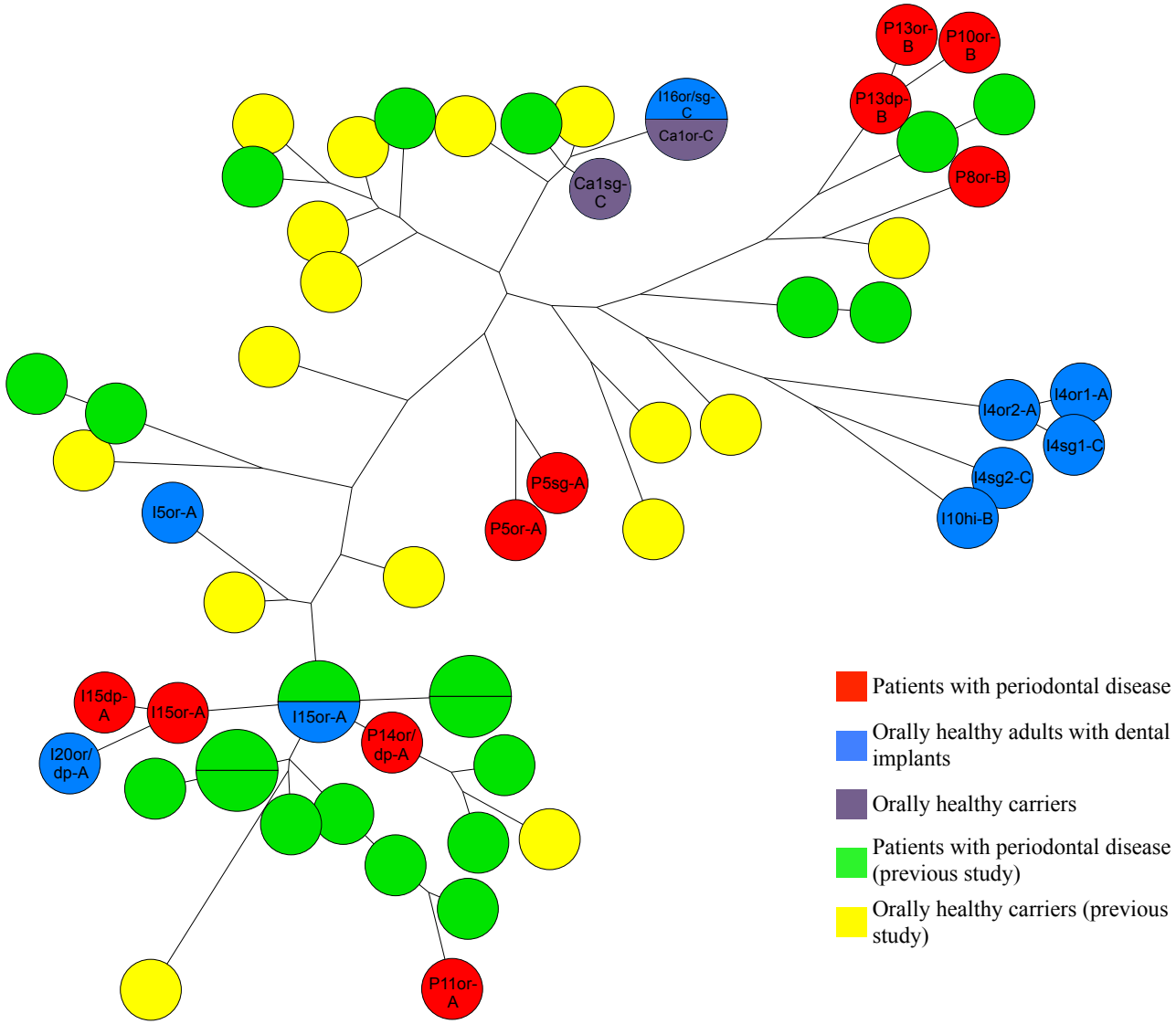
Two *C. albicans* isolates (Clade 4) were recovered from a healthy carrier were assigned to CC2 (Table 6.5; Fig. 6.3).

6.3.4 Epidemiological analysis of recovered isolates

Pairs of *C. albicans* isolates recovered from both oral cavities and subgingival sites of four distinct patients with periodontal disease underwent ABC genotyping and MLST analysis (Table 6.5). These isolates were recovered from the oral cavities and a periodontal pocket of 3/4 patients and isolates recovered from the oral cavity and a healthy subgingival site from the remaining patient were investigated. Each pair of *C. albicans* isolates recovered from the oral cavity and a periodontal pocket of patients with periodontal disease were identified as the same ABC genotype and assigned to the same MLST clade (Table 6.5; Fig. 6.1). Despite this, pairs of isolates recovered from the same patient were identified as the same DST (DST 1133) on one occasion only (Table 6.5). A maximum parsimony tree was constructed based on the concatenated MLST sequences identified amongst the isolates investigated in the present study and 35 DSTs identified amongst oral and subgingival *C. albicans* isolates recovered in Ireland previously (Tables 6.3 & 6.5, Fig. 6.4)(10).

Interestingly, two isolates recovered from the oral cavity (DST3155) and periodontal pocket (DST476) respectively of Patient P13 differed at allele *VPS13* by only one SNP. Similarly, two isolates recovered from the oral cavity (DST79) and periodontal pocket (DST3156) respectively of Patient P15 also differed at allele *VPS13* by one SNP. At both SNP sites, a loss of heterozygosity was observed. The pair of DSTs identified from Patient P5 were found to differ at two alleles (Table 6.5) and within each of these alleles there are multiple nucleotide differences.

Identical DSTs were identified in pairs of *C. albicans* isolates (one recovered from the oral cavity and one recovered from a healthy subgingival site) recovered from two healthy patients (I6 and I20) with implants (I6 and I20; Table 6.5; Fig. 6.1 & 6.4). One healthy patient with implants had four *C. albicans* isolates ABC genotyped and analysed by MLST. A loss of heterozygosity at a SNP in allele *VPS13* was observed between two isolates recovered from the oral cavity (DSTs 3158 & 3157) and one isolate recovered from a subgingival site (DST3159) (Fig. 6.1 & 6.4).



Continued overleaf

Figure 6.4 Maximum parsimony tree illustrating the variation in DSTs identified among *C. albicans* isolates. This tree illustrates the minimum number of evolutionary steps required to generate the variation in DSTs identified among *C. albicans* isolates in the present study and in DSTs identified in a similar group of *C. albicans* isolates recovered in a previous study (Tables 6.3 & 6.5) (10). Red nodes indicate DSTs identified in isolates recovered from patients with periodontal disease, blue nodes indicate DSTs identified in isolates recovered from healthy patients with implants and purple nodes indicate DSTs identified in isolates recovered from orally healthy carriers in the present study. The lettering on each node indicates the patient and anatomical site the DST was recovered from and the ABC genotype of the DST (Table 6.5). Green and yellow nodes indicate DSTs previously identified in isolates recovered from patients with periodontal disease and the orally healthy population, respectively. Larger nodes split in half by a black line represent the same DSTs recovered from two different patients.
Abbreviations: OR, oral rinse; DP, periodontal pocket; SG, subgingival site; HI, healthy subgingival site surrounding a dental implant.

A UPGMA dendrogram was constructed based on the concatenated MLST sequences identified amongst the isolates investigated in the present study and 35 DSTs identified amongst oral and subgingival *C. albicans* isolates recovered from orally healthy *Candida* carriers and patients with periodontal disease in Ireland that had been subjected to MLST previously (Tables 6.3 & 6.5, Fig. 6.1) (10). Overall only one DST was identified in both studies; DST69 was identified in the oral cavity of a healthy patient with implants (isolate I15or) in the present study and in the periodontal pocket of a patient with periodontal disease in the previous study (isolate RMP1dp) (Fig. 6.1).

6.4 Discussion

6.4.1 Study objectives

The objective of this part of the present study was to comparatively characterise the *Candida* population recovered from the oral cavities, subgingival sites and periodontal pockets of patients with periodontal disease, healthy patients with implants and orally healthy *Candida* carriers using ABC genotyping and MLST analysis. Isolates recovered during the present study were used to investigate whether there are any sub-populations of *C. albicans* associated with distinct anatomical sites in patients with periodontal disease, dental implants and orally healthy carriers. These isolates were comparatively characterised to investigate if there was any association of DSTs or ABC genotype with each of the two patient groups and orally healthy carriers, or with a range of sample sites including periodontal pockets, subgingival sites around natural teeth and oral implants and the oral cavity in general. To date, no population analysis of *C. albicans* has been carried out on isolates recovered from multiple distinct oral environments within multiple different patient groups, and no such studies have examined the population of *C. albicans* in the oral cavities of healthy patients with implants.

6.4.2 Population structure based on ABC genotyping and MLST

Previous research identified *C. albicans* as the predominant *Candida* species recovered from the mouths of individuals with oral disease and orally healthy *Candida* carriers (10, 135, 138, 139). Similarly, the present study identified *C. albicans* as the most prevalent oral *Candida* species (Chapter 3, Section 3.3.1).

In total, 93 *C. albicans* isolates recovered during this study were subjected to ABC genotyping analysis. The most prevalent ABC genotype identified amongst isolates recovered from the oral cavities, subgingival sites and periodontal pockets of the two patient groups and orally healthy carriers was genotype A (Table 6.4). Previously ABC genotyping analysis has not been carried out on *C. albicans* isolates recovered from subgingival sites of healthy patients with implants or orally healthy carriers. This result is in agreement with previous research that showed that ABC genotype A isolates predominated among oral isolates of patients with oral or underlying diseases such as periodontal disease or APECED, respectively (10, 137).

Twenty four *C. albicans* isolates selected as representatives of different patient groups, distinct anatomical sites and each ABC genotype detected underwent MLST

analysis. A total of 23/24 of the *C. albicans* isolates subjected to MLST were assigned to previously described MLST clades (207). Interestingly, the ABC genotypes and clades to which these 23 isolates belonged (Table 6.5) correlated with a previous large-scale population analysis of *C. albicans* (207). This previous investigation revealed that Clades 1 and 3 are enriched with ABC genotype A and B isolates, respectively. In the present study, all isolates belonging to Clade 1 were identified as ABC genotype A (9/9, 100%) (Table 6.5) and all isolates belonging to Clade 3 were identified as ABC genotype B (4/4, 100%). Five isolates recovered from two healthy patients with implants and an orally healthy *Candida* carrier were identified as ABC genotype C and these isolates were assigned to Clades 4 (4/5, 80%) and 12 (1/5, 20%) (Table 6.5) which have previously been associated with isolates recovered from blood samples (207). Interestingly, 3/5 ABC genotype C isolates were recovered from subgingival sites, which are proximal to the gingival blood supply. It is possible a correlation exists between ABC genotype C isolates and subgingival sites, however further research is needed to establish a connection due to the limited number of isolates investigated in the present study.

Previously, researchers revealed that isolates recovered from oral rinse samples and periodontal pockets of patients with periodontal disease belonged predominantly to Clade 1, and ABC genotype C isolates were not recovered from these sites (10). Similarly, the present study revealed that almost half (5/11, 45.5%) of isolates recovered from patients with periodontal disease grouped within this clade and identified no genotype C isolates (Fig. 6.1). A previous study reported an enrichment of ABC genotype B isolates from the oral cavities of patients with fixed orthodontic devices compared to orally healthy participants (202). In contrast, only 1/11 (9.1%) isolates recovered from healthy patients with implants in the present study were ABC genotype B (Table 6.5). Five ABC genotype C isolates were detected (three isolates recovered from two healthy patients with implants and two isolates from an orally healthy carrier) in the present study and 4/5 of these isolates were closely related to each other according to MLST (Fig. 6.1).

6.4.3 Epidemiological analysis of *C. albicans* in patients with periodontal disease and healthy implants

Candida albicans isolates recovered from the oral cavity, subgingival sites and periodontal pockets of the same patient with periodontal disease were typically identified as distinct but closely related DSTs (maximum difference of 10 bp between pairs of isolates) in the majority of patients. Due to the small number of isolates subjected to MLST analysis during the present study, MLST data from isolates collected from patients with periodontal disease during a previous study were compared with the isolates collected during the present study (10). Both of these sets of isolates were collected within the Dublin Dental University Hospital and both studies were based on patients recruited according to similar inclusion and exclusion criteria (10). However, it is important to note that these two sets of isolates were recovered five years apart.

Four patients with periodontal disease from the present study and four patients with periodontal disease from the previous study provided two or more *C. albicans* isolates from multiple anatomical sites for MLST analysis (Tables 6.3 & 6.5) (10).

Seven patients with periodontal disease (7/8, 87.5%) yielded isolates from the oral cavity and subgingival sites and/or periodontal pockets that were identified as distinct DSTs, differing at between one and four loci and at a maximum of 10 bp in total (Tables 6.3 & 6.5) (10). The differences between loci in pairs of isolates recovered from 6/7 patients were due to LOH, but there was no association between sample site and LOH. Pairs of isolates recovered in both studies from patients with periodontal disease contained similar numbers of SNPs, with the exception of a pair of isolates recovered from one patient during the previous study, which differed at four loci. The remaining seven pairs of isolates (recovered during both the past and present studies) consisted of DLVs and SLVs of each other only (Tables 6.3 & 6.5) (10). One healthy patient with implants (1/3, 33.3%) and another orally healthy carrier (1/1, 100%) yielded isolates from the oral cavity and subgingival sites that were identified as distinct DSTs (both sets of isolates differed at six loci by 21-27 bp) (Table 6.5).

In the present study multiple isolates recovered from subgingival sites of one patient (Patient I4) were analysed by MLST (Table 6.5). The four isolates (two recovered from the oral cavity and two recovered from subgingival sites) from Patient I4 were identified as different DSTs, however three of the isolates differed at only one locus by 1 bp, the fourth isolate (I4sg2) differed to the other three isolates at six loci by

27 bp in total (Table 6.5). It would be interesting to analyse multiple isolates recovered from the same periodontal pocket or subgingival site to investigate if these sites are enriched with different DSTs unlike the oral cavity, which tends to have one predominant DST.

Based on the *C. albicans* isolates analysed in the present study, no clade or ABC genotype enrichment could be confirmed in isolates recovered from multiple distinct oral sites in patients with periodontal disease, healthy patients with implants and orally healthy carriers, although the findings of the present study did support the results of previous investigations (10). MLST analysis on a greater number of isolates from the current patient cohort is required to confirm any trends identified in the present study, particularly in healthy patients with implants.

Few studies have specifically compared the population of *C. albicans* recovered from subgingival sites and periodontal pockets in comparison to those recovered from the rest of the oral cavity. The present study identified a diverse range of ABC genotypes recovered from different anatomical sites and patient groups. Population analysis on a greater number of isolates is needed to identify any existing *C. albicans* subpopulations in periodontal pocket and subgingival site environments, such as the possible association of ABC genotype C isolates and subgingival sites.

Unfortunately, an association between *C. albicans* DSTs and periodontal pockets that could be used as a marker for periodontal disease progression has not been identified to date. The high prevalence of Clade 1 ABC genotype A isolates recovered from periodontal pockets is most probably due to trafficking of commensal isolates from the oral cavity. As previously mentioned the abundance of *C. albicans* was higher in the oral cavities of patients with periodontal disease compared to the other participant groups (Chapter 3, Section 3.3.2.4). It is possible the larger size of the periodontal pocket compared to subgingival sites could enable greater trafficking of oral commensals. A larger study to characterise the population of *C. albicans* in subgingival sites in orally healthy carriers could identify if there is a diversity of ABC genotypes in these sites that is being lost during periodontal disease.

Chapter 7

General Discussion

7.1 Prevalence and abundance of staphylococcal and *Candida* species in the oral cavity

Many of the previously published studies that investigated oral staphylococci species typically investigated the prevalence of specific staphylococcal species (usually *S. aureus*) in the oral cavity, or typically investigated only one specific anatomical site such as periodontal pockets (24, 126, 128, 130). This has somewhat distorted perceptions of staphylococcal populations, especially CoNS species such as *S. epidermidis*, in the oral cavity during health and disease. The possible influence of CoNS and *Candida* species in the oral cavity as part of the oral microbiome and the potential contribution of these species to the progression of periodontal disease has been, to a large extent, overlooked to date.

The present study is the first to investigate and compare staphylococcal and *Candida* species recovered from the oral cavity in general and from distinct anatomical sites (e.g. subgingival sites around natural teeth and healthy dental implants, and periodontal pockets) across different participant groups including patients with periodontal disease, healthy patients with implants, and orally healthy participants. This study is also the first to identify oral staphylococcal isolates unequivocally using MALDI-TOF. Many previous studies used a variety of phenotypic and/or genotypic methods that were significantly less discriminatory. Furthermore, the majority of these studies failed to carry out any detailed population or genotypic analysis of the recovered isolates. *Staphylococcus aureus* and *S. epidermidis* isolates recovered during this study underwent detailed genotypic analysis by DNA microarray profiling, while a selection of *C. albicans* isolates recovered were investigated by ABC genotyping MLST as a means of population analysis.

The results of the present study showed that staphylococcal and *Candida* species were always more prevalent in oral rinse samples compared to subgingival sites or periodontal pockets (Table 3.2). The higher prevalence of both these species in oral rinse samples suggests that the oral trafficking of these organisms occurs in the direction from oral cavity into subgingival sites or periodontal pockets. *Staphylococcus epidermidis* was the predominant staphylococcal species identified across all distinct sample sites and participant groups. This species was almost significantly more prevalent (p = 0.0501) and significantly more abundant (p = 0.0326) in oral rinse samples of patients with periodontal disease compared to orally healthy participants.

Staphylococcus epidermidis was also significantly more prevalent (p = 0.0189) and abundant (p = 0.0024) in periodontal pocket of patients with periodontal disease compared to subgingival sites in orally healthy participants (Tables 3.2 and 3.3). A similar increased association between *S. epidermidis* and oral disease could also be identified between patients with peri-implantitis and healthy patients with implants. A previous study from this laboratory reported the mean cell density of *S. epidermidis* around peri-implant pockets and subgingival sites in patients with peri-implantitis to be 17 times higher (132) than the abundance of *S. epidermidis* recovered from subgingival sites of healthy patients with implants in the present study (Table 3.3). This previous study used similar sampling techniques to the present study, however samples were cultured on MSA rather than Sa*Select*™.

Periodontal disease and peri-implantitis are both chronic inflammatory diseases that develop in response to an increased prevalence of oral biofilm (i.e. plaque) and dysbiosis (7, 14, 20). The increased prevalence and abundance of *S. epidermidis* in both of these disease states compared to orally healthy individuals strongly suggests that *S. epidermidis* could readily build and contribute to biofilm formation in the diseased environment. However, a direct role for *S. epidermidis* in periodontal disease and peri-implantitis cannot be extrapolated based on the findings of the present study. It is more likely that this species plays an indirect role in disease progression, or could out-compete other microorganisms due to its ability for biofilm formation at the disease site.

Candida albicans is considered an opportunistic pathogen of the oral cavity and an oral commensal in approximately 40% of healthy individuals (98, 135). In stark contrast, *S. epidermidis* is not considered an oral commensal despite the recovery of this species in 74.9% of all oral rinse samples investigated in the present study. The prevalence of *S. epidermidis* in the oral cavity ranges from 41.1 – 84% amongst previous studies depending on the detection and identification methods used (125, 133, 150). In the present study, the use of a chromogenic medium (i.e. Sa*Select*™) that efficiently discriminated *S. epidermidis* from *S. aureus* and other CoNS species, and MALDI-TOF based identification (29, 144) enabled very accurate determination of the prevalence of *S. epidermidis*. This revealed that this species should not only be considered a commensal of the skin, but also of the oral cavity, being detected in 67.2% of oral rinse samples from orally healthy participants in the present study. Additional independent research investigations into the prevalence of this species in the oral cavity using standardised methodology should be undertaken to facilitate determination of the

prevalence of *S. epidermidis* in the oral cavity of healthy individuals of different ages and ethnic backgrounds.

Another important finding of this research was the frequent co-isolation of *S. epidermidis* and *C. albicans* in oral rinse samples, particularly from patients with periodontal disease and healthy patients with implants. Both these species are commonly associated with biofilm formation and are most likely better adapted to the oral cavity. It is possible that they can out-compete other species, such as *S. aureus*, *C. glabrata* and CoNS species to form biofilm on natural teeth or on the titanium-alloy surface of dental implants. Interestingly, in the present study the average abundance of *C. albicans* was 4-6.2 fold higher in subgingival sites of both healthy patients with dental implants and patients with periodontal disease compared to periodontal pockets of patients with periodontal disease (Table 3.5). In contrast, the abundance of *S. epidermidis* was two-fold higher in the periodontal pockets compared to subgingival sites of patients with periodontal disease investigated (Table 3.3). The anaerobic environment of the periodontal pocket influences the types of species that can thrive in it, and the findings of the present study may indicate that *S. epidermidis* is better adapted to this environment than *C. albicans*, despite the fact that *S. epidermidis* is primarily an aerobic organism. It is highly likely that carriage of the ACME element by *S. epidermidis* facilitates its ability to persist in this anaerobic environment.

7.2 Population and genotypic analysis

Previous studies that investigated the prevalence of staphylococcal species in the oral cavities of patients with periodontal disease unfortunately did not undertake any molecular-based typing or characterisation of the species present (123–126, 128). Molecular typing and characterisation provides reliable epidemiological data that can be used to identify patterns of transmission, or if there is enrichment of species-specific clones in association with disease. The present study utilised DNA microarray profiling to investigate the population of 78 MSSA isolates recovered from the oral cavity, and MLST analysis on a representative 26 *S. epidermidis* isolates recovered from oral cavities across the three participant groups (Table 4.1). Overall, no CC enrichment was identified in the oral cavity among the MSSA isolates investigated and similarly the 26 *S. epidermidis* isolates investigated belonged to 10 distinct STs. These findings indicate there is likely no enrichment of specific staphylococcal CCs or STs in the oral cavity,

either in health or disease and therefore these could not be used as a predictive marker for oral health status. Similarly, 20/24 of the *C. albicans* isolates investigated belonged to different DSTs, and no clade enrichment was associated with specific sample sites. Overall, the population analyses from the present study showed there is are diverse populations of *S. aureus*, *S. epidermidis,* and *C. albicans* in the oral cavity. This research did not detect any specific ST enrichment of any of the species investigated in oral rinse samples, subgingival sites, or periodontal pockets.

The present study is one of the first to genotypically characterise *S. aureus* and *S. epidermidis* isolates recovered from the oral rinse samples, subgingival sites and periodontal pockets of patients with periodontal disease, and the oral rinse samples and subgingival sites of healthy patients with implants and orally healthy controls using DNA microarray technology. Only 1.3% of *S. aureus* isolates investigated were identified as MRSA and the MSSA isolates investigated predominantly lacked genes encoding resistance to antimicrobial agents. The most prevalent virulence factor genes in MSSA isolates were associated with the lysogenic bacteriophage encoded IEC, which is responsible for evasion of the host's immune response.

In contrast to the MSSA isolates, a greater diversity of genes associated with antimicrobial resistance were detected in the MSSE and MRSE isolates profiled. Genes encoding resistance to methicillin, fusidic acid, macrolide, tetracycline, mercury, quaternary ammonium compounds, mupirocin and trimethoprim were detected in the *S. epidermidis* isolates analysed. It should be noted that previous studies have also shown that antimicrobial resistance genes are more prevalent among *S. epidermidis* isolates than MSSA isolates (27, 33).

As a typical commensal organism, *S. epidermidis* harbours fewer virulence factor genes than *S. aureus.* In the present study, DNA microarray profiling technology was used to detect the prevalence of virulence factor genes such as *fnbB* and the ACME-*arc* operon in *S. epidermidis*. The *fnbB* gene encodes the fibronectin-binding protein B (FnbB), which is an MSCRAMM and can bind to the mammalian extracellular protein fibronectin and has been shown to significantly contribute to tissue colonisation (70). The prevalence of *fnbB* harboured by *S. epidermidis* isolates was higher in isolates recovered from periodontal pocket (5/9, 55%) from patients with periodontal disease compared to isolates recovered from subgingival sites of the other participant groups [0/9 (0%) healthy patients with implants, and 0/5 (0%) orally healthy participants] (Fig. 4.2). This suggests that *fnbB* may possibly play a role in colonisation

of, and biofilm formation in periodontal pockets, further investigation into the prevalence of this gene in a greater number of isolates recovered from these oral sites could establish if there is an enrichment of *S. epidermidis* isolates harbouring *fnbB* in periodontal pockets.

The most important and significant finding from the molecular characterisation of the *S. epidermidis* isolates investigated was the higher prevalence of the ACME-*arc* detected in *S. epidermidis* isolates from subgingival sites and periodontal pockets of patients with periodontal disease compared to isolates recovered from subgingival sites in healthy patients with implants or orally healthy controls. This suggests that ACME may be a contributing factor to the increased prevalence of *S. epidermidis* in these sites, particular in cases of disease. Interestingly, a previous study also reported a significantly increased prevalence of ACME in *S. epidermidis* recovered from peri-implant pockets (132). In order to investigate the potential role of this element in isolates in subgingival sites, the prevalence of different types of ACMEs harboured by these isolates was investigated further.

7.3 The prevalence and characterisation of ACME types

Although ACME was first described in *S. aureus*, the element is thought to have first been assembled in a modular, stepwise manner in *S. epidermidis* (82). Harbouring ACME is thought to increase the competitive fitness of *S. aureus* by contributing to transmission, ability for skin colonisation, and persistence of the isolate (54). Previous studies that investigated the prevalence of ACME in *S. epidermidis* isolates typically screened isolates recovered from clinical infections (154, 169, 170), and currently there is only one published study that investigated the prevalence of the three distinct ACME types in nasal carriage *S. epidermidis* isolates (186). The present study is the first thorough investigation into the prevalence and characterisation of ACME types I, II, and III harboured by *S. epidermidis* isolates recovered from specific anatomical sites in the oral cavities of patients with periodontal disease, healthy patients with implants and orally healthy participants.

The highest prevalence of ACME in *S. epidermidis* isolates was observed among isolates recovered from subgingival sites and periodontal pockets within each participant group investigated (Table 5.2). The highest overall prevalence of ACME was detected in *S. epidermidis* isolates recovered from subgingival sites and periodontal

pockets of patients with periodontal disease. Importantly, ACME-positive isolates were significantly more prevalent (p = 0.017) in periodontal pockets of patients with periodontal disease compared to subgingival sites of orally healthy participants (Fig. 4.2). These findings correlate with previous research from this laboratory that showed the prevalence of ACME in *S. epidermidis* isolates was higher in isolates recovered from peri-implant pockets compared to isolates recovered from the oral cavity (132). These findings suggest harbouring ACME could confer a fitness advantage to *S. epidermidis* in anaerobic and semi-anaerobic subgingival sites in the oral cavity and particularly in diseased sites.

Further investigations into the prevalence of ACME types I (harbouring both the ACME-*arc* and *opp3* operons), II (harbouring the ACME-*arc* operon and lacking *opp3*), and III (lacking ACME-*arc* operon but containing *opp3*) among the *S. epidermidis* isolates recovered from distinct anatomical sites across the three participant groups uncovered the predominance of ACME type II. Overall, ACME type II was detected in 54/69 (78.3%) *S. epidermidis* isolates recovered from the oral cavities of participants in the present study, followed by ACME I (15/69, 21.7%) and ACME III (7/69, 10.1%) (Table 5.2). However, the most striking result to emerge from this investigation was the significantly (p = 0.0001) lower prevalence of *S. epidermidis* harbouring ACME type III from subgingival sites or periodontal pockets (2/22, 9%) compared to ACME types I and II combined (20/22, 90.9%) (Table 5.2).

The higher prevalence of *S. epidermidis* isolates harbouring ACME recovered from subgingival sites and periodontal pockets compared to the oral cavities sampled across all participant groups, in conjunction with the finding that only ACME types I and II predominated in these sites, strongly implicate the *arc*-operon as a contributor to fitness in these distinct oral environments. The *arc*-operon encodes an arginine deaminase pathway, composed of enzymes that catabolise L-arginine and as a result, regulates the internal pH of the bacteria (83, 193). This function could be critical in enabling *S. epidermidis* to persist and traffic to distinct environments in the oral cavity such as periodontal pockets and saliva, both of which can be subject to pH variation (210). Another important advantage of harbouring ACME-*arc* is that in nutrient and oxygen poor environments such as in periodontal pockets, it is expressed constitutively and the generation of ATP from L-arginine catabolism could provide an energy source for *S. epidermidis*. Finally, the catabolism of L-arginine in subgingival sites or periodontal pockets is likely to greatly reduce the availability of this amino acid for

conversion to nitric oxide by the host, which is required to amplify an immune response. Based on the findings of the present study, it could be hypothesised that one of the primary function of ACME-*arc* is to increase the fitness of *S. epidermidis* in semi-anaerobic or anaerobic environments by pH regulation, energy production and host immune response evasion.

Another novel aspect of the present study was the use of WGS to characterise the entire ACME element in a selection of 25 *S. epidermidis* isolates recovered from oral rinse samples, subgingival sites, and periodontal pockets across all participant groups investigated in this study (Figs. 5.3, 5.4 and 5.5). This study was also the first to characterise the structural organisation of ACME type III elements comprehensively using two distinct WGS-based techniques. Based on the location of DRs and similar to ACME types I and II, ACME type III appears to have been assembled in modular stepwise fashion. However, in contrast to the ACME types I and II investigated, the *copA* and SE_0128 genes were internalised in all three ACME III composite elements (Fig. 5.6). Surprisingly all three isolates harbouring ACME type III belonged to ST329, suggesting there could be a possible association between ACME III and ST329. The function of ACME-*opp3* is unknown however, previously this operon has been associated with amino acid uptake, environmental sensing, and possibly a role in antimicrobial resistance (211). The ACME-*opp3* could possibly be remnant of previous genomic rearrangements that occurred in ST329, but did not expand throughout the *S. epidermidis* population because it appears to confer little fitness advantage, instead remaining associated with the original ST329.

Previous studies have reported that the *arcA* gene is highly conserved (>99% nucleotide identity) amongst different *S. epidermidis* isolates (154, 170, 186). Based on the sequences obtained from WGS in the present study, the *arc*-operon was highly conserved, exhibiting 99.8 – 100% nucleotide sequence identity (Table 5.4). The high conservation of ACME-*arc* further suggests that it confers a selective advantage to *S. epidermidis*. Another interesting finding was that although three *S. epidermidis* isolates (120PPC, I9OR1, and I14OR4) recovered from the oral cavities of three distinct participants harboured the same ACME type II, they each harboured different genes associated with resistance to antimicrobial agents. This finding may further support the hypothesis that ACME provides a fitness advantage to *S. epidermidis* in the oral cavity, likely acting as a MGE and antimicrobial resistance gene reservoir in the oral environment also.

7.4 Future work

Recent advances in WGS technologies have led to the development of core genome MLST (cgMLST), enabling more accurate population analysis to be carried out on collections of isolates. The use of cgMLST would be highly beneficial in the investigation of *S. epidermidis* isolates trafficking between distinct anatomical sites such as oral cavity and nares. Such an investigation could also determine if trafficking occurs more frequently in patients with oral diseases such as periodontal disease, peri-implantitis, or angular cheilitis. It would be interesting to investigate if the increased prevalence and abundance of staphylococcal species in the oral cavity associated with these diseases corresponded to a greater number of trafficking events into the nares.

The present study revealed that the three *S. epidermidis* isolates harbouring ACME type III all belonged to the rarely identified ST329. Future research should identify the STs of the remaining six *S. epidermidis* isolates harbouring ACME III recovered during the present study. As all nine isolates harbouring ACME type III were recovered from nine distinct participants, such analysis would further strengthen the suggestion of an association between ACME type III and ST329.

On a wider scale, the association of *S. epidermidis* harbouring ACME and semi-anaerobic or anaerobic environments should be investigated further. It is possible that ACME also contributes to the success of *S. epidermidis* recovered from other clinical infections that provide semi-anaerobic environments. Staphylococcal infections associated with biofilm formation have previously been frequently reported in connection with prosthetic joint replacements and diabetic ulcers. In addition to collecting the clinical *S. epidermidis* isolates, collecting an oral rinse sample from the same patient would allow for a thorough investigation into the possibly trafficking of *S. epidermidis* from the oral cavity to the site of infection using cgMLST.

In the present study, ACME type II was the most prevalent ACME type identified in *S. epidermidis* isolates. An *in vitro* anaerobic biofilm model could be utilised to compare the rate of biofilm formation by three distinct *S. epidermidis* isolates harbouring each of the ACME types, in comparison to a control isolate lacking ACME. This experiment could possibly indicate if the ACME-*arc* operon confers a fitness advantage in anaerobic environments.

7.5 Conclusion

Based on the overall prevalence of *S. epidermidis* detected in the oral cavities of participants across all participant groups in the present study, *S. epidermidis* should be considered a commensal of the oral cavity during both oral health and disease states (Table 3.2). The increased prevalence and abundance of this species along with *C. albicans* during periodontal disease suggests these species could be inadvertently contributing to periodontal disease progression, perhaps through the formation and development of biofilm in subgingival and periodontal pockets.

The present study was the first to investigate specific staphylococcal and *Candida* species populations recovered from the oral cavities of three distinct groups of participants, with or without oral disease. This study revealed the genetic diversity of these species populations, not only between different participant groups, but also within each of the participant groups investigated. This study was also the first to investigate the prevalence of antimicrobial and virulence factor genes in *S. aureus* and *S. epidermidis* isolates recovered from the oral cavities of distinct participant groups. The findings correlate with previous research that has shown that *S. epidermidis* harbours a more diverse range of antimicrobial resistance genes compared to *S. aureus* (27, 33). One of the most important findings to emerge from the present study was that ACME is highly prevalent among *S. epidermidis* from subgingival and periodontal pockets compared to those from oral rinse samples. The further characterisation of a selection of the ACMEs using WGS revealed the diversity of these elements, despite the high conservation of the *arc*-operon itself. This study was also the first to investigate the genetic characterisation of ACME III using WGS. This study confirms previous findings and provides additional evidence that ACME-*arc* provides a fitness advantage to *S. epidermidis* isolates in semi-anaerobic and anaerobic environments.

Bibliography

1. **Aas JA**, **Paster BJ**, **Stokes LN**, **Olsen I**, **Dewhirst FE**. 2005. Defining the Normal Bacterial Flora of the Oral Cavity. J. Clin. Microbiol. **43**:5721–5732.
2. **Xu X**, **He J**, **Xue J**, **Wang Y**, **Li K**, **Zhang K**, **Guo Q**, **Liu X**, **Zhou Y**, **Cheng L**, **Li M**, **Li Y**, **Li Y**, **Shi W**, **Zhou X**. 2015. Oral cavity contains distinct niches with dynamic microbial communities. Environ. Microbiol. **17**:699–710.
3. **Schaudinn C**, **Gorur A**, **Keller D**, **Sedghizadeh PP**, **Costerton JW**. 2009. Periodontitis: an archetypical biofilm disease. J. Am. Dent. Assoc. **140**:978–986.
4. **Takahashi N**. 2005. Microbial ecosystem in the oral cavity: Metabolic diversity in an ecological niche and its relationship with oral diseases. Int. Congr. Ser. **1284**:103–112.
5. **Seymore GJ**, **Ford PJ**, **Cullinan MP**, **Leishman S**, **Yamazaki K**. 2007. Relationship between periodontal infections and systemic disease. Clin. Microbiol. Infect. **13**:3–10.
6. **Igari K**, **Kudo T**, **Toyofuku T**, **Inoue Y**, **Iwai T**. 2014. Association between periodontitis and the development of systemic diseases. Oral Biol. Dent. **2**:4.
7. **Hajishengallis G**. 2014. Periodontitis: from microbial immune subversion to systemic inflammation. Nat. Rev. Immunol. **15**:30–44.
8. **Scannapieco F**, **Bush RB**, **Paju S**. 2003. Associations between periodontal disease and risk for nosocomial bacterial pneumonia and chronic obstructive pulmonary disease: a systematic review. Ann. Periodontol. **8**:54–69.
9. **Kim J**, **Amar S**. 2006. Periodontal disease and systemic conditions: A bidirectional relationship. Odontology **94**:10–21.
10. **McManus BA**, **Maguire R**, **Cashin PJ**, **Claffey N**, **Flint S**, **Abdulrahim MH**, **Coleman DC**. 2012. Enrichment of multilocus sequence typing clade 1 with oral *Candida albicans* isolates in patients with untreated periodontitis. J. Clin. Microbiol. **50**:3335–3344.
11. **Pihlstrom BL**, **Michalowicz BS**, **Johnson NW**. 2005. Periodontal diseases. Lancet **366**:1809–1820.
12. **Loe H, Theilade E JS**. 1965. Experimental Gingivitis in Man. J. Periodontol. **36**:177–187.
13. **Sbordone L**, **Bortolaia C**. 2003. Oral microbial biofilms and plaque-related diseases: microbial communities and their role in the shift from oral health to disease. Clin. Oral Investig. **7**:181–188.
14. **Gatto MR**, **Montevecchi M**, **Paolucci M**, **Landini MP**, **Checchi L**. 2014.

Prevalence of six periodontal pathogens in subgingival samples of Italian patients with chronic periodontitis. New Microbiol. **37**:517–524.

15. **Garcia R**, **Henshaw M**, **Krall E**. 2001. Relationship between periodontal disease and systemic health. Periodontol. 2000 **25**:21–36.
16. **Albandar JM**, **Rams TE**. 2002. Global epidemiology of periodontal diseases : an overview. Periodontol. 2000 **29**:7–10.
17. **Armitage GC**. 1999. Development of a classification system for periodontal diseases and conditions. Ann. Periodontol. **4**:1–6.
18. **Highfield J**. 2009. Diagnosis and classification of periodontal disease. Aust. Dent. J. **54 Suppl 1**:S11-26.
19. **Pye AD**, **Lockhart DEA**, **Dawson MP**, **Murray CA**, **Smith AJ**. 2009. A review of dental implants and infection. J. Hosp. Infect. **72**:104–110.
20. **Belibasakis GN**. 2014. Microbiological and immuno-pathological aspects of peri-implant diseases. Arch. Oral Biol. **59**:66–72.
21. **Mombelli A**, **Lang NP**. 1998. The diagnosis and treatment of peri-implantitis Evidence for a microbial cause of peri-implant infections. Periodontology **17**:63–76.
22. **Algraffee H**, **Borumandi F**, **Cascarini L**. 2012. Peri-implantitis. Br. J. Oral Maxillofac. Surg. **50**:689–694.
23. **Mombelli A**, **Müller N**, **Cionca N**. 2012. The epidemiology of peri-implantitis. Clin. Oral Implants Res. **23**:67–76.
24. **Zhuang L-F**, **Watt RM**, **Mattheos N**, **Si M-S**, **Lai H-C**, **Lang NP**. 2014. Periodontal and peri-implant microbiota in patients with healthy and inflamed periodontal and peri-implant tissues. Clin. Oral Implants Res. **0**:1–9.
25. **Saaby M**, **Karring E**, **Schou S**, **Isidor F**. 2014. Factors influencing severity of peri-implantitis. Clin. Oral Implants Res. **0**:1–6.
26. **Leonhardt A**, **Renvert S**, **Dahlén G**. 1999. Microbial findings at failing implants. Clin. Oral Implants Res. **10**:339–345.
27. **Becker K**, **Heilmann C**, **Peters G**. 2014. Coagulase-negative staphylococci. Clin. Microbiol. Rev. **27**:870–926.
28. **Baird-Parker AC**. 1962. An improved diagnostic and selective medium for isolating coagulase positive staphylococci. J. Appl. Bacteriol. **25**:12–19.
29. **Hirvonen JJ**, **Kerttula AM**, **Kaukoranta SS**. 2014. Performance of Sa*Select*, a chromogenic medium for detection of staphylococci in clinical specimens. J.

Clin. Microbiol. **52**:1041–1044.

30. **Méric G**, **Miragaia M**, **De Been M**, **Yahara K**, **Pascoe B**, **Mageiros L**, **Mikhail J**, **Harris LG**, **Wilkinson TS**, **Rolo J**, **Lamble S**, **Bray JE**, **Jolley KA**, **Hanage WP**, **Bowden R**, **Maiden MCJ**, **Mack D**, **De Lencastre H**, **Feil EJ**, **Corander J**, **Sheppard SK**. 2015. Ecological overlap and horizontal gene transfer in *Staphylococcus aureus* and *Staphylococcus epidermidis*. Genome Biol. Evol. **7**:1313–1328.
31. **El Garch F**, **Hallin M**, **De Mendonça R**, **Denis O**, **Lefort A**, **Struelens MJ**. 2009. StaphVar-DNA microarray analysis of accessory genome elements of community-acquired methicillin-resistant *Staphylococcus aureus*. J. Antimicrob. Chemother. **63**:877–885.
32. **Wielders CLC**, **Vriens MR**, **Brisse S**, **De Graaf-Miltenburg LAM**, **Troelstra A**, **Fleer A**, **Schmitz FJ**, **Verhoef J**, **Fluit AC**. 2001. Evidence for *in-vivo* transfer of *mecA* DNA between strains of *Staphylococcus aureus*. Lancet **357**:1674–1675.
33. **Otto M**. 2009. *Staphylococcus epidermidis*-the "accidental" pathogen. Nat. Rev. Microbiol. **7**:555–567.
34. **Shopsin B**, **Kreiswirth BN**. 2001. Molecular epidemiology of methicillin-resistant *Staphylococcus aureus*. Emerg. Infect. Dis. **7**:323–326.
35. **Shore AC**, **Deasy EC**, **Slickers P**, **Brennan G**, **O'Connell B**, **Monecke S**, **Ehricht R**, **Coleman DC**. 2011. Detection of staphylococcal cassette chromosome *mec* type XI carrying highly divergent *mecA, mecI, mecR1, blaZ,* and *ccr* genes in human clinical isolates of clonal complex 130 methicillin-resistant *Staphylococcus aureus*. Antimicrob. Agents Chemother. **55**:3765–3773.
36. **Wu Z**, **Li F**, **Liu D**, **Xue H**, **Zhao X**. 2015. Novel type XII staphylococcal cassette chromosome *mec* harboring a new cassette chromosome recombinase, CcrC2. Antimicrob. Agents Chemother. **59**:7597–7601.
37. **Katayama Y**, **Ito T**, **Hiramatsu K**. 2000. A new class of genetic element, staphylococcus cassette chromosome *mec*, encodes methicillin resistance in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **44**:1549–1555.
38. **Diep BA**, **Stone GG**, **Basuino L**, **Graber CJ**, **Miller A**, **des Etages S-A**, **Jones A**, **Palazzolo-Ballance AM**, **Perdreau-Remington F**, **Sensabaugh GF**, **DeLeo FR**, **Chambers HF**. 2008. The arginine catabolic mobile element and staphylococcal chromosomal cassette *mec* linkage: convergence of virulence and

resistance in the USA300 clone of methicillin-resistant *Staphylococcus aureus*. J. Infect. Dis. **197**:1523–1530.

39. **Malachowa N**, **Deleo FR**. 2010. Mobile genetic elements of *Staphylococcus aureus*. Cell. Mol. Life Sci. **67**:3057–3071.
40. **Li S**, **Skov RL**, **Han X**, **Larsen AR**, **Larsen J**, **Sørum M**, **Wulf M**, **Voss A**, **Hiramatsu K**, **Ito T**. 2011. Novel types of staphylococcal cassette chromosome *mec* elements identified in clonal complex 398 methicillin-resistant *Staphylococcus aureus* strains. Antimicrob. Agents Chemother. **55**:3046–3050.
41. **Monecke S**, **Coombs G**, **Shore AC**, **Coleman DC**, **Akpaka P**, **Borg M**, **Chow H**, **Ip M**, **Jatzwauk L**, **Jonas D**, **Kadlec K**, **Kearns A**, **Laurent F**, **O'Brien FG**, **Pearson J**, **Ruppelt A**, **Schwarz S**, **Scicluna E**, **Slickers P**, **Tan HL**, **Weber S**, **Ehricht R**. 2011. A field guide to pandemic, epidemic and sporadic clones of methicillin-resistant *Staphylococcus aureus*. PLoS One e17936.
42. **Ito T**, **Hiramatsu K**, **Oliveira DC**, **De Lencastre H**, **Zhang K**, **Westh H**, **O'Brien F**, **Giffard PM**, **Coleman DC**, **Tenover FC**, **Boyle-Vavra S**, **Skov RL**, **Enright MC**, **Kreiswirth B**, **Kwan SK**, **Grundmann H**, **Laurent F**, **Sollid JE**, **Kearns AM**, **Goering R**, **John JF**, **Daum R**, **Soderquist B**. 2009. Classification of staphylococcal cassette chromosome *mec* (SCC*mec*): Guidelines for reporting novel SCC*mec* elements. Antimicrob. Agents Chemother. **53**:4961–4967.
43. **Ito T**, **Okuma K**, **Ma XX**, **Yuzawa H**, **Hiramatsu K**. 2003. Insights on antibiotic resistance of *Staphylococcus aureus* from its whole genome: Genomic island SCC. Drug Resist. Updat. **6**:41–52.
44. **Wisplinghoff H**, **Rosato AE**, **Enright MC**, **Noto M**, **Craig W**, **Archer GL**. 2003. Related clones containing SCC*mec* type IV predominate among clinically significant *Staphylococcus epidermidis* isolates **47**:3574–3579.
45. **Zong Z**, **Peng C**, **Lü X**. 2011. Diversity of SCC*mec* elements in methicillin-resistant coagulase-negative staphylococci clinical isolates. PLoS One e20191.
46. **McManus BA**, **Coleman DC**, **Deasy EC**, **Brennan GI**, **Connell BO**, **Monecke S**, **Ehricht R**, **Leggett B**, **Leonard N**, **Shore AC**. 2015. Comparative genotypes, staphylococcal cassette chromosome mec (SCC*mec*) genes and antimicrobial resistance amongst *Staphylococcus epidermidis* and *Staphylococcus haemolyticus* isolates from infections in humans and companion animals. PLoS One e0138079.
47. **Monecke S**, **Müller E**, **Schwarz S**, **Hotzel H**, **Ehricht R**. 2012. Rapid microarray-based identification of different *mecA* alleles in staphylococci.

Antimicrob. Agents Chemother. **56**:5547–5554.

48. **Kinnevey PM**, **Shore AC**, **Brennan GI**, **Sullivan DJ**, **Ehricht R**, **Monecke S**, **Slickers P**, **Coleman DC**. 2013. Emergence of sequence type 779 methicillin-resistant *Staphylococcus aureus* harboring a novel pseudo staphylococcal cassette chromosome *mec* (SCC*mec*)-SCC-SCC-CRISPR composite element in Irish Hospitals. Antimicrob. Agents Chemother. **57**:524–531.
49. **Luong TT**, **Ouyang S**, **Bush K**, **Lee CY**. 2002. Type 1 capsule genes of *Staphylococcus aureus* are carried in a staphylococcal cassette chromosome genetic element. J. Bacteriol. **184**:3623–3629.
50. **Holden MTG**, **Feil EJ**, **Lindsay J a**, **Peacock SJ**, **Day NPJ**, **Enright MC**, **Foster TJ**, **Moore CE**, **Hurst L**, **Atkin R**, **Barron A**, **Bason N**, **Bentley SD**, **Chillingworth C**, **Chillingworth T**, **Churcher C**, **Clark L**, **Corton C**, **Cronin A**, **Doggett J**, **Dowd L**, **Feltwell T**, **Hance Z**, **Harris B**, **Hauser H**, **Holroyd S**, **Jagels K**, **James KD**, **Lennard N**, **Line A**, **Mayes R**, **Moule S**, **Mungall K**, **Ormond D**, **Quail M a**, **Rabbinowitsch E**, **Rutherford K**, **Sanders M**, **Sharp S**, **Simmonds M**, **Stevens K**, **Whitehead S**, **Barrell BG**, **Spratt BG**, **Parkhill J**. 2004. Complete genomes of two clinical *Staphylococcus aureus* strains: Evidence for the rapid evolution of virulence and drug resistance. Proc. Natl. Acad. Sci. **101**:9786–9791.
51. **Chongtrakool P**, **Ito T**, **Ma XX**, **Trakulsomboon S**, **Tiensasitorn C**, **Jamklang M**, **Chavalit T**, **Song J**, **Kondo Y**. 2006. Staphylococcal cassette chromosome *mec* (SCC*mec*) typing of methicillin-resistant *Staphylococcus aureus*: a proposal for a new nomenclature for SCC*mec* elements. Antimicrob. Agents Chemother. **50**:1001–1012.
52. **Frank DN**, **Feazel LM**, **Bessesen MT**, **Price CS**, **Janoff EN**, **Pace NR**. 2010. The human nasal microbiota and *Staphylococcus aureus* carriage. PLoS One e10598.
53. **Donlan RM**. 2001. Biofilms and device-associated infections. Emerg. Infect. Dis. **7**:277–281.
54. **Diep BA**, **Gill SR**, **Chang RF**, **Phan TH**, **Chen JH**, **Davidson MG**, **Lin F**, **Lin J**, **Carleton HA**, **Mongodin EF**, **Sensabaugh GF**, **Perdreau-Remington F**. 2006. Complete genome sequence of USA300, an epidemic clone of community-acquired meticillin-resistant *Staphylococcus aureus*. Lancet **367**:731–739.
55. **Grundmann H**, **Aanensen DM**, **Van Den Wijngaard CC**, **Spratt BG**,

Harmsen D, **Friedrich AW**, **Sabat AJ**, **Muilwijk J**, **Monen J**, **Tami A**, **Donker T**, **Mittermayer H**, **Krziwanek K**, **Stumvoll S**, **Koller W**, **Denis O**, **Struelens M**, **Nashev D**, **Budimir A**, **Kalenic S**, **Pieridou-Bagatzouni D**, **Jakubu V**, **Zemlickova H**, **Westh H**, **Sorum M**, **Skov R**, **Laurent F**, **Ettienne J**, **Strommenger B**, **Witte W**, **Vourli S**, **Vatopoulos A**, **Vainio A**, **Vuopio-Varkila J**, **Fuzi M**, **Ungvari E**, **Murchan S**, **Rossney A**, **Miklasevics E**, **Balode A**, **Haraldsson G**, **Kristinsson KG**, **Monaco M**, **Pantosti A**, **Borg M**, **Van Santen-Verheuvel M**, **Huijsdens X**, **Marstein L**, **Jacobsen T**, **Simonsen GS**, **Aires-de-Sousa M**, **De Lencastre H**, **Luczak-Kadlubowska A**, **Hryniewicz W**, **Straut M**, **Codita I**, **Perez-Vazquez M**, **Cuevas O**, **Spik VC**, **Mueller-Premru M**, **Haegman S**, **Olsen-Liljequist B**, **Ellington M**, **Kearns A**, **Kock R**, **Mellmann A**, **Becker K**, **Vogel U**. 2010. Geographic distribution of *Staphylococcus aureus* causing invasive infections in Europe: A molecular-epidemiological analysis. PLoS Med. e1000215.

56. **David MZ**, **Daum RS**. 2010. Community-associated methicillin-resistant *Staphylococcus aureus*: Epidemiology and clinical consequences of an emerging epidemic. Clin. Microbiol. Rev. **23**:616–687.

57. **Rossney AS**, **Shore AC**, **Morgan PM**, **Fitzgibbon MM**, **O'Connell B**, **Coleman DC**. 2007. The emergence and importation of diverse genotypes of methicillin-resistant *Staphylococcus aureus* (MRSA) harboring the panton-valentine leukocidin gene (*pvl*) reveal that *pvl* is a poor marker for community-acquired MRSA strains in Ireland. J. Clin. Microbiol. **45**:2554–2563.

58. **Tenover FC**, **Goering R V.** 2009. Methicillin-resistant *Staphylococcus aureus* strain USA300 : origin and epidemiology. J. Antimicrob. Chemother. **64**:441–446.

59. **Stefani S**, **Chung DR**, **Lindsay JA**, **Friedrich AW**, **Kearns AM**, **Westh H**, **MacKenzie FM**. 2012. Meticillin-resistant *Staphylococcus aureus* (MRSA): Global epidemiology and harmonisation of typing methods. Int. J. Antimicrob. Agents **39**:273–282.

60. **Chen J**, **Luo Y**, **Zhang S**, **Liang Z**, **Wang Y**, **Zhang Y**, **Zhou G**, **Jia Y**, **Chen L**, **She D**. 2014. Community-acquired necrotizing pneumonia caused by methicillin-resistant *Staphylococcus aureus* producing Panton-Valentine leukocidin in a Chinese teenager: Case report and literature review. Int. J. Infect. Dis. **26**:17–21.

61. **Khokhlova O**, **Tomita Y**, **Hung WC**, **Takano T**, **Iwao Y**, **Higuchi W**, **Nishiyama A**, **Reva I**, **Yamamoto T**. 2015. Elderly infection in the community due to ST5/SCC*mec*II methicillin-resistant *Staphylococcus aureus* (the New York/Japan clone) in Japan: Panton-Valentine leukocidin-negative necrotizing pneumonia. J. Microbiol. Immunol. Infect. **48**:335–339.

62. **Brennan GI**, **Abbott Y**, **Burns A**, **Leonard F**, **McManus BA**, **O'Connell B**, **Coleman DC**, **Shore AC**. 2016. The emergence and spread of multiple livestock-associated clonal complex 398 methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* strains among animals and humans in the Republic of Ireland, 2010-2014. PLoS One e0149396.

63. **Seybold U**, **Kourbatova E V**, **Johnson JG**, **Halvosa SJ**, **Wang YF**, **King MD**, **Ray SM**, **Blumberg HM**. 2006. Emergence of community-associated methicillin-resistant *Staphylococcus aureus* USA300 genotype as a major cause of health care-associated blood stream infections. Clin. Infect. Dis. **42**:647–656.

64. **Otter JA**, **French GL**. 2011. Community-associated meticillin-resistant *Staphylococcus aureus* strains as a cause of healthcare-associated infection. J. Hosp. Infect. **79**:189–193.

65. **Earls MR**, **Kinnevey PM**, **Brennan GI**, **Lazaris A**, **Skally M**, **O'Connell B**, **Humphreys H**, **Shore AC**, **Coleman DC**. 2017. The recent emergence in hospitals of multidrug-resistant community-associated sequence type 1 and *spa* type t127 methicillin-resistant *Staphylococcus aureus* investigated by whole-genome sequencing: Implications for screening. PLoS One e0175542.

66. **McCormick JK**, **Yarwood JM**, **Schlievert PM**. 2001. Toxic shock syndrome and bacterial superantigens: An update. Ann. Rev. Microbiol. **55**:77–104.

67. **Verkaik NJ**, **Benard M**, **Boelens HA**, **De Vogel CP**, **Nouwen JL**, **Verbrugh HA**, **Melles DC**, **Van Belkum A**, **Van Wamel WJB**. 2011. Immune evasion cluster-positive bacteriophages are highly prevalent among human *Staphylococcus aureus* strains, but they are not essential in the first stages of nasal colonization. Clin. Microbiol. Infect. **17**:343–348.

68. **Diep BA**, **Otto M**. 2008. The role of virulence determinants in community-associated MRSA pathogenesis. Trends Microbiol. **16**:361–369.

69. **Foster TJ**, **Höök M**. 1998. Surface protein adhesins of *Staphylococcus aureus*. Trends Microbiol. **6**:484–488.

70. **Arciola CR**, **Campoccia D**, **Gamberini S**, **Baldassarri L**, **Montanaro L**. 2005.

Prevalence of *cna*, *fnbA* and *fnbB* adhesin genes among *Staphylococcus aureus* isolates from orthopedic infections associated to different types of implant. FEMS Microbiol. Lett. **246**:81–86.

71. **Locke JB**, **Zuill DE**, **Scharn CR**, **Deane J**, **Sahm DF**, **Denys GA**, **Goering R V.**, **Shawa KJ**. 2014. Linezolid-resistant *Staphylococcus aureus* strain 1128105, the first known clinical isolate possessing the *cfr* multidrug resistance gene. Antimicrob. Agents Chemother. **58**:6592–6598.
72. **Shore AC**, **Lazaris A**, **Kinnevey PM**, **Brennan OM**, **Brennan GI**, **Connell BO**. 2016. First report of *cfr*-carrying plasmids in the pandemic sequence type 22 methicillin-resistant *Staphylococcus aureus* staphylococcal cassette chromosome *mec* type IV clone. Antimicrob. Agents Chemother. **60**:3007–3015.
73. **Kassis C**, **Rangaraj G**, **Jiang Y**, **Hachem RY**, **Raad I**. 2009. Differentiating culture samples representing coagulase-negative staphylococcal bacteremia from those representing contamination by use of time-to-positivity and quantitative blood culture methods. J. Clin. Microbiol. **47**:3255–3260.
74. **Huebner J**, **Goldmann DA**. 1999. Coagulase-negative staphylococci: role as pathogens. Annu. Rev. Med. **50**:223–236.
75. **Gara JPO**, **Humphreys H**. 2001. *Staphylococcus epidermidis* biofilms : importance and implications. J. Med. Microbiol. **50**:582–587.
76. **Harris LG**, **Richards RG**. 2006. Staphylococci and implant surfaces: a review. INT. J. Care Inj. **37**:3–14.
77. **Ziebuhr W**, **Hennig S**, **Eckart M**, **Kränzler H**, **Batzilla C**, **Kozitskaya S**. 2006. Nosocomial infections by *Staphylococcus epidermidis*: how a commensal bacterium turns into a pathogen. Int. J. Antimicrob. Agents **28 Suppl 1**:S14-20.
78. **Fürst MM**, **Salvi GE**, **Lang NP**, **Persson GR**. 2007. Bacterial colonization immediately after installation on oral titanium implants. Clin. Oral Implants Res. **18**:501–508.
79. **Renvert S**, **Lindahl C**, **Renvert H**, **Persson GR**. 2008. Clinical and microbiological analysis of subjects treated with Brånemark or AstraTech implants: A 7-year follow-up study. Clin. Oral Implants Res. **19**:342–347.
80. **Büttner H**, **Mack D**, **Rohde H**. 2015. Structural basis of *Staphylococcus epidermidis* biofilm formation: mechanisms and molecular interactions. Front. Cell. Infect. Microbiol. **5:**14.
81. **Rogers KL**, **Fey PD**, **Rupp ME**. 2009. Coagulase-negative staphylococcal

infections. Infect. Dis. Clin. N. Am. **23**:73–98.

82. **Planet PJ**, **Larussa SJ**, **Dana A**. 2013. Emergence of the epidemic methicillin-resistant *Staphylococcus aureus* strain USA300 coincides with horizontal transfer of the arginine catabolic mobile element and *speG*-mediated adaptations for survival on skin. MBio 4(6):e00889-13.

83. **Thurlow LR**, **Joshi GS**, **Clark JR**, **Spontak JS**, **Neely CJ**, **Maile R**, **Richardson AR**. 2013. Functional modularity of the arginine catabolic mobile element contributes to the success of USA300 methicillin-resistant *Staphylococcus aureus*. Cell Host Microbe **13**:100–107.

84. **Joshi GS**, **Spontak JS**, **Klapper DG**, **Richardson AR**. 2011. Arginine catabolic mobile element encoded *speG* abrogates the unique hypersensitivity of *Staphylococcus aureus* to exogenous polyamines. Mol. Microbiol. **82**:9–20.

85. **Pi B**, **Yu M**, **Chen Y**, **Yu Y**, **Li L**. 2009. Distribution of the ACME-*arcA* gene among meticillin-resistant *Staphylococcus haemolyticus* and identification of a novel *ccr* allotype in ACME-*arcA*-positive isolates. J. Med. Microbiol. **58**:731–736.

86. **Otto M**. 2013. Coagulase-negative staphylococci as reservoirs of genes facilitating MRSA infection: Staphylococcal commensal species such as *Staphylococcus epidermidis* are being recognized as important sources of genes promoting MRSA colonization and virulence. Bioessays **35**:4–11.

87. **Shore AC**, **Coleman DC**. 2013. Staphylococcal cassette chromosome *mec*: Recent advances and new insights. Int. J. Med. Microbiol. **303**:350–359.

88. **Kratzer C**, **Rabitsch W**, **Hirschl AM**, **Graninger W**, **Presterl E**. 2007. *In vitro* activity of daptomycin and tigecycline against coagulase-negative staphylococcus blood isolates from bone marrow transplant recipients. Eur. J. Haematol. **79**:405–409.

89. **Shen J**, **Wang Y**, **Schwarz S**. 2013. Presence and dissemination of the multiresistance gene *cfr* in Gram-positive and Gram-negative bacteria. J. Antimicrob. Chemother. **68**:1697–1706.

90. **Flamm RK**, **Mendes RE**, **Ross JE**, **Sader HS**, **Jones RN**. 2013. An international activity and spectrum analysis of linezolid: ZAAPS Program results for 2011. Diagn. Microbiol. Infect. Dis. **76**:206–213.

91. **Pfaller MA**, **Diekema DJ**. 2007. Epidemiology of invasive candidiasis: A persistent public health problem. Clin. Microbiol. Rev. **20**:133–163.

92. **Sardi JCO**, **Scorzoni L**, **Bernardi T**, **Fusco-Almeida AM**, **Mendes Giannini MJS**. 2013. *Candida* species: Current epidemiology, pathogenicity, biofilm formation, natural antifungal products and new therapeutic options. J. Med. Microbiol. **62**:10–24.

93. **McManus BA**, **Coleman DC**. 2014. Molecular epidemiology, phylogeny and evolution of *Candida albicans*. Infect. Genet. Evol. **21**:166–178.

94. **Wisplinghoff H**, **Bischoff T**, **Tallent SM**, **Seifert H**, **Wenzel RP**, **Edmond MB**, **Edmond M**. 2003. Nosocomial bloodstream infections in US hospitals: Analysis of 24,179 cases from a prospective nationwide surveillance study **19**:309–317.

95. **Tapper-Jones LM**, **Aldred MJ**, **Walker DM**, **Hayes TM**. 1981. Candidal infections and populations of *Candida albicans* in mouths of diabetics. J. Clin. Pathol. **34**:706–11.

96. **Schmidt-Westhausen A**, **Schiller HR-A**, **Pohle HD**, **Reichart PA**. 1991. Oral *Candida* and *Entembacteriaceae* in HIV-1 infection: correlation with clinical candidiasis and antimycotic therapy. J. Oral Pathol. Med. **20**:469–472.

97. **Peltroche-Llacsahuanga H**, **Dohmen H**, **Haase G**. 2002. Recovery of *Candida dubliniensis* from sputum of cystic fibrosis patients. Mycoses **45**:15–18.

98. **Akpan A**, **Morgan R**. 2002. Oral Candidiasis. Postgr. Med J **78**:455–459.

99. **Yapar N**. 2014. Epidemiology and risk factors for invasive candidiasis. Ther. Clin. Risk Manag. **10**:95–105.

100. **Odds FC**, **Bernaerts R**. 1994. CHROMagar Candida, a new differential isolation medium for presumptive identification of clinically important *Candida* species. J. Clin. Microbiol. **32**:1923–1929.

101. **Gilfillna GD**, **Sullivan DJ**, **Haynes K**, **Parkinson T**, **Coleman DC**, **Gow NAR**. 1998. *Candida dubliniensis*: phylogeny and putative virulence factors. Microbiology 829–838.

102. **Sullivan DJ**, **Westerneng TJ**, **Haynes K**, **Bennett DE**, **Coleman DC**. 1995. *Candida dubliniensis* sp-nov - Phenotypic and molecular characterization of a novel species associated with oral candidosis in HIV-infected individuals. Microbiology-UK **141**:1507–1521.

103. **Kirkpatrick WR**, **Revankar SG**, **McAtee RK**, **Lopez-Ribot JL**, **Fothergill AW**, **McCarthy DI**, **Sanche SE**, **Cantu RA**, **Rinaldi MG**, **Patterson TF**. 1998. Detection of *Candida dubliniensis* in oropharyngeal samples from human immunodeficiency virus-infected patients in North America by primary

CHROMagar Candida screening and susceptibility testing of isolates. J. Clin. Microbiol. **36**:3007–3012.

104. **Williams DW**, **Wilson MJ**, **Lewis MO**, **Potts JC**. 1985. Identification of *Candida* species by PCR and restriction fragment length polymorphism analysis of intergenic spacer regions of ribosomal DNA. J Clin Microbiol. **33**:2476–2479.
105. **McManus BA**, **Coleman DC**, **Moran G**, **Pinjon E**, **Diogo D**, **Bougnoux ME**, **Borecka-Melkusova S**, **Bujdakova H**, **Murphy P**, **D'Enfert C**, **Sullivan DJ**. 2008. Multilocus sequence typing reveals that the population structure of *Candida dubliniensis* is significantly less divergent than that of *Candida albicans*. J. Clin. Microbiol. **46**:652–664.
106. **Donnelly SM**, **Sullivan DJ**, **Shanley DB**, **Coleman DC**. 1999. Phylogenetic analysis and rapid identification of *Candida dubliniensis* based on analysis of *ACT1* intron and exon sequences. Microbiology **145**:1871–1882.
107. **Murchan S**, **Kaufmann ME**, **Deplano A**, **Struelens M**, **Zinn CE**, **Fussing V**, **Salmenlinna S**, **Vuopio-varkila J**, **Solh E**, **Cuny C**, **Witte W**, **Tassios PT**, **Legakis N**, **Vindel A**, **Laconcha I**, **Garaizar J**, **Haeggman S**, **Olsson-liljequist B**, **Ransjo U**, **Coombes G**, **Cookson B**. 2003. Harmonization of pulsed-field gel electrophoresis protocols for epidemiological typing of strains of methicillin-resistant *Staphylococcus aureus*: a single approach developed by consensus in 10 European laboratories and its application for tracing the spread of related strains. Society **41**:1574–1585.
108. **Maiden MC**, **Bygraves JA**, **Feil E**, **Morelli G**, **Russell JE**, **Urwin R**, **Zhang Q**, **Zhou J**, **Zurth K**, **Caugant DA**, **Feavers IM**, **Achtman M**, **Spratt BG**. 1998. Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proc. Natl. Acad. Sci. U. S. A. **95**:3140–3145.
109. **Feil EJ**, **Li BC**, **Aanensen DM**, **Hanage WP**, **Spratt BG**. 2004. eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. J. Bacteriol. **186**:1518–1530.
110. **Enright MC**, **Day NPJ**, **Davies CE**, **Peacock SJ**, **Spratt BG**. 2000. Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. J. Clin. Microbiol. **38**:1008–1015.
111. **Thomas JC**, **Vargas MR**, **Miragaia M**, **Peacock SJ**, **Archer GL**, **Enright MC**. 2007. Improved multilocus sequence typing scheme for *Staphylococcus*

epidermidis. J. Clin. Microbiol. **45**:616–619.

112. **Bougnoux M**, **Tavanti A**, **Bouchier C**, **Gow NAR**, **Davidson AD**, **Maiden MCJ**, **D'Enfert C**, **Odds FC**. 2003. Collaborative consensus for optimized multilocus sequence typing of *Candida albicans*. J. Clin. Microbiol. **41**:5265–5267.
113. **Shopsin B**, **Gomez M**, **Montgomery SO**, **Smith DH**, **Dodge DE**, **Bost DA**, **Riehman M**, **Kreiswirth BN**, **Waddington M**. 1999. Evaluation of protein A gene polymorphic region DNA sequencing for typing of *Staphylococcus aureus* strains. J. Clin. Invest. **37**:3556–3563.
114. **Kondo Y**, **Ito T**, **Ma XX**, **Watanabe S**, **Kreiswirth BN**, **Etienne J**, **Hiramatsu K**. 2007. Combination of multiplex PCRs for staphylococcal cassette chromosome *mec* type assignment: Rapid identification system for *mec*, *ccr*, and major differences in junkyard regions. Antimicrob. Agents Chemother. **51**:264–274.
115. **Milheiriço C**, **Oliveira DC**, **De Lencastre H**. 2007. Update to the multiplex PCR strategy for assignment of *mec* element types in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **51**:3374–3377.
116. **Monecke S**, **Slickers P**, **Ehricht R**. 2008. Assignment of *Staphylococcus aureus* isolates to clonal complexes based on microarray analysis and pattern recognition. FEMS Immunol. Med. Microbiol. **53**:237–251.
117. **Soll DR**. 2000. The ins and outs of DNA fingerprinting the infectious fungi. Clin. Microbiol. Rev. **13**:332–370.
118. **Pujol C**, **Pfaller M**, **Soll DR**. 2002. Ca3 fingerprinting of *Candida albicans* bloodstream isolates from the United States, Canada, South America, and Europe reveals a European clade. J. Clin. Microbiol. **40**:2729–2740.
119. **McCullough MJ**, **Clemons K V.**, **Stevens D a.** 1999. Molecular and phenotypic characterization of genotypic *Candida albicans* subgroups and comparison with *Candida dubliniensis* and *Candida stellatoidea*. J. Clin. Microbiol. **37**:417–421.
120. **Bougnoux ME**, **Morand S**, **D'Enfert C**. 2002. Usefulness of multilocus sequence typing for characterization of clinical isolates of *Candida albicans*. J. Clin. Microbiol. **40**:1290–1297.
121. **Tavanti A**, **Gow NAR**, **Senesi S**, **Maiden MCJ**, **Odds FC**. 2003. Optimization and validation of multilocus sequence typing for *Candida albicans*. J Clin Microbiol **41**:3765–3776.

122. **Lott TJ**, **Scarborough RT**. 2008. Development of a MLST-biased SNP microarray for *Candida albicans*. Fungal Genet. Biol. **45**:803–811.
123. **Murdoch FE**, **Sammons RL**, **Chapple ILC**. 2004. Isolation and characterization of subgingival staphylococci from periodontitis patients and controls. Oral Dis. **10**:155–162.
124. **Rams TE**, **Feik D**, **Slots J**. 1990. Staphylococci in human periodontal diseases. Oral Microbiol.Immunol. **5**:29–32.
125. **Loberto JCS**, **De Paiva Martins CA**, **Ferreira Dos Santos SS**, **Cortelli JR**, **Cardoso Jorge AO**. 2004. *Staphylococcus* spp. in the oral cavity and periodontal pockets of chronic periodontitis patients. Brazilian J. Microbiol. **35**:64–68.
126. **Cuesta AI**, **Jewtuchowicz V**, **Brusca MI**, **Nastri ML**, **Rosa AC**. 2010. Prevalence of *Staphylococcus spp* and *Candida spp* in the oral cavity and periodontal pockets of periodontal disease patients. Acta Odontol. Latinoam. **23**:20–26.
127. **Albertini M**, **López-Cerero L**, **O'Sullivan MG**, **Chereguini CF**, **Ballesta S**, **Ríos V**, **Herrero-Climent M**, **Bullón P**. 2014. Assessment of periodontal and opportunistic flora in patients with peri-implantitis. Clin. Oral Implants Res. **0**:1–5.
128. **Rafaela B**, **Demeda CF**, **Novaes E**, **Helena M**, **Fonseca M**, **Lima KC**, **Celeste M**, **Melo N De**. 2014. Prevalence of subgingival *staphylococcus* at periodontally healthy and diseased sites. Braz. Dent. J. **25**:271–276.
129. **Ohman SC**, **Dahlén G**, **Möller A**, **Ohman A**. 1986. Angular cheilitis: a clinical and microbial study. J. Oral Pathol. **15**:213–217.
130. **Fritschi BZ**, **Albert-Kiszely A**, **Persson GR**. 2008. *Staphylococcus aureus* and other bacteria in untreated periodontitis. J. Dent. Res. **87**:589–593.
131. **Tuzuner-Oncul AM**, **Ungor C**, **Dede U**, **Kisnisci RS**. 2009. Methicillin-resistant *Staphylococcus aureus* (MRSA) osteomyelitis of the mandible. Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod. **107**:e1-4.
132. **Cashin PJ**. 2013. Molecular characterisation of *Staphylococcus aureus* and *Staphylococcus epidermidis* populations from healthy and diseased oral implant and natural tooth sites in a cohort of patients with periimplantitis. University of Dublin, Trinity College.
133. **Smith AJ**, **Jackson MS**, **Bagg J**. 2001. The ecology of *staphylococcus* species in the oral cavity. J. Med. Microbiol. **50**:940–946.

134. **Jackson MS**, **Bagg J**, **Gupta MN**, **Sturrock RD**. 1999. Oral carriage of staphylococci in patients with rheumatoid arthritis. Rheumatology **38**:572–575.

135. **Arendorf TM**, **Walker DM**. 1980. The prevalence and intra-oral distribution of *Candida albicans* in man. Arch. Oral Biol. **25**:1–10.

136. **Bougnoux M**, **Diogo D**, **François N**, **Sendid B**, **Colombel JF**, **Bouchier C**, **Kruiningen H Van**, **Enfert C**, **Poulain D**, **Franc N**. 2006. Multilocus sequence typing reveals intrafamilial transmission and microevolutions of *Candida albicans* isolates from the human digestive tract **44**:1810–1820.

137. **McManus BA**, **McGovern E**, **Moran GP**, **Healy CM**, **Nunn J**, **Fleming P**, **Costigan C**, **Sullivan DJ**, **Coleman DC**. 2011. Microbiological screening of Irish patients with autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy reveals persistence of *Candida albicans* strains, gradual reduction in susceptibility to azoles, and incidences of clinical signs of oral candidiasis without culture evidence. J. Clin. Microbiol. **49**:1879–1889.

138. **Abdulrahim MH**, **McManus BA**, **Flint SR**, **Coleman DC**. 2013. Genotyping *Candida albicans* from *Candida leukoplakia* and non-*Candida leukoplakia* shows no enrichment of multilocus sequence typing clades but enrichment of ABC genotype C in *Candida* leukoplakia. PLoS One e73738.

139. **Canabarro A**, **Valle C**, **Farias MR**, **Santos FB**, **Lazera M**, **Wanke B**. 2013. Association of subgingival colonization of *Candida albicans* and other yeasts with severity of chronic periodontitis. J. Periodontal Res. **48**:428–432.

140. **Gainza-Cirauqui ML**, **Nieminen MT**, **Novak Frazer L**, **Aguirre-Urizar JM**, **Moragues MD**, **Rautemaa R**. 2013. Production of carcinogenic acetaldehyde by *Candida albicans* from patients with potentially malignant oral mucosal disorders. J. Oral Pathol. Med. **42**:243–249.

141. **Wingard JR**, **Merz WG**, **Saral R**. 1979. *Candida tropicalis*: A major pathogen in immunocompromised patients. Ann. Intern. Med. **91**:539–543.

142. **Bodey GP**, **Mardani M**, **Hanna HA**, **Boktour M**, **Abbas J**, **Girgawy E**, **Hachem RY**, **Kontoyiannis DP**, **Raad I**. 1999. The epidemiology of *Candida glabrata* and *Candida albicans* fungemia in immuncompromised patients with cancer. Am. J. Med. **9343**:2–7.

143. **Jewtuchowicz VM**, **Brusca MI**, **Mujica MT**, **Gliosca LA**, **Finquelievich JL**, **Lovannitti CA**, **Rosa AC**. 2007. Subgingival distribution of yeast and their antifungal susceptibility in immunocompetent subjects with and without dental

devices. Acta Odontol. Latinoam. **20**:17–22.

144. **Daurel C**, **Leclercq R**. 2007. P1604 Evaluation of a new chromogenic medium Sa*Select* for identification of *Staphylococcus aureus*. Int. J. Antimicrob. Agents **29**:p.S451.

145. **Lavigne JP**, **Espinal P**, **Dunyach-Remy C**, **Messad N**, **Pantel A**, **Sotto A**. 2013. Mass spectrometry: A revolution in clinical microbiology? Clin. Chem. Lab. Med. **51**:257–270.

146. **Fell JW**. 1993. Rapid identification of yeast species using three primers in a polymerase chain reaction. Mol. Mar. Biol. Biotechnol. **2**:174–180.

147. **Coco BJ**, **Bagg J**, **Cross LJ**, **Jose A**, **Cross J**, **Ramage G**. 2008. Mixed *Candida albicans* and *Candida glabrata* populations associated with the pathogenesis of denture stomatitis. Oral Microbiol. Immunol. **23**:377–383.

148. **Salvi GE**, **Fürst MM**, **Lang NP**, **Persson GR**. 2008. One-year bacterial colonization patterns of *Staphylococcus aureus* and other bacteria at implants and adjacent teeth. Clin. Oral Implants Res. **19**:242–248.

149. **Canullo L**, **Peñarrocha-Oltra D**, **Covani U**, **Rossetti P**. 2015. Microbiologic and clinical findings of implants in healthy condition and with peri-implantitis. Int. J. Oral Maxillofac. Implants **30**:834–842.

150. **Ohara-Nemoto Y**, **Haraga H**, **Kimura S**, **Nemoto TK**. 2008. Occurrence of staphylococci in the oral cavities of healthy adults and nasal-oral trafficking of the bacteria. J. Med. Microbiol. **57**:95–99.

151. **Biswas SK**, **Chaffin WL**. 2005. Anaerobic growth of *Candida albicans* does not support biofilm formation under similar conditions used for aerobic biofilm. Curr. Microbiol. **51**:100–104.

152. **Muñoz P**, **Hortal J**, **Giannella M**, **Barrio JM**, **Rodríguez-Créixems M**, **Pérez MJ**, **Rincón C**, **Bouza E**. 2008. Nasal carriage of *S. aureus* increases the risk of surgical site infection after major heart surgery. J. Hosp. Infect. **68**:25–31.

153. **Passariello C**, **Puttini M**, **Iebba V**, **Pera P**, **Gigola P**. 2012. Influence of oral conditions on colonization by highly toxigenic *Staphylococcus aureus* strains. Oral Dis. **18**:402–409.

154. **Miragaia M**, **de Lencastre H**, **Perdreau-Remington F**, **Chambers HF**, **Higashi J**, **Sullam PM**, **Lin J**, **Wong KI**, **King KA**, **Otto M**, **Sensabaugh GF**, **Diep BA**. 2009. Genetic diversity of arginine catabolic mobile element in *Staphylococcus epidermidis*. PLoS One e7722.

155. **McDougal LK**, **Steward CD**, **George E**, **Chaitram JM**, **Mcallister SK**, **Tenover C**, **Killgore GE**, **Tenover FC**. 2003. Pulsed-field gel electrophoresis typing of oxacillin-resistant *Staphylococcus aureus* isolates from the United States : establishing a national database. J. Clin. Microbiol. **41**:5113–5120.

156. **NMRSARL**. 2015. National MRSA Reference Laboratory Annual Report. http://www.stjames.ie/Departments/DepartmentsA-Z/N/NationalMRSAReferenceLaboratory/DepartmentinDepth/AnnRpt2015.pdf.

157. **Kelly S**, **Collins J**, **Maguire M**, **Gowing C**, **Flanagan M**, **Donnelly M**, **Murphy PG**. 2008. An outbreak of colonization with linezolid-resistant *Staphylococcus epidermidis* in an intensive therapy unit. J. Antimicrob. Chemother. **61**:901–907.

158. **Koukos G**, **Sakellari D**, **Arsenakis M**, **Tsalikis L**, **Slini T**, **Konstantinidis A**. 2015. Prevalence of *Staphylococcus aureus* and methicillin resistant *Staphylococcus aureus* (MRSA) in the oral cavity. Arch. Oral Biol. **60**:1410–1415.

159. **Rams TE**, **Degener JE**, **van Winkelhoff AJ**. 2014. Antibiotic resistance in human chronic periodontitis microbiota. J. Periodontol. **85**:160–9.

160. **Monecke S**, **Jatzwauk L**, **Weber S**, **Slickers P**, **Ehricht R**. 2008. DNA microarray-based genotyping of methicillin-resistant *Staphylococcus aureus* strains from Eastern Saxony. Clin. Microbiol. Infect. **14**:534–545.

161. **Monecke S**, **Ehricht R**. 2005. Rapid genotyping of methicillin-resistant *Staphylococcus aureus*. Clin. Microbiol. Infect. **11**:825–833.

162. **Thomas JC**, **Godfrey PA**, **Feldgarden M**, **Robinson DA**. 2012. Draft genome sequences of *Staphylococcus aureus* sequence type 34 (ST34) and ST42 hybrids. J. Bacteriol. **194**:2740–2741.

163. **Yamada Y**, **Hideka K**, **Shiota S**, **Kuroda T**, **Tsuchiya T**. 2006. Gene cloning and characterization of SdrM, a chromosomally-encoded multidrug efflux pump, from *Staphylococcus aureus*. Biol. Pharm. Bull. **29**:554–556.

164. **Lina G**, **Quaglia A**, **Reverdy M**, **Leclercq R**. 1999. Distribution of genes encoding resistance to macrolides, lincosamides, and streptogramins among staphylococci. Antimicrob. Agents Chemother. **43**:1062–1066.

165. **Shore AC**, **Brennan OM**, **Deasy EC**, **Rossney AS**, **Kinnevey PM**, **Ehricht R**, **Monecke S**, **Coleman DC**. 2012. DNA microarray profiling of a diverse collection of nosocomial methicillin-resistant *Staphylococcus aureus* isolates

assigns the majority to the correct sequence type and staphylococcal cassette chromosome *mec* (SCC*mec*) type and results. Antimicrob. Agents Chemother. **56**:5340–5355.

166. **Speller DCE**, **Johnson AP**, **James D**, **Marples RR**, **Charlett A**, **George RC**. 1997. Resistance to methicillin and other antibiotics in isolates of *Staphylococcus aureus* from blood and cerebrospinal fluid, England and Wales, 1989-95. Lancet **350**:323–325.

167. **Shore AC**, **Tecklenborg SC**, **Brennan GI**, **Ehricht R**, **Monecke S**, **Coleman DC**. 2014. Panton-Valentine leukocidin-positive *Staphylococcus aureus* in Ireland from 2002 to 2011: 21 clones, frequent importation of clones, temporal shifts of predominant methicillin-resistant *S. aureus* clones, and increasing multiresistance. J. Clin. Microbiol. **52**:859–870.

168. **Gatermann SG**, **Koschinski T**, **Friedrich S**. 2007. Distribution and expression of macrolide resistance genes in coagulase-negative staphylococci. Clin Microbiol Infect **13**:777–781.

169. **Barbier F**, **Lebeaux D**, **Hernandez D**, **Delannoy AS**, **Caro V**, **François P**, **Schrenzel J**, **Ruppé E**, **Gaillard K**, **Wolff M**, **Brisse S**, **Andremont A**, **Ruimy R**. 2011. High prevalence of the arginine catabolic mobile element in carriage isolates of methicillin-resistant *Staphylococcus epidermidis*. J. Antimicrob. Chemother. **66**:29–36.

170. **Onishi M**, **Urushibara N**, **Kawaguchiya M**, **Ghosh S**, **Shinagawa M**, **Watanabe N**, **Kobayashi N**. 2013. Prevalence and genetic diversity of arginine catabolic mobile element (ACME) in clinical isolates of coagulase-negative staphylococci: identification of ACME type I variants in *Staphylococcus epidermidis*. Infect. Genet. Evol. **20**:381–388.

171. **Bartels MD**, **Hansen LH**, **Boye K**, **Sørensen SJ**, **Westh H**. 2011. An unexpected location of the arginine catabolic mobile element (ACME) in a USA300-related MRSA strain. PLoS One e16193.

172. **Goering R V.**, **Mcdougal LK**, **Fosheim GE**, **Bonnstetter KK**, **Wolter DJ**, **Tenover FC**. 2007. Epidemiologic distribution of the arginine catabolic mobile element among selected methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* isolates. J. Clin. Microbiol. **45**:1981–1984.

173. **Shore AC**, **Rossney AS**, **Brennan OM**, **Kinnevey PM**, **Humphreys H**, **Sullivan DJ**, **Goering R V.**, **Ehricht R**, **Monecke S**, **Coleman DC**. 2011.

Characterization of a novel arginine catabolic mobile element (ACME) and staphylococcal chromosomal cassette *mec* composite Island with significant homology to *Staphylococcus epidermidis* ACME type II in methicillin-resistant *Staphylococcus aureus* genotype ST22-MRSA-IV. Antimicrob. Agents Chemother. **55**:1896–1905.

174. **Sabat AJ**, **Ilczyszyn WM**, **Van Rijen M**, **Akkerboom V**, **Sinha B**, **Kluytmans J**, **Miedzobrodzki J**, **Grundmann H**, **Friedrich AW**. 2014. Genome-wide analysis reveals two novel mosaic regions containing an ACME with an identical DNA sequence in the MRSA ST398-t011 and MSSA ST8-t008 isolates. J. Antimicrob. Chemother. **70**:1298–1302.
175. **Moinard C**, **Cynober L**, **de Bandt JP**. 2005. Polyamines: Metabolism and implications in human diseases. Clin. Nutr. **24**:184–197.
176. **Podbielski A**, **Pohl B**, **Woischnik M**, **Körner C**, **Schmidt KH**, **Rozdzinski E**, **Leonard BA**. 1996. Molecular characterization of group A streptococcal (GAS) oligopeptide permease (Opp) and its effect on cysteine protease production. Mol. Microbiol. **21**:1087–1099.
177. **Mardis ER**. 2008. Next-Generation DNA Sequencing Methods. Annu. Rev. Genomics Hum. Genet. **9**:387–402.
178. **Metzker ML**. 2010. Sequencing technologies - the next generation. Nat. Rev. Genet. **11**:31–46.
179. **Buermans HPJ**, **den Dunnen JT**. 2014. Next generation sequencing technology: Advances and applications. Biochim. Biophys. Acta - Mol. Basis Dis. **1842**:1932–1941.
180. **Rhoads A**, **Au KF**. 2015. PacBio sequencing and its applications. Genomics, Proteomics Bioinforma. **13**:278–289.
181. **Chin C-S**, **Alexander DH**, **Marks P**, **Klammer AA**, **Drake J**, **Heiner C**, **Clum A**, **Copeland A**, **Huddleston J**, **Eichler EE**, **Turner SW**, **Korlach J**. 2013. Nonhybrid, finished microbial genome assemblies from long-read SMRT sequencing data. Nat. Methods **10**:563–569.
182. **Branton D**, **Deamer DW**, **Marziali A**, **Bayley H**, **Benner SA**, **Butler T**, **Di Ventra M**, **Garaj S**, **Hibbs A**, **Huang X**, **Jovanovich SB**, **Krstic PS**, **Lindsay S**, **Ling XS**, **Mastrangelo CH**, **Meller A**, **Oliver JS**, **Pershin Y V**, **Ramsey JM**, **Riehn R**, **Soni G V**, **Tabard-Cossa V**, **Wanunu M**, **Wiggin M**, **Schloss JA**. 2008. The potential and challenges of nanopore sequencing. Nat. Biotechnol.

26:1146–1153.

183. **Shore AC**, **Brennan OM**, **Ehricht R**, **Monecke S**, **Schwarz S**, **Slickers P**, **Coleman DC**. 2010. Identification and characterization of the multidrug resistance gene *cfr* in a panton-valentine leukocidin-positive sequence type 8 methicillin-resistant *Staphylococcus aureus* IVa (USA300) isolate. Antimicrob. Agents Chemother. **54**:4978–4984.
184. **Li H**, **Durbin R**. 2009. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics **25**:1754–1760.
185. **Rutherford K**, **Parkhill J**, **Crook J**, **Horsnell T**, **Rice P**, **Rajandream MA**, **Barrell B**. 2000. Artemis: sequence visualization and annotation. Bioinformatics **16**:944–945.
186. **Du X**, **Zhu Y**, **Song Y**, **Li T**, **Luo T**, **Sun G**, **Yang C**, **Cao C**, **Lu Y**, **Li M**. 2013. Molecular analysis of *Staphylococcus epidermidis* strains isolated from community and hospital environments in China. PLoS One e62742.
187. **Mettraux GR**, **Gusberti FA**, **Graf H**. 1984. Oxygen tension (pO_2) in untreated human periodontal pockets. J Periodontol **55**:516–521.
188. **Matsuhashi M**, **Song MD**, **Ishino F**, **Wachi M**, **Doi M**, **Matsuhisa I**, **Kimiko U**, **Noako Y**, **Masatoshi K**. 1986. Molecular cloning of the gene of a penicillin-binding protein supposed to cause high resistance to beta-lactam antibiotics in *Staphylococcus aureus*. J. Bacteriol. **167**:975–980.
189. **Ito T**, **Takeuchi F**, **Okuma K**, **Yuzawa H**, **Hiramatsu K**. 2004. Novel Type V Staphylococcal Cassette Chromosome. Society **48**:2637–2651.
190. **Xue T**, **You Y**, **Hong D**, **Sun H**, **Sun B**. 2011. The *Staphylococcus aureus* KdpDE two-component system couples extracellular K+ sensing and Agr signaling to infection programming. Infect. Immun. **79**:2154–2167.
191. **Hanssen AM**, **Ericson Sollid JU**. 2006. SCC*mec* in staphylococci: Genes on the move. FEMS Immunol. Med. Microbiol. **46**:8–20.
192. **Kolenbrander PE**, **Palmer RJ**, **Periasamy S**, **Jakubovics NS**. 2010. Oral multispecies biofilm development and the key role of cell-cell distance. Nat. Rev. Microbiol. **8**:471–480.
193. **Lindgren JK**, **Thomas VC**, **Olson ME**, **Chaudhari SS**, **Nuxoll AS**, **Schaeffer CR**, **Lindgren KE**, **Jones J**, **Zimmerman MC**, **Dunman PM**, **Bayles KW**, **Fey PD**. 2014. Arginine deiminase in *Staphylococcus epidermidis* functions to augment biofilm maturation through pH homeostasis. J. Bacteriol. **196**:2277–

2289.

194. **Sun J-L**, **Zhang S-K**, **Chen J-Y**, **Han B-Z**. 2012. Metabolic profiling of *Staphylococcus aureus* cultivated under aerobic and anaerobic conditions with ^{1}H NMR-based nontargeted analysis. Can. J. Microbiol. **58**:709–718.

195. **Krcmery V**, **Barnes AJ**. 2002. Non-albicans *Candida* spp. causing fungaemia: Pathogenicity and antifungal resistance. J. Hosp. Infect. **50**:243–260.

196. **Bassetti M**, **Righi E**, **Costa A**, **Fasce R**, **Molinari M**, **Rosso R**, **Pallavicini F**, **Viscoli C**. 2006. Epidemiological trends in nosocomial candidemia in intensive care. BMC Infect. Dis. **6**:21.

197. **Schmid J**, **Odds FC**, **Wiselka MJ**, **Nicholson KG**, **Soll DR**. 1992. Genetic similarity and maintenance of *Candida albicans* strains from a group of AIDS patients, demonstrated by DNA fingerprinting. J. Clin. Microbiol. **30**:935–941.

198. **Siikala E**, **Rautemaa R**, **Richardson M**, **Saxen H**, **Bowyer P**, **Sanglard D**. 2010. Persistent *Candida albicans* colonization and molecular mechanisms of azole resistance in autoimmune polyendocrinopathy –candidiasis– ectodermal dystrophy (APECED) patients. J. Antimicrob. Chemother. **65**:2505–2513.

199. **Glick M**, **Muzyka BC**, **Lurie D**, **Salkin LM**. 1994. Oral manifestations associated with HIV-related disease as markers for immune suppression and AIDS. Oral Surgery, Oral Med. Oral Pathol. **77**:344–349.

200. **Ranganathan K**, **Hemalatha R**. 2006. Oral Lesions in HIV Infection in Developing Countries: an Overview. Int. Assoc. Dent. Res. 1963-68 **19**:63–68.

201. **Chávez EM**, **Borrell LN**, **Taylor GW**, **Ship JA**. 2001. A longitudinal analysis of salivary flow in control subjects and older adults with type 2 diabetes. Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod. **91**:166–173.

202. **Al-Karaawi ZM**, **Manfredi M**, **Waugh ACW**, **McCullough MJ**, **Jorge J**, **Scully C**, **Porter SR**. 2002. Molecular characterization of *Candida* spp. isolated from the oral cavities of patients from diverse clinical settings. Oral Microbiol. Immunol. **17**:44–49.

203. **Sangeorzan JA**, **Bradley SF**, **He X**, **Zarins LT**, **Ridenour GL**, **Tiballi RN**, **Kauffman CA**. 1994. Epidemiology of oral candidiasis in HIV-infected patients: Colonization, infection, treatment, and emergence of fluconazole resistance. Am. J. Med. **97**:339–346.

204. **Odds FC**, **Davidson AD**, **Jacobsen MD**, **Tavanti A**, **Whyte JA**, **Kibbler CC**, **Ellis DH**, **Maiden MCJ**, **Shaw DJ**, **Gow NAR**. 2006. *Candida albicans* strain

maintenance, replacement, and microvariation demonstrated by multilocus sequence typing. J. Clin. Microbiol. **44**:3647–3658.

205. **Da Matta DA**, **Melo AS**, **Guimarães T**, **Frade JP**, **Lott TJ**, **Colombo AL**. 2010. Multilocus sequence typing of sequential *Candida albicans* isolates from patients with persistent or recurrent fungemia. Med. Mycol. **48**:757–762.
206. **Coste A**, **Selmecki A**, **Forche A**, **Diogo D**, **Bougnoux ME**, **D'Enfert C**, **Berman J**, **Sanglard D**. 2007. Genotypic evolution of azole resistance mechanisms in sequential *Candida albicans* isolates. Eukaryot. Cell **6**:1889–1904.
207. **Odds FC**, **Bougnoux M-E**, **Shaw DJ**, **Bain JM**, **Davidson AD**, **Diogo D**, **Jacobsen MD**, **Lecomte M**, **Li S-Y**, **Tavanti A**, **Maiden MCJ**, **Gow NAR**, **D'Enfert C**. 2007. Molecular phylogenetics of *Candida albicans*. Eukaryot. Cell **6**:1041–1052.
208. **Tavanti A**, **Davidson AD**, **Fordyce MJ**, **Gow N a R**, **Maiden MCJ**, **Odds FC**. 2005. Population structure and properties of *Candida albicans*, as determined by multilocus sequence typing. J. Clin. Microbiol. **43**:5601–5613.
209. **Odds FC**, **Jacobsen MD**. 2008. Multilocus sequence typing of pathogenic *Candida* species. Eukaryot. Cell **7**:1075–1084.
210. **Edgar WM**. 1976. The role of saliva in the control of pH changes in human dental plaque. Caries Res. **10**:241–254.
211. **Yu D**, **Pi B**, **Yu M**, **Wang Y**, **Ruan Z**, **Feng Y**, **Yu Y**. 2014. Diversity and evolution of oligopeptide permease systems in staphylococcal species. Genomics **104**:8–13.

Appendix 1

AMERICAN SOCIETY FOR MICROBIOLOGY | Antimicrobial Agents and Chemotherapy®

First Detailed Genetic Characterization of the Structural Organization of Type III Arginine Catabolic Mobile Elements Harbored by *Staphylococcus epidermidis* by Using Whole-Genome Sequencing

Brenda A. McManus,[a] Aoife M. O'Connor,[a] Peter M. Kinnevey,[a] Michael O'Sullivan,[b] Ioannis Polyzois,[b] David C. Coleman[a]

Microbiology Research Unit, Division of Oral Biosciences, Dublin Dental University Hospital, Trinity College Dublin, University of Dublin, Dublin, Republic of Ireland[a]; Division of Restorative Dentistry and Periodontology, Dublin Dental University Hospital, Trinity College Dublin, University of Dublin, Dublin, Republic of Ireland[b]

ABSTRACT The type III arginine catabolic mobile element (ACME) was detected in three *Staphylococcus epidermidis* oral isolates recovered from separate patients (one healthy, one healthy with dental implants, and one with periodontal disease) based on ACME-*arc*-operon- and ACME-*opp3*-operon-directed PCR. These isolates were subjected to whole-genome sequencing to characterize the precise structural organization of ACME III for the first time, which also revealed that all three isolates were the same sequence type, ST329.

KEYWORDS ACME, *Staphylococcus epidermidis*, oral cavity, ST329

The arginine catabolic mobile element (ACME) was first described in the *Staphylococcus aureus* strain USA300 (1) and is thought to aid colonization and persistence on skin. Since it was first described, ACMEs ranging from 30 to 34 kb in size have been identified in other staphylococcal species, including *Staphylococcus epidermidis* (1, 2–5). The element is primarily characterized by the presence of two distinct operons: the *arc* operon (*arcR/A/D/B/C*), which encodes an arginine deaminase pathway, and the *opp3* operon (*opp3A/B/C/D/E*), which encodes an oligopeptide permease ABC transporter. To date, three distinct types of ACME have been described based on (i) the presence of both *arc* and *opp3* operons (type I), (ii) the *arc* operon only (type II), and (iii) the *opp3* operon only (type III). The genetic structure and organization of ACME types I and II in staphylococci have been elucidated in detail previously, including by the use of whole-genome sequencing (WGS) (1, 4). In contrast, the corresponding genetic structural organization of ACME type IIIs have not been comprehensively characterized to date. What is known about ACME IIIs in staphylococci is based on PCR-based scanning/tiling methods using primer pairs designed against the reference ACME type I in USA300 (6, 7) or based on PCR amplification and subsequent sequence analysis of ACME-*arc* and -*opp3* genes (2, 3, 5, 8). Comprehensive characterization of ACME III could yield useful information regarding important features of ACME and its conservation, evolution and spread, such as into the epidemic methicillin-resistant *S. aureus* strain USA300.

We detected ACME III in 9/142 (6.3%) oral methicillin-susceptible *S. epidermidis* isolates from separate patient groups who (i) were orally healthy, (ii) had dental implants, or (iii) had periodontal disease, using PCR primers directed toward ACME-*arcA* (6) and ACME-*opp3* (ACME-opp3B_F, 5′-GGATTCGCCCAAGTGATGACC-3′ and ACME-opp3B_R, 5′-GACTGCTGGGTATGACGT-3′), using the USA300 strain M05/0060 (9), which harbors both the ACME-*arc* and *opp3* operons, as a positive PCR control. We did not

Received 12 June 2017 **Returned for modification** 9 July 2017 **Accepted** 15 July 2017

Accepted manuscript posted online 31 July 2017

Citation McManus BA, O'Connor AM, Kinnevey PM, O'Sullivan M, Polyzois I, Coleman DC. 2017. First detailed genetic characterization of the structural organization of type III arginine catabolic mobile elements harbored by *Staphylococcus epidermidis* by using whole-genome sequencing. Antimicrob Agents Chemother 61:e01216-17. https://doi.org/10.1128/AAC.01216-17.

Address correspondence to David C. Coleman, david.coleman@dental.tcd.ie.

B.A.M. and A.M.O. contributed equally to this work.

detect ACME III in any of the 54 *S. aureus* isolates investigated from the same three patient groups. The genetic structure of three of these ACME IIIs harbored by *S. epidermidis* isolates recovered by oral rinse sampling of three separate patients (one with periodontal disease [P16OR1], one healthy patient [204OR1], and one healthy patient with a dental implant [I11OR1]) were characterized in detail using WGS. To our knowledge, this is the first comprehensive description of the structural organization of ACME III. Isolates were first sequenced using a MiSeq sequencer (Illumina, Essex, United Kingdom) with genomic DNA extraction and library construction performed as previously described (10). Reads were checked for quality, trimmed, and contigs were generated by *de novo* assembly using SPAdes version 3.6 (http://cab.spbu.ru/software/spades/). For each isolate subjected to MiSeq-based WGS, ACME-associated genes were identified on four different contigs. As the genes in these contigs differed considerably in composition and orientation to those previously described in ACME types I and II and an appropriate reference ACME to use as a sequence scaffold was lacking, these isolates were also sequenced using a Pacific Biosciences (PacBio) RS sequencing system (CA, USA) with subsequent hierarchal genome assembly process (HGAP.3) analysis (The Genome Analysis Centre [TGAC], Norwich, United Kingdom) at an average coverage of 265×. For each isolate, all ACME-associated genes were identified on the same contig, thus confirming the orientation and synteny of all ACME III-associated genes.

The bioinformatic tools used for annotation and analysis were the BioNumerics Genome Analysis Tool (GAT) plug-in version 7.6 (Applied Maths, Sint-Martens-Latem, Belgium), Artemis sequence viewer (11), Artemis Comparison Tool (12) and BLAST software (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Final elucidated genomic structures were confirmed using specific PCR primers (Table S1). The multilocus sequence types (MLST) of all three isolates investigated were determined by submitting the relevant genomic regions to the *S. epidermidis* MLST online database (https://pubmlst.org/sepidermidis/).

Each ACME III harbored the *opp3* genes but lacked the *arc* operon and ranged from 21.2 to 21.5 kb in size. Adjacent staphylococcal cassette chromosome (SCC) elements were identified upstream of ACME III in two isolates (Fig. 1). Five distinct direct repeat sequences (DRs) (1-A, 1-B, and 2-4) were identified among the ACMEs characterized. Four (DR1-A and DR2-4; Fig. 1) were identified in the ACMEs harbored by isolates 204OR1 and I11OR1, whereas three (DR1-B, DR3, and DR4) were detected in the isolate P16OR1ACME (Fig. 1). There were four nucleotide differences identified between DR1-A and DR1-B (Fig. 1).

A comparative BLAST analysis of the DNA sequence for ACME III (the region between DRs 3 and 4 of isolate I11OR1) with ACME types I and II revealed that although the DNA sequence identity with ACME I (GenBank accession number FPR3757) and ACME II (GenBank accession number AE015929) was 99% and 96%, respectively, the query cover was only 54% and 60%, respectively, indicating high genetic similarity in distinct genomic regions only. These findings were confirmed using the Artemis Comparison Tool.

The *copA* gene and the *ars* operon were located directly upstream of ACME III for the first time. Previous studies described their location near the 3′ end and immediately downstream of ACME types I and II (1). In two of the elements sequenced (204OR1 and I11OR1), the *copA* and *ars* genes were located between DRs 2 and 3, whereas in the third ACME these genes were in the same location but DR2 was absent (Fig. 1). The genomic regions from the *copA* gene to DR4 exhibited >99% DNA sequence identity in all three ACMEs characterized. The relocation of these antimicrobial resistance genes has not been reported previously, although other genes encoding tetracycline, cadmium, mercury, and beta-lactam resistance have been detected previously within ACME-SCC composite elements (1).

Genes previously associated with the SCC*pbp4*-ACME II composite element in *S. epidermidis* (1) were identified in two isolates investigated (Fig. 1), including the cassette chromosome recombinase (*ccr*) and *pbp4* genes. Together, these findings highlight the ability of ACMEs to accumulate antimicrobial resistance genes, particularly

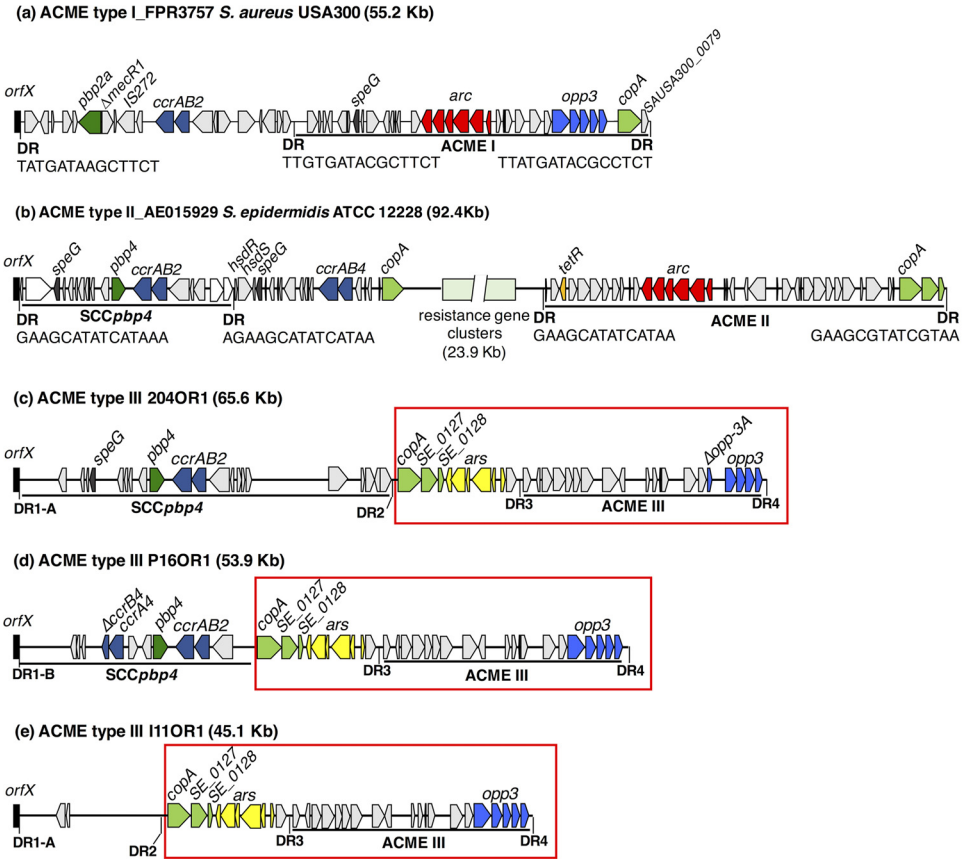


FIG 1 Schematic diagram showing the genetic organization of previously described ACME type I (a) and II (b) elements and the comparative organization of the three ACME III elements (c-e) determined by whole-genome sequencing in the present investigation. Arrows indicate the position and orientation of open reading frames. Genes commonly associated with antimicrobial resistance, SCC, or ACME are shaded in color; ACME-*arc* (red), *opp3* (blue), *speG* (dark gray), *copA* (lime green), *ars* operon (yellow), *pbp* (dark green), *ccr* (navy) and *tetR* (mustard). The resistance gene clusters encoding mercury and cadmium resistance in ACME type II_AE015929 are indicated in pale green. For each ACME, *orfX* is indicated in black and specific direct repeat sequences (DRs) identified are indicated (DR1-A, GAAGCGTATCACAAATAA; DR1-B, GAAGCATATCATAAGTGA; DR2, GAAGGGTATCATAAATAA; DR3, GAAGCG TATAATAAGTAA; DR4 GAAGCGTATCGTAAGTGA). Genomic regions from *copA* to DR4 in each ACME III exhibited >99% DNA sequence homology to each other and are enclosed in red rectangles.

within composite elements, and their potential to facilitate the spread of these genes to different strains and species.

The *speG* gene conferring polyamine resistance was identified in only one ACME III sequenced and previous research has suggested an association of this gene with *arcA*, which is absent in ACME III (13).

The main feature of ACME III is considered to be the presence of the *opp3* operon in the absence of the *arc* operon. The function of ACME-*opp3* has not been fully elucidated to date, but multiple different *opp* operons have been identified in bacterial species and are reportedly involved in nutrient uptake, host cell attachment, cell wall metabolism, resistance to antimicrobial peptides, and chemotaxis (11, 12). This operon was detected 510 bp upstream of DR4 in all three ACMEs characterized; however, a nucleotide deletion identified at the +384 position of the *opp3A* gene in isolate 204OR1 resulted in a frameshift mutation and the premature truncation of the encoded protein. These ACME-*opp3* genes likely contribute little advantage, perhaps due to the presence of two native *opp* operons in staphylococci, and perhaps represent remnants from previous ACME rearrangements.

The elements characterized were divided into modular segments by DRs (Fig. 1) in which the genomic regions between the *copA* gene and DR4 were highly conserved. Only eight of the 20 open reading frames observed in ACME III shared >97% sequence

homology with the *opp3* operon and surrounding genomic regions of previously described ACME I (1); however, the *copA* and SE_0128 genes (corresponding to *copA* and SAUSA300_0079 in FPR3757) at the 3′ end of ACME I have been internalized in these ACME III-SCC composite elements (Fig. 1). Previous research has suggested a stepwise assembly of modular ACME segments in *S. epidermidis* prior to transfer to USA300 (14). The results of the present study support this hypothesis, demonstrate how mobile genetic elements can be constructed in a stepwise manner at this genomic region, and suggest that ACME III is most likely a genetic remnant of these processes. Surprisingly, all three isolates were identified as belonging to multilocus sequence type ST329. Previous MLST-based studies from this laboratory (unpublished) that investigated 36 independent oral *S. epidermidis* isolates identified 18 distinct STs, not including ST329. ST329 has been identified in only 3/1068 (0.3%) allelic profiles currently listed in the *S. epidermidis* MLST database (accessed 8 June 2017), suggesting that this ST is rare and is possibly the ST in which ACME rearrangements resulting in ACME type III originally occurred.

Accession number(s). The nucleotide sequences of the three ACME-SCC composite elements 204OR1, P16OR1, and I11OR1 have been submitted to GenBank under accession numbers MF346683, MF346684, and MF346685, respectively.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.01216-17.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

ACKNOWLEDGMENTS

We thank Peter Slickers at the InfectoGnostics Research Campus, Jena, Germany, for technical assistance with *de novo* assemblies using SPAdes software.

We declare no conflicts of interest.

This work was supported by the Microbiology Research Unit, Dublin Dental University Hospital. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

REFERENCES

1. Diep BA, Gill SR, Chang RF, Phan TH, Chen JH, Davidson MG, Lin F, Lin J, Carleton HA, Mongodin EF, Sensabaugh GF, Perdreau-Remington F. 2006. Complete genome sequence of USA300, an epidemic clone of community-acquired meticillin-resistant *Staphylococcus aureus*. Lancet 367:731–739. https://doi.org/10.1016/S0140-6736(06)68231-7.
2. Barbier F, Lebeaux D, Hernandez D, Delannoy AS, Caro V, Francois P, Schrenzel J, Ruppe E, Gaillard K, Wolff M, Brisse S, Andremont A, Ruimy R. 2011. High prevalence of the arginine catabolic mobile element in carriage isolates of methicillin-resistant *Staphylococcus epidermidis*. J Antimicrob Chemother 66:29–36. https://doi.org/10.1093/jac/dkq410.
3. Pi B, Yu M, Chen Y, Yu Y, Li L. 2009. Distribution of the ACME-*arcA* gene among meticillin-resistant *Staphylococcus haemolyticus* and identification of a novel *ccr* allotype in ACME-*arcA*-positive isolates. J Med Microbiol 58:731–736. https://doi.org/10.1099/jmm.0.007351-0.
4. Shore AC, Rossney AS, Brennan OM, Kinnevey PM, Humphreys H, Sullivan DJ, Goering RV, Ehricht R, Monecke S, Coleman DC. 2011. Characterization of a novel arginine catabolic mobile element (ACME) and staphylococcal chromosomal cassette *mec* composite island with significant homology to *Staphylococcus epidermidis* ACME type II in methicillin-resistant *Staphylococcus aureus* genotype ST22-MRSA-IV. Antimicrob Agents Chemother 55:1896–1905. https://doi.org/10.1128/AAC.01756-10.
5. Onishi M, Urushibara N, Kawaguchiya M, Ghosh S, Shinagawa M, Watanabe N, Kobayashi N. 2013. Prevalence and genetic diversity of arginine catabolic mobile element (ACME) in clinical isolates of coagulase-negative staphylococci: identification of ACME type I variants in *Staphylococcus epidermidis*. Infect Genet Evol 20:381–388. https://doi.org/10.1016/j.meegid.2013.09.018.
6. Diep BA, Stone GG, Basuino L, Graber CJ, Miller A, des Etages SA, Jones A, Palazzolo-Ballance AM, Perdreau-Remington F, Sensabaugh GF, DeLeo FR, Chambers HF. 2008. The arginine catabolic mobile element and staphylococcal chromosomal cassette *mec* linkage: convergence of virulence and resistance in the USA300 clone of methicillin-resistant *Staphylococcus aureus*. J Infect Dis 197:1523–1530. https://doi.org/10.1086/587907.
7. Miragaia M, de Lencastre H, Perdreau-Remington F, Chambers HF, Higashi J, Sullam PM, Lin J, Wong KI, King KA, Otto M, Sensabaugh GF, Diep BA. 2009. Genetic diversity of arginine catabolic mobile element in *Staphylococcus epidermidis*. PLoS One 4:e7722. https://doi.org/10.1371/journal.pone.0007722.
8. Goering RV, McDougal LK, Fosheim GE, Bonnstetter KK, Wolter DJ, Tenover FC. 2007. Epidemiologic distribution of the arginine catabolic mobile element among selected methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* isolates. J Clin Microbiol 45:1981–1984. https://doi.org/10.1128/JCM.00273-07.
9. Shore AC, Rossney AS, Kinnevey PM, Brennan OM, Creamer E, Sherlock O, Dolan A, Cunney R, Sullivan DJ, Goering RV, Humphreys H, Coleman DC. 2010. Enhanced discrimination of highly clonal ST22-methicillin-resistant *Staphylococcus aureus* IV isolates achieved by combining *spa*, *dru*, and pulsed-field gel electrophoresis typing data. J Clin Microbiol 48:1839–1852. https://doi.org/10.1128/JCM.02155-09.
10. Earls MR, Kinnevey PM, Brennan GI, Lazaris A, Skally M, O'Connell B, Humphreys H, Shore AC, Coleman DC. 2017. The recent emergence in hospitals of multidrug-resistant community-associated sequence type 1 and *spa* type t127 methicillin-resistant *Staphylococcus aureus* investigated by whole-genome sequencing: Implications for screening. PLoS One 12:e0175542. https://doi.org/10.1371/journal.pone.0175542.

11. Berriman M, Rutherford K. 2003. Viewing and annotating sequence data with Artemis. Brief Bioinform 4:124–132. https://doi.org/10.1093/bib/4.2.124.
12. Carver TJ, Rutherford KM, Berriman M, Rajandream MA, Barrell BG, Parkhill J. 2005. ACT: the Artemis Comparison Tool. Bioinformatics 21:3422–3423. https://doi.org/10.1093/bioinformatics/bti553.
13. Planet PJ, LaRussa SJ, Dana A, Smith H, Xu A, Ryan C, Uhlemann AC, Boundy S, Goldberg J, Narechania A, Kulkarni R, Ratner AJ, Geoghegan JA, Kolokotronis SO, Prince A. 2013. Emergence of the epidemic methicillin-resistant *Staphylococcus aureus* strain USA300 coincides with horizontal transfer of the arginine catabolic mobile element and *speG*-mediated adaptations for survival on skin. mBio 4:e00889–e00813. https://doi.org/10.1128/mBio.00889-13.
14. Thurlow LR, Joshi GS, Clark JR, Spontak JS, Neely CJ, Maile R, Richardson AR. 2013. Functional modularity of the arginine catabolic mobile element contributes to the success of USA300 methicillin-resistant *Staphylococcus aureus*. Cell Host Microbe 13:100–107. https://doi.org/10.1016/j.chom.2012.11.012.

Appendix 2

Application form for ethical approval and approval letter

COLÁISTE NA TRÍONÓIDE, BAILE ÁTHA CLIATH

Dámh na nEolaíochtaí Slainte,
Foirgneamh na Ceimice
Colaiste na Tríonóide,
Baile Átha Cliath 2, Éire.

TRINITY COLLEGE DUBLIN

Faculty of Health Sciences,
Chemistry Building,
Trinity College,
Dublin 2, Ireland.
T:- +353 (0)1 8964255

Ms.Brenda McManus
Microbiology Research Unit,
Dublin Dental University Hospital,
Lincoln Place,
Dublin 2.

21st January 2014

Re: Characterisation of Candida & Staphylococcal Populations recovered from healthy & diseased subgingival & implant sites

Dear Applicant (s),

Further to a meeting of the Faculty of Health Sciences Ethics Committee held in December 2013, we are pleased to inform you that the above project (as amended) has been approved without further audit.

Yours sincerely,

pp. Sonya McConnon

__ _____

Dr. Ruth Pilkington
Chairperson
Faculty Research Ethics Committee

Microbiology Research Unit
Dublin Dental University Hospital
Lincoln Place
Dublin 2

Dear Participant,

My name is Dr. Brenda McManus and I am employed as a Postdoctoral Research Fellow by the Dublin Dental University Hospital. I am currently carrying out research examining specific yeast and bacterial communities present in both healthy and diseased sub-gingivae (i.e. the space between gums and teeth), and oral implants (i.e. artificial teeth supported by a post inserted in the jaw bone). *Candida* are yeasts that are naturally present in 40 - 60% of healthy mouths and may be associated with gum disease, but are rarely found in healthy sub-gingivae. Recent studies reported that some bacteria such as staphylococci (i.e. natural skin bacteria) are prevalent around diseased teeth and oral implants. We wish to examine the abundance of both *Candida* and staphylococci in each of these sites and to determine their association with the progression of disease or implant failure.

In order to carry out this study, we wish to briefly assess the health of patient's sub-gingivae or oral implants, and to obtain some clinical samples from patients with both healthy and diseased sub-gingivae, or from patients with oral implants. These samples would consist of a nasal swab, an oral rinse and some periopaper samples of sub-gingival sites. These sampling methods are non-invasive, risk-free, will not cause any discomfort and will take under 2 minutes to perform. **Sampling would be carried out during your next scheduled appointment.**

- Participants must be over 18 years old and be able to provide informed consent. Patients without implants must have at least 10 natural teeth.
- Individuals who are carrying blood-borne illnesses, who are pregnant or breastfeeding, have diabetes or asthma, have had steroid treatment during the last year, or have had antibiotic or anti-fungal treatment in the previous two months will not be suitable for this study.

Participation in this study is entirely voluntary, there are no repercussions should you decide not to participate. All information will remain anonymous and confidential, and any samples you provide will be destroyed upon completion of the study.

I hope you will consider assisting me in my research endeavors. If you have any questions regarding this study or if you would like to participate, please do not hesitate to contact me (01-6127608 or brenda.mcmanus@dental.tcd.ie) or Dr. Mary O'Donnell (01-6127269 or mary.odonnell@dental.tcd.ie).

Best wishes,
Dr. Brenda McManus

PARTICIPANT INFORMATION LEAFLET

1. **Title of study: Characterisation of *Candida* and staphylococci populations recovered from healthy and diseased subgingival and implant sites.**
2. **Introduction:** *Candida* are yeasts that are naturally present in 40 to 60% of healthy mouths. They are rarely found in healthy sub-gingivae (i.e. the space between gums and teeth) although they may be associated with gum disease. Recent studies suggest that natural skin bacteria such as staphylococci are prevalent around diseased teeth and oral implants (i.e. artifical teeth supported by a post inserted in the jaw bone). The purpose of this study is to examine the *Candida* and staphylococcal species present in both healthy and diseased sub-gingival and implant sites and to determine their abundance in each condition.
3. **Procedures:** Each participant will be asked to provide an oral rinse, a nasal swab and PerioPaper samples of both healthy and diseased sub-gingival or implant sites. All samples will be taken in the DDUH clinics by a qualified dental professional. Nasal swabs will be obtained by gently rubbing a cotton swab on the inside of the nose. For the oral rinses each person will be supplied with 10 ml sterile saline in a universal container and will be requested to rinse the mouth thoroughly for 60 seconds. The person will then return the mouth rinse to the container. Periopaper samples will be obtained using one PerioPaper strip per sub-gingival site which will then be immediately introduced into a sterile 1.5 ml plastic tube containing 1 ml of nutrient broth and sealed. The procedures will not cause any discomfort and will take less than two minutes to perform. All samples will be brought to the microbiology laboratory in DDUH for microbiological analysis.
4. **Benefits:** Your participation will provide research material for a DDUH Ph.D. research project that will help to improve our understanding of sub-gingival *Candida* and staphylococcal populations.
5. **Risks:** There are no risks associated with providing PerioPaper samples, nasal swabs or oral rinses.
6. **Exclusion from participation:** Patients and healthy subjects will be excluded from the study if they meet any of the following criteria: carriage of blood-borne illnesses, pregnancy or lactation, have diabetes or asthma, have received steroid treatment during the last year, have received antibiotic or anti-fungal treatment in the previous two months. Participants must be over 18 years old and be able to provide informed consent for the study. Patients without implants must have at least 10 natural teeth.
7. **Confidentiality:** Your identity will remain confidential. Your name will not be published and will not be disclosed to anyone outside the study group. All samples will be labelled with a number and no details will be recorded apart from your age range, gender and sub-gingival health. The procedures will not cause any discomfort and will take less than two minutes to perform. All samples will be destroyed on completion of the study.
8. **Compensation:** This study is covered by standard institutional indemnity insurance. Nothing in this document restricts or curtails your rights.
9. **Voluntary Participation:** Participation in this study is entirely voluntary. If you decide not to participate you will not be penalised and will not give up any benefits that you had before entering the study.
10. **Permission:** These procedures and the research project have been approved by the Faculty of Health Sciences Ethics Group in TCD.
11. **Further information:** You can obtain more information about the study, your participation and your rights, from Dr. Mary O' Donnell (Gatekeeper) at 6127269/mary.odonnell@dental.tcd.ie and Dr. Brenda McManus at 61272608/brenda.mcmanus@dental.tcd.ie. If the study team learns of important new information that might affect your desire to remain in the study, you will be informed at once.

INFORMED CONSENT FORM

Research Project title:
Characterisation of *Candida* and Staphylococci populations recovered from healthy and diseased subgingival and implant sites.

Principle investigators:
Investigator/Supervisor: Dr. Brenda McManus

Background
The purpose of this study is to characterise the *Candida* and staphylococcal communities present in both healthy and diseased sub-gingival or implant sites, to determine their abundance in each condition. As part of this study each participant will be asked to provide an oral rinse, a nasal swab and PerioPaper samples of both healthy and diseased sub-gingival or implant sites. All samples will be taken in the DDUH clinics by a qualified dental professional. Nasal swabs will be obtained by gently rubbing a cotton swab on the inside of the nose. For the oral rinses, each person will be supplied with 10 ml sterile saline in a universal container and will be requested to rinse the mouth thoroughly for 60 seconds. The person will then return the mouth rinse to the container. PerioPaper samples will be obtained using separate PerioPaper strips per sub-gingival site, which will then be immediately introduced into a sterile 1.5 ml plastic tube containing 1 ml of nutrient broth and sealed. The procedures will not cause any discomfort and will take less than two minutes to perform. Your identity will remain confidential. All samples will be brought to the microbiology laboratory in DDUH for microbiological analysis and will be destroyed on completion of the study.

DECLARATION:
I have read this consent form and I agree to partipate by allowing my nose to be swabbed, my sub-gingival sites to be sampled using PerioPaper strips and by performing the oral rinse requested. I have had the opportunity to ask questions and all my questions have been answered to my satisfaction. I freely and voluntarily agree to be part of this research study without prejudice to my legal and ethical rights. I have received a copy of this agreement and I understand that, if there is a sponsoring company, a signed copy will be sent to that sponsor.

PARTICIPANT'S NAME: ……

CONTACT DETAILS: ……

PARTICIPANT'S SIGNATURE: ……

DATE: ……

Statement of investigator's responsibility: I have explained the nature and purpose of this research study, the procedures to be undertaken and any risks that may be involved. I have offered to answer any questions and fully answered such questions. I believe that the participant understands my explanation and has freely given informed consent.

INVESTIGATOR'S SIGNATURE: ……
Date:……
(Keep the original of this form in the investigator's file, give one copy to the participant, and send one copy to the sponsor (if there is a sponsor).

PARTICIPANT QUESTIONAIRE: 1. CHARACTERISATION OF CANDIDA AND STAPHYLOCOCCI POPULATIONS RECOVERED FROM HEALTHY AND DISEASED SUBGINGIVAL AND IMPLANT SITES.

5. **Age range (circle the appropriate age range):**
   18-24
   25-34
   35-44
   45-54
   55-64
   65-74

6. **Gender (circle the appropriate gender):**
   Male
   Female

7. **Periodontal or peri-implant health (circle where appropriate)**
   Gingival redness
   Bleeding on probing
   Plaque accumulation
   Suppuration
   Healthy

8. **Smoking status (circle where appropriate)**
   Smoker
   Non-smoker
   Former smoker

9. **Dental Status (circle where appropriate)**
   Dentate
   Edentulous

10. **Denture Wearer**
    Yes
    No

11. **List any underlying disorders**