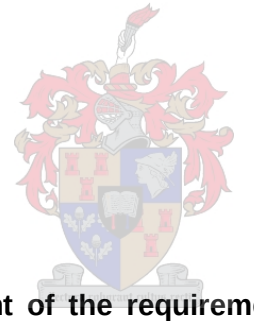
The role of the accessory gene regulator system on biofilm formation and stress response in *Staphylococcus aureus*



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Declaration

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Abstract

Background: Biofilm formation is a key contributor to *Staphylococcus aureus* virulence and pathogenicity. It is regulated by the accessory gene regulator (*agr*) operon, which may become dysfunctional due to genetic mutations. These mutations may affect the expression of key genes like *RNAIII* and *icaA* that are involved in key pathogenesis pathways. Previous studies have associated *agr* dysfunctional strains with strong biofilm formation, persistent infections, and treatment failure. Therefore, this study aimed to determine the impact of *agr* functionality status on biofilm development and antibiotic stress tolerance in clinical *S. aureus* isolates.

Methods: Twelve previously characterized (phenotypically and genotypically) blood culture *S. aureus* isolates, collected from February 2015 to March 2017 at Tygerberg Hospital were selected for this study. Crystal Violet biofilm assay was then performed to assess biofilm formation over a 24-hour period at different time points in the presence and absence of oxacillin, vancomycin, and rifampicin at sub-minimum inhibitory concentrations [sub-MIC: 0.25 µg/ml (oxacillin and vancomycin), 0,005 µg/ml rifampicin] and clinically relevant concentrations (10 µg/ml). The minimum inhibitory concentration (MIC) was determined using the gradient diffusion assay (E-test). Reverse-transcription real-time PCR was used to measure the expression of *RNAIII* and *icaA* genes. Whole genome sequence data were analyzed for genetic differences in the *agr* locus including the *bap*, *icaA,* and *icaD* regions for the 12 isolates, using online platforms (Prokka, Artemis, and BioEdit 7.2).

Result: There was statistically an insignificance increase in the overall biofilm formation levels in *agr* dysfunctional isolates than in *agr* functional isolates in the absence and presence of antibiotics, except for when exposed to sub-MIC of oxacillin (p=0.007). Similarly, an increase in the overall biofilm formation level in *agr* I isolates was observed when compared to *agr* II and *agr* III isolates in the absence and presence of antibiotics. Furthermore, overall methicillin-resistant *S. aureus* (MRSA) isolates produced more biofilm, especially at time point 6 and 8 hours after incubation in the absence of antibiotics; while methicillin-sensitive *S. aureus* (MSSA) isolates formed more biofilm in the presence of antibiotics overall time points. Furthermore, a significant increase in the expression levels of both *RNAIII* (p=0.041) and *icaA* *(p=0.020)* was observed in *agr* dysfunctional isolates when compared to *agr* functional isolates. A significant increase in the expression of *icaA* *(*p=*0.008)* was observed in MRSA isolates; and dysfunctional isolates had more mutations on the *agr*-related gene than functional isolates.

Conclusion: An increase in biofilm formation based on phenotypic *agr* functionality, *agr* type, and methicillin susceptibility profile in the absence or presence of antibiotics was not statistically significant. Additionally, mutations observed on the *agr* locus in *agr* dysfunctional

isolates confirmed the role mutations play on *agr* functionality. The study analyzed 12 isolates, which may decrease statistical power. Therefore, future studies with a larger sample size should confirm or refute these study findings about the role *agr* functionality, *agr* type, and methicillin susceptibility profile have on the ability of clinical *S. aureus* isolates to produce biofilm in the absence and in the presence of antibiotics.

Opsomming

Inleiding: Biofilmvorming is 'n belangrike bydraende faktor vir *Staphylococcus aureus* virulensie en patogenisiteit. Dit word gereguleer deur die geassosieerde geen reguleerder (*agr*) lokus, wat disfunksioneel kan raak as gevolg van genetiese mutasies. Hierdie mutasies beïnvloed die uitdrukking van belangrike gene soos *RNAIII* en *icaA* wat betrokke is by patogenisiteit. Vorige studies het *agr*-disfunksionele stamme geassosieer met sterk biofilmvorming, aanhoudende infeksies en behandelingsmislukking. Hierdie studie het gepoog om die impak van *agr*-funksionaliteit status op biofilm ontwikkeling en antibiotika-stresverdraagsaamheid in kliniese *S. aureus* isolate te bepaal.

Metodes: Twaalf voorheen gekarakteriseerde (fenotipies en genotipies) *S. aureus* isolate is vir hierdie studie geselekteer. Die isolate is van bloedkulture vanaf Februarie 2015 tot Maart 2017 by Tygerberg Hospitaal versamel. ‘n Kristal Violet biofilm-toets is uitgevoer om biofilmvorming oor 'n 24-uur periode op verskillende tydpunte te bepaal in die teenwoordigheid en afwesigheid van oksassilien, vankomisien en rifampisien by sub-minimum inhiberende konsentrasies [sub-MIK: 0.25 µg/ml (oksasillien en vankomisien), 0,005 µg/ml rifampisien] en klinies relevante konsentrasies (10 µg/ml). Die minimum inhiberende konsentrasie (MIK) is bepaal met behulp van die gradiëntdiffusietoets (E-toets). Omgekeerde transkripsie intydse PKR is gebruik om die uitdrukking van die *RNAIII* en *icaA* gene te meet. Heelgenoomvolgordedata van die 12 isolate is ontleed met behulp van aanlyn platforms (Prokka, Artemis en BioEdit 7.2) om genetiese verskille in die *agr*-lokus, insluitend die *bap*-, *icaA*- en *icaD*-streke, te identifiseer.

Resultate: Daar was 'n statisties onbeduidende toename in die algehele biofilmvormingsvlakke in *agr*-disfunksionele isolate in vergelyking met *agr*-funksionele isolate in die afwesigheid en teenwoordigheid van antibiotika, met die uitsondering van blootgestelling aan sub-MIK van oksasillien (p=0.007). 'n Toename in die algehele biofilmvormingsvlak in *agr* I isolate in vergelyking met *agr* II en *agr* III isolate is waargeneem in die afwesigheid en teenwoordigheid van antibiotika. Verder, metisillien-weerstandige *S. aureus* (MWSA) isolate het meer biofilms geproduseer, veral by tydpunte 6 en 8 uur na inkubasie in die afwesigheid van antibiotika, terwyl metisillien-sensitiewe *S. aureus* (MSSA) isolate meer biofilms in die teenwoordigheid van antibiotika by alle tydpunte gevorm het. Verder is 'n betekenisvolle toename in die uitdrukkingsvlakke van beide *RNAIII* (p=0.041) en *icaA* (p=0.020) in *agr* disfunksionele isolate waargeneem in vergelyking met *agr* funksionele isolate. 'n Betekenisvolle toename in die uitdrukking van *icaA* (p=0.008) is in MWSA-isolate waargeneem; en disfunksionele isolate het meer mutasies op die *agr*-verwante gene gehad as funksionele isolate.

Gevolgtrekking: 'n Toename in biofilmvorming gebaseer op fenotipiese *agr* funksionaliteit, *agr* tipe en metisillien-vatbaarheidsprofiel in die afwesigheid of teenwoordigheid van antibiotika was nie statisties betekenisvol nie. Boonop, mutasies wat op die *agr*-lokus in *agr*-disfunksionele isolate waargeneem is, het die rol van mutasies op *agr*-funksionaliteit bevestig. Die studie het 12 isolate ontleed wat statistiese krag kan verminder, daarom is toekomstige studies met 'n groter steekproefgrootte nodig om hierdie studiebevindinge te bevestig of te weerlê.

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List of abbreviations

Agr	Accessory gene regulator
AIP	Auto-inducing peptide
Bap	Biofilm-associated protein
BURP	Based upon repeat patterns
cDNA	Complementary DNA
CI	Confidence interval
CLSI	Clinical and Laboratory Standard Institute
CV	Crystal violet
*eta*A	Exfoliative toxin A
EPS	Extracellular polymeric substance
FnbpA	Fibronectin-binding proteins A
GFs	Growth factors
hla, *hlb*, *hld*	α-, β-, δ-haemolysin
HREC	Health Research Ethics Committee
Ica	Intercellular adhesion cluster
IQR	Interquartile range
MLST	Multilocus sequence typing
MLVA	Multilocus variable tandem repeat analysis
mRNA	Messenger RNA
MRSA	Methicillin-resistant *Staphylococcus aureus*
CA-MRSA	Community-acquired methicillin-resistant *Staphylococcus aureus*
HA-MRSA	Health acquired methicillin-resistant *Staphylococcus aureus*
LA-MRSA	Livestock-associated methicillin-resistant *Staphylococcus aureus*
MSSA	Methicillin susceptible *Staphylococcus aureus*
MSCRAMM	Microbial surface component recognizing adhesive matrix molecules

NCBI	National Center for Biotechnology Information
NGS	Next-generation sequencing
NO	Nitric oxide
OD	Optical density
P2	Promoter 2
P3	Promoter 3
PBP2a	Penicillin-binding protein 2a
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PIA	Polysaccharide intercellular adhesions
PSMs	Phenol soluble modulins
QS	Quorum sensing
Rep-PCR	Repetitive element palindromic polymerase chain reaction
RT-qPCR	Real-time polymerase chain reaction
S. aureus	*Staphylococcus aureus*
SNP	Single-nucleotide polymorphisms
sRNA	Small RNA
SS*mec*	Staphylococcal cassette chromosome mec
Sspa	Staphylococcal serine protease
ST	Sequence Type
Spa	*Staphylococcus aureus* protein A
Spa-CC	*Staphylococcus aureus* protein A – Clonal complexes
Sar	Staphylococcal accessory regulator
SNPs	Single nucleotide polymorphisms
TSB	Tryptic soy broth
TBA	Tryptone bile agar

TSST	Toxic shock syndrome toxin
VNTR	Variable-number tandem repeat
VRSA	Vancomycin-resistant *Staphylococcus aureus*
WSG	Whole genome sequencing
WHO	World health organization

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1. CHAPTER 1: Literature review

1.1. *Staphylococcus aureus*

Staphylococcus aureus (*S. aureus*) is a gram-positive, coccoid, immobile bacterium that often forms grape-like clusters when viewed under the microscope (Lee et al. 2018). *S. aureus* belongs to the firmicutes phylum and under the Staphylococcus genus, which comprises 52 species and 28 subspecies of which *S. aureus*, is the most clinically relevant, as it remains a significant health-care burden and its incidence has been rising over time (Lee et al. 2018). *Staphylococcus aureus* is a facultative organism that can grow in aerobic and anaerobic conditions between 18°C and 40°C and it can also grow in 10% salt concentrations on media and colonies often appear golden or yellowish (Lowy 1998). Typical biochemical identification tests include the catalase test, of which most Staphylococcus species are positive except for *S. aureus* subsp. *anaerobius* and *S. saccharolyticus*. The coagulase test differentiates *S. aureus* from coagulase-negative Staphylococci (CoNS) (Grüner et al. 2007; Lowy 1998; Rasigade, Dumitrescu, and Lina 2014). The Mannitol fermentation test is also used to differentiate *S. aureus* (mannitol positive) from the CoNS (mannitol negative) (Boucher and Corey 2008; Lowy 1998).

Staphylococcus aureus is one of the most concerning pathogens in the world and is currently the leading cause of bacterial infections in humans and animals globally (Lehar et al. 2015). Infections caused by *S. aureus* pose a major health threat in both hospital and community settings worldwide (Lehar et al. 2015). The natural history of humans' colonization with *S. aureus* throughout life is remarkable (Conly and Johnston 2003). Shortly after birth, many infants become colonized with *S. aureus* through human and environmental contact (Conly and Johnston 2003). *Staphylococcus aureus* colonization is usually on the skin, umbilical stump, or gastrointestinal tract in infants and persistently but asymptomatically found on 30% of all humans on the nasopharynx and/or other body sites in healthy individuals (Cogen, Nizet, and Gallo 2008). Although contact with contaminated objects and surfaces might play a role in transmission of *S. aureus* (Vahabi, Nadri, and Izadi 2014). About 20% of the healthy population have persistent nasal colonization with *S. aureus*, and 80% have intermittent colonization (Burian, Wolz, and Goerke 2010). Upon disturbance of the symbiotic balance between microorganism and host, localised or disseminated invasive infection can occur (Ellis and Ong 2014). To date, *S. aureus* is responsible for causing various infections, these include skin and soft tissue infections such as folliculitis, furuncles and carbuncles, impetigo, mastitis, and wound infections (Jeong et al. 2019; Lowy 1998). Skin and soft tissue infections represent approximately 90% of *S. aureus* infections and can lead to the spread of *S. aureus* to other

parts of the body, often resulting in life-threatening complications such as bacteraemia or pneumonia (Jeong et al. 2019). Immunosuppressed or immunocompromised individuals are associated with an increased risk of colonization by *S. aureus*, thus increasing the risk of infections and morbidity (Id et al. 2022). *Staphylococcus aureus's* ability to rapidly adapt to antibiotic pressure and develop antibiotic resistance has generated a lot of interest in the organism in the last half a century (Wangai et al. 2019).

1.2. Methicillin-resistance *Staphylococcus aureus*

Staphylococcus aureus has become increasingly difficult to treat due to the emergence and rapid spread of methicillin-resistant *S. aureus* (MRSA), a strain of *S. aureus* which is resistant to all known β -lactam antibiotics (Lehar et al. 2015). These MRSA strains develop resistance to methicillin by the acquisition of the *mec*A gene, not native to this species, which codes a modified penicillin-binding protein (PBP2a) with low affinity for β -lactams (Stefani et al. 2012). *S. aureus* strains that are resistant to methicillin but lack the *mec*A gene have been found in human and bovine populations in the UK and Denmark (Stefani et al. 2012). MRSA strains resistant to methicillin without the presence of the *mec*A gene were found to carry a *mec*A homolog (*mec*ALGA251) which is approximately 69% similar to the classical *mec*A gene (Cikman et al. 2019). This newly identified gene encodes a protein similar to the PBP2a protein by approximately 63% and the new gene was named *mec*C in 2012 (Cikman et al. 2019).

MRSA infections were first discovered in hospitals and were referred to as hospital-acquired MRSA (HA-MRSA) infections (Fukunaga et al. 2016). However, MRSA infections have since emerged in communities and are now referred to as community-acquired MRSA (CA-MRSA) infections. In addition, MRSA infections have been described in livestock and these are referred to as livestock-associated MRSA (LA-MRSA) infections (Stefani et al. 2012). As a result, MRSA can no longer be considered an exclusive healthcare-associated problem and the fight against such a pathogen is challenging (Stefani et al. 2012). A one-health approach might be warranted (Correia et al. 2019). MRSA infections are spreading globally and they are not caused by a single pandemic strain (Turner et al. 2019). Instead, MRSA tends to occur in waves of infection, often characterized by the serial emergence of predominant strains (Turner et al. 2019). Children, elderly individuals, athletes, military personnel, individuals who inject drugs, persons with an indigenous background or in urban, underserved areas, individuals with HIV or cystic fibrosis, those with frequent health-care contact and those in institutionalized populations, including prisoners are at higher risk of MRSA infections (Turner et al. 2019).

1.3. Epidemiology of *Staphylococcus aureus*

It has been more than a century since *S. aureus* was described but continues to be a serious pathogen for humans (Cheung, Bae, and Otto 2021; Lowy 1998). Despite constant

development and improvement in patient care, *S. aureus* infections remain associated with sizeable morbidity and mortality, both in hospitals and in communities (Cheung et al. 2021; Lowy 1998). The emergence of CA-MRSA has changed the clinical and molecular epidemiology of *S. aureus* over the past two decades. Community-acquired MRSA clones lack classic epidemiological risk factors for MRSA infection, such as healthcare contact, suggesting that CA-MRSA epidemics could originate from unexpected areas and populations (Rasigade et al. 2014).

In Europe, the prevalence of MRSA shows trends of increasing from the northern countries of the continent (Netherlands, Norway, Sweden and Denmark) with a prevalence of <5% compared to the southern countries of the continent (Portugal, *Spa*in, Italy and Greece) with a prevalence of 25–50% (Lee et al. 2018). After years of observing a rise in MRSA prevalence, a steady or decreasing prevalence has been observed in a number of countries in Europe, since the early 2000s due to improved national control interventions (Lee et al. 2018). Moreover, most European countries have seen a decline in HA-MRSA, an increase in human MRSA reservoirs, and an increase in LA-MRSA infections, especially in food-producing animals such as pigs, cattle and poultry especially in the Netherlands, north-western Germany, and *Spa*in, among those who work with these animals (van Alen et al. 2017; Garcia-Alvarez et al. 2012; Lozano et al. 2012).

In the Americas, specifically in the United States the prevalence of MRSA was roughly 53% and this is largely attributed to the emergence of CA-MRSA (Lee et al. 2018; Styers et al. 2006). Similar to what happened in Europe there was also a decline in HA-MRSA (Dantes et al. 2013).

A study by Chen & Huang done in 2014 showed that the dynamics of MRSA in Asian countries have followed those observed in western countries to some extent, including the emergence of CA-MRSA, which now accounts for >50% of *S. aureus* strains in some regions (Chen and Huang 2014). In the Middle East MRSA accounts for 45% of *S. aureus* strains, this is mostly from nasal carriage strains both in children and in adult patients living in the Gaza strip (Tokajian 2014).

As the first case of CA-MRSA was discovered in isolated indigenous communities in Australia in the early 1990s, and as distinct CA-MRSA clones have emerged and spread in Australia and New Zealand, Oceania can be considered the region where CA-MRSA originated. From 2000 to 2011, the burden of staphylococcal disease also increased significantly (Williamson, Coombs, and Nimmo 2014).

In Africa, MRSA prevalence data was variable in coverage and quality (Lee et al. 2018). Most published data came from South Africa, Nigeria, and countries from the Mediterranean basin

like Egypt, Tunisia, and Algeria. Moreover, there was some paucity of data from other countries like Libya, Botswana, Ethiopia, Ivory Coast, Tanzania, and Madagascar (Falagas et al. 2013; Lee et al. 2018). The prevalence of MRSA was lower than 50% in most of the African countries, except for countries like Egypt and the Ivory Coast which had a prevalence of 52% and 55% respectively (Falagas et al. 2013; Lee et al. 2018). This changed as the prevalence of MRSA has risen since 2000 in many African countries, except for South Africa which has had a decrease in MRSA prevalence from 36% in 2006 to 24% during 2007–2011 (Brink et al. 2007; Jansen Van Rensburg, Whitelaw, and Elisha 2012).

1.4. Virulence in *S. aureus*

Staphylococcus aureus is a versatile and highly adaptive opportunistic pathogen capable of colonizing and infecting most tissues in the human body, resulting in a multitude of different clinical outcomes (Cheung et al. 2021; Clarke and Foster 2006; Tong et al. 2015). The success of this organism as a pathogen can be attributed its specific virulence factors. The ability of the organism to adhere to host tissues, which in most cases, infections result from colonization of the host skin or mucosal surfaces.. The organism is able to attach to host tissues by a well-described group of staphylococcal adhesins, the cell wall–anchored surface (CWA) proteins. The adhesions of *S. aureus* are crucial for both host colonization and for the establishment of infections. However, the CWA proteins are part of a complex and diverse arsenal of adhesins (Berry et al. 2022).

Staphylococcus aureus was classically considered an extracellular pathogen; however, it is now known that it invades and persists in non-professional phagocytes. It is capable of evading the immune system resulting in the its internalization by host cells (Alexander and Hudson 2001). The organism does not form a capsule, but has virulence surface components during the infection process. One of the classical components is the ability to form biofilms on both the inserted or implanted biomaterials and on host tissues, which once established are difficult to eradicate and tend to recur. *Staphylococcus aureus* is also equipped with various virulence factors (Cruz et al. 2021), which include factors such as haemolysins, leukocidins, Proteases, Enterotoxins, exfoliating Toxins, immune-modulatory factors, and surface Proteins (Oogai et al. 2011).

These virulence factors are expressed and regulated by complex regulatory networks that allow *S. aureus* to adapt to different host environments (Cruz et al. 2021; Oogai et al. 2011). The accessory gene regulator (agr) and the staphylococcal accessory regulator (sar) systems are the two regulatory systems that are part of an important network modulating the expression of *S. aureus* virulence genes (Bien, Sokolova, and Bozko 2011). The agr and sar systems are activated at specific times during bacterial growth and as a response to a change in the

local environment, to ensure the survival and pathogenicity of *S. aureus* (Archer 1998). The system is responsible for the regulation of virulence genes like the haemolysin genes (hla and hlb) and is intimately associated with the pathogenesis and biofilm formation of *S. aureus* (Gomes-Fernandes et al. 2017)

1.5. Pathogenesis of *S. aureus* infection

Staphylococcus aureus infections are initiated when there is a breach of the skin or mucosal barrier, which allows the bacteria to access adjoining tissues or the bloodstream. In addition, the dissemination of biofilm that can form on indwelling medical devices is also a major facilitator of *S. aureus* infections (Feng et al. 2007; Liu 2010).

For invasive infections, *S. aureus* adheres to endothelial cells and binds through adhesion-receptor interactions (Lowy 1998). It may be phagocytized by endothelial cells, however, *S. aureus* can form small-colony variants within the intra-endothelial-cell milieu fosters to escape host defence mechanisms leading to the development of persistent or recurrent infections (Feng et al. 2007; Lowy 1998).

For skin and soft tissue infections when *S. aureus* enters the skin, neutrophils and macrophages migrate to the site of infection (Tong et al. 2015). To evade the immune response *S. aureus* responds in a multitude of ways, including blocking chemotaxis of leukocytes, sequestering host antibodies, hiding from detection via polysaccharide capsule or biofilm formation, and resisting destruction after ingestion by phagocytes (Tong et al. 2015).

To maintain infection *S. aureus* forms abscesses to ensure that there's bacterial proliferation and infiltration of a large number of leukocytes (Tong et al. 2015). They also form a biofilm to protect the bacteria from phagocyte attacks (Tong et al. 2015).

1.6. Treatment of *S. aureus* infections

Staphylococcus aureus infections are associated with significant morbidity and mortality and are becoming more common and severe (Diering, Maxson & Mitchell, and Freeman 2018). In recent decades, treatment for MRSA has become more difficult due to the evolution of *S. aureus* and the overuse of antibiotics (Guo et al. 2020). Penicillin was the first successful treatment for patients with *S. aureus* infections until resistant strains emerged (Lee et al. 2018). Today, 70% to 90% of *S. aureus* strains are resistant to the drug (Lee et al. 2018). There are currently seven antibiotics in common use against MRSA, which includes vancomycin, daptomycin, linezolid, sulfamethoxazole and trimethoprim (TMP-SMZ), quinupristin-dalfopristin, clindamycin and tigecycline (Okwu et al. 2019). These antibiotics are gradually losing their efficiency as MRSA strains are developing resistance against them (Okwu et al. 2019). Combination therapy with rifampicin has been used as a solution to treat

S. aureus infections as rifampicin can penetrate biofilms and kill organisms in the sessile growth phase (J. Perlroth et.al 2008). Its use as monotherapy has been abandoned because of the rapid development of resistance (J. Perlroth et.al 2008). This is concerning as the globe is running short of drugs/antibiotics available for therapy against *S. aureus* infections (Okwu et al. 2019). There is a global urgency for the development of novel drugs that will be effective in the treatment of *S. aureus* infections (Guo et al. 2020; Okwu et al. 2019).

1.7. *Staphylococcus aureus* genotyping techniques

Genotyping techniques are essential to understand the molecular epidemiology of *S. aureus* within a setting. Applications such as outbreak source tracing in hospitals are important to stop the transmission of the pathogen. In addition, molecular genotyping methods helped in establishing epidemiological investigations, e.g. comparing strains across continents and describing the distribution and prevalence of specific strains (Arakere et al. 2005). The ideal method for *S. aureus* typing should be easy, rapid, reliable, highly discriminatory, and reproducible (Belkum, Peeters, and Buiting 1999). Furthermore, it should be suitable for widespread use, so that the genotyping results obtained in different laboratories could be compared (Mazen M. Jamil Al-Obaidi 2018; Stephens 2008). *Staphylococcus aureus* is frequently genotyped using polymerase chain reaction (PCR)-based techniques like staphylococcal cassette chromosome *mec* (SCC*mec*) typing, multilocus variable tandem repeat analysis (MLVA), and repetitive element palindromic PCR (rep-PCR), as well as sequence typing techniques like *spa* typing and multilocus sequence typing (MLST), whole genome-based techniques like pulsed-field gel electrophoresis (PFGE) and finally whole genome sequencing (WGS) (Mazen M. Jamil Al-Obaidi 2018).

1.7.1. *Spa* typing

Staphylococcus aureus-specific staphylococcal protein A typing (*Spa* typing) is a targeted sequence-based method that targets tandem repeats (about 24 bp in length) on regions of the protein A gene (Alkharsah et al. 2019). The polymorphism of this region is based on the deletions, insertions or duplications of these repeats and punctual mutations (Al-Tam et al. 2012). Each unique sequence of repeats is defined by a value, and a unique combination of values identifies a *spa*-type (Al-Tam et al. 2012). A software called the Random StaphType which uses the based upon repeat patterns (BURP) algorithm enables straightforward sequence analysis and designation of *spa* types via synchronization to a central server (Alkharsah et al. 2019; Elçi 2018; Strommenger et al. 2006).

The advantages of *spa* tying are that it is based on sequencing of a single locus, is less expensive and less time-consuming than other typing methods. Moreover, it has more

discriminative power compared to MLST (Alkharsah et al. 2019). The disadvantage of *spa* typing is it can misclassify particular types due to recombination and/or sequencing errors (Satta et al. 2013).

1.7.2. Multilocus sequence typing

Multilocus sequence typing (MLST) is a highly discriminatory method of characterizing bacterial isolates based on the sequences of ~450-bp internal fragments of seven housekeeping genes (Kingdom 1998). For each gene fragment, the different sequences are assigned as distinct alleles, and each isolate is defined by the alleles at each of the seven housekeeping loci (the allelic profile or sequence type [ST]) (Enright et al. 2000; Kingdom 1998). As there are many alleles at each of the seven loci, isolates are highly unlikely to have identical allelic profiles, by chance isolates with the same allelic profile can be assigned as members of the same clone (Enright et al. 2000; Kingdom 1998). Multilocus sequence typing is fully portable and data can be stored in a single expanding central multilocus sequence database that can be interrogated electronically online (Kingdom 1998). However, it is very expensive to perform and requires a big computational capacity (Patiño et al. 2018).

1.7.3. Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) is considered the “gold standard” for bacterial typing (Neoh et al. 2019). The method involves enzyme restriction of bacterial DNA, and separation of the restricted DNA bands using a pulsed-field electrophoresis chamber, followed by clonal assignment of bacteria based on PFGE banding patterns (Neoh et al. 2019). Pulsed-field gel electrophoresis is a very discriminative approach for *S. aureus* typing (Alkharsah et al. 2019). However, it is laborious, time-consuming and lacks reproducibility among different laboratories in addition to ineffectiveness (Alkharsah et al. 2019).

1.7.4. Other genotyping methods

SCC*mec* typing is a single-step multiplex PCR method used to provide a maximum resolution of the various structural variants of the SCC*mec* element (Kim et al. 2007). SCC*mec* element is a mobile genetic element that plays a role in resistance to methicillin in staphylococcus bacterial species (Hashemizadeh et al. 2019). It encodes for recombinases that mediate the integration and excision of SCC*mec* into and from the chromosome (Saber et al. 2017). Furthermore, SCC*mec* also contains a few other genes such as insertion sequences, transposons, and plasmids (Zong, Peng, and Lü 2011). This method is simple and easy to

perform, but it is limited by its inability to detect the newly identified SCC*mec* types (Kim et al. 2007).

The repetitive element palindromic PCR (rep-PCR) method uses primers that target noncoding repetitive sequences interspersed throughout the bacterial genome and is an established approach for subspecies classification and strain delineation of bacteria (Healy et al. 2005). Repetitive element palindromic PCR is a discriminatory and reproducible tool for microbial subtype analyzes and microbial ecology investigations (Healy et al. 2005).

Multilocus variable tandem repeat analysis (MLVA) is a method that analyzes multiple variable-number tandem repeat (VNTR) loci, which are areas of the bacterial genome that evolve quickly (Noller et al. 2003). It is fast and portable (Noller et al. 2003). The limitation is that it often fails to identify closely and distantly related isolates (Ahlstrom et al. 2015).

Single-nucleotide polymorphisms (SNPs) are single genetic code variations and are usually biallelic (two alternative bases occur) (Vallejos-Vidal et al. 2020). The SNPs are the most common form of variation in the genome and they are extensively used to study genetic differences between individuals and populations. These SNPs may contribute to changes in the genomic sequence, either in the coding (exons), intergenic, or noncoding (introns) region (Vallejos-Vidal et al. 2020). SNPs may also have a great influence on the immune response to pathogenic challenges and diseases outcome, contributing to a range of susceptibility to infections among individuals. Thus, some SNPs may have a protective role, influence the rate of disease progression or even the type of cellular immune response evoked by pathogens The limitation is that next-generation sequencing (NGS) analysis tools are error prone and can lead to false-positive SNPs (Hande Morgil 2020).

Lastly, whole genome sequencing (WGS) is a technique that determines the order of bases in the genome of an organism in one process (CDC 2016). WGS provides comprehensive genetic information which includes all possible genomic targets, as well as additional valuable information on drug resistance, virulence determinants, and genome evolution (Roetzer et al. 2013). Ongoing technological developments are contributing to decreasing costs, and WGS has the potential to become the ultimate tool for diagnostics and pathogen typing and to dramatically amplify the impact of molecular diagnostics on clinical microbiology (Roetzer et al. 2013). Limitation of WGS is that the identification of abnormal variants is dependent on the presence of these sequence variants in the sequencing data. Additionally, certain types of sequence variation are difficult to identify and have not been validated to be reliably detected for current clinical use (El et al. 2013).

1.8. The Accessory gene regulator system: structure and function

The *agr* locus was first identified in 1988 by Orlandi et al. and described in numerous staphylococcal species (Orlandi et al. 1988). The *agr* system is a five-gene locus that is a global regulator of virulence in *S. aureus*. It encodes a two-component transcriptional quorum-sensing (QS) system activated by an auto-inducing, thiolactone-containing cyclic peptide (AIP). The *agr* operon is approximately 3.5 kb in size and consists of two divergent transcriptional units, *RNAII* and *RNAIII*, whose transcription processes are driven by promoters P2 and P3, respectively (Le and Otto 2015). *RNAII* is a 3.5 kb polycistronic mRNA that encodes four polypeptides including sensors and the response regulatory proteins (Abdelnour et al. 1993). The four polypeptides encoded by *RNAII* are *Agr*B, *Agr*D, *Agr*C and *Agr*A (Le and Otto 2015). The *Agr*C polypeptide is a membrane histidine kinase sensor and *Agr*A is the response regulator together they form the *Agr*AC two-component signal transduction system (Novick 2000). The *Agr*D polypeptide encodes the auto-inducing peptide (AIP) and *Agr*B is a multifunctional endopeptidase and chaperone protein that contributes to the maturation and export of AIP (Novick 2000). The *RNAIII* is a 0.5 kb mRNA vital in the regulation of expression of various virulence factors (Abdelnour et al. 1993).

The *agr* locus is activated when the extracellular AIP concentration reaches a threshold due to unfavourable environmental signals and the activity of a variety of other regulators, such as SarA or SrrAB (Le and Otto 2015). Upon binding to the AIP, *Agr*C phosphorylates *Agr*A, which in turn activates expression from the promoters P2 for *RNAII* and P3 for *RNAIII* in addition to several other transcriptional targets (Le and Otto 2015; Novick 2000). The structure of the *agr* system and active pathways used by *agr* system in the regulation of virulence genes is shown in figure 1.

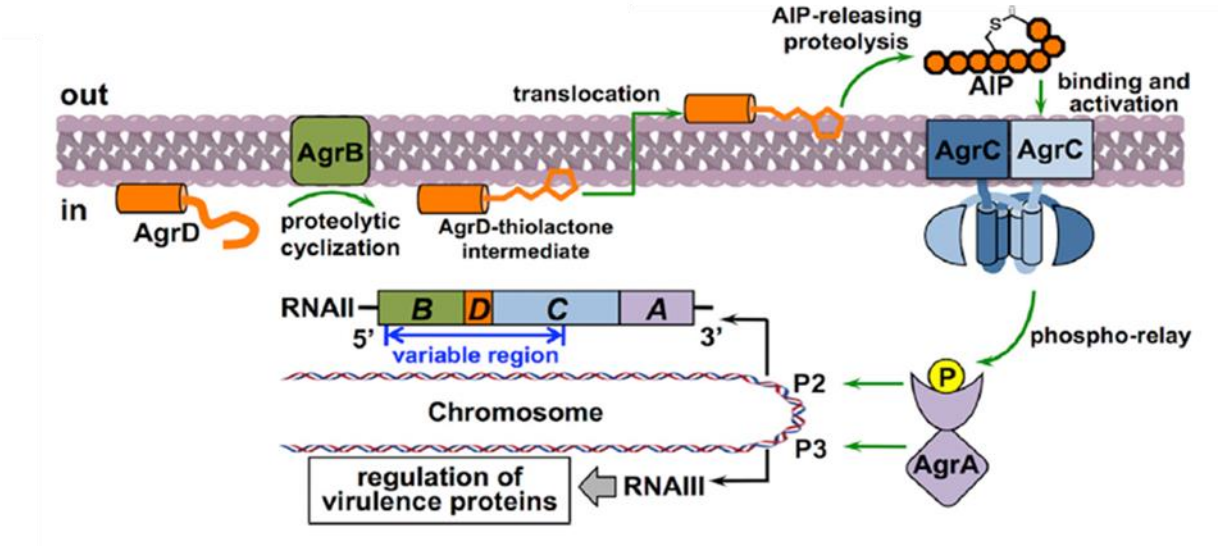

Figure 1.1: The structure and activation of *agr* system which plays a role in the regulation of virulence (source: Wang and Muir 2016).

1.8.1. *RNA III*

RNAIII is one of the largest in the small RNA (sRNAs) family with 514 nucleotides. It regulates virulence gene expression as the intracellular effector of the *agr* system (Gupta, Luong, and Lee 2015).. The structure of *RNAIII*, it contains 14 potential stem-loop structures. Regions in the folded molecule participate in two long-distance interactions (Bronesky et al. 2016; Gupta et al. 2015). The 3' end of *RNAIII* contains several C-rich sequence motifs and unpaired regions that contribute to the initiation of *RNAIII* binding to ribosome binding sites of multiple target mRNAs (Bronesky et al. 2016). A domain at the 3' end of *RNAIII* suppresses the synthesis of several *S. aureus*-specific surface and secreted proteins. (Gupta et al. 2015).

1.8.2. Regulation of virulence genes in *S. aureus*

The *agr* system has shown dual regulatory activity in relation to staphylococcal virulence (Arvidson and Tegmark 2001; Bronner, Monteil, and Prévost 2004). It has the ability to upregulate the expression of a number of exoproteins (such as α-, β-, γ-hemolysin, and leucotoxins), lipases, phenol-soluble modulins (PSMs), and toxic shock syndrome toxins (TSST), and to repress the transcription of a number of cell wall-associated proteins (such as protein A, coagulase, and fibronectin binding protein) (Bronner et al. 2004).

The *agr* system can both directly and indirectly influence the expression of virulence factors (Tan et al. 2018). For instance, the *agr* system controls the expression of PSM by directly binding *Agr*A to PSM promoter regions. Additionally, the *agr* system regulates *RNAIII* to control the expression of the genes for protein A (*spa*), exfoliative toxin A (*etaA*), toxic shock

syndrome toxin-1 (*tsst*), alpha-hemolysin (*hla*), beta-hemolysin (*hlb*), and staphylococcal serine protease (*sspa*). *RNAIII* can upregulate or downregulate the expression of virulence genes by directly base-pairing with target gene populations or indirectly controlling transcriptional regulators like Rot, SarT, and SarS (Arvidson and Tegmark 2001; Bronner et al. 2004; Le and Otto 2015).

1.9. *Agr* types

Staphylococcus aureus isolates can be categorized based on the sequence variation within the *agrC* and *agrD* genes into four types namely *agr* I, *agr* II, *agr* III & *agr* IV (Bibalan et al. 2014). *Agr* typing is a PCR assay used to target the hypervariable regions of the *agr* locus to distinguish *agr* types (Javdan et al. 2019).

Different *agr* types encode distinct AIPs with seven to nine amino acids containing a pentpeptide thiolactone macrocycle and all AIPs can bind to the *agr*C receptors of all four types. However, only an intra*agr*oup AIP–*agr*C interaction leads to activation of the *agr* response (George and Muir 2007). Remarkably, cross-type AIP–*agr*C interactions are inhibitory and can block activation by intratype AIPs (Thoendel et al. 2011). The only exception to this intergroup interference is the cross-activation observed between *agr* I and *agr* IV, which show a single amino acid difference (George and Muir 2007).

Strains belonging to different *agr* types are shown to be associated with different diseases, have different properties and their prevalence varies in different regions (Javdan et al. 2019). Most clinical *S. aureus* strains belonging to *agr* I have been described to be associated with community acquired-MRSA genotypes. While strains belonging to the *agr* II and *agr* III are the main biofilm producers and are associated with HA-MRSA in human isolates (Tan et al. 2018). Moreover, *agr* I and *agr* II are associated with endocarditis and suppurative infections, while *agr* III and *agr* IV are associated with toxic shock syndrome toxin (TSST-1) and *agr* IV is also associated with the generalized exfoliative syndrome and bullous impetigo (Tan et al. 2018).

1.10. *Agr* functionality

The *agr* system is known to be genetically unstable and thus results in spontaneous mutations mostly in the *agrA* and *agrC* genes during *S. aureus* infections (Gor et al. 2019). These mutations are single mutations which are irreversible (Gor et al. 2019). Additionally, these mutations within the *agr* locus tend to alter the coding genes linked to many virulence factors like autolysins and haemolysins leading to altered expression levels. This results in an impaired or dysfunctional *agr* system in some clinical samples (Gomes-Fernandes et al. 2017; Schweizer et al. 2011). *Agr* dysfunctional strains are associated with high patient mortality,

persistent bacteraemia, resistance to antibiotics like vancomycin and a thicker biofilm compared to *agr* functional strains (Gomes-Fernandes et al. 2017; Gor et al. 2019).

Agr functionality status can be determined by the production and presence of delta (δ)-haemolysin, a translational product of *RNAIII* (Novick 2016; Seidl et al. 2011). Production of δ- haemolysin is considered a surrogate for the functionality of the *agr* system and could be detected in-vitro using several phenotypic and genotypic methods like the phenotypic synergistic *agr* functionality assay (Gomes-Fernandes et al. 2017).

1.11. Antibiotic tolerance in *Staphylococcus aureus*

Tolerance and resistance are two different ways by which bacteria evade antibiotic treatment (Levin-Reisman et al. 2019). Tolerance is a population-level phenomenon that enables the population to survive the duration of a transient antibiotic treatment several times above the minimum inhibitory concentration (MIC) without a resistance mechanism (Balaban et al. 2019). Resistance is the inherited or acquired ability of microorganisms to grow in the presence of antibiotics, regardless of the duration of treatment (Levin-Reisman et al. 2019). The formation of multi-layered biofilms is one other mechanism used by *S. aureus* for antibiotic tolerance, while mechanisms like acquisition and mutations on genes like the *mecA* gene are used in antibiotic resistance in these microorganisms (Pinto et al. 2019).

Staphylococcus aureus is one of the many organisms on the World Health Organization (WHO) priority pathogens list for research and development of new antibiotics (Amann, Neef, and Kohl 2019). According to the World Health Organization, new therapeutic agents against *S. aureus* infections are urgently needed due to the increase in antibiotic tolerance and resistance resulting in the need for new therapies is particularly important in biofilm-associated infections (Amann et al. 2019). Last antibiotics alone, such as vancomycin and daptomycin, are no longer effective in treating biofilm-associated infections (Pinto et al. 2019).

1.11.1. Vancomycin

Vancomycin is an antibiotic that is biosynthesized from *Nocardia orientalis* which are organisms found mostly in the soil and belongs to a class of antibiotics called glycopeptides (Sattur et al. 2000). It was first developed 50 years ago as an alternative to penicillin-resistant *S. aureus*. It is now one of the most widely used antibiotics against serious infections caused by gram-positive organisms particularly methicillin-resistant *S. aureus* (Levine 2006).

The mode of action of vancomycin is to inhibit the cell wall synthesis of susceptible bacteria by targeting the (L-Lys)-D-alanyl-D-alanine terminal peptide of the cell wall precursor (Sattur et al. 2000). The interaction prevents the precursor from being added to the growing cell wall

of the bacteria. Moreover, vancomycin alters bacterial cell membrane permeability and RNA synthesis (Sattur et al. 2000).

Vancomycin resistance was first discovered in Enterococci species and the resistance was mediated by transposons mainly found on plasmids (Cong, Yang, and Rao 2020). Vancomycin-resistant *S. aureus* (VRSA) were isolated less than a decade ago and the resistance in these organisms is mediated by a *vanA* gene cluster, which can be transferred from vancomycin-resistant enterococcus to vancomycin-susceptible *S. aureus* (Cong et al. 2020).

1.11.2. Rifampicin

Rifampicin is an antibiotic that belongs to the class of rifamycins and it is a semisynthetic antibiotic derived from *Amycolatopsis mediterranei* (originally classified as *Streptomyces mediterranei*) (Scarpignato and Pelosini 2005). It is a broad-spectrum lipophilic antibiotic primarily used to treat *Mycobacterium tuberculosis* and *Mycobacterium leprae* but can also be used to treat chronic staphylococcal infections (Scarpignato and Pelosini 2005).

The mode of action for rifampicin is to inhibit bacterial RNA synthesis by binding to the β subunit of DNA-dependent RNA polymerase, thus blocking RNA transcription (Somasundaram, Ram, and Sankaranarayanan 2014). Mutations on the RNA polymerase β subunit (*rpoB*) gene on the 81bp region results in resistance and also the alterations in codon526 or codon531 result in high resistance while alterations in codons 511, 516, 518 and 522 result in a low resistance (Somasundaram et al. 2014).

1.11.3. Oxacillin

Oxacillin is a semi-synthetic penicillin-derived antibiotic used to treat a wide range of bacterial infections, particularly staphylococcal infections caused by methicillin-susceptible strains (Gujral, Haque, and Shanker 2009).

The mode of action for oxacillin sodium is that it inhibits the biosynthesis of the bacterial cell wall during bacterial replication by inhibiting penicillin-binding proteins (PBPs) that are involved in the synthesis of peptidoglycan (Tipper 1979).

Oxacillin resistance like other β -lactams antibiotics is mediated by the acquisition of the *mecA* gene which encodes a modified penicillin-binding protein (PBP2a) with a low affinity for β- lactams (Stefani et al. 2012).

1.12. Biofilm formation and regulation

1.12.1. Quorum sensing and biofilm formation

Quorum sensing also referred to as intercellular signalling in *S. aureus* is encoded by *agr* locus (Yarwood et al. 2004; Yarwood and Schlievert 2003). It was developed by staphylococci to improve its ability to cause a variety of human diseases and to occupy numerous niches within the host to enable cell-to-cell communication and regulation of numerous colonization and virulence factors (Yarwood et al. 2004; Yarwood and Schlievert 2003). In *S. aureus* the presence of an active quorum-sensing impedes attachment and development of a biofilm, unlike organisms such as *Pseudomonas aeruginosa* an active quorum sensing promotes attachment and development of robust biofilm under some growth conditions (Yarwood et al. 2004).

Biofilm formation involves the establishment of one or more microorganisms into structured communities of microbial cells embedded in an extracellular polymeric matrix that adheres to implants and host tissues (Chung et al. 2007). The formation of biofilm occurs in stages as shown and explained in figure 1.6.

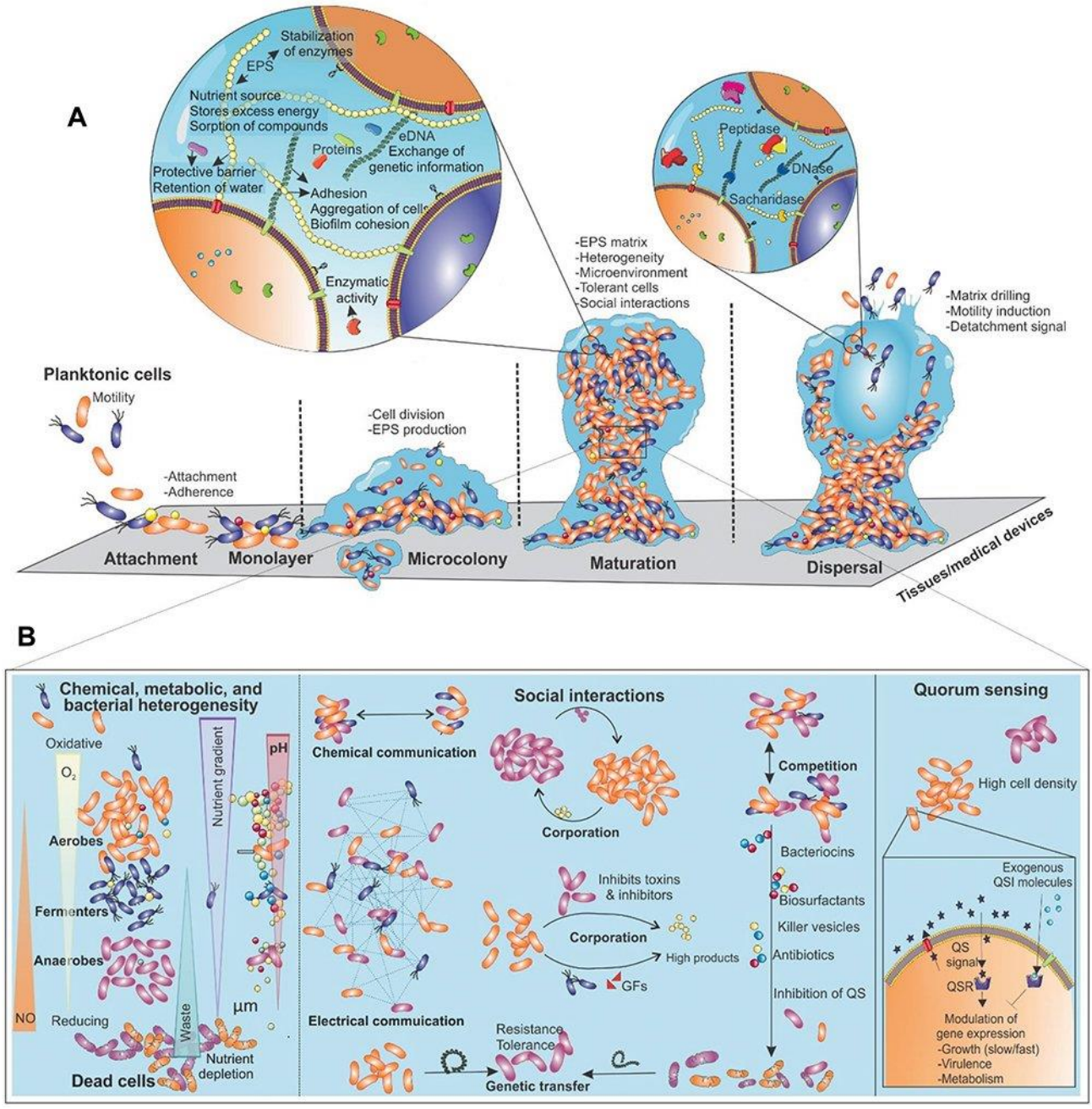

Figure 1.2: The stages and complex structure of bacterial biofilms. (A) Biofilm formation has different stages during which many changes occur. These stages include initial attachment, micro colonisation, maturation, and dispersal. Detachment allows bacteria to colonize new niches. (B) Formation of the EPS matrix leads to the establishment of stable nutrient, pH, waste, and oxygen gradients, forming small-scale and distinct local habitats. Biofilm social connectivity involves positive (competitive or cooperative) and negative (competitive) interactions between bacterial cells that lead to remodelling of the biofilm community. Cooperation is mediated by electrical and chemical communication between cells within the biofilm, whereas competition is mediated by a variety of mechanisms, including bacteriocins, antibiotics, enzymes, and the generation of growth-inhibitory mechanisms such as QS prevention and nutrient depletion. It is mediated by a deadly killing strategy (source: Barzegari et al. 2020).

For *S. aureus* to form biofilms, different virulence factors must be deployed depending on the growth stage (Kong, Vuong, and Otto 2006; Wang and Muir 2016). During the lag phase and early exponential phase, *S. aureus* produces cell wall-associated factors. This factor facilitates attachment and evasion of the host's immune system, allowing the bacteria to aggregate and form biofilms (Kong et al. 2006; Wang and Muir 2016). Still, in the exponential phases, rapid growth occurs at the expense of surrounding nutrients (Roger, Bhakoo, and Zhang 2008). The initial attachment ends and biological processes such as cell division begin to take over. The secretion of polysaccharide intercellular adhesion (PIA) polymers and the presence of divalent cations interact to form stronger bonds between cells. During the stationary phase, a number of cell signalling mechanisms are used by biofilms, collectively termed quorum sensing. Quorum sensing stimulates gene expression of both mechanical and enzymatic processors of alginate, which forms a fundamental part of the extracellular matrix (Roger et al. 2008). The death phase results in biofilm degradation. By breaking down the polysaccharides binding the biofilm together with the help of enzymes produced by the community, surface bacteria are actively released for the colonization of new substrates or surface (Roger et al. 2008).

There are two types of biofilm formations involved in *S. aureus* isolates namely polysaccharide intercellular adhesions (PIA)-dependent biofilm formation and PIA-independent biofilm formation (Nourbakhsh and Namvar 2016). The PIA-dependent biofilm formation requires the presence of the intercellular adhesion cluster (*ica*) locus to form a biofilm, while PIA-independent biofilm formation requires biofilm-associated proteins (*bap*) to form a biofilm (Fajardo et al. 2016).

Biofilms contribute to virulence by protecting pathogens from host defences and impeding the delivery of antibiotics to bacterial cells which results, in treatment failure of biofilm-related infections (Neopane et al. 2018). Biofilm-forming strains are associated with several infections particularly chronic nosocomial infections that are difficult to treat using antimicrobial therapy and require surgical interventions (Beenken et al. 2004; Cramton et al. 1999). The development of biofilms and antibiotic resistance are functionally related in *S. aureus*, as extracellular matrix of the biofilm enables communities of bacteria to exist in close proximity and provides an ideal reservoir for the cellular exchange of plasmids encoding for resistance to antibiotics, thus potentially promoting the spread of bacterial resistance (Bowler, Murphy, and Wolcott 2020; Lade et al. 2019). Horizontal transfer of resistance-conferring genes between bacterial cells within biofilm and has been reported as being 700 times more efficient than among free-living, planktonic bacterial cells (Bowler et al. 2020). Biofilm phenotype expressed can be influenced by the acquisition of antimicrobial resistance (Lade et al. 2019). A study by (Pozzi et al. 2012) showed that the acquisition of methicillin resistance appears to

repress polysaccharide-type biofilm formation and promote formation of proteinaceous-type biofilms (Mccarthy et al. 2015).

1.12.2. Biofilm regulation

Biofilm formation is regulated by global regulators like *agr* and SarA (Nourbakhsh and Namvar 2016). Upregulation of the two-component system arlRS (regulated by the SarA and *agr* systems), is shown to prevent biofilm formation (Fajardo et al. 2016; Nourbakhsh and Namvar 2016). The sarA transcripts (sarA P2, sarA P3, and sarA P1) are elevated during biofilm formation than in the planktonic state and mutations in the SarA locus are associated with reduced biofilm formation (Nourbakhsh and Namvar 2016). The *agr* system regulates biofilm formation by down-regulating expression of cell wall-associated adhesion factors like the microbial surface component recognizing adhesive matrix molecules (MSCRAMMs) which are crucial in the initiation of biofilm formation, for example, in the case of colonisation (Fajardo et al. 2016). Repression of the *agr* system is necessary for the initiation of biofilm formation (Nourbakhsh and Namvar 2016).

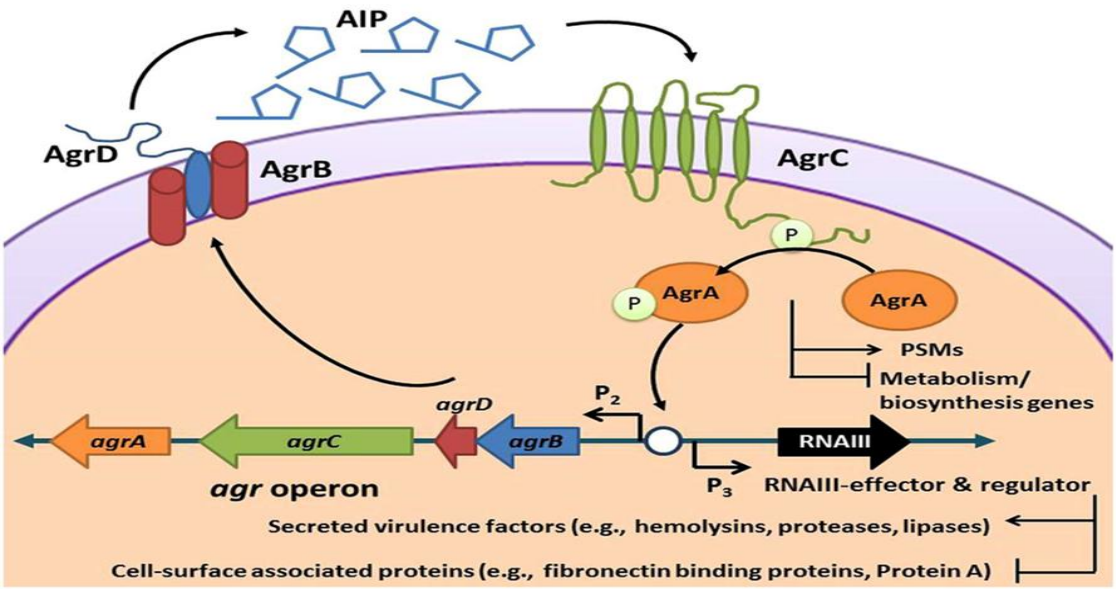

Figure 1.3: Regulation of biofilm formation by the *agr* system. The regulation of biofilm by the *agr* system first P2 drives the transcription of *RNAII* which results in four polypeptides namely the *Agr*D, *Agr*B, *Agr*C, and *Agr*A. *Agr*B transfers the *agr*D that encodes for AIP outside the cell. The AIP accumulates outside due to external signals. When the AIP reaches the threshold, it binds to *agr*C, which phosphorylates *Agr*A, which activates P3, which drives the transcription of *RNAIII*, which is the effector and regulator. Secreted virulence factors like proteases are upregulated to break down the mature biofilm multi-layered structure and cell surface-associated proteins such as protein A and fibronectin proteins responsible for the adhesion of cells to the surface during the start of biofilm formation are downregulated. Before phosphorylation, *Agr*A promotes the upregulation of PSMs which play a role in the dissemination of biofilm during biofilm formation and downregulates the metabolism and biosynthesis of genes need during biofilm formation (source: Quave and Horswill, 2014).

Other regulators of biofilm formation include the sigma operon which upregulates factors like fibronectin-binding protein A (FnbpA) and coagulase which are necessary for the early stages (attachment) of biofilm formation (Nourbakhsh and Namvar 2016).

1.13. The effects of antibiotics on biofilm

Bacterial isolates at the stage of biofilm formation tend to display an increased level of antibiotic resistance because of the extra layer given by the biofilm as compared to their planktonic state and are therefore a major obstacle to infectious disease treatment. Nearly 80% of biofilm-related infections do not respond to antibiotic treatment (Penesyan et al. 2020).

Beta-lactam antibiotics (methicillin, ampicillin, amoxicillin, and cloxacillin) have shown to promote biofilm formation in both MSSA and MRSA strains. This effect has been associated to autolysin-dependent release of eDNA which is an important constituent of biofilms (Kaplan et al. 2012). Additionally, a study by Wen Yu et al. in 2018 demonstrated that sub-MIC concentrations of bacterial cell-wall targeting antibiotics led to enhanced biofilm formation and increased density of biofilm cells in the prominent nosocomial pathogen *E. faecalis* (Wen Yu, Kelsey M. Hallinen 2018). This effect was associated with increased cell lysis accompanied by a surge in eDNA levels, suggesting that such effects may take place in a variety of Gram-positive pathogens (Penesyan et al. 2020).

Aminoglycosides (Amikacin and Gentamicin) did not promote biofilm formation in *S. aureus*, *E.coli* and *P. aeruginosa* (Aranjani and Manuel 2014). Earlier reports show that the sub inhibitory concentration of aminoglycoside antibiotics induces biofilm formation in *P. aeruginosa* and *E.coli* (Aranjani and Manuel 2014). For fluoroquinolones, norfloxacin and ofloxacin were shown to be least potent against biofilm of MRSA, while ciprofloxacin and ofloxacin were the most potent (Masadeh, Alzoubi, and Ahmed 2019).

Antibiotics like daptomycin and vancomycin have shown promising results in the disruption of biofilm of *S. aureus* in combination with other antibiotics like ceftaroline, rifampicin, and fosfomycin (Barber et al. 2015; Christina, Jorgensen, and Rybak 2018; Fujimura et al. 2015; Kamble, Sanghvi, and Pardesi 2022; Shi et al. 2014). Rifampicin has the capacity to kill metabolically dormant sessile bacteria, making it highly useful for biofilm related infections (Munteanu et al. 2017). However, in order to prevent emergence of resistance to rifampicin, it should be only used in combination with another antibiotic agent that is active against *S. aureus*, such as vancomycin or one of the fluroquinolones (Munteanu et al. 2017).

1.14. Biofilm quantification

Quantification of biofilm in cultures is one of the most basic and most commonly acquired types of bacterial measurements; it informs how much biofilm is present. Quantification of

biofilm can be achieved through a variety of direct and indirect methods (Wilson et al. 2018). Direct counting methods permit the enumeration of cells that can be cultured, including plate counts, microscopic cell counts, Coulter cell counting, flow cytometry, and fluorescence microscopy (Wilson et al. 2018). Indirect measurement methods include the determination of dry mass, total organic carbon, microtiter plate assays (Tetrazolium salt and Crystal violet), ATP bioluminescence, total protein, and quartz crystal microbalance (Wilson et al. 2018).

Dye-based methods such as crystal violet (CV) and tetrazolium-based dyes are commonly used to quantify biofilm growth in static microplate assays because they are easy to perform and inexpensive (Haney 2018). The CV assay has become the “gold standard” for quantifying biofilms in a microtiter plate because it is an inexpensive assay that can be routinely performed with relative ease in the lab (Haney 2018). They offer limited high throughput and direct comparison between single- and multi-species biofilms using these methods alone is unlikely to be without bias (Rajamani et al. 2019; Rosca et al. 2022).

1.15. Problem Statement

The *agr* system is key in the regulation of virulence in *S. aureus*. The functionality of this system may be affected due to mutations leading to variations in bacterial physiology by affecting the downstream regulation of several virulence factors, including those involved in biofilm formation. Compared to *agr* functional strains, *agr* dysfunctional strains tend to be associated with resistant to antibiotics like vancomycin and daptomycin leading to treatment failure and persistent infections that are associated with worse clinical outcomes. It is therefore critical to understand the effect of *agr* functionality status on biofilm formation to better improve the management of infections caused by *agr*-impaired isolates.

1.16. Aim

To determine the impact of *agr* functionality status on biofilm development and stress tolerance in clinical *S. aureus* isolates.

1.17. Objectives

- Determine the effect of *agr* functionality status on biofilm formation in *S. aureus* isolates with different genetic backgrounds.
- Investigate the effect of *agr* functionality status on the expression of both *agr* and biofilm related genes.
- Investigate the impact of *agr* functionality status on biofilm production in the presence of antibiotics.
- Investigate the possible genetic changes in both agr and biofilm related genes that might explain the in-vitro phenotypic responses in the different isolates.

2. CHAPTER 2: Biofilm production under antibiotics stress in *S. aureus* isolates with different phenotypic and genotypic characteristics

2.1. Introduction

The *agr* operon plays an important role in the regulation of *S. aureus* virulence including biofilm formation (Jenul and Horswill 2019). *Agr* functionality is determined *in-vitro* by the presence or absence of the delta (δ)-haemolysin toxin (Vuong et al. 2000). *Agr* functional isolates (also known as wild-type strains) have successfully expressed the δ- haemolysin toxin (Boles and Horswill 2008; Vuong et al. 2000). The suppression of the *agr* system is key to the initiation of biofilm formation as it promotes the genes involved in cellular attachment and development of biofilm (Vuong et al. 2000).

Genetic mutations on the *agr* operon may affect the functionality of the regulatory system (Jenul and Horswill 2019). The *agr*A-9A or *agr*A-8A mutations have been associated with *agr* dysfunction (Chong et al. 2013). *Agr* dysfunction (phenotype) has been associated with increased biofilm formation when compared to *agr* function (Barzani, Abdul, and Suleiman 2018). With regard to genotypes, isolates from *agr* II have been associated with high and strong biofilm formation capabilities, followed by isolates from *agr* III (Cafiso et al. 2007; Fabres-klein et al. 2015). MRSA isolates are known to cause a wide range of infections and are associated with robust biofilm formation compared to MSSA isolates (Bat- and Schnall 2022).

Biofilm-forming bacteria such as *S. aureus* are known to exhibit approximately 100-1000 times higher antibiotic resistance than planktonic bacteria (Shin, Yang, and Lim 2021). The formation of biofilm in *S. aureus* has been linked with decreased susceptibility to antimicrobials and immune defences, making it difficult to eradicate most chronic infections caused by biofilm forming *S. aureus* (Lister and Horswill 2014). The relatively slow growth of bacteria in biofilms, the metabolic differences between biofilms and planktonic cells, the poor penetration of antibiotics into biofilms, the relatively slow growth of bacteria in biofilms, and the facilitation of horizontal gene transfer in biofilms all contribute to the evasion of the host immune response and lead to more antibiotic resistance in biofilms than in planktonic bacteria, according to (Shin et al. 2021).

There is lack of research focusing on the effect of *agr* functionality on biofilm formation. *Agr* dysfunction is associated with negative outcomes in hospital settings. There is also a lack of information on how biofilm-forming *S. aureus* behaves in the presence and absence of antimicrobials. Given the dreadful association between biofilm formation and antimicrobial resistance, it is important to understand the behaviour of biofilm formation under antibiotics

stress in *S. aureus* with different phenotypic and genotypic characteristics. This will better improve the management of biofilm-associated infections.

This chapter aims to determine the effects of *agr* functionality on biofilm formation in clinical *S. aureus* isolates with different *agr* types, *spa* types, and methicillin susceptibility profiles and to investigate the impact of *agr* functionality status on biofilm production in the presence of antibiotics.

2.2. Methods

2.2.1. Study design and bacterial isolate selection

This study was a laboratory-based descriptive study that investigated the role of the *agr* functionality status on biofilm formation in response to antibiotic stress. *S. aureus* isolates included in the study were selected from bio-banked clinical isolates stored at the division of Medical Microbiology. A total of twelve isolates collected from blood cultures as part of a previous study from 2015 to 2017 at the Tygerberg Hospital were selected based on *agr* functionality status, *agr* type, *spa* type, and methicillin susceptibility profile (i.e., sensitive or resistant). *Agr* functionality was determined using the phenotypic synergistic *agr* functionality assay, while *agr* type and *spa* type were determined by multiplex PCR assays. Methicillin susceptibility was determined by using the cefoxitin disc diffusion test and confirmed methicillin resistance using the Vitek 2 AES (BioMérieux, France) system.

Table 2.1: Study isolates selection criteria.

Isolate #	*Agr* type	Phenotype	*Spa* type	*Spa*-CC	Methicillin susceptibility
2	I	functional	t037	*Spa*-CC012	MRSA
10	I	functional	t318	*Spa*-CC012	MSSA
66*	I	dysfunctional	t148	*Spa*-CCNF9	MSSA
98	I	dysfunctional	t037	*Spa*-CC012	MRSA
9	II	functional	t509	*Spa*-CC002	MSSA
80	II	dysfunctional	t045	*Spa*-CC002	MRSA
153	II	functional	t045	*Spa*-CC002	MRSA
199	II	dysfunctional	t045	*Spa*-CC002	MSSA
1	III	functional	t012	*Spa*-CC012	MSSA
29	III	functional	t012	*Spa*-CC012	MRSA
46	III	dysfunctional	t012	*Spa*-CC012	MRSA
140	III	dysfunctional	t012	*Spa*-CC012	MSSA

*Isolate re-assigned as functional isolate after analysis of qPCR and WGS results and after repeating the haemolysin assay.

2.2.2. Ethical consideration

The Health Research Ethics Committee (HREC) at the Faculty of Medicine and Health Sciences, Stellenbosch University approved the study. The ethics number is N14/06/065.

2.2.3. Phenotypic synergistic *agr* functionality assay

The phenotypic synergistic *agr* functionality assay was performed as part of a previous study (Abdulgader et al. 2020) and the phenotypic synergistic *agr* functionality assay was used to determine the *agr* functionality of *S. aureus* isolates (Sakoulas et al. 2002) used in this study. The phenotypic synergistic *agr* functionality assay is based on the synergistic activity of β-haemolysin and δ-haemolysin in the haemolysis of sheep red blood cells. *S. aureus* RN4220 is a β-haemolytic control strain that causes partial haemolysis of red blood cells. When a δ-haemolysin producing (*agr* functional) isolate is cross-streaked with RN4220, the synergistic activity of δ- and β-haemolysin results in enhanced or complete haemolysis within the β-haemolysis zone of *S. aureus* RN4220 on sheep blood agar plates, allowing its classification as an *agr* functional isolate (Sakoulas et al. 2002). In this study, we needed to confirm the results of the previous study (Abdulgader et al. 2020) since this assay is generally objective and mostly dependant on the experience of the reader and therefore the assay was repeated due to conflicting results observed between the Real-time PCR, WGS results with the previously performed synergistic assay.

S. aureus strain RN4420 was streaked out vertically down the middle of a sheep blood agar plate (Green Point Media Lab, South Africa) and the test isolates were streaked out perpendicular to RN4220 with the haemolysis zones overlapping. The plates were incubated aerobically at 37°C overnight. The δ-haemolysin activity was indicated by enhanced or complete haemolysis within the β-haemolysis zone of *S. aureus* RN4220. The experiment was performed in duplicate for each isolate and read by at least two individuals to ensure that the results were properly documented. Positive and negative controls, NRS 149 and NRS 155 respectively, were included for each batch**.**

2.2.4. Bacterial growth curve

Growth curves were performed to select time points at which measurement of the planktonic cells and biofilm cells would be taken at 595 nm and 490 nm respectively. At least one-time point at each growth phase (i.e., lag phase, mid-log phase, late-log phase, mid-stationary phase, and late-stationary phase) was selected.

The isolates were cultured overnight on blood agar plates (Green Point Media Lab, South Africa) at 37°C aerobically. Single colonies were picked for each isolate, then inoculated into 5 ml of tryptic soy broth (TSB) (Sigma-Aldrich, USA), and incubated aerobically overnight at 37°C. The overnight cultures of each isolate were diluted with fresh TSB to make 1:200 dilutions. Furthermore, 200µl of each diluted culture was aliquoted into 96 Well Polystyrene Microplates, Clear (Lasec, South Africa), in quadruplicates for each isolate. Twelve (12) 96-well microtiter plates were prepared and pipetted in the same manner. The first plate was taken for optical density (OD) reading at zero hours. The other microtiter plates were incubated aerobically at 37°C and were taken for OD readings at intervals of one hour and the OD readings were observed at 595nm using an iMark™ Microplate Absorbance Reader (Bio-Rad Laboratories; California; USA)**.**

2.2.5. Crystal violet biofilm assay in the absence of antibiotics

This method was performed to describe the biofilm formation between the different *S. aureus* isolates at the chosen time points. The time points were selected using bacterial growth curves as previously described in (2.2.4.). The planktonic and biofilm OD readings were recorded and analyzed.

The twelve (12) isolates were cultured overnight using blood agar plates at 37 °C aerobically to get single colonies for the different isolates. The single colonies were inoculated into 20ml sterilin containers with 5m of fresh Tryptic Soy Broth (TSB) (Sigma-Aldrich, USA), and incubated overnight at 37 °C aerobically. The overnight cultures were diluted with fresh TSB in new Sterilin containers (Thermo Fisher Scientific; USA) to make 1:200 dilutions and 200µl of each diluted culture was transferred onto 96-well microtiter plates in quadruplicates and incubated at 37 °C aerobically.

Two 96-well plates (labelled A and B) were prepared for each time-selected point, incubated for the target duration, and taken out for OD readings. The planktonic cells (supernatant) were transferred onto a new microtiter plate to measure the OD at 595nm for each selected time point. For biofilm analysis, the primary plates with biofilm cells were washed 3 times after planktonic cells were aspirated out. The plates were washed with distilled water, and then the plates were heat-fixed and dried at 60°C for an hour in the incubator. Crystal violet (CV) was added to each dry well containing the fixed biofilm and incubated for 15 minutes at room temperature (+/- 26-28 ^{o}C). The excess crystal violet was discarded, and the plate was washed again, with distilled water and allowed to dry for 30 minutes near an open flame. Acetic acid (30%) was added to the wells and the plate was taken for an OD reading at 490nm. Fresh TSB media was used as a negative control, while the *S. aureus* RN4220, a well-characterized

biofilm-producing strain, was used as a positive control. All isolates were categorized on biofilm-forming capacity, as either non-biofilm formers, weak-biofilm formers, moderate biofilm formers or strong-biofilm formers as previously described by (Singh et al. 2017).

Equations: The following equations were used to categorize isolates based on biofilm-forming capacity.

OD_{cut} = OD_{avg} of negative control + 3 x standard deviation (SD) of ODs of negative control

1. *$OD \leq OD_{cut}$ = Non-biofilm-former (NBF).*
2. *$OD_{cut} < OD \leq 2 \times OD_{cut}$ = Weak biofilm-former (WBF)*
3. *$2 \times OD_{cut} < OD \leq 4 \times OD_{cut}$ = Moderate biofilm-former (MBF)*
4. *$OD > 4 \times OD_{cut}$ = Strong biofilm-former*

2.2.6. Selections of the Sub-clinical-minimum inhibitory (sub-MICs) and Serum-level concentrations

The arbitrary subclinical MICs were selected based on the MICs obtained by an E-test. The lowest MICs measured when exposed to vancomycin, oxacillin, and rifampicin were selected and rounded off to the nearest 100^{th} of the lowest MICs for both vancomycin and oxacillin, but for rifampicin, it was rounded off to the nearest 1000^{th} of the lowest MICs due to low MICs. The Serum-level concentrations were selected based on clinically acceptable doses of vancomycin, oxacillin, and rifampicin, oxacillin as described in the literature by (Garnham et al. 1976; Https://www.accessdata.fda.gov/drugsatfda_docs/label/2016/050638s019lbl.pdf 2016; Rybak et al. 2009).

2.2.7. Sub-clinical-minimum inhibitory concentrations (sub-MICs)

The gradient diffusion assay (E-test) was performed to determine bacterial MICs of the different antibiotics (vancomycin, oxacillin, and rifampicin) for all isolates.

Before performing this method, the E-test strips were taken out of the fridge and left on a bench to reach room temperature. The isolates were cultured overnight in a blood agar plate at 37 °C aerobically. The next day, pure single colonies were picked and suspended in sterile saline to make a 0.5 M McFarland solution. The standardised suspension was inoculated and spread on Mueller Hinton agar plates. The E-test strips of each antibiotic (vancomycin, oxacillin, and rifampicin) were placed on top of the spread on the Mueller Hinton agar plates and then incubated overnight at 37 °C aerobically. The Clinical and Laboratory Standard Institute (CLSI) guidelines were used for MICs interpretations.

2.2.8. Crystal violet biofilm assay in the presence of antibiotics

This method was performed to determine biofilm formation in the presence of different antibiotics (vancomycin, oxacillin, and rifampicin) at the selected time points amongst the different isolates.

The twelve isolates were cultured overnight on blood agar plates at 37 °C aerobically to get single colonies of each isolate. The single colonies were inoculated into 20ml sterilin containers containing 5m of fresh TSB, the containers were then incubated overnight at 37 °C aerobically. The overnight cultures were diluted with fresh TSB in new Sterilin containers to make 1:200 dilutions. From each diluted culture 200µl was aliquoted onto eight 96-well microtiter plates in triplicates and then incubated at 37 °C aerobically. The plates were incubated for 4 hours, to allow the bacteria to grow. After 4 hours the different antibiotics (vancomycin, oxacillin, daptomycin, and rifampicin) with different concentrations [Sub-minimum inhibitory concentrations (sub-MICs) and serum levels concentrations] were added to the plates and then incubated. Time-point 4 (4 hours after incubation) was recorded as the start point for the bacteria. The planktonic cells were transferred onto a new microtiter plate to measure the OD and performed CV assay as described in section 2.2.4.

2.3. Statistical analysis

Microsoft Excel was used for basic descriptive statistics and graphs. STATA BE: Basic edition v.17 software was used to analyze all OD data. A p-value of <0.05 was considered statistically significant. The mean±standard deviation was reported for normally distributed OD data, while the median and interquartile range was reported for not normally distributed OD data. Linear regression test was used to assess the differences in means/medians of ODs in biofilm and planktonic cells by *agr* functionality (*agr* functional and *agr* dysfunctional), *agr* types (*agr* I, *agr* II and *agr* III) and methicillin susceptibility profile (MSSA and MRSA) at individual time points. Even though most of the OD data was not normally distributed the linear regression and mixed-effects (ML) regression model use the differences in means to get a coefficient value, which indicates the increase or decrease compared to reference variable. *Agr* functional isolates, MSSA and *agr* I isolates respectively were used as reference groups when comparing to the other isolates in their selected criteria. Difference in means at each time point was calculated using the formula e.g., mean = mean $_{(agr\ \text{dysfunctional at t4})}$ − mean $_{(agr\ \text{functional at t4})}$. A positive difference in mean/median meant that there was a decrease in the reference group than the group it's compared to while a negative difference meant the opposite. Mixed-effects ML regression model was used to check for significant differences in the overall (the overall median/mean across all the time points combined) biofilm and planktonic cell levels between *agr* functional and *agr* dysfunctional isolates, MRSA and MSSA, and the different *agr* types.

In this study overall or across all time points in the absence of antibiotics is defined as total biofilm formed during the OD reading at time point 2, 4, 6, 8 and 24 hours after incubation. Overall or across all time points in the presence of antibiotics is defined as the total biofilm formed at the time of OD reading at time point 6, 8 and 24 hours after the addition of the antibiotics.

2.4. Results

2.4.1. The phenotypic synergistic *agr* functionality assay

The *agr* functionality of 11 out of the 12 isolates used in this study matched what was previously observed by Abdulgader and co-workers (Abdulgader et al. 2020). However, one isolate (sample ID 66) was reported previously to be *agr* dysfunctional, while in this study it was *agr* functional. We therefore reassigned it as a functional isolates throughout the study, as described in the methods section under 2.2.3.

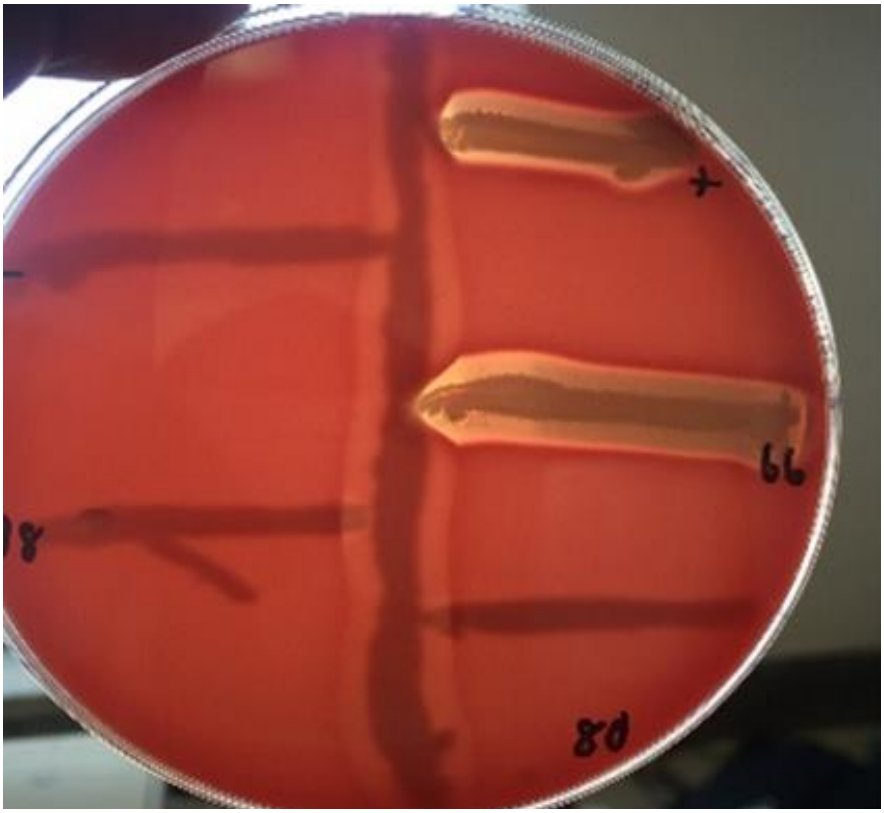

Figure 2.1: The phenotypic synergistic *agr* functionality assay results. There is complete haemolysis (*agr* functional) for the positive control NRS 149 and isolate 66. While no haemolysis (*agr* dysfunctional) for the negative control NRS 155, isolate 98 and isolate 80.

2.4.2. Bacterial growth curve

A time point was defined as the time that had lapsed after initial bacterial inoculation and plate incubation. Five time points were selected for the study using the bacterial growth curves. The time points selected were at 2, 4, 6, 8, and 24 hours, respectively as shown in (Figure 2.1). The time points represented specific bacterial state; time point 2 (t2) represents the lag phase, time point 4 (t4) represents the log phase, time point 6 (t6) represents late log phase/start of the stationary phase, time point 8 (t8) represents the stationary phase and time point 24 (t24) represents the late stationary phase.

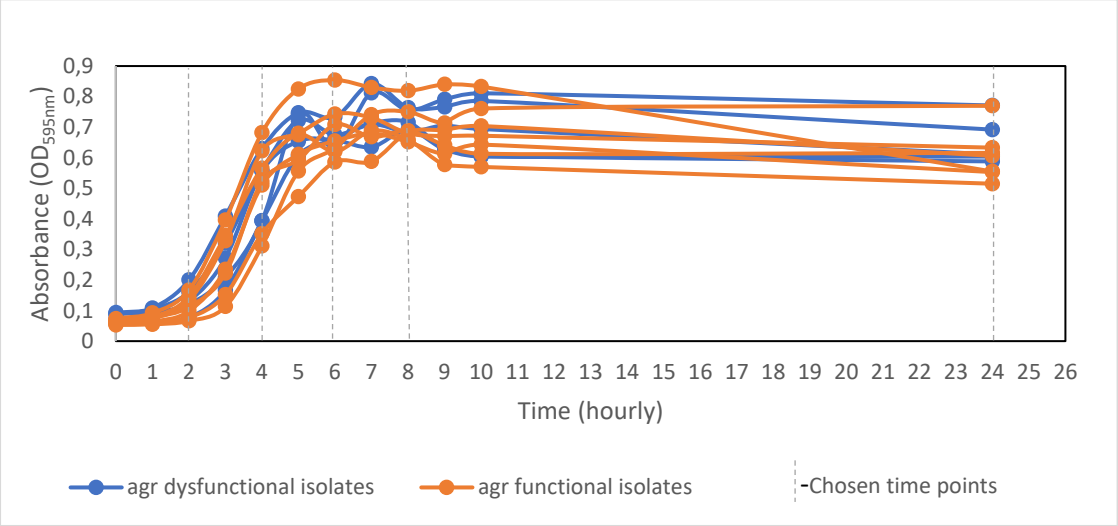

Figure 2.2: Bacterial growth curves of all twelve (12) isolates categorized based on *agr* functionality and chosen time points. *Agr* functional isolates are shown in orange while dysfunctional in blue. The dotted line indicates the selected time points.

2.4.3. Crystal violet biofilm assay in the absence of antibiotics

2.4.3.1. Biofilm formation based on Phenotypic characteristic

Overall, although not significant (p=0.879), there was an increase in biofilm formation in *agr* dysfunctional isolates compared to *agr* functional isolates by 0.0025 OD (Table 2.2).

Table 2.2: The difference in biofilm formation between functional and dysfunctional isolates at different time points and overall.

N=12	Time points	*Agr* functionality	Median (IQR)	OD difference in mean (individual time points)	OD difference in mean (Overall)	*p*-value	Overall *p*-value
7	2	functional	0.012 (0.007-0.016)	0.0004	0.0025	0.889	0.879
5		dysfunctional	0.013 (0.010-0.013)				
7	4	functional	0.043 (0.035-0.048)	0.0074		0.572	
5		dysfunctional	0.065 (0.051-0.066)				
7	6	functional	0.068 (0.050-0.122)	0.0407		0.323	
5		dysfunctional	0.070 (0.062-0.185)				
7	8	functional	0.110 (0.095-0.134)	0.0904		0.326	
5		dysfunctional	0.125 (0.086-0.411)				
7	24	functional	0.262 (0.182-0.400)	0.2234		0.308	
5		dysfunctional	0.181 (0.15-0.871)				

Note: OD; optical density, IQR; interquartile range. p-value of <0.05 was considered statistically significant.

2.4.3.2. Categorization of isolates based on biofilm-forming capacity

After 24 hours, 9 of the 12 (75%) isolates were categorized as strong biofilm formers, five of which were *agr* functional (OD_{495} = 0.182-0.527) and four were *agr* dysfunctional (OD_{495} = 0.181-1.302). The remaining three (3) isolates were moderate biofilm formers one being *agr* functional (OD_{495} = 0.126) and two *agr* dysfunctional (OD_{495} = 0.112 and 0.15 respectively).

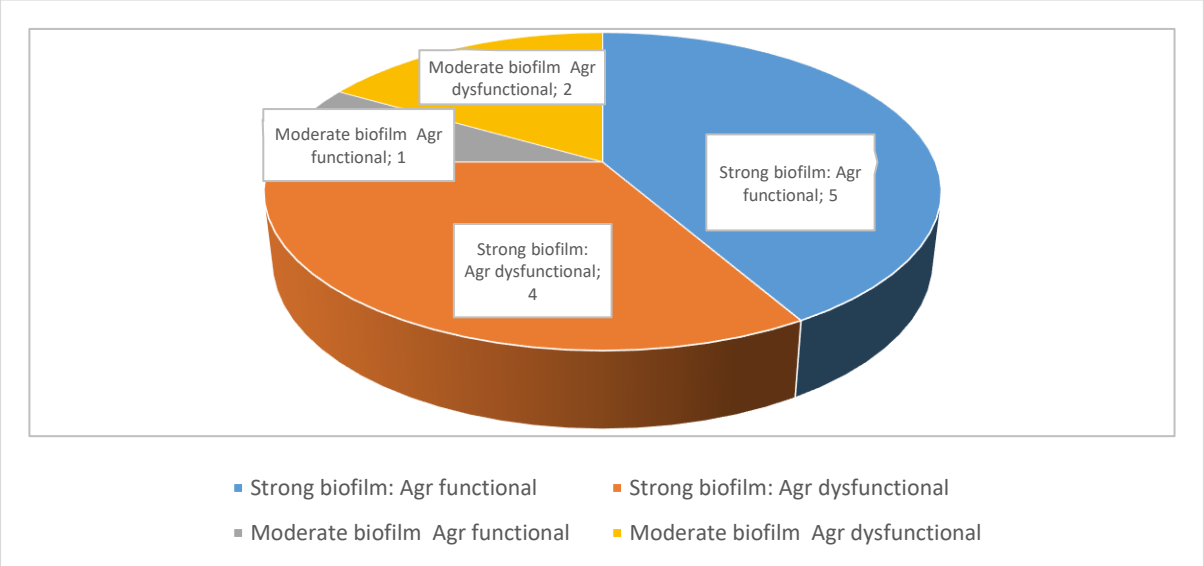

Figure 2.3: Categorization of biofilm formation capacity between *agr*-functional and *agr*-dysfunctional isolates.

2.4.3.3. Biofilm formation based on genetic background

For *agr* types, *agr* I was used as a reference to compare biofilm formation between *agr* II and *agr* III. We noted an increase in the overall biofilm formation in *agr* I isolates compared to *agr* II isolates by 0.031 OD, however, this did not reach statistical significance (p=0.111). A similar pattern was observed at the individual time points (t2, t4, t6, t8 and t24) (Table 2.3). For *agr* I and *agr* III, there was a significant increase in the overall biofilm formation in *agr* I isolates compared to *agr* III isolates by 0.042 OD (p=0.029). This increase was only evident at t2 (p=0.002) and t6 (p=0.034) for *agr* I isolates.

Table 2.3: The difference in biofilm formation between *agr* I, *agr* II and *agr* III isolates at different time points.

N=12	Time points	*Agr* type	Median IQR)	OD difference in mean (individual time points)	OD difference in mean (overall)		p value	Overall p value	
					I vs II	I vs III		I vs II	I vs III
4	2	II	0.012 (0.011-0.013)	-0.0048	-0.0305	-0.0418	0.055	0.111	0.029
4		I	0.017 (0.015-0.019)	-0.0095			**0.002**		
4		III	0.006 (0.004-0.01)						
4	4	II	0.047 (0.043-0.061)	-0.0138			0.354		
4		I	0.057 (0.047-0.085)	-0.0255			0.104		
4		III	0.034 (0.031-0.05)						
4	6	II	0.065 (0.056-0.127)	-0.0605			0.166		
4		I	0.126 (0.110-0.194)	-0.1005			**0.034**		
4		III	0.051 (0.034-0.069)						
4	8	II	0.103 (0.091-0.303)	-0.0608			0.569		
4		I	0.248 (0.129-0.386)	-0.1678			0.137		
4		III	0.091 (0.064-0.115)						
4	24	II	0.222 (0.147-0.782)	-0.0353			0.895		
4		I	0.435 (0.301-0.699)	-0.2855			0.300		
4		III	0.166 (0.138-0.291)						

Note: OD; optical density, IQR; interquartile range. A p-value of <0.05 was considered statistically significant. Statistically significant observations are bolded.

Based on methicillin susceptibility, MRSA isolates had an increase in the overall biofilm formation level compared to MSSA isolates by 0.021 OD, this did not reach statistical significance (p=0.201). This increase was evident at t6 and t8 (Table 2.4).

Table 2.4: The difference in biofilm formation between MRSA and MSSA isolates at different individual time points and overall.

N=12	Time point (hours)	Methicillin susceptibility	Median IQR	OD difference in mean (individual time points)	OD difference in mean (overall)	*p* value	Overall *p* value
6	2	MSSA	0.013 (0.012-0.016)	-0.0025	0.0211	0.403	0.201
6		MRSA	0.011 (0.004-0.013)				
6	4	MSSA	0.057 (0.043-0.071)	-0.1467		0.245	
6		MRSA	0.044 (0.035-0.051)				
6	6	MSSA	0.083 (0.067-0.122)	0.0055		0.895	
6		MRSA	0.066 (0.05-0.129)				
6	8	MSSA	0.109 (0.076-0.134)	0.3717		0.689	
6		MRSA	0.118 (0.105-0.361)				
6	24	MSSA	0.303 (0.258-0.4)	-0.1297		0.262	
6		MRSA	0.166 (0.126-0.527)				

Note: OD; optical density, IQR; interquartile range. A p-value of <0.05 was considered statistically significant.

2.4.4. Crystal violet biofilm assay in the presence of antibiotics

2.4.4.1. Selection of sub-MICs and Serum-level concentrations

MIC testing was done on all 12 isolates for oxacillin, vancomycin and rifampicin (Supplementary Table 1.1). Accordingly, the sub-MICs 0.25 µg/ml, 0.25 µg/ml and 0.005 µg/ml were selected for oxacillin, vancomycin and rifampicin, respectively. For Serum-level concentrations, 10 µg/ml was selected for all three antibiotics (Table 2.6).

Table 2.5: The clinically acceptable doses, Serum-level concentrations and duration the Serum-level concentrations last of vancomycin, Oxacillin and rifampicin.

Antibiotic	Acceptable dose*	Serum-level concentrations	duration	Reference
Vancomycin	15-20 mg	15µg/ml	8-12h	(Rybak et al. 2009)
Oxacillin	500 mg	10.9µg/m	30 minutes	(Container et al. 2016)
Rifampicin	600 mg	8.2-11.7µg/ml	2-4h	(Garnham et al. 1976)

*Concentration used in clinical practice

Table 2.6: Selected sub-MICs and Serum-level concentrations.

Antibiotic	Selected sub-MICs (µg/ml)	Selected Serum-level concentrations (µg/ml)
Vancomycin	0.25	10
Oxacillin	0.25	10
Rifampicin	0.005	10

2.4.4.2. Sub-MICs of Oxacillin, vancomycin and rifampicin on biofilm based on phenotypic characteristic

When *S. aureus* cultures with different *agr* functionality were exposed to sub-MICs of vancomycin and rifampicin, there was an increase in biofilm formation by *agr* dysfunctional isolates at all individual time points (t6, t8 and t24) and across all time points (overall) when compared to *agr* functional isolates. However, this increase in biofilm formation at individual time points or overall was not statistically significant (Table 2.7). Moreover, a significant increase in the overall biofilm formation was observed (p=0.007) in *agr* dysfunctional isolates compared to *agr* functional isolates when exposed to the sub-MICs of oxacillin. However, no significant increase in biofilm formation was observed between *agr* functional isolates and *agr* dysfunctional isolates at individual time points (t6, t8 and t24) when exposed to the sub-MICs of oxacillin (Table 2.7).

Table 2.7: The difference in biofilm formation in the presence of sub-MIC concentrations of oxacillin, vancomycin & rifampicin between functional and dysfunctional isolates at different time points and overall.

N=12	Time points in the presence of antibiotics (hours)	*Agr* functionality		Oxacillin				Vancomycin					Rifampicin				
			Median (IQR)	OD difference in mean (individual time points)	OD difference in mean (overall)	*p*-value	Overall *p-value*	Median (IQR)	OD difference in mean (individual time points)	*OD difference in mean (overall*	*p*-value	Overall *p*-value	Median (IQR)]	OD difference in mean (individual time points)	OD difference in mean (overall)	*p*-value	Overall *p*-value
7	T6	functional	0.100 (0.071-0.164)	0.119	0.139	0.185		0.066 (0.052-0.074)	0.091		0.085		0.081 (0.049-0.108)	0.122		0.220	
5		dysfunctional	0.123 (0.081-0.296)					0.114 (0.070-0.261)					0.076 (0.067-0.269)				
7	T8	functional	0.111 (0.081-0.179)	0.181		0.059	**0.007**	0.174 (0.119-0.243)	0.112	0.089	0.277	0.087	0.133 (0.109-0.191)	0.128	0.139	0.204	0.091
5		dysfunctional	0.405 (0.122-0.466)					0.152 (0.140-0.389)					0.160 (0.147-0.312)				
7	T24	functional	0.311 (0.228-0.493)	0.165		0.399		0.258 (0.252-0.370)	0.124		0.416		0.302 (0.257-0.372)	0.146		0.138	
5		dysfunctional	0.413 (0.165-0.642)					0.202 (0.157-0.539)					0.341 (0.311-0.586)				

Note: AB; antibiotic, OD; optical density, IQR; interquartile range. A p-value of <0.05 was considered statistically significant. Statistically significant observations are bolded.

2.4.4.3. Sub-MICs of Oxacillin, vancomycin and rifampicin on biofilm based on genetic background

When exposing *S. aureus* cultures to sub-MICs of oxacillin, vancomycin and rifampicin, we observed an increase in biofilm formation at individual time points (t6, t8 and t24) and overall in *agr* I isolates when compared to *agr* III isolates by 0.042 OD, 0.073 OD and 0.116 OD, respectively (Table 2.8). However, the increase in biofilm formation observed between *agr* I isolates and *agr* III was not significant at individual time points and overall. Furthermore, an increase in biofilm formation in *agr* I isolates was observed when compared to *agr* II isolates overall when exposed to sub-MICs of oxacillin and vancomycin respectively. The opposite was observed for the exposure of sub-MICs of rifampicin, an increase in biofilm formation was observed in *agr* II when compared to *agr* I isolates overall. Moreover, the increase in biofilm formation between *agr* I and *agr* II was not significant at individual time points and across all time points when exposed to sub-MICs of oxacillin, vancomycin and rifampicin (Table 2.8).

Table 2.8: The difference in biofilm formation between *agr* I, *agr* II and *agr* III in the presence of sub-MICs of oxacillin, vancomycin and rifampicin at individual time points and overall.

N=12	Time points in the presence of antibiotics (hours)	*Agr* type	Oxacillin					Vancomycin					Rifampicin				
			Median (IQR)	OD difference in mean (individual time points)	p-value	OD difference in mean (overall)	Overall p-value	Median (IQR)	OD difference in mean (individual time points)	p-value	OD difference in mean (overall)	Overall p-value	Median (IQR)	OD difference in mean (individual time points)	p-value	OD difference in mean (overall)	Overall p-value
						I vs II / I vs III	I vs II / I vs III				I vs II / I vs III	I vs II / I vs III				I vs II / I vs III	I vs II / I vs III
4	T6	II	0.109 (0.083-0.354)	0.026	0.809			0.07 (0.061-0.196)	-0.001	0.988			0.058 (0.046-0.342)	0.024	0.844		
4		I	0.166 (0.153-0.232)	-0.122	0.274			0.100 (0.063-0.195)	-0.067	0.339			0.154 (0.106-0.234)	-0.103	0.412		
4		III	0.074 (0.051-0.091)					0.051 (0.035-0.09)					0.072 (0.054-0.079)				
4	T8	II	0.313 (0.121-0.492	0.081	0.481			0.107 (0.09-0.401)	-0.023	0.857			0.154 (0.082-0.435)	0.061	0.631		
4		I	0.199 (0.103-0.347)	-0.123	0.296	-0.045	-0.093	0.257 (0.209-0.33)	-0.110	0.405	-0.076	-0.058	0.173 (0.135-0.26)	-0.069	0.587	0.041	-0.083
4		III	0.097 (0.054-0.151)			0.522	0.180	0.146 (0.133-0.185)			0.258	0.389	0.128 (0.104-0.154)			0.691	0.423
4	T24	II	0.453 (0.412-0.87)	0.263	0.216			0.317 (0.207-0.705	0.109	0.544			0.314 (0.275-0.580)	0.039	0.762		
4		I	0.367 (0.183-0.574)	-0.187	0.372			0.312 (0.239-0.454)	-0.140	0.442			.356 (0.280-0.498)	-0.068	0.596		
4		III	0.212 (0.101-0.285					0.227 (0.158-0.256)					0.326 (0.284-0.357)				

Note: OD; optical density, IQR; interquartile range. A p-value of <0.05 was considered statistically significant.

Table 2.9: The difference in biofilm formation in the presence of sub-MICs of oxacillin, vancomycin & rifampicin between MRSA and MSSA isolates at different time points and overall.

N=12	Time points in the presence of antibiotics (hours)	Methicillin susceptibility profile	Oxacillin					Vancomycin					Rifampicin				
			Median (IQR)	OD difference in mean (individual time points)	p-value	OD difference in mean (overall)	Overall p-value	Median (IQR)	OD difference in mean (individual time points)	p-value	OD difference in mean (overall)	Overall p-value	Median (IQR)	OD difference in mean (individual time points)	p-value	OD difference in mean (overall)	Overall p-value
6	T6	MSSA	0.132 (0.081-0.168)	-0.069	0.453			0.072 (0.055-0.114)	-0.009	0.877			0.091 (0.076-0.108)	-0.058	0.567		
6		MRSA	0.109 (0.066-0.141)					0.068 (0.052-0.130)					0.067 (0.042-0.199)				
6	T8	MSSA	0.103 (0.081-0.179)	0.071	0.488			0.157 (0.126-0.243)	-0.046	0.659			0.149 (0.133-0.191)	-0.080	0.431		
6		MRSA	0.224 (0.122-0.405)			-0.026	0.671	0.185 (0.094-0.271)			0.003	0.955	0.132 (0.099-0.208)			-0.085	0.333
6	T24	MSSA	0.244 (0.137-0.412)	0.035	0.859			0.257 (0.224-0.376)	-0.081	0.594			0.294 (0.257-0.341)	0.004	0.972		
6		MRSA	0.453 (0.311-0.506)					0.255 (0.202-0.370)					0.357 (0.311-0.409)				

Note: OD; optical density, IQR; interquartile range. A p-value of <0.05 was considered statistically significant.

Exposure to sub-MICs of oxacillin, vancomycin and rifampicin resulted in an increase in the overall biofilm formation in MSSA isolates compared to MRSA. However, MRSA isolates had notable increase in biofilm formation at individual time points at t8 & t24 and t24 when exposed to sub-MICs of oxacillin and rifampicin respectively (Table 2.9).

2.4.4.4. Serum-level concentrations of oxacillin, vancomycin and rifampicin on biofilm based on phenotypic characteristic

When *S. aureus* cultures were exposed to the Serum-level concentrations of oxacillin, vancomycin and rifampicin an increase in the overall biofilm formation was observed in *agr* dysfunctional isolates compared to *agr* functional isolates as well as at the individual time points t6, t8 and t24 (Table 2.10). However, this increase did not reach statistical significance. (p=0.633, 0.128 and 0.491, respectively).

Table 2.10: The difference in biofilm formation in the presence of Serum-level concentrations of oxacillin, vancomycin & rifampicin between functional and dysfunctional isolates at different time points and overall.

N=12	Time points in the presence of antibiotics (hours)	*Agr* functionality		Oxacillin				Vancomycin					Rifampicin				
			Median (IQR)	OD difference in mean (individual time points)	OD difference in mean (overall)	*p*-value	Overall *p-value*	Median (IQR)	OD difference in mean (individual time points)	OD difference in mean (overall	*p*-value	Overall *p*-value	Median IQR)	OD difference in mean (individual time points)	OD difference in mean (overall)	*p*-value	Overall *p*-value
7	T6	functional	0.112 (0.102-0.205)	0.033	0.044	0.502	0.264	0.143 (0.126-0.19)	0.080	0.081	0.471	0.268	0.137 (0.049-0.217)	0.107	0.116	0.225	0.049
5		dysfunctional	0.182 (0.155-0.226)					0.085 (0.043-0.406)					0.095 (0.084-0.431)				
7	T8	functional	0.070 (0.064-0.169)	0.049		0.331		0.219 (0.196-0.265)	0.164		0.153		0.223 (0.155-0.273)	0.107		0.133	
5		dysfunctional	0.143 (0.124-0.154)					0.237 (0.208-0.652)					0.233 (0.206-0.337)				
7	T24	functional	0.339 (0.167-0.383)	0.080		0.496		0.338 (0.270-0.428)	0.160		0.369		0.324 (0.272-0.4)	0.065		0.554	
5		dysfunctional	0.327 (0.146-0.512)					0.330 (0.141-0.772)					0.311 (0.233-0.637)				

Note: AB; antibiotic, OD; optical density, IQR; interquartile range. A p-value of <0.05 was considered statistically significant.

2.4.4.5. Serum level concentration of Oxacillin, vancomycin and rifampicin on biofilm based on genetic background

We observed an increase in biofilm formation in *agr* I isolates when compared to *agr* III isolates across all time points when exposed to serum level of concentrations of oxacillin, vancomycin and rifampicin. This increase in biofilm formation in *agr* I isolates compared to *agr* III isolates was evident all individual time points (t6, t8 and t24) when exposed to Serum-level concentrations of oxacillin and vancomycin respectively and t6 and t24 when exposed to Serum-level concentrations of rifampicin. However, no significant difference was observed between *agr* I and *agr* III overall or at individual time points (Table 2.11). Moreover, we observed an increase in biofilm formation in *agr* I isolates compared to *agr* II isolates across all time points when exposed to serum level of concentrations of oxacillin and vancomycin. On the other hand, we observed an increase in biofilm formation in *agr* II isolates compared to *agr* I isolates across all time points when exposed to serum level of concentrations of rifampicin. Moreover, to support what was observed overall, an increase in biofilm formation was observed in *agr* I isolates compared to *agr* II isolates at t8 after exposure to Serum-level concentrations of oxacillin; t6 & t8 after exposure to Serum-level concentrations of vancomycin and t24 after exposure to Serum-level concentrations of rifampicin. Similarly, no significant difference was observed between *agr* I and *agr* II overall or at individual time points (Table 2.11).

Table 2.11: The difference in biofilm formation between *agr* I, *agr* II and *agr* III in the presence of serum level of oxacillin, vancomycin and rifampicin at individual timepoints and overall.

N=12	Time points in the presence of antibiotics (hours)	*Agr* type	Oxacillin					Vancomycin					Rifampicin				
			Median (IQR)	OD difference in mean (individual time points)	p-value	OD difference in mean (overall)	Overall p-value	Median (IQR)	OD difference in mean (individual time points)	p-value	OD difference in mean (overall)	Overall p-value	Median (IQR)	OD difference in mean (individual time points)	p-value	OD difference in mean (overall)	Overall p-value
						I vs II / I vs III	I vs II / I vs III				I vs II / I vs III	I vs II / I vs III				I vs II / I vs III	I vs II / I Vs III
4	T6	II	0.168 (0.121-0.253)	0.010	0.869	-0.012 / -0.077	0.783 / 0.079	0.343 (0.275-0.386)	0.050	0.337	0.031 / 0.055	0.361 / 0.102	0.385 (0.302-0.429)	0.020	0.735	0.031 / 0.055	0.361 / 0.102
4		I	0.155 (0.104-0.249	-0.024	0.699			0.277 (0.258-0.303)-	0.056	0.283			0.336 (0.270-0.421)	0.060	0.320		
4		III	0.142 (0.106-0.199)					0.328 (0.288-0.386)					0.399 (0.380-0.431)				
4	T8	II	0.149 (0.103-0.161)	-0.056	0.300			0.377 (0.252-0.392)	0.018	0.804			0.410 (0.277-0.452)	0.043	0.583		
4		I	0.179 (0.104-0.272	-0.115	0.050			0.279 (0.236-0.373)	0.084	0.256			0.332 (0.245 (0.3975	-0.008	0.921		
4		III	0.058 (0.053-0.094)					0406 (0.359-0.418)					0.323 (0.283-0.344)				
4	T24	II	0.355 (0.247-0.577)	0.054	0.690			0.111 (0.0.96-0.159)	0.032	0.357			0.324 (0.220-0.332)	0.095	0.107		
4		I	0.386 (0.243-0.473)	-0.146	0.293			0.088 (0.048 (0.142-	0.052	0.149			0.163 (0.132-0.231)	0.005	0.923		
4		III	0.182 (0.13-0.293)					0.148 (0.132-0.164)					0.182 (0.162-0.210)				

Note: OD; optical density, IQR; interquartile range. A p-value of <0.05 was considered statistically significant.

Table 2.12: The difference in biofilm formation in the presence of Serum-level concentrations of oxacillin, vancomycin & rifampicin between MRSA and MSSA isolates at different time points and overall.

N=12	Time points in the presence of antibiotics (hours)	Methicillin susceptibility profile	Oxacillin					Vancomycin					Rifampicin				
			Median (IQR)	OD difference in mean (individual time points)	p-value	OD difference in mean (overall)	Overall p-value	Median (IQR)	OD difference in mean (individual time points)	p-value	OD difference in mean (overall)	Overall p-value	Median (IQR)	OD difference in mean (individual time points)	p-value	OD difference in mean (overall)	Overall p-value
6	T6	MSSA	0.133 (0.106-0.226)	-0.057	0.118			0.457 (0.369-0.482)	-0.075	0.219			0.382 (0.347-0.428)	-0.065	0.168		
6		MRSA	0.177 (0.099-0.205)					0.355 (0.244-0.465)					0.330 (0.238-0.361)				
6	T8	MSSA	0.131 (0.07-0.154)	0.058	0.082			0.414 (0.395-0.506)	0.007	0.907			0.375 (0.302-0.428)	-0.028	0.548		
6		MRSA	0.103 (0.064-0.222)			-0.007	0.777	0.456 (0.319-0.467)			-0.007	0.885	0.350 (0.282-0.352)			-0.046	0.236
6	T24	MSSA	0.192 (0.148-0.436)	-0.031	0.416			0.179 (0.158-0.295)	0.003	0.949			0.196 (0.155-0.261)	-0.044	0.403		
6		MRSA	0.354 (0.327-0.383)					0.269 (0.095-0.292)					0.176 (0.110-0.217)				

Note: OD; optical density, IQR; interquartile range. A p-value of <0.05 was considered statistically significant.

An increase in overall biofilm formation was observed in MSSA compared to MRSA when exposed to serum-level concentrations of oxacillin, vancomycin and rifampicin. However, at individual time points, MRSA isolates produced more biofilm at t6, t8 & t24 after exposure to oxacillin, at t8 after exposure to vancomycin and rifampicin (Table 2.12).

2.4.4.6. Planktonic growth based on phenotypic characteristics in the presence and absence of antibiotics

Although we did not observe statistically significant in biofilm formation overall and at individual time points (t2, t4, t6, t8 and t24). It was noted that in the absence of antibiotics, the overall planktonic cells were abundant in *agr* functional isolates and at individual time points (t2, t4, t6, t8) except for t24, where they had less planktonic cells (Table 2.13). Furthermore, in the presence of antibiotics, there was an increase in planktonic cells levels overall in *agr* functional isolates when exposed to the sub-MIC of oxacillin, vancomycin and rifampicin. This was supported by an increase in planktonic cell levels in *agr* functional isolates at t6 when exposed to sub-MIC of oxacillin; t6 and t8 when exposed to sub-MIC of vancomycin and rifampicin (Table 2.14). When exposed to Serum-level concentrations of vancomycin and rifampicin an increase in planktonic cell levels was observed in *agr* functional isolates overall and at individual time points (t6, t8 and t24). When exposed to Serum-level concentrations of oxacillin agr dysfunctional isolates had more planktonic cells overall and at t8 (Table 2.15). However, no significant difference in planktonic cells was observed overall and at individual times points (t6, t8 and t24) between agr functional and agr dysfunctional isolates in the presence of antibiotics (Table 2.14 and 2.15).

Table 2.13: The difference in planktonic cells between functional and dysfunctionalisolates at different time points and overall.

N=12	Time points	*Agr* functionality	Median (IQR)	OD Difference in mean (individuall time points)	OD Difference in mean (overall)	*p-value*	Overall *p*-value
7	2	functional	0.043 (017-0.047)	-0.0063	-0.019	0.533	0.579
5		dysfunctional	0.025 (0.017-0.04)				
7	4	functional	0.236 (0.138-0.264)	-0.0371		0.460	
5		dysfunctional	0.130 (0.104-0.220)				
7	6	functional	0.373 (0.248-0.380)	-0.0711		0.304	
5		dysfunctional	0.247 (0.211-0.392)				
7	8	functional	0.299 (0.265-0.365)	-0.0360		0.469	
5		dysfunctional	0.297 (0.247-0.334)				
7	24	functional	0.089 (0.078-0.131)	0,0197		0.385	
5		dysfunctional	0.105 (0.100-0.155)				

Note: OD; optical density, IQR; interquartile range. p-value of <0.05 was considered statistically significant.

Table 2.14: The difference in level of planktonic cells in the presence of sub-MICs of oxacillin, vancomycin & rifampicin between *agr* functional and *agr* dysfunctional isolates at different time points and overall.

N=12	Time points in the presence of antibiotics (hours)	*Agr* functionality	Oxacillin					Vancomycin					Rifampicin				
			Median (IQR)	OD difference in mean (individual time points)	p-value	OD difference in mean (overall)	Overall p-value	Median (IQR)	OD difference in mean (individual time points)	p-value	OD difference in mean (overall)	Overall p-value	Median (IQR)	OD difference in mean (individual time points)	p-value	OD difference in mean (overall)	Overall p-value
7	T6	Functional	0.201 (0.191-0.270)	-0.046	0.224			0.451 (0.369-0.473)	-0.056	0.372			0.356 (0.347-0.428)	-0.065	0.197		
5		Dysfunctional	0.170 (0.112-0.176)					0.323 (0.281-0.465)					0.309 (0.235-0.361)				
7	T8	Functional	0.233 (0.213-0.308)	0.007	0.841			0.431 (0.395-0.506)	-0.053	0.382			0.350 (0.302-0.423)	-0.037	0.418		
5		Dysfunctional	0.249 (0.195-0.286)			-0.013	0.621	0.448 (0.264-0.467)			-0.010	0.824	0.349 (0.235-0.369)			-0.067	0.090
7	T24	Functional	0.158 (0.077-0.186)	0.030	0.438			0.170 (0.148-0.293)	0.015	0.782			0.184 (0.144-0.208)	0.020	0.720		
5		Dysfunctional	0.102 (0.069-0.166)					0.257 (0.189-0.281)					0.217 (0.099-0.222)				

Note: OD; optical density, IQR; interquartile range. A p-value of <0.05 was considered statistically significant.

Table 2.15: The difference in level of planktonic cells in the presence of serum level of concentration of oxacillin, vancomycin & rifampicin between *agr* functional and *agr* dysfunctional isolates at different time points and overall.

N=12	Time points in the presence of antibiotics (hours)	*Agr* functionality	Oxacillin					Vancomycin					Rifampicin				
			Median (IQR)	OD difference in mean (individual time points)	p-value	OD difference in mean (overall)	Overall p-value	Median (IQR)	OD difference in mean (individual time points)	p-value	OD difference in mean (overall)	Overall p-value	Median (IQR)	OD difference in mean (individual time points)	p-value	OD difference in mean (overall)	Overall p-value
7	T6	Functional	0.155 (0.119-0.240)	-0.008	0.820			0.326 (0.273-0.344)	-0.014	0.742			0.400 (0.367-0.458)	-0.054	0.250		
5		Dysfunctional	0.207 (0.094-0.207)					0.314 (0.244-0.343)					0.369 (0.252-0.392)				
7	T8	Functional	0.114 (0.103-0.269)	0.045	0.519			0.386 (0.292-0.417)	-0.058	0.329			0.325 (0.259-0.456)	-0.029	0.640		
5		Dysfunctional	0.254 (0.142-0.290)			0.019	0.544	0.370 (0.206-0.395)			-0.027	0.352	0.339 (0.339-0.349)			-0.030	0.481
7	T24	Functional	0.134 (0.073-0.207)	-0.048	0.250			0.124 (0.112-0.162)	-0.025	0.405			0.182 (0.139-0.319)	-0.005	0.917		
5		Dysfunctional	0.096 (0.078-0.115)					0.110 (0.081-0.139)					0.187 (0.182-0.238)				

Note: OD; optical density, IQR; interquartile range. A p-value of <0.05 was considered statistically significant.

2.4.4.7. Planktonic growth based on genetic background in the presence and absence of antibiotics

There was an increase in planktonic cells in agr II and agr III respectively compared to agr I mostly overall and at individual time points in the absence and presence of antibiotics. Overall and at individual time points (t6, t8 and t24) there was no significant difference in planktonic cells in the absence of antibiotics amongst the different *agr* types, except between *agr* II and *agr* I at time points t8 (p=0.022) and t24 (p=0.041) (Table 2.16). There was also no significant difference in the presence of antibiotics overall and at individual time points (t6, t8 and t24) (Table 2.17 and 2.18)) except overall (0.009) when exposed to sub-MIC of rifampicin between *agr* I and *agr* II and at t24 (p=0.010) (Table 2.17).

Table 2.16: The difference in level of planktonic cells between *agr* I, *agr* II and *agr* III isolatesat different time points.

N=12	Time points (hours)	*Agr* type	Median IQR	OD difference in mean (individual time points)	OD difference in mean (overall)		*p* value	Overall *p* value	
					I vs II	I vs III		I vs II	I vs III
4	2	II	0.039 (0.024-0.042)	-0.0005	0.0647	0.0397	0.969	0.116	0.335
4		I	0.036 (0.018-0.049)						
				0.0005			0.969		
4		III	0.032 (0.017-0.051)						
4	4	II	0.223 (0.162-0.232)	0.0368			0.562		
4		I	0.159 (0.07-0.25)						
				0.0480			0.452		
4		III	0.203 (0.134-0.282)						
4	6	II	0.384 (0.293-0.464)	0.1088			0.209		
4		I	0.280 (0.17-0.369)						
				0.0525			0.530		
4		III	0.314 (0.248-0.4)						
4	8	II	0.373 (0.296-0.431)	0.1258			**0.022**		
4		I	0.255 (0.201-0.274)						
				0.0865			0.090		
4		III	0.317 (0.298-0.35)						
4	24	II	0.152 (0.113-0.162)	0.0525			**0.041**		
4		I	0.076 (0.058-0.112)						
				0.0110			0.629		
4		III	0.095 (0.089-0.103)						

Note: OD; optical density, IQR; interquartile range. A p-value of <0.05 was considered statistically significant. Statistically significant observations are bolded.

Table 2.17: The difference in levels of planktonic cells between *agr* I, *agr* II and *agr* III in the presence of sub-MICs of oxacillin, vancomycin and rifampicin at individual time point and overall.

N=12	Time points in the presence of antibiotics (hours)	*Agr* type	Oxacillin					Vancomycin					Rifampicin				
			Median (IQR)	OD difference in mean (individual time points)	p-value	OD difference in mean (overall)	Overall p-value	Median (IQR)	OD difference in mean (individual time points)	p-value	OD difference in mean (overall)	Overall p-value	Median (IQR)	OD difference in mean (individual time points)	p-value	OD difference in mean (overall)	Overall p-value
						I vs II / I vs III	I vs II / I vs III				I vs II / I vs III	I vs II / I vs III				I vs II / I vs III	I vs II / I vs III
4	T6	II	0.181 (0.141-0.236)	0.013	0.783			0.469 (0.373-0.477)	0.085	0.283			0.371 (0.298-0.416)	0.051	0.407		
4		I	0.163 (0.115-0.235)	0.046	0.342			0.347 (0.223-0.457)	0.053	0.491			0.297 (0.22-0.392)	0.048	0.439		
4		III	0.215 (0.186-0.256)					0.377 (0.346-0.441)					0.349 (0.328-0.379)				
4	T8	II	0.294 (0.231-0.331)	0.069	0.113			0.44 (0.337-0.456)	0.026	0.704			0.361 (0.273-0.396)	0.016	0789		
4		I	0.213 (0.201-0.223)	0.037	0.366	0.072	0.038 / **0.009** / 0.161	0.357 (0.291-0.450)	0.112	0.126	-0.076	-0.058 / 0.258 / 0.389	0.304 (0259-0.379)	0.039	0.514	0.041	-0.083 / 0.656 / 0.177
4		III	0.267 (0.201-0.297)					0.487 (0.432-0.533)					0.35 (0.326-0.389)				
4	T24	II	0.176 (0.166-0.203)	0.106	**0.010**			0.223 (0.173-0.275)	0.066	0.277			0.176 (0.105-0.217)	0.005	0.940		
4		I	0.367 (0.183-0.574)	0.035	0.313			0.133 (0.084-0.233)	0.100	0.114			0.133 (0.105-0.208	0.095	0.138		
4		III	0.090 (0.077-0.150)					0.286 (0.214-0.301)					0.212 (0.196-0.308)				

Note: OD; optical density, IQR; interquartile range. A p-value of <0.05 was considered statistically significant. Statistically significant observations are bolded.

Table 2.18: The difference in levels of planktonic cells between *agr* I, *agr* II and *agr* III in the presence of Serum-level concentrations of oxacillin, vancomycin and rifampicin at individual time points and overall.

N=12	Time points in the presence of antibiotics (hours)	*Agr* type	Oxacillin					Vancomycin					Rifampicin				
			Median (IQR)	OD difference in mean (individual time points)	p-value	OD difference in mean (overall)	Overall p-value	Median (IQR)	OD difference in mean (individual time points)	p-value	OD difference in mean (overall)	Overall p-value	Median (IQR)	OD difference in mean (individual time points)	p-value	OD difference in mean (overall)	Overall p-value
						I vs II / I vs III	I vs II / I vs III				I vs II / I vs III	I vs II / I vs III				I vs II / I vs III	I vs II / I vs III
4	T6	II	0.208 (0.141-0.240)	0.064	0.123			0.343 (0.275-0.386)	-0.005	0.337			0.386 (0.302-0.429)	0.064	0.735		
4		I	0.130 (0.104-0.149)	0.066	0.113			0.277 (0.258-0.303)	-0.152	0.283			0.336 (0.27-0.421)	-0.126	0.320		
4		III	0.207 (0.161-0.226)					0.328 (0.288-0.386)					0.399 (0.38-0.431)				
4	T8	II	0.178 (0.087-0.261)	0.046	0.526			0.378 (0.252-0.392)	-0.001	0.804			0.410 (0.277-0.452)	0.072	0.583		
4		I	0.125 (0.075-0.181)	0.155	0.055	0.017	0.063	0.279 (0.236-0.373)	-0.133	0.256	-0.101	-0.032	0.332 (0.245-0.398)	0.043	0.921	0.114	0.001
4		III	0.319 (0.202-0.363)					0.406 (0.359-0.406)					0.323 (0.283-0.344))				
4	T24	II	0.093 (0.084-0.115)	-0.031	0.574			0.111 (0.096-0.159)	0.096	0.357			0.324 (0.22-0.332)	-0.107	0.107		
4		I	0.121 (0.032-0.227)	0.025	0.963			0.088 (0.048-0.142)	-0.256	0.149			0.163 (0.132-0.231)	-0.236	0.923		
4		III	0.129 (0.094-0.17)					0.148 (0.132-0.164)					0.148 (0.132-0.164)				

Note: OD; optical density, IQR; interquartile range. A p-value of <0.05 was considered statistically significant.

Abundant planktonic cells were observed in MSSA isolates than MRSA isolates mostly overall and at individual time points in the absence and presence of antibiotics. The overall planktonic cell levels did not significantly differ between MSSA and MRSA isolates neither in the absence nor in the presence of oxacillin, vancomycin and rifampicin both at sub-MICs and serum-level concentration (Table 2.19, 2.20 and 2.21). Except in the presence of oxacillin serum-level concentrations at t8 MRSA significantly formed more planktonic cells than MSSA (Table 2.21).

Table 2.19: The difference in level of planktonic cells levels betweenMRSA and MSSA isolates at different individual time points and overall.

N=12	Time point (hours)	Methicillin susceptibility	Median IQR	OD difference in mean (individual time points)	OD difference in mean (overall)	*p* value	Overall *p* value
6	2	MSSA	0.047 (0.037-0.05)	-0.0155	-0.0289	0.095	0.397
6		MRSA	0.021 (0.017-0.04)				
6	4	MSSA	0.25 (0.226-0.267)	-0.0882		0.054	
6		MRSA	0.134 (0.082-0.22)				
6	6	MSSA	0.374 (0.365-0.38)	-0.5917		0.389	
6		MRSA	0.248 (0.194-0.392)				
6	8	MSSA	0.300 (0.247-0.344)	0.0160		0.747	
6		MRSA	0.298 (0.283-0.401)				
6	24	MSSA	0.097 (0.078-0.131)	0.0025		0.913	
6		MRSA	0.96 .089-0.148)				

Note: OD; optical density, IQR; interquartile range. A p-value of <0.05 was considered statistically significant.

Table 2.20: The difference in level of planktonic cells in the presence of sub-MICs concentrations of oxacillin, vancomycin & rifampicin between MRSA and MSSA isolates at different time points and overall.

N=12	Time points in the presence of antibiotics (hours)	Methicillin susceptibility profile	Oxacillin					Vancomycin					Rifampicin				
			Median (IQR)	OD difference in mean (individual time points)	p-value	OD difference in mean (overall)	Overall p-value	Median (IQR)	OD difference in mean (individual time points)	p-value	OD difference in mean (overall)	Overall p-value	Median (IQR)	OD difference in mean (individual time points)	p-value	OD difference in mean (overall)	Overall p-value
6	T6	MSSA	0.235 (0.197-0.279)	-0.057	0.118			0.457 (0.369-0.482)	-0.075	0.219			0.382 (0.347-0.428)	-0.065	0.168		
6		MRSA	0.173 (0.126-0.191)					0.355 (0.244-0.465)					0.330 (0.238-0.361)				
6	T8	MSSA	0.223 (0.195-0.249)	0.058	0.082			0.414 (0.395-0.506)	0.007	0.907			0.375 (0.302-0.428)	-0.028	0.548		
6		MRSA	0.297 (0.214-0.323)			-0.007	0.777	0.456 (0.319-0.467)			-0.007	0.885	0.350 (0.282-0.352)			-0.046	0.236
6	T24	MSSA	0.158 (0.077-0.186)	-0.031	0.416			0.179 (0.158-0.295)	0.003	0.949			0.196 (0.155-0.261)	-0.044	0.403		
6		MRSA	0.09 (0.053-0.166)					0.269 (0.095-0.292)					0.176 (0.110-0.217)				

Note: OD; optical density, IQR; interquartile range. A p-value of <0.05 was considered statistically significant.

Table 2.21: The difference in level of planktonic cells in the presence of serum level concentrations of oxacillin, vancomycin & rifampicin between MRSA and MSSA isolates at different time points and overall.

N=12	Time points in the presence of antibiotics (hours)	Methicillin susceptibility profile	Oxacillin					Vancomycin					Rifampicin				
			Median (IQR)	OD difference in mean (individual time points)	p-value	OD difference in mean (overall)	Overall p-value	Median (IQR)	OD difference in mean (individual time points)	p-value	OD difference in mean (overall)	Overall p-value	Median (IQR)	OD difference in mean (individual time points)	p-value	OD difference in mean (overall)	Overall p-value
6	T6	MSSA	0.149 (0.116-0.207)	0.017	0.625			0.303 (0.261-0.429)	-0.013	0.766			0.420 (0.367-0.458)	-0.042	0.370		
6		MRSA	0.198 (0.119-0.227)					0.328 (0.273-0.343)					0.381 (0.288-0.400)				
6	T8	MSSA	0.105 (0.072-0.114)	0.147	**0.015**			0.361 (0.292-0.418)	0.003	0.957			0.332 (0.307-0.435)	-0.011	0.863		
6		MRSA	0.261 (0.219-0.349)			0.023	0.428	0.378 (0.267-0.395)			-0.032	0.274	0.344 (0.259-0.385)			-0.008	0.858
6	T24	MSSA	0.125 (0.078-0.207)	-0.044	0.285			0.132 (0.123-0.162)	-0.032	0.274			0.191 (0.125-0.274)	0.022	0.661		
6		MRSA	0.093 (0.035-0.143)					0.111 (0.053-0.157)					0.185 (0.182-0.329)				

Note: OD; optical density, IQR; interquartile range. A p-value of <0.05 was considered statistically significant. Statistically significant observations are bolded

2.5. Discussion

In this chapter, twelve isolates collected from blood cultures between 2015 and 2017 from Tygerberg Hospital as part of a previous study, were selected based on *agr* functionality, *agr* type, methicillin susceptibility profiles and *spa* types. The phenotypic synergistic *agr* functionality assay results of all isolates included in this study were in concordance with the previous study by Abdulgader et al in 2020 except for isolate 66, which was previously phenotypically described as an *agr* dysfunctional isolate, but the repeated phenotypic synergistic *agr* functionality assay results in this study assigned it as an *agr* functional isolate. A previous study by Gor et al in 2019 justified the difference in results reported in this study and by Abdulgader et al by reporting that certain isolates contained a mixed population of both *agr* functional and *agr* dysfunctional colonies from a single colony or short-lived mutations that resulted in *agr* dysfunction. This indicates that these isolates have the ability to produce mutants that cam oscillate between the functional and dysfunctional state, depending on the abundance of specific mutants during the time of testing.

For biofilm formation, although no significant difference in the overall biofilm formation level was observed between *agr* functional and *agr* dysfunctional isolates (as well as between individual time points); we noted a trend towards an increase in biofilm production in *agr* dysfunctional isolates. This is supported by previous studies which also used isolates collected from blood samples (Ferreira et al. 2013; Lade et al. 2019; Yang et al. 2019). The significant increase of biofilm formation in *agr* dysfunctional isolates was evident in the study by Ferreira and co-workers (Ferreira et al. 2013). This is maybe due to the technical differences between our study and theirs. They performed the biofilm assays on surfaces covered with human fibronectin and treated the biofilm with sodium metaperiodate or proteinase K before treatment with crystal violet. These were all done to enhance biofilm formation in their study. The majority of isolates included in this study were strong biofilm formers regardless of the *agr* functionality status. While previous studies reported that *agr* dysfunctional isolates were strong biofilm formers (Lade et al. 2019; Yarwood and Schlievert 2003). Another study reported that glucose addition to TSB promoted robust biofilm formation and resulted in improved assay results (Lade et al. 2019). However, no glucose was added to the TSB in this study, but majority of isolates were strong biofilm formers suggesting that any catalyst did not promote the strong biofilm formation observed in this study.

Previous studies conducted on a human clinical *S. aureus* isolate and bovine mastitis cases respectively reported that biofilm formation is greater at later time points during the growth curve than the earlier time points (Osmon et al. 2013; Vaezi et al. 2020). This was also the case in this study more biofilm formation was observed at later time points (8 hours onwards)

than at early time points. The reason for the increase in biofilm at later time points might be because at that time biological processes such as cell division have taken over and crucial biofilm proteins such as polysaccharide intercellular adhesion (PIA) polymers are secreted to help strengthen the biofilm (Roger et al. 2008). Another study conducted on human clinical isolates reported no *agr*-dependent difference in biofilm formation was observed until t24 where the *agr* functional strain significantly formed more biofilm at this time (Jordan, Hall, and Daly 2022). This is in line with what was observed in this study i.e., no *agr*-dependent difference was observed in biofilm production before t24. However, in this study at t24, there was still no *agr*-dependent difference and *agr* dysfunctional isolates formed more biofilm than *agr* functional isolates. The reason for the difference in result at t24 might have been the small sample size used in the study by Jordan, Hall, and Daly (2022) two isolates were used (one functional and one dysfunctional). Besides the study by Jordan and co-workers (2022), it appears that no studies to date have specifically investigated the relationship between *agr* status and biofilm formation across multiple time points at different bacterial growth stages.

For *agr* types, we observed that isolates from *agr* I formed more biofilm compared to both *agr* II and *agr* III isolates. Previous studies supported this observation, they showed that *agr* I type *S. aureus* isolated from bovine mastitis formed more biofilm when compared to the other *agr* types in that study (Bardiau et al. 2013, 2014; Khoramrooz et al. 2016). However, other studies reported that *agr* II isolates were stronger biofilm formers than the other *agr* types (Cafiso et al. 2007; Ikonomidis et al. 2009). Cafiso et al. (2007) used only MRSA isolates, while Ikonomidis et al. (2009) had a big sample size, which might explain the difference in results observed. Based on methicillin susceptibility, previous studies also conducted on human clinical isolates reported that MRSA isolates significantly formed more biofilm than MSSA isolates (Leshem et al. 2022; Piechota et al. 2018). This supported what we observed in the absence of antibiotics, that MRSA isolates formed more biofilm than MSSA isolates, even though no statistical significance was observed. The difference in statistical difference observed in this study and those previous studies could be due to the difference in sample size, the previous studies had a larger sample size. Additionally, the study by Leshem and co-workers (2022) used a different method to quantify biofilm formation they used the Congo red agar assay (Leshem et al. 2022) while the other by Piechota and co-workers coated the wells with tissues cells (Piechota et al. 2018).

When we exposed *S. aureus* cultures to both sub-MICs and Serum-level concentrations of oxacillin, vancomycin and rifampicin we observed a non-significant increase in the overall biofilm formation in *agr* dysfunctional isolates, except for the exposure to sub-MICs of oxacillin where a significant increase in biofilm formation was observed in *agr* dysfunctional isolates. Previous studies have reported that exposure to sub-MICs of oxacillin, vancomycin and

rifampicin increases biofilm formation (Lima-e-Silva et al. 2017; Mirani and Jamil 2011). While another study reported that lower concentrations of the isolates MICs of oxacillin had significant inhibitory effects on biofilm formation (Majidpour et al. 2017). This might explain the significant increase in biofilm formation observed overall when exposed to sub-MICs of oxacillin, but due to the small number of isolates we used in this study, we were not able to observe statistical significance at individual time points. To our knowledge, there were no studies that have investigated, in detail, the effect of exposure of sub-MICs and or Serum-level concentrations based on *agr* functionality or *agr* types.

For methicillin susceptibility profiles, a study conducted on food borne *S. aureus* isolates reported that MRSA isolates formed enhance biofilm formation at t24 after exposure to oxacillin (Mirani et al. 2013). This supported what was observed in this study, exposure to sub-MICs of oxacillin showed an increase in biofilm formation at t24 in MRSA isolates. No further studies were available to support or dis*agr*ee with our findings on this topic in literature to our knowledge.

Increased biofilm formation resulted in decreased planktonic cells and vice versa. This was observed in *agr* functionality, *agr* types and methicillin susceptibility profiles in the absence and presences of antibiotics. However, at specific time points like t24 in the absence of antibiotics *agr* dysfunctional isolates formed more biofilm and had more planktonic cells. Reasons for that was that some biofilm cells might have detached from the surface and floated in the planktonic state and were measured, hence the increase in both planktonic and biofilm cells.

2.6. Conclusion

In conclusion, the small sample size used in this study may have failed to show statistically significant differences in biofilm formation in the absence and presence of antibiotics in different *S. aureus* isolates with different *agr* functionality, *agr* types and methicillin susceptibility profiles. However, statistically significant difference were observed between agr functional and agr dysfunctional isolates when exposed to sub-MIC of oxacillin suggesting that *agr* functionality may impact biofilm formation in the presence of specific antibiotics, like oxacillin as seen in this study. A study with a larger sample size is needed to prove or refute this hypothesis.

3. CHAPTER 3: Molecular impact of biofilm and *agr*-related genes on *agr* functionality status

3.1. Introduction

The *Agr* system involves four genes namely the *agrA*, *agrB*, *agrC*, and *agrD* which all work together to induce the transcription of *RNAIII* (Tan et al. 2022). *RNAIII* is the effector molecule of the *agr* system, it contributes to the regulation of virulence genes and biofilm formation (Gupta et al. 2015). However, the *agr* system is known to be genetically unstable and prone to mutations (Traber et al. 2008) resulting in the dysfunction of the *agr* system (Gor et al. 2019). The dysfunction in the *agr* system may arise during the course of infection *in-vivo* (Shopsin et al. 2010). There has been an increase in the detection of *agr* dysfunctional isolates in clinical and laboratory settings (Gor et al. 2019). The *agrA* and *agrC* genes are the regions most affected by genetic changes including frameshift insertion/deletions, nonsynonymous SNPs, and poly(A) tract alterations (Gor et al. 2019; Shopsin et al. 2010).

Various genes are involved in the formation and maintenance of biofilms by *S. aureus* (Arciola et al. 2015). Biofilm formation in *S. aureus* mainly uses the PIA mechanism, which involves the *ica* operon, of which *icaA* and *icaD* are the most extensively studied and they encode main components of the exopolysaccharide matrix surrounding the bacterial cells within biofilm like N-acetylglucosamine (Arciola et al. 2015). However, numerous studies of *S. aureus* biofilm formation have shown alternative PIA-independent mechanism of biofilm formation (Arciola et al. 2015). The *bap* gene which encodes for biofilm-associated proteins is mainly involved in the PIA-independent mechanism by enabling biofilm formation even in the absence of production of the exopolysaccharide component (Arciola et al. 2015).

The lack of information around molecular and genetic changes on *agr* and biofilm related genes is concerning. Particularly when these changes turn to affect the function of a key regulatory operon like the *agr* system, which regulates virulence and biofilm formation.

This section aims to investigate differences in the genetic structure and expression of biofilm-related genes in *agr* functional and dysfunctional isolates and Investigate differences in the genetic structure and expression of the *agr* locus in *agr* functional and dysfunctional isolates.

3.1. Materials and methods

3.1.1. RNA Extraction

The twelve (12) isolates were cultured overnight on blood agar plates and incubated at 37 °C aerobically to get single colonies of each isolate the following day. The single colonies were inoculated into 20ml sterilin containers containing 5ml of fresh TSB, and incubated overnight

at 37 °C aerobically. The overnight liquid cultures were diluted with fresh TSB in new Sterilin containers to make 1:200 dilutions, and incubated for four (4) hours at 37°C. After 4 hours, 700µl of the cultures was aliquoted in 2ml microcentrifuge tubes, and 1.3ml RNAprotect Bacteria Reagent (Qiagen, Hilden, Germany) was added to each 2ml microcentrifuge tube containing the 700µl cultures. The 2ml microcentrifuge tubes were then centrifuged at 5000xg for 10 minutes to get pellets, the supernatants were discarded and the pellets were frozen at -80°C. Before the RNA extraction, the pellet was re-suspended in 100 µl nuclease free-water and 200µl RNAprotect Bacteria Reagent and centrifuged at 5000xg for 10 minutes. The RNeasy mini kit (Qiagen, Hilden, Germany) was used for RNA extraction which was done according to the manufacturer's instructions, with slight modification; the on-column genomic DNA (gDNA) removal step was done at 37°C for 30 minutes instead of 25 °C for 15 minutes in the original protocol. The RNA was stored at -80°C for later use.

3.1.2. Complementary DNA (cDNA) synthesis and off-column DNA treatment

RevertAid RT Reverse Transcription kit (ThermoFisher Scientific) was used for the removal of genomic DNA from the frozen extracted RNA using the RNeasy mini kit and for the synthesis of cDNA. The protocols for the removal of the genomic DNA and cDNA synthesis were followed according to the manufacturer's instructions. For removal of genomic DNA 0.5µg of RNA was used as a start material. The manufacturer allows up to 1µg. The concentrations of isolates we observed after RNA extraction using a BioDrop spectrophotometer in (µg/ml) was divide by 500 to get the starting volume in microliters (µl). The volume calculated was add into an RNA-free tube containing: 1µl 10X Reaction Buffer with $MgCl_2$, 1µl DNase I and nuclease-free water to make up to 10µl. The tube was then incubated at 37°C for 30 minutes recommend by the manufacturer. For cDNA synthesis, 2µl of the genomic DNA free RNA was used as starting material. After cDNA synthesis using a BioDrop spectrophotometer the cDNA concentrations were measured before running the PCR and were observed to be too high (1000-2000 µg/ml) for real-time PCR. To get the cDNA concentrations to be within range of what is acceptable for real-time PCR, the cDNA were diluted using a 1:100 ratio in nuclease-free water.

3.1.3. Gene quantification

Real-time PCR assays were used to detect DNA contamination after the removal of genomic DNA and to quantify the expression of the *icaA* and *RNAIII*. The *icaA* is part of the intercellular adhesion cluster (*ica*) operon responsible for mediating the production of polysaccharide intercellular adhesions (PIA) which are proteins needed for biofilm formation (Marques et al. 2021). The *icaA* encodes for N-acetylglucosaminyl transferase which is essential in biofilm

formation (Marques et al. 2021). *RNAIII* is the effector of the *agr* system in *S. aureus*, it plays a vital role in the regulation of virulence genes (Gupta et al. 2015). The r*poB* was used as a reference gene. The *rpoB* is a universal gene for bacteria and it encodes for the RNA polymerase which is essential for transcriptional process (Drancourt and Raoult 2008). The primer sequences are shown in Table 4.1.

Table 3.1: Primer sequences of the *icaA*, *RNAIII,* and *rpoB* genes respectively.

Gene	Nucleotide sequence of primers (5'-3')	References
icaA	F: CAATACTATTTCGGGTGTCTTCACTCT R: CAAGAAACTGCAATATCTTCGGTAATCAT	(Kot, Sytykiewicz, and Sprawka 2018)
*rpo*B	F: CAGCTGACGAAGAAGATAGCTATGT R: ACTTCATCATCCATGAAACGACCAT	(Kot et al. 2018)
RNAIII	F: GCCATCCCAACTTAATAACCA R: TGTTGTTTACGATAGCTTACATGC	(Seidl et al. 2011)

The KAPA SYBR® FAST Master Mix (2x) Universal kit (Merck KGaA, Darmstadt, Germany) was used for qPCR reactions. For each qPCR run, the total volume was 20µl. The master mix was 19µl, it contained 10 µl of KAPA SYBR® FAST, 0.2 µM each of the forward and reverse primers, and 8.6µl of nuclease-free water. The extracted RNA (after the removal of the genomic DNA) was used to check for DNA contamination, and synthesised cDNA detection of was the RNA after the removal of the genomic DNA step and for gene expression quantification, it was. DNA dilutions of 10^{-2} and 10^{-5} from one of the isolate 199 used in the study were used as positive controls. The DNA was extracted from the positive control using the Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research, California) and the protocol was followed according to the manufacturer's instructions. Nuclease-free water was used as a negative template. Quantification of the gene expression levels was done by a singleplex real-time PCR on a RotorgeneQ real-time PCR cycler (Qiagen, Hilden, Germany). The same cycling conditions were used for *rpoB* and *RNAIII* PCRs: an initial cycle at 95°C for 3 min, followed by 40 cycles at 95°C for 15s, the annealing temperature of 58°C for the 20s, extending temperature of 72°C for 20s. The cycling conditions for *icaA* PCR consisted of an initial cycle at 95°C for 15 min, followed by 40 cycles at 95°C for the 30s, an annealing temperature of 55°C for 20s, the extending temperature of 72°C for 20s. Melting curves were constructed in the range of 60 to 95°C to verify the specificity of the amplified products.

For the calculations: To get ΔCt we calculated the averages of the three biological replicates PCRs {i.e. $[(PCR_{1RNAIII}\text{-}PCR_{1rpoB}) + (PCR_{2RNAIII}\text{-}PCR_{1rpoB}) + PCR_{3RNAIII}\text{-}PCR_{1rpoB})] \div 3 = \Delta Ct$}.

To calculate $\Delta\Delta CT = \Delta Ct_{\textit{agr}\text{ dysfunctional isolate}} - \Delta Ct_{\textit{agr}\text{ functional isolate.}}$ To calculate the fold change we used this equation: Fold change=$2^{-\Delta\Delta CT}$.

3.1.4. Genetic analysis

Whole genome sequencing (WGS) was done by Abdulgader et al. in 2020 as part of that study. In this study, the genome sequences of the chosen isolates were available in FASTA format. WGS in that study by Abdulgader et al. in 2020 was done using Illumina MiSeq at The Sanger Institute, United Kingdom (as part of a collaboration). *De novo* assembly was done using the velvet algorithm package. The assembled genome sequences were annotated in this study using the Prokka a software tool used to annotate bacterial genomes. The *icaA*, *icaD*, *bap*, *agrA*, *agrB*, *agrC*, *agrD,* and *RNAIII* regions were analyzed for nucleobases and amino acid changes. Artemis a software used to browse bacterial genomes and an annotation tool that allows visualisation of sequence features from next generation data was used to visualize sequence files and to extract the FASTA files of the genes of interest for all study isolates. In cases where Prokka annotation was inaccurate, the sequences were blasted on The National Center for Biotechnology Information (NCBI) database to confirm gene identification. Once confirmed they were renamed using Artemis. All the genes were saved in FASTA file format.

BioEdit 7.2 was used to perform sequence alignment. Isolates with different *agr* functionality but the same *agr* type, methicillin susceptibility, *spa* type, and *spa*-CC were aligned to investigate any nucleobases and amino acid changes that might affect the structure of specific proteins.

3.2. Statistical analysis

Microsoft excel was used to calculate the fold changes and draw bar graphs. One-sample t-test was used to the mean, 95% confidence interval and p value. A p value of >was considered statistically significant, while a p value of >0.05 was not considered statistically significant. OD data that was normally distributed a mean±standard deviation was reported. While OD data that was not normally distributed a median and interquartile range was reported.

3.3. Results

3.3.1. Quantification of *RNAIII* relative to *rpoB*

The ΔCt of *RNAIII* expression relative to *rpoB* for each isolate was calculated and isolates were grouped based of their *agr* functionality, this is shown in (Figure 3.1.). *Agr* functional isolates had average ΔCt data that was normally distributed with a mean±SD of -7.22±3.29.

While *agr* dysfunctional isolates had average ΔCt data that was not normally distributed with a median and interquartile range of -2.45 [-6.48-(-2.33)]. *Agr* dysfunctional isolates showed a significant increase (0.332, 95%CI 0.02-0.64) in the level of *RNAIII* expression relative to *rpoB* when compared to *agr* functional isolates (Table 3.2). Furthermore, when comparing isolates 66 and 10 (both functional), a 294-fold change in the expression of *RNAIII* was observed between these isolates.

Table 3.2: Fold changes in *RNAIII* relative to *rpoB* between functional and dysfunctional isolates; fold changes between MRSA and MSSA isolates.

Sample ID	**Fold change (FC=2-ΔΔCt)**	**Mean**	**95% confidence interval**	***p*-value**
agr dysfunctional isolates vs *agr* functional isolates				
1 vs 140	0.50	0.332	0.02-0.64	0.041
46 vs 29	0.43			
98 vs 2	0.59			
80 vs 153	0.122			
199 vs 9	0.018			
66 vs 10	294,07			
MRSA isolates vs MSSA isolates				
Sample ID	**Fold change (FC=2-ΔΔCt)**	**Mean**	**95% confidence interval**	***p*-value**
46 vs 1	0.016	1.34	-1.56-4.24	0.289
29 vs 140	0.019			
98 vs 66	0.014			
2 vs 10	6.93			
153 vs 9	0.13			
80 vs 199	0.92			

Moreover, MRSA isolates showed an increase in the level of *RNAIII* expression (1.34, 95%CI 0.02-0.64) relative to *rpoB* compared to MSSA. However, there was no significant difference in the fold change expression of *RNAIII* relative to *rpoB* between MRSA and MSSA isolate (p=0.289).

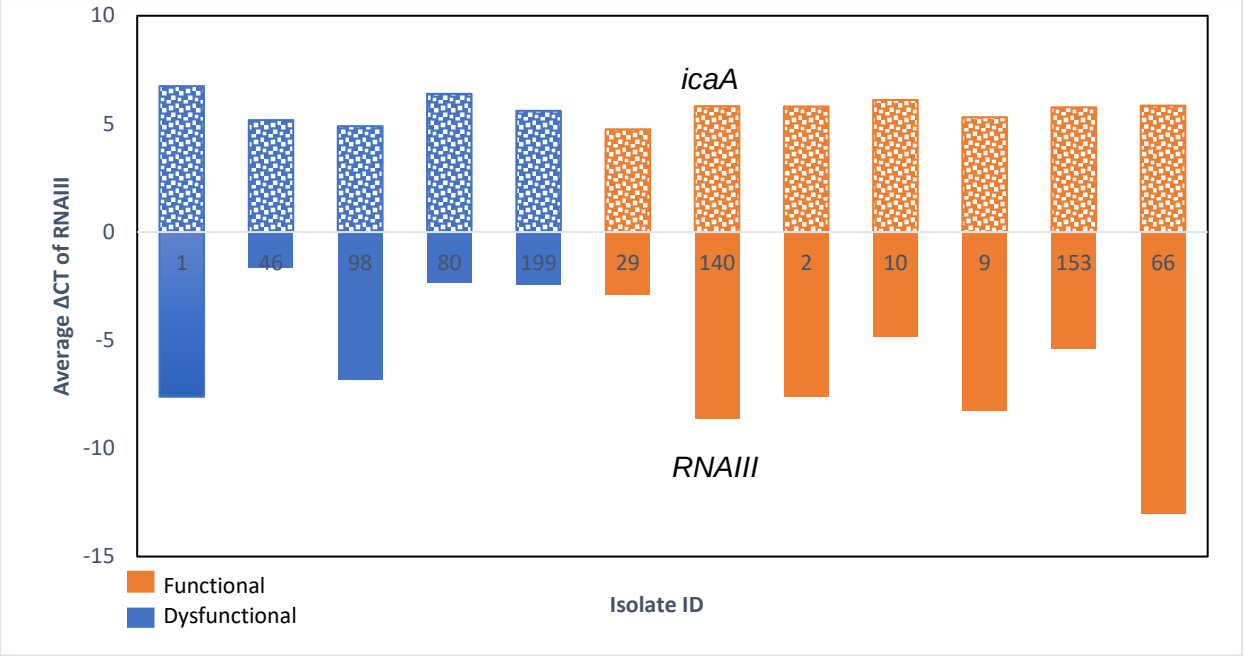

Figure 3.1: Average ΔCt of *icaA* and *RNAIII* relative to rpoB between functional and dysfunctional isolates after four hours.

3.3.2. Quantification of *icaA* relative to *rpoB*

The ΔCt of *icaA* expression relative to r*poB* for each isolate was calculated and isolates were group based of their *agr* functionality, this is also shown in (Figure 3.1.). The average ΔCt data for both *agr* functional and dysfunctional isolates was normally distributed with mean±SD of -5.59±0.49 and 5.77±0.71 respectively. *Agr* dysfunctional isolates showed a significant increase (0.92, 95%CI 0.24-1.60) in the level of *icaA* expression relative to *rpoB* when compared to *agr* functional isolates (Table 3.3). Moreover, when comparing isolates 66 and 10 (both functional), a 1.20-fold change in the expression of *icaA* was observed between these isolates.

MRSA isolates showed a significant increase (1.59, 95%CI 0.63-2.55) in the level of *icaA* expression relative to *rpoB* when compared to MSSA isolates (p=0.008).

Table 3.3: Fold changes in *icaA* relative to *rpoB* between functional and dysfunctional isolates; fold changes between MRSA and MSSA isolates.

Sample ID	Fold change (FC=2-ΔΔCt)	Mean	95% confidence interval	*p*-value
agr dysfunctional isolates vs *agr* functional isolates				
1 vs 140	0.52	0.92	0.24-1.6	0.02
46 vs 29	0.74			
98 vs 2	1.88			
80 vs 153	0.65			
199 vs 9	0.81			
66 vs 10	1.20			
MRSA isolates vs MSSA isolates				
Sample ID	**Fold change (FC=2-ΔΔCt)**	**Mean**	**95% confidence interval**	***p*-value**
46 vs 1	2.98	1.59	0.63-2.55	0.008
29 vs 140	2.10			
98 vs 66	1.93			
2 vs 10	1.22			
153 vs 9	0.73			
80 vs 199	0.58			

3.3.3. Genetic analysis of the *icaA*, *icaD*, *bap*, *agrA*, *agrB*, *agrC*, *agrD*, and *RNAIII* regions

The *bap* gene which encodes for biofilm-associated proteins which are responsible for biofilm production in strains without the PIAs was not present in all the twelve (12) isolates used in this study, we therefore, excluded *bap* gene from the gene expression (qRT-PCR) analysis. We observed no genetic changes in both *agr* (*agrA, agrB, agrC, agrD, and RNAIII*) and biofilm-related genes (*icaA* and *icaD*) between isolates 2 & 98 (*agr* type I) (Supplementary Table 2.1). For the alignment of isolates 10 & 66 also belonging to *agr* I, several changes were observed on *agr* related genes. We observed a nine (9) nucleotide deletion and six SNPs in the *agrA* gene in isolate 66, resulting in three amino acids deletion on isolate 66 from position 82-84 and an amino acid change at position R136K. Moreover, we observed one SNP on *agrB* which resulted in an amino acid change at position G12S. Furthermore, seven SNPs were observed on *agrC*, which resulted in an amino acid change at position Q236K. No changes were observed on biofilm related genes (*icaA* and *icaD*) and the *RNAIII* region for the alignment of isolate 10 and 66.

We also observed no genetic changes in both *agr* and biofilm-related genes between isolates 153 & 80 (*agr* type II). For isolates 9 and 199 (*agr* II), we observed two SNPs in *agrC* which resulted in two amino acids changes at F29K and G393D. No other changes were observed on biofilm related genes (*icaA* and *icaD*) and *agr* related genes (*agrA*, *agrB*, *AgrD,* and *RNAIII*).

For Isolates 140 and 1 (*agr* type III), we observed a deletion of a nucleotide base at position 437 on isolate 1 on the *RNAIII* and there were no other changes observed on biofilm related genes (*icaA* and *icaD*) and *agr* related (*agrA*, *agrB*, *agrC* and *agrD*). When aligning isolate 29 and 46 (*agr* type III), we observed a number of changes on both *agr* and biofilm related genes. For biofilm related genes, we observed five SNPs on *icaA* which resulted in no amino acid changes. Moreover, we also observed five SNPs on *icaD* which resulted in two amino acids changes on I17V and K97R. Furthermore, we observed 16 SNPs on *agrA* which did not change the encoded amino acid. We further observed diverse number of SNPs and amino acid changes on *agrB*, *agrC,* and *agrD*. However, no changes were observed on the *RNAIII* region for the isolates 29 and 46 (Supplementary Table 2.1.). To further investigate these diverse changes observed between isolate 29 and 46. We aligned all the biofilm and *agr* related genes from these pair of isolates with other isolates (1 and 140) from the same *agr* group (*agr* III). We observed similarities between isolate 1, 29 and 140 in both biofilm and *agr* related genes. We also observed diverse changes on isolate 46 when compared to the other three isolates in the same *agr* group on both biofilm and *agr* related genes. We then aligned both biofilm and *agr* related genes from isolate 46 with isolates from other *agr* groups (*agr* I and *agr* II), we observed partial similarities in both the biofilm and *agr* related genes in both *agr* I and *agr* II isolates. All these observations and changes on isolates 46 informed us that isolate 46 might be a result of *agr* recombination between *agr* I and *agr* II.

3.4. Discussion

In this chapter, RT-qPCR was used to to investigate the expression levels of *agr* related genes (*RNAIII*) and biofilm related genes (*icaA*) between *agr* functional and *agr* dysfunctional and MSSA and MRSA isolates. It was observed that isolate 66 which was assigned as *agr* dysfunctional expressed more *RNAIII* compared to most *agr* functional isolates. However, this was uncommon in literature. According to literature *agr* functional isolates expressed more *RNAIII* than *agr* dysfunction isolates (Laabei et al. 2014). Based on that and the fact the phenotypic synergistic *agr* functionality assay is subjective. The phenotypic synergistic agr functionality assay was repeated to confirm the *agr* functionality of all isolates. Previous Studies suggested that late onset of *RNAIII* expression (at 4h and later) may result in failure to translate delta-hemolysin leading to an *agr* dysfunctional phenotype (Traber et al. 2008; Traber and Novick 2006). This was not observed in this study, after 4h of incubation *agr* dysfunctional isolates expressed a significant increase in *RNAIII* compared to *agr* functional. The difference in isolate profiles (methicillin susceptibility profile, spa type, agar functionality) used in this study might have influenced the differences observed for this study. Furthermore, no studies directly compared *RNAIII* expression between *agr* functional and *agr* dysfunctional isolates as far as the literature that was reviewed for this study. For methicillin susceptibility,

an increase in *RNAIII* expression was observed in MRSA isolates than MSSA isolates. Previous studies conducted on human clinical isolates reported that *RNAIII* was expressed more in MRSA isolates compared to MSSA isolates which supported what was observed in this study (Dehbashi et al. 2021; Seidl et al. 2011; Skinner et al. 2013). For the expression of *icaA*, *agr* dysfunctional isolates were associated with significant increase *icaA* expression compared to *agr* functional isolates in this study. Moreover, a similar result was observed in MRSA isolates with a significant increase in *icaA* expression compared to MSSA isolates. However, no studies were available to our knowledge to support these observations. Most studies focused on how *icaA* is associated to biofilm formation. According to these studies, *icaA* is associated with an increase biofilm formation in *S. aureus* isolates (Kot et al. 2018; Marques et al. 2021). This study has identified a gap in literature, which explores and describes the expression levels of *icaA* between *agr* functional and *agr* dysfunctional isolates.

Genetic analysis of these biofilm related genes (*icaA* and *icaD*) and *agr* related genes (*agr*A, *agr*B, *agr*C, *agr*D and *RNAIII*) between *agr* functional and *agr* dysfunctional isolates, showed that all the twelve isolates did not have the *bap* gene. Since all the isolates were biofilm formers that meant all of them were PIA-dependent biofilm formers. Both the *icaA* and *icaD* were present in all the isolates and they are known to play a significant role in biofilm formation. In this study, we observed only one change on biofilm related genes (*icaD*) and the mutations were missense. As far as we know no known amino acid changes are reported on *icaD* in *S. aureus*. For *agr* related genes, in this study, we observed mutations on the *agrC* gene. Most of these mutations observed were missense mutations. So, most of the mutations observed in this study on *agr* related genes might be the reason for the difference in *agr* functionality. A previous study conducted on human clinical isolates comparing an *agr* functional and *agr* dysfunctional colony from the same isolate reported common mutations on the *agr* system which were responsible for *agr* dysfunction were *agr*A-8A and *agr*C-T399P (Traber et al. 2008).

3.5. Conclusion

In conclusion, it is better to use both the qRT-PCR expression of *RNAIII* and the phenotypic synergistic *agr* functionality assay, when determining *agr* functionality to get results that are more accurate. *Agr* dysfunctional isolates expressed more biofilm and *agr* related genes (*icaA* and *RNAIII*). While MRSA isolates expressed more biofilm related genes (*icaA*). Mutations on the *agr* locus especially on *agr*C might explain the difference in *agr* functionality observed. Future studies need to be conducted to investigate if mutations that resulted in amino acid changes affected protein functions and whether they are linked to *agr* functionality.

4. CHAPTER 4: Concluding remarks

The effect of *agr* functionality status on biofilm formation in *S. aureus* isolates with different genetic backgrounds.

This study provided a critical preliminary finding on the possible effect of *agr* functionality status on biofilm formation. An increase in biofilm formation in *agr* dysfunctional isolates was observed throughout the time points and overall. There were also increased levels of biofilm formation in *agr* I isolates compared to *agr* II and *agr* III isolates respectively, although the increases in biofilm formation were not statistically significant. This observation is concerning as *agr* I isolates have been described well in literature as those predominantly isolated in clinical settings and were shown to be associated with bacteraemia and invasive infections. In this study it was observed that some clinical isolates contained mixed populations of both *agr* functional and *agr* dysfunctional strains. Mixed populations are of concern because of their ability to oscillate between the functional and dysfunctional state, which can help evade the host's immune system and further cause persistent infections, which lead to poor outcomes.

The effect of *agr* functionality status on the expression of both *agr* and biofilm-related genes.

The significant increase in the expressions of *RNAIII* in dysfunctional isolates did not match the biofilm results observed. *RNAIII* is an effector molecule for the *agr* system; it mediates up regulation of genes that suppress biofilm formation, so an increased in the expression of *RNAIII* results in a decrease in biofilm formation. Planktonic cells were inversely proportional to biofilm formation in the absence and presence of antibiotics.

The impact of *agr* functionality status on biofilm production in the presence of antibiotics.

Agr dysfunctional isolates formed more levels of biofilm overall in the absence and presence of oxacillin, vancomycin, and rifampicin as well as at specific time points compared to *agr* functional isolates. The increase was however not statistically significant, except when exposed to sub-MICs of oxacillin overall ($p=0.007$).

The possible genetic changes in the core genome that might explain the in-vitro phenotypic responses in the different isolates.

Mutations on *agr* related genes (*agr*A, *agr*B. *agr*C, *agr*D, and *RNAIII*) were on *agr*C. Since mutations on *agr*C are associated to *agr* dysfunction. Some mutations might have been due to difference in the genetic background like in the case of isolates 10 and 66. While others were due to *agr* recombination like what we observed in isolates 29 and 46. The were no

mutations or changes observed in biofilm related genes (*icaA* and *icaD*), which might explain why the consistent level of expression across isolates.

Limitations of the study and future studies

The main limitation of this study was the small sample number of the isolates included, which might have influenced the significance of our findings. When reporting the mean OD, we did not have cut-off values to inform us whether the increase or decrease observed was sufficient to be reported. We reported any increase or decrease we observed no matter how small. Due to time constraints.

Future studies may need to include a larger sample size to overcome the shortcoming experienced in this study. Future studies on biofilm formation need address some technical aspect of the CV assay to improve the adherence of the biofilm cells and avoid washing away the biofilm during washing steps, the 96-well plates used for biofilm formation analysis may be coated with plasma or tissue cells for better accuracy and reproducibility of the results. Moreover, other future studies need to investigate the difference in biofilm formation between PIA-dependent and PIA-independent biofilm formers in clinical *S. aureus* isolates, as there is a gap in literature on the topic. In the absence of a standard cut-off for what is considered a difference in the OD values (whether it being an increase or a decrease) we reported our results as observed regardless of the extent of the difference seen in the study results. A standardized cut-off is needed to report legitimate differences observed between OD readings. This will resolve the issue of reporting non-significant differences. Proteomic studies are need to be conducted as a follow to this study to confirm or refute the association of mutations with amino acids changes and the possible effect on the synthesis proteins coded for.

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Appendixes

Chapter 2

1.1. Crystal violet biofilm assay in the presence of antibiotics
1.1.1. Supplementary Tables

Supplementary Table 1.1: Measured E-test results and CLSI breakpoints.

Sample ID	Vancomycin		Rifampicin		Oxacillin	
	MIC (Ug/ml)	Breakpoints ≤ 2 - S 4-8 - I ≥ 16 - R	MIC (ug/ml)	Breakpoints ≤ 1 - S 2 - I ≥ 4 - R	MIC (ug/ml)	Breakpoints ≤ 2 - S - ≥ 4 - R
1	0.38	S	0.06	S	0.50	S
29	0.50	S	0.016	S	>256	R
46	0.38	S	0.016	S	>256	R
140	0.50	S	0.016	S	0.50	S
2	0.50	S	0.016	S	>256	R
10	0.50	S	0.016	S	0.75	S
66	0.50	S	0.016	S	0.75	S
98	0.75	S	0.016	S	>256	R
9	0.50	S	0.012	S	0.38	S
80	1.0	S	0.012	S	>256	R
153	1.0	S	>256	R	>256	R
199	0.75	S	>256	R	0.75	S
*ATCC25923	1.0	S	0.012	S	0.50	S

Note: *ATCC2592 was a positive control. S: Susceptible; I: intermediate; R: Resistant.

Chapter 3

2.1. Genetic analysis

2.1.1. Supplementary Table

Supplementary Table 2.1: Genetic difference observed by aligning biofilm related genes (*icaA* and *icaD*) and *agr* related genes (*agrA*, *agrB*, *agrC*, *agrD* and *RNAIII*) sequences between *agr* functional and *agr* dysfunctional isolates.

Agr type	Sample ID	*Agr* functionality	*Spa* type	*Spa*-CC	Methicillin	*ica-A*	*ica-D*	*agrA*	*agrB*	*agrC*	*agrD*	*RNAIII*
I	2	Functional	t037	*spa*-CC012	MRSA	No changes observed						
	98	Dysfunctional	t037	*spa*-CC012	MRSA							
	10	Functional	t318	*spa*-CC012	MSSA	No changes observed		Amino acid change at position 136 (R to K)	Amino acid at position 12 (G to S)	Amino acid at 236 (Q to K)	No changes observed	There was a SNP at position 448 (G-A)
	66	Functional	t148	*spa*-CCNF9	MSSA							
II	9	Dysfunctional	t509	*spa*-CC002	MSSA	No changes observed				Amino acid changes at 29 (F to K) and 393 (G to D)	No changes observed	
	199	Functional	t045	*spa*-CC002	MSSA							
	80	Dysfunctional	t045	*spa*-CC002	MRSA	No changes observed						
	153	Functional	t045	*spa*-CC002	MRSA							
III	1	Dysfunctional	t012	*spa*-CC012	MSSA	No changes observed						Deletion of nucleotide base at position 437 on isolate 1 on the *RNAIII*
	140	Functional	t012	*spa*-CC012	MSSA							
	29	Functional	t012	*spa*-CC012	MRSA	No change observed	Amino acid changes at positions 17 (I to V) and 97 (K to R).	No change observed	There were diverse number of amino acid changes	There were diverse number of amino acid changes	There were diverse number of amino acid changes	No changes observed
	46	Dysfunctional	t012	*spa*-CC012	MRSA							