Persistent *Staphylococcus aureus* infection through the selection of alternative lifestyles

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I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program Scholarship.

James Lee

Date

14/04/2022

Signature

Acknowledgements

My first and most appreciative acknowledgement go to my supervisors Dr Stephen Kidd and Dr Peter Zilm. Your advice, endless patience, and drive for me to do my best and set me up to become a better researcher truly got me to get through my experiments, the difficulties I endured and altogether the completion of my PhD. I appreciate all of your guidance to establish my foundation in academia and all my future successes I may have.

To all my colleagues who worked alongside me in the Kidd Lab and ACARE: Tania Veltman, Jan Bell, Dr Mathu Indren, Nick Gunn, Brady Welsh, Christopher Keneally, Hang Nguyen, Afshin Karami, Andrew Hayles, Dehui Kong, Yu Han and so many others that went through our lab group. I have so much appreciation to have colleagues to talk to and interact with and for the students who I had the chance to teach and guide through their own journey in the laboratory. The opportunity to be able to teach others was a unexpected joy during my time in the lab. But of all my colleagues, I especially am grateful for Dr Alexandra Tikhomirova. My first steps in laboratory work were a daunting and nervous time but I had the guidance of her to cover everything from basic laboratory etiquette to efficient experimental techniques. Even after moving to a new lab, I could always come to you for advice.

A huge thanks to Dr Elizabeth Tran who I had constantly had to turn to for assistance with many various experiments. You were always a happy and positive face to turn to when I needed help and I only wish there was more I could to return the favour.

To all the collaborators from SA Health: Professor Robert Fitridge, Dr Joe Dawson, Dr Beatrice Kuang, Dr Guilherme Pena, Dr Matipaishe Mashayamombe, Dr David Jesudason and all the registrars from the clinics. Everyone had assisted me in a new and unfamiliar environment outside the lab to collect samples for our clinical study despite their already hectic workloads.

I would like to give special thanks to Konstantinos Bogias and Dr Nathan Watson-Haigh for teaching me the basics of bioinformatics and coding, a completely new field of study I otherwise would have no success with.

All my friends from undergraduate studies, especially those who were also going through their own Masters and PhD studies. To be able to talk and discuss about the difficulties of a PhD outside of the workplace and for others to relate to my exact situation was cathartic and necessary to get through any hardships I faced.

To the Adelaide University Hockey Club and all my teammates I played with. To be able to play hockey, have a mental break from research, a reason to stay physically fit was needed to keep my mental health positive.

And finally, I would like to thank my family who supported me throughout my life shaped me to the person I am now. A person who was able to go through this journey at university and life in general. I hope I've made you all proud with my achievements and all the success I will have in the future.

List of abbreviations

AIP	Auto-inducing Peptide
ALE	Adaptive Laboratory Evolution
ATP	Adenosine Triphosphate
BMI	Body Mass Index
bp	base pair
CA-MRSA	Community Acquired Methicillin Resistant *Staphylococcus aureus*
CDM	Chemically Defined Media
CF	Cystic Fibrosis
d	day(s)
DFI	Diabetic Foot Infection
DFI-OM	Diabetic Foot Infection - Osteomyelitis
DFI-W	Diabetic Foot Infection - Wound only
DFU	Diabetic Foot Ulcer
DNA	deoxyribonucleic acid
ECM	Extracellular Matrix
eDNA	Extracellular Deoxyribonucleic Acid
EPS	Extracellular Polymeric Substance
FnBP	Fibronectin Binding Protein
h	hour(s)
HA-MRSA	Hospital Acquired Methicillin Resistant *Staphylococcus aureus*
L	Large (colony size)
LDDP	Length Dependent Diabetic Neuropathy
MGE	Mobile Genetic Element
min	minute(s)
MRSA	Methicillin Resistant *Staphylococcus aureus*
MSCRAMMs	Microbial Surface Components Recognising Adhesive Matrix Molecules
MSSA	Methicillin Sensitive *Staphylococcus aureus*
nm	nanometer
NP	Non-pigmented
nsSCV	non-stable Small Colony Variant
nt	nucleotide
OD	optical density
P	Pigmented
PCR	polymerase chain reaction
PIA	Polysaccharide Intracellular Adhesion
PJI	Prosthetic Joint Infection
QEH	Queen Elizabeth Hospital
RAH	Royal Adelaide Hospital
RNA	ribonucleic acid
ROS	Reactive Oxygen Species
rpm	revolutions per minute
S	Small (colony size)
SCV	Small Colony Variant
SEM	Scanning Electron Microscopy
SNP	Single Nucleotide Polymorphism

sSCV	stable Small Colony Variant
TCA	Tricarboxylic Acid Cycle/Citric Acid Cycle
TCS	Two Component System
TSA	Tryptic Soya Agar
TSB	Tryptic Soya Broth
VRSA	Vancomycin Resistant *Staphylococcus aureus*
WCH	Women's and Children's Hospital

List of publications

Lee, J., Zilm, P.S., Kidd, S.P. (2020). "Novel Research Models for *Staphylococcus aureus* Small Colony Variants (SCV) Development: Co-pathogenesis and Growth Rate", *Front Microbiol* **11** (321)

Conference presentations

Lee, J., Bui, L.M.G., Crosby, H., Horswill, A.., Zilm, P.S. and Kidd, S.P. *Novel research models of Staphylococcus aureus Small Colony Variants (SCV) in Persistent Infections*. Gordon Research Conference on Staphylococcal Diseases, 2019, Castelldefels, Spain. Poster Presentation

Lee, J., Bui, L.M.G., Crosby, H., Horswill, A.., Zilm, P.S. and Kidd, S.P. *Novel research models of Staphylococcus aureus Small Colony Variants (SCV) in Persistent Infections*. Gordon Research Seminars on Staphylococcal Diseases, 2019, Castelldefels, Spain. Poster Presentation

Abstract

Staphylococcus (S.) aureus is an important human pathogen and is notorious for its ability to survive stressful environments. The classic mechanism of survival is through expression of virulence factors that can neutralise the immune response and the widespread transfer of antibiotic resistance that hinders clinical antibiotic treatments. However, cases occur where initially a clinical infection has cleared only for the infection to relapse. This can be via a new infection or as a result of changes in bacterial lifestyles of the original infection that can create sub-populations of *S. aureus* with varied mechanisms to survive different conditions of host and medically induced stressors. These lifestyles include the formation of Small Colony Variants (SCV), a cell type characterised by impeded metabolism and reduced virulence which confers evasion of the immune response and antibiotic tolerance. This phenotype is largely unstable and often reverts to the normal cell type which hinders research into the mechanism of SCV formation.

To overcome this hurdle, stable SCVs (sSCV) have been generated by researchers through genetic mutations which cause impedance of the electron transport chain, create metabolic defects, and produces the SCV phenotype. Previous research has generated these within a laboratory setting and with a single mutation. This is not a complete representation of the complex, adaptative mechanisms which lead to the SCV phenotype within a clinical setting where adaptation occurs in response to nutritional limitations, immune response, antibiotic stress, and selection over long-term growth. Given there are multiple pathways which can lead to the SCV phenotype, our project investigates two different paradigms of SCV: firstly, that conditions of stress that cause changes within a cell and result in a SCV and when the stress is removed, the cells that revert to the normal cell type. Alternatively, SCVs continually form stochastically within a bacterial population and introduction of conditions of stress select for

the SCVs and they dominate the population but when those stressors are removed, the normal cell types are selected.

This project utilises alternative research models for investigating SCV formation and clinical isolates of *S. aureus* from diabetic foot infection (DFI) to study both the mechanisms which define the switch to SCV and the population dynamics which select for SCV. We have used physiological and genetic methods to assess the SCV generated through different methods.

Continuous culture within a chemostat was used to grow *S. aureus* in a controlled, low growth rate over a prolonged duration. In these conditions, we have selected for alternative cell types with decreased metabolic demands within the population.

In a similar theme, we have used long-term infection of the osteocyte cell line SaOS-2 provided an *ex vivo* model of long-term, persistent bone infection. Infection of SaOS-2 resulted in viable but non-culturable *S. aureus* cells that were able to silently persist within the osteocyte and over time, these cells switched to viable and culturable cells.

Adaptive laboratory evolution was used to expose *S. aureus* to continual antibiotic stress at an inhibitory concentration over time. Over multiple generations of growth within these inhibitory conditions we observed changes in cell type, and development of cells with increased antibiotic resistance and antibiotic tolerance.

We have undertaken a specific clinical study isolating *S. aureus* from patients with DFI. The complications of diabetes are notorious for establishing persistent infections within the foot ulcer and this can spread to the bone causing osteomyelitis. We have identified and extracted pairs of different *S. aureus* cell types within the same patient and notably we have isolated a pair of isolates comprising of a non-stable SCV and sSCV.

From all these research models of *S. aureus* we have identified alternate cell types selected for in response to the respective stressful conditions. These cell types had phenotypic changes,

with colonies of decreased pigmentation and size, decreased growth rate, increased biofilm formation, and increased resistance to antibiotics. Using whole genome sequencing, we identified a large variety of SNPs associated with these phenotypes, including carbohydrate metabolism, metal ion regulation, virulence regulation and cell wall biosynthesis. We found there was a lack of a common pathway associated with the introduced stress associated with our research models. This suggests the adaptations to these stressors are through selection of stochastic genetic variants with greater fitness instead of a defined pathway which lead to the observed cell types in response to the stress.

Chapter 1: Review of *Staphylococcus aureus* pathogenesis and the mechanisms underlying persistence through alternative lifestyles.

This chapter is comprised of a review of the literature detailing the background of *S. aureus* including the . This review is comprised of a published mini-review "Novel Research Models for *Staphylococcus aureus* Small Colony Variants (SCV) Development Co-pathogenesis and Growth Rate" [1] which describes the background of SCVs and the current research models used to study SCVs.

Statement of Authorship

Title of Paper	Functional *mgrA* influences genetic changes within a *Staphylococcus aureus* cell population over time
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Lee, J., Zilm, P.S., Kidd, S.P. (2020). "Novel Research Models for *Staphylococcus aureus* Small Colony Variants (SCV) Development: Co-pathogenesis and Growth Rate." *Front. Microbiol.* 11(321)

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Name of Principal Author (Candidate)	James Lee		
Contribution to the Paper	Carried out continuous culture experiments, experiments to characterise bacterial isolates, data analysis and drafted and wrote the manuscript		
Overall percentage (%)	80		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	8/4/22

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Stephen Kidd		
Contribution to the Paper	Review and editing of manuscript, conceived and designed the study. Facilitated design of methodologies		
Signature		Date	11 APR, 2022

Name of Co-Author	Peter Zilm		
Contribution to the Paper	Review and editing of manuscript, conceived and designed the study. Developed methodology for continuous culture experiments and characterising biofilm/growth of isolates.		
Signature		Date	12/04/22

Background

Staphylococcus (S.) aureus is an important human pathogen responsible for morbidity and mortality worldwide. In Australia, there are currently 180,000 hospital acquired infections a year, with approx. 8,000 *S.aureus* infections, with an estimated cost of $180 million a year, resulting in 1,700 deaths a year directly by *S. aureus* infection [2]. Clinically, *S. aureus* has become a huge burden, being involved in hospital acquired methicillin resistant *S. aureus* (HA-MRSA) and community acquired methicillin resistant *S. aureus* (CA-MRSA). *S. aureus* can adapt to many harsh conditions presented by host-specific environments, resulting in chronic/relapsing infection.

S. aureus is a Gram positive, facultative anaerobe and non-motile opportunistic bacteria. It is identified by its characteristic carotenoid pigment staphlyoxanthin, which gives the colonies a bright yellow colour, and its haemolytic activity [3]. The anterior nares serve as a major reservoir for *S. aureus* and a source for relapsing systemic infections, where 30% of the population are asymptomatic carriers and 60% are intermittent carriers [4, 5]. However, *S. aureus* is able to colonise many other anatomical niches where either a change in the *S. aureus* physiological response and/or the host environment results in a pathogenic cell type and disease symptoms. A major issue of *S. aureus* related diseases is the high incidence of chronic and recurrent infections. These arise from antibiotic treatments being ineffective due to two mechanisms: (A) antibiotic resistances (MRSA and VRSA: vancomycin resistant *S. aureus*) and (B) subpopulations within the original infection. of persister cells which tolerate killing by antibiotic treatments and host immune responses. Selection of antimicrobial resistant *S. aureus* by antibiotic treatments is a common basis of chronic infection [6]. There are a series of infections by *S. aureus* that are difficult to clear and have a high rate of reoccurrence.

Osteomyelitis

Osteomyelitis is a bone infection which causes inflammation and bone destruction. *S. aureus* a predominant cause of osteomyelitis, and this is mediated by its surface proteins which bind to bone matrix and collagen in a complex pathway of pathogenesis [7]. Furthermore, this interaction can lead to formation of *S. aureus* biofilms, conferring protection from antimicrobials. *S. aureus* have been associated with specific bone cell types: osteoblasts and osteocytes. *S. aureus* SCVs have been identified during chronic bone infections. SCVs internalisation within osteoblasts or osteocytes allows them to be even more protected from antimicrobials and hidden from the host immune response [4, 7]. Long-term antibiotic treatments are associated with relapsing and chronic infection. Infected dead bone fragments can develop in chronic osteomyelitis, which requires surgical bone debridement to clear the infection However this reduces bone stock, creating issues in fracture stability and prosthesis stability in prosthetic joint infection. An established *S. aureus* infection in a contaminated prosthetic requires revision surgery to remove in many cases, which has greatly associated inherent risks, especially considering reduced bone stock [6].

Foreign body-related infections

Foreign body-related infections, as found in pacemakers, prosthetic valve infectious endocarditis, heart assist devices and prosthetic joint infections (PJI), have been associated with the presence of SCV populations. After implementation of a prosthetic, the surface is covered in the hosts proteins which are targets of adhesion for *S. aureus* [8]. Infection occurs after manipulation of the prosthetic body, and formation of a biofilm on the prosthetic surface, which facilitates tolerance to antimicrobials and the host immune response and adhesion [4, 6].

Cystic fibrosis

Cystic fibrosis (CF) patients are predisposed to bacterial infection of the airways, which produces a vicious mucous layer which facilitate colonisation and infection by opportunistic pathogens. *S. aureus* is usually one of the first pathogens of CF airways, which facilitates co-infection with *Pseudomonas aeruginosa*, a highly prevalent CF pathogen which infects over 80% of adult CF patients. Such pathogens can establish lifelong persistent and chronic infection, with reoccurring infections and significant decline in lung function. Long-term antibiotic treatment with aminoglycosides in CF also creates a selection pressure for SCVs with corresponding resistances, further contributing to bacterial persistence [6].

Infective endocarditis

There is an elevated risk to infective endocarditis in patients with prosthetic valves due to a high probability of seeding of *S. aureus* in the bloodstream. Incomplete recovery of endothelialisation of prosthetic valve after valve replacement surgery leads to development of infective endocarditis. Inflammation of cardiac endothelium causes sub-endothelium to produce extracellular matrix proteins, tissue factors, fibrin and platelets which *S. aureus* can attach, bind and colonise with specific surface proteins [4].

Diabetic foot infection

Complications in diabetes can predispose patients to diabetic foot ulcers (DFU) which results in a wound that is slow to heal and susceptible to infection. DFUs are a highly exposed site which can allow infection and progress to a diabetic foot infection (DFI), cellulitis and osteomyelitis [9-11]. The persistent and chronic nature of DFUs means there is constant exposure of (the deeper epithelial tissues, reference) to the environment. *S. aureus* is among the most common and prevalent pathogens which colonise a DFU [9, 12, 13] with prevalence of *S. aureus* in DFI varying between 15-38% depending on geography [14-17].

These complications of diabetes create an immunosuppressed site that facilitate persistent *S. aureus* colonisation. In combination with the high prevalence of antibiotic resistance, formation of biofilms and intracellular persistence within host cells, DFIs are incredibly difficult to clear with infections lasting for months.

Pathogenesis

Infection and colonisation

Asymptomatic carriage is now recognised as being a vital stage for *S. aureus* to establish an invasive or chronic infection. Its ability to adapt and survive in many environmental conditions allow infection of various host-specific niches, including skin and soft tissues, joints, heart valves to cause endocarditis, meninges to cause meningitis and systemic infections to cause sepsis [4].

Colonisation by *S. aureus* is as a commensal in the anterior nares (in additionally the skin and other musical surfaces), however it can disseminate through the circulatory system, where it can adhere to host extracellular matrix proteins and form biofilms [18]. Open wounds also present opportunities for external sources of *S. aureus* to colonise [19].

Adhesion is mediated by a subset of surface proteins, called Microbial Surface Components Recognising Adhesive Matrix Molecules (MSCRAMMs), which attach to the host extracellular matrix components such as fibrinogen, fibronectin and collagen. Two clumping factors (ClfA and ClfB) enable binding to fibrinogen and aggregates cells together. Clumped cells are more able to resistant clearance by the immune response where the increased size clump cannot be phagocytosed and the fibrinogen coating the clump reduces antibody binding and complement deposition [20].

Colonisation and attachment are required to prevent clearance from the host immune response and establish a population within the host. This process in pathogenesis is associated with

increased expression of adhesions and downregulation of secreted virulence factors. The exposure to other host tissues generates a switch in regulation which signals the switch from adhesive, non-invasive *S. aureus* to planktonic, virulent cells which can access deeper tissues and obtain nutrients from host molecules [21]. The determinant of the switch between these two cell lifestyles is reliant on the global regulator of virulence *agr*. The Agr system is a quorum sensing two component system (TCS) and a major regulator of virulence in *S. aureus* involved in the expression of toxins, proteases and leukocidins [22, 23]. Agr is self-regulated through the expression of an autoinducing peptide (AIP). Once the colonising cell population has grown enough to reach a threshold amount of AIP, or the quorum, the cell population upregulates *agr* expression and switches to the virulence, pathogenic cell type.

Virulence factors

Humans have many defence mechanisms in the form of the immune response to fight off bacterial invasion. For any pathogen to invade, colonise and grow within the human host, they must be able to overcome these mechanisms. *S. aureus* has an incredible ability to adapt and survive amidst these host factors.

Toxin Production: *S. aureus* can produce a multitude of toxins to overcome host factors to colonise and evade the immune response. Most *S. aureus* toxins belong in three major toxin groups; Pore-forming toxins which lyse host cells, such as Panton-Valentine leukocidins which lyses phagocytes and facilitates survival in the blood; Exfoliative toxins facilitate bacterial skin infection; Enterotoxins which belong to the family of superantigens (Sag) which over induces cytokine production from T-lymphocytes and macrophages causing inflammation, and apoptosis in host cells [24]. Essential nutrients such as iron are sequestered as a mechanism of limiting bacterial pathogenesis in the human body. The use of cytolytic toxins is required for acquisition of iron from host-sequestered iron sources [25].

Antibiotic resistance: Antibiotic resistance is highly prevalent in *S. aureus* populations and complicates treatments of many *S. aureus* infections, especially within hospital acquired infections. The staphylococcal cassette chromosome (SCC*mec*) is a mobile genetic element (MGE) which facilitates the spread of resistance through horizontal gene transfer through *S. aureus* populations [26]. These genetically encoded antibiotic resistances have become highly widespread in *S. aureus* populations with a high prevalence of resistance to β-lactam antibiotics, which are referred to as methicillin resistant *S. aureus* (MRSA). There is also the prevalence of resistances to tetracycline, erythromycin, clindamycin and mupirocin [27].

Antibiotic tolerance is distinct from classic mechanisms of resistance. Resistance permits growth in the presence of antibiotics and relates to the minimum inhibitory concentration (MIC), the concentration of antibiotic which inhibits bacterial growth. Tolerance occurs when there is no cell growth and no cell death in the presence of antibiotics. This occurs through a state of dormancy where antibiotic targets are either ineffective or unable to be targeted [28] and the lack of downstream pathways prevents lethal outcomes [29]. This relates to the minimum bactericidal concentration (MBC), the concentration of antibiotic required to actively kill bacteria.

Immune evasion: Many mechanisms of clearance by the host need to be circumvented for *S. aureus* to colonise and infect host tissues. This involves reactive oxygen species (ROS) produced from metabolic processes, oxidative stresses in the host inflammation response and phagocytosis [30, 31]. Reactive oxygen species targets DNA, RNA, proteins, and lipids to disrupt the cell membrane and vital cell processes, of which can cause bacterial death.

Neutrophils attack on *S. aureus* induces a change in gene expression to protect from clearance. Leukocidins generated by *S. aureus* can lyse neutrophils, and survival within neutrophils is achieved through expression of oxidative stress molecules (catalases, superoxide dismutase,

glutathione peroxidase and others), capsule synthesis and inhibition of metabolism. Downregulation of cell division indicates a focus on survival rather than growth [24, 32].

Alternative lifestyles

There are sub-populations of *S. aureus* with reduced metabolic activity, virulence and growth, among other properties, which creates an alternative lifestyle, facilitating persistent and relapsing infection. These properties arise from defects in metabolic processes and provide greater fitness in highly stressful environments.

While the mechanisms that alternative lifestyles arise may differ, these sub-populations generally create a reservoir of *S. aureus* which are difficult to clear either through the immune response or therapeutic means and can revert to the planktonic cell type. Thus, the infection is incredibly difficult to completely clear, resulting in chronic and/or relapsing infection which is currently a heavy burden in healthcare. Understanding these lifestyles is important in a clinical setting, as many aspects of these *S. aureus* lifestyles is poorly understood.

Persister cells: It is recognised that bacterial cultures include a sub-population of cells with phenotypic variations including a tolerance to antibiotics, and this is different to conventional resistance mechanisms. This can occur stochastically by epigenetic variants (persisters are not necessarily due to a genetic mutation). These are referred to as persister cells. Formation of persisters is growth stage dependent, with increased persister populations beginning in early to mid-exponential phase [33]. The mechanism of persister cell formation is still poorly understood, however the main basis of the persister state is a switch to metabolic dormancy. The current model of inducing the dormant state can be through the mechanism of toxin-antitoxin (TA) pairs. These toxins disrupt an essential cellular process of its own cell but concurrently produced is its antitoxin which prevents the action of the toxin, in a specific manner. There are multiple persister cell mechanisms involving TA pairs, which leads to

decreased ATP production, reduced growth and dormancy [34]. *S. aureus* TA system utilises the *mazEF* operon (MazF toxin, MazE anti-toxin) where greater mazF toxin leads to slow growth, decreased pigment and persistent in stressed conditions [35]. The *mazEF* operon is upstream of the *sigB* operon, and the two are closely regulated. The *sigB* operon requires *mazEF* promoter (P_{mazEF}) for full activity, and *sigB* negatively regulates P_{mazEF}, which creates a negative feedback loop. There is further control of *mazEF* with activation in response to stresses such as antibiotics and heat [36].

Biofilm: Biofilms are microbial derived community embedded within an extracellular matrix. This matrix is comprised of extracellular polymeric substances (EPS): extracellular DNA (eDNA), proteins and polysaccharides [37], which can sequester and concentrate environmental nutrients and for *S. aureus* much work has been done on the EPS being mediated by its polysaccharide intercellular adhesion (PIA) [38]. However, there are numerous reports of PIA-independent biofilms in *S. aureus* [39, 40]. This matrix creates a physical barrier which lead to decreased or incomplete diffusion of antibiotics reducing its effectiveness [41]. The thickness of this matrix creates a gradient of oxygen and nutrients through the biofilm to create a heterogenous microbial population with four distinct metabolic states: aerobic, fermentative, dormant and dead. This diverse cell type population of *S. aureus* possess altered phenotypes and gene expression which allows survival against immune responses, host stressors and antibiotic treatments [18]. In a biofilm (especially important in a polymicrobial biofilm) there is also horizontal gene transfer, allowing for passage of important genetic material for cells to acquire traits for their future survival, such as antibiotic resistance [42]. Biofilm formation itself also confers alternate mechanisms of antibiotic resistances, which are not inherited resistances, but conferred by the biofilm itself as evidence by restored antibiotic susceptibility when bacteria disperse from the biofilm.

Intracellular survival: *S. aureus* can facilitate intracellular survival in both non-professional phagocytic cells [43-45] and professional phagocytes [43], which promotes evasion of the immune response and antimicrobial therapies. *In vitro* cell culture models in non-professional phagocytic cells by *S. aureus* shows a dependence on FnBPs for adhesion and activating cytoskeletal remodelling. Down regulation of α-toxin is required to keep the host cell alive and avoid an immune response. The intracellular population creates a reservoir of *S. aureus* which are difficult to detect and treat and creates relapsing infections [44, 46].

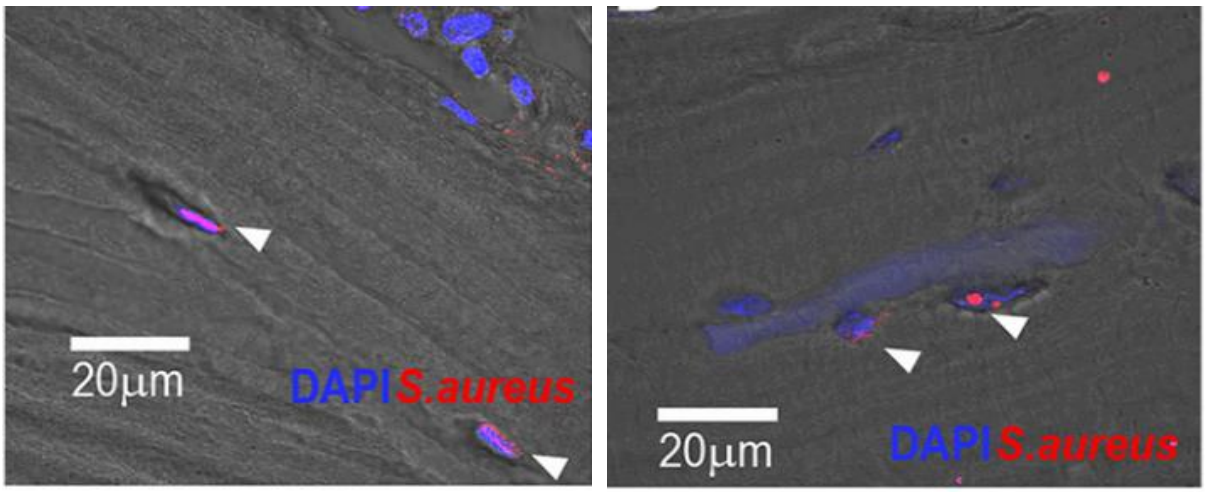

Figure 1. Electron microscopy of intracellular *S. aureus* localised within osteocytes (left) and acetabular bone of a prosthetic join infection (right) (Yang, et al. 2018).

Small colony variants (SCV)

Novel Research Models for Staphylococcus aureus Small Colony Variants (SCV) Development: Co-pathogenesis and Growth Rate.

We have published a mini-review "Novel Research Models for *Staphylococcus aureus* Small Colony Variants (SCV): Development Co-pathogenesis and Growth Rate" in Frontiers in Microbiology which has 13 citations at the time this thesis was submitted. This mini-review describes the background of *S. aureus* SCVs, the pathways that underly this cell type and the methods used to study SCVs within laboratory settings.

frontiers in Microbiology

MINI REVIEW
published: 28 February 2020
doi: 10.3389/fmicb.2020.00321

Novel Research Models for *Staphylococcus aureus* Small Colony Variants (SCV) Development: Co-pathogenesis and Growth Rate

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Staphylococcus aureus remains a great burden on the healthcare system. Despite prescribed treatments often seemingly to be successful, *S. aureus* can survive and cause a relapsing infection which cannot be cleared. These infections are in part due to quasi-dormant sub-population which is tolerant to antibiotics and able to evade the host immune response. These include Small Colony Variants (SCVs). Because SCVs readily revert to non-SCV cell types under laboratory conditions, the characterization of SCVs has been problematic. This mini-review covers the phenotypic and genetic changes in stable SCVs including the selection of SCVs by and interactions with other bacterial species.

Keywords: *Staphylococcus aureus*, small colony variants, continuous culture, prolonged growth, co-colonization

OPEN ACCESS

Edited by:
Lorena Tuchscherr,
Jena University Hospital, Germany

Reviewed by:
Daniel O. Sordelli,
University of Buenos Aires, Argentina
Karsten Becker,
University Medicine Greifswald, Germany

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Specialty section:
This article was submitted to Infectious Diseases, a section of the journal Frontiers in Microbiology

Received: *10 December 2019*
Accepted: *13 February 2020*
Published: *28 February 2020*

Citation:
Lee J, Zilm PS and Kidd SP (2020) Novel Research Models for Staphylococcus aureus Small Colony Variants (SCV) Development: Co-pathogenesis and Growth Rate. Front. Microbiol. 11:321. doi: 10.3389/fmicb.2020.00321

INTRODUCTION

Staphylococcus aureus is a Gram positive, facultative anaerobe and non-motile opportunistic bacterium. Approximately 30% of the human population are asymptomatic carriers and 60% are intermittent carriers. The anterior nares serve as the major reservoir and thereby becomes a source for systemic infections (Tong et al., 2015) as *S. aureus* is also an important human pathogen responsible for morbidity and mortality worldwide (Tong et al., 2015; Kahl et al., 2016). These infections can be difficult to clear due to a persistent reservoir of *S. aureus* which survives antibiotic treatment.

Even within a genetically clonal population, it is now recognized that there exists a variety of phenotypes which can be referred to as alternative lifestyles (Balaban et al., 2004; Wood et al., 2013). In contrast to classical resistance mechanisms, these phenotypes confer tolerance to antimicrobials, where there is limited or no growth, but not cell death (Keren et al., 2004). The alternative lifestyles arise through disruptions to cellular activities and not through the acquisition of new virulence genes (Balaban et al., 2004; Wood et al., 2013). These lifestyles involve forming quasi-dormant sub-populations during infection which have increased fitness in unfavorable conditions. While the mechanisms may differ, these sub-populations generally create a reservoir of *S. aureus* within an anatomical niche which are difficult to clear by the host immune response or therapeutic means and can revert to their parental, active cell type (Keren et al., 2004; Singh et al., 2009). These phenotypic switches in *S. aureus* include formation of biofilms, persister cells and Small Colony Variants (SCVs).

S. aureus Small Colony Variants

Staphylococcus aureus SCVs are characterized by small colony size, impeded growth, loss of carotenoid pigment (Proctor et al., 2006; Melter and Radojevič, 2010) non-hemolytic and coagulase negative (Thomas, 1955; Quie, 1969; Melter and Radojevič, 2010; Bui et al., 2015). SCVs exhibit changes in structural morphology and dysfunction in cell separation (Kahl et al., 2003a). While they have been extensively studied there remain unknown nuances to their development and reversion. It is known that they form through auxotrophy in elements of the electron transport chain and ATP production (Kahl et al., 2003a; Proctor et al., 2006). These are either single or a combination of auxotrophy in the biosynthesis of menadione, hemin or thymidine (Kahl et al., 2003a; Kohler et al., 2008; Melter and Radojevič, 2010; Maduka-Ezeh et al., 2012; Dean et al., 2014; Horiuchi et al., 2015) CO_2 (Thomas, 1955) and fatty acids (Schleimer et al., 2018). Many SCV isolates have no defined auxotrophism (Edwards, 2012) and various mutations in the electron transport chain that result in SCV (Proctor, 2019) are not observed in clinical isolates.

Although complex, reduced ATP production is associated with variations in cell-wall biosynthesis and carotenoid pigment (Proctor et al., 2006, 2014). Global genetic change has been observed in the switch to SCVs, with increased biofilm formation (Singh et al., 2010), autolysis (Bui et al., 2015), expression of adhesive proteins (Mirani et al., 2015) and decreased expression of secreted virulence factors (Melter and Radojevič, 2010; Tuchscherr et al., 2010; Ou et al., 2016).

Small Colony Variants persist intracellularly, avoiding clearance by the host immune response (Melter and Radojevič, 2010; Kahl et al., 2016). Examples of SCV infections include the lungs in patients with cystic fibrosis (Kahl et al., 1998, 2003b; Schwerdt et al., 2018), bovine mastitis (Atalla et al., 2011), osteomyelitis (Proctor et al., 1995), and foreign body infections (Baddour and Christensen, 1987; Von Eiff et al., 1999) including prosthetic-periprosthetic joint infection (PJI) (Yang et al., 2018). SCVs have an innate tolerance of antibiotics which is not associated with resistance genes (Edwards, 2012). The lack of an electrochemical gradient in their cell wall prevents aminoglycosides from entering the cell and the growth dormancy reduces the effectiveness of antibiotics that target metabolic processes of actively growing cells (Proctor et al., 1998; Melter and Radojevič, 2010; Garcia et al., 2013). This allows selection of SCV and chronic infection in antibiotic treatment, such as the use of gentamicin in bone cement for prosthetic joint implants (Chang et al., 2013). SCVs also have greater intracellular uptake and survival within non-professional phagocytic cells (fibroblast, epithelial, endothelial, osteoblast, osteocytes, keratinocytes) and professional phagocytes (Garzoni and Kelley, 2009). *In vitro* cell culture models of non-professional phagocytic cells shows a dependence on fibronectin binding proteins (FbNPs) for adhesion and activating cytoskeletal remodeling (Sendi and Proctor, 2009). Many surface adhesion molecules are upregulated in SCVs (Gómez-González et al., 2010; Bui and Kidd, 2015). Down regulation of α-toxin is required to keep the host cell alive and allow persistence intracellularly (Garzoni and Kelley, 2009; Sendi and Proctor, 2009).

Clinically isolated SCVs are often not stable and revert to their non-SCV cell type when cultured in the laboratory (Edwards, 2012; Kriegeskorte et al., 2014a; Bui et al., 2015). Also, clinical isolates of SCVs from diseased tissues give no indication of the parental type, leaving questions as to the genetic and molecular mechanisms involved during the transition from the original cell to its SCV phenotype. While SCVs can be abundant in persistent infections (Bates et al., 2003), identifying and culturing clinical samples is indeed difficult. The use of genetically stable SCVs (sSCV) has greatly improved our understanding of SCVs (Kriegeskorte et al., 2014a; Bui et al., 2015). Models of sSCV *S. aureus* include mutants auxotrophic to menadione (Schaaff et al., 2003; Lannergård et al., 2008; Dean et al., 2014; Pader et al., 2014), hemin (Balwit et al., 1994; Von Eiff et al., 1997; Schaaff et al., 2003), thymidine (Balwit et al., 1994; Von Eiff et al., 1997; Besier et al., 2007; Chatterjee et al., 2008; Kriegeskorte et al., 2014a; Kittinger et al., 2019), fatty acids (Bazaid et al., 2018; Schleimer et al., 2018) CO_2 (Thomas, 1955; Gómez-González et al., 2010), chorismite synthesis (precursor for aromatic amino acids and menaquinone biosynthesis) (Zhang et al., 2017), selection in gentamicin (Balwit et al., 1994) and serial passage in mice models immunized against capsular polysaccharide (Tuchscherr et al., 2008).

Induction of Stable SCV by Prolonged Slow Growth Under Nutrient Limitation

Alternative methods of inducing the transition to SCV and formation of sSCV have been reported. Bui et al., grew the clinical blood isolate, WCH-SK2 for a prolonged time-period by continuous culture under nutrient limiting conditions and a low growth rate using an *in vitro* system allows single parameters, outside the complexity of *in vivo* conditions (Bui et al., 2015). Introduced oxidative stress and growth over 209 generations (60 days) produced a sSCV which dominated the population. Accumulated oxidative stress causes damage to DNA, and the SOS response creates mismatch repairs and increased rate of mutation. Previous studies have shown the frequency of SCV formation increases with the rate of mutation (Schaaff et al., 2003; Vestergaard et al., 2015; Lacoma et al., 2019).

This methodology has been replicated to select for sSCV in other *S. aureus* strains; such as MW2, community acquired MRSA blood isolate (Lee et al., unpublished). Unlike sSCV created in the laboratory (by site directed mutations of specific genes), continuous culture considers the phenotypic and genetic responses in a time-dependent manner within nutrient limiting conditions. This enables one strain to be observed in transition from a parental population to one with a diversity of cell types and then dominated by SCVs.

Whole genome sequencing of WCH-SK2 and WCH-SK2-SCV revealed 24 genetic events; single nucleotide polymorphisms (SNPs) or insertions-deletions (indels) which could be implicated with the switch to a sSCV while under nutrient starved, stressed conditions (Bui et al., 2015; **Table 1**). The instability of some clinical SCV isolates suggests their phenotype is transcriptionally controlled (and/or post-transcription) rather than by stable SNPs.

TABLE 1 | The switch to a Small Colony Variant (SCV) by WCH-SK2 through continuous culture was associated with genetic events (SNPs) in global regulators of virulence.

	Agr	MgrA	ArlRS	SigB
Extracellular components				
cap5A-P	+	+	+	+
icaABCD		+	+	
ebh				
clfA				+
fnbA	−			+
fnbB	−			+
SpA	−	−	−	+
Leukocidins				
lukDE	+	+	−	−
lukSF	+	+		−
lukM		+		−
Exotoxins				
hla	+	+		−
hlb	+	+		−
seb	+	+		−
sea, seb, sec	+			
etaA, etaB	+			
tst-1	+			
Secretory proteases				
splA, splB, splC, splD	+	+	−	−
sspA	+	+		−
sspBC	+		−	−
nuc1	+	+		−
srtA		+		−
Autolysis				
lytM		−		+
lytN		−	−	
lytSR			+	
atl		−		
cidA		−		
lgrAB		+	+	−
Metabolism				
Lactose metabolism		−	−	
Urea metabolism		−	−	
Arginine deaminase		−	−	

Shown are the change in expression of genes regulated in S. aureus pathogenesis through these global regulators. +, increased expression, −, decreased expression.

The *in vivo* environment with combined stressors likely pressures *S. aureus* to become intracellular and remain as a SCV.

MgrA –A Global Regulator of Virulence

The transition of WCH-SK2 to an sSCV was suggested to be associated with a SNP in the DNA binding domain of *mgrA* (R92C change in MgrA). This mutation could impact the binding kinetics (protein-DNA interaction); previous mutations studied in MgrA suggests mutations at this point would make MgrA non-functional. The loss of *mgrA* function has not been previously reported in any SCV clinical isolates, but present a new perspective for sSCVs. The global regulator MgrA is known to function downstream to the two-component ArlRS (Crosby et al., 2016; Kwiecinski et al., 2019) and controls genes including the upregulation of capsular polysaccharide, α-toxin, leukocidins, coagulase and protein A (Luong et al., 2006; Lei et al., 2019), all of which are virulence factors downregulated in SCVs (Proctor et al., 2014; Bui and Kidd, 2015). The loss of *mgrA* enhances autolysis (Ingavale et al., 2003, 2005), invasion of HeLa cells (Lei et al., 2019), increases biofilm formation (Trotonda et al., 2008; Crosby et al., 2016) and increases expression of microbial surface components recognizing adhesive matrix molecules (MSCRAMM) such as Ebh, a large 1.1-MDa protein (Crosby et al., 2016) and indeed other surface proteins (Kwiecinski et al., 2019). While the loss of function of MgrA has not been characterized in the context of formation or stability of SCVs, the downregulation of other homologous SarA family proteins have been associated with the formation of SCV (Kahl et al., 2005; Kriegeskorte et al., 2014b; Mirani et al., 2015).

Agr – The Quorum Sensing System

Another major determinant for the production of toxins and extracellular enzymes is the quorum sensing two component system (TCS), Agr. Quorum sensing is controlled by secretion of the *agr* inducing peptide (AIP) to upregulate *agr* expression in surrounding *S. aureus*. The downregulation or loss of *agr* has previously been reported in clinical SCVs (Vaudaux et al., 2002; Kahl et al., 2005; Moisan et al., 2006; Kriegeskorte et al., 2014b). RNAIII is the effector molecule of the Agr TCS and controls the upregulation of secreted proteins and toxins and downregulation of cell surface proteins (Rescei et al., 1986; Abdelnour et al., 1993; Arvidson and Tegmark, 2001; Novick, 2003; Cheung et al., 2004). SCVs can display changes in RNAIII in persistent infections through the RNA degrasome, production of small RNAs and toxin-antitoxins (Proctor et al., 2014).

RsbU – A Regulator of SigB

In stressful environments, *S. aureus* employs alternative sigma factor B (SigB) to sense changes in the environment and alter its gene expression profile accordingly. The SigB expression profile acts in opposition to the Agr TCS where there is an increased expression of cell surface proteins for colonization and decreased expression of secreted proteins and toxins (Kullik et al., 1998; Bischoff et al., 2004; Jonsson et al., 2004). RsbU positively regulates *sigB* in a growth phase dependent manner, with SigB expression highest during late exponential phase (Senn et al., 2005). SigB can regulate the switch to dormancy in *S. aureus* in opposition to *agr* and its regulation of virulence. High levels of SigB have been found in clinical SCVs isolated from cystic fibrosis (Moisan et al., 2006; Mitchell et al., 2008, 2013), osteomyelitis (Tuchscherr et al., 2017) and bovine mastitis (Mitchell et al., 2010a). SigB activity is associated with downregulation of the *agr* system (Yarwood and Schlievert, 2003) and immunogenic virulence factors (enterotoxins, hemolysins, secreted proteases) and shown to allow SCVs to persist intracellularly within human endothelium (Tuchscherr et al., 2015). This also is associated with the increase in expression of FnBPs such as FnbA which then contributes to biofilm formation, adhesion

and intracellular persistence. Furthermore, *sigB* expression is required for intracellular replication of SCVs, and was shown to confer greater fitness in a pulmonary mouse model (Mitchell et al., 2013). SigB and subsequent *agr* repression is required for formation of SCVs in response to aminoglycoside stress (Mitchell et al., 2010a).

SELECTION OF SCV WITHIN POLYMICROBIAL ENVIRONMENTS

Presence of *Pseudomonas aeruginosa* Selects for SCV

The diverse population of bacteria within the human microbiome means *S. aureus* is rarely in isolation during infection or commensal carriage. The specific nature of the microbial population within a niche creates a vast array of complex interactions between *S. aureus* and other bacterial species and this affects its ability to colonize, acquire nutrients and proliferate. The local bacteria are known to impact on *S. aureus* cell types. One such interaction is a co-culture of *S. aureus* and *P. aeruginosa* which has been found to select for SCV or persisters of *S. aureus*. Various *S. aureus* infections are frequently isolated alongside *P. aeruginosa* such as in soft tissue infections, diabetic foot infections, osteomyelitis and within cystic fibrosis airways (Kahl et al., 2016). These two species can act competitively or cooperatively, such as *P. aeruginosa* secretions of LasB; an elastase which removes lung surfactant and prevents uptake by macrophages to allow effective colonization and persistence in the lung (Hotterbeekx et al., 2017).

Conversely, the secretion of an antistaphyloccocal metabolite, 4-hydroxy-2-heptylquinoline-N-oxide (HQNQ), by *P. aeruginosa* inhibits *S. aureus* growth through interrupting its electron transport chain and ATP production (Machan et al., 1992; Proctor, 2019). Long term exposure to physiological concentrations of HQNQ in combination with aminoglycosides *in vitro* resulted in high proportions of menadione SCVs (Hoffman et al., 2006). Indeed, in the co-existence of these bacteria, *S. aureus* becomes less susceptible to vancomycin and other antibiotics (Orazi and O'Toole, 2017; Radlinski et al., 2017). This has also been recognized through clinically relevant analyses (Mitchell et al., 2010b; Fugère et al., 2014).

No other interaction between *S. aureus* and other bacterial species has been found to directly induce SCV formation. However, if we consider the selective pressures which allow SCV to survive and dominate a population of cells through increased fitness over their parental cell types; including a reduced nutrient availability, antibiotics, phagocytosis, extreme pH; we can deduce that the interactions between *S. aureus* and other bacterial species which negatively affect *S. aureus* pathogenesis and survival may also be selecting for SCV (or indeed, other quasi-dormant cell types). *Corynebacterium* spp. and other Staphylococci are known to inhibit *S. aureus* virulence (in particular, through blocking *agr* function), nutrient acquisition and adhesion (Iwase et al., 2010; Wollenberg et al., 2014; Ramsey et al., 2016). *Streptococcus* spp. and *Staphylococcus lugdunensis* produce exoproducts which actively kill *S. aureus* (Zipperer et al., 2016; Wu et al., 2019). Many of these species we describe later are carried in the nares (Lina et al., 2003; Huttenhower et al., 2012), a potentially ideal anatomical niche for selecting and forming SCVs. Thymidine auxotroph SCVs have been isolated from the nares in a patient with AIDS (von Eiff et al., 2004) and pulmonary fibrosis (Cleeve et al., 2006). There are cases of *S. aureus* progressing from nasal colonization to bacteremia without the acquisition of additional virulence genes and SNPs in *arlS* and *agrA* (Benoit et al., 2018); genetic profiles previously reported in clinical SCVs (Kahl et al., 2005; Kohler et al., 2008; Kriegeskorte et al., 2014b). However, in contrast, *S. aureus* nasal colonization has been shown to favor dispersed cell-types rather than biofilm formation (Krismer and Peschel, 2011) which may imply SCV are less fit in the nares. The interactions between *S. aureus* and other bacterial species has not been researched in detail, and so we review the interactions in the context of selection of SCV.

Staphylococcus epidermidis is commonly found to outcompete *S. aureus* in the nares (Lina et al., 2003; Frank et al., 2010; Lee et al., 2019). *S. epidermidis* can block *agr* quorum sensing in *S. aureus* (Otto et al., 1999) and production of the serine protease, Esp, which inhibits *S. aureus* colonization through inhibiting biofilm formation and synergistically increasing the ability of Human-beta defensin 2 to clear *S. aureus* (Iwase et al., 2010). In a similar fashion, *Staphylococcus caprae*, a skin commensal, also interferes with *S. aureus* colonization and produces an AIP which blocks *S. aureus agr* sensing (Paharik et al., 2017).

Staphylococcus lugdunensis produces a peptide antibiotic, lugdunin, which has bactericidal effects against *S. aureus* (Zipperer et al., 2016). The mechanism of action of lugdunin against *S. aureus* has not been determined, and so whether this bactericidal effect selects for SCV is unclear. However, it has been shown to act synergistically with the innate immune response, where lugdunin increases recruitment of monocytes and neutrophils to keratinocytes (Bitschar et al., 2019).

Propionibacterium spp. produces an exoproduct coproporphyrin III (CIII) which induces aggregation and biofilm formation in *S. aureus* within acidic conditions (pH 4–6) (Wollenberg et al., 2014). SarA was shown to be involved in CIII mediated biofilm, however, the role of other biofilm regulators was not tested in this study.

Streptococcus pneumoniae production of hydrogen peroxide is able to kill *S. aureus* within *in vitro* conditions (Uehara et al., 2001; Regev-Yochay et al., 2006; Wu et al., 2019). Hydrogen peroxide can oxidize iron groups to damage proteins, or generate OH^- to cause DNA damage (Keyer and Imlay, 1996). However, a study using the nasal cavities of neonatal rats showed this hydrogen peroxide is not enough to affect *S. aureus* colonization (Margolis, 2009). It is known that over prolonged periods of time *S. aureus* in its SCV state can tolerate hydrogen peroxide (Painter et al., 2015) and so *S. aureus* may switch to a SCV when assaulted by *S. pneumoniae* generated hydrogen peroxide.

Both *S. aureus* and *Cornyebacterium* spp. are common nasal colonizers (Frank et al., 2010) and *in vitro* co-colonization of *S. aureus* with *Corynebacterium* spp. results in a shift of *S. aureus* from a virulent to a commensal state, with a strongly inhibited *agr* (Ramsey et al., 2016). Co-cultures with *Corynebacterium* spp.

(including nasal strains *C. striatum*, *C. amycolatum*, *C. accolens*, *C. pseudodiptheriticum* and a soil strain *C. glutamicum*) interferes with AIP-1 (*agr* activator molecule) and increases clearance of *S. aureus* within a murine model of infection (Ramsey et al., 2016). This co-culture is associated with a global change in gene regulation, with a notably increased expression of *spa* (260-fold) and down-regulation of *agr*. The loss of *agr* function has been associated with resistance to the bacteriocidal effects of *C. pseudodiptheriticum* (Hardy et al., 2019), however, the bacteriocidal mechanism by *C. pseudodiptheriticum* has not been established. The loss of *agr* activity and increased expression of adhesive surface proteins are common characteristics of SCV and this is an indicator that *S. aureus* may resist clearance by *Corynebacterium* spp. by switching to SCV.

CONCLUSION

To advance both diagnosis and treatment protocols for *S. aureus* infections, it is vital to understand the molecular mechanisms that select for or induce the formation of SCVs. Current therapeutic treatments are becoming less effective against persistent *S. aureus* infections. Given the tendency for SCVs to revert, and the difficulty in their culturing, means research into *S. aureus* SCVs still remains a challenge. The use of continuous culture to select for SCVs amongst a population of cells has a great potential in discovering the molecular mechanisms involved in the transition into a SCV state which other sSCV models cannot clearly define.

Another aspect of SCV formation to consider is the influence of the microbiota co-existing with *S. aureus*. Studies with *S. aureus* and *P. aeruginosa* have shown this interaction selects for quasi-dormant cell types. The conditions in the nares, interactions with other bacterial species and their ability to persist intracellularly may favor the formation of SCVs. These can then transit around the human body and evade the host immune response. This provides a new perspective on the nasal carriage of *S. aureus* and the increased risk of endogenous *S. aureus* infection (Von Eiff et al., 2001; Kluytmans and Wertheim, 2005; Stanaway et al., 2007; Haleem et al., 2014; Dunyach-Remy et al., 2017). This includes immunocompromised sites such as the diabetic foot (Lavery et al., 2006) or a foreign body surface for attachment and biofilm production (Kahl et al., 2016; Yang et al., 2018).

AUTHOR CONTRIBUTIONS

JL, PZ, and SK each contributed to the design, construction, and writing of this manuscript.

REFERENCES

Abdelnour, A., Arvidson, S., Bremell, T., Ryden, C., and Tarkowski, A. (1993). The accessory gene regulator (agr) controls *Staphylococcus aureus* virulence in a murine arthritis model. *Infect. Immun.* 61, 3879–3885. doi: 10.1128/iai.61.9.3879-3885.1993

Arvidson, S., and Tegmark, K. (2001). Regulation of virulence determinants in *Staphylococcus aureus*. *Int. J. Med. Microbiol.* 291, 159–170. doi: 10.1078/1438-4221-00112

Atalla, H., Gyles, C., and Mallard, B. (2011). *Staphylococcus aureus* small colony variants (SCVs) and their role in disease. *Anim. Health Res. Rev.* 12, 33–45. doi: 10.1017/S1466252311000065

Baddour, L. M., and Christensen, G. D. (1987). Prosthetic valve endocarditis due to small-colony staphylococcal variants. *Rev. Infect. Dis.* 9, 1168–1174. doi: 10.1093/clinids/9.6.1168

Balaban, N. Q., Merrin, J., Chait, R., Kowalik, L., and Leibler, S. (2004). Bacterial persistence as a phenotypic switch. *Science* 305, 1622–1625. doi: 10.1126/science.1099390

Balwit, J. M., van Langevelde, P., Vann, J. M., and Proctor, R. (1994). Gentamicin-resistant menadione and hemin auxotrophic *Staphylococcus aureus* persist within cultured endothelial cells. *J. Infect Dis.* 170, 1033–1037. doi: 10.1093/infdis/170.4.1033

Bates, D. M., Von Eiff, C., McNamara, P. J., Peters, G., Yeaman, M. R., and Bayer, A. S. (2003). *Staphylococcus aureus menD* and *hemB* mutants are as infective as the parent strains, but the menadione biosynthetic mutant persists within the kidney. *J. Infect. Dis.* 187, 1654–1661. doi: 10.1086/374642

Bazaid, A. S., Forbes, S., Humphreys, G. J., Ledder, R. G., OCualain, R., and McBain, A. J. (2018). Fatty acid supplementation reverses the small colony variant phenotype in triclosan-adapted *Staphylococcus aureus*: genetic, proteomic and phenotypic analyses. *Sci. Rep.* 8:3876. doi: 10.1038/s41598-018-21925-6

Benoit, J. B., Frank, D. N., and Bessesen, M. T. (2018). Genomic evolution of *Staphylococcus aureus* isolates colonizing the nares and progressing to bacteremia. *PLoS One* 13:e0195860. doi: 10.1371/journal.pone.0195860

Besier, S., Ludwig, A., Ohlsen, K., Brade, V., and Wichelhaus, T. A. (2007). Molecular analysis of the thymidine-auxotrophic small colony variant phenotype of *Staphylococcus aureus*. *Int. J. Med. Microbiol.* 297, 217–225. doi: 10.1016/j.ijmm.2007.02.003

Bischoff, M., Dunman, P., Kormanec, J., Macapagal, D., Murphy, E., and Mounts, W. (2004). Microarray-based analysis of the *Staphylococcus aureus* σB regulon. *J. Bacteriol.* 186, 4085–4099. doi: 10.1128/jb.186.13.4085-4099.2004

Bitschar, K., Sauer, B., Focken, J., Dehmer, H., Moos, S., and Konnerth, M. (2019). Lugdunin amplifies innate immune responses in the skin in synergy with host- and microbiota-derived factors. *Nat. Commun.* 10:2730. doi: 10.1038/s41467-019-10646-7

Bui, L. M. G., Hoffmann, P., Turnidge, J. D., Zilm, P. S., and Kidd, S. P. (2015). Prolonged growth of a clinical *Staphylococcus aureus* strain selects for a stable small-colony-variant cell type. *Infect. Immun.* 83, 470–481. doi: 10.1128/IAI.02702-14

Bui, L. M. G., and Kidd, S. P. (2015). A full genomic characterization of the development of a stable small colony variant cell-type by a clinical *Staphylococcus aureus* strain. *Infect. Genet. Evol.* 36, 345–355. doi: 10.1016/j.meegid.2015.10.011

Chang, Y., Tai, C. L., Hsieh, P. H., and Ueng, S. W. (2013). Gentamicin in bone cement: a potentially more effective prophylactic measure of infectionin joint arthroplasty. *Bone Joint Res.* 2, 220–226. doi: 10.1302/2046-3758.210.2000188

Chatterjee, I., Kriegeskorte, A., Fischer, A., Deiwick, S., Theimann, N., Proctor, R. A., et al. (2008). In vivo mutations of thymidylate synthase (Encoded by *thyA*) are responsible for thymidine dependency in clinical small-colony variants of *Staphylococcus aureus*. *J. Bacteriol.* 190, 834–842. doi: 10.1128/jb.00912-07

Cheung, A. L., Bayer, A. S., Zhang, G., Gresham, H., and Xiong, Y. Q. (2004). Regulation of virulence determinants in vitro and in vivo in *Staphylococcus aureus*. *FEMS Immun. Med. Microbiol.* 40, 1–9. doi: 10.1016/s0928-8244(03)00309-2

Cleeve, V. J., Perry, J. D., Cresswell, G., and Orr, K. E. (2006). Thymidine-dependent meticillin-resistant *Staphylococcus aureus*: a potentially unrecognized reservoir of MRSA in hospital patients? *J. Hosp. Infect.* 63, 228–229. doi: 10.1016/j.jhin.2005.11.009

Crosby, H. A., Schlievert, P. M., Merriman, J. A., King, J. M., Salgado-Pabon, W., and Horswill, A. R. (2016). The *Staphylococcus aureus* global regulator mgra modulates clumping and virulence by controlling surface protein expression. *PLoS Pathog.* 12:e1005604. doi: 10.1371/journal.ppat.1005604

Dean, M. A., Olsen, R. J., Wesley Long, S., Rosato, A. E., and Musser, J. M. (2014). Identification of point mutations in clinical *Staphylococcus aureus* strains that produce small-colony variants auxotrophic for menadione. *Infect. Immun.* 82, 1600–1605. doi: 10.1128/IAI.01487-13

Dunyach-Remy, C., Courtais-Coulon, C., DeMattei, C., Jourdan, N., Schuldiner, S., and Sultan, A. (2017). Link between nasal carriage of *Staphylococcus aureus* and infected diabetic foot ulcers. *Diabetes Metab.* 43, 167–171. doi: 10.1016/j.diabet.2016.09.003

Edwards, A. M. (2012). Phenotype switching is a natural consequence of *Staphylococcus aureus* replication. *J. Bacteriol.* 194, 5404–5412. doi: 10.1128/JB.00948-12

Frank, D. N., Feazel, L. M., Bessesen, M. T., Price, C. S., Janoff, E. N., and Pace, N. R. (2010). The human nasal microbiota and *Staphylococcus aureus*. *PLoS One* 5:e10598. doi: 10.1371/journal.pone.0010598

Fugère, A., Lalonde Séguin, D., Mitchell, G., Déziel, E., Dekimpe, V., and Cantin André, M. (2014). Interspecific small molecule interactions between clinical isolates of *Pseudomonas aeruginosa* and *Staphylococcus aureus* from adult cystic fibrosis patients. *PLoS One* 9:e86705. doi: 10.1371/journal.pone.0086705

Garcia, L. G., Lemaire, S., Kahl, B. C., Becker, K., Proctor, R. A., and Denis, O. (2013). Antibiotic activity against small-colony variants of *Staphylococcus aureus*: review of in vitro, animal and clinical data. *J. Antimicrob. Chemother.* 68, 1455–1464. doi: 10.1093/jac/dkt072

Garzoni, C., and Kelley, W. L. (2009). *Staphylococcus aureus*: new evidence for intracellular persistence. *Trends Microbiol.* 17, 59–65. doi: 10.1016/j.tim.2008.11.005

Gómez-González, C., Acosta, J., Villa, J., Barrado, L., Sanz, F., Orellana, M. A., et al. (2010). Clinical and molecular characteristics of infections with CO2-dependent small-colony variants of *Staphylococcus aureus*. *J. Clin. Microbiol.* 48, 2878–2884. doi: 10.1128/JCM.00520-10

Haleem, A., Schultz, J. S., Heilmann, K. P., Dohrn, C. L., Diekema, D. J., and Gardner, S. E. (2014). Concordance of nasal and diabetic foot ulcer staphylococcal colonization. *Diagn. Microbiol. Infect. Dis.* 79, 85–89. doi: 10.1016/j.diagmicrobio.2014.01.014

Hardy, B. L., Dickey, S. W., Plaut, R. D., Riggins, D. P., Stibitz, S., and Otto, M. (2019). *Corynebacterium pseudodiphtheriticum* exploits *Staphylococcus aureus* virulence components in a novel polymicrobial defense strategy. *mBio* 10:e02491-18. doi: 10.1128/mBio.02491-18

Hoffman, L. R., Déziel, E., DArgenio, D. A., Lépine, F., Emerson, J., and McNamara, S. (2006). Selection for *Staphylococcus aureus* small-colony variants due to growth in the presence of *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. U.S.A.* 103, 19890–19895. doi: 10.1073/pnas.0606756104

Horiuchi, K., Matsumoto, T., Ota, Y., Kasuga, E., Negishi, T., and Yaguchi, T. (2015). Addition of thymidine to culture media for accurate examination of thymidine-dependent small-colony variants of methicillin-resistant *Staphylococcus aureus*: a pilot study. *J. Microbiol. Methods* 110, 40–44. doi: 10.1016/j.mimet.2015.01.007

Hotterbeekx, A., Kumar-Singh, S., Goossens, H., and Malhotra-Kumar, S. (2017). In vivo and In vitro interactions between *Pseudomonas aeruginosa* and *Staphylococcus* spp. *Front. Cell. Infect. Microbiol.* 7:106. doi: 10.3389/fcimb.2017.00106

Huttenhower, C., Gevers, D., Knight, R., Abubucker, S., Badger, J. H., and Chinwalla, S. T. (2012). Structure, function and diversity of the healthy human microbiome. *Nature* 486, 207–214. doi: 10.1038/nature11234

Ingavale, S. S., Van Wamel, W., and Cheung, A. L. (2003). Characterization of RAT, an autolysis regulator in *Staphylococcus aureus*. *Mol. Microbiol.* 48, 1451–1466. doi: 10.1046/j.1365-2958.2003.03503.x

Ingavale, S. S., van Wamel, W., Luong, T. T., Lee, C. Y., and Cheung, A. L. (2005). Rat/MgrA, a regulator of autolysis, is a regulator of virulence genes in *Staphylococcus aureus*. *Infect. Immun.* 73, 1423–1431. doi: 10.1128/iai.73.3.1423-1431.2005

Iwase, T., Uehara, Y., Shinji, H., Tajima, A., Seo, H., and Takada, K. (2010). *Staphylococcus epidermidis* Esp inhibits *Staphylococcus aureus* biofilm formation and nasal colonization. *Nature* 465, 346–349. doi: 10.1038/nature09074

Jonsson, I. M., Arvidson, S., Foster, S., and Tarkowski, A. (2004). Sigma factor B and RsbU are required for virulence in *Staphylococcus aureus*-induced arthritis and sepsis. *Infect. Immun.* 72, 6106–6111. doi: 10.1128/iai.72.10.6106-6111.2004

Kahl, B. C., Becker, K., and Löffler, B. (2016). Clinical significance and pathogenesis of staphylococcal small colony variants in persistent infections. *Clin. Microbiol. Rev.* 29, 401–427. doi: 10.1128/CMR.00069-15

Kahl, B. C., Belling, G., Becker, P., Chatterjee, I., Wardecki, K., and Hilgert, K. (2005). Thymidine-dependent *Staphylococcus aureus* small-colony variants are associated with extensive alterations in regulator and virulence gene expression profiles. *Infect. Immun.* 73, 4119–4126. doi: 10.1128/iai.73.7.4119-4126.2005

Kahl, B. C., Belling, G., Reichelt, R., Herrmann, M., Proctor, R. A., and Peters, G. (2003a). Thymidine-dependent small-colony variants of *Staphylococcus aureus* exhibit gross morphological and ultrastructural changes consistent with impaired cell separation. *J. Clin. Microbiol.* 41, 410–413. doi: 10.1128/jcm.41.1.410-413.2003

Kahl, B. C., Duebbers, A., Lubritz, G., Haeberle, J., Koch, H. G., and Ritzerfeld, B. (2003b). Population dynamics of persistent *Staphylococcus aureus* isolated from the airways of cystic fibrosis patients during a 6-year prospective study. *J. Clin. Microbiol.* 41, 4424–4427. doi: 10.1128/jcm.41.9.4424-4427.2003

Kahl, B. C., Herrmann, M., Everding, A. S., Koch, H. G., Becker, K., and Harms, E. (1998). Persistent infection with small colony variant strains of *Staphylococcus aureus* in patients with cystic fibrosis. *J. Infect. Dis.* 177, 1023–1029. doi: 10.1086/515238

Keren, I., Kaldalu, N., Spoering, A., Wang, Y., and Lewis, K. (2004). Persister cells and tolerance to antimicrobials. *FEMS Microbiol. Lett.* 230, 13–18. doi: 10.1016/s0378-1097(03)00856-5

Keyer, K., and Imlay, J. A. (1996). Superoxide accelerates DNA damage by elevating free-iron levels. *Proc. Natl. Acad. Sci. U.S.A.* 93, 13635. doi: 10.1073/pnas.93.24.13635

Kittinger, C., Toplitsch, D., Folli, B., Masoud, L. L., and Zarfel, G. (2019). Phenotypic Stability of *Staphylococcus aureus* small colony variants (SCV) isolates from cystic fibrosis (CF) Patients. *Int. J. Env. Res. Public Health* 16:1940. doi: 10.3390/ijerph16111940

Kluytmans, J. A., and Wertheim, H. F. (2005). Nasal carriage of *Staphylococcus aureus* and prevention of nosocomial infections. *Infection* 33, 3–8. doi: 10.1007/s15010-005-4012-9

Kohler, C., von Eiff, C., Liebeke, M., McNamara, P. J., Lalk, M., and Proctor, R. A. (2008). A defect in menadione biosynthesis induces global changes in gene expression in *Staphylococcus aureus*. *J. Bacteriol.* 190, 6351–6364. doi: 10.1128/JB.00505-08

Kriegeskorte, A., Block, D., Drescher, M., Windmüller, N., Mellmann, A., Baum, C., et al. (2014b). Inactivation of thyA in *Staphylococcus aureus* attenuates virulence and has a strong impact on metabolism and virulence gene expression. *mBio* 5:e01447-14. doi: 10.1128/mBio.01447-14

Kriegeskorte, A., Grubmüller, S., Huber, C., Kahl, B. C., von Eiff, C., and Proctor, R. A. (2014a). *Staphylococcus aureus* small colony variants show common metabolic features in central metabolism irrespective of the underlying auxotrophism. *Front. Cell. Infect. Microbiol.* 4:141. doi: 10.3389/fcimb.2014.00141

Krismer, B., and Peschel, A. (2011). Does *Staphylococcus aureus* nasal colonization involve biofilm formation? *Future Microbiol.* 6, 489–493. doi: 10.2217/fmb.11.37

Kullik, I., Giachino, P., and Fuchs, T. (1998). Deletion of the alternative sigma factor σ(B) in *Staphylococcus aureus* reveals its function as a global regulator of virulence genes. *J. Bacteriol.* 180, 4814–4820. doi: 10.1128/jb.180.18.4814-4820.1998

Kwiecinski, J. M., Crosby, H. A., Valotteau, C., Hippensteel, J. A., Nayak, M. K., and Chauhan, A. K. (2019). *Staphylococcus aureus* adhesion in endovascular infections is controlled by the arlRS–MgrA signaling cascade. *PLoS Pathog.* 15:e1007800. doi: 10.1371/journal.ppat.1007800

Lacoma, A., Edwards, A. M., Young, B. C., Domínguez, J., Prat, C., and Laabei, M. (2019). Cigarette smoke exposure redirects *Staphylococcus aureus* to a virulence profile associated with persistent infection. *Sci. Rep.* 9:10798. doi: 10.1038/s41598-019-47258-6

Lannergård, J., Von Eiff, C., Sander, G., Cordes, T., Seggewiß, J., and Peters, G. (2008). Identification of the genetic basis for clinical menadione-auxotrophic small-colony variant isolates of *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* 52, 4017–4022. doi: 10.1128/AAC.00668-08

Lavery, L. A., Armstrong, D. G., Wunderlich, R. P., Mohler, M. J., Wendel, C. S., and Lipsky, B. A. (2006). Risk factors for foot infections in individuals with diabetes. *Diabetes Care* 29, 1288–1293. doi: 10.2337/dc05-2425

Lee, D. C., Kananurak, A., Tran, M. T., Connolly, P. A., Polage, C. R., and Iwase, T. (2019). Bacterial colonization of the hospitalized newborn: competition between *Staphylococcus aureus* and *Staphylococcus epidermidis*. *Paediatr. Inf. Dis. J.* 38, 682–686. doi: 10.1097/INF.0000000000002285

Lei, M. G., Gudeta, D. D., Luong, T. T., and Lee, C. Y. (2019). MgrA negatively impacts *Staphylococcus aureus* invasion by regulating capsule and FnbA. *Infect. Immun.* 87:e00590-19. doi: 10.1128/IAI.00590-19

Lina, G., Boutite, F., Tristan, A., Bes, M., Etienne, J., and Vandenesch, F. (2003). Bacterial competition for human nasal cavity colonization: role of staphylococcal *agr* alleles. *Appl. Environ. Microbiol.* 69, 18–23. doi: 10.1128/aem.69.1.18-23.2003

Luong, T. T., Dunman, P. M., Murphy, E., Projan, S. J., and Lee, C. Y. (2006). Transcription profiling of the *mgrA* regulon in *Staphylococcus aureus*. *J. Bacteriol.* 188, 1899–1910. doi: 10.1128/jb.188.5.1899-1910.2006

Machan, Z. A., Graham, W. T., Pitt, T. L., Cole, P. J., and Wilson, R. (1992). 2-heptyl-4-hydroxyquinoline N-oxide, an antistaphylococcal produced by *Pseudomonas aeruginosa*. *J. Antimicrob. Chemother.* 30, 615–623. doi: 10.1093/jac/30.5.615

Maduka-Ezeh, A., Seville, M. T., Kusne, S., Vikram, H. R., Blair, J. E., and Greenwood-Quaintance, K. (2012). Thymidine auxotrophic *Staphylococcus aureus* small-colony variant endocarditis and left ventricular assist device infection. *J. Clin. Microbiol.* 50, 1102–1105. doi: 10.1128/JCM.01170-11

Margolis, E. (2009). Hydrogen peroxide-mediated interference competition by *Streptococcus pneumoniae* has no significant effect on *Staphylococcus aureus* nasal colonization of neonatal rats. *J. Bacteriol.* 191, 571–575. doi: 10.1128/JB.00950-08

Melter, O., and Radojevič, B. (2010). Small colony variants of *Staphylococcus aureus*—review. *Folia Microbiol.* 55, 548–558. doi: 10.1007/s12223-010-0089-3

Mirani, Z. A., Aziz, M., and Khan, S. I. (2015). Small colony variants have a major role in stability and persistence of *Staphylococcus aureus* biofilms. *J. Antibiot.* 68, 98–105. doi: 10.1038/ja.2014.115

Mitchell, G., Brouillette, E., Séguin, D. L., Asselin, A. E., Jacob, C. L., and Malouin, F. (2010a). A role for sigma factor B in the emergence of *Staphylococcus aureus* small-colony variants and elevated biofilm production resulting from an exposure to aminoglycosides. *Microb. Pathog.* 48, 18–27. doi: 10.1016/j.micpath.2009.10.003

Mitchell, G., Fugère, A., Gaudreau, K., Brouillette, E., Frost, E. H., and Cantin, A. M. (2013). SigB is a dominant regulator of virulence in *Staphylococcus aureus* small-colony variants. *PLoS One* 8:e65018. doi: 10.1371/journal.pone.0065018

Mitchell, G., Lamontagne, C. A., Brouillette, E., Grondin, G., Talbot, B. G., and Grandbois, M. (2008). *Staphylococcus aureus* SigB activity promotes a strong fibronectin-bacterium interaction which may sustain host tissue colonization by small-colony variants isolated from cystic fibrosis patients. *Mol. Microbiol.* 70, 1540–1555. doi: 10.1111/j.1365-2958.2008.06511.x

Mitchell, G., Séguin, D. L., Asselin, A. E., Déziel, E., Cantin, A. M., and Frost, E. H. (2010b). *Staphylococcus aureus* sigma B-dependent emergence of small-colony variants and biofilm production following exposure to *Pseudomonas aeruginosa* 4-hydroxy-2-heptylquinoline-N-oxide. *BMC Microbiol.* 10:33. doi: 10.1186/1471-2180-10-33

Moisan, H., Brouillette, E., Jacob, C. L., Langlois-Bégin, P., Michaud, S., and Malouin, F. (2006). Transcription of virulence factors in *Staphylococcus aureus* small-colony variants isolated from cystic fibrosis patients is influenced by SigB. *J. Bacteriol.* 188, 64–76. doi: 10.1128/jb.188.1.64-76.2006

Novick, R. P. (2003). Autoinduction and signal transduction in the regulation of staphylococcal virulence. *Mol. Microbiol.* 48, 1429–1449. doi: 10.1046/j.1365-2958.2003.03526.x

Orazi, G., and O'Toole, G. A. (2017). *Pseudomonas aeruginosa* alters *Staphylococcus aureus* sensitivity to vancomycin in a biofilm model of cystic fibrosis infection. *mBio* 8:e00873-17. doi: 10.1128/mBio.00873-17

Otto, M., Süßmuth, R., Vuong, C., Jung, G., and Götz, F. (1999). Inhibition of virulence factor expression in *Staphylococcus aureus* by the *Staphylococcus epidermidis* agr pheromone and derivatives. *FEBS Lett.* 450, 257–262.

Ou, J. J., Drilling, A. J., Cooksley, C., Bassiouni, A., Kidd, S. P., and Psaltis, A. J. (2016). Reduced innate immune response to a *Staphylococcus aureus* small colony variant compared to its wild-type parent strain. *Front. Cell. Infect. Microbiol.* 6:187. doi: 10.3389/fcimb.2016.00187

Pader, V., James, E. H., Painter, K. L., Wigneshweraraj, S., and Edwards, A. M. (2014). The agr quorum-sensing system regulates fibronectin binding but not hemolysis in the absence of a functional electron transport chain. *Inf. Immun.* 82, 4337–4347. doi: 10.1128/IAI.02254-14

Paharik, A. E., Parlet, C. P., Chung, N., Todd, D. A., Rodriguez, I., and Van Dyke, M. J. (2017). Coagulase-negative staphylococcal strain prevents *Staphylococcus aureus* colonization and skin infection by blocking quorum sensing. *Cell Host Microbe* 22, 746.e5–756.e5. doi: 10.1016/j.chom.2017.11.001

Painter, K. L., Strange, E., Parkhill, J., Bamford, K. B., Armstrong-James, D., and Edwards, A. M. (2015). *Staphylococcus aureus* adapts to oxidative stress by producing H2O2 resistant small-colony variants via the SOS response. *Inf. Immun.* 83, 1830–1844. doi: 10.1128/IAI.03016-14

Proctor, R. A. (2019). Respiration and small colony variants of *Staphylococcus aureus*. *Microbiol. Spectr.* 7, 1–15. doi: 10.1128/microbiolspec.GPP3-0069-2019

Proctor, R. A., Kahl, B., Von Eiff, C., Vaudaux, P. E., Lew, D. P., and Peters, G. (1998). Staphylococcal small colony variants have novel mechanisms for antibiotic resistance. *Clin. Infect. Dis.* 27(Suppl. 1), S68–S74.

Proctor, R. A., Kriegeskorte, A., Kahl, B. C., Becker, K., Löffler, B., and Peters, G. (2014). *Staphylococcus aureus* small colony variants (SCVs): a road map for the metabolic pathways involved in persistent infections. *Front. Cell. Infect. Microbiol.* 4:99. doi: 10.3389/fcimb.2014.00099

Proctor, R. A., Van Langevelde, P., Kristjansson, M., Maslow, J. N., and Arbeit, R. D. (1995). Persistent and relapsing infections associated with small-colony variants of *Staphylococcus aureus*. *Clin. Infect. Dis.* 20, 95–102. doi: 10.1093/clinids/20.1.95

Proctor, R. A., von Eiff, C., Kahl, B. C., Becker, K., McNamara, P., and Herrmann, M. (2006). Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections. *Nat. Rev. Microbiol.* 4, 295–305. doi: 10.1038/nrmicro1384

Quie, P. G. (1969). Microcolonies (G-Variants) of *Staphylococcus aureus*. *Yale J. Bio. Med.* 41, 394–403.

Radlinski, L., Rowe Sarah, E., Kartchner Laurel, B., Maile, R., Cairns Bruce, A., and Vitko, P. (2017). *Pseudomonas aeruginosa* exoproducts determine antibiotic efficacy against *Staphylococcus aureus*. *PLoS Biol.* 15:e2003981. doi: 10.1371/journal.pbio.2003981

Ramsey, M. M., Freire, M. O., Gabrilska, R. A., Rumbaugh, K. P., and Lemon, K. P. (2016). *Staphylococcus aureus* shifts toward commensalism in response to *Corynebacterium* species. *Front. Microbiol.* 7:1230. doi: 10.3389/fmicb.2016.01230

Regev-Yochay, G., Trzciński, K., Thompson, C. M., Malley, R., and Lipsitch, M. (2006). Interference between *Streptococcus pneumoniae* and *Staphylococcus aureus*: In Vitro hydrogen peroxide-mediated killing by *Streptococcus pneumoniae*. *J. Bacteriol.* 188, 4996–5001. doi: 10.1128/jb.00317-06

Rescei, R., Kreiswirth, B., Reilly, M. O., Schlievert, P., Gruss, A., and Novick, R. P. (1986). Regulation of exoprotein gene expression is *Staphylococcus aureus* by agr. *Mol. Gen. Genet.* 202, 58–61. doi: 10.1007/bf00330517

Schaaff, F., Bierbaum, G., Baumert, N., Bartmann, P., and Sahl, H. G. (2003). Mutations are involved in emergence of aminoglycoside-induced small colony variants of *Staphylococcus aureus*. *Int. J. Med Microbiol.* 293, 427–435. doi: 10.1078/1438-4221-00282

Schleimer, N., Kaspar, U., Drescher, M., Seggewiß, J., von Eiff, C., and Proctor, R. A. (2018). The energy-coupling factor transporter module EcfAAT, a novel candidate for the genetic basis of fatty acid-auxotrophic small-colony variants of *Staphylococcus aureus*. *Front. Microbiol.* 9:1863. doi: 10.3389/fmicb.2018.01863

Schwerdt, M., Neumann, C., Schwartbeck, B., Kampmeier, S., Herzog, S., and Görlich, D. (2018). *Staphylococcus aureus* in the airways of cystic fibrosis patients – A retrospective long-term study. *Int. J. Med. Microbiol.* 308, 631–639. doi: 10.1016/j.ijmm.2018.02.003

Sendi, P., and Proctor, R. A. (2009). *Staphylococcus aureus* as an intracellular pathogen: the role of small colony variants. *Trends Microbiol.* 17, 54–58. doi: 10.1016/j.tim.2008.11.004

Senn, M. M., Giachino, P., Homerova, D., Steinhuber, A., Strassner, J., and Kormanec, J. (2005). Molecular analysis and organization of the σB operon in *Staphylococcus aureus*. *J. Bacteriol.* 187, 8006–8019. doi: 10.1128/jb.187.23.8006-8019.2005

Singh, R., Ray, P., Das, A., and Sharma, M. (2009). Role of persisters and small-colony variants in antibiotic resistance of planktonic and biofilm-associated *Staphylococcus aureus*: an in vitro study. *J. Med. Microbiol.* 58(Pt 8), 1067–1073. doi: 10.1099/jmm.0.009720-0

Singh, R., Ray, P., Das, A., and Sharma, M. (2010). Enhanced production of exopolysaccharide matrix and biofilm by a menadione-auxotrophic *Staphylococcus aureus* small-colony variant. *J. Med. Microbiol.* 59(Pt 5), 521–527. doi: 10.1099/jmm.0.017046-0

Stanaway, S., Johnson, D., Moulik, P., and Gill, G. (2007). Methicillin-resistant *Staphyloccocus aureus* (MRSA) isolation from diabetic foot ulcers correlates with nasal MRSA carriage. *Diabetes Res. Clin. Pract.* 75, 47–50. doi: 10.1016/j.diabres.2006.05.021

Thomas, M. E. (1955). Studies on a CO2-dependent staphylococcus. *J. Clin. Pathol.* 8, 288–291. doi: 10.1136/jcp.8.4.288

Tong, S. Y., Davis, J. S., Eichenberger, E., Holland, T. L., and Fowler, V. G. Jr. (2015). *Staphylococcus aureus* infections: epidemiology, pathophysiology, clinical manifestations, and management. *Clin. Microbiol. Rev.* 28, 603–661. doi: 10.1128/cmr.00134-14

Trotonda, M. P., Tamber, S., Memmi, G., and Cheung, A. L. (2008). MgrA represses biofilm formation in *Staphylococcus aureus*. *Inf. Immun.* 76, 5645–5654. doi: 10.1128/IAI.00735-08

Tuchscherr, L., Bischoff, M., Lattar, S. M., Noto Llana, M., Pförtner, H., and Niemann, S. (2015). Sigma factor SigB is crucial to mediate *Staphylococcus aureus* adaptation during chronic infections. *PLoS Pathog.* 11:e1004870. doi: 10.1371/journal.ppat.1004870

Tuchscherr, L., Buzzola, F. R., Alvarez, L. P., Lee, J. C., and Sordelli, D. O. (2008). Antibodies to capsular polysaccharide and clumping factor a prevent mastitis and the emergence of unencapsulated and small-colony variants of *Staphylococcus aureus* in mice. *Infect. Immun.* 76, 5738–5744. doi: 10.1128/IAI.00874-08

Tuchscherr, L., Geraci, J., and Löffler, B. (2017). *Staphylococcus aureus* regulator sigma B is important to develop chronic infections in hematogenous murine osteomyelitis model. *Pathogens* 6:31. doi: 10.3390/pathogens6030031

Tuchscherr, L., Heitmann, V., Hussain, M., Viemann, D., Roth, J., and Von Eiff, C. (2010). *Staphylococcus aureus* small-colony variants are adapted phenotypes for intracellular persistence. *J. Infect. Dis.* 202, 1031–1040. doi: 10.1086/656047

Uehara, Y., Kikuchi, K., Nakamura, T., Nakama, H., Agematsu, K., Kawakami, Y., et al. (2001). H2O2 Produced by viridans group streptococci may contribute to inhibition of methicillin-resistant *Staphylococcus aureus* colonization of oral cavities in newborns. *Clin. Infect. Dis.* 32, 1408–1413. doi: 10.1086/320179

Vaudaux, P., Francois, P., Bisognano, C., Kelley, W. L., Lew, D. P., and Schrenzel, J. (2002). Increased expression of clumping factor and fibronectin-binding proteins by *hemB* mutants of *Staphylococcus aureus* expressing small colony variant phenotypes. *Inf. Immun.* 70, 5428–5437. doi: 10.1128/iai.70.10.5428-5437.2002

Vestergaard, M., Paulander, W., and Ingmer, H. (2015). Activation of the SOS response increases the frequency of small colony variants. *BMC Res. Notes* 8:749. doi: 10.1186/s13104-015-1735-2

Von Eiff, C., Becker, K., Machka, K., Stammer, H., and Peters, G. (2001). Nasal carriage as a source of *Staphylococcus aureus* bacteremia. *N. Engl. J. Med.* 344, 11–16.

Von Eiff, C., Heilmann, C., Proctor, R., Woltz, C., Peters, G., and Götz, F. (1997). A site-directed *Staphylococcus aureus hemB* mutant is a small colony variant which persists intracellularly. *J. Bacteriol.* 179, 4706–4712. doi: 10.1128/jb.179.15.4706-4712.1997

von Eiff, C., Lubritz, G., Heese, C., Peters, G., and Becker, K. (2004). Effect of trimethoprim-sulfamethoxazole prophylaxis in AIDS patients on the formation of the small colony variant phenotype of *Staphylococcus aureus*. *Diagn. Microbiol. Infect. Dis.* 48, 191–194. doi: 10.1016/j.diagmicrobio.2003.10.010

Von Eiff, C., Vaudaux, P., Kahl, B., Lew, D., Emler, S., and Schmidt, A. (1999). Bloodstream infections caused by small-colony variants of coagulase-negative staphylococci following pacemaker implantation. *Clin. Infect. Dis.* 29, 932–934. doi: 10.1086/520462

Wollenberg, M. S., Claesen, J., Escapa, I. F., Aldridge, K. L., Fischbach, M. A., and Lemon, K. P. (2014). *Propionibacterium*-produced coproporphyrin III induces *Staphylococcus aureus* aggregation and biofilm formation. *mBio* 5:e01286-14. doi: 10.1128/mBio.01286-14

Wood, T. K., Knabel, S. J., and Kwan, B. W. (2013). Bacterial persister cell formation and dormancy. *Appl. Environ. Microbiol.* 79, 7116–7121. doi: 10.1128/AEM.02636-13

Wu, X., Gordon, O., Jiang, W., Antezana, B. S., Angulo-Samudio, U. A., del Rio, C., et al. (2019). Interaction between *Streptococcus pneumoniae* and *Staphylococcus aureus* generates OH radicals that rapidly kill *Staphylococcus aureus* strains. *J. Bacteriol.* 201:e00474-19. doi: 10.1128/JB.00474-19

Yang, D., Wijenayaka, A. R., Solomon, L. B., Pederson, S. M., Findlay, D. M., and Kidd, S. P. (2018). Novel insights into *Staphylococcus aureus* deep bone infections: the involvement of osteocytes. *mBio* 9:e00415-18. doi: 10.1128/mBio.00415-18

Yarwood, J. M., and Schlievert, P. M. (2003). Quorum sensing in *Staphylococcus* infections. *J. Clin. Invest.* 112, 1620–1625. doi: 10.1172/jci200320442

Zhang, P., Wright, J. A., Osman, A. A., and Nair, S. P. (2017). An aroD Ochre mutation results in a *Staphylococcus aureus* small colony variant that can undergo phenotypic switching via two alternative mechanisms. *Front. Microbiol.* 8:1001. doi: 10.3389/fmicb.2017.01001

Zipperer, A., Konnerth, M. C., Laux, C., Berscheid, A., Janek, D., and Weidenmaier, C. (2016). Human commensals producing a novel antibiotic impair pathogen colonization. *Nature* 535, 511–516. doi: 10.1038/nature18634

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Continuing on from our mini-review, we build on this review of the literature to cover the clinical impacts associated with SCVs in the context of diabetic foot infection.

SCV clinical impact

Within a population of *S. aureus* is a heterogeneity of cells, where SCVs are present even in the absence of stress, with a consistently maintained level of slow growing, gentimicin resistant SCV sub-population. This sub-population provides *S. aureus* populations with the ability to adapt and expand an SCV population in response to environmental stress [47] to cause chronic and relapsing infections. SCVs are less susceptible to clearance by the immune response, by anti-microbial therapeutics, can switch to their virulent parental cell type to create a relapsing infection, and are difficult to detect [6].

SCVs are commonly isolated in cases of persistent infection, with the frequency of isolation of SCVs as high as 40% in osteomyelitis patients and 30% from bronchial secretions or throat swabs from cystic fibrosis patients [6, 48]. Up to 70% of cystic fibrosis airways were persistently colonised with both normal and SCV *S. aureus* [49] which is likely given the environmental niche of cystic fibrosis airways (hostile environment stressors, hypoxia, and multiple antibiotics) tend to lead to the emergence of SCVs [6]. Despite antibiotic treatment and surgical interventions, SCV infections are still incredibly difficult to clear. The use of antibiotics is normally required to treat *S. aureus* infections, however in combination with the high prevalence of antibiotic resistant strains, there is evidence long-term antibiotic treatments select for SCV populations in chronic osteomyelitis [28, 50, 51].The dormancy and asymptomatic virulence profile of SCVs presents a challenge for clinicians, where even after initial intervention has seemingly resulted in clearance of infection, a reservoir of SCVs can persist which are highly resistant to clearance. The unstable nature of SCV hinders isolation of clinical samples and research within a laboratory.

S. aureus in Diabetic Foot Infections

Diabetes mellitus is a group of chronic metabolic diseases characterized by elevated levels of blood sugar (hyperglycemia) due to insulin resistance or reduced insulin production. There are two types of diabetes, of which Type 2 is the most prevalent worldwide. Type 1 is an autoimmune response to pancreatic β-cells, where insufficient levels of insulin produced. Type 2 is caused by interactions between genetic factors, environmental and lifestyle risk factors resulting in resistances to the insulin produced, and rendering tissues non-responsive to insulin [52, 53]. In either case, there is decreased glucose transport to the tissues, where the lack of uptake of glucose stays in the blood resulting in hyperglycemia.

These are debilitating conditions responsible for an increase in morbidity and mortality worldwide. It affects an estimated 422 million people in 2014, with the prevalence of diabetes rising from 4.7% in 1980 to 8.5% of the adult population [54], with 1.2 million in Australia alone (5.1% of the population). It is the seventh most prevalent major long-term condition affecting Australians [55]. Patients with diabetes are at high risk of many short term and long term complications which may often lead to a premature death [52]. A common complication of diabetes is the susceptibility of ulcers on the plantar or dorsal foot, with incidence of diabetic foot ulcers (DFU) as high as 25% [10, 56]. These foot ulcers are a result of lower limb neuropathy and endothelial dysfunction, and are susceptible to infections, causing a diabetic foot infection (DFI).

Endothelial dysfunction

Endothelium is the inner lining of blood vessels and plays a key role in controlling the flow of blood through the production of vasodilators and vasoconstrictors to maintain arterial tone [57]. Endothelial dysfunction results in an imbalance of substances controlling these vasodilator and vasoconstrictors, of which can occur during the manifestation of diabetes [57, 58]. The

endothelium has roles in regulating bloody supply to the tissues around the body, allowing coagulation, platelet adhesion and immune responses, which are vital processes for wound healing [53]. Nitric oxide (NO) is a signalling molecule produced by vascular endothelial cells required for maintenance of vascular tone, anti-inflammatory states and other physiological process [57]. In a diabetic state, these processes can be disrupted through increased production of ROS and decreased availability of NO, causing dysfunction of endothelial processes, inflammation, and other pathologies [53, 57].

Neuropathy

Length dependent diabetic neuropathy (LDDP) is the most usual form of diabetic neuropathy. 80% of patients with diabetic neuropathy have distal symmetrical form of LDDP, with signs and symptoms starting in the proximal lower limbs, where the effects are most usually most prominent. [59, 60]. Neuropathy is associated with impaired glucose tolerance and hyperglycemia associated with diabetes. Hyperglycemia leads to increased ROS production which can damage neurons and lead to degeneration [60], and advance glycation products which act on receptors producing cytokines and adhesion molecules to damage nerve fibres [60, 61]. Endothelium dysfunction also creates deficits in substances required for nerve fibre function [62].

Diabetic foot ulcers (DFU) and diabetic foot infection (DFI).

The combination of endothelial dysfunction and neuropathy create environment where the diabetic foot is susceptible to long-term, chronic foot wounds defined as diabetic foot ulcers (DFU). These ulcers are debilitating, painful and are a heavy burden physically and emotionally to the patient. LDDP is most prominent in the foot, and while there are initial symptoms of LDDP (numbness, burning feet, pins-and-needles), there is usually a lack of medical intervention, which means constant foot damage can be occurring without feeling [60].

The endothelial dysfunction decreases the blood flow to the wound site, which can hinder the process of wound healing, and lead to persistent foot ulcers.[53, 56, 63]. Lack of perfusion to the foot is also a factor in the formation of callus, which can increase the pressure on the foot and hence the severity of DFUs [64]

The DFU is a highly exposed site which can allow infection and progress to a diabetic foot infection (DFI), cellulitis and osteomyelitis [9-11]. The persistent and chronic nature of DFUs means there is constant exposure of (the deeper epithelial tissues, reference) to the environment. The endothelial dysfunction decreases the ability for leukocytes to access the wound site, which leaves the DFU an immunocompromised site [53] in conditions more ideal for opportunistic pathogens to colonise and survive compared to a healthy individual. Consequently, the presence of a DFU is a high-risk factor for amputation where foot ulcers preceded as high as 84% of amputations in one study [65]. The progression to a DFI is a greater risk factor for amputation, with DFI preceding approximately two-thirds of lower-extremity amputations, second to only gangrene [66]. The pathophysiology of DFI is complex and is largely related to the prevalence of host-related disturbances. including immunopathy, neuropathy and endothelium dysfunction [67]. Recruitment of macrophages and monocytes to the site of infection is required to fight off pathogenic bacteria. Dysfunction of perfusion from hyperglycemia decreases the ability of these cells to migrate to the site of infection [67]. This in combination with the ability of *S. aureus* to evade the immune response result in DFI being an immunocompromised site high prone to persistent infection and a decreased ability to heal damaged tissue [9, 24].

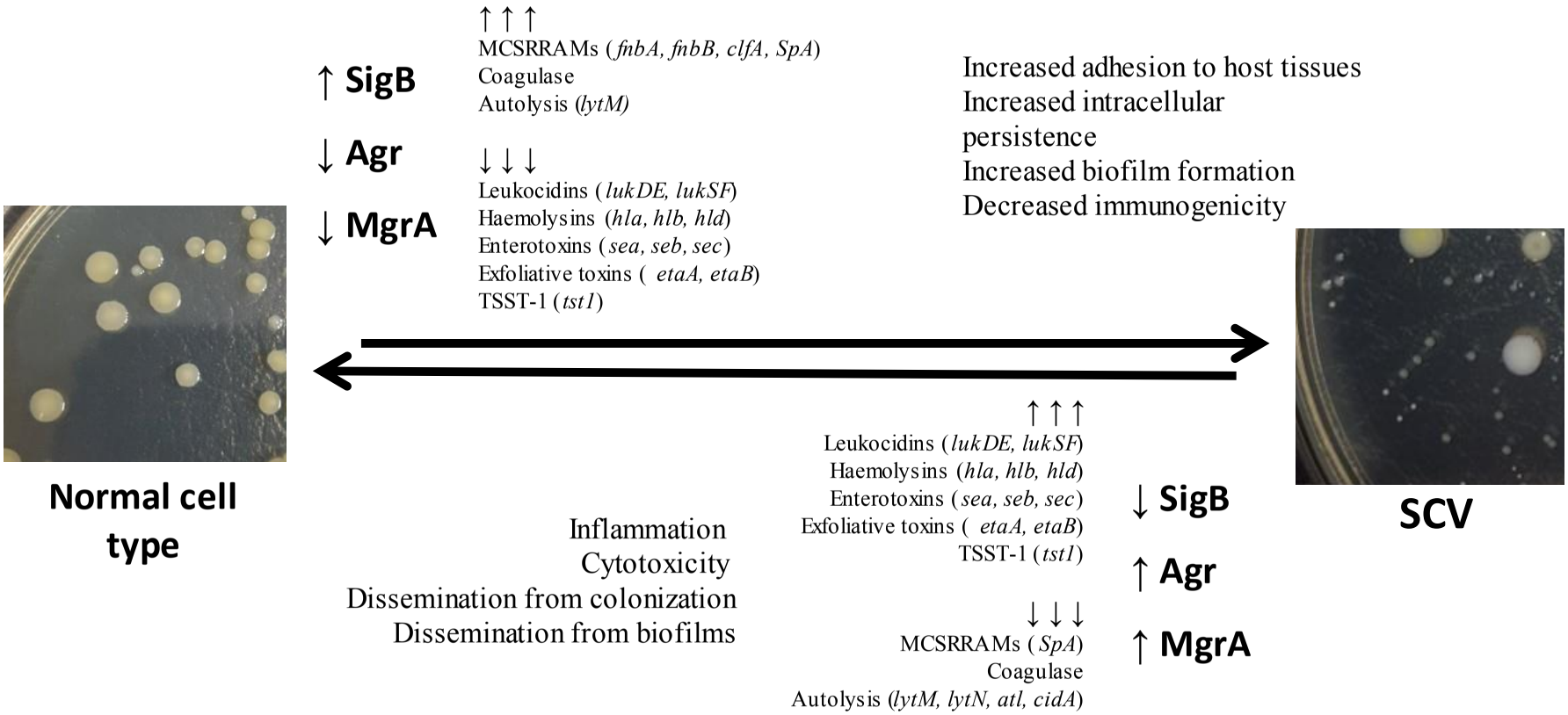

Figure 2. The switch from a normal cell type to a SCV from continuous culture in low growth conditions was associated with changes in the activity of global virulence regulators (SigB, Agr and MgrA), expression of virulence factors and overall changes in pathogenesis

S. aureus in Diabetic Foot Infection

S. aureus is among the most common and prevalent pathogens which colonise a DFU [9, 12, 13]. Prevalence of *S. aureus* in DFI can vary between 15-38% depending on geography [14-17] and is often in co-infection with other pathogens, although these can include strains that do not necessarily contribute to a worse outcome [11]. The severity of *S. aureus* infections in DFIs is associated with certain virulence profiles, with more severe ulcer infections are associated with *S. aureus* with increased prevalence of leukocidins, haemolysins, capsule and enterotoxins [68, 69]. *S. aureus* strains with decreased cytotoxic virulence profiles were found to be more prevalent in diabetics than non-diabetics, as diabetics are less able to clear infections [70]. The secretion of toxins facilitate the acquisition of nutrients from the host and dissemination to other tissues and result in the clinical symptoms of infection [71].

The progression to osteomyelitis is a common complication of DFI with osteomyelitis occurring in 10-15% of moderate infections and 50% in severe infections [72]. This is a consequence of soft tissue infections spreading to the bone and attaching to surfaces through MSCRAMMs. Protein A is secreted by *S. aureus* which can bind to osteoblasts which mediates apoptosis, inhibition of bone formation and activates bone resorption [7]. Adhesion of FnBPs to osteoblasts are required for internalisation into osteoblasts [73, 74] which results in either activation of osteoblast apoptosis or long-term intracellular persistence through the switch to a SCV [7, 75]. The switch to SCV is associated with decreased expression of virulence factors which would otherwise activate apoptosis or lyse the cell [76]. This intracellular environment favours selection of SCV with decreased virulence, cytotoxicity, and impeded metabolism [35, 77].

Endogenous *S. aureus* infection

The high prevalence of commensal *S. aureus* in the global population is an issue as a risk factor of transmitting and/or establishing *S. aureus* infection. An international study found in a cohort of 1166 orthopaedic surgeons, on average, 28% had *S. aureus* nasal colonisation and 2% carrying MRSA (with geographic variations: up to 6.1% in Asia, low as 0% in North America) [78]. The nasal carriage of *S. aureus* is an endogenous source of infection (where infective *S. aureus* strains has the same genotype as the nasal strain) with carriage frequently associated with *S. aureus* bacteremia [79]. Interestingly, nasal carriage is also associated with lower mortality rates by *S. aureus* related bacteremia infections compared to non-carriers of *S. aureus* although the prevalence of *S. aureus* infection is greater [79].

Meta-analyses of studies from 1979 to 2016 show nasal carriage of *S. aureus* is associated with increased prevalence of disease compared to people not colonised with *S. aureus* [80]. Many of these studies reveal the patients own *S. aureus* as an endogenous source of infection [79, 81]. Nasal carriage of *S. aureus* as a source of infection is further established as an endogenous source of infection with removal of *S. aureus* from the anterior nares showed a decrease in *S. aureus* infections [81, 82]. Approximately 30% of the global population carries *S. aureus*, of which are commensal, asymptomatic carriers or pathogenic carriers [4, 5]. Multiple body sites can be colonised by *S. aureus* including the skin and mucosa, with the nose the most prevalent site of carriage. Carriage of nasal *S. aureus* is associated with a greater prevalence of *S. aureus* carriage on the skin and other mucosal surfaces [5].

A review of the literature found only a few studies which have investigated the relationship between nasal carriage of commensal *S. aureus* and the *S. aureus* present in DFI. A study of 79 patients with DFUs attempted to investigate the association of nasal *S. aureus* and DFU *S. aureus* [83]. Confounding factors (polymicrobial infection, Body Mass Index, mean tissue oxygen) and small sample size believed to have contributed to poor correlations and the lack

of significant conclusions of the role of *S. aureus* carriage and infection. There was a trend of a longer duration of ulcer more likely to be colonised with MRSA. A study in France found nasal carriage of *S. aureus* was a risk factor for endogenous *S. aureus* infection of DFUs, with 65.3% of patients carrying the same strain of *S. aureus* in both the nares and wound [84]. A UK study found patients carrying MRSA in the nares were more likely to have a MRSA positive DFI (58%) compared to a MRSA negative DFI (8%) which suggests there may be some SCCmec specific mechanisms involved in dissemination from the nares to a foot ulcer [85].

These studies studied the role of *S. aureus* in isolation. There are often many other species of bacteria which *S. aureus* interact with in a polymicrobial environment. Many studies show the interplay between *S. aureus* and its interaction with other bacterial species, which can be cooperative (*Candida albicans*, *Enterococcus faecalis, Haemophilus influenzae, influenza virus*) and competitive (*Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, *Lactobacillus sp.* and *Corynebacteriam sp*.) [86]. This interplay of *S. aureus* and other bacterial species has significant effects on the pathogenesis of *S. aureus* and clinical outcomes which we had previously covered in our mini-review [1].

The progression from commensal to pathogenic

The patients nasal *S. aureus* is one of the most common sources of infection in bacteremia [79, 81], however the actual mechanism on how *S. aureus* progresses from a commensal population in the nares to entering the bloodstream is poorly understood.

A case study on patients with bacteremia with same *S. aureus* strain in both nose and blood. In all 8 cases, there was no acquisition of new virulence genes in the progression from colonisation to bloodstream infection. SNPs that were acquired were associated with membrane transport functions. Study found the SNPs compared between different time points

suggests sequence variation is due to strain evolution instead of selection of variable strains within the population [87]. The competition of other bacteria strains within the polymicrobial environment also effects *S. aureus* nasal colonisation [86, 88, 89]. These factors include whether host is a persistent or intermittent carrier (an established population is more difficult to displace), competition for adhesion sites, interaction with other species, competition for nutrients and competition by the induction of the host defence. Depending on the nature of the nasal environment, *S. aureus* may have more difficulty in colonising the nares which can lead to dissemination to the epithelial and blood.

An uncharacterised mechanism of *S. aureus* disseminating to the blood is through the formation of SCVs. Case studies show the genetic changes to *S. aureus* which progresses to the blood [87] have similarities to the changes seen in the switch to a sSCV by prolonged growth [31]. Adherence to the nasal epithelium during commensal carriage is controlled by *clfB* and *sasG* [90] and inhibition of *S. aureus* adherence to epithelial cells has been linked to *S. aureus* entering the bloodstream [5]. Studies with *mgrA* mutants show a down-regulation of *clfB* and *sasG* via down-regulation of the Agr system [20] which may result in an inability to colonise the nares and disseminate to other tissues. Indeed, our lab has previously isolated a SCV associated with a mutation in *mgrA* [91] which opens the potential that *S. aureus* is able to disseminate from commensal carriage in the nares to other sites of the body through the switch to a SCV. We had previously described the interactions of *S. aureus* with other species of bacteria which are potentially associated with the switch to alternate cell types within our mini-review [1].

Research Project

The switch to alternate cell types presents methods for *S. aureus* to establish persistent infections that are incredibly difficult to clear. This research investigates the clinically relevant factors involved in the selection of these cell types within bacterial populations and how they contribute to *S. aureus* persistence. Our research identifies two different paradigms of the shift to a population of SCV: 1) the molecular mechanisms which define the change from a metabolic active, normal cell type to the SCV and 2) the selection of SCV within a population with greater selective fitness within stressful conditions.

The vast majority of research in SCV is focused on the specific pathways which give rise to SCV. These involve generating a mutation which results in an impeded metabolism and the SCV phenotype. This creates a limited perspective on the formation of SCV focusing on a clonal population with a single mutation. However, the conditions of clinical infection are nuanced and highly complex with a multitude of factors that apply selective pressures on *S. aureus* rather than a single mutation giving rise to SCV. We postulate that the different cell types of *S. aureus* fall in a spectrum ranging from fast-growing, virulent profiles to dormant, non-pathogenic cell types. Through this spectrum, there is an accumulation of genetic changes and selective pressures such as reduced availability of nutrients, competition by other bacterial species, presence of antibiotics which contribute to the dormant phenotypes. The extremes in this end of the spectrum result in the SCV phenotype.

Our research identifies how population dynamics of *S. aureus* gives rise to SCV through the selection of these cell types. SCVs form stochastically within normal growth and are selected for in conditions of stress. Isolating and studying stochastically formed SCVs are difficult and so instead we identify the mechanisms which dictate selection of these SCVs.

Aim 1

To characterise the role of MgrA in the population dynamics and the selection of small colony variants of *S. aureus* in a controlled, low growth rate. Previous research reported that over a prolonged culture time, under stress, SCVs dominated a heterogenous population of cell types and these harboured a mutation in mgrA. The objectives of this aim involve:

- Generation a *mgrA* knockout mutation in WCH-SK2 for a model to investigate the effects of MgrA
- Culturing the WCH-SK2-Δ*mgrA* isolate in continuous culture as described previously to identify the effect of MgrA in an entire population of *S. aureus* and how it affects the population dynamics and selection of SCV
- Isolate sSCVs with genetically stable changes that we can identify using whole genome sequencing

Aim 2

To characterise the microbiology of different cell types of *S. aureus* associated with persistent diabetic foot infections. The objectives of this aim involve:

- A clinical study in collaboration with SA Health to recruit patients with diabetes. These included patients with uninfected foot ulcers, infected foot ulcers and hospitalised patients with osteomyelitis.
- Epidemiological and bacteriological data will be obtained from each patient to identify the association of population data and bacteria present with worse outcomes of diabetic foot infections.
- The pathogenic bacteria from patients with DFI and osteomyelitis were extracted from their wounds and bone (if applicable). Pairs of *S. aureus* from the bone and

wound of the same patient were selected to identify the genetic mechanisms involved in colonisation within the bone against colonisation in the wound.

- To identify the genetic and phenotypic changes in the switch to a SCV from pairs of *S. aureus* extracted from the same patient

Aim 3

To characterise the genetic mechanisms that underly the long-term evolution of *Staphylococcus aureus* to adapt to clinically relevant conditions. The objectives of this aim involve:

- Long-term infection of SaOS osteocytes with the *S. aureus* strain WCH-SK3 to identify the mechanisms underlying intracellular persistence.
- Long-term growth using Adaptive Laboratory Evolution. Using a clinical strain of *S. aureus* from DFI, we will expose this isolate to antibiotics at an inhibitory concentration

Reference list

1. Lee J, Zilm PS, Kidd SP. Novel Research Models for *Staphylococcus aureus* Small Colony Variants (SCV) Development: Co-pathogenesis and Growth Rate. *Front Microbiol*. 2020;**11**(321).

2. Turnidge JD, Nimmo GR, Pearson J, Gottlieb T, Collignon PJ, Resistance AGoA. Epidemiology and outcomes for *Staphylococcus aureus* bacteraemia in Australian hospitals. The Australian Government Department of Health; 2007.

3. Cohen ML. *Staphylococcus aureus*: biology, mechanisms of virulence, epidemiology. *J Pediatr*. 1986;**108**(5):796-9.

4. Tong SY, Davis JS, Eichenberger E, Holland TL, Fowler VG, Jr. *Staphylococcus aureus* infections: epidemiology, pathophysiology, clinical manifestations, and management. *Clin Microbiol Rev*. 2015;**28**(3):603-61.

5. Wertheim HFL, Melles DC, Vos MC, van Leeuwen W, van Belkum A, Verbrugh HA, et al. The role of nasal carriage in *Staphylococcus aureus* infections. *Lancet Infect Dis*. 2005;**5**(12):751-62.

6. Kahl BC, Becker K, Löffler B. Clinical significance and pathogenesis of staphylococcal small colony variants in persistent infections. *Clin Microbiol Rev*. 2016;**29**(2):401-27.

7. Josse J, Velard F, Gangloff SC. *Staphylococcus aureus* vs. Osteoblast: Relationship and Consequences in Osteomyelitis. *Front Cell Infect Microbiol*. 2015;**5**:85.

8. Neut D, Van Der Mei HC, Bulstra SK, Busscher HJ. The role of small-colony variants in failure to diagnose and treat biofilm infections in orthopedics. *Acta Orthopaedica*. 2007;**78**(3):299-308.

9. Lipsky BA, Berendt AR, Deery HG, Embil JM, Joseph WS, Karchmer AW, et al. Diagnosis and treatment of diabetic foot infections. *Clin Infect Dis*. 2004;**39**(7):885-910.

10. Armstrong DG, Lipsky BA. Diabetic foot infections: Stepwise medical and surgical management. *Int Wound J*. 2004;**1**(2):123-32.

11. Uckay I, Aragon-Sanchez J, Lew D, Lipsky BA. Diabetic foot infections: what have we learned in the last 30 years? *Int J Infect Dis*. 2015;**40**:81-91.

12. Akhi MT, Ghotaslou R, Memar MY, Asgharzadeh M, Varshochi M, Pirzadeh T, et al. Frequency of MRSA in diabetic foot infections. *Int J Diabetes in Dev Ctries*. 2017;**37**(1):58-62.

13. Bülent Ertuğrul M, Uyar-Güleç G, Baktıroğlu S, Çörekli E, Türe M. The distribution of causative microorganisms in diabetic foot infection: Has there been any alterations? *Klimik Dergisi*. 2017;**30**(1):27-31.

14. Hobizal KB, Wukich DK. Diabetic foot infections: current concept review. *Diabet Foot Ankle*. 2012;**3**.

15. Ge Y, MacDonald D, Hait H, Lipsky B, Zasloff M, Holroyd K. Microbiological profile of infected diabetic foot ulcers. *Diabet Med*. 2002;**19**(12):1032-4.

16. Abdulrazak A, Ibrahim Bitar Z, Ayesh Al-Shamali A, Ahmed Mobasher L. Bacteriological study of diabetic foot infections. *J Diabetes Complications*. 2005;**19**(3):138-41.

17. Tentolouris N, Jude EB, Smirnof I, Knowles EA, Boulton AJM. Methicillin-resistant *Staphylococcus aureus*: An increasing problem in a diabetic foot clinic. *Diabet Med*. 1999;**16**(9):767-71.

18. Archer NK, Mazaitis MJ, William Costerton J, Leid JG, Powers ME, Shirtliff ME. *Staphylococcus aureus* biofilms: Properties, regulation and roles in human disease. *Virulence*. 2011;**2**(5):445-59.

19. Liu GY. Molecular pathogenesis of *Staphylococcus aureus* infection. *Pediatr Res*. 2009;**65**(5 Pt 2):71R-7R.

20. Crosby HA, Schlievert PM, Merriman JA, King JM, Salgado-Pabon W, Horswill AR. The *Staphylococcus aureus* Global Regulator MgrA Modulates Clumping and Virulence by Controlling Surface Protein Expression. *PLoS Pathog*. 2016;**12**(5):e1005604.

21. Novick RP. Autoinduction and signal transduction in the regulation of staphylococcal virulence. *Mol Microbiol*. 2003;**48**(6):1429-49.

22. Abdelnour A, Arvidson S, Bremell T, Ryden C, Tarkowski A. The accessory gene regulator (agr) controls *Staphylococcus aureus* virulence in a murine arthritis model. *Infect Immune*. 1993;**61**(9):3879-85.

23. Jenul C, Horswill AR. Regulation of *Staphylococcus aureus* Virulence. *Microbiol Spectr*. 2019;**7**(2):10.1128/microbiolspec.GPP3-0031-2018.

24. Dunyach-Remy C, Essebe CN, Sotto A, Lavigne JP. *Staphylococcus aureus* toxins and diabetic foot ulcers: Role in pathogenesis and interest in diagnosis. *Toxins*. 2016;**8**(7):1-20.

25. Hammer ND, Skaar EP. Molecular mechanisms of *Staphylococcus aureus* iron acquisition. *Annu Rev Microbiol*. 2011;**65**:129-47.

26. Hanssen AM, Ericson Sollid JU. SCCmec in staphylococci: genes on the move. *FEMS Immunol Med Microbiol*. 2006;**46**(1):8-20.

27. Alekshun MN, Levy SB. Molecular Mechanisms of Antibacterial Multidrug Resistance. *Cell*. 2007;**128**(6):1037-50.

28. Garcia LG, Lemaire S, Kahl BC, Becker K, Proctor RA, Denis O, et al. Antibiotic activity against small-colony variants of *Staphylococcus aureus*: Review of in vitro, animal and clinical data. *J Antimicrob Chemother*. 2013;**68**(7):1455-64.

29. Pu Y, Ke Y, Bai F. Active efflux in dormant bacterial cells – New insights into antibiotic persistence. *Drug Resistance Updates*. 2017;**30**:7-14.

30. Cabiscol E, Tamarit J, Ros J. Oxidative stress in bacteria and protein damage by reactive oxygen species. *International Microbiology*. 2000;**3**:3-8.

31. Bui LMG, Kidd SP. A full genomic characterization of the development of a stable Small Colony Variant cell-type by a clinical *Staphylococcus aureus* strain. *Infect Genet Evol*. 2015;**36**:345-55.

32. Voyich JM, Braughton KR, Sturdevant DE, Whitney AR, Said-Salim B, Porcella SF, et al. Insights into Mechanisms Used by *Staphylococcus aureus* to Avoid Destruction by Human Neutrophils. *J Immunol*. 2005;**175**(6):3907-19.

33. Keren I, Kaldalu N, Spoering A, Wang Y, Lewis K. Persister cells and tolerance to antimicrobials. *FEMS Microbiol Lett*. 2004;**230**(1):13-8.

34. Wood TK, Knabel SJ, Kwan BW. Bacterial persister cell formation and dormancy. *Appl Environ Microbiol*. 2013;**79**(23):7116-21.

35. Proctor RA, Kriegeskorte A, Kahl BC, Becker K, Löffler B, Peters G. *Staphylococcus aureus* Small Colony Variants (SCVs): A road map for the metabolic pathways involved in persistent infections. *Front Cell Infect Microbiol*. 2014;**4**.

36. Donegan NP, Cheung AL. Regulation of the mazEF toxin-antitoxin module in *Staphylococcus aureus* and its impact on *sigB* expression. *J Bacteriol*. 2009;**191**(8):2795-805.

37. Bui LMG, Turnidge JD, Kidd SP. The induction of *Staphylococcus aureus* biofilm formation or Small Colony Variants is a strain-specific response to host-generated chemical stresses. *Microbes and Infection*. 2015;**17**(1):77-82.

38. Foulston L, Elsholz AK, DeFrancesco AS, Losick R. The extracellular matrix of *Staphylococcus aureus* biofilms comprises cytoplasmic proteins that associate with the cell surface in response to decreasing pH. *MBio*. 2014;**5**(5):e01667-14.

39. Trotonda MP, Tamber S, Memmi G, Cheung AL. MgrA represses biofilm formation in *Staphylococcus aureus*. *Inf Immune*. 2008;**76**(12):5645-54.

40. Mirani ZA, Aziz M, Khan SI. Small colony variants have a major role in stability and persistence of *Staphylococcus aureus* biofilms. *J Antibiot*. 2015;**68**(2):98-105.

41. Stewart PS, William Costerton J. Antibiotic resistance of bacteria in biofilms. *The Lancet*. 2001;**358**(9276):135-8.

42. Madsen JS, Burmølle M, Hansen LH, Sørensen SJ. The interconnection between biofilm formation and horizontal gene transfer. *FEMS Immunol Med Microbiol*. 2012;**65**(2):183-95.

43. Horn J, Stelzner K, Rudel T, Fraunholz M. Inside job: *Staphylococcus aureus* host-pathogen interactions. *Int J Med Microbiol*. 2018;**308**(6):607-24.

44. Garzoni C, Kelley WL. *Staphylococcus aureus*: new evidence for intracellular persistence. *Trends Microbiol*. 2009;**17**(2):59-65.

45. Loffler B, Tuchscherr L, Niemann S, Peters G. *Staphylococcus aureus* persistence in non-professional phagocytes. *Int J Med Microbiol*. 2014;**304**(2):170-6.

46. Sendi P, Proctor RA. *Staphylococcus aureus* as an intracellular pathogen: the role of small colony variants. *Trends Microbiol*. 2009;**17**(2):54-8.

47. Edwards AM. Phenotype switching is a natural consequence of *Staphylococcus aureus* replication. *J Bacteriol*. 2012;**194**(19):5404-12.

48. Bates DM, Von Eiff C, McNamara PJ, Peters G, Yeaman MR, Bayer AS, et al. *Staphylococcus aureus menD* and *hemB* mutants are as infective as the parent strains, but the menadione biosynthetic mutant persists within the kidney. *J Infect Dis*. 2003;**187**(10):1654-61.

49. von Eiff C. *Staphylococcus aureus* small colony variants: a challenge to microbiologists and clinicians. *Int J Antimicrob Agents*. 2008;**31**(6):507-10.

50. Tuchscherr L, Kreis CA, Hoerr V, Flint L, Hachmeister M, Geraci J, et al. *Staphylococcus aureus* develops increased resistance to antibiotics by forming dynamic small colony variants during chronic osteomyelitis. *J Antimicrob Chemother*. 2016;**71**(2):438-48.

51. Proctor RA, Kahl B, Von Eiff C, Vaudaux PE, Lew DP, Peters G. Staphylococcal small colony variants have novel mechanisms for antibiotic resistance. *Clin Infect Dis*. 1998;**27**(SUPPL.1):S68-S74.

52. Olokoba AB, Obateru OA, Olokoba KB. Type 2 Diabetes Mellitus - A Review of Current Trends. *Oman Medical Journal*. 2012;**27**:269-73.

53. Kolluru GK, Bir SC, Kevil CG. Endothelial dysfunction and diabetes: effects on angiogenesis, vascular remodeling, and wound healing. *Int J Vasc Med*. 2012;**2012**:918267.

54. WHO. Global Report of Diabetes. 2016.

55. Statistics ABo. National Health Survey: First Results, 2014-15 2015 [Available from: http://www.abs.gov.au/ausstats/abs@.nsf/PrimaryMainFeatures/4364.0.55.001?OpenDocument.

56. Singh N, Armstrong DG, Lipsky BA. Preventing Foot Ulcers in Patients With Diabetes. *JAMA*. 2005;**293**:217-28.

57. Tabit CE, Chung WB, Hamburg NM, Vita JA. Endothelial dysfunction in diabetes mellitus: molecular mechanisms and clinical implications. *Rev Endocr Metab Disord*. 2010;**11**(1):61-74.

58. Hadi HAR, Suwadi JAL. Endothelial dysfunction in diabetes mellitus. *Vascular Health and Risk Management*. 2007;**3**:853-76.

59. Tesfaye S, Boulton AJ, Dyck PJ, Freeman R, Horowitz M, Kempler P, et al. Diabetic neuropathies: update on definitions, diagnostic criteria, estimation of severity, and treatments. *Diabetes Care*. 2010;**33**(10):2285-93.

60. Said G. Diabetic neuropathy--a review. *Nat Clin Pract Neurol*. 2007;**3**(6):331-40.

61. Vincent AM, Russell JW, Low P, Feldman EL. Oxidative stress in the pathogenesis of diabetic neuropathy. *Endocr Rev*. 2004;**25**(4):612-28.

62. Cameron NE, Eaton SEM, Cotter MA, Tesfaye S. Vascular factors and metabolic interactions in the pathogenesis of diabetic neuropathy. *Diabetologia*. 2001;**44**(11):1973-88.

63. Jeffcoate WJ, Harding KG. Diabetic foot ulcers. *The Lancet*. 2003;**361**(9368):1545-51.

64. Pitei DL, Foster A, Edmonds M. The Effect of Regular Callus Removal on Foot Pressures. *J Foot Ankle Surg*. 1999;**38**:251-5.

65. Pecoraro RE, Reiber GE, Burgess EM. Pathways to Diabetic limb amputation. *Diabetic Care*. 1990;**13**:513-21.

66. Lavery LA, Armstrong DG, Wunderlich RP, Mohler MJ, Wendel CS, Lipsky BA. Risk factors for foot infections in individuals with diabetes. *Diabetes Care*. 2006;**29**(6):1288-93.

67. Damir A. Why diabetic foot ulcers do not heal? *JIMSA*. 2011;**24**.

68. Sotto A, Lina G, Richard JL, Combescure C, Bourg G, Vidal L, et al. Virulence potential of *Staphylococcus aureus* strains isolated from diabetic foot ulcers. *Diabetes Care*. 2008;**31**:2318-24.

69. Shettigar K, Jain S, Bhat DV, Acharya R, Ramachandra L, Satyamoorthy K, et al. Virulence determinants in clinical *Staphylococcus aureus* from monomicrobial and polymicrobial infections of diabetic foot ulcers. *J Med Microbiol*. 2016;**65**(12):1392-404.

70. Tuchscherr L, Korpos È, van de Vyver H, Findeisen C, Kherkheulidze S, Siegmund A, et al. *Staphylococcus aureus* requires less virulence to establish an infection in diabetic hosts. *International Journal of Medical Microbiology*. 2018.

71. Dinges MM, Orwin PM, Schlievert PM. Exotoxins of *Staphylococcus aureus*. *Clin Microbiol Rev*. 2000;**13**(1):16-34, table of contents.

72. Lipsky BA, Berendt AR, Deery HG, Embil JM, Joseph WS, Karchmer AW, et al. Diagnosis and treatment of diabetic foot infections. *Plastic and reconstructive surgery*. 2006;**117**(7 Suppl):212s-38s.

73. Ahmed S, Meghji S, Williams RJ, Henderson B, Brock JH, Nair SP. *Staphylococcus aureus* fibronectin binding proteins are essential for internalization by osteoblasts but do not account for differences in intracellular levels of bacteria. *Infect Immun*. 2001;**69**(5):2872-7.

74. Edwards AM, Potts JR, Josefsson E, Massey RC. *Staphylococcus aureus* host cell invasion and virulence in sepsis is facilitated by the multiple repeats within FnBPA. *PLoS Pathog*. 2010;**6**(6):e1000964.

75. Yang D, Wijenayaka AR, Solomon LB, Pederson SM, Findlay DM, Kidd SP, et al. Novel insights into *Staphylococcus aureus* deep bone infections: The involvement of osteocytes. *mBio*. 2018;**9**(2).

76. Josse J, Velard F, Gangloff SC. Staphylococcus aureus vs. Osteoblast: Relationship and Consequences in Osteomyelitis. *Front Cell Infect Microbiol*. 2015;**5**(85).

77. Kalinka J, Hachmeister M, Geraci J, Sordelli D, Hansen U, Niemann S, et al. *Staphylococcus aureus* isolates from chronic osteomyelitis are characterized by high host cell invasion and intracellular adaptation, but still induce inflammation. *Int J Med Microbiol*. 2014;**304**(8):1038-49.

78. Morgenstern M, Erichsen C, Hackl S, Mily J, Militz M, Friederichs J, et al. Antibiotic Resistance of Commensal *Staphylococcus aureus* and Coagulase-Negative Staphylococci in an International Cohort of Surgeons: A Prospective Point-Prevalence Study. *PLoS One*. 2016;**11**(2):e0148437.

79. Wertheim HFL, Vos MC, Ott A, Van Belkum A, Voss A, Kluytmans JAJW, et al. Risk and outcome of nosocomial *Staphylococcus aureus* bacteraemia in nasal carriers versus non-carriers. *Lancet*. 2004;**364**(9435):703-5.

80. Kim MW, Greenfield BK, Snyder RE, Steinmaus CM, Riley LW. The association between community-associated *Staphylococcus aureus* colonization and disease: a meta-analysis. *BMC Infectious Diseases*. 2018;**18**(1).

81. Von Eiff C, Becker K, Machka K, Stammer H, Peters G. Nasal carriage as a source o*f Staphylococcus aureus* bacteremia. *N Engl J Med*. 2001;**344**:11-6.

82. Kluytmans JA, Wertheim HF. Nasal carriage of *Staphylococcus aureus* and prevention of nosocomial infections. *Infection*. 2005;**33**(1):3-8.

83. Haleem A, Schultz JS, Heilmann KP, Dohrn CL, Diekema DJ, Gardner SE. Concordance of nasal and diabetic foot ulcer staphylococcal colonization. *Diagn Microbiol Infect Dis*. 2014;**79**(1):85-9.

84. Dunyach-Remy C, Courtais-Coulon C, DeMattei C, Jourdan N, Schuldiner S, Sultan A, et al. Link between nasal carriage of *Staphylococcus aureus* and infected diabetic foot ulcers. *Diabetes Metab*. 2017;**43**(2):167-71.

85. Stanaway S, Johnson D, Moulik P, Gill G. Methicillin-resistant *Staphyloccocus aureus* (MRSA) isolation from diabetic foot ulcers correlates with nasal MRSA carriage. *Diabetes Res Clin Pract*. 2007;**75**(1):47-50.

86. Nair N, Biswas R, Gotz F, Biswas L. Impact of *Staphylococcus aureus* on pathogenesis in polymicrobial infections. *Infect Immun*. 2014;**82**(6):2162-9.

87. Benoit JB, Frank DN, Bessesen MT. Genomic evolution of *Staphylococcus aureus* isolates colonizing the nares and progressing to bacteremia. *PLoS ONE*. 2018;**13**(5).

88. Mulcahy ME, McLoughlin RM. Host–Bacterial Crosstalk Determines *Staphylococcus aureus* Nasal Colonization. *Trends Microbiol*. 2016;**24**(11):872-86.

89. Krismer B, Weidenmaier C, Zipperer A, Peschel A. The commensal lifestyle of *Staphylococcus aureus* and its interactions with the nasal microbiota. *Nature Rev Microbiol*. 2017;**15**(11):675-87.

90. Roche FR, Meehan M, Foster TJ. The *Staphylococcus aureus* surface protein SasG and its homologues promote bacterial adherence to human desquamated nasal epithelial cells. *Microbiology*. 2003;**149**(10):2759-67.

91. Bui LMG, Hoffmann P, Turnidge JD, Zilm PS, Kidd SP. Prolonged growth of a clinical *Staphylococcus aureus* strain selects for a stable small-colony-variant cell type. *Infect Immun*. 2015;**83**(2):470-81.

Chapter 2: The bacteriology of patients with diabetic foot ulcers and diabetic foot infections and incidence of *Staphylococcus aureus* Small Colony Variants.

Diabetes is a highly prevalent metabolic disease and the complications of diabetes lead to lower extremities susceptible to ulceration and infection. These form DFU which can progress to DFI and can spread to the bone to cause osteomyelitis. The complications of diabetes result in an immunocompromised site with ulcers that can persist for months and can relapse even after initial clearance. *S. aureus* is one of the most prevalent pathogens isolated from DFI. However, DFIs are often found with a polymicrobial infection, and the interactions of other bacterial species can modulate *S. aureus* pathogenesis. A notable interaction occurs between *S. aureus* and *P. aeruginosa*, where the presence of *P. aeruginosa* selects for *S. aureus* SCVs. We propose that there are other unique interactions between *S. aureus* and other bacterial species within the diabetic foot which facilitate the selection of SCV. The switch to SCV creates slow growing cells with a reduced expression of virulence factors and the ability to persist intracellularly within osteocytes. This can form highly resilient reservoirs of dormant *S. aureus* which can revert to the normal, virulent cell type to cause a relapsing infection.

This chapter is presented as a manuscript (*European Journal of Clinical Microbiology*) and describes the clinical study undertaken to identify the epidemiology and bacteriology associated with the progression of patients with diabetes, patients with uninfected foot ulcers (DFU), patients with infection (DFI) and patients with osteomyelitis (DFI-OM).

This manuscript has been submitted to the European Journal of Clinical Microbiology for publication.

Statement of Authorship

Title of Paper	The bacteriology of patients with diabetic foot ulcers and diabetic foot infections and incidence of *Staphylococcus aureus* Small Colony Variants
Publication Status ✓	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Submitted for publication in the European Journal of Clinical Microbiology and Infectious Diseases

Principal Author

Name of Principal Author (Candidate)	James Lee		
Contribution to the Paper	Carried out sample collection, processing of samples, data analysis and drafted and wrote the manuscript		
Overall percentage (%)	75		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	8/4/22

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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The bacteriology and incidence of *Staphylococcus aureus* Small Colony Variants in patients with diabetic foot ulcers and infections

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Running head: **Hidden *S. aureus* in Diabetic Foot Infections.**

Summary

Purpose: Diabetic foot infections (DFIs) are a prevalent complication for patients with diabetes which can often progress to osteomyelitis (DFI-OM). *Staphylococcus aureus* is the most common pathogen in DFIs and DFI-OM and these infections can relapse in 40-70% of cases despite initial clearance of the infection. For *S. aureus,* relapse can be attributed to it hiding within adjacent, healthy tissue by adopting the quasi-dormant Small Colony Variant (SCV) state. The aim of this study was to investigate the patient risk factors and bacterial factors which facilitate these persistent infections.

Methods: Samples were taken from patients with diabetes to compare their bacterial composition in foot complications: those without foot ulcer or infection, uninfected diabetic foot ulcer (DFU), infected foot ulcers (DFI) and those with DFI-OM (both extracellular and from intracellular bone).

Results: *S. aureus* was identified as the most prevalent pathogen (24%) of DFI and DFI-OM patients. Also present in DFUs were *Pseudomonas aeruginosa* (17.74%) and *Escherichia coli* (8.06%). *S. aureus* was isolated as different bacterial colony types, with a notable presence of SCV (20% of all infections). Three DFI-OM patients presented with SCVs intracellular of clinically uninfected bone samples. Additionally, two of these patients with SCVs were found to be in co-infection with *Corynebacterium striatum*.

Conclusion: The presence of SCVs highlights the potential of these cells to cause persistent infection through the formation of dormant, non-pathogenic reservoirs. The survival of these cells in intracellular bone is important. The interactions of *C. striatum* with *S. aureus* are a potential risk factor in developing these reservoirs.

Keywords: *Staphylococcus aureus*, Diabetic Foot Ulcer (DFU), Diabetic Foot Infection (DFI), osteomyelitis.

Introduction

Diabetes mellitus affects an estimated 422 million people (in 2014) worldwide, with the prevalence of diabetes rising from 4.7% in 1980 to 8.5% of the global adult population [1]. There are 1.2 million patients with diabetes in Australia (5.1% of the population). A common complication for these patients is the susceptibility of ulcers on the plantar or dorsal foot, with incidence of diabetic foot ulcers (DFU) as high as 25% [2, 3]. The reasons for ulceration are multi-faceted, but include lower-limb neuropathy and endothelial dysfunction which results in foot ulcers that are slow to heal and are susceptible to infection [4]. DFU therefore progresses to a diabetic foot infection (DFI) and then osteomyelitis (DFI-OM) [2, 5, 6]. DFIs often relapse, even after the infection has seemingly cleared.

Staphylococcus aureus is among the most common pathogen associated with DFU and the cause of DFI [5, 7, 8]. *S. aureus* is known to possess an ability to infect bone and survive intracellularly within different bone cell types (namely, osteoblasts and osteocytes). This can often lead to osteomyelitis, a common complication of DFI [9, 10]. *S. aureus* is notable for being highly recalcitrant to treatment through the occurrence of methicillin resistance strains (MRSA) and their widespread prevalence within healthcare facilities. Prevalence of MRSA in DFI has been reported to be 15-38% (depending on geography) [11-17], often in co-infection with other pathogens.

In addition to antibiotic resistance, *S. aureus* can cause persistent and relapsing infections through its ability to form biofilms, persister cells, survive intracellularly and/or switch to the quasi-dormant Small Colony Variants (SCV) state [9, 18, 19]. Each of these are quiescent cell types that generally have a reduced or no expression of virulence factors and immune mediators. This allows *S. aureus* colonization of an anatomical niche without clinical symptoms. This "dormancy" also allows for antibiotic tolerance where cells can remain despite

the presence of antibiotics, even when the bacteria lack genetically encoded resistance mechanisms [20]. As a result, treatments which seemingly clear *S. aureus* from an infection may indeed leave a sub-population as an asymptomatic reservoir of dormant cells that can later revert to a pathogenic cell type to cause relapsing infection. The identification of these quasi-dormant bacterial cells within clinical samples is highly significant to develop strategies to combat relapsing infection especially in cases where amputation is required.

S. aureus SCV can survive intracellularly in human osteocytes [21], and other bone cell types. This provides a protected niche, safe from antibiotics or the immune response [22] creating an undetected reservoir of *S. aureus* for its long-term survival and eventual relapse of disease. We hypothesize that in cases of DFI-OM, SCVs facilitate the formation of a quasi-dormant reservoir within uninfected bone osteocytes distinct from the infected site. After surgical intervention to remove the infected tissue, the reservoir remains and may result in the relapse of infection.

Carriage of commensal *S. aureus* within the nares presents a vast array of inter-bacterial interactions that can shift *S. aureus* to a dormant, non-pathogenic phenotype [23] and we postulate that these interactions can select for SCV that contribute to persistent infection. The switch to SCV has previously been linked to co-infection of *S. aureus* with *Pseudomonas aeruginosa* [24, 25]. There may be other inter-bacterial interactions with *S. aureus* and other bacterial species within the nares that induce the switch to SCV [26]. Commensal carriage is both a risk factor for invasive infection [12-14, 27-30] and a source of endogenous infection, with carriage frequently associated with *S. aureus* bacteremia [31] and surgical site infection [27]. The mechanisms on how *S. aureus* in the nares leads to infections elsewhere in the body is equivocal. *S. aureus* can potentially transit from the nose to other sites of the body by switching to SCV enabling the bacteria to transit through the body without eliciting an immune

response. Within an immunocompromised site, (e.g. DFU) the selective pressures which favored SCVs are absent and actively growing, pathogenic cell types would be selected for.

Similar studies in France [32] and Australia [33, 34] have found carriage of nasal *S. aureus* was associated with the prevalence of *S. aureus* in DFI. We have undertaken a study that identified the bacteria present in uninfected DFU and DFI/DFI-OM with focus on the interactions of the skin microbiome and the effect on the switch to the *S. aureus* SCV cell type in diseased and healthy tissue of these patients. This study aimed to collect clinical samples from patients with DFU and DFI from local hospitals (South Australia) and assess the associated patient factors and bacterial populations. Our methodology primarily focused on isolation of *S. aureus* and isolation of all possible bacterial species was not the of this study.

Materials and methods

Study population: The human ethics for this study was approved by the Human Research Ethics Committee at the Royal Adelaide Hospital (Approval number: HREC/17/RAH/242).

Data was collected from 153 participants between March 2018 to the August 2021. All participants had either Type 1 or Type 2 diabetes mellitus, no viral illness and no critical limb ischemia. Informed, written consent was obtained. Controls for this study were participants with diabetes and no foot ulcer or infection. Patients with a DFU were further classified as having a clinically uninfected foot wound. Then there were DFI patients with a clinically infected foot wound and patients with DFI-OM (with both bone and wound samples). Control participants were sampled at the Queen Elizabeth Hospital (QEH) Endocrine Clinic (Adelaide, South Australia). DFU and DFI patients were sampled for skin and wound samples at the QEH Multidisciplinary Foot Clinic. DFI-OM patients undergoing surgical amputation were sampled from the Royal Adelaide Hospital (RAH) Department of Vascular Surgery (Adelaide, South

Australia). DFI patients with infected foot wounds but not undergoing surgical amputation (DFI-W) were sampled from the QEH (Fig. 1).

From power calculations, a minimum of 41 DFU and DFI participants were needed with 41 controls. This was calculated based on previous studies; *S. aureus* prevalence of 44.7% in DFU and 67.7% in DFI, and a false positive rate of 0.05 and an error of not detecting a difference when one exists of 0.2.

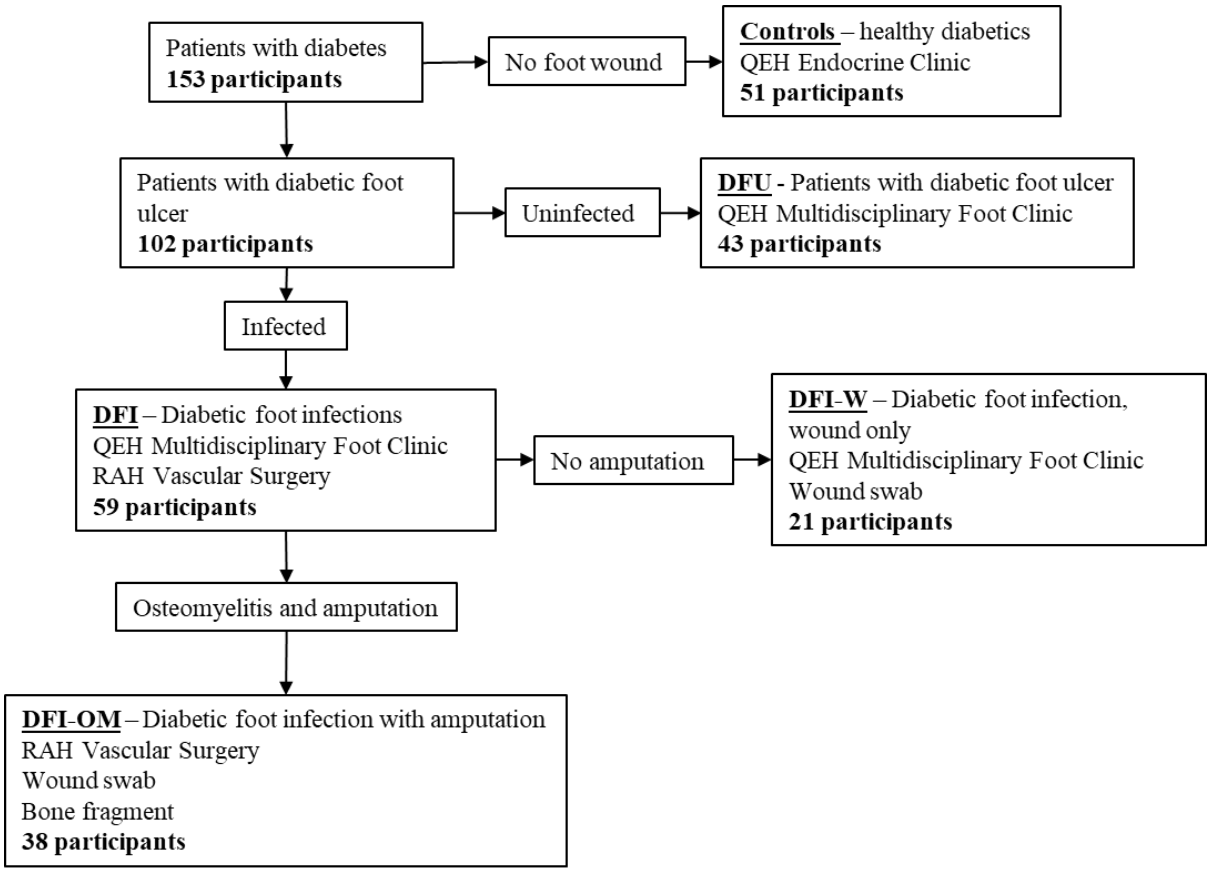

Fig 1. Flowchart showing the selection criteria of patients for each sample group and the samples taken for the current study. QEH – Queen Elizabeth Hospital; RAH – Royal Adelaide Hospital

Inclusion and exclusion criteria for study groups: The DFI group as described above had an inclusion of hospital admission for surgical treatment of DFI at the RAH. Inclusion for the DFU group was clinically diagnosed active, diabetic, neuropathic DFU. The control group was defined as presence of diabetes with no current ulceration or foot ulcer in last 12-months. Exclusion criteria from the study (in any group) included pregnancy, current viral illness, critical limb ischaemia (inadequate perfusion for wound healing to occur), the inability to understand English or the inability to provide consent. Specific exclusion for DFU group was recent (within one-month) foot infection, and specific exclusions for the control group was a history of DFI or DFU (within 12-months), or previous pedal amputation. Patients with recent antibiotic use were included within the study due to the severity of infection in all cases of DFI had required antibiotic treatment.

Diabetic foot ulcer/wound/vascular classification: For the DFU and DFI participants, the Lower Extremity Threatened Limb Classification System (WIfI) was used to stratify wounds from grade 0 to 3 [35].

Patient social and diabetes history and assessment: To describe the pathology and patient sample, we have recorded: age, sex, race, duration of diabetes, average glycated hemoglobin (% HbA1c) over the previous 6-months, antibiotic use over the past 12-months, and smoking status. Presence of protective sensation was assessed with a 5.07 Semmes-Weinstein monofilament. Height and weight were measured with a digital scale and stadiometer, from these data body mass index (BMI) was calculated. The One-Way ANOVA test and Turkey Multiple Comparison Test was used to determine statistically significant differences between pairs of populations (where significant differences was determined by a threshold of $p<0.05$). Wound, infection and ischemia data were collected for patients with a foot wound. Swabs of the wound were cultured on Tryptic Soy Agar (TSA, Oxoid, Australia) and incubated for up to 72h at 37°C to obtain single colonies of bacterial isolates. *S. aureus* SCV were also identified

through increased incubation times as well as growth on a chemically defined media (CDM) which is based on previous media design for slime production by coagulase negative Staphylococci [36].

Bacterial extraction from bone samples: Two types of bone samples were taken from each DFI-OM patients; distal bone which was taken from infected tissue and proximal bone, which was taken from clean, clinically uninfected bone subsequently after amputation. Bone samples were transferred to 1mL Phosphate Buffer Solution (PBS). Extracellular bacteria were isolated by plating 100 μL of PBS onto TSA and incubated for 72 h at 37 °C to obtain single colonies. To extract intracellular bacteria, bone fragments were washed twice with PBS and then sonicated with 4 cycles at 180 Hz for 10 seconds in PBS. Then, 100 μL was cultured on TSA for up to 72 h at 37 °C.

Bacterial identification: From all sampling (as described) bacterial species from colonies were identified using MALDI-TOF Biotyper (Bruker). Bacterial samples from each colony were grown in Tryptic Soya Broth (TSB, Oxoid, Australia) to mid-log phase (OD_{600} ~ 0.3), centrifuged at 4000 X *g*/4 °C for 10 min and resuspended in 30% glycerol and stored at -80 °C.

***S. aureus* colony types:** Colony types were recorded after 24 h growth on TSA at 37 °C (or a greater incubation time when colonies were first observable). *S. aureus* colonies were identified as large (L, greater than 10 mm in diameter), or small (S, between 1 mm to 10 mm in diameter). Pigmentation of colonies were recorded as pigmented (P) or non-pigmented (NP). SCV were classified as *S. aureus* colonies on TSA <1 mm in diameter, non-pigmented and non-haemolytic. The stability of the SCV cell type was determined by performing 5 cycles of sub-culturing of the SCV colony onto fresh TSA and incubated for 48 h at 37 °C. A colony was described as a non-stable SCV (nsSCV) if reversion of the colony to a non-SCV occurred

within the 5 sub-cultures on TSA while if colonies remained as a SCV they were described as stable SCV (sSCV).

Whole genome sequencing: Whole genomic DNA was extracted and purified using QIAGEN Genomic-tip 500/G columns (QIAGEN, Australia) according to manufacturer protocols. Quality and quantity of genomic DNA was determined using FEMTO Pulse (SA Pathology). Genomes were sequenced using an Oxford Nanopore MinION device. The MinION library construction and sequencing were performed by FISABIO University of Valencia's sequencing service, using the Oxford Nanopore PCR barcoding kit following the manufacturer's instructions. Sequence data have been deposited with links to BioProject accession number PRJNA821238 in the NCBI BioProject database.

Results

This study recruited 153 participants, with cohorts of 51 controls, 43 DFUs, 21 DFI-W and 38 DFI-OM. The participant cohort was predominately Caucasian (95.10%) and 73.42% of samples were collected from men (Table 1). The average age and HbA1c was greater in the DFI cohorts (Table 1). Within the DFI cohort, HbA1c levels were greater in DFI-OM patients while the average age and duration of diabetes was greatest in DFI-W patients. The One-Way ANOVA test found the HbA1c between the controls and the DFI cohorts were the only populations with statistically significant differences. DFI patients had the highest proportion of patients who were current smokers (25.45% of the total number of patients sampled). DFU patients had the lowest proportion of current smokers and the highest proportion of past smokers (Table 1). Of the 102 patients with a foot ulcer (patients in the DFU, DFI-W and DFI-OM cohorts), 62 (60.78%) had cultivable bacteria present in either their bone fragment or wound swab (Table 1). The most common bacterial species found from all participants with culturable bacteria was *S. aureus* (25% of the total samples) followed by *P. aeruginosa* (15.28% of total samples) and *Escherichia coli* (9.72%) (Table 2, Fig. 2).

Table 1. Demographic and clinical data of patients with a DFI, DFU or health diabetic with no foot ulcer (control). Mean HbA1c % was average reading over the previous 6 months when available. Number of cultivable bacteria defined as whether growth was observed from bone/wound sample from patient. DFI: diabetic foot infection. DFI-W: diabetic foot infection, wound only and no amputation. DFI-OM: diabetic foot infection with osteomyelitis, undergoing amputation. BMI: body mass index, ratio of body weight over body height. Standard deviation in brackets.

	Control	DFU	DFI-W	DFI-OM	DFI (all)	Total
No. of participants	51	42	21	39	60	153
No. of participants with culturable bacteria	-	8	16	26	54	62
Mean age (years)	66.14 (12.36)	67.64 (12.43)	67.38 (13.23)	74.90 (10.63)	70.01 (12.81)	68.07
Mean Diabetes duration (years)	18.54 (12.16)	17.85 (12.32)	16.26 (8.52)	23.27 (16.89)	18.86 (12.61)	18.36
Mean HbA1c (%)	7.68 (2.15)	7.60 (1.45)	8.94 (1.92)	8.26 (1.37)	8.75 (1.77)	7.91
BMI (kg/m^2)	31.83 (6.64)	28.69 (4.82)	30.99 (7.81)	28.07 (3.68)	30.06 (6.87)	30.39

		No.	Proportion (%)				No.
Smokers	**DFI**	21	38.18	**Ethnicity**	**DFI**	Caucasian	41
		20	36.36			Other	0
		14	25.45				
	DFU	14	34.1		**DFU**	Caucasian	42
		25	60.97			Other	1
		2	4.87				
	Controls	25	49,01		**Controls**	Caucasian	26
		18	43.9			Other	8
		8	19.51				
	Total	60	40.81		**Total**	Caucasian	131
		63	42.85			Other	9
		24	16.32			N/A	7

Additionally, *S. aureus* was the most prevalent bacterial isolate in both DFI (24.19%) and DFU (40.00%) cohorts (Table 3, Fig. 2). Within the DFI cohort, *S. aureus* was also the most prevalent isolate (31.58%) in DFI-OM patients (Table 4, Fig. 2). *P. aeruginosa* was the most prevalent (29.17%) in DFI-W patients. All DFI-W patients identified with *S. aureus* in the wound either had a previous relapsed foot infection or a previous amputation from osteomyelitis infected with *S. aureus*. Of all the samples studied, 23.80% of DFU patients had cultivable bacteria which could be isolated from wound swabs. *S. aureus* isolates from clinically uninfected DFUs all produced large, golden colonies (Table 5, Fig. 3). Different *S. aureus* cell types were associated with differences in their genetic backgrounds. Whole genome sequences were obtained from four *S. aureus* isolates presenting with altered colony types (large and pigmented, large and non-pigmented, small and non-pigmented and a SCV) and the presence of notable virulence factors associated with toxin production, adhesion and antibiotic resistance were identified (Table 7).

While all DFI-OM patients presented with osteomyelitis were diagnosed by clinicians, four patients presented with no culturable bacteria from the bone samples (Table 1). Additionally, two of these patients had no culturable bacteria present within their wound. Four patients with osteomyelitis who presented with *S. aureus* had a heterogenous population of cell types which included the presence of SCV from their wound and/or bone cultures. This accounted for 25.00% of DFI patients infected with *S. aureus* carrying observable SCV and 20.00% of the *S. aureus* isolated (including patients with a DFU). Within osteomyelitis patients infected with *S. aureus*, seven were found within a polymicrobial infection with other bacterial species while four were an axenic infection (Table 6). Two of the SCV isolated were in co-infection with other bacterial species; one was the sSCV with *Corynebacterium striatum* and the other was a nsSCV with *C. striatum* and *Staphylococcus epidermidis*.

Table 2. Bacterial isolates across all patients with a foot ulcer, including uninfected foot ulcers (DFU), patients with osteomyelitis (DFI-OM) and patients with only an infected foot ulcer (DFI-W).

	Species	**No.**
	S. aureus	18
	P. aeruginosa	11
	E. coli	7
	E. cloacae	4
	P. mirabilis	4
	S. epidermidis	4
	A. pitti	2
	C. striatum	2
	E. faecalis	2
	K. oxytoca	2
	S, marcescans	2
	S. capitis	2
	S. maltophilia	2
	S. simulans	2
	A. iwoffi	1
	B. pumilis	1
	K. pneumoniae	1
	K. rhizophilia	1
	P. rettgeri	1
	S. caprae	1
	S. lugdunesis	1
	S. oralis	1
	Total	72

Table 3. Proportion of bacterial isolates between DFI and DFU sample groups

	Species	No.	Proportion (%)		Species	No.	Proportion (%)
DFI	*S. aureus*	15	24.19	**DFU**	*S. aureus*	4	40
	P. aeruginosa	11	17.74		*E. coli*	2	20
	E. coli	5	8.06		*S. epidermidis*	1	10
	E. cloacae	4	6.45		*S. maltophilia*	1	10
	P. mirabillis	4	6.45		*A. iwoffi*	1	10
	S. epidermidis	3	4.84		*S. caprae*	1	10
	A. pitti	2	3.22		**Total**	10	
	C. striatum	2	3.22				
	E. faecalis	2	3.22				
	K. oxytoca	2	3.22				
	S. marcescans	2	3.22				
	S. simulans	2	3.22				
	S. capitis	2	3.22				
	B. pumilis	1	1.61				
	K. pneumoniae	1	1.61				
	K. rhizophilia	1	1.61				
	S. lugdunensis	1	1.61				
	S. maltophilia	1	1.61				
	S. oralis	1	1.61				
	Total	**62**					

Discussion

Increased age, duration of diabetes and HbA1c have been established as risk factors for progression to DFU [3] and DFI [33, 37]. This is also consistent with the progression to DFI being associated with other diabetic complications, particularly endothelial dysfunction and lower-limb neuropathy. We identified an increase in all these risk factors in DFI patients compared to DFU patients. However, the only statistically significant difference was the HbA1c between the controls and DFI cohorts. An ability to control HbA1c may therefore be an opportunity in controlling pathogens in foot infections and the development of deeper and persistent infection.

A significant disparity is present in the number of cigarette smokers between DFU and DFI cohorts. Cigarette smoking is known as an important factor in glycemic control and the development of diabetic complications [38]. This is consistent with our data, where there was a greater proportion of current smokers in patients with DFI compared to the control group, and patients with DFU only. A high proportion of patients with a DFU were past smokers and understanding how this may affect the progression of bacterial infection is complex as the number of cigarettes smoked and the length of cessation from smoking was highly variable.

For a bacterial infection to establish within the foot ulcer, the endothelial function of the patient is likely to be dysfunctional so that the immune response is compromised to allow infection. The differences in HbA1c, age, duration of diabetes and BMI were not statistically significant in our dataset and therefore we could not identify these as possible risk factors for the progression from DFU to DFI-OM. We can conclude within the parameters we measured, that the smoking status and HbA1c were the major patient factors associated with worsening clinical manifestations, likely associated with their role in exacerbating endothelial dysfunction [39-41]. We had also identified notable virulence factors associated with different *S. aureus*

cell types (Table 7). All cell types were positive for α-hemolysin and the β-lactamase *blaZ* and lacked the adhesion *clfA* and most major leukocidins. The *mecA* gene conferring methicillin resistance was only present in UA-DI-117 (isolated from a DFI-W) and thus the lack of methicillin resistance may not be a determining factor for establishing persistent infection. In this case, the switch to SCV can compensate for the lack of antibiotic resistance through alternate pathways.

Table 4. Proportion of bacterial isolates from DFI patients between those with osteomyelitis (DFI-OM) and those with only an infected foot ulcer (DFI-W).

	Species	**No.**	**Proportion (%)**		**Species**	**No.**	**Proportion (%)**
DFI-OM	*S. aureus*	12	31.58	**DFI-W**	*P. aeruginosa*	7	29.17
	E. coli	4	10.53		*S. aureus*	3	12.50
	P. aeruginosa	4	10.53		*P. mirabilis*	2	8.33
	E. cloacae	3	7.89		*A. pitti*	2	8.33
	S. epidermidis	3	7.89		*E. cloacae*	1	4.16
	C. striatum	2	5.26		*E. coli*	1	4.16
	P. mirabillis	2	5.26		*E. faecalis*	1	4.16
	S. marcescans	2	5.26		*K. oxytoca*	1	4.16
	B. pumilis	1	2.63		*K. pneumoniae*	1	4.16
	C. simulans	1	2.63		*K. rhizophilia*	1	4.16
	E. faecalis	1	2.63		*S. capitis*	1	4.16
	K. oxytoca	1	2.63		*S. lugdunesis*	1	4.16
	S. capitis	1	2.63		*S. maltophilia*	1	4.16
	S. oralis	1	2.63		*S. simulans*	1	4.16
	Total	38			**Total**	24	

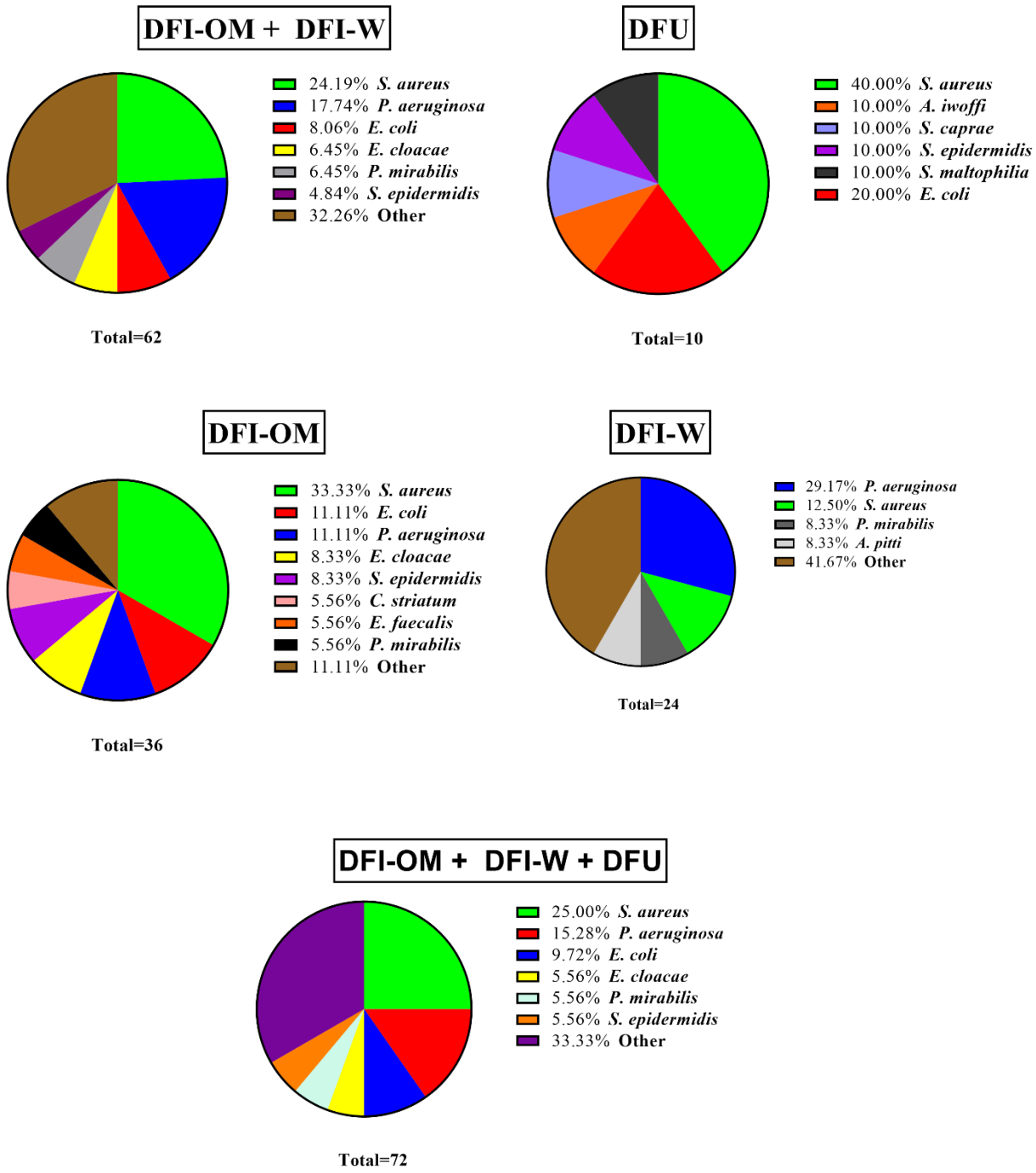

Fig 2. Proportion of bacterial species isolated from the total number of patients with cultivable bacterial present in each cohort. Bacterial species with the lowest prevalence were included within the other category

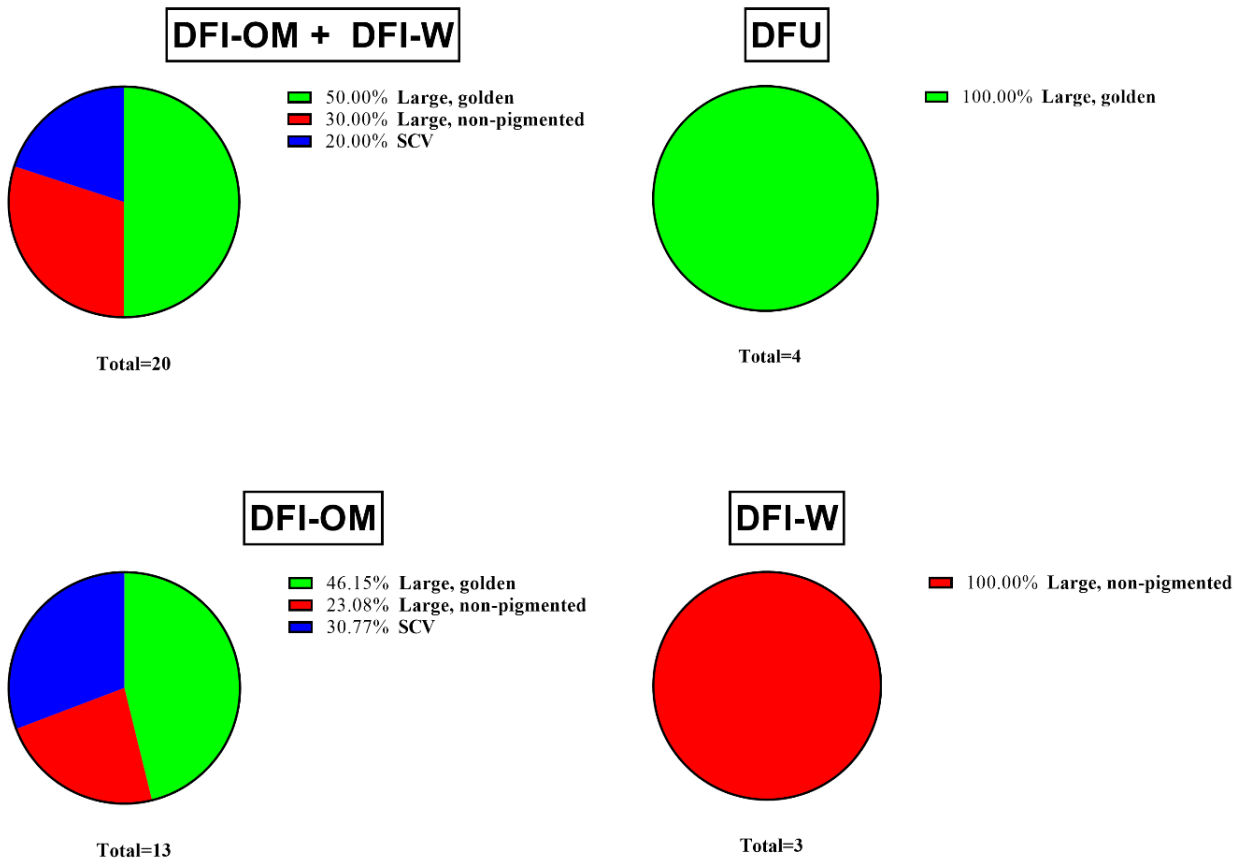

Fig. 3. Proportion of the different cell types of *S. aureus* present in each cohort.

We had isolated bacteria from DFU lesions, even though the criteria for DFU recruitment was a clinically uninfected lesion. These isolates are likely skin commensals rather than more pathogenic strains. This implicates those bacterial isolates obtained from DFI wounds which could also be skin commensals, however from our current data it cannot be determined whether an isolate is indeed pathogenic or commensal.

There were interesting cases in which bacteria could not be isolated. The lack of any bacterial isolates from both the DFI-W and DFI-OM is likely due to different factors: (1) our culture media was unable to detect strictly anaerobic species, which are common in bone infection [42, 43], or (2) our method of sampling lesions with cotton flock swabs may be an inefficient method to identify bacterial species requiring a deeper tissue biopsy [44-46]. Alternative culture methods exist to accommodate growth of various other bacterial species, however this was not the principal aim of this study. Proximal bone samples adjacent to the infected bone were obtained as part of our study to detect quiescent, non-pathogenic cell populations within "healthy" bone which could result in relapsing infection after amputation of the infected tissue. Indeed, there were DFI-OM patients that had *S. aureus* isolated from both the infected tissue and the proximal bone which supports our hypothesis on how relapsing infections may occur. Notably, these patterns of bacterial colonization were associated with the presence of *S. aureus* SCV.

Intriguingly, we isolated *S. aureus* SCVs from both the bone and the wound in four DFI-OM patients. A sSCV was obtained from one patient, which suggests there were some stable genetic events which enabled the sSCV to remain stable on nutrient rich TSA. Osteocytes are the most predominant bone cell type and have been established as an intracellular reservoir for SCV [19, 21].

Table 5. Number of different cell types of *S. aureus* based on the colony phenotype after 24 hours of growth in TSA.

	DFU	DFI-OM	DFI-W	DFI-OM + DFI-W	Total	Proportion (%)
Large, pigmented	4	6	0	6	10	50.00
Large, non-pigmented	0	3	3	6	6	30.00
SCV	0	4	0	4	4	20.00

Table 6. *S. aureus* isolates was found in DFI and DFU patients as the sole bacterial species (axenic) or in co-infection with other bacterial species (polymicrobial)

	DFU	DFI-OM	DFI-W	DFI-OM + DFI-W	Total
Axenic	3	4	0	4	7
Polymicrobial	1	7	3	10	11

Indeed, three of the *S. aureus* isolates which had presented as SCV were obtained from intracellular proximal bone. These bone samples were taken from healthy, uninfected bone after amputation of infected tissue. This is consistent with our hypothesis that SCVs facilitate quasi-dormant reservoirs in clinically uninfected bone tissue (not diseased) leading to a relapse of infection. Previous research induced sSCV in a clinical strain of *S. aureus* by continuous culture under conditions which were highly representative of the environmental stresses and selective factors present in diabetic foot infections [47, 48]. The switch to SCV was indeed a mechanism utilized by the *S. aureus* strain to enable survival and proliferation intracellularly in osteocytes [21].

Two of DFI-OM patients that presented with a sSCV and a non-stable SCV were in co-infection with other bacterial species including *C. striatum*. Co-infection with *C. striatum* have been reported to downregulate *S. aureus* that results in a shift towards a commensal like cell type [49]. This involved decreased activity of the major virulence regulator, *agr*, which is responsible for the expression of various secreted virulence factors associated with disease symptoms and adhesion to host surfaces [49]. These changes in virulence factors are also signature characteristics of SCV and thus the co-infection in our clinical samples may favour SCV cell types [26]. These interactions with *C. striatum* may also have a major role in the selection of sSCV and are a potential avenue of research for sSCV. The basis of most research in sSCV rely on laboratory generated sSCV and a new model of sSCV based on interactions

with other bacterial species can provide valuable insight into the mechanisms of SCV within a clinically relevant environment.

All *S. aureus* isolated from the DFI-W had been from a patient undergoing a previously recurrent infection or amputation that highlights *S. aureus* association with persistent infection. Additionally, *S. aureus* were co-infected with other bacterial species, however the low sample size of *S. aureus* in DFI-W means no definite conclusions could be made.

As expected, fewer bacterial isolates were detected in DFU samples compared to DFI cohorts. Patients in these cohorts had long-term foot ulcers that suggest diabetes had resulted in impairment of wound healing but was not immunocompromised for opportunistic infection.

In conclusion, our study highlights the link between *S. aureus* in patients with diabetes and their foot complications. In addition, the presence of quasi-dormant SCV in DFU and DFI-OM patients suggests a direct link with persistent and relapsing infections. While treatment of infective tissue in DFI and DFI-OM is a priority, care must be given to prevent recurrent infections by identifying *S. aureus* presence in adjacent to diseased tissue after initial clearance of infection thereby reducing the future burden to the patient.

Table 7. Whole genome sequences were obtained from *S. aureus* isolates displaying different colony types (large and pigmented, large and non-pigmented and SCV). For each isolate, the presence of absence of virulence genes involved in toxin production, adhesion and antibiotic resistance were identified. An X indicates the presence of the gene

	UA-DI-113 **DFU** **L, G**	**UA-DI-117** **DFI-W** **L, NP**	**UA-DI-86** **DFI-W** **S, NP**	**UA-DI-47** **DFI-OM** **SCV**
Collagen adhesion ***can***		X		X
Clumping factor A ***clfA***				
Clumping factor B ***clfB***	X		X	X
α-hemolysin ***hla***	X	X	X	X
β-hemolysin ***hlb***				
δ-hemolysin ***hld***	X			X
Leukotoxin E ***lukE***				
Leukotoxin D ***lukD***	X		X	
Leukocodin M ***lukM***				
Penicillin binding protein ***mecA***		X		
β-lactamase ***blaZ***	X	X	X	X

Acknowledgements

We acknowledge the assistance of Tania Veltman (ACARE) in use of the *Biotyper* (Bruker), the Registrars of the RAH Department of Vascular Surgery and the QEH Diabetes and Endocrinology for recruitment of patients for the clinical study.

Statement of Ethics

The human ethics for this study was approved by the Human Research Ethics Committee at the Royal Adelaide Hospital (Approval number: HREC/17/RAH/242).

Funding

This research was in part funded by the Vascular Foundation (Australia) to JD and SPK. The was also funding from the South Australian (SA) Health Allied Health Research Seed Funding Grant to SPK, TPW and RF.

References

1. WHO. Global Report of Diabetes. 2016.

2. Armstrong DG, Lipsky BA. Diabetic foot infections: Stepwise medical and surgical management. *Int Wound J*. 2004;**1**(2):123-32.

3. Singh N, Armstrong DG, Lipsky BA. Preventing Foot Ulcers in Patients With Diabetes. *JAMA*. 2005;**293**:217-28.

4. Lavery LA, Armstrong DG, Wunderlich RP, Mohler MJ, Wendel CS, Lipsky BA. Risk factors for foot infections in individuals with diabetes. *Diabetes Care*. 2006;**29**(6):1288-93.

5. Lipsky BA, Berendt AR, Deery HG, Embil JM, Joseph WS, Karchmer AW, et al. Diagnosis and treatment of diabetic foot infections. *Clin Infect Dis*. 2004;**39**(7):885-910.

6. Uckay I, Aragon-Sanchez J, Lew D, Lipsky BA. Diabetic foot infections: what have we learned in the last 30 years? *Int J Infect Dis*. 2015;**40**:81-91.

7. Akhi MT, Ghotaslou R, Memar MY, Asgharzadeh M, Varshochi M, Pirzadeh T, et al. Frequency of MRSA in diabetic foot infections. *Int J Diabetes in Dev Ctries*. 2017;**37**(1):58-62.

8. Bülent Ertuğrul M, Uyar-Güleç G, Baktıroğlu S, Çörekli E, Türe M. The distribution of causative microorganisms in diabetic foot infection: Has there been any alterations? *Klimik Dergisi*. 2017;**30**(1):27-31.

9. Tong SY, Davis JS, Eichenberger E, Holland TL, Fowler VG, Jr. *Staphylococcus aureus* infections: epidemiology, pathophysiology, clinical manifestations, and management. *Clin Microbiol Rev*. 2015;**28**(3):603-61.

10. Waldvogel FA, Medoff G, Swartz MN. Osteomyelitis: A Review of Clinical Features, Therapeutic Considerations and Unusual Aspects. *NEJM*. 1970;**282**(6):316-22.

11. Hobizal KB, Wukich DK. Diabetic foot infections: current concept review. *Diabet Foot Ankle*. 2012;**3**.

12. Al-Allak MH, Al-Khafaji NSK, Al-Dahmoshi HOM. Microbial and resistance profile among diabetic foot-infections. *EurAsian J of Biosci*. 2019;**13**(2):1953-9.

13. Lin SY, Lin NY, Huang YY, Hsieh CC, Huang YC. Methicillin-resistant *Staphylococcus aureus* nasal carriage and infection among patients with diabetic foot ulcer. *J Microbiol Immunol Infect*. 2020;**53**(2):292-9.

14. Viswanathan V, Pendsey S, Radhakrishnan C, Rege TD, Ahdal J, Jain R. Methicillin-Resistant *Staphylococcus aureus* in Diabetic Foot Infection in India: A Growing Menace. *Int J Low Extrem Wounds*. 2019;**18**(3):236-46.

15. Abdulrazak A, Ibrahim Bitar Z, Ayesh Al-Shamali A, Ahmed Mobasher L. Bacteriological study of diabetic foot infections. *J Diabetes Complications*. 2005;**19**(3):138-41.

16. Ge Y, MacDonald D, Hait H, Lipsky B, Zasloff M, Holroyd K. Microbiological profile of infected diabetic foot ulcers. *Diabet Med*. 2002;**19**(12):1032-4.

17. Tentolouris N, Jude EB, Smirnof I, Knowles EA, Boulton AJM. Methicillin-resistant *Staphylococcus aureus*: An increasing problem in a diabetic foot clinic. *Diabet Med*. 1999;**16**(9):767-71.

18. Kahl BC, Becker K, Löffler B. Clinical significance and pathogenesis of staphylococcal small colony variants in persistent infections. *Clin Microbiol Rev*. 2016;**29**(2):401-27.

19. Kalinka J, Hachmeister M, Geraci J, Sordelli D, Hansen U, Niemann S, et al. *Staphylococcus aureus* isolates from chronic osteomyelitis are characterized by high host cell invasion and intracellular adaptation, but still induce inflammation. *Int J Med Microbiol*. 2014;**304**(8):1038-49.

20. Keren I, Kaldalu N, Spoering A, Wang Y, Lewis K. Persister cells and tolerance to antimicrobials. *FEMS Microbiol Lett*. 2004;**230**(1):13-8.

21. Yang D, Wijenayaka AR, Solomon LB, Pederson SM, Findlay DM, Kidd SP, et al. Novel insights into *Staphylococcus aureus* deep bone infections: The involvement of osteocytes. *mBio*. 2018;**9**(2).

22. Tuchscherr L, Kreis CA, Hoerr V, Flint L, Hachmeister M, Geraci J, et al. *Staphylococcus aureus* develops increased resistance to antibiotics by forming dynamic small colony variants during chronic osteomyelitis. *J Antimicrob Chemother*. 2016;**71**(2):438-48.

23. Krismer B, Weidenmaier C, Zipperer A, Peschel A. The commensal lifestyle of *Staphylococcus aureus* and its interactions with the nasal microbiota. *Nature Rev Microbiol*. 2017;**15**(11):675-87.

24. Hoffman LR, Déziel E, D'Argenio DA, Lépine F, Emerson J, McNamara S, et al. Selection for *Staphylococcus aureus* small-colony variants due to growth in the presence of *Pseudomonas aeruginosa*. *P NATL ACAD SCI USA*. 2006;**103**(52):19890-5.

25. Hotterbeekx A, Kumar-Singh S, Goossens H, Malhotra-Kumar S. In vivo and In vitro interactions between *Pseudomonas aeruginosa* and *Staphylococcus* spp. *Front Cell Infect Microbiol*. 2017;**7**(APR).

26. Lee J, Zilm PS, Kidd SP. Novel Research Models for *Staphylococcus aureus* Small Colony Variants (SCV) Development: Co-pathogenesis and Growth Rate. *Front Microbiol*. 2020;**11**(321).

27. Berthelot P, Grattard F, Cazorla C, Passot JP, Fayard JP, Meley R, et al. Is nasal carriage of *Staphylococcus aureus* the main acquisition pathway for surgical-site infection in orthopaedic surgery? *Eur J Clin Microb Infect Dis*. 2010;**29**(4):373-82.

28. Gagnaire J, Botelho-Nevers E, Martin-Simoes P, Morel J, Zéni F, Maillard N, et al. Interplay of nasal and rectal carriage of *Staphylococcus aureus* in intensive care unit patients. *Eur J Clin Microbiol Infect Dis*. 2019;**38**(10):1811-9.

29. Lederer SR, Riedelsdorf G, Schiffl H. Nasal carriage of meticillin resistant *staphylococcus aureus*: The prevalence, patients at risk and the effect of elimination on outcomes among outclinic haemodialysis patients. *Eur J Med Res*. 2007;**12**(7):284-8.

30. Taha AB. Relationship and susceptibility profile of *Staphylococcus aureus* infection diabetic foot ulcers with Staphylococcus aureus nasal carriage. *Foot*. 2013;**23**(1):11-6.

31. Wertheim HFL, Vos MC, Ott A, Van Belkum A, Voss A, Kluytmans JAJW, et al. Risk and outcome of nosocomial *Staphylococcus aureus* bacteraemia in nasal carriers versus non-carriers. *Lancet*. 2004;**364**(9435):703-5.

32. Dunyach-Remy C, Courtais-Coulon C, DeMattei C, Jourdan N, Schuldiner S, Sultan A, et al. Link between nasal carriage of *Staphylococcus aureus* and infected diabetic foot ulcers. *Diabetes Metab*. 2017;**43**(2):167-71.

33. Jeyaraman K, Berhane T, Hamilton M, Chandra AP, Falhammar H. Mortality in patients with diabetic foot ulcer: A retrospective study of 513 cases from a single Centre in the Northern Territory of Australia. *BMC Endocr Disord*. 2019;**19**(1).

34. Commons RJ, Robinson CH, Gawler D, Davis JS, Price RN. High burden of diabetic foot infections in the top end of Australia: An emerging health crisis (DEFINE study). *Diabetes Res Clin Pract*. 2015;**110**(2):147-57.

35. Mills JL, Sr., Conte MS, Armstrong DG, Pomposelli FB, Schanzer A, Sidawy AN, et al. The Society for Vascular Surgery Lower Extremity Threatened Limb Classification System: risk stratification based on wound, ischemia, and foot infection (WIfI). *Journal of vascular surgery*. 2014;**59**(1):220-34.e1-2.

36. Hussain M, Hastings JGM, White PJ. A chemically defined medium for slime production by coagulase-negative staphylococci. *Journal of Medical Microbiology*. 1991;**34**(3):143-7.

37. Wang SH, Sun ZL, Guo YJ, Yang BQ, Yuan Y, Wei Q, et al. Meticillin-resistant *Staphylococcus aureus* isolated from foot ulcers in diabetic patients in a Chinese care hospital: Risk factors for infection and prevalence. *J Med Microbiol*. 2010;**59**(10):1219-24.

38. Eliasson B. Cigarette smoking and diabetes. *Prog Cardiovasc Dis*. 2003;**45**(5):405-13.

39. Messner B, Bernhard D. Smoking and cardiovascular disease: mechanisms of endothelial dysfunction and early atherogenesis. *Arterioscler Thromb Vasc Biol*. 2014;**34**(3):509-15.

40. Tabit CE, Chung WB, Hamburg NM, Vita JA. Endothelial dysfunction in diabetes mellitus: molecular mechanisms and clinical implications. *Rev Endocr Metab Disord*. 2010;**11**(1):61-74.

41. Tepper OM. Human Endothelial Progenitor Cells From Type II Diabetics Exhibit Impaired Proliferation, Adhesion, and Incorporation Into Vascular Structures. *Circulation*. 2002;**106**(22):2781-6.

42. Lewis RP, Sutter VL, Finegold SM. Bone infections involving anaerobic bacteria. *Medicine*. 1978;**57**(4).

43. Walter G, Vernier M, Pinelli PO, Million M, Coulange M, Seng P, et al. Bone and joint infections due to anaerobic bacteria: an analysis of 61 cases and review of the literature. *Eur J Clin Microb Infect Dis*. 2014;**33**(8):1355-64.

44. Nelson A, Wright-Hughes A, Backhouse MR, Lipsky BA, Nixon J, Bhogal MS, et al. CODIFI (Concordance in Diabetic Foot Ulcer Infection): a cross-sectional study of wound swab versus tissue sampling in infected diabetic foot ulcers in England. *BMJ Open*. 2018;**8**(1):e019437.

45. Smith ME, Robinowitz N, Chaulk P, Johnson K. Comparison of chronic wound culture techniques: swab versus curetted tissue for microbial recovery. *Br J Community Nurs*. 2014;**Suppl**(9 0):S22-S6.

46. Huang Y, Cao Y, Zou M, Luo X, Jiang Y, Xue Y, et al. A Comparison of Tissue versus Swab Culturing of Infected Diabetic Foot Wounds. *Int J Endocrinol*. 2016;**2016**:8198714.

47. Bui LMG, Hoffmann P, Turnidge JD, Zilm PS, Kidd SP. Prolonged growth of a clinical *Staphylococcus aureus* strain selects for a stable small-colony-variant cell type. *Infect Immun*. 2015;**83**(2):470-81.

48. Bui LMG, Kidd SP. A full genomic characterization of the development of a stable Small Colony Variant cell-type by a clinical *Staphylococcus aureus* strain. *Infect Genet Evol*. 2015;**36**:345-55.

49. Ramsey MM, Freire MO, Gabrilska RA, Rumbaugh KP, Lemon KP. *Staphylococcus aureus* Shifts toward commensalism in response to *Corynebacterium* species. *Front Microbiol*. 2016;**7**(AUG).

Chapter 3: Functional *mgrA* influences changes in *Staphylococcus aureus* cell population over time

SCVs are a quasi-dormant cell type associated with reduced growth, increased adhesion and decreased virulence. These create sub-populations with alternate mechanisms that can tolerate antibiotics and evade the immune response. This phenotype is unstable and readily reverts when cultured in laboratory conditions which hinders experiments with SCV. Previous research in SCV utilise *S. aureus* isolates with mutations that result in auxotrophism to hemin, menadione and thymidine. These result in impeded electron transport chain, reduced growth and the sSCV phenotype. However, these models of SCV are not truly representative of the induction of SCV in clinical settings.

Bui, et al. had previously used continuous culture to grow the MRSA bloodstream isolate WCH-SK2 within a chemostat. With the stressful conditions of a controlled low growth rate and limited availability of nutrients, over time metabolic active cell types are selected out in favour of metabolic dormant alternative cell types such as SCV. Over time, this selected for a sSCV which implied there were stable genetic events mediating the switch to a stable phenotype. Whole genome sequences were obtained for the cell populations after 1 day and 55 days of growth. SNP analysis found a total of 24 genetic events associated with the progression from the parental WCH-SK2 to the sSCV. Most notable of these was a base substitution in the DNA binding domain (amino acid change of Arg92Cys) of the regulator of virulence *mgrA*. MgrA is highly relevant in *S. aureus* pathogenesis as it controls expression of over 300 genes which include upregulation of secreted toxins, capsule polysaccharide and antibiotic resistance and downregulation of adhesions and biofilm formation. Previous research based on genetically generated auxotrophic models of SCV has not reported the role of MgrA in SCV development.

This submitted manuscript (*Journal of Bacteriology*) describes the research into identifying the role of MgrA in the selection of SCV within a population. This was investigated using the *S. aureus* isolate WCH-SK2 with a knockout of *mgrA*. This isolate was grown in continuous culture in the same conditions and parameters previously used by Bui, et al. to determine the effect of the loss of *mgrA* activity and the adaption of the population to limited nutrient availability over a prolonged duration.

This manuscript has been submitted to the Journal of Bacteriology for publication.

Statement of Authorship

Title of Paper	Functional *mgrA* influences genetic changes within a *Staphylococcus aureus* cell population over time
Publication Status ✓	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Submitted for publication in the Journal of Bacteriology

Principal Author

Name of Principal Author (Candidate)	James Lee		
Contribution to the Paper	Carried out continuous culture experiments, experiments to characterise bacterial isolates, data analysis and drafted and wrote the manuscript		
Overall percentage (%)	80		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Stephen Kidd		
Contribution to the Paper	Review and editing of manuscript, conceived and designed the study. Facilitated design of methodologies		
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Functional *mgrA* influences genetic changes within a *Staphylococcus aureus* cell population over time.

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Abstract

Prolonged survival in the host-bacteria microenvironment drives the selection of alternative cell types in *Staphylococcus aureus* permitting quasi-dormant sub-populations to develop. These facilitate antibiotic tolerance, long-term growth, and relapse of infection. Small Colony Variants (SCV) are an important cell type associated with persistent infection but are difficult to study *in vitro* due to the instability of the phenotype and reversion to the normal cell type.

We have previously reported that under conditions of growth in continuous culture over a prolonged culture time, SCVs dominated a heterogenous population of cell types and these SCVs harboured a mutation in the DNA binding domain of the gene for the transcription factor, *mgrA*. To investigate this specific cell type further, *S. aureus* WCH-SK2-Δ*mgrA* itself was assessed with continuous culture. Compared to the wild type, the *mgrA* mutant strain required fewer generations to select for SCVs. There was an increased rate of mutagenesis within the Δ*mgrA* strain compared to the wild type, which we postulate is the mechanism explaining the increased rate of SCV selection. The *mgrA* derived SCVs had impeded metabolism, altered minimum inhibitory concentration (MIC) to specific antibiotics and an increased biofilm formation. Whole genomic sequencing detected single nucleotide polymorphisms (SNP) in the phosphoglucosamine mutase *glmM*. In addition, several genomic rearrangements were detected which affected genes involved in important functions such as antibiotic and toxic metal resistance and pathogenicity. Thus, we propose a direct link between *mgrA* and the SCV phenotype.

Importance

Within a bacterial population, having a stochastically generated heterogeneity of genotypic-based phenotypes, allows continual survival of the population against current and future stressors. The generation of a sub-population of quasi-dormant Small Colony Variants (SCV) in *Staphylococcus aureus* is such a mechanism, allowing for persistent or relapse of infection even after initial intervention seemingly results in clearance of infection. The use of continuous culture has allowed us to introduce time to the growth system and select for SCV within the population under clinically relevant conditions. This study provides valuable insights into the generation of SCV which are not addressed in standard laboratory generated models and reveals new pathways for understanding persistent *S. aureus* infection which can potentially be targeted in future treatments of persistent *S. aureus* infection.

Introduction

Staphylococcus aureus is commonly associated with persistent infections such as prosthetic joint infection (PJI), endocarditis, and diabetic foot infection (DFI) that are recalcitrant to treatment regimens and persist for months or even years [1]. This is in part due to the incredible ability of *S. aureus* to survive and adapt to stresses generated from the host or clinical treatment through the formation of alternative cell types [2]. Small Colony Variants (SCV) are characterised as slow growing cells with a non-pathogenic virulence profile [3, 4]. SCVs are also known to survive intracellularly in different human cell types and these traits result in the evasion of the immune response, intracellular persistence and avoidance of antimicrobials that would otherwise clear actively growing populations [5-9]. SCV can exist as a minor sub-population within an active infection, perhaps even undetectable amongst the active cells when grown under standard laboratory growth methods, but then they can become the dominant or indeed the only cell type, such as when *S. aureus* is intracellular. These cell type dynamics can vary as a result of stressors or conditions of the host-pathogen environment and the subsequent selective pressures on the bacteria. These could include the immune response, growth parameters and antimicrobials which positively select for SCVs. The removal of these stress factors from the micro-environment then permits the cell population dynamics to return to one where the active cells dominate the profile. In addition, SCVs are often unstable and revert to a normal pathogenic cell type. SCV dominance in the population creates reservoirs of *S. aureus* within an anatomical niche but without clinical symptoms. The infection may have seemingly been cleared, but there remains a capacity for the cell population to shift to one dominated by the pathogenic cell type causing a relapse of infection [4, 10].

The understanding of SCVs is originally based on laboratory generated mutant strains of *S. aureus* that are genetically stable (sSCV). These involved defects in the electron transport chain through mutations in *hemB* [11-13], *menD* [13-16] and *thyA* [17-19]. Other models include

auxotrophism to CO_2 [20, 21], fatty acids [22, 23], chorismite synthesis [24] and selection with gentamicin [12]. However, these mutations may not be wholly representative of all the features *S. aureus* adopts during the clinical process of infection. There are other pathways that have been shown to be part of the development of SCV, such as that driven by *sigB* [25]. Our research investigates an alternate perspective of SCV where we address a shift in the paradigm from the mechanisms that define the switch to a SCV within a cell to the population dynamics which facilitate selection of SCV from a population. This model presents SCV as being able to appear stochastically within *S. aureus* populations during normal growth in the absence of any selective pressures [5], but only being detectable when there are conditions which favour their survival while being non-permissive to the active cell types. Stochastic changes within a microbial population that permit survival in changing environments have previously been reported in various species of bacteria [26]. This would suggest that the shift to a population of SCVs during stressful conditions is through selection of stochastically generated SCVs rather than a direct phenotypic switch in response to stress. Consequently, when selective pressures are removed, the population shifts back to the metabolic active normal cell types. These population dynamics would form the basis of relapsing infection by SCV.

An important factor within the host-pathogen interplay during adaptation by the bacterial cell population critically involves the time-dependent feature of colonisation and infection and the associated temporal context for genetic events. Our laboratory has developed a growth platform for selecting sSCV within a bacterial cell population through prolonged continuous culture while in low growth rate conditions that resemble those in the host, and are not growth phase dependent [27]. We grew the methicillin resistant (MRSA) blood isolate *S. aureus* WCH-SK2 over a prolonged time frame (up to 180 generations) and produced a diversity of cell phenotypes that included stable SCV (sSCV) [27].

Whole genome sequencing (WGS) of the WCH-SK2 and its sSCV cells that were generated at the end of the experiment, revealed this phenotype was associated with specific genetic changes, most notably a single nucleotide polymorphism (SNP) in the DNA binding domain of the global virulence regulator *mgrA* (resulting in protein shift; Arg92Cys) [28]. MgrA controls over 300 different genes in *S. aureus* and is well-known for affecting virulence and antibiotic resistance [29] and is closely linked with the regulation of the Agr system of virulence [30]. MgrA is associated with the progression of infection in animal models [31, 32] and down-regulation of cell invasion [33], adhesion to host molecules and biofilm formation [31, 33-35]. The virulence factors affected by MgrA include the upregulation of capsular polysaccharide, leukocidins and exotoxins, all of which are downregulated in SCVs [36]. The role of *mgrA* had not previously been reported in S. aureus SCV. We have now conducted experiments using WCH-SK2-Δ*mgrA* to characterise the role of *mgrA* when grown under clinically relevant conditions in a chemostat. Using an *mgrA* knockout, we observed the effects of beginning a continuous culture with this mutation as the entire bacterial cell population of *S. aureus* under low growth conditions to investigate the role of this gene in the selection of SCV. We therefore grew WCH-SK2-Δ*mgrA* under the same prolonged, controlled growth conditions as previously described to push the cell population to a SCV type [27]. We have identified the genetic and genomic changes associated with sSCV and their effect on growth, biofilm formation and antibiotic resistance.

Results

Continuous culture of WCH-SK2-Δ*mgrA* selects for SCV. A WCH-SK2-Δ*mgrA* knockout was generated with a tetracycline insert as previously described [31]. WCH-SK2-Δ*mgrA* was cultured in CDM in continuous culture over 53 generations (30 d) (Fig. 1a). The maximum growth rate (μ_{max}) of WCH-SK2-Δ*mgrA* in chemically defined media (CDM) was calculated as μ_{max} = 0.338 h^{-1}. The growth of WCH-SK2-Δ*mgrA* was then limited to 15% of the μ_{max} which is μ_{rel} = 0.051 h^{-1} to give a generation time (T_g) of 13.66 h^{-1}.. As the number of generations increased, the proportion of SCV cells also increased (%SCV of total colonies (colony forming units, CFU)/mL). The parental cell type WCH-SK2-Δ*mgrA* produced large, golden colonies when cultured on tryptic soya agar (TSA) (Fig. 1b). Non-pigmented colonies were observed after approximately 6 generations (3 d) and colonies of smaller size were observed after approximately 11 generations (6 d) within the population (Fig. 1c). SCVs first appeared after 16 generations, (9 d) however, these were non-stable and increased in size after sub-culture on TSA but retained the lack of pigment (Fig. 1d). An sSCV was isolated after 50 generations (28 d) which was used in further experiments (WCH-SK2-Δ*mgrA*-SCV). The proportion of SCVs within the population was over 40% after 55 generations (31 d).

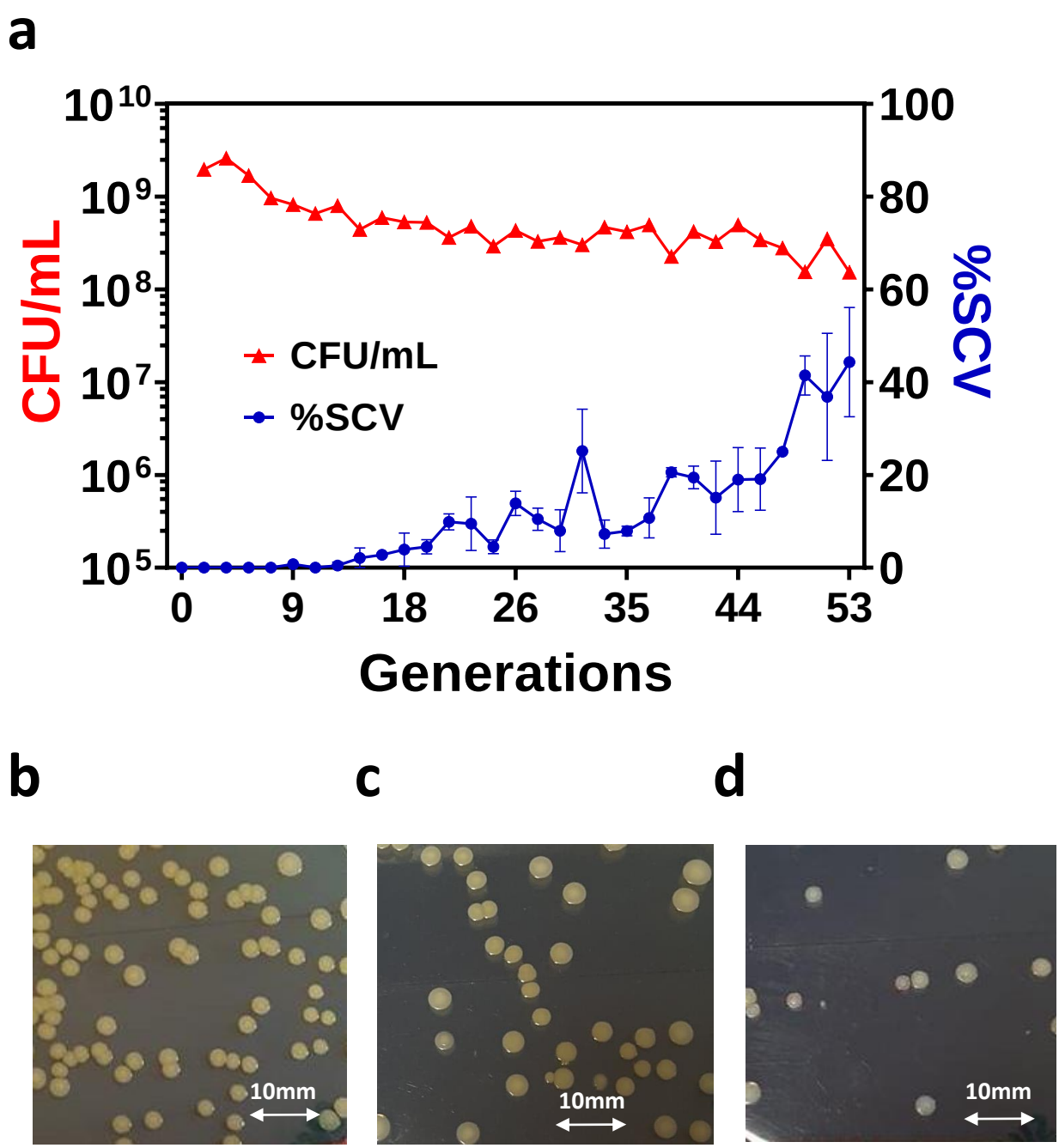

FIG 1. Prolonged growth of WCH-SK2-Δ*mgrA* with limited growth selects for SCV. WCH-SK2-Δ*mgrA* was cultured in CDM within a chemostat over 53 generations of growth (30 d) at a reduced growth rate of μ_{rel} = 0.15. CFU/mL (red, left axis) and proportion of SCV in the population (blue, right axis) was recorded every 24 h. Shown are the cultures plated onto TSA after 3 generations (b), 16 generations (c) and 50 generations (d).

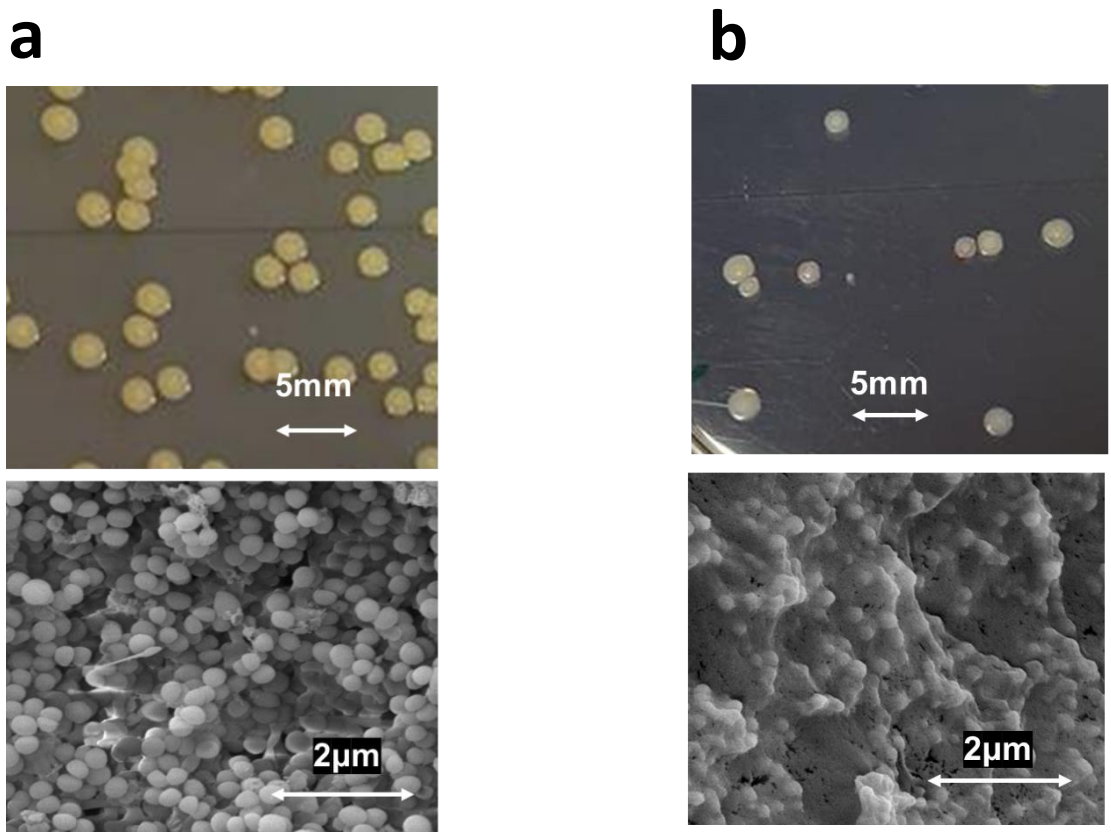

FIG 2. The switch to a SCV in WCH-SK2-Δ*mgrA* is associated with an extracellular matrix. Cell cultures of the parental WCH-SK2-Δ*mgrA* displaying the normal cell type (**a**, left) and the SCV isolated after 50 generations (**b**, right) were imaged using Scanning Electron Microscopy imaging at 20 000x magnification.

Features of the cell type variations were associated with the *mgrA* mutant phenotypic change to SCV. The change in cell type of WCH-SK2-Δ*mgrA* in response to low growth rate (μ_{rel} = 0.051 h^{-1}) was associated with cellular phenotypic differences. SEM imaging of WCH-SK2-Δ*mgrA*-SCV revealed that cells were embedded within an extracellular matrix (Fig. 2). No significant differences were observed between the planktonic growth of WCH-SK2 and WCH-SK2-Δ*mgrA* (as measured by optical density at 600 nm for 20h). In contrast to these results, the WCH-SK2-Δ*mgrA*-sSCV had an extended lag phase (4 h) compared to the parental cell types (less than 1 h- Fig. 3a). The mid exponential phase occurs after 2.25 h for WCH-SK2 andWCH-SK2Δ*mgrA* and 6.7 h for WCH-SK2Δ*mgrA*-SCV (Fig. 3).

Biofilm formation analysis in the impedance-based xCELLigence system enabled the kinetic features of biofilm growth to be measured and compared between strains. Biofilm formation differed in both the kinetics and end-point quantity between WCH-SK2, WCH-SK2-Δ*mgrA* and WCH-SK2-Δ*mgrA*-SCV (Fig. 3). Biofilm formation over time displayed distinct phases; lag, exponential phase and stationary phases. Therefore, there is both an indicator of the rate of biofilm formation, as the mid-point of biofilm exponential phase, and the final end-point value (total biofilm). WCH-SK2-Δ*mgrA*-SCV produced the highest endpoint biofilm followed by WCH-SK2-Δ*mgrA*, and then WCH-SK2, which reached a biofilm mass three times lower than the SCV. Comparing the mid-exponential phase of growth and biofilm formation (Fig. 3) revealed differences in the growth phase dependency of biofilm formation. The mid-exponential phase of biofilm formation for WCH-SK2-ΔmgrA-SCV occurred 4 h after WCH-SK2 and WCH-SK2-Δ*mgrA*. In fact, WCH-SK2-ΔmgrA-SCV started to form biofilms only around 15h while WCH-SK2-ΔmgrA reached a similar biofilm mass (Cell Index value of 0.2) already at 7h.

Table 1. The switch to a SCV in WCH-SK2-Δ*mgrA* is associated with changes in MIC to antibiotics. MIC to antibiotics was determined using microbroth dilution assay with 1/2 factor serial dilutions. 2000 μg/mL was the highest concentration antibiotic used in assay and MIC value above this were not identified.

	Penicillin (μg/mL)	**Gentamicin (μg/mL)**	**Erythromycin (μg/mL)**	**Ciprofloxacin (μg/mL)**
WCH-SK2	>2000	125	>2000	62.5
WCH-SK2-ΔmgrA	>2000	62.5	>2000	62.5
WCH-SK2-ΔmgrA-SCV	62.5	62.5	1000	500

Comparison of antibiotic minimum inhibitory concentration (MIC) between these variants revealed significant changes in their response to penicillin and ciprofloxacin by WCH-SK2-Δ*mgrA*-SCV (Table 1). The parental cell type WCH-SK2 and WCH-SK2-Δ*mgrA* had an unchanged MIC to penicillin, erythromycin and ciprofloxacin and a minor decrease to gentamicin. WCH-SK2-Δ*mgrA*-SCV demonstrated a 20-fold decrease in resistance to penicillin and an 8-fold increase in resistance to ciprofloxacin (Table 1).

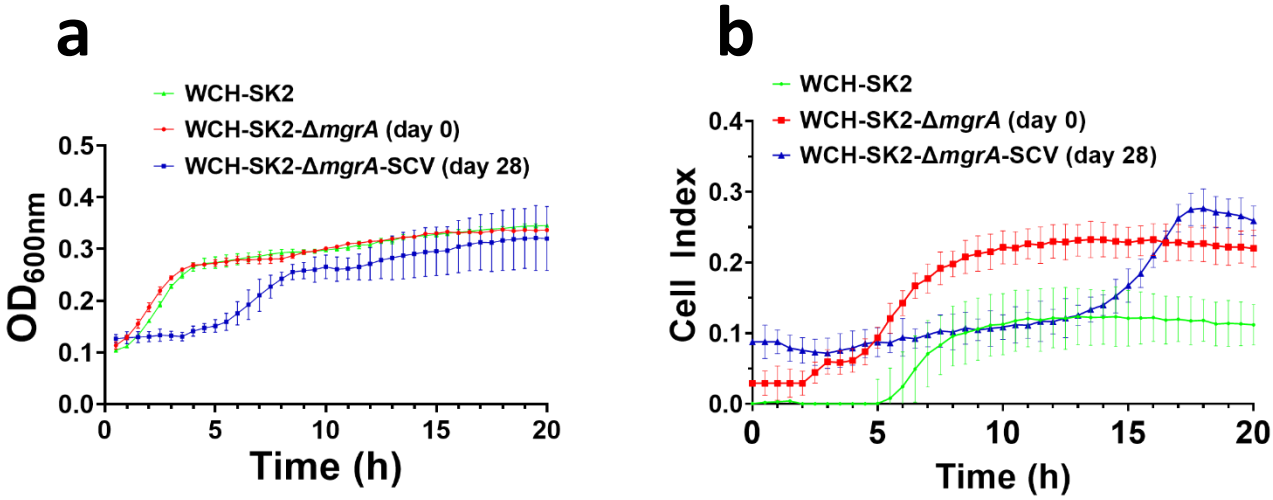

FIG 3. The switch to SCV in WCH-SK2-Δ*mgrA* is associated with changes in growth and biofilm formation. Growth was measured spectrophotometrically at 600nm (**a**) and biofilm formation was measured using xCELLigence RTCA (**b**) in WCH-SK2 (green), WCH-SK2-Δ*mgrA* (red) and WCH-SK2-Δ*mgrA*-SCV (blue)

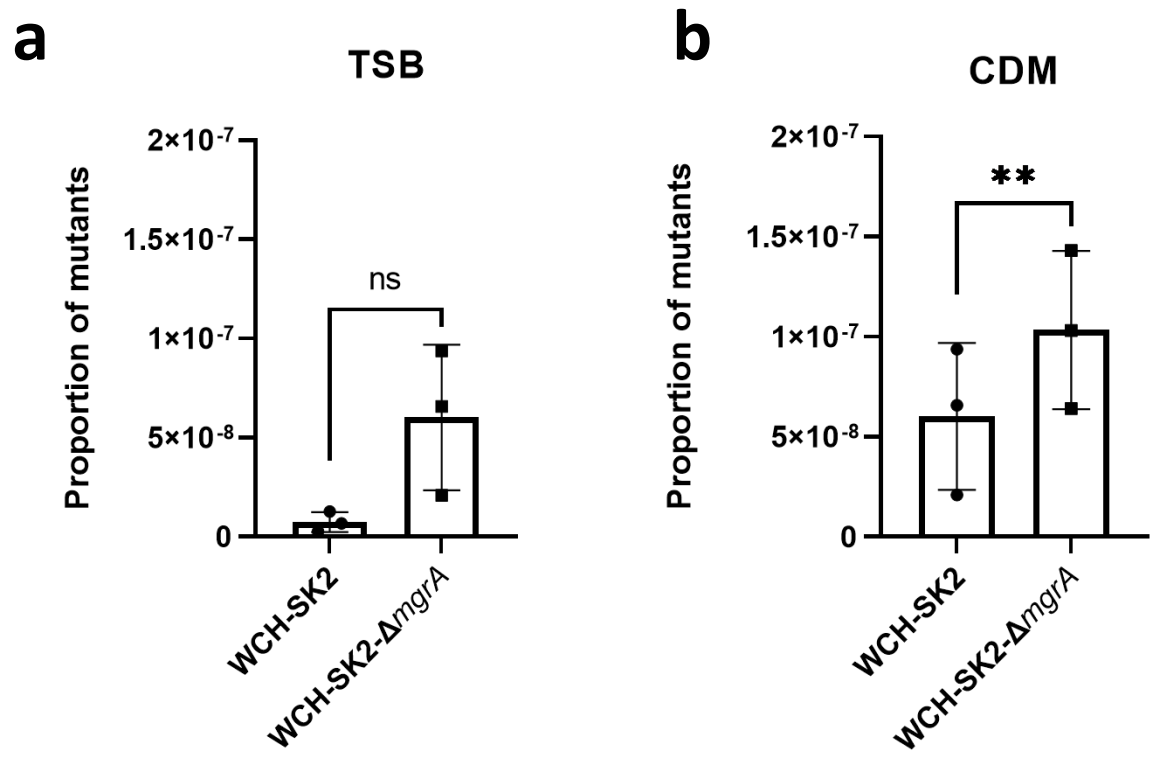

FIG 4. The loss of MgrA activity leads to an increased rate of mutation. The rate of mutation in WCH-SK2 and WCH-SK2-Δ*mgrA* was determined through the number of spontaneous mutations after 24 h of growth in TSB (**a**) and CDM (**b**) which resulted in resistance against gentamicin. A mutational event was defined as a single colony growing on TSA with gentamicin above the MIC. Proportion of mutants was defined as number of mutations over the total number of CFU.

** = p-value < 0.005

Cell type variants in a *mgrA* mutant is associated with increased mutation rate. There was a significant difference in the number of generations that selected for SCVs within the population. For WCH-SK2-Δ*mgrA*, SCVs first appeared in continuous culture after 11 generations and an sSCV was isolated after 49 generations (Fig. 1). In the same growth conditions for WCH-SK2, SCVs first appeared after 52 generations and sSCV were isolated after 174 generations [27]. We postulated that there was an intrinsic alteration in the permissible level of mutations in the WCH-SK2-Δ*mgrA* strain that allowed mutations that drive SCV formation within the population [27]. The rate of mutation was determined by the enumeration of spontaneous mutations in a bacterial cell grown in gentamicin above the MIC. Growth in tryptic soya broth (TSB) resulted in no statistically significant differences in the mutation rate between WCH-SK2 and WCH-SK2-Δ*mgrA*. However, a significantly increased rate of mutation in WCH-SK2-Δ*mgrA* (mean of $1.03x10^{-7}$ mutation events per CFU, p-value = 0.160) was observed compared to WCH-SK2 when cultured in CDM (mean of $6.01x10^{-8}$ mutational events per CFU, p-value = 0.006) (Fig. 4).

Genomic alignments reveal genetic events associated with SCV. The continuous culture of WCH-SK2-ΔmgrA created a niche comprised of limited nutrients and hence a low growth rate. In this environment, cell types with differences in metabolic demands may have greater selective fitness, and the imposed slow growth rate would enable slow growing bacteria to increase in proportion. As noted, the SCV phenotype accounted for a significant portion of the population over time (Fig. 2).

After 50 generations (28 days) of WCH-SK2-Δ*mgrA* growing on the chemostat, two phenotypes which were clearly differentiated, namely a large non-pigmented colony and a SCV, were fully sequenced. This allowed us to detect single nucleotide polymorphisms (SNPs) and genomic rearrangements (GRAs) i.e. duplications, translocations, insertions or deletions. For this, we combined NextSeq (Illumina) with Minion (Oxford Nanopore) technologies to

Table 2. List of *S. aureus* isolates selected for whole genome sequencing and genome alignment

Isolate name	Colony description	Notes
WCH-SK2-ΔmgrA day 0	Large, pigmented	Parental strain used to inoculate the continuous culture
WCH-SK2-ΔmgrA d28	Large, non-pigmented	Isolated after 50 generations in continuous culture
WCH-SK2-ΔmgrA-SCV d28	SCV	Isolated after 50 generations in continuous culture

obtain complete, fully-closed genomes of WCH-SK2-Δ*mgrA*-28d (the non-pigmented strain) and WCH-SK2-Δ*mgrA*-SCV. Briefly, an average of 3.7 million reads of 150 bp of length sequenced by NextSeq were combined with 150,000 reads of 9.7±0.5 Kbp of length sequenced with Minion technology (Oxford Nanopore Technology) per genome. Consequently, genomes were closed with >500x coverage, where detected polymorphisms could unequivocally be assigned to mutations and not be derived from sequencing errors.

The results revealed a missense mutation in the phosphoglucosamine mutase *glmM* (Gly6Val) and three small indels in intergenic regions (Table 3). The non-synonymous mutation at the glmM gene was not located in the active site of the protein. GlmM catalyses the conversion of glucosamine-6-phosphate to glucosamine-1-phosphate for the biosynthesis of the cell-wall peptidoglycan. It is important to note that WCH-SK2-Δ*mgrA*-SCV was a single colony obtained from an entire population of cells and may not be representative of all the SCVs present within the population. Genome alignments revealed sections of genome varying between WCH-SK2-Δ*mgrA*-28d and WCH-SK2-Δ*mgrA*-SCV. Notable genes missing in WCH-SK2-Δ*mgrA*-SCV are involved in the SOS response and DNA repair (*gloB, recF*), peptidoglycan synthesis (*nagC*) and antibiotic resistance (*mecA, mecI, tetM*).

Table 3. SNPs detected between WCH-SK2-Δ*mgrA* isolates taken after 50 generations (28 d) within a chemostat in controlled growth, low nutrient conditions. For SNP analysis, a large, non-pigmented colony type was aligned against a sSCV.

Genome position	Gene	Nucleotide change	Amino acid change	Product
1273471	*glmM*	17C>A	Gly6Val	Phosphoglucosamine mutase
1336128	Non-coding			
2653422	Non-coding			
2653489	Non-coding			

Secondly, the complete genomes were compared by blastN in order to detect potential Genomic Rearrangements (GRA) that could correspond to added, deleted or reoriented genomic segments. Only one GRA was found when comparing WCH-SK2-Δ*mgrA* (time 0) and WCH-SK2-Δ*mgrA*-28d. Interestingly, this GRA was not found in WCH-SK2-Δ*mgrA*-SCV. On the other hand, when the WCH-SK2-Δ*mgrA*-SCV strain was compared to the original WCH-SK2-Δ*mgrA*, 54 GRAs were detected (Table 4). Among these GRAs, 46 affected fewer than 10 genes whereas the remaining varied between 14 and 49 genes. In addition, there were four "high-divergence" zones where some genes appear to be missing in the SCV genome, but where part of the genes were homologous to the reference ancestral genome but with a dramatically low level of sequence similarity **(FIG. 5)**. These highly-divergent regions contained, among others, genes encoding Type VII secretion systems or antibiotic resistance, such as a methicillin resistance regulatory protein (MecI), a kanamycin nucleotidyltransferase, and a bleomycin resistance protein.

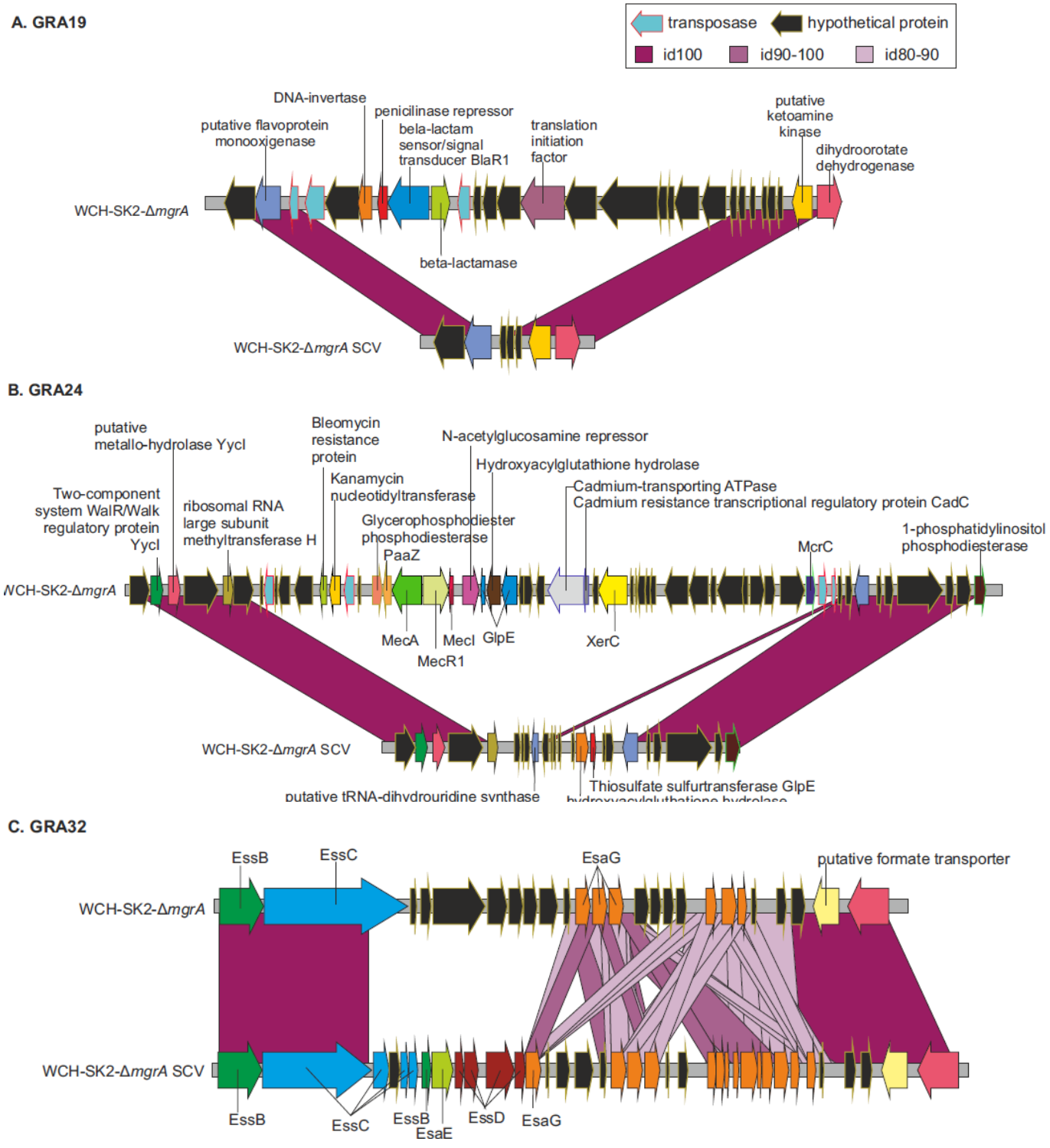

FIG. 5. Segments of genome were associated with GRAs. Three segments were identified as high divergence zones where there was a dramatically increased presence of GRAs in WCH-SK2-Δ*mgrA*-SCV with regions homologous to the parental strain WCH-SK2-Δ*mgrA* but a low level of sequence similarity

To unravel the potential cause of these GRAs, we studied the genomic features present at the edges of the rearranged segments and identified some known to cause genomic instability including transposases, invertases, integrases, recombinases and/or repetitions at the beginning and the end of the GRAs (Suppl. Table 3). Interestingly, we detected transposases in 41 cases (71%). In addition, we detected the tyrosine recombinase XerC seven times at adjacent positions, another seven cases where a repeated DNA sequence was detected at the beginning and the end of the GRA and one case containing a prophage integrase. No mobile or repetitive elements were found in four cases. In addition, in 14 GRAs, sequences annotated as miscellaneous RNA (also known as non-coding RNA or bacterial introns) were included and we hypothesize that this might also be involved in generating the rearrangements (1, 2). Thus, the origin of most GRAs could be explained by genomic instability imposed by mobile or repetitive genetic elements.

In most cases, inserted/deleted proteins were hypothetical. However, there were some mutated genes with a known function. For example, one of the GRAs affecting both strains had genes involved in the type VII secretion system. This system has been associated with pathogenesis and the mutation of the entire secretion system or any subunit (EsxA, EssB, EssC, EsxC, EsxB, EsaB, EsaD or EsaE) has been shown to decrease *S. aureus* virulence (3–8). Other genes that are known to have a role in *S. aureus* infection and were found in the GRAs were enterotoxin type A (10), α-hemolysin (11) or the ATP-dependent Clp protease proteolytic subunit (12). It must be underlined that some of these genes were translocated in the genome but not lost. In addition, staphylocoagulase was found to have several mutations and the automatic annotation by Prokka software split the protein in 3 ORFs, suggesting a potential lack of functionality.

Additionally, mutations in other genes could influence important functions such as antibiotic or toxic metals resistance. For example, the β-lactam sensor/signal transducer BlaR1, a penicillinase repressor, and a β-lactamase were translocated in the WCH-SK2-Δ*mgrA*-28d

strain and the β-lactam sensor/signal transducer MecR1 and a PBP2a family β-lactam-resistant peptidoglycan were lost. These genes are important for resistance to the β-lactamase group antibiotics. Interestingly, the 3 copies of streptomycin 3'-adenylyltransferase, which is a gene responsible for streptomycin and/or spectinomycin resistance, were lost in WCH-SK2-Δ*mgrA*-28d. Furthermore, cadmium resistance transcriptional regulatory protein CadC and cadmium-transporting ATPase were also translocated. On the other side, arsenate reductase, arsenical pump membrane protein, arsenical pump membrane protein, Copper-exporting P-type ATPase B and multicopper oxidase (*mco*) were lost in WCH-SK2-Δ*mgrA*-SCV.

Discussion

Comparison of the data between WCH-SK2 and WCH-SK2-Δ*mgrA* in continuous culture showed the impact of MgrA on population dynamics in response to prolonged growth in nutrient limited conditions. Wildtype WCH-SK2 required 56 generations (16 d) until SCVs were first observed and an sSCV was first isolated after 192 generations (55 d) [27]. The genetic profile of the sSCV included a missense mutation leading to a R92C amino acid in the DNA binding domain of MgrA. MgrA is homologous to MarR in *Escherichia coli* and previous research identified a R94C SNP in MarR that prevented DNA binding [37]. This previously described SNP in MgrA closely aligns to the R94C SNP in WCH-SK2 which implies the SNP in WCH-SK2-SCV would be detrimental to MgrA activity. In addition to a SNP in *mgrA*, WCH-SK2 sSCV after 56 generations also showed the up-regulation of Ebh and this was consistent with cells embedded in a proteinaceous extracellular matrix.

In comparison to WCH-SK2, WCH-SK2-Δ*mgrA* took 16 generations (9 d) to reveal SCVs and an sSCV was first isolated after 50 generations (28 d). The loss of MgrA decreased the number of generations required for the formation or selection of SCV. MgrA has been shown to sense reactive oxygen species (ROS) including hydrogen peroxide and potassium superoxide that

results in activation of MgrA targets [38]. We speculate that this results in increased intracellular ROS and thereby an increased rate of mutation. This would result in fewer generations being required to accumulate the necessary genetic events that lead to SCV formation and thus allow populations of *S. aureus* to adapt to stresses more efficiently. We did determine the rate of mutation was significantly increased in WCH-SK2-Δ*mgrA* when cultured in CDM compared to the wildtype WCH-SK2. Previous research have identified increased rates of mutagenesis through the SOS DNA repair response was associated with increased frequency of SCV [39]. As the same difference in rate of mutation did not occur when cultured in TSB, this rate of mutation is likely dependent on the limited nutrients in CDM. Previous research identified glucose, arginine, glutamic acid and proline as the growth-limiting nutrients for *S. aureus* within this CDM [27]. The knockout of *mgrA* alone does not confer the colony type and growth characteristics of SCV in WCH-SK2, but instead creates a population with a greater disposition for genetic events that can result in SCV. In addition, the gene encoding the methylglyoxal detoxification protein *gloB* is missing in WCH-SK2-Δ*mgrA*-SCV. Methylglyoxal is a by-product of metabolism that can create ROS stress and is utilised by the innate immune response to clear pathogens. The loss of methylglyoxal detoxification can increase the presence of ROS, DNA damage and thus mutations through DNA repair mechanisms.

WCH-SK2-Δ*mgrA*-SCV had an impeded growth rate compared to WCH-SK2 and WCH-SK2-Δ*mgrA*. Reduced growth is a defining characteristic of SCV that facilitates persistence during clinical infection. *In silico* analysis of WCH-SK2-Δ*mgrA*-SCV and WCH-SK2-Δ*mgrA* did not detect SNPs in genes that have previously been commonly associated with metabolism in SCV (central carbon metabolism and the electron transport chain). However, GlmM, in addition to DacA and YbbR, are involved in the regulation of the secondary messenger molecule c-di-

AMP which controls the osmotic stress response and virulence. Increased intracellular c-di-AMP levels has been shown to result in growth impedence and increased resistance to β-lactams and acid stress [40-42]. DacA is a diadenylate cyclase and the sole producer of c-di-AMP in S. aureus [43]. Production of c-di-AMP was inhibited when DacA was complexed with GlmM. In addition, YbbR also forms complexes with DacA/GlmM however the function of YbbR on c-di-AMP production is still unknown. Therefore, the loss of glmM expression would prevent the repression of c-di-AMP production (or indirect activation of c-di-AMP production). The lack of c-di-AMP production has previously resulted in suppressed growth in complex media such as TSB through the repression of amino acid and osmolyte transporters [44]. Additionally, low c-di-AMP levels have been reported to be associated with increased ROS production [44] and so the loss of GlmM activity and increased c-di-AMP may provide another pathway to survive oxidative stresses.

Kinetic readings of biofilm formation using xCELLigence found WCH-SK2-Δ*mgrA*-SCV produced less biofilm at a slower rate but produced a greater biofilm during late stationary phase compared to WCH-SK2-Δ*mgrA*. Studies with an *mgrA* mutant found the loss of *mgrA* resulted in increased biofilm formation where early biofilm was dependent on extracellular DNA [35]. Additionally, we identified a significantly increased biofilm formation in WCH-SK2-Δ*mgrA* compared to its parental cell type WCH-SK2. Mutations in *mgrA* have been associated with increased biofilm formation and reduced clumping through upregulation of large surface proteins such as Ebh and SasG [31]. The loss of *glmM* does not inhibit peptidoglycan synthesis but modifies the properties of the cell wall which reduces the ability for PBP-2 to act against antibiotics [45]. This modified cell wall may affect the activity of other cell wall proteins such as Ebh and SasG and the observed changes in extracellular matrix and biofilm formation.

Table 4. Two cell types comprising of a large, non-pigmented producing colony (WCH-SK2-*ΔmgrA* d28) and a SCV (WCH-SK2-*ΔmgrA*-SCV d28) were isolated after 50 generations (28 d) in continuous growth. Alignment of whole genome sequences were analysed for genetic GRAs associated with added, deleted or reoriented genomic segments

Genomic Rearrangement (GRA) #	# Genes	Type of GRA	Encoded Genes	Mobile/Repetitive element	Type of element/Motif
GRA1	2	deletion	IS256 family transposase IS256 x2	transposase	IS256
GRA2	3	insertion	ISL3 family transposase IS1181 x2 hypothetical protein	transposase	IS1181
GRA3	3	deletion	ISL3 family transposase IS256 x2 hypothetical protein	transposase	IS256
GRA4	2	deletion	IS200/IS605 family transposase ISSep3 hypothetical protein	transposase	ISSep3
GRA5	2	deletion	hypothetical protein IS256 family transposase IS256	transposase	IS256
GRA6	1+1RNA	insertion	IS200/IS605 family transposase ISSep3	transposase + misc RNA	ISSep3 tsr24
GRA7	14+1RNA	insertion	beta-lactam sensor/signal transducer BlaR1 Beta-lactamase hypothetical protein x8 Penicillinase repressor Tyrosine recombinase XerC x2	TR + misc RNA+recombinase	tsr24 GAAGAAGAA XerC
GRA8	2	deletion	IS256 family transposase IS256 hypothetical protein	transposase	IS256
GRA9	1+1RNA	deletion	Enterotoxin type A	misc RNA	rli28
GRA10	49	deletion	ATP-dependent Clp protease proteolytic subunit Chromosome partition protein Smc x2 hypothetical protein x43 putative HTH-type transcriptional regulator Single-stranded DNA-binding protein A Tyrosine recombinase XerC	recombinase	XerC

GRA11	2	deletion	ISL3 family transposase IS256 x2	transposase	IS256
GRA12	21	deletion	hypothetical protein x20 Tyrosine recombinase XerC	TR + recombinase	TTTTACATCATTCCTGGCAT XerC
GRA13	2	deletion	ISL3 family transposase IS256 x2	transposase	IS256
GRA14	40+3RNA	insertion	Arsenate reductase Arsenical pump membrane protein Arsenical pump membrane protein Cadmium resistance transcriptional regulatory protein CadC Cadmium-transporting ATPase Copper-exporting P-type ATPase B x3 DNA-invertase hin HTH-type transcriptional regulator CysL hypothetical protein x21 IS1182 family transposase ISSau3 x3 IS6 family transposase IS431mec Multicopper oxidase mco Potassium-transporting ATPase ATP-binding subunit putative ABC transporter ATP-binding protein YknY Putative transposon Tn552 DNA-invertase bin3	transposase + misc RNA	ISSau3 tsr24 x2 fstAT
GRA15	2	deletion	ISL3 family transposase IS256 x2	transposase	IS256
GRA16	6	deletion	30S ribosomal protein S9 Holliday junction ATP-dependent DNA helicase RuvB hypothetical protein x4	TR	ATCGAGGTGTTTTGTATATAT
GRA17	1+1RNA	insertion	ISL3 family transposase IS1181	transposase + misc RNA	IS1181 sau_5949
GRA18	2	insertion	IS200/IS605 family transposase ISSep3 hypothetical protein	transposase	ISSep3
GRA19	7	deletion	IS30 family transposase ISSau5 beta-lactam sensor/signal transducer BlaR1 Beta-lactamase DNA-invertase hin hypothetical protein ISNCY family transposase ISBli29 Penicillinase repressor	transposase	ISSau5 ISBli29

GRA20	14	deletion	IS30 family transposase ISSau5 hypothetical protein x12 Translation initiation factor IF-2	transposase	ISSau5
GRA21	2	deletion	ISL3 family transposase IS256 x2	transposase	IS256
GRA22	1	deletion	Collagen adhesin	-	-
GRA23	4	deletion	IS30 family transposase IS1252 hypothetical protein x3	transposase	IS1252
GRA24	45 14	High-divergence Zone	beta-lactam sensor/signal transducer MecR1 Bifunctional protein PaaZ Bleomycin resistance protein Cadmium resistance transcriptional regulatory protein CadC Cadmium-transporting ATPase Glycerophosphodiester phosphodiesterase, cytoplasmic Hydroxyacylglutathione hydrolase hypothetical protein x26 IS256 family transposase IS256 x2 IS6 family transposase IS431mec x2 Kanamycin nucleotidyltransferase Methicillin resistance regulatory protein MecI N-acetylglucosamine repressor PBP2a family beta-lactam-resistant peptidoglycan transpeptidase MecA Protein McrC Thiosulfate sulfurtransferase GlpE x2 Tyrosine recombinase XerC Hydroxyacylglutathione hydrolase hypothetical protein x11 putative tRNA-dihydrouridine synthase Thiosulfate sulfurtransferase GlpE	transposase+recombinase	IS256 IS431mec XerC
GRA25	2+1RNA 2	High-divergence Zone	hypothetical protein Replication initiation protein hypothetical protein x2	misc RNA	S35
GRA26	2	deletion	ISL3 family transposase IS256 x2	transposase	IS256

GRA27	9 9	High-divergence Zone	2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-acetyltransferase hypothetical protein x5 IS256 family transposase IS256 x2 2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-acetyltransferase hypothetical protein x8	transposase	IS256
GRA28	2	deletion	IS256 family transposase IS256 x2	transposase	IS256
GRA29	1	deletion	Staphylocoagulase	-	-
GRA30	1	deletion	hypothetical protein	-	-
GRA31	1	deletion	hypothetical protein	TR	TATTACTTTTTACTAGG
GRA32	28 17	High-divergence Zone	hypothetical protein x7 Type VII secretion system extracellular protein B Type VII secretion system extracellular protein C Type VII secretion system extracellular protein D Type VII secretion system protein EsaE Type VII secretion system protein EsaG x13 Type VII secretion system protein EssC Type VII secretion system protein EssD x3 hypothetical protein x12 Type VII secretion system protein EsaG x5	-	-
GRA33	15+1RNA	insertion	hypothetical protein x12 IS30 family transposase ISSau5 x2 Translation initiation factor IF-2	transposase + misc RNA	ISSau5 tsr26
GRA34	28	deletion	ATP-dependent DNA helicase Rep DNA replication and repair protein RecF hypothetical protein x22 IS21 family transposase ISCth9 IS256 family transposase ISLgar5 tetracycline resistance ribosomal protection protein Tet(M) Tyrosine recombinase XerC	transposase+recombinase	ISCth9 ISLgar5 XerC
GRA35	1+1RNA	insertion	ISL3 family transposase IS1181	transposase + misc RNA	IS1181 sau_5949

GRA36	2	deletion	Serine-aspartate repeat-containing protein D Serine-aspartate repeat-containing protein E	TR	AAAACACCAAAATATAGTTTAC TATGTTTGGTACGACAGTAATA
GRA37	2	deletion in WCH-SK2-ΔmgrA-28d	IS256 family transposase IS256 hypothetical protein	transposase	IS256
GRA38	2 1	deletion insertion	IS256 family transposase IS256 hypothetical protein hypothetical protein	transposase	IS256
GRA39	2	deletion	IS256 family transposase IS256 x2 hypothetical protein	transposase	IS256
GRA40	3	insertion	ISL3 family transposase IS1181 x3	transposase	IS1181
GRA41	1+1RNA	insertion	ISL3 family transposase IS1181	transposase + misc RNA	IS1181 sau-5949
GRA42	3	deletion	IS256 family transposase IS256 x2 hypothetical protein	transposase	IS256
GRA43	25	deletion	Putative prophage phiRv2 integrase hypothetical protein x22 Iron(3+)-hydroxamate-binding protein FhuD	prophage integrase + TR	TCCCGCCGTCTCCAT
GRA44	8	deletion	Tyrosine recombinase XerC x2 hypothetical protein x2 Streptomycin 3"-adenylyltransferase rRNA adenine N-6-methyltransferase 2-methoxy-6-polyprenyl-1,4-benzoquinol methylase, mitochondrial putative protein YwqG	misc RNA + recombinase	S35 XerC
GRA45	4 4	deletion // insertion	hypothetical protein x4 hypothetical protein x4	-	-
GRA46	7	deletion	hypothetical protein x4 IS256 family transposase IS256 x3	transposase	IS256
GRA47	3	deletion	hypothetical protein IS256 family transposase IS256 x2	transposase	IS256

GRA48	2	deletion	IS256 family transposase IS256 Alpha-hemolysin	transposase	IS256
GRA49	3	deletion	hypothetical protein x2 IS256 family transposase IS256	transposase	IS256
GRA50	2+1RNA	insertion	IS1182 family transposase ISSau3 hypothetical protein	transposase + misc RNA	ISSau3 tsr24
GRA51	1	deletion	hypothetical protein	TR	CTTTATTT
GRA52	3	deletion	hypothetical protein x2 IS256 family transposase IS256	transposase	IS256
GRA53	2	deletion	hypothetical protein IS256 family transposase IS256	transposase	IS256
GRA54	3	deletion	IS256 family transposase IS256 x2 GlcA/glcB genes antiterminator	transposase	IS256
GRA55	3+1RNA	deletion	IS200/IS605 family transposase ISSep3 hypothetical protein x2	transposase + misc RNA	ISSep3 tsr25
GRA56	2	deletion	IS256 family transposase IS256 x2	transposase	IS256
GRA57	1+1RNA	insertion	ISL3 family transposase IS1181	transposase + misc RNA	IS1181 sau_5949
GRA58	8	deletion	hypothetical protein x3 rRNA adenine N-6-methyltransferase Streptomycin 3"-adenylyltransferase x2 Tyrosine recombinase XerC x2	recombinase	XerC

It is interesting to note that contrary to the non-pigmented strain isolated after 28 days of growth in the chemostat, which only had one genomic rearrangement compared to the ancestral WCH-SK2-Δ*mgrA* strain, the SCV isolate generated during the same amount of time had more than 50 genomic rearrangements comprising up to 49 genes. These involved deletions, insertions, translocations and at least four regions where genes had undergone extraordinary divergence within 50 generations. Most rearrangements were flanked by mobile or repetitive elements, which are known to generate considerable genomic instability [46]. Having this considerable number of mutations, many of which affect genes responsible for the pathogenesis or antibiotic resistance, could be due to cells growing in the absence of a host and in an antibiotic-free environment. SCVs are characterised as slow growing cells with a non-pathogenic virulence profile [3, 4]. However, clones carrying these mutations would be expected to be unsuccessful to proliferate in the host or during antibiotic treatment. A plausible explanation to why these are more commonly lost after just a few generations is because they might not be relevant for growing in the chemostat and their loss could contribute to outcompete other clones with lower fitness. It is remarkable that some regions appeared to have a large sequence divergence compared to the original ancestor. This, together with the large number of genomic regions that vary between WCH-SK2-Δ*mgrA*-28d and WCH-SK2-Δ*mgrA*-SCV but the almost complete genomic stability between WCH-SK2-Δ*mgrA* and WCH-SK2-Δ*mgrA*-28d, suggest that the population of SCVs will be highly heterogenous, favoring the future success of any of the coexisting, highly mutated clones. Heterogenous populations have been reported to maximize the mean fitness of the population in various environments where sub-populations are more fit to survive in unpredictable stressors. Other bacterial species have been reported to create heterogenous populations through bet-hedging including endospore formation in *Bacillus subtillis* endospore formation and alteration of metabolism in *Dictyostelium discoiduem*, *Escherichia coli* and *Lactococcus lactis* [26].

In summary, this study utilised an *mgrA* mutant to demonstrate that a dysfunctional MgrA is an important factor in adaptation to unfavourable conditions by facilitating the generation of different cell types. These cell types include SCV cells which are associated with impeded growth and modulated biofilm formation that result in greater fitness under clinically relevant conditions. While previous research has focused on the direct effects of MgrA on pathogenesis, we identified a potential role of MgrA in the population dynamics in stressful conditions that may lead to persistent and highly resilient infections.

Materials and Methods

Generation of *mgrA* knockout mutations. To construct the *mgrA* deletion plasmid, ~700 bp regions flanking the gene were amplified with primer pairs HC116/HC117 and HC118/119. The products were column purified and fused in a second PCR using primers HC116 and HC119. This fusion product was gel purified, digested with SacI and SalI, and ligated into pJB38 [81] to generate pHC34. This plasmid was electroporated in RN4220, selecting on TSA plates containing Cam at 30°C. The plasmid was then transduced into the MRSA isolate WCH-SK2. Individual colonies were streaked on tryptic soya agar (TSA) Cam plates and incubated at 42°C to select for integration into the chromosome. Single colonies were grown in TSB at 30°C and diluted 1:500 in fresh media for four successive days before diluting to 10−6 and plating on TSA containing 0.2 μg/mL anhydrotetracycline to select for loss of the plasmid. Colonies were screened for resistance to Cam, and CamS colonies were screened by PCR for deletion of *mgrA*.

Establishing continuous culture conditions. Growth of *S. aureus* was preformed within. Growth was controlled through the flow of chemically defined media medium (CDM, Supplementary Table S1) into the culture vessel so that the growth rate was relative (μ_{rel}) to

the maximum growth rate (0.15% of the μ_{max}). The maximum growth rate was determined by batch culture in CDM within the chemostat vessel. The greatest change in CFU/mL was between 5 and 6 h with a change of $1.4x10^8$ CFU/mL. Monod's equation (Supplementary Equation S1**)** was used to calculate the maximum growth rate of μ_{max} = 0.338h. The dilution rate (D) was derived from the growth rate (μ) and working volume (V) (Supplementary equation S2). To achieve 15% of the maximum growth rate (μ_{max} = 0.338), a flowrate of 25.3 mL/h was required to give a generation time of 13.66 h^{-1} .

CDM was prepared as previously described [47] and connected to the chemostat vessel with the inflow of CDM mediated by a peristaltic pump.

Continuous culture of *S. aureus.* 10 mL of an overnight culture grown in TSB was washed twice by centrifugation (1900 x g, 4°C, 10min) and resuspended in 10 mL CDM before inoculated into the chemostat. *S. aureus* was grown by batch culture in a chemostat vessel (Sartorius Biostat B) with a working volume of 500mL to determine the maximum growth rate. OD_{600} readings were measured at hour intervals, and total cell numbers enumerated (N_{cells} = OD_{600} x 0.8 x 109) for each sample to determine the maximum growth rate (μ_{max}) and generation time (T_g) using the Monod equation. The greatest change in CFU/mL was between 5 and 6 h with a change of $1.4x10^8$ CFU/mL. Monod's equation (Supplementary Equation S1**)** was used to calculate the maximum growth rate of μ_{max} = 0.338h. The dilution rate (D) was derived from the growth rate (μ) and working volume (V) (Supplementary equation S2). To achieve 15% of the maximum growth rate (μ_{max} = 0.338), a dilution rate of 0.05 h^{-1} was achieved with a flowrate of 25.3 mL/h of CDM to the chemostat to give a generation time of 13.66 h^{-1}. The chemostat vessel was kept at pH 7.4 with the addition of ammonia (7.5%). A ring sparger provided aeration directly into the culture medium. Agitation was achieved with a Rushton turbine (6 blade disk impeller) at 250 rpm. The temperature was kept at 37°C with a double-jacketed glass vessel with thermostat control.

Sample collection and analysis. Aliquots were obtained from the chemostat aseptically with a slip tip syringe connected to a lure-lock sampling tube. To determine colony morphology and CFU/mL, culture broth was serially diluted and plated onto TSA. SCV were classified as non-pigmented colonies with diameter <1mm after 48 h incubation at 37°C. A SCV was considered stable if it retained the SCV phenotype after 5 sub-cultures on TSA. Samples of the chemostat were stored by directly centrifuging 5 mL of culture supernatant (1900 x g, 4°C, 10min) and resuspending in 30% glycerol and stored at -80°C.

Growth kinetics. Growth kinetics assays were performed in 96-well microtitre plates. Cells were incubated in media at 37°C to log phase (OD_{600} ~ 0.2). 20 μL of log phase culture was added to a well containing 180 μL of media. Plates were incubated at 37°C for 18 h and OD_{600} readings taken at 30 min intervals with a Sunrise Absorbance Microplate Reader (Tecan).

Determining antibiotic MIC. Antibiotics were serially diluted into TSB by a factor of 1:2. Cells were incubated in TSB at 37°C to log phase (OD_{600} ~ 0.2). 20 μL of log phase culture was added to wells containing 180 μL TSB with antibiotic. Cultures were incubated at 37°C for 18 h and OD_{600} readings were taken. Cultures were serially diluted to determine CFU/mL. MIC was determined to be the lowest concentration of antibiotic which resulted in no visual bacterial growth and change in OD_{600}.

Real-time xCELLigence biofilm assay. Real-time biofilm assays were performed with an xCELLigence RTCA (ACEA Biosciences) as previously described [48, 49]. Biofilm assay was performed in 16-well ePlates (ACEA Biosciences). 150 μL of CDM was placed into each well and sterile distilled water was placed into the surrounding evaporation control troughs as recommended by the manufacturers. The plate was inserted into the RTCA Plate Analyzer and placed inside a 37°C incubator for 30 min. Bacteria were grown separately to log phase (OD_{600} ~ 0.2) and 50 μL was added to ePlate wells in triplicate. Impedance readings were taken at 15 min for 20 h. Cell-sensor impedance was expressed as an arbitrary unit called cell index (CI)

according to the manufacturer's instructions. CI at a timepoint was defined as Z_n-Z_b where Z_n is the cell-electrode impedance of the well that contains the cells and Z_b is the background impedance with media alone. CI values have been shown to correlate with total biofilm mass in *S. aureus* [49].

Rate of mutation assay. *S. aureus* cells were incubated overnight in 5 mL of TSB at 37°C. Approximately $1x10^6$ cells were inoculated into a series of five 50 mL tubes with 5 mL of fresh TSB. Cultures were incubated at 37°C for 18 h and CFU/mL was enumerated by serial dilution. Cultures were centrifuged (1900 x g, 4°C, 10min), supernatant was discarded, and cells were resuspended in 100 μL of sterile PBS. Cell suspensions were plated out onto TSA containing gentimicin at a concentration above the MIC for each strain. Colonies which grew on the selective media were defined as a mutational event. The rate of mutation was defined as the proportion of mutational events over the total CFU in the culture.

Scanning electron microscopy (SEM). Cells were filtered through a 0.2 μm Milipore filter paper and fixed with a fixative solution (4% paraformaldehyde, 1.25% glutaraldehyde, 4% sucrose in PBS). Filters were washed twice with 4% sucrose in PBS, post-fixed with 0.1% osmium tetraoxide for 60 min and washed twice with 4% sucrose in PBS. Cells were dehydrated with a series of two 10-minute ethanol washes, each in 70%, 90% and 100% ethanol solutions. Cells were dried in a 1:1 mix HMDS and 100% ethanol for 20 min and then in HMDS for 15 min. Samples were mounted onto titanium stubs and coated with 2 mm platinum. Images were taken using a Phillips XL30 FEG scanning electron microscope (Adelaide Microscopy).

Whole genome sequencing. Whole genomic DNA was extracted and purified using QIAGEN Genomic-tip 500/G columns (QIAGEN, Australia) according to manufacturer protocols. Quality and quantity of genomic DNA was determined using FEMTO Pulse (SA Pathology). Genomes were sequenced using PacBio SMRT (Single Molecule Real Time) sequencing

preformed by the Ramciotti Centre for Genomics (Sydney, Australia) or an Oxford Nanopore MinION device. The MinION library construction and sequencing were performed by FISABIO University of Valencia's sequencing service, using the Oxford Nanopore PCR barcoding kit following the manufacturer's instructions. Sequence data have been deposited with links to BioProject accession number PRJNA821238 in the NCBI BioProject database.

Bioinformatics analysis. Annotation of complete genomes was achieved using Prokka v1.14.6. Virulence factors were identified using VFanalyzer, an automatic pipeline analysis to screen FASTA sequences against the VFanalyzer database for known and/or potential virulence factors in a genome. Antibiotic resistances were identified using the Comprehensive Antibiotic Resistance Database (CARD) and ResFinder 4.0 which uses BLAST to annotate resistance genes and Resistance Gene Identifier (RGI) to detect chromosomal mutations to leading to antibiotic resistance. Genes involved in metabolism were identified using BlastKOALA KEGG annotation tool which annotates genes within a FASTA sequence based on their score against the KEGG genes database. MUMmer v3.0, an open software package which allows rapid alignments of FASTA sequences and has a SNP detection pipeline to identify all SNPs in regions of sequence similarity. Mobile genetic elements were identified by pipelining the whole genome FASTA sequences through relevant databases using PHAST (prophage), ISfinder, (insertion sequences) IslandViewer (genomic islands) and SCCmec Finder 1.2 (SCCmec Type).

Acknowledgements

We thank Adelaide Microscopy (University of Adelaide, Adelaide, Australia) with their contribution in assisting with the SEM imaging. We thank Andrew Hayles with his assistance with the xCELLigence protocols.

References

1. Tong SY, Davis JS, Eichenberger E, Holland TL, Fowler VG, Jr. *Staphylococcus aureus* infections: epidemiology, pathophysiology, clinical manifestations, and management. *Clin Microbiol Rev*. 2015;**28**(3):603-61.

2. Tuchscherr L, Medina E, Hussain M, Volker W, Heitmann V, Niemann S, et al. *Staphylococcus aureus* phenotype switching: an effective bacterial strategy to escape host immune response and establish a chronic infection. *EMBO Mol Med*. 2011;**3**(3):129-41.

3. Proctor RA, Van Langevelde P, Kristjansson M, Maslow JN, Arbeit RD. Persistent and relapsing infections associated with small-colony variants of *Staphylococcus aureus*. *Clin Infect Dis*. 1995;**20**(1):95-102.

4. Proctor RA, von Eiff C, Kahl BC, Becker K, McNamara P, Herrmann M, et al. Small colony variants: A pathogenic form of bacteria that facilitates persistent and recurrent infections. *Nature Rev Microbiol*. 2006;**4**(4):295-305.

5. Edwards AM. Phenotype switching is a natural consequence of *Staphylococcus aureus* replication. *J Bacteriol*. 2012;**194**(19):5404-12.

6. von Eiff C, Peters G, Becker K. The small colony variant (SCV) concept-the role of staphylococcal SCVs in persistent infections. *Injury*. 2006;**37**(2 SUPPL.):S26-S33.

7. Garcia LG, Lemaire S, Kahl BC, Becker K, Proctor RA, Denis O, et al. Antibiotic activity against small-colony variants of *Staphylococcus aureus*: Review of in vitro, animal and clinical data. *J Antimicrob Chemother*. 2013;**68**(7):1455-64.

8. Proctor RA, Kahl B, Von Eiff C, Vaudaux PE, Lew DP, Peters G. Staphylococcal small colony variants have novel mechanisms for antibiotic resistance. *Clin Infect Dis*. 1998;**27**(SUPPL.1):S68-S74.

9. Forbes S, Latimer J, Bazaid A, McBain AJ. Altered competitive fitness, antimicrobial susceptibility, and cellular morphology in a triclosan-induced small-colony variant of *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy*. 2015;**59**(8):4809-16.

10. Kittinger C, Toplitsch D, Folli B, Landgraf LM, Zarfel G. Phenotypic stability of *Staphylococcus aureus* small colony variants (SCV) isolates from cystic fibrosis (CF) patients. *International Journal of Environmental Research and Public Health*. 2019;**16**(11).

11. Von Eiff C, Heilmann C, Proctor R, Woltz C, Peters G, Götz F. A Site-Directed *Staphylococcus aureus hemB* Mutant Is a Small Colony Variant which persists intracellularly. *J Bacteriol*. 1997;**179**(15):4706-12.

12. Balwit JM, van Langevelde P, Vann JM, Proctor R. Gentamicin-resistant menadione and hemin auxotrophic *Staphylococcus aureus* persist within cultured endothelial cells. *J Infect Dis*. 1994;**170**(4):1033-7.

13. Schaaff F, Bierbaum G, Baumert N, Bartmann P, Sahl HG. Mutations are involved in emergence of aminoglycoside-induced small colony variants of *Staphylococcus aureus*. *Int J Med Microbiol*. 2003;**293**(6):427-35.

14. Lannergård J, Von Eiff C, Sander G, Cordes T, Seggewiß J, Peters G, et al. Identification of the genetic basis for clinical menadione-auxotrophic small-colony variant isolates of *Staphylococcus aureus*. *Antimicrob Agents Ch*. 2008;**52**(11):4017-22.

15. Pader V, James EH, Painter KL, Wigneshweraraj S, Edwards AM. The agr quorum-sensing system regulates fibronectin binding but not hemolysis in the absence of a functional electron transport chain. *Inf Immune*. 2014;**82**(10):4337-47.

16. Dean MA, Olsen RJ, Wesley Long S, Rosato AE, Musser JM. Identification of point mutations in clinical *Staphylococcus aureus* strains that produce small-colony variants auxotrophic for menadione. *Infect Immun*. 2014;**82**(4):1600-5.

17. Kittinger C, Toplitsch D, Folli B, Masoud Landgraf L, Zarfel G. Phenotypic Stability of *Staphylococcus aureus* Small Colony Variants (SCV) Isolates from Cystic Fibrosis (CF) Patients. *Int J Environ Res Public Health*. 2019;**16**(11):1940.

18. Chatterjee I, Kriegeskorte A, Fischer A, Deiwick S, Theimann N, Proctor RA, et al. In vivo mutations of thymidylate synthase (Encoded by *thyA*) are responsible for thymidine dependency in clinical small-colony variants of *Staphylococcus aureus*. *J Bacteriol*. 2008;**190**(3):834-42.

19. Besier S, Ludwig A, Ohlsen K, Brade V, Wichelhaus TA. Molecular analysis of the thymidine-auxotrophic small colony variant phenotype of *Staphylococcus aureus*. *International Journal of Medical Microbiology*. 2007;**297**(4):217-25.

20. Gómez-González C, Acosta J, Villa J, Barrado L, Sanz F, Orellana MÁ, et al. Clinical and molecular characteristics of infections with CO_2-dependent small-colony variants of *Staphylococcus aureus*. *J Clin Microbiol*. 2010;**48**(8):2878-84.

21. Thomas ME. Studies on a CO_2-dependent staphylococcus. *J Clin Pathol*. 1955;**8**(4):288-91.

22. Bazaid AS, Forbes S, Humphreys GJ, Ledder RG, O'Cualain R, McBain AJ. Fatty Acid Supplementation Reverses the Small Colony Variant Phenotype in Triclosan-Adapted *Staphylococcus aureus*: Genetic, Proteomic and Phenotypic Analyses. *Scientific Reports*. 2018;**8**(1).

23. Schleimer N, Kaspar U, Drescher M, Seggewiß J, von Eiff C, Proctor RA, et al. The energy-coupling factor transporter module EcfAA'T, a novel candidate for the genetic basis of fatty acid-auxotrophic small-colony variants of *Staphylococcus aureus*. *Front Microbiol*. 2018;**9**(AUG).

24. Zhang P, Wright JA, Osman AA, Nair SP. An aroD Ochre mutation results in a *Staphylococcus aureus* small colony variant that can undergo phenotypic switching via two alternative mechanisms. *Front Microbiol*. 2017;**8**(MAY).

25. Tuchscherr L, Löffler B. *Staphylococcus aureus* dynamically adapts global regulators and virulence factor expression in the course from acute to chronic infection. *Current Genetics*. 2016;**62**(1):15-7.

26. Grimbergen AJ, Siebring J, Solopova A, Kuipers OP. Microbial bet-hedging: the power of being different. *Curr Opin Microbiol*. 2015;**25**:67-72.

27. Bui LMG, Hoffmann P, Turnidge JD, Zilm PS, Kidd SP. Prolonged growth of a clinical *Staphylococcus aureus* strain selects for a stable small-colony-variant cell type. *Infect Immun*. 2015;**83**(2):470-81.

28. Bui LMG, Kidd SP. A full genomic characterization of the development of a stable Small Colony Variant cell-type by a clinical *Staphylococcus aureus* strain. *Infect Genet Evol*. 2015;**36**:345-55.

29. Luong TT, Dunman PM, Murphy E, Projan SJ, Lee CY. Transcription Profiling of the *mgrA* Regulon in *Staphylococcus aureus*. *J Bacteriol*. 2006;**188**(5):1899-910.

30. Ingavale SS, van Wamel W, Luong TT, Lee CY, Cheung AL. Rat/MgrA, a regulator of autolysis, is a regulator of virulence genes in *Staphylococcus aureus*. *Inf Immune*. 2005;**73**(3):1423-31.

31. Crosby HA, Schlievert PM, Merriman JA, King JM, Salgado-Pabon W, Horswill AR. The *Staphylococcus aureus* Global Regulator MgrA Modulates Clumping and Virulence by Controlling Surface Protein Expression. *PLoS Pathog*. 2016;**12**(5):e1005604.

32. Jonsson IM, Lindholm C, Luong TT, Lee CY, Tarkowski A. *mgrA* regulates staphylococcal virulence important for induction and progression of septic arthritis and sepsis. *Microbes Infect*. 2008;**10**(12-13):1229-35.

33. Lei MG, Gudeta DD, Luong TT, Lee CY. MgrA Negatively Impacts *Staphylococcus aureus* Invasion by Regulating Capsule and FnbA. *Infect Immun*. 2019;**87**(12).

34. Kwiecinski JM, Crosby HA, Valotteau C, Hippensteel JA, Nayak MK, Chauhan AK, et al. *Staphylococcus aureus* adhesion in endovascular infections is controlled by the arlRS–MgrA signaling cascade. *PLoS Pathogens*. 2019;**15**(5).

35. Trotonda MP, Tamber S, Memmi G, Cheung AL. MgrA represses biofilm formation in *Staphylococcus aureus*. *Inf Immune*. 2008;**76**(12):5645-54.

36. Lee J, Zilm PS, Kidd SP. Novel Research Models for *Staphylococcus aureus* Small Colony Variants (SCV) Development: Co-pathogenesis and Growth Rate. *Front Microbiol*. 2020;**11**(321).

37. Alekshun MN, Kim YS, Levy SB. Mutational analysis of MarR, the negative regulator of marRAB expression in *Escherichia coli*, suggests the presence of two regions required for DNA binding. *Mol Microbiol*. 2000;**35**(6):1394-404.

38. Chen PR, Bae T, Williams WA, Duguid EM, Rice PA, Schneewind O, et al. An oxidation-sensing mechanism is used by the global regulator MgrA in *Staphylococcus aureus*. *Nature Chemical Biology*. 2006;**2**(11):591-5.

39. Vestergaard M, Paulander W, Ingmer H. Activation of the SOS response increases the frequency of small colony variants. *BMC Res Notes*. 2015;**8**(1).

40. Bowman L, Zeden MS, Schuster CF, Kaever V, Gründling A. New Insights into the Cyclic Di-adenosine Monophosphate (c-di-AMP) Degradation Pathway and the Requirement of the Cyclic Dinucleotide for Acid Stress Resistance in *Staphylococcus aureus**. *Journal of Biological Chemistry*. 2016;**291**(53):26970-86.

41. Corrigan RM, Bellows LE, Wood A, Gründling A. ppGpp negatively impacts ribosome assembly affecting growth and antimicrobial tolerance in Gram-positive bacteria. *Proc Natl Acad Sci U S A*. 2016;**113**(12):E1710-9.

42. Dengler V, McCallum N, Kiefer P, Christen P, Patrignani A, Vorholt JA, et al. Mutation in the C-Di-AMP Cyclase dacA Affects Fitness and Resistance of Methicillin Resistant *Staphylococcus aureus*. *PLOS ONE*. 2013;**8**(8):e73512.

43. Tosi T, Hoshiga F, Millership C, Singh R, Eldrid C, Patin D, et al. Inhibition of the *Staphylococcus aureus* c-di-AMP cyclase DacA by direct interaction with the phosphoglucosamine mutase GlmM. *PLoS Pathogens*. 2019;**15**(1).

44. Zeden MS, Schuster CF, Bowman L, Zhong Q, Williams HD, Gründling A. Cyclic di-adenosine monophosphate (c-di-AMP) is required for osmotic regulation in *Staphylococcus aureus* but dispensable for viability in anaerobic conditions. *J Biol Chem*. 2018;**293**(9):3180-200.

45. Glanzmann P, Gustafson J, Komatsuzawa H, Ohta K, Berger-Bächi B. glmM operon and methicillin-resistant glmM suppressor mutants in *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy*. 1999;**43**(2):240-5.

46. Alibayov B, Baba-Moussa L, Sina H, Zdeňková K, Demnerová K. *Staphylococcus aureus* mobile genetic elements. *Molecular biology reports*. 2014;**41**(8):5005-18.

47. Hussain M, Hastings JGM, White PJ. A chemically defined medium for slime production by coagulase-negative staphylococci. *Journal of Medical Microbiology*. 1991;**34**(3):143-7.

48. Abrantes P, Africa CWJ. Measuring *Streptococcus mutans*, *Streptococcus sanguinis* and *Candida albicans* biofilm formation using a real-time impedance-based system. *J Microbiol Methods*. 2020;**169**:105815.

49. Ferrer MD, Rodriguez JC, Álvarez L, Artacho A, Royo G, Mira A. Effect of antibiotics on biofilm inhibition and induction measured by real-time cell analysis. *Journal of Applied Microbiology*. 2017;**122**(3):640-50.

Supplementary Material

Supplementary Table S1. Components of chemically defined media (CDM) for limiting *S. aureus* growth based on previous experiments for growing staphylococci slime production

Ingredients	**Concentration (mg/L)**	**Concentration (mmol/L)**
Group 1 (18 Amino acids)		
L- Alanine	100	1.12
L- Arginine	100	0.57
L- Aspartic acid	150	1.13
L- Cystine	50	0.21
L- Glutamic acid	150	1.02
L- Glycine	100	1.33
L- Histidine	100	0.65
L- Isoleucine	150	1.14
L- Leucine	150	1.14
L- Lysine	100	0.68
L- Methionine	100	0.67
L- Phenylalanine	100	0.6
L- Proline	150	1.3
L- Serine	100	0.95
L- Threonine	150	1.26
L- Tryptophan	100	0.49
L- Tyrosine	100	0.55
L- Valine	150	1.28
Group 2 (Glucose)		
Glucose	2500	13.9
Group 3 (Salts+minerals)		
MgSO4	500	4.15
Na2HPO4	10000	70.44
KH2PO4	3000	22.04
CaCl2	10	0.09
MnCl2	5	0.04
(NH4)2SO4	6	0.05
Group 4 (Vitamins)		
Biotin	1	0.004
Nicotinic acid	2	0.016
D- Pantothenic acid, Ca salt	2	0.004
Pyridoxal	4	0.016
Riboflavin	2	0.005
Thiamin	2	0.007

(1) $$\mu_{max} = 2.303\left(\frac{log_{10}N_2 - log_{10}N_1}{t_1 - t_0}\right)$$

Supplementary Equation S1. Monods equation: the mathematical model which relates the growth rate of bacteria to the concentration of a limiting nutrient

μ_{max} = maximum growth rate (h^{-1}), *N* = number of CFU at timepoint, *t* = timepoint (h)

(2) $$T_g = \frac{ln2}{D}$$

$$D = \frac{F}{V}$$

Supplementary Equation S2. Derivations of equations for determining the parameters for continuous derivations of flowrate calculations. At steady state growth, the dilution rate is equal to the growth rate. The generation time and flowrate can be determined at a specific growth rate.

T_g = generation time (h^{-1}), μ_{max} = maximum growth rate (h^{-1}), D = dilution rate (h^{-1}), F = flow rate ($mL.h^{-1}$), V = volume of vessel (mL),

Chapter 4: The mechanisms that underly alternate cell types in *Staphylococcus aureus* through long-term culture models

Changes in *S. aureus* lifestyles is a mechanism to form sub-populations with alternate adaptations to stress. The presence of various sub-populations means at least a proportion of the population can survive otherwise lethal stress and persistent population would then be able to grow and cause infection again. These pathways of adaption to stress are the basis of persistent and relapsing infection. *S. aureus* is a common pathogen involved in osteomyelitis where *S. aureus* can interact with the bone during infection which can result in either cell death or persistence. One of these outcomes is internalisation and persistence within the bone cell. The expression of immunogenic virulence factors can result in lysis of the cell or induction of apoptosis. The switch to a small colony variant (SCV) can mediate the downregulation of virulence factors that allow persistence within the bone. Within the bone, *S. aureus* is protected from the immune response and antibiotic exposure which creates a dormant and highly resistant reservoir of *S. aureus* during infection.

The prevalence of antibiotic tolerances further complicate treatment of persistent *S. aureus* infections. Unlike antibiotic resistance, which is a genetically conferred mechanism that allow growth in the presence of antibiotics, antibiotic tolerance is the lack of cell death in antibiotics. This is generally a result of metabolic dormancy that leads to downregulation of antibiotic targets such as cell wall formation and protein synthesis. These dormant, alternative cell types are associated with reduced expression of virulence factors and do not cause signs of infection. Consequently, an infection can seemingly be cleared after treatment, but a reservoir of tolerant *S. aureus* is still present. Identifying the mechanisms on how tolerance evolves is crucial for more complete treatment of persistent infections.

This manuscript describes the experiments that utilise long-term culture methods to identify the time dependency for the adaptation to clinically relevant stressors. Adaptive laboratory evolution (ALE) was used to induce the evolution of antibiotic tolerance in the clinical *S. aureus* isolate UA-DI-55. ALE allows long-term growth of *S. aureus* in parallel, serial cultures and aliquots of the culture are transferred to fresh medium every day. By repeated sub-cultures in antibiotic stress over a prolonged duration, we can select for different cell types with greater antibiotic resistance and tolerance. To determine the adaptations that facilitate persistence within osteocytes, the human bone cell line SaOS-2 was infected with the clinical *S. aureus* isolate WCH-SK3. Intracellular bacteria were extracted over time to find *S. aureus* persisted within the bone as a viable, but non-culturable cell type. *S. aureus* reverted to a culturable cell type in one set of SaOS-2 osteocytes which was representative of an adaptation leading to a relapsing infection in a clinical context.

Whole genome sequencing was used to characterise the genotypes that underly these alternate cell types observed in these experiments to determine a pathway in which alternative cell types and tolerance are formed.

Statement of Authorship

Title of Paper	The mechanisms that underly alternate cell types in *Staphylococcus aureus* through long-term culture models
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ✓ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Prepared for submission to the *Journal of Bacteriology*

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Name of Principal Author (Candidate)	James Lee		
Contribution to the Paper	Carried out experiments characterising bacterial isolates, SEM imaging, data analysis, ALE experiments and drafted the manuscript.		
Overall percentage (%)	75		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	8/4/22

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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The mechanisms that underly alternate cell types in *Staphylococcus aureus* through long-term culture models

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Abstract

Staphylococcus aureus has an incredible ability to survive stresses from the either host cellular or immune response or clinical treatments. Genetically encoded antibiotic resistances are highly prevalent in *S. aureus* and their mechanism of action and spread through horizontal gene transfer are well characterised. Antibiotic tolerance results from bacterial switch to a limited or no growth state (or "lifestyle") and thus avoiding of cell death in the presence antibiotics. This is known to permit *S. aureus* to survive while being assaulted by otherwise lethal stress from either the host or clinical treatment, by switching "lifestyles" over prolonged periods of time. We used two models of long-term infection mirroring these two sources of stressors to investigate the time-dependent adaptions to stressful conditions: Adaptive Laboratory Evolution (ALE) with antibiotics and persistent intracellular infection of the bone cell type (osteocyte) SaOS-2 cell line.

ALE was used to determine how a *S. aureus* isolate from a bone infection *S. aureus* UA-DI-55 adapted to limited nutrients with ciprofloxacin stress over multiple generations. We found over time UA-DI-55 developed an increased resistance to ciprofloxacin as well as tolerance to other classes of antibiotics. The growth kinetics of UA-DI-55 was significantly reduced which suggests antibiotic tolerance was conferred through a switch to a dormant cell population. Unusually, whole genome sequencing revealed single nucleotide polymorphisms (SNPs) in *fur* and *ptsH* in these cells, indicating; defects in iron acquisition and carbohydrate uptake.

The SaOS-2 cells were infected with the *S. aureus* isolate WCH-SK3. After 7 days the SaOS-2 cells had no viable *S. aureus* but comprised of intracellular viable but non-culturable cells until after 24 days of infection when *S. aureus* were again culturable. Whole genome sequencing on intracellular, culturable *S. aureus* from day 1 and day 24 from SaOS-2 cells revealed the progression from non-culturable to a culturable cell type was associated with an

SNP in *srrB* and major genetic rearrangements resulting in the loss of genes involved in virulence and the SOS response.

Importance

Clearance of *Staphylococcus aureus* infections are difficult due to their ability to adapt to stresses. This can lead to highly debilitating and persistent infections that relapse even after the infection has initially been seemingly cleared. We have identified a subtle pathway for *S. aureus* evolving over multiple generations of growth to adapt to ciprofloxacin stress. Also, during infection, *S. aureus* is known to sit intracellularly. We examine a long-term, intracellular adaptation and reveal novel genetic switches that enable *S. aureus* to be viable but non-culturable and then return to an active lifestyle. Understanding the mechanisms underpinning *S. aureus* adaptation to stress over time is important to improve diagnosis and to treat persistent and relapsing *S. aureus* infection.

Keywords: *Staphylococcus aureus*, adaptive laboratory evolution, antibiotic tolerance, antibiotic resistance, ciprofloxacin

Introduction

The high prevalence of antibiotic resistance in *Staphylococcus aureus* is a major contributor in the difficulty to treat persistent *S. aureus* infections (in particular are infections notoriously chronic, such as osteomyelitis, lung infection in cystic fibrosis patients, endocarditis, and wound infections) . The transfer of resistance genes through horizontal gene transfer facilitates the rapid spread of genetically-encoded antibiotic resistance and indeed the increased prevalence of methicillin resistant *S. aureus* (MRSA) and hospital-acquired strains (HA-MRSA and vancomycin resistance, VRSA). However, in various diseases such as diabetic foot infections (DFI) and its progression to osteomyelitis [1], the infection can

relapse despite the treatment initially seemingly being successful in clearing the infection. This is now understood to be in part due to sub-populations of the original *S. aureus* population which confers antibiotic tolerance.

Antibiotic tolerance is the ability for a small percentage of the cell populations of bacteria to survive lethal exposure to antibiotics without a change in their minimum inhibitory concentration (MIC) to that antibiotic. These do not necessarily arise from genetic events (the acquisition of gene cassettes), but rather through phenotypic changes in "lifestyle" (which involve pathways to cell growth, surface composition and management of intracellular conditions, such as pH and redox) or cell type that creates a dormant or quasi-dormant sub-populations [2]. For instance, metabolically dormant cells tolerate antibiotics through slowing down processes which are targeted by antibiotics and so require a longer minimum treatment duration to be killed [3]. In contrast to antibiotic resistance, tolerance is associated with changes in the minimum bactericidal concentration (MBC) rather than the MIC. In clinical settings of infection, these sub-populations can survive otherwise lethal treatments and create reservoirs of bacteria that can revert to the virulent cell type to cause infection again [2, 4].

The switch to Small Colony Variants (SCV) is an important change in *S. aureus* cell type that confers antibiotic tolerance and thus permits persistent infection. SCV are characterised as slow growing cells, quasi-dormant cells with a non-pathogenic virulence profile [5]. They have down regulation of many surface structures and proteins known to be toxins, all known as immune mediators. This results in evasion of the immune response at the same time as having tolerance to antimicrobials that would otherwise clear actively growing populations [6]. SCVs form stochastically during growth of *S. aureus* which provides sub-populations that can survive and be selected for in conditions of stress [6]. This can create reservoirs of *S.*

aureus without clinical symptoms. While the infection has seemingly been cleared, due to the instability of the SCV cell type that can revert to pathogenic types, the infection after some time can return to a clinical state [7].

These characteristics complicate treatment of osteomyelitis where *S. aureus* is one of the most commonly isolated pathogens from DFI [8-10]. *S. aureus* cell populations are dominated by metabolic active, virulent cell types which cause death of osteoblasts (and other bone cell types, such as osteocytes) `and activation of bone resorption that result in loss of bone stock [11]. A major complication is the relapse of infection which occurs in up to 10% of osteomyelitis cases [12, 13].

The instability of the SCV phenotype hampers research on the mechanisms of SCV formation and physiology. Research of SCV is largely based on genetically modified strains with defects in the electron transport chain through mutations in *hemB* [14-16], *menD* [16-19] and *thyA* [20-22] that create genetically stable SCV (sSCV). Other models include auxotrophism to CO_2 [23, 24], fatty acids [25, 26], chorismite synthesis [27] and selection in gentamicin [15]. These experiments are based on laboratory generated SCVs that focus on the effect of a single gene that confers the phenotype. There is no common gene involved in all SCVs and indeed there are SCVs in which the pathway to the cell type is unknown [16]. Alternative experiments have been utilized to select for SCV which takes in account time-dependent infection and environmental stresses that better represent the formation of SCV in response to clinical infection. This includes long-term growth in limited growth conditions in continuous culture [28, 29], long-term infection and intracellular persistence within osteocytes [30] and serial passage within mice [31]. Our research utilizes multiple methods of long-term growth to identify the mechanisms of evolution that allow *S. aureus* to adapt to stressful

environments. Adaptive laboratory evolution (ALE) is the process of culturing bacteria over many generations [32] which has been previously used to characterise the long-term evolution of resistances in *Escherichia coli* to various antibiotics [33]. ALE can be used to characterise how a *S. aureus* evolves over many generations of growth to adapt to antibiotic stress and the development of resistance and tolerance through changes in cell types. We have used this methodology to determine how a clinical isolate of *S. aureus* adapts to long-term ciprofloxacin exposure.

In addition, we have used the osteocyte cell line SaOS-2 as an *ex vivo* model of infection to simulate intracellular bone infection [34]. The outcome of internalisation is either activation of the immune response or long-term persistence. *S. aureus* virulence factors activate the chemokine response and cause inflammation and bone loss [11, 35]. However, the switch to a SCV can down-regulate expression of Agr controlled virulence factors [36] which can hide the internalized *S. aureus* from the immune response and facilitate long-term persistence [30, 35, 37, 38]. Indeed, the intracellular environment favours cells with decreased virulence, cytotoxicity and impeded metabolism [39, 40]. The use of laboratory generated stable SCV (sSCV) have been shown to be well adapted to persist within the intracellular environment [41]. We aimed to determine the pathways that allow persistence of *S. aureus* within an *ex vivo* model of bone infection.

Results

UA-DI-55: a *S. aureus* isolated from osteomyelitis. The *S. aureus* isolate UA-DI-55 was isolated from a DFI patient with osteomyelitis undergoing amputation to treat the infected tissue. The patient was treated with intravenous amoxicillin during their hospital stay. From this patient, a shaving of uninfected bone distal from the site of infection was taken and the

intracellular bacteria was extracted from which UA-DI-55 was taken. UA-DI-55 formed a non-stable SCV which had reverted to form a large, non-pigmented colony. The parental UA-DI-55 strain conferred strong ciprofloxacin resistance with a MIC of 250 µg/mL and MBC of 1000 µg/mL **(Table 1)**. Additionally, UA-DI-55 also presented with resistance to penicillin (MIC of 250 mg/mL) and gentamicin (MIC of 62 mg/mL).

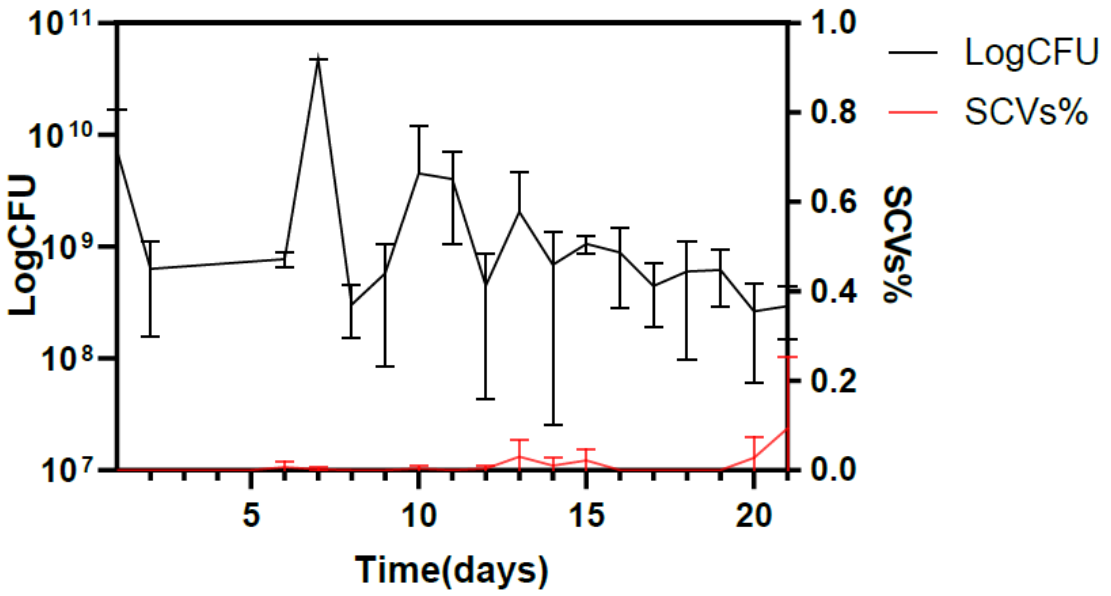

FIG. 1. UA-DI-55 in adaptive laboratory evolution. The *S. aureus* isolate UA-DI-55 was cultured in CDM at the minimum inhibitory concentration (MIC) of ciprofloxacin and sub-cultured to fresh media daily to identify the evolution of alternative cell types that are more fit to survive in the ALE conditions. Each day CFU/mL (left axis, black) and the proportion of SCV (right axis, red) were recorded.

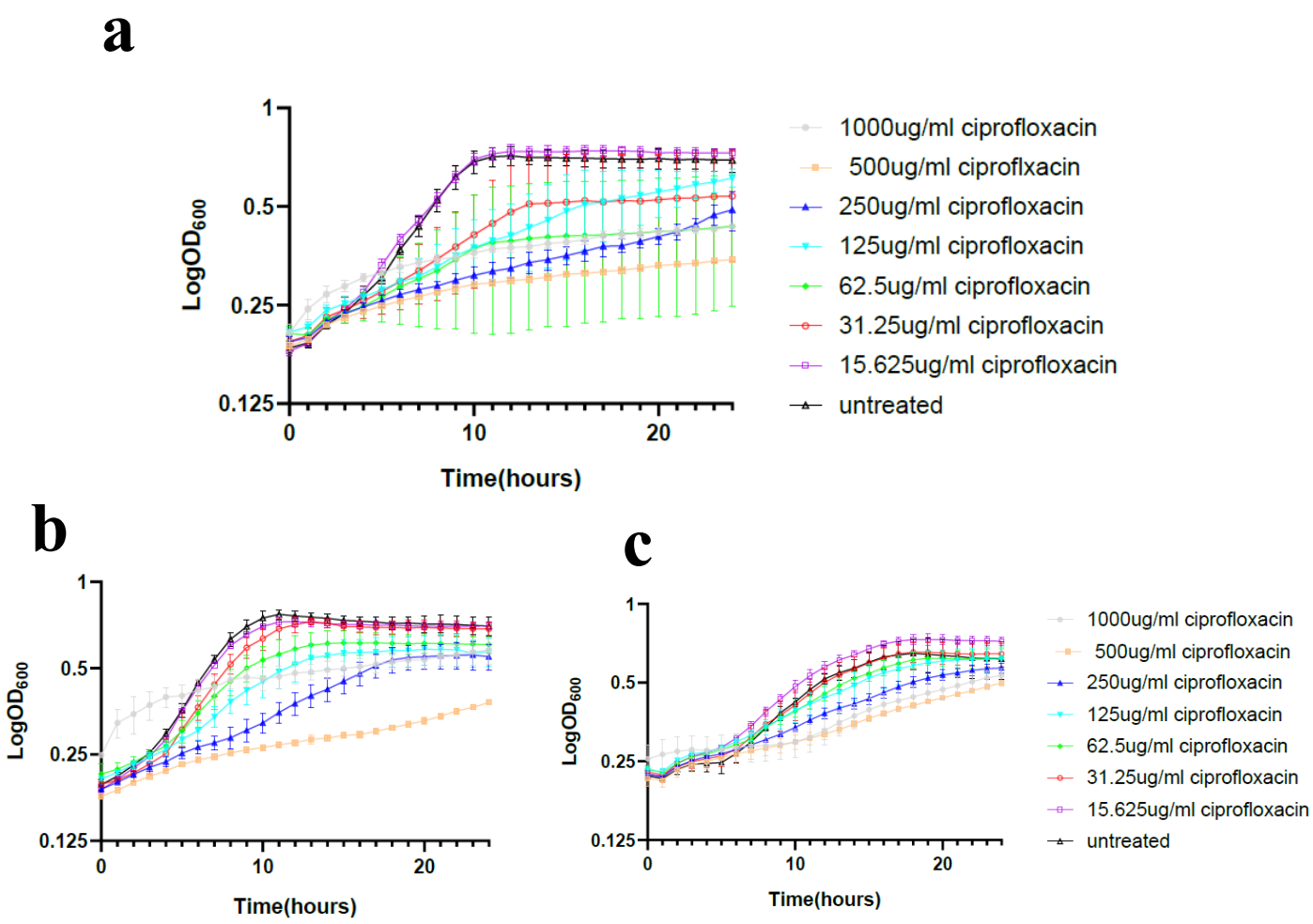

FIG. 2. Growth kinetics of UA-DI-55 in ciprofloxacin. Growth was measured by OD_{600} recorded every 0.5 hours. UA-DI-55 (a), UA-DI-55 post 7 days ALE (b) and UA-DI-55 post 20 days ALE (c) was cultured in CDM and the addition of ciprofloxacin serially diluted by a factor of 2.

ALE with UA-DI-55 selects for cell types more fit to survive in ciprofloxacin.

Approximately $5x10^8$ cells were inoculated into CDM and after the first 24 hours of growth the culture had approximately $1x10^{10}$ CFU/mL. The CFU/mL had significant fluctuation in the first 10 days of ALE but began to converge to a constant, steady state like concentration. After 10 days there was a steady decrease in CFU/mL for the remainder of the culture **(Fig. 1)**. The CFU/mL decreased over the first 7 days (7d) of culturing after which the CFU/mL began to increase. The population switched from the large, non-pigmented forming cell types of the parental UA-DI-55 to cells producing non-pigmented colonies with variations in the colony size. SCVs were observed sporadically during ALE with less than 5% of the population forming SCV. After 19 days of ALE, the proportion of SCV in the population began to increase before the experiment was concluded **(Fig. 1)**.

SEM imaging revealed the parental UA-DI-55 isolate had a population of clumped cells and cells within an extracellular matrix. After 20 days of ALE the cells were mostly comprised of cells embedded within an extracellular matrix **(Fig. 2)**.

Table 1. The MIC and MBC of ciprofloxacin in UA-DI-55 in the parental cell type and after 7 days and 20 days of culturing in chemically defined media at the minimum inhibitory concentration of ciprofloxacin in adaptive laboratory evolution. MICS and MBCs at concentrations greater than 1000μg/mL were unable to be determined.

	MIC (μg/mL)	**MBC (μg/mL)**
UA-DI-55	250	1000
UA-DI-55 d7	>1000	>1000
UA-DI-55 d20	>1000	>1000

Adaptation to long-term ciprofloxacin and nutritional stress selects for cells with increased resistance and tolerance. Samples from two timepoints were chosen for further analysis; day 7 where the CFU/mL began to converge towards steady state growth and day 20 at the conclusion of ALE. After 7 days of the ALE the MIC and MBC to ciprofloxacin increased to at least 1000μg/mL; the MIC and MBC of ciprofloxacin were at concentrations above 1000μg/mL which could not be evaluated **(Table 1)**.

Differences in tolerance to ciprofloxacin were further identified through the differences in growth kinetics of the different strains. UA-DI-55 at 7 days and 20 days of ALE had MICs to ciprofloxacin of at least 1000μg/mL however the dynamics of the growth phases differed. In the absence of ciprofloxacin, similar growth rates were observed in the parental UA-DI-55 and UA-DI-55 post 7 days ALE **(Fig. 2)**. In contrast, UA-DI-55 post 20 days ALE had an extended lag phase and exponential phase of growth. At the same concentrations of ciprofloxacin, we observed a decreased growth rate in UA-DI-55-d7, where the increase of OD_{600} during the exponential phases of growth was greater in UA-DI-55 at 7 days compared to UA-DI-55 at 20 days **(Fig. 2)**. In all strains, the rate of growth decreased in proportion to the concentration of ciprofloxacin present. Scanning electron microscopy (SEM) revealed these phenotypic changes were also associated with cells embedded within an extracellular matrix **(Fig. 3)**.

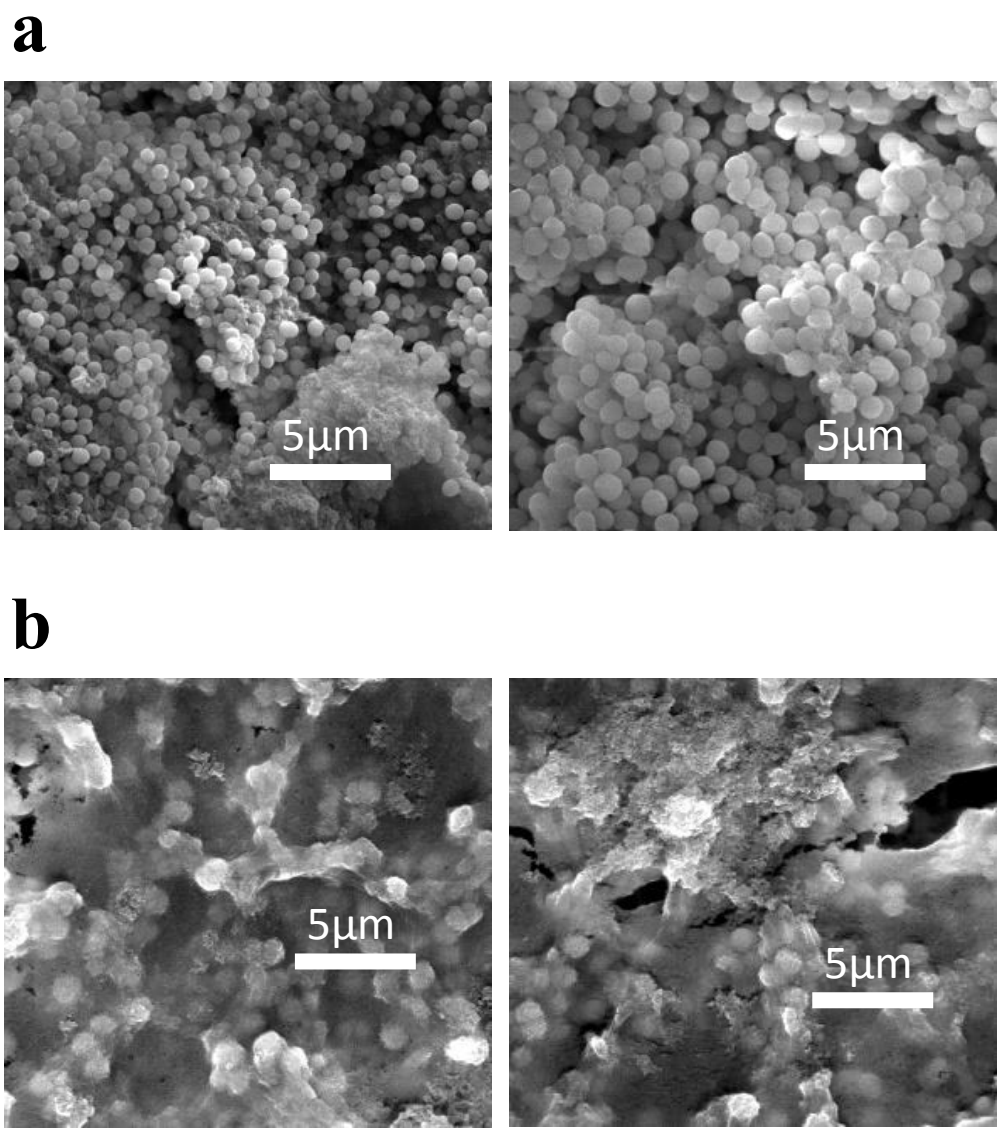

FIG. 3. Scanning electron microscopy (SEM) imaging of **(a)** UA-DI-55 and **(b)** UA-DI-55 post 20 days of subcultures in chemically defined media (CDM) and minimum inhibitory concentration of ciprofloxacin in adaptive laboratory evolution (ALE). Imaging was taken at x10 000 magnification

Table 2. SNPs detected through alignment between the parental *S. aureus* strain UA-DI-55 and UA-DI-55 post 20 days of sub-culturing in CDM at the MIC of ciprofloxacin during ALE.

Gene	Nucleotide change	Amino acid change	Product
fur	20G>C	Arg7Pro	Ferric uptake regulation protein
Hypothetical	475A>G	Lys159Glu	
ptsH	236_238dupACG	Asp79dup	Phosphocarrier protein
Hypothetical	548A>T	Glu183Val	

Changes in cell types were associated with iron acquisition and carbon metabolism.

Given the unique nature of the phenotypic antibiotic responses, we investigated the underlying genetic changes. A SNP was detected in in *ptsH*, which encodes for a non-specific component of the phosphoenolpyruvate-dependent sugar phosphotransferase system (PTS). This resulted in a duplication mutation which added an additional asparagine to the protein in UA-DI-55 d20 (**Table 2)**. PtsH is involved in the uptake and phosphorylation of carbohydrates and the conversion of phosphoenolpyruvate to pyruvate as a by-product in a reversible five step process [42].

Furthermore, a SNP was detected in *fur*, a global regulator which senses iron-limited environments. This SNP resulted in a missense mutation (presumptively at amino acid 7) from the non-polar amino acid proline to a positively charged arginine which is within the DNA binding domain (amino acids 1-85) in UA-DI-55 d20 (**Table 4)**. Upon binding to Fe^{2+} (iron available in the intracellular environment), Fur is active (Fur-Fe) and binds to its DNA

operator targets (Fur boxes) to activate/repress the required genes. While the lack of Fe^{2+} (iron limited environments) results in unbound Fur (apo-Fur) and no binding to its operator sites. Fur-Fe represses genes involved in iron acquisition, biofilm formation and antioxidative stress proteins [43].

Long-term infection of SaOS osteocytes was associated with viable but not culturable cells.

After seeding of SaOS cells with the strain WCH-SK3, cells were lysed after 7, 16 and 24 days (d) of infection to isolate the presence of intracellular bacteria. No colonies were observed from d7 to d16 of infection, however Droplet Digital PCR (ddPCR) continued to identify *S. aureus* DNA from the cell lysate which suggests the presence of viable but non-culturable (VBNC) cells. After d24 of infection, culturable bacterial cells were again able to be isolated from the cell lysates (from two wells). The remaining wells of infected SaOS cells were lysed after d28, d38 , d43 and d50 post infection and these cell lysates all contained VBNC cells.

Whole genome sequencing revealed genetic events associated with culturable cells

A single colony from the parental *S. aureus* isolate WCH-SK3 and from the cell population isolated post d24 infection was selected for whole genome sequencing (WGS) to identify the genetic events associated with the progression from a VBNC state intracellularly to return to a viable cell state.

Alignment of the WGS between the parental cell type WCH-SK3 (d0 and WCH-SK3 post d 24 infection identified a single SNP in *srrB* comprised of a T to A base substitution resulting in a missense mutation (Ile161Lys) **(Table 3)**. This mutation was located within the extracellular domain and not located within the histidine kinase or HAMP domains.

Alignment also revealed sections of genome missing between WCH-SK3 and WCH-SK3 post 24d infection. The list of coding genes missing within these sequences are listed in **Table 4**.

Discussion

ALE experiments

Studies in *E. coli* found a knockout of *fur* and the subsequent increase in intracellular iron facilitated the evolution of ciprofloxacin resistance within an *in vitro* setting [44]. Exposure to ciprofloxacin induces physiological changes resulting in a hyperactivated electron transport chain which produces superoxides (O_2^-). Reduced function of superoxide dismutases increases intracellular O_2^- levels that result in increased intracellular ferrous iron and hydroxyl radicals that increase the rate of oxidative damaged induced mutagenesis. This increased rate of mutagenesis can promote the rate antibiotic resistances evolve in response to ciprofloxacin [44].

Table 3. SNPs detected through alignment between the parental *S. aureus* strain WCH-SK3 d0 and WCH-SK3 d24 which was selected for after 24 days of intracellular persistence within the human osteocyte cell line SaOS-2.

Gene	Nucleotide change	Amino acid change	Product
srrB	482T>A	Ile161Lys	Sensor component of the SrrAB TCS virulence regulator

PTS-dependent uptake systems are utilised (but not essential) for growth in glucose by *S. aureus.* The loss of PTS uptake of glucose has no significant effects on growth in rich media in either aerobic or anaerobic conditions. Conversely, in conditions of anaerobic growth the loss of PTS uptake significantly reduced growth [45]. This suggests that PTS uptake is a major pathway for the acquisition of carbohydrates in nutrient limited conditions as observed in clinical niches of infection. Indeed, the loss of glucose uptake in a mice model of infection was associated with reduced bacterial burden as *S. aureus* must uptake host carbohydrates to accommodate the increased glycolytic flux [45]. Our data revealed the evolution of UA-DI-55 in adaption to limited media and ciprofloxacin was associated with a reduction in growth in response to increased ciprofloxacin concentrations. Ciprofloxacin is a fluoroquinolone mechanism of action targets the DNA gyrase to inhibit cell division [46]. PtsH, or PTS transport of carbohydrates in general, has no direct role in resistance to ciprofloxacin. However, the mutation in *ptsH* may still contributed to survival against ciprofloxacin by conferring antibiotic tolerance via reduced growth kinetics. The limited nutrients available in the CDM mean UA-DI-55 may rely on PTS uptake of glucose as a source of carbohydrates and thus growth and so the mutation of *pstH* may give rise to slower growing cell types. The reduction of growth has been established as a basis of antibiotic tolerance in *S. aureus* [2]. Slower growth can potentially provide tolerance to ciprofloxacin which specifically targets growing cell populations.

Antibiotic tolerance is an important aspect of bacterial survival that contributes to persistent infection. The association of tolerance and non-virulent, dormant sub-populations creates reservoirs of bacteria that are difficult to diagnose whether the infection has truly cleared. Understanding the mechanisms of antibiotic tolerance and the switch to dormant cell types are vital to identify methods to clear long-term, persistent infection. Here, we show a

potential mechanism of antibiotic resistance and tolerance through a cell type with defects in iron acquisition and carbohydrate metabolism.

WCH-SK3 osteocyte infection

There have been a number of studies that describe genomic rearrangements as the factor in the switch of a bacterial cell to a dormant lifestyle (persister, VBNC or SCV) [47-49]. Significantly, in one specific study that created *S. aureus* SCV cells (using *hemB* and *menD* mutants) suppressor mutants were created that returned the SCV to a normal cell type, they continuously identified mutants in *srrB* [50]. This highlights a pathway during infection where the *S. aureus*, for instance, infects and is associated with bone, switches to a SCV (or VBNC state) via genetic rearrangements and then returns to an active growth state by mutation in *srrB*. *S. aureus* WCH-SK3 was able to facilitate intracellular persistence within the bone as a VBNC cell type. This persistent cell type was associated with a SNP in *srrB* **(Table 3)** and missing genes encoding for virulence (*agr*, *entABCDG*, *lukD*) (**Table 4)**. These changes are potentially resulting in a decreased virulence profile which can facilitate intracellular persistence. SrrAB is a two-component system involved in the down-regulation of virulence in low-oxygen conditions. The SrrA response regulator binds to promotors regions of *agr*, *spa*, *srr* and *tst* which modifies virulence through *agr*-dependent and *agr*-independent pathways. Both *in vitro* models and *in vivo* rabbit models of endocarditis show modulation of virulence through repression of RNAIII, the effector molecule of the *agr* system and the exotoxin TSST-1 [51, 52]. SrrAB regulation of virulence is dependent on the environmental oxygen present. Promoter activity is increased in hypoxic conditions such as the environment intracellular of osteocytes [53]. Indeed, SrrAB has been identified as the sole TCS required for survival and growth within osteocytes [54]. The intracellular environment of osteocytes is hypoxic and even more so during osteomyelitis that increase SrrAB activity.

The effects of SrrAB activity is known to lead to protection against oxidative and nitrosative stresses from neutrophils [54-56] and decreased expression of *tst, spa* and *icaR* [53].

S. aureus WCH-SK3 can internalize and persist intracellularly in the bone (osteocytes) but growth may not be permissible. Thus, WCH-SK3 persists in a non-growing state. The switch from a VBNC cell type to a culturable cell type is associated with the restoration of a functional SrrB and genes involved in virulence and the oxidative stress response. Culturable cells were associated with the presence of various toxins which normally need to be down-regulated prevent cell death and establish intracellular persistence [57]. However, the expression of α-toxin has been identified to mediate persistence in human monocytes by permeabilization of phagosome membranes [58]. The activity of these toxins may have functions such as nutrient acquisition that facilitate the switch to a culturable phenotype. These changes are a combination of SNPs **(Table 3)** and genetic rearrangements **(Table 4)**. Previous research had identified genetic rearrangements as a driving force for genetic changes that lead to alternate cell types including SCVs [49].

Table 4. The genomes of WCH-SK3 d0 and WCH-SK3 d24 were aligned to identify the presence/absence of genes due to genetic rearrangements. Genes present in an isolate but missing in the other are listed with their respective function.

Gene	Missing in WCH-SK3 day 0	Missing in WCH-SK3 day 24	Function
agrA	x		accessory gene regulator protein
bacC		x	dihydroanticapsin 7-dehydrogenase
ble		x	bleomycin resistance protein
btuD		x	vitamin B12 import ATP-binding protein
cadA		x	cadmium transporting ATPase
clpB		x	chaperone protein
cshA	x		DEAD-box ATP-dependent RNA helicase
dapG	x		2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-acetyltransferase
dinG	x		3'-5' exonuclease
eamA	x		putative amino acid metabolite efflux pump
entA	x		enterotoxin
entB	x		enterotoxin
entC	x		enterotoxin
entD	x		enterotoxin
entG	x		enterotoxin
epiD		x	epidermin decarboxylase
essC		x	Type VII secretion system protein
essE	x		Type VII secretion system protein
essG	x		Type VII secretion system protein
essG		x	Type VII secretion system protein
esxB	x		Type VII secretion system extracellular protein
esxC	x		Type VII secretion system extracellular protein
esxD	x		Type VII secretion system extracellular protein
fecD	x		Fe(3+) dicitrate transport system permease protein

fni	**x**		**isopentenyl-diphosphate delta-isomerase**
gdmA		x	lantibiotic gallidermin
gloB	x		hydroxyacylglutathione hydrolase
gloC		x	hydroxyacylglutathione hydrolase
glpE	x		thiosulfate sulfurtransferase
glpE		x	thiosulfate sulfurtransferase
glpQ		x	glycerophosphodiester phosphodiesterase
hin		x	DNA invertase
hly	x		alpha-hemolysin
*hly**		x	alpha-hemolysin
knt		x	kanamycin nucleotidyltransferase
lexA	x		LexA repressor
lpl	x		putative lipoprotein
lpl		x	putative lipoprotein
lukD	x		Leukocidin
lukD		x	Leukocidin
macB	x		macrolide export ATP-binding/permease protein
mcrB		x	5-methylcytosine-specific restriction enzyme B
mecI		x	methicillin resistance regulatory protein
mecR		x	methicillin resistance mecR1 protein
nanK		x	N-acetylmannosamine kinase
natA	x		ABC transporter ATP-binding protein
norG	x		HTH type transcriptional regulator
paaZ		x	bifunctional protein
pbp		x	beta-lactam inducible penicillin binding protein
rplM		x	50S ribosomal protein L13
rpsI		x	30S ribosomal protein S9
rpsI		x	30S ribosomal protein S9
sraP		x	serine rich adhesin for platelets

tarP	**x**		**poly(ribitol-phosphate) beta-N-acetylglucosaminetransferase**
ugpQ		x	cytoplasmic glycerophosphodiester phosphodiesterase
xerC	x		tyrosine recombinase
xerC		x	tyrosine recombinase
xerC		x	tyrosine recombinase
yitU	x		5-amino-6-(5-phospho-D-ribitylamino)-uracil-phosphotase
yknY	x		putative ABC transporter ATP-binding protein
ywpJ	x		phosphotase
ywpJ		x	phosphotase

Materials and Methods

Strains and culture media. Chemically defined media (CDM) composition was adapted from media used for coagulase-negative staphylococci slime production [59]. Components of CDM are listed in Supplementary Table S1.

Adaptive laboratory evolution (ALE). ALE protocols were adapted from methods previously described [33]. UA-DI-55 was cultured in 5mL CDM to log phase (OD_{600} ~0.2). 500µL of culture was inoculated into a 250mL glass conical flask of 50mL CDM with a sub-MIC concentration of ciprofloxacin (250µg/mL) and incubated at 37°C. Every 24 hours, 100µL of culture was serially diluted and plated to TSA to determine CFU/mL and colony phenotypes. 500µL of culture was aliquoted to a fresh flask of 50mL CDM with sub-MIC ciprofloxacin. The remaining culture was centrifuged at 4000rpm at 4°C and resuspended in 80% glycerol for storage at -80°C.

Growth kinetics. Growth kinetics assays were performed in 96-well microtitre plates. Cells were incubated in TSB at 37°C to log phase (OD_{600} ~ 0.2). 20µL of log phase culture was added to a well containing 180µL of media. Culture was incubated at 37°C for 18 hours and OD_{600} readings taken every 30 minutes with a Sunrise Absorbance Microplate Reader (Tecan).

Minimum bacteriostatic and bactericidal concentrations of antibiotics. Antibiotics were serially diluted into TSB by a factor of 1:2. Cells were incubated in TSB at 37°C to log phase (OD_{600} ~ 0.2). 20µL of log phase culture was added to wells containing 180µL of TSB with antibiotic. Cultures were incubated at 37°C for 18 hours and OD_{600} readings were taken. Cultures were serially diluted to determine CFU/mL. Minimum inhibitory concentration (MIC) was determined to be the lowest concentration of antibiotic which resulted in no visual bacterial growth and change in OD_{600}. Minimum bactericidal concentration (MBC) was

determined to be the lowest concentration of antibiotic which resulted in a decrease in CFU/mL.

Scanning electron microscopy. Cells were filtered through a 0.2μm Milipore filter paper and fixed with a fixative solution (4% paraformaldehyde, 1.25% glutaraldehyde, 4% sucrose in PBS). Filters were washed twice with 4% sucrose in PBS, post-fixed with 0.1% osmium tetraoxide for 60 minutes and washed twice with 4% sucrose in PBS. Cells were dehydrated with a series of two 10-minute ethanol washes, each in 70%, 90% and 100% ethanol solutions. Cells were dried in a 1:1 mix HMDS and 100% ethanol for 20 minutes and then in HMDS for 15 minutes. Samples were mounted onto titanium stubs and coated with 2mm platinum. Images were taken using a Phillips XL30 FEG scanning electron microscope (Adelaide Microscopy).

Infection of SaOS osteocytes. Inner walls of a 48-well plate with $1.5x10^4$ SaOS-2 cells per well. SaOS cells were infected at a M.O.I of 0.97 (target of 1) for 2h followed by overnight lysostaphin treatment to remove extracellular cell population. The presence of *S. aureus* within SaOS-2 cells was determined using Droplet Digital PCR according to manufacturers protocols. At various timepoints, a well was lysed and 2μL of media was drop plated to tryptic soya agar (TSA) and single colonies were inoculated into tryptic soya broth (TSB). Cultures were incubated at 37°C to mid-log phase (OD_{620nm} ~ 0.2), pelleted by centrifugation (1900 x g, 4°C, 10min), resuspended in 30% glycerol and stored at -80°C.

Whole genome sequencing. Whole genomic DNA was extracted and purified using QIAGEN Genomic-tip 500/G columns (QIAGEN, Australia) according to manufacturer protocols. Quality and quantity of genomic DNA was determined using FEMTO Pulse (SA Pathology). Genomes were sequenced using an Oxford Nanopore MinION device. The MinION library construction and sequencing were performed by FISABIO University of

Valencia's sequencing service, using the Oxford Nanopore PCR barcoding kit following the manufacturer's instructions. Sequence data have been deposited with links to BioProject accession number PRJNA821238 in the NCBI BioProject database.

Acknowledgements

We thank Adelaide Microscopy (University of Adelaide, Adelaide, Australia) with their contribution in assisting with the SEM imaging. We thank Andrew Hayles with his assistance with the xCELLigence protocols.

Reference List

1. Tong SY, Davis JS, Eichenberger E, Holland TL, Fowler VG, Jr. *Staphylococcus aureus* infections: epidemiology, pathophysiology, clinical manifestations, and management. *Clin Microbiol Rev*. 2015;**28**(3):603-61.

2. Bui LM, Conlon BP, Kidd SP. Antibiotic tolerance and the alternative lifestyles of *Staphylococcus aureus*. *Essays in biochemistry*. 2017;**61**(1):71-9.

3. Brauner A, Fridman O, Gefen O, Balaban NQ. Distinguishing between resistance, tolerance and persistence to antibiotic treatment. *Nature Reviews Microbiology*. 2016;**14**(5):320-30.

4. Conlon BP. *Staphylococcus aureus* chronic and relapsing infections: Evidence of a role for persister cells: An investigation of persister cells, their formation and their role in *S. aureus* disease. *Bioessays*. 2014;**36**(10):991-6.

5. Proctor RA, Van Langevelde P, Kristjansson M, Maslow JN, Arbeit RD. Persistent and relapsing infections associated with small-colony variants of *Staphylococcus aureus*. *Clin Infect Dis*. 1995;**20**(1):95-102.

6. Edwards AM. Phenotype switching is a natural consequence of *Staphylococcus aureus* replication. *J Bacteriol*. 2012;**194**(19):5404-12.

7. Proctor RA, von Eiff C, Kahl BC, Becker K, McNamara P, Herrmann M, et al. Small colony variants: A pathogenic form of bacteria that facilitates persistent and recurrent infections. *Nature Rev Microbiol*. 2006;**4**(4):295-305.

8. Lipsky BA, Berendt AR, Deery HG, Embil JM, Joseph WS, Karchmer AW, et al. Diagnosis and treatment of diabetic foot infections. *Clin Infect Dis*. 2004;**39**(7):885-910.

9. Akhi MT, Ghotaslou R, Memar MY, Asgharzadeh M, Varshochi M, Pirzadeh T, et al. Frequency of MRSA in diabetic foot infections. *Int J Diabetes in Dev Ctries*. 2017;**37**(1):58-62.

10. Bülent Ertuğrul M, Uyar-Güleç G, Baktıroğlu S, Çörekli E, Türe M. The distribution of causative microorganisms in diabetic foot infection: Has there been any alterations? *Klimik Dergisi*. 2017;**30**(1):27-31.

11. Kahl BC, Becker K, Löffler B. Clinical significance and pathogenesis of staphylococcal small colony variants in persistent infections. *Clin Microbiol Rev*. 2016;**29**(2):401-27.

12. Chang FY, Peacock JE, Jr., Musher DM, Triplett P, MacDonald BB, Mylotte JM, et al. *Staphylococcus aureus* bacteremia: recurrence and the impact of antibiotic treatment in a prospective multicenter study. *Medicine (Baltimore)*. 2003;**82**(5):333-9.

13. Garcia del Pozo E, Collazos J, Carton JA, Camporro D, Asensi V. Factors predictive of relapse in adult bacterial osteomyelitis of long bones. *BMC Infectious Diseases*. 2018;**18**(1):635.

14. Von Eiff C, Heilmann C, Proctor R, Woltz C, Peters G, Götz F. A Site-Directed *Staphylococcus aureus hemB* Mutant Is a Small Colony Variant which persists intracellularly. *J Bacteriol*. 1997;**179**(15):4706-12.

15. Balwit JM, van Langevelde P, Vann JM, Proctor R. Gentamicin-resistant menadione and hemin auxotrophic *Staphylococcus aureus* persist within cultured endothelial cells. *J Infect Dis*. 1994;**170**(4):1033-7.

16. Schaaff F, Bierbaum G, Baumert N, Bartmann P, Sahl HG. Mutations are involved in emergence of aminoglycoside-induced small colony variants of *Staphylococcus aureus*. *Int J Med Microbiol*. 2003;**293**(6):427-35.

17. Lannergård J, Von Eiff C, Sander G, Cordes T, Seggewiß J, Peters G, et al. Identification of the genetic basis for clinical menadione-auxotrophic small-colony variant isolates of *Staphylococcus aureus*. *Antimicrob Agents Ch*. 2008;**52**(11):4017-22.

18. Pader V, James EH, Painter KL, Wigneshweraraj S, Edwards AM. The agr quorum-sensing system regulates fibronectin binding but not hemolysis in the absence of a functional electron transport chain. *Inf Immune*. 2014;**82**(10):4337-47.

19. Dean MA, Olsen RJ, Wesley Long S, Rosato AE, Musser JM. Identification of point mutations in clinical *Staphylococcus aureus* strains that produce small-colony variants auxotrophic for menadione. *Infect Immun*. 2014;**82**(4):1600-5.

20. Kittinger C, Toplitsch D, Folli B, Masoud Landgraf L, Zarfel G. Phenotypic Stability of *Staphylococcus aureus* Small Colony Variants (SCV) Isolates from Cystic Fibrosis (CF) Patients. *Int J Environ Res Public Health*. 2019;**16**(11):1940.

21. Chatterjee I, Kriegeskorte A, Fischer A, Deiwick S, Theimann N, Proctor RA, et al. In vivo mutations of thymidylate synthase (Encoded by *thyA*) are responsible for thymidine dependency in clinical small-colony variants of *Staphylococcus aureus*. *J Bacteriol*. 2008;**190**(3):834-42.

22. Besier S, Ludwig A, Ohlsen K, Brade V, Wichelhaus TA. Molecular analysis of the thymidine-auxotrophic small colony variant phenotype of *Staphylococcus aureus*. *International Journal of Medical Microbiology*. 2007;**297**(4):217-25.

23. Gómez-González C, Acosta J, Villa J, Barrado L, Sanz F, Orellana MÁ, et al. Clinical and molecular characteristics of infections with CO_2-dependent small-colony variants of *Staphylococcus aureus*. *J Clin Microbiol*. 2010;**48**(8):2878-84.

24. Thomas ME. Studies on a CO_2-dependent staphylococcus. *J Clin Pathol*. 1955;**8**(4):288-91.

25. Bazaid AS, Forbes S, Humphreys GJ, Ledder RG, O'Cualain R, McBain AJ. Fatty Acid Supplementation Reverses the Small Colony Variant Phenotype in Triclosan-Adapted *Staphylococcus aureus*: Genetic, Proteomic and Phenotypic Analyses. *Scientific Reports*. 2018;**8**(1).

26. Schleimer N, Kaspar U, Drescher M, Seggewiß J, von Eiff C, Proctor RA, et al. The energy-coupling factor transporter module EcfAA'T, a novel candidate for the genetic basis of fatty acid-auxotrophic small-colony variants of *Staphylococcus aureus*. *Front Microbiol*. 2018;**9**(AUG).

27. Zhang P, Wright JA, Osman AA, Nair SP. An aroD Ochre mutation results in a *Staphylococcus aureus* small colony variant that can undergo phenotypic switching via two alternative mechanisms. *Front Microbiol*. 2017;**8**(MAY).

28. Bui LMG, Hoffmann P, Turnidge JD, Zilm PS, Kidd SP. Prolonged growth of a clinical *Staphylococcus aureus* strain selects for a stable small-colony-variant cell type. *Infect Immun*. 2015;**83**(2):470-81.

29. Bui LMG, Kidd SP. A full genomic characterization of the development of a stable Small Colony Variant cell-type by a clinical *Staphylococcus aureus* strain. *Infect Genet Evol*. 2015;**36**:345-55.

30. Yang D, Wijenayaka AR, Solomon LB, Pederson SM, Findlay DM, Kidd SP, et al. Novel insights into *Staphylococcus aureus* deep bone infections: The involvement of osteocytes. *mBio*. 2018;**9**(2).

31. Tuchscherr L, Buzzola FR, Alvarez LP, Lee JC, Sordelli DO. Antibodies to capsular polysaccharide and clumping factor a prevent mastitis and the emergence of unencapsulated and small-colony variants of *Staphylococcus aureus* in mice. *Infection and Immunity*. 2008;**76**(12):5738-44.

32. Dragosits M, Mattanovich D. Adaptive laboratory evolution – principles and applications for biotechnology. *Microbial Cell Factories*. 2013;**12**(1):64.

33. Jahn LJ, Munck C, Ellabaan MMH, Sommer MOA. Adaptive Laboratory Evolution of Antibiotic Resistance Using Different Selection Regimes Lead to Similar Phenotypes and Genotypes. *Frontiers in Microbiology*. 2017;**8**(816).

34. Prideaux M, Wijenayaka AR, Kumarasinghe DD, Ormsby RT, Evdokiou A, Findlay DM, et al. SaOS2 Osteosarcoma cells as an in vitro model for studying the transition of human osteoblasts to osteocytes. *Calcified tissue international*. 2014;**95**(2):183-93.

35. Josse J, Velard F, Gangloff SC. Staphylococcus aureus vs. Osteoblast: Relationship and Consequences in Osteomyelitis. *Front Cell Infect Microbiol*. 2015;**5**(85).

36. Tuchscherr L, Bischoff M, Lattar SM, Noto Llana M, Pförtner H, Niemann S, et al. Sigma Factor SigB Is Crucial to Mediate *Staphylococcus aureus* Adaptation during Chronic Infections. *PLoS Pathog*. 2015;**11**(4).

37. Tuchscherr L, Medina E, Hussain M, Volker W, Heitmann V, Niemann S, et al. *Staphylococcus aureus* phenotype switching: an effective bacterial strategy to escape host immune response and establish a chronic infection. *EMBO Mol Med*. 2011;**3**(3):129-41.

38. Josse J, Velard F, Gangloff SC. *Staphylococcus aureus* vs. Osteoblast: Relationship and Consequences in Osteomyelitis. *Front Cell Infect Microbiol*. 2015;**5**:85.

39. Kalinka J, Hachmeister M, Geraci J, Sordelli D, Hansen U, Niemann S, et al. *Staphylococcus aureus* isolates from chronic osteomyelitis are characterized by high host cell

invasion and intracellular adaptation, but still induce inflammation. *Int J Med Microbiol*. 2014;**304**(8):1038-49.

40. Proctor RA, Kriegeskorte A, Kahl BC, Becker K, Löffler B, Peters G. *Staphylococcus aureus* Small Colony Variants (SCVs): A road map for the metabolic pathways involved in persistent infections. *Front Cell Infect Microbiol*. 2014;**4**.

41. Tuchscherr L, Heitmann V, Hussain M, Viemann D, Roth J, Von Eiff C, et al. *Staphylococcus aureus* small-colony variants are adapted phenotypes for intracellular persistence. *J Infect Dis*. 2010;**202**(7):1031-40.

42. Kotrba P, Inui M, Yukawa H. Bacterial phosphotransferase system (PTS) in carbohydrate uptake and control of carbon metabolism. *Journal of Bioscience and Bioengineering*. 2001;**92**(6):502-17.

43. Torres VJ, Attia AS, Mason WJ, Hood MI, Corbin BD, Beasley FC, et al. *Staphylococcus aureus* fur regulates the expression of virulence factors that contribute to the pathogenesis of pneumonia. *Infection and Immunity*. 2010;**78**(4):1618-28.

44. Méhi O, Bogos B, Csörgő B, Pál F, Nyerges A, Papp B, et al. Perturbation of iron homeostasis promotes the evolution of antibiotic resistance. *Mol Biol Evol*. 2014;**31**(10):2793-804.

45. Vitko NP, Grosser MR, Khatri D, Lance TR, Richardson AR. Expanded glucose import capability affords *Staphylococcus aureus* optimized glycolytic flux during infection. *mBio*. 2016;**7**(3).

46. LeBel M. Ciprofloxacin: chemistry, mechanism of action, resistance, antimicrobial spectrum, pharmacokinetics, clinical trials, and adverse reactions. *Pharmacotherapy*. 1988;**8**(1):3-33.

47. Gao W, Monk IR, Tobias NJ, Gladman SL, Seemann T, Stinear TP, et al. Large tandem chromosome expansions facilitate niche adaptation during persistent infection with drug-resistant *Staphylococcus aureus*. *Microbial genomics*. 2015;**1**(2):e000026.

48. Giulieri SG, Baines SL, Guerillot R, Seemann T, Goncalves da Silva A, Schultz M, et al. Genomic exploration of sequential clinical isolates reveals a distinctive molecular signature of persistent *Staphylococcus aureus* bacteraemia. *Genome Med*. 2018;**10**(1):65.

49. Guérillot R, Kostoulias X, Donovan L, Li L, Carter GP, Hachani A, et al. Unstable chromosome rearrangements in *Staphylococcus aureus* cause phenotype switching associated with persistent infections. *Proceedings of the National Academy of Sciences*. 2019.

50. Cao S, Huseby DL, Brandis G, Hughes D. Alternative evolutionary pathways for drug-resistant small colony variant mutants in *Staphylococcus aureus*. *mBio*. 2017;**8**(3).

51. Pragman AA, Yarwood JM, Tripp TJ, Schlievert PM. Characterization of virulence factor regulation by SrrAB, a two-component system in *Staphylococcus aureus*. *J Bacteriol*. 2004;**186**(8):2430-8.

52. Yarwood JM, McCormick JK, Schlievert PM. Identification of a novel two-component regulatory system that acts in global regulation of virulence factors of *Staphylococcus aureus*. *Journal of bacteriology*. 2001;**183**(4):1113-23.

53. Pragman AA, Ji Y, Schlievert PM. Repression of *Staphylococcus aureus* SrrAB Using Inducible Antisense srrA Alters Growth and Virulence Factor Transcript Levels. *Biochemistry*. 2007;**46**(1):314-21.

54. Wilde AD, Snyder DJ, Putnam NE, Valentino MD, Hammer ND, Lonergan ZR, et al. Bacterial Hypoxic Responses Revealed as Critical Determinants of the Host-Pathogen Outcome by TnSeq Analysis of *Staphylococcus aureus* Invasive Infection. *PLoS Pathog*. 2015;**11**(12):e1005341.

55. Kinkel TL, Roux CM, Dunman PM, Fang FC. The *Staphylococcus aureus* SrrAB two-component system promotes resistance to nitrosative stress and hypoxia. *mBio*. 2013;**4**(6).

56. Ulrich M, Bastian M, Cramton SE, Ziegler K, Pragman AA, Bragonzi A, et al. The staphylococcal respiratory response regulator SrrAB induces ica gene transcription and polysaccharide intercellular adhesin expression, protecting *Staphylococcus aureus* from neutrophil killing under anaerobic growth conditions. *Mol Microbiol*. 2007;**65**(5):1276-87.

57. Loffler B, Tuchscherr L, Niemann S, Peters G. *Staphylococcus aureus* persistence in non-professional phagocytes. *Int J Med Microbiol*. 2014;**304**(2):170-6.

58. Melter O, Radojevič B. Small colony variants of *Staphylococcus aureus*—review. *Folia microbiol*. 2010;**55**(6):548-58.

59. Hussain M, Hastings JGM, White PJ. A chemically defined medium for slime production by coagulase-negative staphylococci. *Journal of Medical Microbiology*. 1991;**34**(3):143-7.

Supplementary data

Supplementary Table 1. Components of chemically defined media (CDM) for limiting *S. aureus* growth based on previous experiments for growing staphylococci slime production [1].

Ingredients	Concentration (mg/L)	Concentration (mmol/L)
Group 1 (18 Amino acids)		
L- Alanine	100	1.12
L- Arginine	100	0.57
L- Aspartic acid	150	1.13
L- Cystine	50	0.21
L- Glutamic acid	150	1.02
L- Glycine	100	1.33
L- Histidine	100	0.65
L- Isoleucine	150	1.14
L- Leucine	150	1.14
L- Lysine	100	0.68
L- Methionine	100	0.67
L- Phenylalanine	100	0.6
L- Proline	150	1.3
L- Serine	100	0.95
L- Threonine	150	1.26
L- Tryptophan	100	0.49
L- Tyrosine	100	0.55
L- Valine	150	1.28
Group 2 (Glucose)		
Glucose	2500	13.9
Group 3 (Salts+minerals)		
MgSO4	500	4.15
Na2HPO4	10000	70.44
KH2PO4	3000	22.04
CaCl2	10	0.09
MnCl2	5	0.04
(NH4)2SO4	6	0.05
Group 4 (Vitamins)		
Biotin	1	0.004
Nicotinic acid	2	0.016
D- Pantothenic acid, Ca salt	2	0.004
Pyridoxal	4	0.016
Riboflavin	2	0.005
Thiamin	2	0.007

Chapter 5: Characterisation of the transition to alternative cell types of *Staphylococcus aureus* from wounds and bones of patients with osteomyelitis

Diabetic Foot Ulcers (DFU) are susceptible to infection (DFI) and are very slow to heal. These are a common complication of patients with diabetes. The infection can be recalcitrant to treatments and then spread to the bone resulting in osteomyelitis (OM). A pressing issue in DFI is the difficulty in clearing the infection. The rising prevalence of antibiotic resistance and persistent cell types conferring antibiotic tolerance render antibiotic treatments ineffective and consequently often require amputation of the infected tissue. However, even after the infection has initially cleared the infection can relapse from the previous pathogen and cause re-infection. It is important to understand the mechanisms of how bacteria can persist to cause long-term infection and relapse even despite amputations.

Staphylococcus aureus is the most prevalent pathogen isolated from DFI and its incredible ability to adapt and survive under stressful conditions is a major contributor to persistent cases of DFI. Antibiotic resistance is widespread in *S. aureus* with populations of MRSA and the rise of VRSA. The switch to alternate lifestyles creates sub-populations of *S. aureus* with different characteristics and selective fitness. These include biofilms, invasion and internalisation into host cells and a discrete switch to cell types such as SCV. Multiple sub-populations of *S. aureus* provide numerous strategies to survive otherwise lethal stresses that allow reservoirs of *S. aureus* to persist during clinical treatment.

We had previously conducted a clinical study identifying the epidemiology and bacteriology of patients with diabetes (Chapter 3). These involved patients with a DFI that had progressed to OM which was associated with persistent *S. aureus* infections. *S. aureus* displaying different cell types such as SCVs were isolated from both their wounds and bone. These cell types

represented different adaptations utilised by *S. aureus* within a clinically relevant environment. We investigated the microbiology of pairs of *S. aureus* cells isolated from the same patient but different anatomical sites (wound and bone) and compared their genetic profile which may underlie their survival in their niche and the switch to different cell types. From this set of 4 different patients with osteomyelitis we isolated a pair of *S. aureus* comprised of a non-stable SCV and a stable SCV (sSCV). We investigated their genotype and phenotype associated with the switch to a sSCV in a clinically relevant setting.

This manuscript was drafted for submission to the journal Microbiological Research.

Statement of Authorship

Title of Paper	Characterisation of the transition to alternative cell types of *Staphylococcus aureus* from wounds and bones of patients with osteomyelitis
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ✓ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Prepared for submission to the journal *Microbiological Research*

Principal Author

Name of Principal Author (Candidate)	James Lee		
Contribution to the Paper	Carried out sample collection, growth and biofilm assays, enolase activity assay, SEM imaging, genome alignments, genome data analysis and drafted the manuscript		
Overall percentage (%)	80		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Signature		Date	11/04/2022

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Contribution to the Paper	Preformed whole genome extractions		
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Signature		Date	11th APR, 2022

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Signature		Date	13 April 2022

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Signature		Date	13 April 2022

Characterisation of the transition to alternative cell types of *Staphylococcus aureus* from the bone and wounds of patients with osteomyelitis

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Abstract

The switch to alternate cell types in *Staphylococcus aureus* creates sub-populations that are highly resilient, tolerant to antibiotics and lack clinical symptoms of infection. This presents a challenge for clinical treatment where even after initial intervention has seemingly cleared the infection, these alternate cell types persist within tissue to again cause disease.

Pairs of *S. aureus* isolates were obtained from the wound and bones of the same patients with diabetic foot infections. Whole genome sequencing found mutations in genes involved in metabolism and cell wall synthesis. A pair of *S. aureus* isolates comprised of a non-stable SCV (nsSCV) and a stable small colony variant (sSCV) were extracted from a single patient. The switch to the sSCV phenotype was associated with frameshift mutations in the enolase *eno* and the histidine kinase *arlS*. These genetic events resulted in an impeded growth dependent on amino acid catabolism and modulated biofilm. These mutations present new perspectives in the switch to SCV in a clinically relevant environment.

Keywords: *Staphylococcus aureus*, small colony variants, diabetic foot infection, osteomyelitis, intracellular,

1. Introduction

Staphylococcus aureus is commonly associated with persistent infections that can last for months or even years [2]. Despite the infection being seemingly cleared, there is the issue of the high incidence of relapsing infection. Relapse of *S. aureus* infection has been reported to occur in up to 10% of cases in osteomyelitis and bacteremia [3, 4]. Indeed, *S. aureus* is one of the most prevalent pathogens isolated from diabetic foot infections (DFI) [5-7]. There are host factors which have been established as being associated with the incidence of relapse [4, 8], however the molecular mechanisms of relapse in *S. aureus* are poorly understood.

S. aureus has an incredible ability to survive and adapt to a range of stresses generated by the host and clinical treatment and this is, in part, through the formation of alternative cell types. This creates sub-populations with different mechanisms that are more adapted to survive different stresses. Multiple mechanisms of survival then provide the population as a whole to survive dynamic and damaging environments. A notable alternate cell type are Small Colony Variants (SCV), a quasi-dormant sub-population characterised as slow growing cells with a non-pathogenic virulence profile [9]. This results in evasion of the immune response and tolerance to antimicrobials that would otherwise clear actively growing populations [10]. The SCV cells are generally unstable and readily revert to a normal, pathogenic cell type. This can create reservoirs of *S. aureus* remaining within host tissue without clinical symptoms and result in infection that has seemingly been cleared. However, reversion to active infection can occur after some time [11]. Indeed, further to this, a reduced virulence profile of the infecting bacteria is required for infection and pathology in patients with diabetes [12].

There are different models for how SCVs arise within a population. Since the mid-1990s, specific genes have been known to create SCVs and mutant strains with auxotrophisms in metabolic substrates have been used as models to research SCV. *S. aureus* with mutations in *hemB*, [13-15], *menD* [15-18] and *thyA* [19-21] can create stable SCV (sSCV) through auxotrophisms that result in deficiencies in the electron transport chain. These mutant models have led to significant advances, but it is now appreciated they are not a complete representation of the progression of *S. aureus* to an SCV; other isolates have auxotrophisms to CO_2 [22, 23], fatty acids [24, 25], chorismite synthesis [26] and even more without any defined auxotrophism [15, 27]. While these specific mutations may arise spontaneously or as a direct response to environmental stresses, there are broader stochastic changes to the cell that appear over time. There are differences in the cell physiology, gene expression of cell surface metal ion transporters or nutritional requirements that may result from genetic rearrangements or

epigenetic events over time [28, 29]. Steady state growth through continuous culture has been used to select for SCV by growing *S. aureus* at a controlled low growth rate over a prolonged duration [30]. Cells within this population had single nucleotide polymorphisms (SNP) associated with the adaptation to clinically relevant stresses. Mutations in virulence regulators such as *mgrA* and *rsbU* and upregulation of the gene encoding the large (1.1 MDa) surface protein *ebh* were events not previously identified to have been linked to other SCV [31]. The serial passage of *S. aureus* in mice has also selected for sSCV through increased antibodies with specificities against capsular polysaccharide, however the genetic mechanisms underlying the phenotype was not established [32]. Clearly, there is a disparity of the SCV generated through single mutations and SCVs generation through adaptation to clinically relevant conditions.

In this current work we aim to characterise the progression of *S. aureus* to SCV as a mechanism to establish persistent infection to a DFI. We have previously identified patient factors associated with the progression of an uninfected diabetic foot ulcer (DFU) to a DFI and osteomyelitis. Complications of diabetes include endovascular dysfunction that result in an immunocompromised site in the lower limbs that facilitate persistent infections in the foot [33]. However, the mechanism in how *S. aureus* is able to persist and relapse after the infection has been "cleared" is equivocal. We hypothesise that sub-populations of SCV allows *S. aureus* to form non-virulent reservoirs which do not show signs of infection adjacent to the initial site of infection.

In our clinical study, we have isolated *S. aureus* from different niches within the same patient that displayed different cell types. We have used whole genome sequencing to identify the genotype in different cell types, and progression to the intracellular bone. Notably we isolated a pair of *S. aureus* comprising an sSCV and a non-stable small colony variant

(nsSCV). The stability of the sSCV phenotype suggested there were stable genetic events associated with the switch to the sSCV within a clinical infection.

2. Materials and Methods

2.1. Collection of bacterial isolates from DFI patients.

The human ethics for this study was approved by the Human Research Ethics Committee at the Royal Adelaide Hospital (Approval number: HREC/17/RAH/242). Patients with DFI progressing to osteomyelitis and required amputation were recruited from the Royal Adelaide Hospital (RAH). From each patient, a cotton flock swab was used to isolate bacteria from the foot ulcer and cultured on tryptic soya agar (TSA) for single colonies. Extracellular bacteria were isolated by plating 100µL of PBS onto TSA and incubated for 72h at 37°C to obtain single colonies. To assess bacteria from intracellular bone, bone fragments were obtained from amputations of DFI-OM patients and suspended in Phosphate Buffer Solution (PBS). To extract the intracellular bacteria, bone fragments were washed twice with PBS and then sonicated with 4 cycles at 180Hz for 10 seconds in PBS. 100µL of PBS was cultured on TSA for up to 72h at 37°C. Bacterial species in colonies were identified using MALDI-TOF Biotyper (Bruker).

2.2. Strains and culture media

Growth of *S. aureus* isolates were preformed in a 96-well microtitre plate. Tryptic soya broth (TSB) was compared with Chemically defined media (CDM) adapted from experiments for slime production of coagulase-negative staphylococci [1]. Glucose concentrations of CDM was modified when required. Cells were stored in 30% glycerol at -80°C and streaked onto TSA for single colonies when needed.

2.3. Growth kinetics

Growth kinetics assays were performed in a 96-well microtitre plates. Cells were incubated in TSB, CDM or modified CDM at 37°C to log phase (OD_{600} ~ 0.2). 20µL of log phase culture

was added to a well containing 180µL of media in triplicate. Plates were incubated at 37°C for 18 h and OD_{600} readings taken every 30 minutes with a Sunrise Absorbance Microplate Reader (Tecan).

2.4. Crystal violet biofilm assay

Biofilm assays are performed in flat-welled 96-well microtitre plates. Cells were incubated in TSB at 37°C to log phase (OD_{600} ~ 0.2). 20µL of log phase culture was added to a well containing 180µL of media. Plates were incubated at 37°C for 18 hours and OD_{600} readings taken. Supernatant in each well was gently tipped out and the wells were washed twice with 200µL of PBS. 200µL of 0.1% crystal violet stain was added to each well and incubated at room temperature for 60 mins. Each well was washed twice with 200µL of PBS. Plate was left to dry upside-down overnight. 150µL of 30% acetic acid was added to each well and incubated at room temperature for 15 minutes and then transferred to a new 96-well plate. OD_{600} readings were taken, and each reading was normalised to their initial OD_{600} reading.

2.5. xCELLigence kinetic biofilm assay

The xCELLigence Real-Time Cell Analysis (ACEA) was used to measure formation of biofilm in real-time to quantify biofilm kinetics. The biofilm assay was performed in 16-well ePlates containing gold electrodes in the well surface. Single colonies were incubated in TSB at 37°C to log phase (OD_{600} ~ 0.2). 150µL of media was added to each well, sterile distilled water was added to the evaporation control wells and plate was incubated at 37°C for 30 minutes. 50µL of log phase cultures (OD_{600} ~ 0.15) was added to each well. Biofilm formation was quantified by measuring electrical impedance between electrode sensors in the bottom of the ePlate wells, expressed as cell index (CI). Readings were taken every 15 minutes at 37°C for up to 60 hours. Cell index values were normalised by subtracting the cell index readings from the media only samples.

2.6. Minimum inhibitory concentrations (MIC) of antibiotics

Antibiotics were serially diluted into TSB by a factor of 1:2. Cell cultures were grown in TSB at 37°C to log phase (OD_{600} ~ 0.2). 20µL of log phase culture was added to wells containing 180µL of TSB/antibiotic. Cultures were incubated at 37°C for 18 hours and OD_{600} readings were taken. Cultures were then serially diluted to determine CFU/mL. MIC was determined to be the lowest concentration of antibiotic which resulted in no visual bacterial colonies and change in OD_{600}.

2.7. Scanning electron microscopy

Cells were filtered through a 0.2µm Milipore filter paper and fixed with a fixative solution (4% paraformaldehyde, 1.25% glutaraldehyde, 4% sucrose in PBS). Filters were washed twice with 4% sucrose in PBS, post-fixed with 0.1% osmium tetraoxide for 60 minutes and washed twice with 4% sucrose in PBS. Cells were dehydrated with a series of two 10-minute ethanol washes, each in 70%, 90% and 100% ethanol solutions. Cells were dried in a 1:1 mix HMDS and 100% ethanol for 20 minutes and then in HMDS for 15 minutes. Samples were mounted onto titanium stubs and coated with 2mm platinum. Images were taken using a Phillips XL30 FEG scanning electron microscope (Adelaide Microscopy).

2.8. Enolase activity assay

Cell-free protein extracts were isolated from overnight cultures grown in TSB. Cultures were centrifuged (1900 x g, 4°C, 10 min), supernatant was discarded, and the pellet stored at -80°C. Cells were resuspended in 1 mL of sterile Milli-Q water and lysed with four freeze-thaw cycles in dry-ice/100% ethanol followed by water at 37°C. 1mL of lysis buffer (50 mM Tris, 1 mM ETDA, 10% glycerol, 1% Triton X-100) was added to cells and incubated at 37°C for 30 min. Protein concentrations were measured using the Bradford assay. Concentrations of cell-free extracts were normalised to approximately 2.5 mg/mL between isolates. 100µL of cell free extract was added to 900 µL of a buffer containing excess 2-phosphoglycerate (2-PG) (120mM

KCl, 30mM $MgSO_4$, 4.5mM 2-PG) in UV transmissible cuvettes. The activity of enolase was measured by quantifying the conversion of 2-PG to phosphoenolpyruvate (PEP) by absorption of PEP at 240nm in a Nanodrop 2000c over 1 min.

2.9. Whole genome sequencing.

Whole genomic DNA was extracted and purified using QIAGEN Genomic-tip 500/G columns (QIAGEN, Australia) according to manufacturer protocols. Quality and quantity of genomic DNA was determined using FEMTO Pulse (SA Pathology). Genomes were sequenced using an Oxford Nanopore MinION device. The MinION library construction and sequencing were performed by FISABIO University of Valencia's sequencing service, using the Oxford Nanopore PCR barcoding kit following the manufacturer's instructions. Sequence data have been deposited with links to BioProject accession number PRJNA821238 in the NCBI BioProject database.

3. Results

3.1. DFI patients with osteomyelitis were associated with multiple S. aureus cell types which include SCV.

S. aureus isolates were isolated from four different patients with a DFI which had progressed to osteomyelitis and was admitted to the Royal Adelaide Hospital (RAH). Treatments of these patients involved amputation of the infected tissue and bone fragments were obtained for bacterial isolation. For each patient, bone was taken from the infected tissue (distal bone) and clinically uninfected bone adjacent from the site of infection (proximal bone). Four patients were selected for further study where *S. aureus* was isolated from the wound and bone and displayed heterogenous cell types from the same patient. Whole genomic sequences were obtained for each isolate and isolates from the same patient were used for genomic alignment and SNP analysis **(Table 1)**.

UA-DI-49 (wound isolate) and UA-DI-52 (intracellular bone isolate) were a pair of nsSCV in co-infection with *Staphylococcus epidermidis* and *Corynebacterium striatum*. UA-DI-57 (wound isolate) and UA-DI-59 (intracellular bone isolate) were also a pair of nsSCV which formed large, non-pigmented colonies in co-infection with *S. epidermidis*, *Streptococcus oralis* and *Pseudomonas aeruginosa*. UA-DI-144 (extracellular bone isolate) and UA-DI-145 (wound isolate) were a pair of normal cell types which formed large, non-pigmented colonies. These paired isolates represented the change involved in the switch to colonisation within soft tissue to intracellular of the bone. In these selected isolates, SNPs were identified in genes with known function including the aminoacyltransferase *femA* (cross-linking of peptidoglycan units), the EIIA component of the mannitol specific PTS *manP* (mannitol transport across the cell membrane) and the proline aminoacyltransferase *proS* (attachment of proline to polypeptide chains) **(Table 1)**. No SNPs were detected between UA-DI-57 and UA-DI-59.

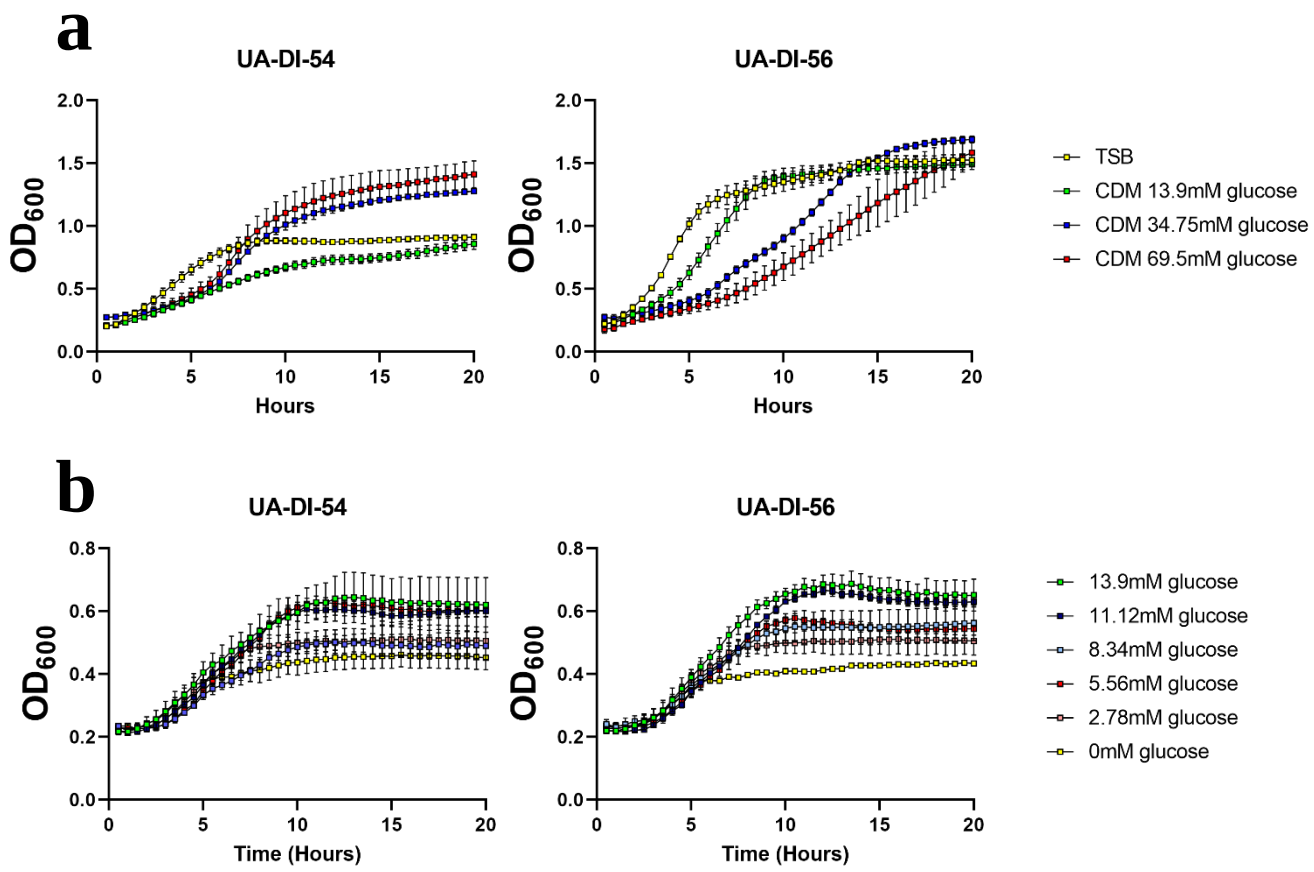

FIG. 1. Growth kinetics at varying concentrations of glucose. Mid-exponential phase cultures of the sSCV UA-DI-54 and the nsSCV UA-DI-56 were cultured in CDM (with a base glucose concentration of 13.9mM) with either an increased in glucose concentration (a) or decrease in glucose concentration (b). Growth was measured spectrophotometrically through changes in OD_{600} over 20 hours.

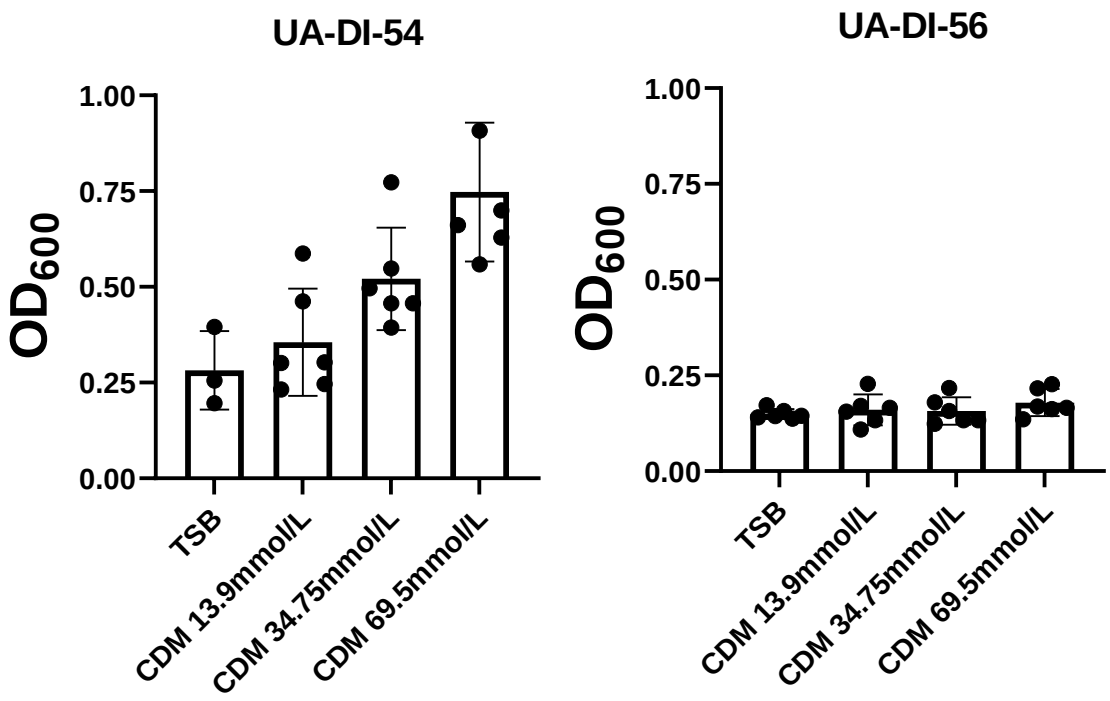

FIG 2. Crystal violet stain biofilm assay. An endpoint measurement of biofilm formation of the sSCV UA-DI-54 and the nsSCV UA-DI-56 was quantified using a crystal violet assay after an overnight culture in CDM with increasing concentrations of glucose.

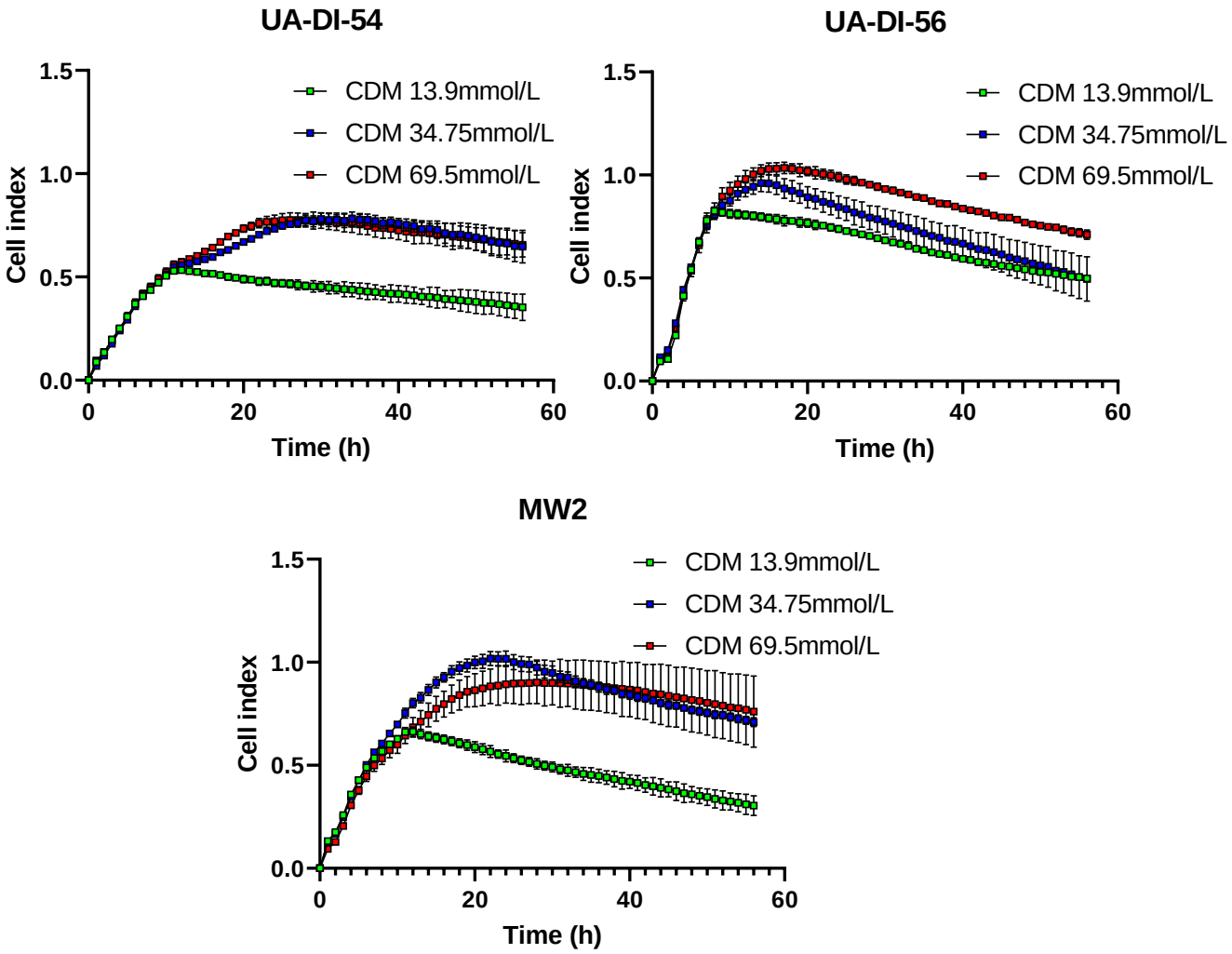

FIG. 3. Biofilm production kinetics. Mid-exponential phase cultures of the sSCV UA-DI-54 and the nsSCV UA-DI-56 were inoculated in CDM and a kinetic measurement of biofilm formation of was quantified using a xCELLigence Real Time Cell Analysis (Agligent) over 56h.

3.2. Transition between the bone and wound is associated with phenotypic and genetic variation

Samples from one patient presented with both nsSCV and a sSCV. The stability of the SCV phenotype indicated that amongst the population of cells there were stable genetic changes involved in the switch from a nsSCV to a sSCV. UA-DI-56 was a nsSCV which formed large, non-pigmented colonies isolated from the intracellular bone and UA-DI-54 was a sSCV isolated from the wound. Both isolates were in the clonal complex CC22, SCCmec Type IV 2B and contained an additional plasmid (**Table. 3**).

Comparing UA-DI-56 to UA-DI-53, SNPs were detected in four genes that encoded for the enolase *eno*, the histidine kinase *arlS*, the ornithine racemase *orr* and a hypothetical oxidoreductase **(Table 1).** These SNPs resulted in frameshift mutations and premature stop codons in their respective genes in UA-DI-54. The frameshift in *eno* lead to a premature stop codon at amino acid sequence 333 which truncated the active and substrate binding sites of enolase. The frameshift in *arlS* lead to a premature stop codon at amino acid sequence 269 which truncated the histidine kinase domain. The frameshift mutation in *orr* lead to a premature stop codon at amino acid sequence 25 which truncated the majority of the active and binding sites. These frameshift mutations would have negative effect on the protein function, and we aimed to determine the nature of the phenotypes of strains with these genetic events.

Table 1. SNP analysis from genome alignments of whole genome sequences. Alignments were preformed between pairs of *S. aureus* isolates sourced from the bone and wound from the same patient. * = reference genome in alignment

UA-DI-56* **UA-DI-54**	**Gene**	**Nucleotide change**	**Amino acid change**	**Product**
	eno	996_997insA	Glu333Lys, Frameshift – premature stop	Enolase
	arlS	792delT	Asp265Ile, frameshift – premature stop	ArlRS histidine kinase sensor element
	orr	61_62insA	Gln23Ser, frameshift – premature stop	Ornithine racemase
	gfo	328delA	Met110X	Hypothetical oxidoreductase
UA-DI-57* **UA-DI-59**	**Gene**	**Nucleotide change**	**Amino acid change**	**Product**
	sdrD	3522C>T	Synonymous	Serine-aspartate repeat containing protein D
	sdrD	2524C>T	Synonymous	
	sdrC	2187T>C	Synonymous	Serine-aspartate repeat containing protein C
	femA	703A>G	Thr235Ala	Aminoacyltransferase A
	ilvB	486G>A	Synonymous	Acetolactate synthase large subunit
UA-DI-144* **UA-DI-145**	**Gene**	**Nucleotide change**	**Amino acid change**	**Product**
	manP	724C>T	Arg242Trp	PTS system mannose-specific EIIBCA component
	proS	1502T>C	Val501Ala	Proline-tRNA ligase
	Hypothetical	437A>G	Asp146Gly	
	grsB	3602C>T	Thr1201Met	Gramicidin S synthase
	nisB	2844T>G	Thre948Thr	Nisin biosynthesis protein
	Hypothetical	109G>A	Ala37Thr	
	Hypothetical	354C>T	Phe118Phe	

Table 2. Notable virulence factors of *S. aureus* missing in the genomes of both UA-DI-54 and UA-DI-56

Gene	Product	Function
splABCDEF	Serine protease operon	Associated with virulence
fnbB	Fibronectin binding protein B	Adhesion, host cell internalisation
clfB	Clumping factor B	Adhesion, nasal colonisation
sdrE	Ser-Asp rich fibrinogen-binding proteins	Inhibit early innate immune response
sak	Staphylokinase	Activate plasminogen and α-defensins
chp	Chemotaxis inhibitory protein	Inhibit neutrophil migration
scn	Staphylococcal complement inhibitor	Inhibits complement
esaDE	Type VII secretion operon	Protection from EsxA toxin
esxBCD	Type VII secretion operon	Defence against antimicrobial fatty acids
hlb	Haemolysin beta	Toxin
lukDEM	Leukocidins	Leukocyte killing
lukFS-PV	Panton-Valentine Leukocidins	Leukocyte killing

Table 3. List of genes carried in the plasmid present in UA-DI-54 and UA-DI-56

Gene	Product	Function
acuI	Putative acrylyl-CoA reductase	YhdH/YhfP family oxidoreductase
bcrR	HTH-type transcriptional activator	Xenobiotic response element
arsC	Arsenate reductase	Catalyses reduction of arsenate to arsenite
arsB	Arsenical pump membrane protein	Intermembrane pump for arsenite
sdpR	Transcriptional repressor	Part of the *sdpIR* operon in sporulation in *Bascillus subtillis*
cadC	Cadmium resistance transcriptional regulatory protein	Repressor of cad operion. Binding of heavy metals (cadmium, zinc) releases cadC, activates operon and cadmium resistance
cadA	Cadmium-transporting ATPase	ATP mediated membrane cadmium pump
mco	Multicopper oxidase	Involved in copper homeostasis and oxidative stress response. Oxidises 3,3′-dimethoxybenzidine in the presence of copper
copB	Copper-exporting P-Type ATPase B	Involved in copper transport

3.3. UA-DI-54 was a sSCV associated with altered metabolism and biofilm formation

The frameshift mutations in both *eno* and *arlS* coding regions (premature stop codons) would have major effects on their ability to function. Enolase catalyses the penultimate step of glycolysis through the conversion of 2-phosphoglycerate (2-PG) to phosphoenolpyruvate (PEP). ArlS is the histidine kinase component of the ArlRS two component system which regulates various genes involved in *S. aureus* pathogenesis. We determined how these genotypes impact the pathogenesis potential and the growth of UA-DI-54.

UA-DI-54 had a reduced ability to grow in the complex, undefined media (TSB). Compared to the nsSCV UA-DI-56, UA-DI-54 had an extended lag phase and a lower OD_{600} at stationary

phase. Enolase is essential for the complete metabolism of glucose to pyruvate through glycolysis. To determine the impact of this SNP in enolase on cell growth, the glucose concentration within CDM was altered to determine how UA-DI-54 responded to changes in glucose concentration. Both UA-DI-54 and UA-DI-56 had a reduction in growth and extended exponential phases when cultured in CDM with the standard glucose concentration of 13.9mmo/L compared to TSB. The growth of UA-DI-56 was reduced in proportion with increased glucose concentrations (**FIG .1)**. In contrast, the growth rate increased proportionally to the glucose concentration after the mid-exponential phase of growth for UA-DI-54. Growth in CDM resulted in an extended exponential phase of growth dependent on the glucose concentration, while the OD_{600} at stationary phase in glucose concentrations above 13.9mM was greater than growth in TSB. Reduction of the concentration of glucose resulted in minor changes in the growth kinetics of UA-DI-54 and UA-DI-56. As the concentration of glucose in CDM decreased, the exponential phase of growth decreased in duration and the peak OD_{600} at stationary phase was lower (**FIG. 1b)**.

The crystal violet biofilm assay showed an endpoint measurement of the production of biofilm. UA-DI-56 produced low level of biofilm comparable to the media controls independent of glucose concentration **(FIG. 2)**. The production of biofilm in UA-DI-54 increased proportionally with glucose concentration. In contrast, the xCELLigence biofilm assay measured the biofilm kinetics, with distinct phases; lag, exponential and stationary phases. Prolonged exponential phase of biofilm formation and peak cell-index was associated with increased glucose concentration in both isolates. UA-DI-56 had a shorter exponential phase with a greater endpoint cell index compared to UA-DI-54 **(FIG. 3)**.

The early stages of biofilm formation were largely unaffected by the concentration of glucose. At glucose concentrations 34.75mM and 69.5mM, all isolates had an extended exponential phase compared to the base concentration of 13.9mM and resulted in a greater endpoint cell-

index. The mapping of the growth cycle against the biofilm kinetic data revealed the timing of biofilm formation. The duration of the exponential phase of biofilm formation coincided with the stationary growth phase of UA-DI-56 and this was regardless of the concentration of glucose **(FIG. 1, FIG. 3)**. In contrast, a significant difference was observed in the stationary phase of growth at 34.75mM and 69.5mM glucose (at approximately 12h) and the respective stationary phase of biofilm (approximately 23h).

3.4. The sSCV was associated with decreased enolase activity

The SNP detected in *eno* caused a frameshift mutation which was predicted to result in a premature stop codon and therefore a truncated protein (Table 1). The activity of enolase was measured by the increase of PEP which was measured spectrophotometrically (Abs_{240nm}). UA-DI-56 had enolase activity comparable to positive controls *S. aureus* MW2 and *E. coli* DH5α with functional enolase activity. UA-DI-54 had a significantly decreased enolase activity compared to UA-DI-56 and had OD_{240} readings significantly greater than the media only controls.

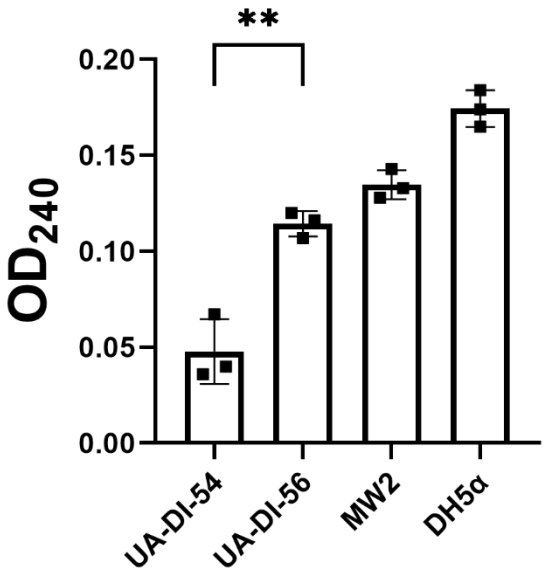

FIG. 4. Enolase assay. Enolase catalyses the conversion of 2-phosphoglycerate (2-PG) to phosphoenolpyruvate (PEP). Cell-free extracts containing enolase was added to a solution containing 2-PG and the formation of PEP was measured at a wavelength of 240nm after 30 seconds.

** = p-value < 0.005

4. Discussion

Different cell types of a clonal population of *S. aureus* exist across a spectrum ranging from virulent and metabolically active to dormant, non-pathogenic cell types. Through this spectrum, over time, there can be an accumulation of genetic changes in response to selective pressures such as reduced availability of nutrients, competition by other bacterial species, the immune response and the presence of antibiotics which contribute to the dormant phenotypes. The extremes in this end of the spectrum result in the SCV phenotype. Our data characterises the different cell types sourced from clinical samples and within this spectrum and thereby provides a deeper analysis on the progression to a sSCV (specifically in strain UA-DI-54).

There were no clear trends in the genes affect by SNPs and infection within the intracellular bone. An overview of the processes affected by SNPs included cell wall cross-linking, sugar metabolism and amino acid metabolism. However, the affected processes occur within independent cases of infections. The lack of a common mutation associated with persistence within the bone indicates the absence of a defined pathway which occurs in response to intracellular bone infection, but rather random, spontaneous mutations which lead to heterogenous cell types. Among these sub-populations are cell types that are more permissible to persist within the bone. However, reversion of the SCV phenotype may have resulted in other genetic events not detected through our methods

A sSCV was associated with SNPs in enolase and *arlS*; novel genes not previously reported in other models of SCV. Enolase is essential for *S. aureus* growth and bacterial glycolysis in general, however growth with a dysfunctional enolase has been reported in specific growth conditions in *E. coli* (supplementation with glycerol and glucose) [34]. Enolase catalyses the penultimate step of glycolysis which metabolises 2PG to phosphoenolpyruvate (PEP). Consequently, complete metabolism of glucose cannot conclude and cannot be utilized as the

major source of carbon and must rely on catabolism of amino acids (as *S. aureus* lacks the enzymes for β-oxidation to cataabolise fatty acids to actyl-CoA) to create pyruvate, acetyl-CoA and other TCA cycle intermediates [35-37] which our data shows when culturing UA-DI-54 in conditions of limited glucose and amino acids **(FIG.1)**. Amino acid catabolism and partial glycolysis pathways predominately rely on substrate level phosphorylation from acetate and lactate for ATP production and these metabolic pathways produce less energy (ATP) compared to glucose dependant pathways and are likely the basis of the reduction of growth in UA-DI-54. Defects in growth are a defining characteristic of SCV and the SNP in *eno* presents a new mechanism which underpins the switch to a SCV. The loss of enolase function has not previously been associated with the switch to SCV and this is in part due to previous research recognizing *eno* as essential in *S. aureus* [38].

UA-DI-54 was isolated from a DFI patient with hyperglycemia. High levels of glucose is toxic to *S. aureus*, with studies finding increased supplementation of glucose was associated with a decrease in CFU/mL in the *S. aureus* isolate Newman [39]. In contrast, UA-DI-54 had increased rate of growth in higher levels of glucose (34.75mM and greater) that otherwise resulted in a reduction of growth in UA-DI-56 and *S. aureus* MW2 **(FIG. 1)**. There is still a reduced level of enolase activity and so the growth patterns in toxic levels of glucose are likely linked to the reduced activity of enolase **(FIG. 4)**. The mechanisms on how this occurs is uncertain without further knowledge on how the loss of enolase affects the regulation of glycolysis and accumulation of intermediates. Carbohydrate transport is mediated through phosphoenolpyruvate dependent carbohydrate phosphotransferase systems (PTS) [40, 41]. Other than gluconeogenesis, there are no other redundant pathways which catalyse the formation of PEP from 2-PG other than enolase. We can postulate that the lack of PEP reduces the transport of glucose into the cell and thus increased levels of glucose saturates glucose transport rendering glycolytic regulation independent of the glucose levels.

The presence of glucose can influence the expression of virulence factors through the action of the carbon catabolite protein CcpA. In the presence of preferential carbon sources such as glucose, CcpA binds to DNA targets at the catabolite response element *cre* sites to inhibit amino acid catabolism. The addition of glucose and consequent CcpA activity resulted in increased virulence and pro-inflammatory responses in *S. aureus*, *Streptococcus pneumoniae* and *Bacillus anthracis* [42-44]. The inhibition of enolase activity only leaves amino acid catabolism as a carbon source for ATP production which must rely on the repression of CcpA and thus resulting in a reduction in virulence.

Defects in metabolism are clinically significant in establishing persistent infection. Dormancy within SCVs is one of the keys to antibiotic tolerance [45, 46], intracellular persistence [47-49] and evasion of the immune response [48, 50]. The SNP in enolase and the defects in growth may exhibit similar clinical manifestations.

5. Conclusions

We identified multiple genetic events that were associated with *S. aureus* persistence within the intracellular bone. However, the lack of a single or common pathway identified in persistent cell types (SCVs and bone isolates) indicates that persistence in the bone is potentially not through a specific response to the environmental factors, but rather selection of spontaneously generated sub-populations with greater fitness for survival within the bone. The sSCV phenotype was associated with a defect in the essential glycolytic enzyme enolase and a diminished growth rate that was dependent on amino acid catabolism.

Acknowledgements

We thank Andrew Hayles with his assistance with the xCELLigence protocols and the Vascular Surgeons of the Central Adelaide Local Health Network who recruited patients with diabetes and provided bone and wound swabs which our bacterial isolates were extracted.

Funding

This research was in part funded by the Vascular Foundation (Australia) to JD and SPK. The was also funding from the South Australian (SA) Health Allied Health Research Seed Funding Grant to SPK, TPW and RF.

Declaration of Competing Interest

The authors declare no conflict of interest

Reference List

1. Hussain M, Hastings JGM, White PJ. A chemically defined medium for slime production by coagulase-negative staphylococci. *Journal of Medical Microbiology*. 1991;**34**(3):143-7.

2. Tong SY, Davis JS, Eichenberger E, Holland TL, Fowler VG, Jr. *Staphylococcus aureus* infections: epidemiology, pathophysiology, clinical manifestations, and management. *Clin Microbiol Rev*. 2015;**28**(3):603-61.

3. Chang FY, Peacock JE, Jr., Musher DM, Triplett P, MacDonald BB, Mylotte JM, et al. *Staphylococcus aureus* bacteremia: recurrence and the impact of antibiotic treatment in a prospective multicenter study. *Medicine (Baltimore)*. 2003;**82**(5):333-9.

4. Garcia del Pozo E, Collazos J, Carton JA, Camporro D, Asensi V. Factors predictive of relapse in adult bacterial osteomyelitis of long bones. *BMC Infectious Diseases*. 2018;**18**(1):635.

5. Lipsky BA, Berendt AR, Deery HG, Embil JM, Joseph WS, Karchmer AW, et al. Diagnosis and treatment of diabetic foot infections. *Clin Infect Dis*. 2004;**39**(7):885-910.

6. Akhi MT, Ghotaslou R, Memar MY, Asgharzadeh M, Varshochi M, Pirzadeh T, et al. Frequency of MRSA in diabetic foot infections. *Int J Diabetes in Dev Ctries*. 2017;**37**(1):58-62.

7. Bülent Ertuğrul M, Uyar-Güleç G, Baktıroğlu S, Çörekli E, Türe M. The distribution of causative microorganisms in diabetic foot infection: Has there been any alterations? *Klimik Dergisi*. 2017;**30**(1):27-31.

8. Montgomery CP, David MZ, Daum RS. Host factors that contribute to recurrent staphylococcal skin infection. *Current opinion in infectious diseases*. 2015;**28**(3):253-8.

9. Proctor RA, Van Langevelde P, Kristjansson M, Maslow JN, Arbeit RD. Persistent and relapsing infections associated with small-colony variants of *Staphylococcus aureus*. *Clin Infect Dis*. 1995;**20**(1):95-102.

10. Edwards AM. Phenotype switching is a natural consequence of *Staphylococcus aureus* replication. *J Bacteriol*. 2012;**194**(19):5404-12.

11. Proctor RA, von Eiff C, Kahl BC, Becker K, McNamara P, Herrmann M, et al. Small colony variants: A pathogenic form of bacteria that facilitates persistent and recurrent infections. *Nature Rev Microbiol*. 2006;**4**(4):295-305.

12. Tuchscherr L, Korpos È, van de Vyver H, Findeisen C, Kherkheulidze S, Siegmund A, et al. *Staphylococcus aureus* requires less virulence to establish an infection in diabetic hosts. *International Journal of Medical Microbiology*. 2018.

13. Von Eiff C, Heilmann C, Proctor R, Woltz C, Peters G, Götz F. A Site-Directed *Staphylococcus aureus hemB* Mutant Is a Small Colony Variant which persists intracellularly. *J Bacteriol*. 1997;**179**(15):4706-12.

14. Balwit JM, van Langevelde P, Vann JM, Proctor R. Gentamicin-resistant menadione and hemin auxotrophic *Staphylococcus aureus* persist within cultured endothelial cells. *J Infect Dis*. 1994;**170**(4):1033-7.

15. Schaaff F, Bierbaum G, Baumert N, Bartmann P, Sahl HG. Mutations are involved in emergence of aminoglycoside-induced small colony variants of *Staphylococcus aureus*. *Int J Med Microbiol*. 2003;**293**(6):427-35.

16. Lannergård J, Von Eiff C, Sander G, Cordes T, Seggewiß J, Peters G, et al. Identification of the genetic basis for clinical menadione-auxotrophic small-colony variant isolates of *Staphylococcus aureus*. *Antimicrob Agents Ch*. 2008;**52**(11):4017-22.

17. Pader V, James EH, Painter KL, Wigneshweraraj S, Edwards AM. The agr quorum-sensing system regulates fibronectin binding but not hemolysis in the absence of a functional electron transport chain. *Inf Immune*. 2014;**82**(10):4337-47.

18. Dean MA, Olsen RJ, Wesley Long S, Rosato AE, Musser JM. Identification of point mutations in clinical *Staphylococcus aureus* strains that produce small-colony variants auxotrophic for menadione. *Infect Immun*. 2014;**82**(4):1600-5.

19. Kittinger C, Toplitsch D, Folli B, Masoud Landgraf L, Zarfel G. Phenotypic Stability of *Staphylococcus aureus* Small Colony Variants (SCV) Isolates from Cystic Fibrosis (CF) Patients. *Int J Environ Res Public Health*. 2019;**16**(11):1940.

20. Chatterjee I, Kriegeskorte A, Fischer A, Deiwick S, Theimann N, Proctor RA, et al. In vivo mutations of thymidylate synthase (Encoded by *thyA*) are responsible for thymidine dependency in clinical small-colony variants of *Staphylococcus aureus*. *J Bacteriol*. 2008;**190**(3):834-42.

21. Besier S, Ludwig A, Ohlsen K, Brade V, Wichelhaus TA. Molecular analysis of the thymidine-auxotrophic small colony variant phenotype of *Staphylococcus aureus*. *International Journal of Medical Microbiology*. 2007;**297**(4):217-25.

22. Gómez-González C, Acosta J, Villa J, Barrado L, Sanz F, Orellana MÁ, et al. Clinical and molecular characteristics of infections with CO_2-dependent small-colony variants of *Staphylococcus aureus*. *J Clin Microbiol*. 2010;**48**(8):2878-84.

23. Thomas ME. Studies on a CO_2-dependent staphylococcus. *J Clin Pathol*. 1955;**8**(4):288-91.

24. Bazaid AS, Forbes S, Humphreys GJ, Ledder RG, O'Cualain R, McBain AJ. Fatty Acid Supplementation Reverses the Small Colony Variant Phenotype in Triclosan-Adapted *Staphylococcus aureus*: Genetic, Proteomic and Phenotypic Analyses. *Scientific Reports*. 2018;**8**(1).

25. Schleimer N, Kaspar U, Drescher M, Seggewiß J, von Eiff C, Proctor RA, et al. The energy-coupling factor transporter module EcfAA'T, a novel candidate for the genetic basis of fatty acid-auxotrophic small-colony variants of *Staphylococcus aureus*. *Front Microbiol*. 2018;**9**(AUG).

26. Zhang P, Wright JA, Osman AA, Nair SP. An aroD Ochre mutation results in a *Staphylococcus aureus* small colony variant that can undergo phenotypic switching via two alternative mechanisms. *Front Microbiol*. 2017;**8**(MAY).

27. Gómez-González C, Acosta J, Villa J, Barrado L, Sanz F, Orellana MÁ, et al. Clinical and molecular characteristics of infections with CO 2-dependent small-colony variants of Staphylococcus aureus. *Journal of Clinical Microbiology*. 2010;**48**(8):2878-84.

28. Proctor RA, Kriegeskorte A, Kahl BC, Becker K, Löffler B, Peters G. *Staphylococcus aureus* Small Colony Variants (SCVs): A road map for the metabolic pathways involved in persistent infections. *Front Cell Infect Microbiol*. 2014;**4**.

29. Tuchscherr L, Löffler B, Proctor RA. Persistence of *Staphylococcus aureus*: Multiple Metabolic Pathways Impact the Expression of Virulence Factors in Small-Colony Variants (SCVs). *Frontiers in Microbiology*. 2020;**11**.

30. Bui LMG, Hoffmann P, Turnidge JD, Zilm PS, Kidd SP. Prolonged growth of a clinical *Staphylococcus aureus* strain selects for a stable small-colony-variant cell type. *Infect Immun*. 2015;**83**(2):470-81.

31. Bui LMG, Kidd SP. A full genomic characterization of the development of a stable Small Colony Variant cell-type by a clinical *Staphylococcus aureus* strain. *Infect Genet Evol*. 2015;**36**:345-55.

32. Tuchscherr L, Buzzola FR, Alvarez LP, Lee JC, Sordelli DO. Antibodies to capsular polysaccharide and clumping factor a prevent mastitis and the emergence of unencapsulated and small-colony variants of *Staphylococcus aureus* in mice. *Infection and Immunity*. 2008;**76**(12):5738-44.

33. Lavery LA, Armstrong DG, Wunderlich RP, Mohler MJ, Wendel CS, Lipsky BA. Risk factors for foot infections in individuals with diabetes. *Diabetes Care*. 2006;**29**(6):1288-93.

34. Irani MH, Maitra PK. Properties of *Escherichia coli* mutants deficient in enzymes of glycolysis. *J Bacteriol*. 1977;**132**(2):398-410.

35. Halsey CR, Lei S, Wax JK, Lehman MK, Nuxoll AS, Steinke L, et al. Amino Acid Catabolism in *Staphylococcus aureus* and the Function of Carbon Catabolite Repression. *mBio*. 2017;**8**(1):e01434-16.

36. Harper L, Balasubramanian D, Ohneck EA, Sause WE, Chapman J, Mejia-Sosa B, et al. *Staphylococcus aureus* Responds to the Central Metabolite Pyruvate To Regulate Virulence. *mBio*. 2018;**9**(1):e02272-17.

37. Parsons JB, Frank MW, Jackson P, Subramanian C, Rock CO. Incorporation of extracellular fatty acids by a fatty acid kinase-dependent pathway in *Staphylococcus aureus*. *Molecular Microbiology*. 2014;**92**(2):234-45.

38. Yu X, Zheng L, Yang J, Lei T, Ji Y. Characterization of essential enolase in Staphylococcus aureus. *World Journal of Microbiology and Biotechnology*. 2011;**27**:897-905.

39. Luo Z, Yue S, Chen T, She P, Wu Y, Wu Y. Reduced Growth of *Staphylococcus aureus* Under High Glucose Conditions Is Associated With Decreased Pentaglycine Expression. *Frontiers in Microbiology*. 2020;**11**(2701).

40. Deutscher J, Francke C, Postma PW. How phosphotransferase system-related protein phosphorylation regulates carbohydrate metabolism in bacteria. *Microbiology and molecular biology reviews : MMBR*. 2006;**70**(4):939-1031.

41. Postma PW, Lengeler JW, Jacobson GR. Phosphoenolpyruvate:carbohydrate phosphotransferase systems of bacteria. *Microbiological reviews*. 1993;**57**(3):543-94.

42. Iyer R, Baliga NS, Camilli A. Catabolite control protein A (CcpA) contributes to virulence and regulation of sugar metabolism in *Streptococcus pneumoniae*. *J Bacteriol*. 2005;**187**(24):8340-9.

43. Chiang C, Bongiorni C, Perego M. Glucose-dependent activation of *Bacillus anthracis* toxin gene expression and virulence requires the carbon catabolite protein CcpA. *Journal of bacteriology*. 2011;**193**(1):52-62.

44. Bischoff M, Wonnenberg B, Nippe N, Nyffenegger-Jann NJ, Voss M, Beisswenger C, et al. CcpA Affects Infectivity of *Staphylococcus aureus* in a Hyperglycemic Environment. *Frontiers in cellular and infection microbiology*. 2017;**7**:172-.

45. Brauner A, Fridman O, Gefen O, Balaban NQ. Distinguishing between resistance, tolerance and persistence to antibiotic treatment. *Nature Reviews Microbiology*. 2016;**14**(5):320-30.

46. Bui LM, Conlon BP, Kidd SP. Antibiotic tolerance and the alternative lifestyles of *Staphylococcus aureus*. *Essays in biochemistry*. 2017;**61**(1):71-9.

47. Garzoni C, Kelley WL. *Staphylococcus aureus*: new evidence for intracellular persistence. *Trends Microbiol*. 2009;**17**(2):59-65.

48. Ou JJ, Drilling AJ, Cooksley C, Bassiouni A, Kidd SP, Psaltis AJ, et al. Reduced Innate Immune Response to a *Staphylococcus aureus* Small Colony Variant Compared to Its Wild-Type Parent Strain. *Front Cell Infect Microbiol*. 2016;**6**:187.

49. Tuchscherr L, Heitmann V, Hussain M, Viemann D, Roth J, Von Eiff C, et al. *Staphylococcus aureus* small-colony variants are adapted phenotypes for intracellular persistence. *J Infect Dis*. 2010;**202**(7):1031-40.

50. Glaser R, Becker K, von Eiff C, Meyer-Hoffert U, Harder J. Decreased susceptibility of *Staphylococcus aureus* small-colony variants toward human antimicrobial peptides. *J Invest Dermatol*. 2014;**134**(9):2347-50.

Chapter 6: Conclusions

The research presented in this thesis encompasses the broad research into the alternative cell types of *Staphylococcus aureus* which can facilitate its survival in stressful environments and thus establish persistent or relapsing infections, in particular this research was utilising DFU and DFI/DFI-OM as model infections. These alternate cell types are complex and can be presented as a result of regulation in response to environmental stresses or more broadly as the selection of stochastically generated cell phenotypes with greater fitness in the specific conditions of a particular niche.

There are various pathways that lead to survival within these stresses through reduced growth and expression of virulence factors. This lifestyle confers antibiotic tolerance and reduces immunogenicity of *S. aureus* allowing it to evade both the immune response and clinical treatment thereby allowing bacterial persistence. An extreme outcome of this cell lifestyle is the formation of Small Colony Variant (SCV).

SCVs are difficult to research for several reasons. One is their extremely slow growth and then the instability of the phenotype results in reversion to the normal cell type. This reversion may occur through stochastic genetic events, and thereby a section of the population of cells will start growing, but not in the original SCV state. Thus, much of the existing research into SCVs has used genetically modified strains of *S. aureus* to generate a stable SCV phenotype. These phenotypes that arise from a single mutation may not truly represent SCV formation within a clinical setting. To address this constricted perspective of SCV formation, we have used research models with a focus on long-term growth to select for SCV. Time-dependent growth allows for the accumulation of genetic events in response to clinically relevant stresses that result in alternate cell types including SCV.

These models involve continuous culture in a chemostat, adaptive laboratory evolution (ALE) and intracellular infection of the SaOS-2 cell line. In addition to using experimental research models to investigate SCV formation, we have conducted a study investigating *S. aureus* in patients with a diabetic foot infection (DFI) to give us a clinical perspective on long-term *S. aureus* infection.

This chapter is partially comprised of a published mini-review.

Chapter 2: The bacteriology of patients with diabetic foot ulcers and diabetic foot infections and incidence of *Staphylococcus aureus* Small Colony Variants.

The conditions of pathogenesis during DFU and DFI parallels our previous research models that selected for SCV. Persistent, long-term infections that are difficult to clear are highly prevalent in cases of DFI as a result of endothelial dysfunction which impairs wound healing and the immune response. *S. aureus* populations are under constant stress from the innate immune response and antibiotic treatments and thus utilises alternate cell types to adapt to these stressful conditions.

This chapter describes data analysis of the epidemiology and bacteriology of patients with diabetes. A total of 153 patients with diabetes were recruited in this study comprised of patients with osteomyelitis (DFI-OM), patients with infected foot ulcers (DFI-W), patients with uninfected foot ulcers (DFU) and healthy patients with no ulcers (controls).

S. aureus was identified as the most prevalent pathogen with 24% of DFI patients harbouring *S. aureus* in the bone (DFI-OM) and wounds of DFI patients. Other bacteria also present were *Pseudomonas aeruginosa* (17.74%) and *Escherichia coli* (8.06%). *S. aureus* infections were represented by differences in their bacterial colony types, with a notable presence of SCV (20% of all infection). Additionally, different colony types were isolated from the patients dependent on the anatomical niche which included the soft tissue of the ulcer, extracellular of the bone

and intracellular of the bone. The high proportion of SCVs highlights the potential to facilitate persistent infection through the formation of dormant, non-pathogenic reservoirs. We characterise the genetic and phenotypic mechanisms that underly the switch to SCVs in Chapter 5 of this thesis.

Chapter 3: Knockout of *mgrA* induces changes in *Staphylococcus aureus* population to adapt to prolonged, low growth conditions

Previous research using continuous culture to grow the *S. aureus* isolate WCH-SK2 at a limited growth rate over a prolonged duration found a cell population dominated by SCVs that were associated with 23 SNPs. Of these genetic events was a notable mutation in the DNA binding domain of the global virulence regulator MgrA.

To determine the role of MgrA in the selection of SCV in these conditions, a knockout mutation of *mgrA* was introduced into WCH-SK2 (WCH-SK2-Δ*mgrA*) with a tetracycline insert. WCH-SK2-Δ*mgrA* was cultured in limited growth conditions within a chemostat over 53 generations (30 days). These conditions select for a highly heterogenous population of cell types. These different cell types fall within a spectrum ranging from metabolic active cells which produce large, pigmented colonies to dormant, slow growing cells producing small, non-pigmented cells.

SCVs were selected within the population after 16 generations (9 days) and a sSCV was isolated after 50 generations (28 days). In comparison, the wildtype WCH-SK2 in the same continuous culture conditions selected for SCV in the population only after 56 generations (16 days) and a sSCV was isolated after 192 generations (55 days). The increased frequency of SCV in WCH-SK2-Δ*mgrA* was associated with an increased rate of mutation which we postulate increases the rate beneficial genetic events that create cell types with greater fitness in limited growth conditions.

A sSCV was isolated after 50 generations (28 days) in continuous culture. This cell type had a reduced rate of growth and increased biofilm production. Whole genome sequences were obtained from this sSCV (WCH-SK2-Δ*mgrA*-SCV d28) and a large, non-pigmented colony (WCH-SK2-Δ*mgrA* d28) to identify the genetic mechanisms which underly the SCV phenotype. A SNP was detected in the phosphoglucosamine mutase *glmM* which controls metabolism and the oxidative stress response via c-di-AMP regulation. Additionally, the WCH-SK2-Δ*mgrA*-SCV had over 50 genomic rearrangements affecting 49 genes compared to the one genetic rearrangement in WCH-SK2-Δ*mgrA*-d28.

Chapter 4: The mechanisms that underly alternate cell types through long-term culture models

Over a period of time under specific conditions such as within host tissue, S. aureus will adapt. We have used two different long-term culture models to investigate the time-dependent adaptations utilised by *S. aureus* survive within clinically relevant conditions. These models address the process of internalisation within osteocytes and tolerance to antibiotics.

SaOS-2 infection

The outcomes of *S. aureus* invasion within the bone are either an inflammatory response or persistence in a dormant state. Persistence in the bone is facilitated by through a down-regulation of virulence factors in the bacteria that limits an immunogenic response. This forms a reservoir of *S. aureus* which are difficult to detect and challenging to clear through the immune response or clinical treatment. We had replicated this phenomenon using the *S. aureus* isolate WCH-SK3 to infect the human osteocyte cell line SaOS-2. Notably, WCH-SK3 was able to persist intracellularly within the osteocytes as a viable but not culturable cell type. Over time, WCH-SK3 was able to revert to a culturable cell type which seemed to be associated with

a restoration in function SNP in the virulence regulator *srrB*. This ability to persist in a dormant, non-viable state and the reversion from a non-culturable to a culturable cell type is a mechanism that facilitates *S. aureus* persisting despite medical intervention seemingly having cleared an infection. SrrB is a component of the SrrAB two component system which represses virulence in low-oxygen conditions. SrrAB is the sole TCS required for survival and growth within osteocytes and so the switch to a functional SrrB may facilitate the culturable cell type. Our research has taken the *S. aureus* cells from osteocytes at day 1 (post-infection) and then when they re-appeared after being in a VBNC state, at day 20 (post-infection). The WGS data uniquely revealed there had been genetic rearrangements and a single SNP (in *srrB*). Consistent with the literature we have postulated that the genetic re-arrangements drive the shift to the VBNC state and the *srrB* SNP has resuscitated the cells to a growing state.

Adaptive laboratory evolution (ALE)

The widespread prevalence of antibiotic resistance in *S. aureus* is a major complication during treatment. However even with increased concentrations of antibiotics, the development of antibiotic tolerance can also create reservoirs of *S. aureus* which are not killed by antibiotics. Thus, despite initial clearance of infection, the process of long-term antibiotic treatments can develop tolerant populations and thus persistent infection.

ALE was used to grow multiple generations of the *S. aureus* isolate UA-DI-55 while exposed to a sub minimum inhibitory concentration (MIC) of the antibiotic ciprofloxacin to identify the subsequent response and adaptations in a time-dependent manner. We found UA-DI-55 developed an increase in both the MIC and minimum bactericidal concentration (MBC). Whole genome sequencing found this evolution of resistance and tolerance was not associated with any genes which directly interacted with ciprofloxacin. This evolution was associated with SNPs in *fur*, an iron response regulator and *ptsH*, a component of a phosphotransferase system.

The loss of *fur* increases intracellular iron and thus increases reactive oxygen species that can cause oxidative damaged induced mutagenesis which can promote the evolution of antibiotic resistances. While *ptsH* has no direct role in resistance against ciprofloxacin, it is involved in carbohydrate transport and inhibition of this activity may lead to reduced metabolic activity and potentially tolerance to antibiotics.

Chapter 5: Characterisation of the transition to alternative cell types of *Staphylococcus aureus* from the bone and wounds of patients with osteomyelitis

In addition to experiments which replicate various aspects of *S. aureus* pathogenesis, we have pairs of *S. aureus* isolates from our clinical study representing alternate cell types which form in response to different anatomical niches. These isolate pairs were cultured from the wound and the bones of DFI patients with osteomyelitis. Whole genome sequences of each isolate identified the genetics associated with the change in cell types. Within the anatomical niche of a DFI, we deduce that the process in which alternate cell types develop is either 1) continuous stochastic changes in the population are selected for in stressful conditions or 2) the environmental conditions signal pathways that result in these genetic events. We have discovered SNPs in genes affecting *S. aureus* pathogenesis in pathways involved in metabolism, cell wall biosynthesis, virulence metal ion regulation and the oxidative stress response. However, there was no common affected pathway which was associated with the different cell types which suggests these genetic events occur stochastically rather than a defined stimulus and response.

Notably, we have a pair of isolates comprising of a stable SCV (sSCV) and a non-stable SCV (nsSCV). The stability of the SCV phenotype suggests there are stable genetic events associated with the cell type that we can identify using whole genome sequencing. SNPs were

identified in the enolase, *eno*, an essential enzyme required for the penultimate step of glycolysis and a defect in this process is potentially the mechanism which gives rise to the SCV phenotype. The inability to complete glycolysis would require ATP to be generated through either partial glycolysis or amino acid catabolism which are much less efficient processes. This mutation in enolase driving a lacking metabolism has not previously been reported in SCV models, or any *S. aureus* strain all together and provides further avenues of research in *S. aureus* metabolism and its role in pathogenesis. In addition to a defect in *eno*, the sSCV was associate with SNPs in *arlS*, the response regulator component of the ArlRS virulence regulator. Defects in virulence are required for evasion of the immune response and are largely responsible for creating hidden, asymptomatic reservoirs that facilitate persistent infection.

Summary

We have used a variety of long-term research models to investigate the development and presence of alternative cell types in a bacterial population and specifically SCV in *S. aureus* and in both in both laboratory and clinical settings. Our research identifies two different paradigms of the shift to a population of SCV: 1) the molecular mechanisms which define the change from a metabolic active, normal cell type to the SCV and 2) the selection of SCV within a population with greater selective fitness within stressful conditions. Much of the previous, existing research on SCV had been based on laboratory generated strains with a single, defined mutation which gives rise to the sSCV phenotype. This limits experimental data on the mechanisms of SCV to these specific mutations when in reality the switch to SCV is not limited to only these pathways. Our long-term research models replicate different aspects of infection which drive the selection of alternative cell types. This data thereby supports novel mechanisms which lead to alternative cell types and SCV.

The use of long-term methods of culturing allowed genetic events to occur in a time-dependent manner in response to clinically relevant stresses including nutrient limitation, antibiotics and internalisation in osteocytes. These environmental stressors selected for different cell types within the population with altered phenotypes. Using whole genome sequencing, we identified pathways involved in metabolism, cell wall biosynthesis, virulence, metal regulation and the oxidative stress response affected by genetic events.

The ultimate goal for this field in research is to establish methods to treat persistent *S. aureus* infections that are facilitated by these alternate cell types. By narrowing research into these laboratory-generated models of SCV, we limit our knowledge to only a small subset of pathways which create SCV. Treatments targeting SCVs based on the current models of SCV actually may not be fully accommodating the breadth of SCVs where there are not all known underlying mechanisms. Our research shifts the focus on the specific mechanisms which lead to SCV formation to the conditions that select for SCV. Through our experiments, we have identified that over time, stresses such as antibiotic stress and nutrient limitation will be overcome through the stochastic formation and selection of alternate cell types. Identifying the pathway of how these develops may provide new research models to better investigate *S. aureus* SCV and novel perspectives into the treatment of persistent and relapsing *S. aureus* infections.