Surface colonisation and survival of *Staphylococcus aureus*

Breno Augusto Barroso Salgado

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Abstract

Staphylococcus aureus nasal persistently colonises the nasal cavity of 20% of the human global population and a further proportion is frequently transiently colonised. Prevention of *S. aureus* colonisation reduces transmission and minimizes infections caused by this microorganism, which range from minor skin infections to life-threatening diseases. In this study, a murine nasopharyngeal colonisation model was established and then used for three serial passages of seven days with *S. aureus*. During the evolutionary timescale, a pronounced genetic variation was identified among the surviving colonising isolates, demonstrating that *S. aureus* mutations are naturally selected against the selective pressures in the nasopharynx niche. DNA Sequence variants were mainly found in genes that encode proteins with roles in DNA and RNA activity and those involved in carbohydrate and amino acid metabolism and stress responses. *S. aureus* nasopharyngeal evolved clones revealed a trend for increased fitness as evidenced by some weak statistical support for increased frequency of colonisation in the murine nasopharynx. This observation provides some support for the selection of sequence variants resulting in adaptive responses to the niche. Over the course of this study, intra-species competition for the nasopharynx niche was observed, whereby a *S. aureus* strain already colonising mice (named SA_MOU) prevented invasion by the experimental *S. aureus* strain after its inoculation. Although the competitor strains are closely related to USA300 (both belong to ST8), the SA_MOU genome sequence did not reveal strong evidence for similar genetic adaptation to the nasopharynx as that identified via the SNPs of USA300 LAC JE2 after serial passages of the murine nasopharynx. This study has increased our understanding of *S. aureus* adaptive responses to selective pressures in the nasopharynx, which could contribute to future novel strategies aimed at inhibiting human nasal carriage of *S. aureus*.

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Contents

List of figures

List of tables

List of abbreviations

Agr	**Accessory gene regulator**
AFLP	**Amplified fragment polymorphism**
BHI	**Brain Heart Infusion**
BLASTn	**Basic Local Alignment Search Tool (Nucleotide)**
bp	**Base Pair**
°C	**Degrees Celsius**
CA-MRSA	**Community-Associated MRSA**
Cfu	**Colony forming unit**
DNA	**Deoxyribonucleic acid**
dNTP	**Deoxynucleotide triphosphate**
ECM	**Extracellular Matrix**
g, mg, µg, ng	**Gram, milligram, microgram, nanogram**
HA-MRSA	**Hospital-Associated MRSA**
mL, µL	**Litre, Mililitre, Microlitre**
m	**Minutes**
MLEE	**Multilocus enzyme electrophoresis**
MLST	**Multilocus Sequence Typing**
mRNA	**Messenger RNA**
MRSA	**Methicillin-Resistant *Staphylococcus aureus***
MSSA	**Methicillin-Susceptible *S. aureus***
NGS	**Next Generation Sequencing**
ORF	**Open reading frame**
PBS	**Phosphate-Buffered Saline**
PCR	**Polymerase Chain Reaction**
pH	**Hydronium ion concentration**
PFGE	**Pulsed-Field Gel Electrophoresis**
RAPD	**Random amplification of polymorphic DNA**
RNA	**Ribonucleic acid**
RPM	**Revolutions per minute**
sRNA	**Small RNA**
rRNA	**Ribosomal RNA**
SNP	**Single Nucleotide Polymorphism**
SCC*mec*	**Staphylococcal Cassette Chromosome *mec***
ST	**Sequence Type**
tRNA	**Transfer RNA**
URT	**Upper Respiratory Tract**

Chapter 1 Introduction

1.1 *Staphylococcus aureus*

S. aureus is one of the 52 species belonging to the *Staphylococcaceae* family (www.bacterio.net/staphylococcus.html, accessed on 18th September 2017) that comprises Gram-positive, catalase-positive cocci with a low GC content (30-35%). *S. aureus* is a facultative anaerobe usually distinguished from other staphylococci by its ability to produce coagulase, an enzyme that interacts with prothrombin resulting in blood clotting by the conversion of fibrinogen to fibrin (Murray, 2012).

S. aureus is occasionally found on the skin and mucosa of mammals and birds where it mostly establishes an asymptomatic colonisation. As an opportunistic pathogen, *S. aureus* has a vast virulence determinant arsenal that enables invasion of host tissues to cause a wide range of infections from superficial, uncomplicated skin infections, including impetigo (Liu *et al*., 2009) and abscesses (López *et al*., 2013), to life-threatening diseases such as pneumonia (Ott *et al*., 2010), meningitis (Pereira *et al*., 2015), endocarditis (Huang *et al*., 2008) and osteomyelitis (Lalikian *et al*., 2017). Food poisoning (Zhao *et al*., 2017) and both scalded skin and toxic shock syndromes (Handler & Schwartz, 2014; Kulhankova *et al*., 2014) are examples of toxin-dependent diseases caused by *S. aureus*.

Treatment of infections caused by *S. aureus* has become an increasing challenge for healthcare facilities after the emergence of strains with high levels of resistance to multiple antibiotics, exemplified by Methicillin-Resistant *S. aureus* (MRSA). Such strains represent a great concern in healthcare units since they are a frequent cause of nosocomial infections that are complicated in their treatment (Al-Talib, 2010). In recent years MRSA strains have surged in healthy humans without previous exposure to healthcare settings, indicating new trends in pathogen evolution (Community-Associated MRSA – CA-MRSA) (Otto, 2013).

S. aureus possess diverse ways to evolve resistance to a vast range of antibiotics. In the 1940s, penicillin-resistant strains were observed (Rammelkamp & Maxon, 19420 and two decades later they reached pandemic levels. These strains produce a penicillinase that interacts with the β-lactam ring of penicillin resulting in drug inactivation. In the 1960s, methicillin was then introduced to overcome the penicillin resistance. Methicillin has less affinity with the β-lactamases produced by *S. aureus* but two years after it was introduced there were reports of methicillin-resistant strains (Peacock & Paterson, 2015). MRSA strains express an additional penicillin-binding protein (PBP2a) that has less affinity to β-lactams. PBP2a is encoded by the *mec* gene (*mecA*, *mecB* and *mecC*) located in the staphylococcal cassette chromosome mec (SCC*mec*). The incorporation of this genetic mobile element led to the evolution of methicillin-susceptible *S. aureus* (MSSA) to MRSA (Deurenberg & Stobberingh, 2009).

SCC*mec* structure consists of the *mec* gene and its regulatory elements (repressor, signal transducer and insertion sequences), cassette recombinase genes (*ccrA, ccrB* and *ccrC*) and the J regions that are located beside *mec* and *ccr*. Different combinations of the *mec* and *ccr* complexes define the SCC*mec* types that can be divided in subtypes according to variations in the J regions in the same type. To date, 11 types have been described which are divided in several subtypes (Lakhundi & Zhang, 2018).

While HA-MRSA is characterised by SCC*mec* type II, CA-MRSA strains frequently carry SCC*mec* type IV or V. Additionally, CA-MRSA possess the Panton-valentine leucocidin (PVL) gene - a cytotoxin that enhances the bacterial virulence. However, it has been suggested that the classification of HA-MRSA and CA-MRSA is no longer valid as infections caused by HA-MSA have been reported in patients from community settings and CA-MRSA infections have been observed in healthcare settings (Choo, 2017).

Inevitably, the need to control *S. aureus* infections, especially MRSA, consumes a considerable part of the health budget and this economic burden has led to hospitals increasing precautions, such as screening for MRSA and isolation of patients, to counter the increased length of hospital stay (Ott *et al*., 2010). It was shown that nosocomial MRSA pneumonia results in an overall cost 50% higher than a similar infection caused by MSSA (Methicillin-Susceptible *S. aureus*) (Ott *et al*., 2010). A separate study performed in Germany demonstrated that although it is not possible to determine the total economic cost of MRSA infections and colonisation, it imposes a high financial impact in the German

healthcare system with direct costs ranging from €350 million to €1.5 billion (Claus *et al*., 2014). In the United States, CA-MRSA cases cost up to $13.8 billion per year on society (Lee *et al*., 2013). The economic impact of MRSA on the UK in 2005 was between £3 to £11 billion (6-20% of the total NHS expenditure ten years earlier), an expense calculated based on changes in productivity, healthcare delivery cost and labour supply (Smith *et al*., 2005).

Thereafter, controlling MRSA infections and its transmission would lead to significant economic savings (Gould *et al*., 2006). The simplest way to avoid *S. aureus* infections is by preventing colonisation. In humans, the most common anatomical site colonised by *S. aureus* are the anterior nares where 20% of the global population is persistently colonised and a further 30% is colonised transiently (van Belkum *et al*., 2009).

1.1.1 Typing, phylogeny and epidemiology of MRSA

The epidemiology of MRSA infections depends on phenotypic and genotypic typing methods which enable investigations of clonal spreading based on characterization of isolates. Multiple criteria are evaluated for the ideal typing technique: typeability, reproducibility, discriminatory power, versatility, rapidity and simplicity of data interpretation. The most suitable technique should possess the majority if not all the characteristics mentioned above (Faria *et al*., 2008).

Phage typing was the first typing technique to demonstrate epidemic spread of MRSA lineages between hospitals as well as intercontinental spread (Mehndiratta & Bhalla, 2012). This method has a great discriminatory power which has helped the identification of MRSA strains in epidemiological studies, however it possess some disadvantages including the lack of reproducibility, the time consumed and a relative high proportion of nontypeable strains (Lakhundi & Zhang, 2018). Pulsed-field gel electrophoresis (PFGE) is a technique that employs digestion of genomic DNA separated in a gel with periodic changes in the orientation of the electric pulse. When carried out with the restriction endonuclease *Sma*I, PFGE is considered the gold standard for MRSA typing due its discriminatory power, reproducibility and ease of execution and interpretation (Goering, 2010). Although this technique has limitations like cost of consumables and labor-intensive protocols, PFGE has helped in the understanding of both endemic and epidemic MRSA strains (Blanc *et al*., 2001; Goering, 2010; Weller, 2000).

PCR-based typing systems have been developed for MRSA including Amplified fragment polymorphism (AFLP) (Vos *et al*., 1995), Random amplification of polymorphic DNA (RAPD) (Raoul & Fouriner, 2009) and repetitive element sequence-based PCR (Rep-PCR) (Sabat *et al*., 2006) with distinct levels of discriminatory power and inter and intra-laboratory reproducibility. Another typing technique based on PCR involves the variable region of the *agr* gene (accessory gene regulator), a global regulator of the staphylococcal virulon (Francois *et al*., 2006). Different biotypes of *agr* (II, III and IV) have been

associated with specific clinical conditions and group I is prevalent between MRSA isolates (Lakhundi & Zhang, 2018).

Multilocus enzyme electrophoresis (MLEE) is a typing technique that involves the extraction of proteins from the cell followed by separation on gels by electrophoresis. It has god reproducibility and typeability in general with moderate usefulness for outbreak investigations (Lakshmi, 2015). Multilocus sequence typing (MLST) is a variation of MLEE which is based on variation of multiple loci. This technique analyses seven housekeeping genes and assigns alleles to all of them resulting in an allelic profile (sequence type or ST). It has provided important insights into the clonal populations of MRSA (Robinson *et al*., 2004). Other techniques involve the characterization of genetic variability. While *spa* typing is based on polymorphism of the X region from the *spa* gene (protein A), SCC*mec* typing is based on variants of the mobile genetic element responsible for β-lactam resistance as mentioned above. They have been used for studies of molecular evolution and hospital outbreaks (O'Hara *et al*., 2016; Satta *et al*., 2013). Ultimately, whole-genome sequencing has the potential to resolve a single bp variation between genomes providing a more accurate characterization of transmission events and outbreaks (Durand *et al*., 2018).

Molecular typing methodologies have supported studies of MRSA epidemiology. Combination of MLST and SCC*mec* typing revealed an evolution of MRSA into phylogenetically distinct lineages over time and that only a few clones are responsible for MRSA diseases (Enright *et al*., 2002). Findings from both techniques also support the theory that SCC*mec* was introduced multiple times

into diverse genetic lineages of *S. aureus* (Enright *et al*., 2002). Although MRSA populations are naturally dynamic, one or two lineages usually predominate in a particular geographical region, where the successful clone reaches a peak before it declines and disappears (Willie *et al*., 2011).

Nowadays, MRSA is pandemic and is represented by a number of strains, including HA-MRSA – disseminated in the 1960s – and CA-MRSA – disseminated in the mid-1990s (Chambers & Deleo, 2009). Although MRSA has emerged into distinct lineages, most of the clones reported belong to five different clonal complexes (CCs): CC5, CC8, CC22, CC30 and CC45. These complexes contain several STs. While the global population of HA-MRSA is represented mainly by CC5 and CC8, CA-MRSA clones are dispersed in several CCs (Chambers & Deleo, 2009; Feil *et al*., 2003). In Asia, the most frequently reported subgroups are CC5-ST5, CC8-ST239 and CC22-ST22 (Chen *et al*., 2010; D'Souza *et al*., 2010). The first two are HA-MRSA and studies have demonstrated the spread of these clones from hospitals into the community (Song *et al*., 2011). Even though distinct STs from CC5, CC8 and CC30 predominate in Latin America and Africa, some STs are common for both regions: ST239, ST8 and ST5 (Breurec *et al*., 2011; Rodriguez-Noriega *et al*., 2010). While multiple CA-MRSA STs have been commonly reported in Europe (ST1, ST5, ST8, ST22, ST30, ST80 and ST88), ST8-IV (USA300) is almost exclusively the cause of infections caused by CA-MRSA in the United States, which replaced ST1-IV (USA400) and became endemic (Lakhundi & Zhang, 2018). ST22, ST36 and in a small proportion ST80 isolates are the major cause of MRSA infection in the United Kingdom (Ellington et al., 2009)

1.2 The anatomy of the human upper respiratory tract

The human respiratory system is responsible not only for air transportation from and to the lungs, but also for olfaction, filtering, humidification, together with other functions. It is divided into upper and lower airways from which the upper respiratory tract (URT) is comprised of the mouth, nose, pharynx (segmented into nasopharynx, oropharynx and laryngopharynx) and larynx. The URT represents the main air-conducting components of the respiratory system and is also the colonisation site for a range of microbes at all levels (Isaacs & Sykes, 2002; Pierce & Worsnop, 1999) and is no longer considered sterile at lower levels in the lungs (Dickson & Huffnagle, 2015).

Air can enter the respiratory system through the nostrils passing the anterior nares, the outermost part of the nose that connects the skin and environment with the nasal vestibule and nasal cavity, the latter further segmented in two by the nasal septum. Each half of the nasal cavity has horizontal bony projections in their lateral walls called turbinates (also known as conchae due to their shape) that are divided into inferior, middle and superior. Turbinates are important structures that increase surface area within the nasal cavity to disrupt the airflow (Dahl & Mygind, 1998).

The nasal vestibule is lined with an epithelium, similar to that of skin (stratified squamous, keratinised), which shifts to a ciliated pseudostratified columnar epithelium (respiratory epithelium) within the nasal cavity (Uraih & Marompot, 1990) (Fig 1). Hairs in the nostrils filter foreign particles from the incoming air

and mucous secretions (produced by globet cells and subepithelial glands). Cilia from the respiratory epithelium aid the filtering mechanism by retaining dust, bacteria and other unwanted particles as the air moves from the nasal vestibule towards the nasal cavity. The mucus helps with humidifying the inspired air that is warmed by capillaries located close to the mucus membrane (Harkema *et al*., 2006). The synchronised beating of cilia on the surface epithelium propels the mucous containing filtered trapped materials towards the nasopharynx (Morgan *et al*., 1986). During uncommon situations, such as intense physical exercise or nasal obstruction from a cold, the mouth becomes the preferred passage for air intake.

Moving through the URT, both left and right nasal cavities open into four different paranasal sinuses (frontal, sphenoid, ethmoidal and adenoid) through narrow channels called ostia (sinus passages). The biological functions of sinuses are still unclear, although they are frequent sites of infections from common cold, causing inflammation (Zollikofer & Weissmann, 2008). The filtered, moistened and heated air coming from the nose reaches the choanae apertures in the posterior part of the nasal cavity that merge into the pharyngeal airway, a funnel shaped tube constituted by respiratory epithelium similar to the tissue from the nasal cavity that conducts inspired air from the URT. Since the nasopharynx houses the pharyngeal ostium of the Eustachian tube, an anatomic structure that connects nasopharynx with middle ear, scraps of stratified squamous epithelium typical of skin is also present (Uraih & Marompot, 1990).

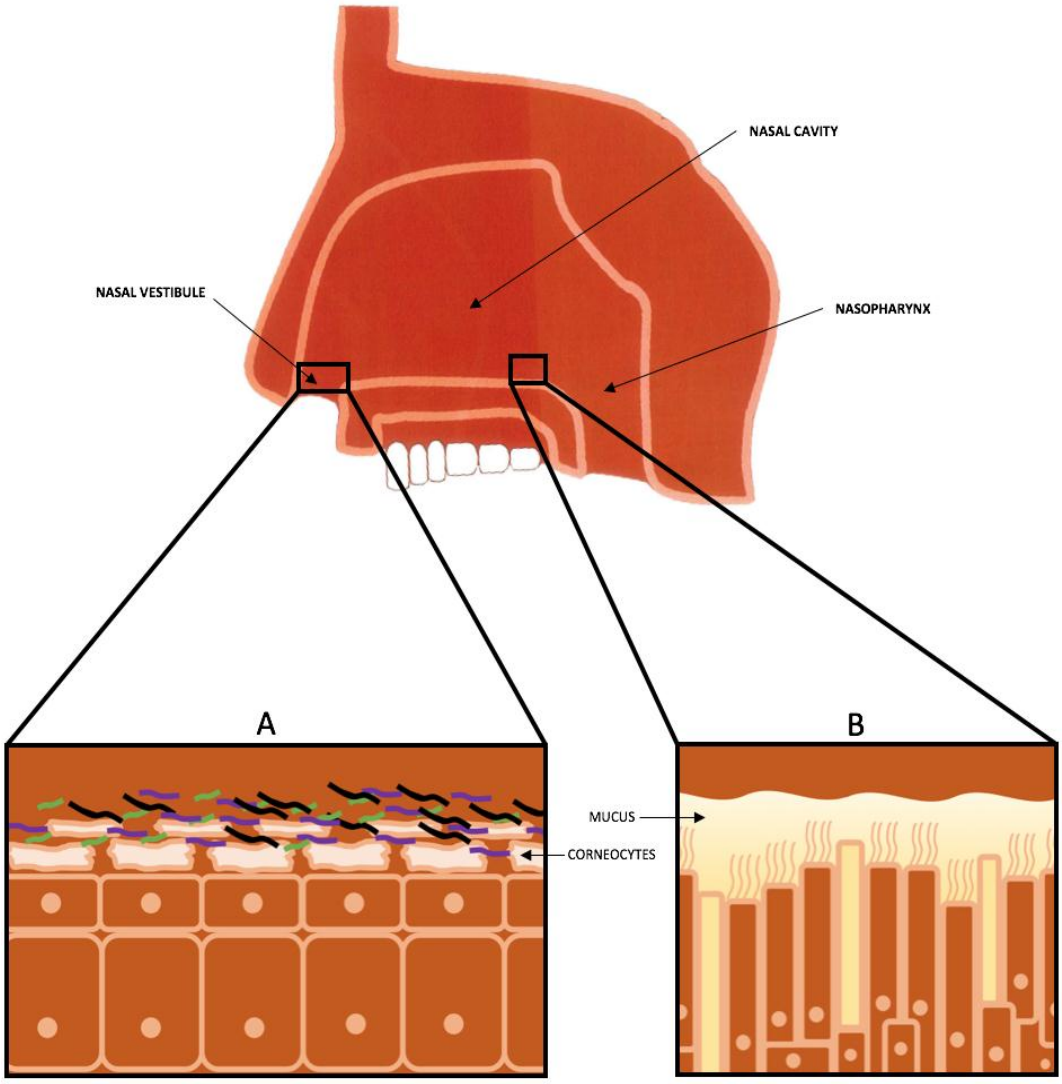

Figure 1.1 Epithelia of the anterior nares (A) and nasal cavity (B).

The anterior nares where the nasal vestibule is located is lined with stratified, keratinised, non-ciliated squamous epithelium. During differentiation, cells become anucleated and the top layer is constituted by corneocytes soaked with a proteinaceous material that contains cytokeratin, involucrin, loricrin (coloured rod shapes) among other proteins. The nasal cavity, including the nasopharynx is mainly lined by pseudostratified columnar ciliated epithelium. Goblet cells (yellow rod cells) produce mucus that coats the top layer of the epithelium.

The adenoid tonsils located at the top of the nasopharynx (posterior end of the nasal cavity) are an aggregate of lymphoid tissue enriched with lymphocytes that destroy pathogens during inhalation. Once the air is collected by the nasopharynx, it passes through the other segments of the pharyngeal airway (oropharynx and laryngopharynx) that also serves as passage for food to be further moved downstream towards the larynx. The epiglottis – one of the cartilage structures located in the larynx - plays a critical role in controlling air entrance into the larynx by blocking breathing during swallowing. The larynx is an organ also lined with respiratory mucosa that connects the inferior part of the laryngopharynx to the trachea and is known as the voice box as it is responsible for vocalisation. The URT terminates at the glottis where vocal cords can be found (Isaacs & Sykes, 2002).

1.3 Indigenous nasal microbiota and *S. aureus* carriage

With a main function to process oxygen and carbon dioxide exchange in human physiology, adult airways comprise an area ~70m^2 (40x larger than the skin surface area). Bacterial species are frequent colonisers of nasal passages based on interactions that vary from beneficial to prejudicial and with responses by the host ranging from tolerance to damage (Man *et al*., 2017). Culture-independent studies have shown high microbiota similarity throughout the nasal passages and nasopharynx, although they harbour different patterns of bacterial community composition that changes according to host age and health status (de Steenhuijen Piters *et al*., 2015.).

A combination of the keratinised squamous epithelium, plus moist, high-oxygen availability conditions in the anterior nares and its proximity to the external environment favours growth of the dominant skin colonisers such as *Corynebacterium* spp., *Propionibacterium* spp. and *Staphylococcus* spp. (Costello *et al*., 2009; de Steenhuijen Piters *et al*., 2015; Wos-Oxley *et al*., 2010) Other examples of bacteria that are often found in the anterior nares are *Dolosigranulum* spp., *Moraxella* spp. and *Streptococcus* spp (Biesbroek *et al*., 2014a; Camarinha-Silva *et al*., 2014) .

The nasopharynx harbours a more diverse bacterial community compared to the anterior nares (Yan *et al*., 2013) possessing high overlap with microbiota from proximate niches, like the anterior nares (notably Gram-positive aerobes such as *Corynebacterium* spp., *Dolosigranulum* spp., *Propionibacterium* spp. and *Staphylococcus* spp.) and oropharynx and oral cavity (*Streptococcus* spp. and Gram-negative anaerobes *Prevotella* spp. and *Veillonella* spp.) (Man *et al*., 2017; de Steenhuijsen Piters *et al*., 2015). Since the oropharynx is linked to a variety of anatomic sites, it generally has a large microbial community constituted by pathobionts such as *Haemophilus* spp., *Neisseria* spp., *Streptococcus* spp. and commensal Gram-negative anaerobes including *Fusobacterium* spp., *Leptotrichia* spp., *Prevotella* spp. and *Veillonella* spp (Charlson *et al*., 2011; Gong *et al*., 2014; Segata *et al*., 2012).

Differences in the nasal microbiota of hosts at different life stages can be observed. Children are usually colonised in their nostrils and nasopharynx by members of the phyla Actinobacteria (notably *Corynebacterium* spp.),

Firmicutes (notably *Streptococcus* spp.), Proteobacteria (notably *Moraxella* spp.) and in a lower frequency by Bacteroidetes (Bogaert *et al*., 2011; Stearns *et al*., 2015). As children reach puberty, a shift occurs and the bacterial community in the anterior nares once comprised by those few dominant taxa is replaced by a more diverse microbiota where Actinobacteria (notably represented by *Corynebacterium* spp. and *Propionibacterium* spp.) and Firmicutes (notably represented *Staphylococcus* spp.) become the most frequent major coloniser phyla and genera (Oh *et al*., 2012). In the nasopharynx, predominance of bacterial species is proposed to be initially derived from the maternal skin or vaginal microbiota in the early stages of life (*Moraxella* spp., *Corynebacterium* spp., *Dolosigranulum* spp., *Staphylococcus* spp. and *Streptococcus* spp.) (Biesbroek *et al*., 2014b). Although a distinct microbiome profile can be clearly described for adults, a broadly similar bacterial nasopharyngeal community was reported, except for the absence of *Moraxella* spp. (Cremers *et al*., 2014; Stearns *et al*., 2015).

Although *S. aureus* is found at multiple sites in the respiratory system, it is mainly localised to the nasal vestibule (Johannessen *et al*., 2012) where the anterior nares are the primary ecological niche of the URT for *S. aureus.* Those permanently and asymptomatically colonised (~20% of the human population) consistently show higher *S. aureus* abundance than those transiently colonised. Due to differences in sampling techniques, it was proposed that those described non-carriers could be transient carriers, but with an insufficient *S. aureus* abundance for cultural detection, resulting in the notion that intermittent and

non-carriers form a single group with the inability to sustain *S. aureus* nasal colonisation (van Belkum *et al*., 2009).

Although *S. aureus* usually acts as an asymptomatic commensal, its colonisation represents a high-risk for subsequent infection (von Eiff *et al*., 2001; van Rijen *et al*., 2008). Success in *S. aureus* nasal colonisation is governed by a complex set of interactions between *S. aureus* and both the host (e.g. epithelium adherence, host immune system evasion) and nasal microbial community (e.g. competition for nutrients and host ligands) (Fig 1.2). Therefore, an increased knowledge and understanding of *S. aureus* nasopharyngeal persistence determinants is a focus of the study in this thesis.

1.4 Determinant factors of *S. aureus* carriage

1.4.1 Host genetic factors

S. aureus carriage patterns support host genetics as an influence on the permanent colonisation by the bacteria. Variation in carriage rates is observed between different ethnic groups indicating that particular races may be more genetically susceptible to *S. aureus* colonisation (Noble, 1974). Screening of random individuals from distinct rural villages in the Netherlands revealed a family predisposition to *S. aureus* carriage, giving indications of influence from host genetics in the success of *S. aureus* colonisation (Noble, 1967). A separate study comprising volunteer isolates from Northern China found that individuals from the Han ethnic group are less susceptible to *S. aureus* nasal carriage (Yan *et al*., 2015).

Comparisons of carriage patterns in sets of twins, to gain insights of host genetic contribution nasal *S. aureus* colonisation, has provided conflicted conclusions. While Hoeksma & Winkler (1963) observed high concordance for *S. aureus* carriage in monozygotic twins, Aly *et al*. (1974) found no significant greater concordance for *S. aureus* carriage in identical twins compared with non-identical twins and non-related children. Besides, no significant heritability contribution was found regarding *S. aureus* carriage in middle-aged and elderly Danish twins and the authors suggested a minor influence of host genetic determinants in colonisation (Andersen *et al*., 2012). Nasal *S. aureus* carriage also seems to be biased by gender, where men show higher colonisation rates than woman (Andersen *et al*., 2012; Andersen *et al*., 2013; Ruimy *et al*., 2010).

Multiple host genetic determinants for *S. aureus* carriage have been associated with the immune system. Polymorphisms in diverse genes encoding for immune components are linked with high risk for *S. aureus* nasal carriage: β-defensin 1 (DEFB1) (Nurjadi *et al*., 2013), interleukin-4 (IL-4) (Emonts *et al*., 2008), C-reactive protein (Ruimy *et al*., 2010), glucocorticoid receptor (van den Akker *et al*., 2006), mannose-binding lectin (Vuononvirta *et al*., 2011) and Toll-like receptor 2 (TLR2) (Vuononvirta *et al*., 2011). For example, the glucocorticoid receptor located in the human epithelial cell surface modulates the transcription of anti-inflammatory genes and a study described that individuals harbouring the 23 lysine allele are more likely to carry *S. aureus* (van den Akker *et al*., 2006). Polymorphism in the DEFB1 promoter region is linked to reduced mRNA expression of β-defensin 1 and 3, which is associated with persistent *S. aureus* carriage (Nurjadi *et al*., 2013; Zanger *et al*., 2011).

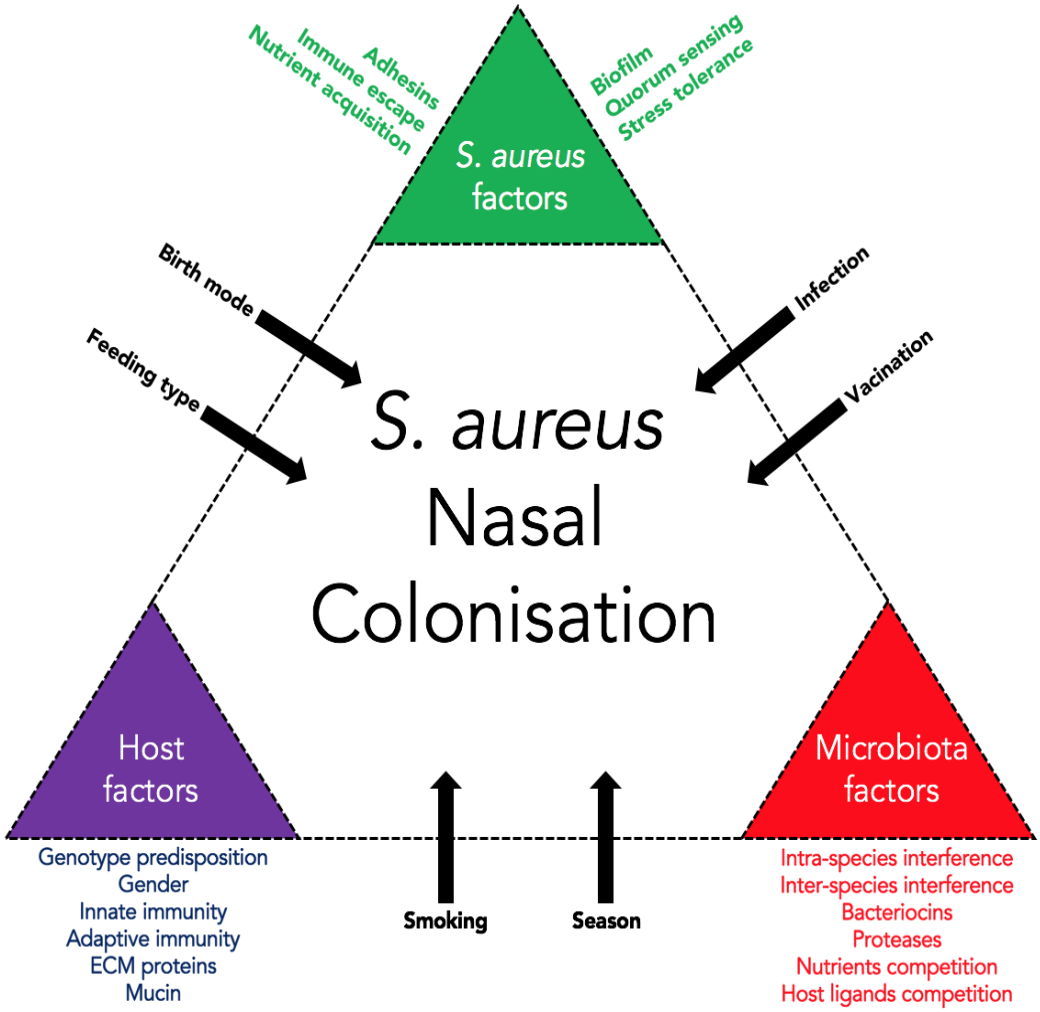

Figure 1.2 Determinants for *S. aureus* nasal carriage.

Schematic representation of the multifactorial nasal colonisation by *S. aureus* and the major components that may affect persistence of *S. aureus* in the nasal passages. A balanced interaction between *S. aureus*, host factors and the nasal microbiome is critical for colonisation by *S. aureus*. Interference from environmental factors and the host lifestyle (black arrows) possibly influence nasal colonisation by *S. aureus*.

Although available studies indicate a correlation between host genetics and nasal carriage of *S. aureus*, a definitive answer of genotypic predisposition to persistent *S. aureus* colonisation cannot be established (Mulcahy & McLoughlin, 2016).

1.4.2 Interaction with the host immune system

Humans have a complex immune system based on diverse mechanisms that activate responses to kill bacteria. Therefore, a potential factor that influences carriage could alter the ability of *S. aureus* to evade the host immune responses. There is emerging evidence for adaptive immune response as a critical determinant of *S. aureus* nasal carriage and for the ability of certain *S. aureus* strains to manipulate the innate signalling pathways in the nose (Mulcahy & McLoughlin, 2016).

The complement cascade is an important feature of the innate immune system and it comprises 25 serum proteins enrolled in bacterial killing by altering the microbe membrane potential via the membrane attack complex. Complement targets microbe cells for phagocytosis via opsonisation and promotes inflammation (Stoermer & Morrison, 2011). *S. aureus* is able to inactivate the complement cascade activation through a wide range of secreted proteins. For example, *Staphylococcus* complement inhibitor protein (SCIN) binds to C3b inactivating C3 convertase and thereby obstructs phagocyte recruitment (Rooijakkers *et al*., 2006). *S. aureus* cell surface anchored protein A (Spa) prevents opsonisation by binding the Fc region of IgG resulting in incorrect

orientation for phagocytosis receptor-mediated uptake (Deisenhofer, 1981). Studies have detected Staphylococcal protein A (Spa) in the exoproteome of a nasal carrier *S. aureus* strain (Muthukrishnan *et al*., 2011) and also demonstrated that Spa-deficient mutants are less virulent in animal models (Patel *et al*., 1987).

Staphylokinase (Sak) activates plasminogen into plasmin enabling bacteria to cleave molecules of complement attached to the bacterial cell surface, such as C3b and IgG (Rooijakkers *et al*., 2005). The gene for Sak was shown to be highly expressed during nasal colonisation. Sak also possesses alpha-defensin binding ability, therefore inhibiting their bactericidal effects (Jin *et al*., 2004). Polysaccharide capsule is present across a range of clinical *S. aureus* isolates and whilst it does not block the attachment of complement molecules to the bacterial cell surface, it limits the phagocytosis, suggesting that the capsule inhibits the interaction between neutrophils and complement factors adhered to the capsule (O'Riordan & Lee, 2004; Thakker *et al*., 1998).

Fibrinogen-binding proteins play an important role in *S. aureus* evasion from the host immune responses. Extracellular fibrinogen binding protein (Efb) has been shown to bind complement factor C3 thereby limiting bacterial opsonisation (Lee *et al*., 2004). The fibrinogen-binding protein, clumping factor A (ClfA), has antiphagocytic activity. ClfA expression leads to the bacterial cell surface being coated with fibrinogen which consequently blocks the placement of opsonins (Bischoff *et al*., 2004; O'Brien *et al*., 2002). Studies have shown that ClfA is present on the cell surface in post exponential phase and this protein not

only inhibits phagocytosis by macrophages (Palmqvist *et al*., 2004) and aids higher virulence of *S. aureus* in murine model (Josefsson *et al*., 2001).

S. aureus reduces phagocytosis by inhibiting neutrophil chemotaxis. Chemotaxis inhibitory protein of *Staphylococcus* (CHIPS) binds to C5a and formylated peptide neutrophil receptors to avoid recognition of these chemoattractant molecules (de Haas *et al*., 2004). Extracellular adherence protein (Eap) inhibits leukocyte recruitment by binding to the adhesive protein receptors like intracellular adhesion molecule 1 (ICAM-1) receptor on endothelial cells, blocking the adhesion of neutrophils and thereby diapedesis and extravasation (Chavakis *et al*., 2002).

S. aureus expresses potent factors that disable the host immune system, including exotoxins. Panton-Valentine leukocidin (PVL) is a *S. aureus* exotoxin associated with high virulent phenotypes such as those of USA300 strains and PVL secretion destroys leukocytes (Genestier *et al*., 2005). *S. aureus* that are engulfed can escape killing within phagosomes via staphyloxanthin, the orange carotenoid pigment in its membrane, which prevents the oxidising effects of reactive oxygen species (Liu *et al*., 2005). *S. aureus* also possess superoxide dismutase enzymes that detoxify reactive oxygen species (Karavolos *et al*., 2003).

Innate immune responses from the host include synthesis of antimicrobial lipids, peptides or proteins and *S. aureus* expresses resistance mechanisms. Lysozyme is an important enzyme from the host innate immune system, which

cleaves the glycosidic bond between N-acetylglucosamine and N-acetyl muramic acid residues, consequently disrupting the cell wall. Production of the cell wall modifying enzyme, O-acetyl transferase (OatA), leads to N-acetyl muramic acid acetylation which combined with the presence of wall teichoic acid (WTA) at this site inhibits access of lysozyme to the glycosidic bond with N-acetyl glucsamine (Bera *et al*., 2005). *S. aureus* escape from the activity of diverse cationic antimicrobial peptides attracted to the anionic bacterial cell surface by reducing the negative charge of its membrane. MprF and Dlt enzymes catalyse changes to the charge of the cell membrane and teichoic acid to limit integration of antimicrobial peptides into the cell surface. Operon mutants for these enzymes have reduced virulence in animal models (Collins *et al*., 2002; Kristian *et al*., 2003). Antimicrobial fatty acids (AFAs) are part of skin defence and present in the nasal cavity; sapienic acid is active on skin while linoleic acid is active in the anterior nasopharynx. *S. aureus* has protection mechanisms via IsdA (iron-regulated determinant A), WTA and an efflux pump for linoleic acid as these components alter cell surface hydrophobicity and limit interaction avoiding bacterial killing by the AFAs (Bera *et al*., 2007; Clarke *et al*., 2007; Kenny *et al*., 2009). Reduced free *cis*-6-hexadecanoic acid on skin has been associated with *S. aureus* carriage among atopic dermatitis patients (Takigawa *et al*., 2005).

1.4.3 Interaction with the nasal epithelium

Bacterial attachment to surface components of the nasal cavity is a critical step in persistence of *S. aureus* in nasal passages. The anterior nares are lined with

keratinised, stratified, squamous epithelium, which undergoes a differentiation that changes their appearance to highly keratinised corneocytes (anucleated cells). These cells are covered by a matrix composed of proteins such as cytokeratin 10 (K10), filaggrin, involucrin and loricrin (Fig 1) (Mulcahy & McLoughlin, 2016). The interaction of *S. aureus* with these proteins is mediated primarily by cell wall-anchored adhesins, of which the MSCRAMM (microbial surface components recognising adhesive matrix molecules) family is the largest group; these enable the bacterial cell to adhere to nasal epithelium (Foster *et al*., 2014).

Clumping factor B (ClfB) is proposed to be a major component in *S. aureus* adherence to the epithelium during colonisation and its role was described in rodent models, being linked to promote nasal colonisation of humans. ClfB adheres to proteins located in the cornified envelope of desquamated nasal epithelial cells such as cytokeratin 10 (K10) and loricrin (Mulcahy *et al*., 2012; Schaffer *et al*., 2006; Wertheim *et al*., 2008). The latter is the primary target for ClfB and loricrin-deficient mice have reduced levels of *S. aureus* colonisation; recombinant loricrin can inhibit *S. aureus* adherence to the nasal epithelium (Haim *et al*., 2010; Mulcahy *et al*., 2012). The iron-regulated IsdA protein promotes adherence to human desquamated epithelial cells through interaction with locrinin, involucrin and K10 (Clarke *et al*., 2009). This protein aids *S. aureus* nasal colonisation in a cotton rat model and IsdA is also expressed during colonisation in human and murine models (Burian *et al*., 2010a; Clarke *et al*., 2006).

Inner nasal cavity epithelium is an important niche for *S. aureus* adherence. The cell-wall anchored macromolecule WTA is composed of repeat units covalently linked to bacterial peptidoglycan. WTA contributes to the initial *S. aureus* colonisation of the nasal cavity in a cotton rat model, with a WTA-deficient mutant strain being unable to persist in the nose after one day, unlike its parental strain (Weidenmaier *et al*., 2004; Wiedenmaier *et al*., 2008). A transcriptomics-based study revealed increased expression of WTA-biosynthesis genes during the early stages of colonisation, while genes encoding ClfB and IsdA showed up-regulation in later growth stages (Burian *et al*., 2010a). WTA adheres to epithelial cells in the nasal cavity by binding its ligand on the host cell surface: SREC-1 (scavenger receptor expressed by endothelial cell-I) (Baur *et al*., 2014). SREC-1 was first associated with bacterial recognition through its mediation of the interaction between *Neisseria gonorrhoeae* with epithelial cells (Rechner *et al*., 2007) and has an important role in both innate immune and inflammatory responses after microbe recognition (Murshid *et al*., 2015). Therewith, it was suggested that WTA manipulates host immune responses (Mulcahy & McLoughlin, 2016).

Further *S. aureus* adhesins are associated with promotion of nasal colonisation, corroborating the multi-factorial nature of interactions during *S. aureus* attachment to the nasal epithelium, although the range of corresponding ligands in the human epithelial cells remain to be characterised (Edwards *et al*., 2012). Serine-aspartate repeat containing proteins SdrC and SdrD were shown to contribute *S. aureus* adhesion to desquamated nasal epithelial cells (Corrigan *et al*., 2009) although their specific roles in colonisation are unknown. A recent

study identified desmoglein 1 (Dsg1) as the keratinocyte receptor of SdrD (Askarian *et al*., 2016). *S. aureus* surface protein X (SasX) was linked with promoting *S. aureus* nasal carriage with a SasX-deficient mutant showing drastically reduced levels of nasal colonisation compared with the parental strain in a mouse model (Liu *et al*., 2015). Separately, SasG was demonstrated to promote *S. aureus* adhesion to squamous cells and although laboratory strains appear not to express SasG at detectable levels, its expression was quantified in clinical isolates (Corrigan *et al*., 2007). Several other *S. aureus* MSCRAMMs and SERAM (secretable expanded repertoire adhesive molecules) adhesins have been identified that interact with host factors, including collagen and elastin, however, their relative contribution to nasal colonisation is yet to be described (Heilmann, 2011).

A further important factor associated with *S. aureus* nasal colonisation is its interaction with nasal mucus. *S. aureus* cells are usually found trapped in the mucus layer in rat and human histological samples (Burian *et al*., 2010a; Peacock *et al*., 2001) and have the ability to bind mucin from multiple origins such as ferret airway mucus, bovine submaxillary gland mucin and purified human mucin (Sanford *et al*., 1989; Shuter *et al*., 1996). Although the cognate ligand for mucin expressed by *S. aureus* remains uncharacterised, pre-treatment with trypsin leads to reduced mucin binding levels, suggesting a cell surface protein has mucin binding activity (Shuter *et al*., 1996).

1.4.4 *S. aureus* interactions with nasal microbiota

For a successful colonisation in the nasal passages, *S aureus* needs to compete with other intra and intergenic species together with other *S. aureus* strains. Simultaneous colonisation with two *S. aureus* strains is considered unusual (Weidenmaier *et al*., 2012) and studies showed it required nasal absence of another *S. aureus* to colonise the niche in a rat model (Margolis *et al*., 2010) due to interference in subsequent colonisation by other coagulase-positive staphylococcal strains (Shinefield *et al*., 1974). *S. aureus* intra-species interference occurs due to polymorphism in *agr* (accessory gene regulator), which leads to a block in virulence gene expression (Ji *et al*., 1997), although *agr* interference has not yet been associated with strain interference during nasal colonisation (Goerke *et al*., 2003).

Antimicrobial peptides produced by bacteria, known as bacteriocins, are usually effective against microbes closely related to the producer strain. Bacteriocins produced by *S. aureus*, also known as aureocins, are active against members of nasal flora, including *Corynebacterium fimi*, *Micrococcus luteus* and *Streptococcus* spp. (Gamon *et al*., 1999). Research about *S. aureus* antimicrobial activity against other common nasal isolates is scarce. In a recent study, staphylococci were tested for their antimicrobial activity against members from the nasal microbiota and *S. aureus* isolates showed inhibitory activity against *Dolosigranulum pseudodipthteriticum*, *Moraxella catarrhalis* and *M. luteus*, but no inhibition was detected against other species including *Propionibacterium acnes*, *Streptococcus pyogenes* and *Haemophillus influenza* (Janek *et al*., 2016).

S. aureus populations were demonstrated to be more susceptible to toxin-producing *Staphylococcus epidermidis* strains than non-producers (Libberton *et al*., 2015). Known bacteriocins produced by *S. epidermidis* with bactericidal activity against *S. aureus* are: Pep5, epidermin, epicidin 280 and Nukacin ISK-1. Both Pep5 and epicidin 280 lantibiotics disrupt the cell membrane and effect release of autolytic enzymes from their inhibitors like the teichoic acid. The combination of a weakened cell wall and increased osmotic pressure leads to cell lysis (McAuliffe *et al*., 2001). Epidermin is one the most frequent staphylococcal lantibiotics, found not only in several *S. epidermidis* strains but also other *Staphylococcus* species (Bastos *et al*., 2009). It acts by interacting with lipids of the membrane resulting in murein biosynthesis inhibition and the formation of a pore. Epidermin also inhibits WTA biosynthesis (Hasper *et al*., 2006, Gotz *et al*., 2014). Nukacin ISK-1 was described in *S. warneri* (Ceotto *et al*., 2009) and, although its mode of action remains unreported, this lantibiotic has bacteriostatic activity against *S. aureus* (Janek *et al*., 2016).

S. epidermidis negatively interferes in *S. aureus* nasal colonisation by secreting extracellular serine protease (Esp) that inhibits *S. aureus* biofilm formation, including MRSA and VISA isolates (Iwase *et al*., 2010). This is however contentious, since the contribution of biofilm to nasal colonisation is not clear and was argued against (Krismer & Peschel, 2011), thereby questioning Esp as a mechanism for the negative association with *S. aureus*.

S. aureus is negatively associated with particular *Corynebacterium* spp. colonising the nose. *Corynebacterium* sp. strain Co304 completely eradicated *S. aureus* carriage from 71% out of 17 carriers in a mechanism proposed to be different to bacteriocin-like activity (Uehara *et al*., 2000). In a separate study, *C accolens* was revealed to convert skin surface triacylglycerides into free fatty acids, including oleic and linoleic acid that inhibited the growth of *Streptococcus pneumoniae* grown adjacent (Bomar *et al*., 2016.). Given the *S. aureus* susceptibility to long chain unsaturated free fatty acid killing (Moran *et al*., 2017), the antagonistic mechanism of *C. accolens* can be extrapolated to *S. aureus*.

Several *Streptococcus* species, including *S. pneumoniae* and *S. mitis,* are negatively correlated with *S. aureus* nasal colonisation (Bessesen *et al*., 2015). *S. pneumoniae* antagonism with *S. aureus* was first suggested to be mediated through hydrogen peroxide production (Regev-Yochay *et al*., 2006) and later associated with the pilus produced by *S. pneumoniae* (Regev-Yochay *et al*., 2009). H_2O_2 toxicity for *S. aureus* was also associated with bacteriophage induction, due to induction of the SOS response activating the lytic cycle (Selva *et al*., 2009), but no significant difference in *S. aureus* nasal colonisation was observed when co-colonised with H_2O_2 producing and non-producing *S. pneumoniae* strains (Margolis, 2009).

Propionibacterium acnes is negatively associated with *S. aureus* in the nares (Frank *et al*., 2010) and it reduces *S. aureus* viability both *in vitro* and *in vivo* (Shu *et al*., 2013). *P. acnes* is able to produce, in the presence of glycerol,

propionic acid which has *S. aureus* killing activity at milimolar concentrations (Shu *et al*., 2013). In the absence of glycerol, a natural product on the skin, *P. acnes* inhibits *S. epidermidis,* presumably via berninamycin A-like thiopeptides (Christensen *et al*., 2016). Given that *S. aureus* is also affected by these antibiotics (Haste *et al*., 2012), it is possible that these mechanisms target *S. aureus*.

Of the other species with described antagonistic properties*, Bacillus subtilis* synthesises diverse antimicrobials active against *S. aureus*, including surfactin and plipastatin. The combination of these antimicrobials could inhibit *S. aureus* growth in a murine skin model (Gonzalez *et al*., 2011; Liu *et al*., 2010). *Pseudomonas aeruginosa* also negatively correlates with *S. aureus* by affecting the *agr* system expression through signalling peptide 3-oxo-C12-HSL. This peptide has been shown to benefit *P. aeruginosa* rather than *S. aureus* in co-colonised sites such as wound infections and the respiratory system of patients with cystic fibrosis (Qazi *et al*., 2006).

1.4.5 *S. aureus* quorum sensing system and the Agr regulatory mechanism

Quorum sensing is a mechanism by which bacteria sense cell density within a population by employing small signal molecules for cell-to-cell communication, through which the cells sense and respond to the signal and alter gene expression. Changes in gene expression are transduced by means of a two-component signal transduction system (TCS). The major staphylococcal TCS is

agr which regulates virulence determinants such as toxins, exoenzymes and adhesins (Le & Otto, 2015).

The *agr* locus is comprised by two transcriptional units: RNAII and RNAIII, regulated by promoters P2 and P3, respectively. While RNAII harbour four genes (*agrA, agrB, agrC* and *agrD*), RNAIII is an effector molecule that controls Agr targets and is also a mRNA that encodes the delta-hemolysin Hdl. An auto-inducing peptide (AIP) signal is produced from the AgrD precursor, maturated by AgrB and exported. AIP detection by AgrC leads to phosphorylation of AgrA which activates both promoters P2 and P3, resulting in an auto-feedback regulation (Tan *et al*., 2018) (Figure 1.3).

RNAIII usually acts blocking the translation of a number of surface proteins. It also blocks the translation of the repressor of toxin (Rot) protein leading to the transcription of many exoproteins and toxins (Boisset *et al*., 2007) (Figure 1.3).

Quorum sensing inhibition can occur by interference between staphylococcal species. AIP amino acid sequence variation determines different classes of *agr*. While *S. aureus* has four *agr* groups (I-IV), *S. epidermidis* has three (I-III) (Chen *et al*., 2011; Thoendel *et al*., 2011). AIP of one strain can inhibt *agr* activation of another strain or staphylococcal species. Quorum sensing antagonism was shown between *S. aureus* and *S. epidermidis* where AIP class I from the latter is able to inhibit all *S. aureus agr* classes, expect for IV. The opposite happens only for *S. aureus* AIP class IV which can inhibit all *S. epidermidis agr* classes.

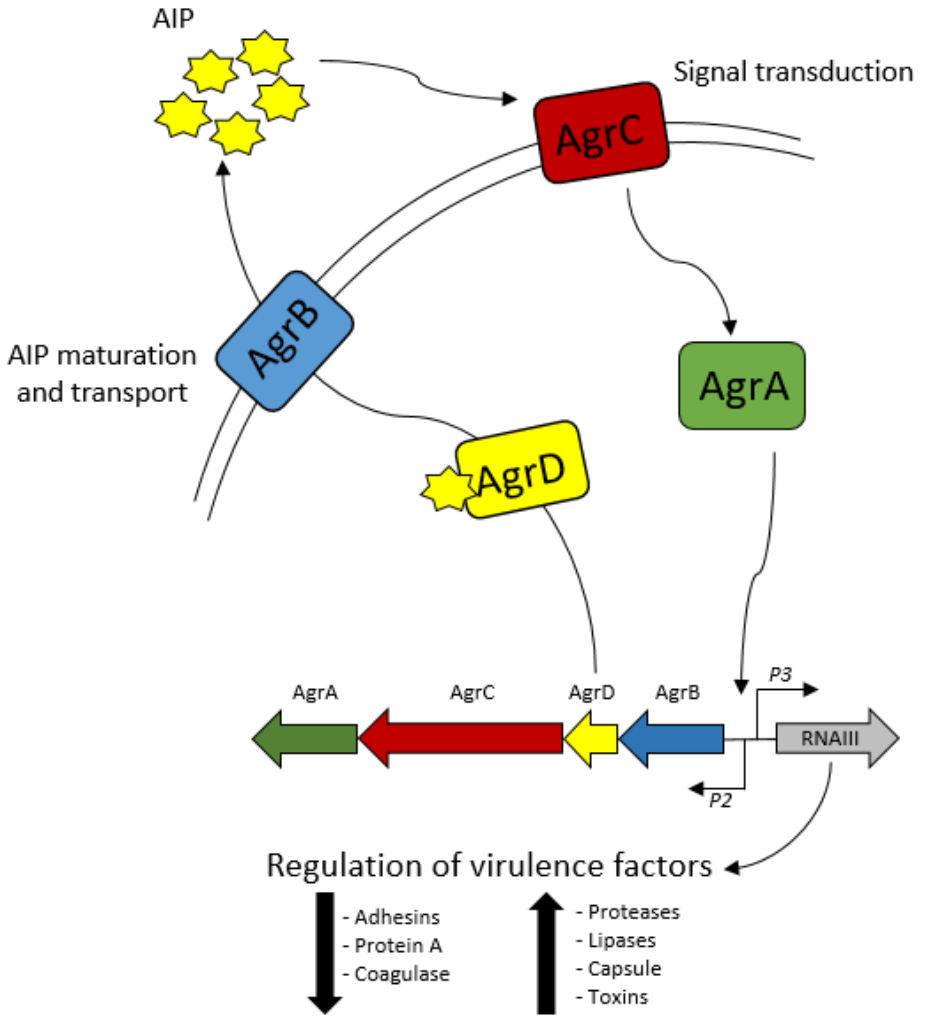

Figure 1.3. Schematic of the *agr* autoinduction circuit in *S. aureus*.
The AgrD peptide processed by the membrane-anchored AgrB is exported in the form of an autoinducing peptide (AIP; tailed thiolactone ring). Ah high extracellular concentrations, AIP binds to AgrC receptor and activates the AgrC kinase, resulting in phosphorylation of the AgrA. Once phosphorylated, AgrA binds and activates promoters P2 and P3. While P2 drives the autoactivation circuit, P3 promotes the transcription of RNAIII, the regulatory effector of the *agr* system.

While antagonism activity results in gene expression alteration, these effects of *agr* in *S. aureus* nasal colonisation are still unclear (Otto *et al*., 2009). Burian *et al*. (2010b) showed that *agr* expression is minimal during nasal colonisation. Canovas et al., (2018) identified other staphylococcal coloniser species interfering with S. aureus *agr* with some of them exhibiting broad antagonistic activity on S. aureus. A separate study of the role of haemoglobin in *S. aureus* nasal colonisation demonstrated that inhibition of the promoter in each of the four classes of *agr* via haemoglobin reduces nasal colonisation of *S. aureus* (Pynnonen *et al*., 2011).

1.4.6 Biofilm and its role in *S. aureus* nasal colonisation

Planktonic cultures of *S. aureus* show bacteria forming small cell clusters (typically with grape-like appearance). Under certain conditions, *S. aureus* communities display the structure of complex dynamic biofilm formations, being characteristically composed of several enclosed bacterial layers immersed into an extracellular matrix that can adhere to biological and non-biological surfaces (Otto, 2008). *S. aureus* biofilm is associated with several chronic diseases, such as endocarditis (Parsek *et al*., 2008), osteomyelitis (Brady *et al*., 2008) and medical device-related infections (Otto, 2008).

S. aureus biofilms are formed upon initial attachment to inert or biotic surfaces in which bacterial components such as WTA, lipotheicoic acids, clumping factors A and b, fibrinogen/fibronectin-binding proteins (FnBPs) and

accumulation-associated protein (Aap) have been reported to play an important role in this event (Speziale *et al*., 2014). After adhesion to a particular tissue, *S. aureus* biofilm undergo a proliferation event leading to the production of an extracellular matrix which can be composed of proteins, polysaccharide and/or eDNA (figure 1.4). Polysaccharide intracellular adhesion (PIA), also known as polymeric N-acetyl-glucosamine (PNAG), is the major constituent of polysaccharide biofilms. It is encoded by the *icaADBC* locus and is a major component for the structural integrity of *S. aureus* biofilms (Lister & Horswill, 2014). *ica*-independent biofilms are primarily constituted by proteins (Foster *et al*., 2014) and eDNA (Montanaro *et al*., 2011), the latter acting as intracellular adhesins in the absence of PIA. Protein A (SpA), FnBPs and Biofilm-association protein (Bap) have been shown to mediate cell-to-cell aggregation in PIA-independent biofilm (Foster *et al*., 2014). Once a mature biofilm is established, detachment of micro-colonies may occur as a genetically controlled response to environmental cues (figure 1.4). The primary biofilm dispersal mechanism is via the production of exo-enzymes that promotes the self-destruction of the matrix. In general, proteases (e.g. Proteinase K, SspA and Aureolysin) and nucleases (e.g. Nuc and Nuc2) have reduced activity against PIA-dependent biofilms (Lister & Horswill, 2014). In contrast, components that target PIA are ineffective against polysaccharide-independent biofilms. Non-specific factors phenol-soluble modulins (PSMs) are effective against most of the biofilms (Peschel & Otto, 2013). Agr system promotes expression of both proteases and PSMs. Studies have demonstrated that Agr is essential for biofilm dissemination (Beenken et al., 2010; Boles et al., 2008), while its inactivation promotes biofilm

formation (Boles et al., 2010). *agr* mutants were reported in thick biofilm-associated infections (Yarwood *et al*., 2004).

S. aureus ability to form biofilm *in vitro* was proposed to be associated with nasal epithelium colonisation (Iwase *et al*., 2010). As mentioned above, proteins associated with staphylococcal biofilms interact with the host to promote *S. aureus* colonisation, such as Spa, Atl, FnBpA, Emp, Eap in addition to further adhesive proteins (Sugimoto *et al*., 2013). These proteins can be degraded by Esp protease produced by *S. epidermidis,* thereby suggesting a mechanism that explains antagonistic correlation between *S. aureus* and *S. epidermidis* mediated by Esp (Iwase *et al*., 2010). However, Fredheim *et al*. (2014) found no interference between Esp protease and biofilm inhibition of *S. aureus*. Moreover, the authors demonstrated a correlation between biofilm formation genes and nasal carriage. Contrastingly, histological studies failed to observe biofilm formation during *S. aureus* nasal colonisation from rat and human tissues (Nouwen *et al*., 2004; ten Broeke-Smits *et al*., 2010). Since the role of biofilm-like *S. aureus* populations during nasal colonisation therefore remains undetermined, inclination for a dispersed life style rather than biofilm formation was proposed (Krismer & Peschel, 2011).

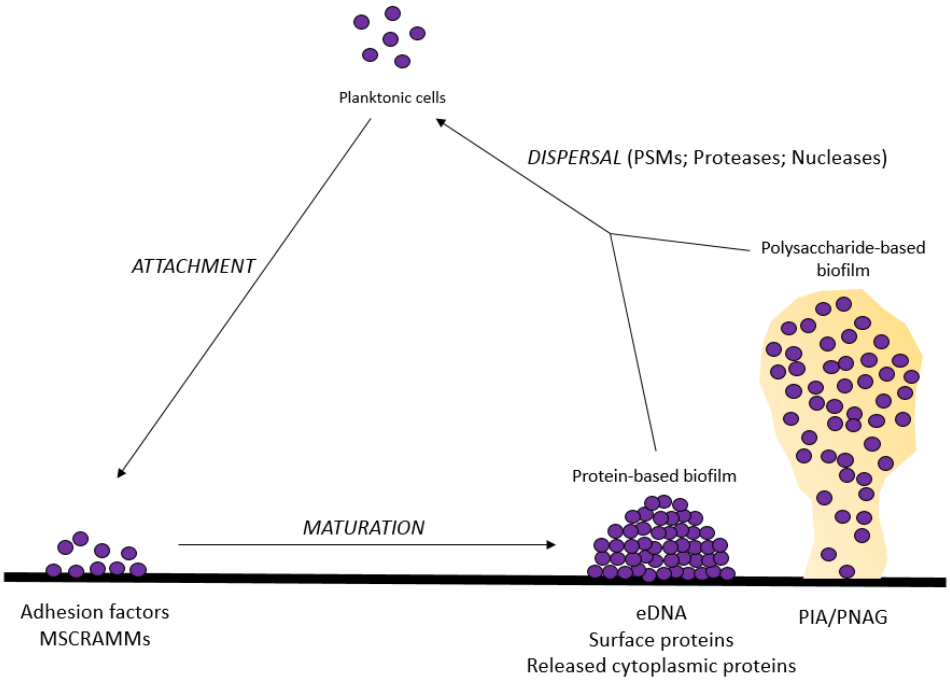

Figure 1.4. Phases of *S. aureus* biofilm formation.

Upon contact with a abiotic or biotic surface, planktonic cells attach via surface-anchored proteins. Microcolonies are formed followed by the production of an extracellular polymeric substance, as part of the biofilm maturation. The matrix can be composed of Polysaccharide Intracellular Adhesin (PIA), extracellular DNA or proteins. Environmental signals trigger dispersal mechanisms where cells re-enter a planktonic mode of growth and can seed new sites for biofilm formation.

1.5 Models to study *S. aureus* nasal colonisation

Experimental models are established to address *S. aureus* colonisation both *in vitro, ex vivo* and *in vivo*. The role of bacterial adhesion components and their respective host ligands during *S. aureus* nasal colonisation were assessed *in vitro* by differences in adherence to human epithelial cells between mutants and their corresponding isogenic parental strains (Baur *et al*. 2014; Clarke *et al*., 2006 Mulcahy *et al*., 2012). Animal models provide more realistic insights about the relevance of such adhesion components, while variations in the establishment of nasal colonisation between *S. aureus* mutant and parental strains have given indications about the role of adhesins (Liu *et al*., 2015; Weidenmaier *et al*., 2008). Similarly, animal models have been used to investigate immune system-related factors and their role in *S. aureus* nasal colonisation (Gonzalez-Zorn *et al*., 2005; Mulcahy *et al*., 2016).

Metagenome sequencing of 16S rRNA from nasal fluid samples produced insights of the nasal microbiome (Choi *et al*., 2014). Nasal microbial flora has been identified with both culture-based methods (Alvarez *et al*., 2013) and multiple culture-independent technologies that include: quantitative polymerase chain reaction (qPCR), fluorescence in situ hybridisation (FISH) mass spectrometry and DNA microarray (Abreu *et al*., 2012; Boase *et al*., 2013; Foreman *et al*., 2009; Frank *et al*., 2010). These methodologies are used to study the population dynamics of the nasal microbiota during *S. aureus* colonisation and interference from other members. Additionally, their combination with

murine models was used to study the correlation between *S. aureus* and potential competitors of the nasal niche (Margolis *et al*., 2010).

Burian *et al*. (2010b) quantified mRNA levels extracted from nose swabs from persistent carriers to investigate the regulatory adaptation of *S. aureus* to the nasal passages and the authors observed a predominant role for the two-component system WalKR during nasal colonisation. Transcriptomic analysis was also used to assess different *in vivo* expression patterns from distinct *S. aureus* carrier strains. This approach showed as a valuable technique to infer essential components for *S. aureus* carriage requiring further investigation for controlling or intervening in colonisation (Chaves-Moreno *et al*., 2016).

Computational modelling is another tool to be used for assessment of interactions during *S. aureus* nasal colonisation. Recently, temporal and spatial predictions of *S. aureus* and *P. aeruginosa* metabolism in a biofilm consortium could be obtained from a computational model (Phalak *et al*., 2016). Therefore, computational modelling coupled with genomic and metabolic data could be important to predict the dynamics of populations in nasal passages during *S. aureus* colonisation.

1.6 Sequencing technologies

More than 50 years ago the first nucleic acid sequence was published where Holley *et al*. (1965) revealed the alanine tRNA sequence from *Saccharomyces cerevisae*. Later, Sanger revolutionised sequencing by presenting a DNA

sequencing technology that would be implemented as the standard sequencing method for decades. Since inception, many aspects have improved in terms of cost, time and effort required to finish a sequencing study, especially after the Human Genome Project was implemented in the 1990s resulting in the publication of the human genome in 2001 (Lander *et al*., 2001; McGinn & Gut, 2013). In terms of bacteria sequencing, the first genome to be published was from *Haemophilus influenzae* in 1995 (Fleischmann *et al*., 1995) with *S. aureus* following 6 years after with the first two genomes published (Kuroda *et al*., 2001).

Nowadays, there are several, diverse platforms able to perform whole genome sequencing that are classified into three generations of DNA sequencers. The first generation of DNA sequencing is attributed to the Sanger sequencing method as the first most significant sequencing technology developed and for decades it was the gold standard for DNA sequencing. Also known as the chain-termination technique, it latterly consists of the generation of multiple fragments of different sizes from a single-stranded DNA template after an amplification reaction mixed with deoxyribonucleotides (dNTPs) and fluorescent-labelled dideoxynucleotides (ddNTPs). The labelled dNTP analogues stop the enzymatic extension due the lack of the group responsible for binding with the next dNTP. Because ddNTPs are randomly incorporated in the strand under extension, fragments of all possible sizes are generated and the fluorescent dye in the terminal ddNTP enables fragments to be separated by gel-electrophoresis with detection by fluorescent imaging (Sanger *et al*., 1977).

The somewhat similar but inferior approach that is considered as the birth of first generation DNA sequencing was proposed by Maxam & Gilbert (1977). Although this technique also uses capillary electrophoresis to separate and visualise fragments generated by strand breakage, such fragments are only available with radiolabelled DNA (usually P^{32}) using different combinations of chemicals to cleave the strand at specific bases, hence explaining why this method is also known as the chemical cleavage sequencing technique (Maxam and Gilbert, 1977).

The need to lower the cost and time for DNA sequencing volume and also to achieve a better accuracy led to the development of second generation sequencing platforms, also referred as next generation sequencing. Unlike the existing methods at the time of their release, the new semi-automated sequencers did not use fluorescent/radio-labelled dNTPs or oligonucleotides although mechanistically there was a reliance on DNA polymerase synthesis. The new approach enabled massive generation of reads by parallel sequencing reactions that resulted in the high efficiency to complete sequencing of a whole genome (Heather & Chain, 2016).

Commercially, 454 Life Sciences licensed the first next generation genome sequencer in 2005, which was later acquired by Roche in 2007. Also referred as DNA pyrosequencing, the library preparation for this technique involves the ligation of a DNA fragmented template to adaptor sequences that are later attached to beads. An emulsion PCR (emPCR) is then performed to coat the beads, resulting in ideally one single DNA amplified fragment immobilised on

one bead. After filtration of beads without an attached fragment, the DNA-coated beads are washed over a picoliter slide that contains millions of wells, each supporting one bead. A sequencing reaction occurs as nucleotides are incorporated into the DNA fragment on the beads in a reaction that releases pyrophosphate. A light signal proportional to the length of the incorporated bases is recorded via sensors on the wells (Margulies *et al*., 2005).

Implementation of 454, parallel-based sequencers became state-of-the-art. Illumina sequencers became the second generation sequence platform most extensively used after their release. Using Solexa technology that was acquired by Illumina in 2007 it is capable of generating millions of highly accurate reads quicker and cheaper than most of the other platforms available (Heather & Chain, 2016). In this technology, single-stranded fragments of DNA have adaptors ligated to their 3' and 5' ends. These adapter-linked DNA fragments are washed across a flow cell which is loaded with primers complimentary to the adaptors. Any DNA fragments that do not attach to the flow cell by one of the adapters are washed away and the single-stranded DNA fragment library is then used in a PCR to synthesise the complementary DNA strand. After removal of the original DNA fragment, a second adapter is annealed to the complementary primer on the flow cell and clonal expansion occurs in a mechanism called "bridge amplification". According to this approach, the recently generated DNA fragment bends and allows the attachment of the second adapter to the primer on the flow cell with subsequent synthesis of complementary strand (identical to the original DNA fragment). This double-strand structure with both ends ligated to the flow cell is then denatured,

exposing both forward and reverse copies of the same DNA fragment. This process is repeated several times generating dense clusters of DNA populations in a confined area (Fedurco *et al*., 2006).

Sequencing is performed in a sequence-by-synthesis manner where the flow cell is coated with fluorescent terminator dNTPs and DNA polymerase to initially amplify the forward strands. After a nucleotide is incorporated into the strand under extension in the clonal cluster, the polymerisation is blocked and the fluorescent dNTPS and polymerase are washed from the flow cell. Repeated cycles are performed until the fragment is read and the sequence is deduced by monitoring the emission of fluorophore signals from each round. Finally, the reverse strand is produced and read according to the same protocol for the forward strand (Turcatti *et al*., 2008). The main platforms from Illumina are HiSeq (greater read length and depth) and MiSeq (lower quality, but cheaper and faster than HiSeq).

Further second-generation sequencing methodologies emerged in the last decade. Applied Biosystems that merged with Invitrogen to form Life Technologies developed SOLiD, a sequencing platform that unlike Illumina is based not on synthesis but on ligation through DNA ligases (McKernan *et al*., 2009). Short read length limits the general use of SOLiD, however the cost per base sequenced is a positive factor from this sequencer (Glen, 2011).

Another remarkable sequencing platform based on ligation was implemented by Complete Genomics from which the nucleotide sequence is obtained from

nanoballs with clonal amplification of a DNA fragment (Drmanac *et al*., 2010) Finally, another notable second-generation sequencing platform is Ion Torrent with similarlities to 454, however it involves neither fluorescence nor luminescence to detect the nucleotides from the DNA fragment under extension. Instead, it measures the pH change during polymerisation and the main advantage of this platform relying on the rapidness of the sequencing (Rothberg *et al*., 2011).

Second generation sequencers, with special mention to Illumina sequencers, have helped researchers to answer questions about diverse biological mechanisms, however for massive projects such as metagenomics and comparative higher eukaryote genomics, the final cost for sequencing of genomes remained high. Third-generation DNA sequencers were established to improve the quality of the finished genome with the challenge to lower the final price of sequencing. Although there is not a consensus about the components of a platform to be included in the second or third generation of DNA sequencing, the most recent methodologies have aimed for longer reads to facilitate the genome assembly. Heather & Chain (2016) considered third-generation sequencers as those technologies capable of sequencing single DNA molecules without the need of a clonal amplification step.

Following these concepts, PacBio from Pacific Biosciences is possibly the most notable third-generation platform. It uses zero-mode waveguides (ZMWs) as nanostructures where the DNA template is immobilised alongside DNA polymerase and phospho-linked nucleotides. As the nucleotides are

incorporated in the DNA strand being synthesised, fluorescence signals can be monitored in real time. Recent improvements in PacBio technology have taken this platform to >99.99% accuracy in the consensus sequence (Flusberg *et al.*, 2010).

Another third-generation sequencing methodology was implemented by Oxford Nanopore when the company released GridION and MinION platforms. Their technology is based on the potential of a DNA fragment (or other molecules) to pass through nanopore channels under when electronically induced. As the DNA travels through the nano holes the ion flow is blocked causing disruptions in the current proportional to the fragment size, allowing detection of the acid nucleic sequence (Clarke *et al.*, 2009). Nanopore sequencing is at its early stages and the technology involved needs much refinement. However, this methodology is expected to potentially revolutionise the sequencing field (Heather & Chain, 2016).

1.7 Thesis aims

S. aureus nasal colonisation is a high-risk factor for transmission and infection caused by this microorganism. Although studies have aimed to elucidate host-microbe interactions and bacterial components relevant for *S. aureus* persistence in the nasopharynx, a profound understanding of *S. aureus* carriage determinants are yet to be characterised. This study investigates *S. aureus* DNA sequence variants selected over an experimental nasopharyngeal and their potential role in bacterial adaptation to this niche.

Chapter three will aim to develop a long-term nasopharyngeal colonisation murine model and its subsequent use to perform serial passages of *S. aureus*. With the model successfully established, the repeated passages are a powerful approach to check if there is selection of genetic variants over an evolution time-frame in the nasopharynx. Chapter four will aim to capture these mutations naturally selected during *S. aureus* colonisation of the mice nasopharynx. It is expected that such DNA sequence variants provide increased fitness for colonisation, which will be addressed in Chapter five. Variation in the colonisation status amongst nasopharyngeal clones indicates that *S. aureus* adapts to this anatomic site by selecting mutations that lead to improved phenotypic responses against environmental stress conditions.

Chapter 2 Materials and methods

2.1 Bacterial strains and growth conditions

The *S. aureus* strain used in this study was USA300 LAC JE2, a derivative of the clinical isolate cured of plasmids p01 and p03. *S. aureus* USA300 LAC was isolated from a skin and soft tissue infection in a detainee from the Los Angeles County Jail (LAC) and is well-studied. This strain harbours a SCC*mec* type IV (Fey *et al*., 2013; McCarthy *et al*., 2016) and epidemiologically it is confined mostly to the Americas, mostly North America, and has gained favour as a strain of choice since isogenic variants in the Nebraska Transposon Mutant Library allow versatile genetic study. USA300 LAC JE2 was selected for the experimental nasopharyngeal evolution work in this study for repeated colonisation of the nasopharynx.

All cultures were grown for 18 h at 37°C with shaking unless stated otherwise. Brain Heart Infusion (BHI) broth or agar was used as the medium for all work, unless otherwise stated.

2.2 Establishing a long-term nasopharyngeal colonisation murine model

Animal experiments were carried out in strict accordance with ethical approval and recommendations from the University of Liverpool Animal Welfare and Ethics Committee. For this study, all experiments with mice were performed in the Ronald Ross Building (Department of Clinical Infection, Microbiology and Immunology, Institute of Infection and Global Health) under guidance and supervision of Dr. Elaine Waters via an ethics licence granted to Prof Aras Kadioglu. The animal model proposed in this work was also meant to be used in separated immunological studies. Therefore, Dr. Elaine Waters investigated the lowest dose capable of colonising the mice nasopharynx so the model could be used for both the immune responses from the host upon *S. aureus* colonisation and the bacterial genomic changes after repeated nasopharyngeal long-term colonisation by *S. aureus*. Dr. Elaine Waters concluded that 5 x 10^4 cfu was the lowest bacterial dose (see details of the experimental procedures below) suitable for both approaches.

The proposed bacterial load was then used for the standardisation of the long-term nasopharyngeal colonisation murine model used in this work. The inoculum was prepared in Lab H (Biosciences building, University of Liverpool) by growing *S. aureus* USA300 LAC JE2 overnight for 18 h at 37°C in BHI and transferring 100 µL of this solution to fresh broth. The culture was allowed to grow until OD_{600} 0.4 from which 16 µL was diluted into 984 µL of sterile PBS (phosphate-buffered saline) in order to achieve a bacterial load of 5 x 10^4

(inoculum was serially diluted and plated on BHI agar plates to confirm the bacterial dose).

The bacterial dose was kept in ice and promptly taken to the Ronald Ross building so the mice could be inoculated in less than 30 min after the inoculum was prepared. Under guidance of Dr. Elaine, fifteen age-matched, 6-8-week-old, outbred BALB/c female mice (Charles River, United Kingdom) divided into groups of three were anesthetised and their nose was horizontally inoculated with 15 μL of the standard bacterial dose prepared as mentioned above. Once the mice were inoculated, they were returned to their cages to be further evaluated regarding *S. aureus* nasopharyngeal colonisation at five time points over a period of 14 d (1, 3, 5, 7 and 14 d).

Groups of three mice were anesthetised prior to sacrifice at different time points. The nasopharynx was then aseptically removed, mixed with PBS and mechanically disrupted. Viable counts from the resulting nasal washes of tissue were performed by plating 3 x 20 μL from the nasopharyngeal material on mannitol salt agar prior to incubation for up to 48 h at 37 °C until colonies were of sufficient size and could be colorimetrically detected on the agar plate.

2.3 Experimental evolution

2.3.1 Nasopharyngeal passages as a selection

The experimental strategy was to perform three serial passages of *S. aureus* USA300 LAC JE2 by repeated colonisation of the nasopharynx of three mice per passage (Fig 2.1). Animal handling and inoculum preparation was performed as described in section 2.2. After inoculation, the mice were maintained with food and water *ad libitum* for 7 d prior to euthanisation then nasopharynx removal.

An inoculum of USA300 LAC JE2 culture was prepared for the first nasopharyngeal passage and instilled in each of three mice. After the 7 d incubation period, each nasopharynx was removed and disrupted in the Ronald Ross Building and the resulting tissue homogenates were spread on three mannitol salt agar plates bin Lab H. For each agar plate cultured overnight, ten clones were randomly selected and subcultured to OD_{600} 4.0. Then, equal volumes (1 mL) of the ten cultures were mixed to generate a new inoculum followed by dilution in PBS as described in section 2.2 to obtain a bacterial load of ~ 5 x 10^4 cfu. This inoculum was used for a second passage of murine nasopharyngeal colonisation. A third and final passage was achieved using this approach one more time. Both control and resulting clones from each of the three nasopharyngeal passages were stored alongside the nasal homogenates at-80 °C with 15% glycerol (v/v).

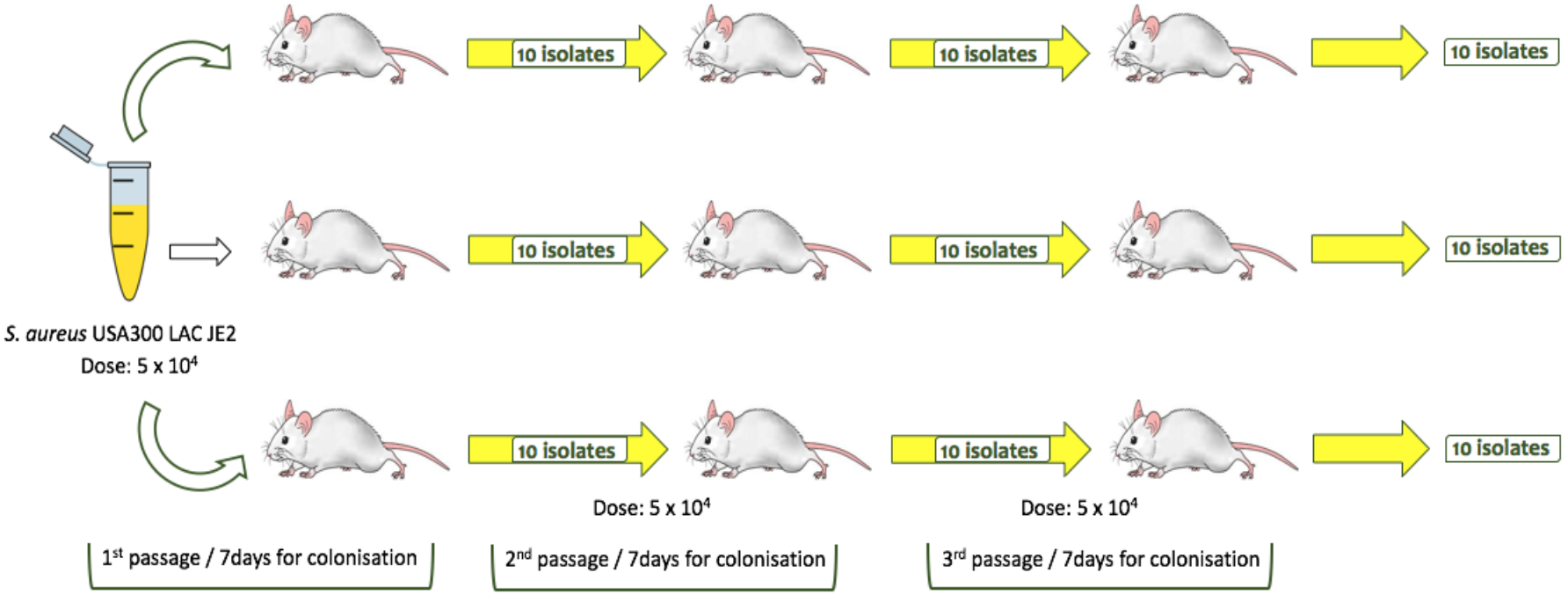

Figure 2.1 Serial passages of *S. aureus* in a murine nasopharyngeal colonisation model.

Schematic demonstration of repeated colonisation of mice nasopharynx by *S. aureus* USA300 LAC JE2. A total of three colonisation serial passages were performed with each lasting 7 d. A standard culture was used as inoculum for the first passage while 10 isolates randomly collected and pooled from passage 1 and 2 served as inoculum for subsequent passages. Three mice were used per colonisation passage.

2.3.2 Strain confirmation

After each of the three serial nasopharyngeal passages was completed, *S. aureus* isolates recovered from the mice nasopharynx were screened by PCR amplification of the staphylococcal protein A (*spa*) gene to confirm that the clones found in the nasal washes after the colonisation period belonged to the same spa type as the inoculum strain. DNA extraction (see section 2.4.1) was followed by PCR amplification of the Short Sequence Repeat (SSR) region of the *spa* gene. The reactions were undertaken using Mastercycler pro S (Eppendorf) and each contained 0.5 μM PA 1095F forward PCR primer, 0.5 μM PA 1517R reverse PCR primer (Shopsin *et al*., 1999), 80 ng DNA, 25 μL of BioMix Red (Bioline) and DEPC-treated water up to a total reaction size of 50 μL mixed into 0.2 mL PCR tubes. The run cycle parameters were an initial 10 min at 95°C and then 30 cycles of 30 s at 95°C, 30 s at 60°C and 45 s at °C with a final 10 min at 72°C.

PCR products were purified by using the Isolate II PCR and gel kit (Bioline), analysed by agarose gel electrophoresis (see section 2.4.2) and then sequenced by GATC Biotech with samples prepared according to the company requirements. The *spa* sequence was then analysed by using BLASTn (Madden, 2002) with default settings. Matching PCR fragment sizes and BLASTn output data for both controls and passaged clones indicated that they belong to the same strain.

2.4 Genome sequencing

2.4.1 DNA extraction

For DNA extractions, 1.5 ml of overnight cultures were pelleted at 6000 rpm for 4 min in sterile Eppendorf tubes. DNeasy Blood and Tissue kit (Qiagen) was then used according to the manufacturer's instructions for Gram-positive bacteria, including the addition of lysostaphin (Sigma-aldrich) at a final concentration of 15 μg/mL and 10 U mutanolysin (Sigma-Aldrich) to the cell lysis buffer. A RNase step was also included in the protocol.

2.4.2 Agarose gel electrophoresis

To analyse gDNA integrity prior to sequencing, 1% (w/v) agarose gels were prepared in TAE buffer. When necessary, small PCR products were analysed in 1.5% (w/v) agarose gels. For visualisation, 2 μL of Midori Green (Nippon Genetics) was added into 100 mL of melted agarose. For gDNA integrity analysis, loading dye was mixed with the samples before loading onto the gel. Samples were electrophoresed in TAE buffer at 90 v for up to 50 min.

2.4.3 DNA quantification - Qubit

For DNA quantification, analysis was performed according to the manufacturer's instructions using the Qubit dsDNA BR assay kit (Life

Technologies). Briefly, a working solution was used to dilute both the samples and standard reagents into Qubit tubes with final volume of 200 μL. After vortexing, the tubes were incubated for 2 min in room temperature and read in Qubit fluorimeter.

2.4.4 DNA purity quantification - Nanodrop analysis

Nanodrop (Thermo Scientific) analysis was performed according to the manufacturer's instructions for DNA samples. Briefly, the equipment was cleaned with 70% ethanol and 2 μL of the same buffer used to dilute DNA was loaded onto the machine to blank it. Analysis of samples was done with 2 μL of DNA.

2.4.5 DNA quality control

To meet the requirements for sequencing at the Centre for Genomic Research (CGR) at the University of Liverpool, the quality of DNA was assessed using agarose gel electrophoresis to detect possible DNA fragmentation, Nanodrop to analyse protein and/or solvent contamination and Qubit for quantification. Prior to sequencing, DNA was transferred to an Eppendorf with ≥ 1000 ng in a volume of ≤ 55 μL as determined by Qubit reads, with none or low DNA fragmentation shown in the agarose gels and Nanodrop 260/230 and 260/280 ratios greater than 1.8.

2.4.6 Pooled DNA and control samples

The DNA of ten isolates randomly picked from nasal homogenates for each mice amongst the three serial nasopharyngeal passages were pooled prior sequencing. To effectively capture frequencies of sequence variance that was selected during the experimental nasopharyngeal evolution, DNA was individually extracted from each clone and then pooled at equimolar concentrations according to the Qubit readings.

Since three serial nasopharyngeal passages were performed and each included three colonised mice, the total number of pooled DNA samples was nine. Additionally, one sample comprised *S. aureus* USA300 LAC JE2 as control DNA.

2.4.7 DNA library preparation

DNA libraries with an insert size of 350 bp were prepared using TruSeq PCR-free sample prep kit (Illumina) according to the manufacturer's instructions. MiSeq (Illumina) was used as sequencing platform for both control and pool of isolates. DNA library preparation and samples sequencing was done by the CGR (Centre for Genomic Research), University of Liverpool.

2.4.8 Genome assembly

Reads from both pooled and single isolate samples were mapped against the reference *S. aureus* USA300 genome (NC_007793.1) using two packages of BWA software – Burrows-Wheeler Aligner version 0.7.13: aln and mem (Li & Durbin, 2009). After the alignment, mapped reads were filtered to remove secondary alignments, PCR duplicates and reads with poor quality by using Samtools software version 1.3.1 (Li *et al*., 2009).

2.4.9 SNP analysis

SAM files generated after genome assembly and quality check of reads were coverted do bcf (binary variant call) with Samtools for SNP (Single Nucleotide Polymorphism) calling using the mpileup package. The bcf output file was then converted to vcf (variant call format). Variants in the genomes were then called by using BCFtools version 1.3 (Li *et al*., 2009) to generate a VCF file with all the polymorphism for each sample.

SNPeff algorithm was then used to annotate the variants called in the VCF files. In order to remove SNPs present in both control and pooled clones from serial passages, a perl script was used so only the variants that appeared during the experimental evolution would be shown. A second perl script was used to the pooled isolates so the frequency of reads harbouring variants could be assessed. To reduce falsely called variants, SNPs not called from both alignments (BWA aln or BWA mem) were removed from the dataset.

Chapter 3 Establishment of a murine long term nasopharyngeal colonisation model

3.1 Introduction

3.1.1 Different approaches to study *S. aureus* nasal colonisation

S. aureus is an opportunistic pathogen commonly found in the anterior nares of humans where the bacterium typically colonises the site in an asymptomatic manner. However, carriage of *S. aureus* represents a high-risk factor for the host as the bacterium can switch from harmless coloniser to pathogen depending on several factors from both bacteria and host and also the environment. Assessing the determinants that enable *S. aureus* to colonise the human nasopharynx is a critical step in controlling the spread of this microorganism, since the eradication of carriage prevents both infection and transmission of *S. aureus* (von Eiff *et al*., 2001; Grothe *et al*., 2014; Liu *et al*., 2017). Experimental models have been developed and tested to assess features of *S. aureus* colonisation for a variety of variable parameters: competition with other bacteria, micronutrients acquisition, adhesion to human cells, defence against the host immune weapons and etc.

The role of specific *S. aureus* determinants in nasal colonisation, such as the MSCRAMM family of adhesins, was evaluated *in vitro* by measuring attachment ability to human epithelial cells in mutant strains compared with wild type (Corrigan *et al*., 2009; Askarian *et al*., 2016). Host factors derived mainly from

the immune system (innate and adaptive response) were assessed regarding their relevance in controlling *S. aureus* nasal colonisation (Simanski *et al*., 2013; Peres *et al*., 2015; Burgey *et al*., 2016).

Further models have been established to check how *S. aureus* competes with other members from the nasal microbiome to persist in this niche. A solid agar model was developed to analyse the competition dynamics of different communities of *S. aureus* and *S. epidermidis* where it was observed that *S. epidermidis* can both prevent and invade *S. aureus* populations via secreted toxins (Libberton *et al*., 2015). An assay combining *in vitro* mono and co-culture with transcriptomics of *S. aureus* and *Corynebacterium striatum* (a common skin and nasal commensal) enabled the analysis of changes in gene expression of *S. aureus* that reflects a shift from a virulent to commensal state when in the presence of *C. striatum* (Ramsey *et al*., 2016). The competition between *S. aureus* and *S. pneumoniae* was assessed *in vitro* through plate and liquid culture-based assays to propose that the latter kill competing bacteria using H_2O_2 by inducing bacteriophage-mediated lysis, while *S. aureus* uses catalase as a counteracting survival mechanism (Park *et al*., 2008). However, one year later it was reported that, using a rat nasal model, catalase production is not significantly relevant for *S. aureus* nasal colonisation regarding the presence or absence of a H_2O_2-producing *S. pneumoniae* strain (Margolis, 2009). Such findings support the need for the establishment of *in vivo* models and greater study to get a more realistic notion of the important features during nasal colonisation by *S. aureus*.

3.1.2 Mice as hosts in *S. aureus* nasopharyngeal *in vivo* colonisation models

S. aureus has been extensively analysed regarding its virulence factors through *in vivo* models developed for a variety of organisms such as fish (Li & Hu, 2012), insect (Prithiviraj *et al*., 2005) and plant (Miyazaki *et al*., 2012). Still, mice are the most common host used for animal models to evaluate *S. aureus* determinants for both infection and colonisation due to several reasons: breeding and maintenance cost, current knowledge about the immune system and availability of knock-out strains for numerous genes (Schulz *et al*., 2017).

Although mice and humans share some expected similarities in their nasal passages due to the fact they are both mammals, their anatomical and physiological differences may influence the interaction with bacteria. Humans can use both nasal and oral cavities to breath while mice breath strictly through their nose. A notable anatomic difference is the airflow pattern in rodents, which harbour a nasal turbinate shape that favours a better protection of the lower respiratory tract and an acute olfaction; the olfactory epithelium in mice has a much greater nasal airway area (Harkema *et al*., 2006).
Mice used in laboratory experiments can be either germ-free or specific pathogen-free. With both populations the fact that the microbiome is known or constrained represents an advantage for scientific purposes. In humans, four main phyla are the major components of the olfactory microbiota: Bacteroidetes, Firmicutes, Proteobacteria and Actinobacteria (François *et al*., 2016).

Particular to *S. aureus*, *in vivo* experiments using murine models were reported that studied specific components relevant for nasal colonisation. Satorres *et al*. (2009) showed in a murine model that IFN-γ contributes to *S. aureus* nasal colonisation even though this cytokine is an important element of the mice immune defences. Another immune system component, IL-17, was assessed for its relevance in the clearance of *S. aureus* nasal colonisation by analysing the production of antimicrobial peptides in a murine model (Archer *et al*., 2016). Adhesins of *S. aureus* and their role in the nasal colonisation were investigated in murine models. The study revealed that the surface proteins, ClfB (Mulcahy *et al*., 2012), IsdA (Burian *et al*., 2010a), SdrC (Corrigan *et al*., 2009), SdrD (Askarian *et al*., 2016), and the glycopolymer wall-teichoic acid (Baur *et al*., 2014) facilitate nasal colonisation. Responses to oxidative stress are also critical for *S. aureus* nasal colonisation and Cosgrove *et al*. (2007) described the relevance of two genes *ahpC* and *katA*, encoding peroxide defence enzymes alkylhydroperoxide reductase and catalase, in successful colonisation of rodent nasal epithelium.

Despite the fact that mice have been extensively used for infection and colonisation models these rodents are not considered a natural host of *S. aureus*. Given that multiple virulence factors expressed by *S. aureus* are human specific means that the physiological conditions from murine models may not be ideal to evaluate nasal colonisation and virulence. Infection models are of course critical to our understanding of pathogenesis and modern developments accentuate the scope of research potential. In this regard, inducing a nasal colonisation in mice is useful given the wide range of knock-out mice strains that can be used to

assess the contribution of both *S. aureus* determinants and individual host components during colonisation (Holtfreter *et al*., 2013).

3.1.3 Serial passages as an experimental evolution approach

Exposing microorganisms to different environments induces changes in their life-cycle. Depending on the microenvironment pressure encountered, a switch in a metabolic pathway might occur, genes can be up or down-regulated, mutations that favour bacterial survival for such new conditions might also be selected. Experimental evolution experiments that select microorganisms that adapt to a specific microenvironment are a useful approach to detect relevant mechanisms in the adaptation process. Several studies describe *S. aureus* evolution *in vitro* by serially passaging isolates over days or even weeks under different circumstances to assess adaptation mechanisms.

Most of these reports describe how *S. aureus* develops resistance to inhibitory or sub-inhibitory doses of antimicrobials after serial passages of the bacteria in culture media. These laboratory-evolved isolates are a good source of genetic information to understand details of the evolution mechanisms specific to a certain antimicrobial. Not every relevant bacterial factor can be observed in these types of experiments as some of the interactions with the host are not reproduced in such *in vitro* assays. Johnston and co-workers (2016) showed that serially-passaged strains in a study of antimicrobial peptide (AMP) resistance using experimental evolution presented SNPs in genes related to AMP susceptibility. Further studies have performed *in vitro* serial passages as an

experimental method to yield better understanding of resistance to other AMPs (Samuelsen *et al*., 2005; Kubicek-Sutherland *et al*., 2016).

Mishra and co-workers (2012) tested the reproducibility of serial passages performed *in vitro*. The authors serially passaged a *S. aureus* strain in media containing a sub-lethal dose of the lipopeptide antibiotic daptomycin and after 20 days they found phenotypic and genotypic changes in the passaged strain similar to those identified in a previous study done with the same conditions. However, of note some of the metabolic changes and mutations that occurred in the evolution process did not match with the first dataset. This discrepancy identifies the potential for multiple pathways to resistance and the potential for subtle differences in the selection placed on the test bacterium.

Such differences in findings could also relate to the fact that *in vitro* assays do not reflect those conditions found *in vivo*. Somerville and co-workers (2002) showed that serial passages *in vitro* was a reason for *S. aureus* having decreased aconitase specific activity. By serially passaging a *S. aureus* clinical isolate they observed that after 6 weeks the aconitase activity decreased by 38%. This insight reinforces the need to use *in vivo* models to have a more robust comprehension of the events that happen in a real scenario.

3.2 Aims

S. aureus is an important opportunistic human pathogen and nasal colonisation by this microorganism is a risk factor for the host to develop infections. Although extensive details of how *S. aureus* establishes infections have been determined, detailed mechanisms of nasal colonisation remain unclear. Individual components from both *S. aureus* and humans have been assessed *in vitro* and *in vivo* but the effects of colonisation as a global genetic event have not been analysed yet using *in vivo* models.

This chapter aims to describe firstly the development of an *in vivo* long-term asymptomatic nasopharyngeal colonisation model using mice as the host. Successful development of the model would enable design and establishment of an experimental evolution method using serial passages of *S. aureus* through the nasopharynx of mice. The hypothesis formed is that *S. aureus* is capable of long-term colonisation of the murine nasopharynx. Further, the establishment of such a model would enable an experimental evolution approach using serial passages of strains. The output set of isolates generated using this approach would then be a good source to investigate genetic changes due to selection that would highlight key contributing components in the colonisation event played of *S. aureus* enabling it to persist in the nasal cavity.

3.3 Results

This chapter explores the experimental details and the technical challenges associated with establishment of a murine model to identify factors contributing to *S. aureus* nasopharyngeal colonisation. The robustness of the model is a requirement to establish serial passages in subsequent experiments.

3.3.1 Development of a long-term nasopharyngeal colonisation murine model

Groups of three mice (6 to 8 week-old, BALB/c) were inoculated in their nose by gently pipetting 15 μL of the inoculum that was prepared with *S. aureus* USA300 LAC JE2 strain (~ 5 x 10^4 cfu). The mice were returned to their cages and maintained in their previous environment for a period of up to 14 d. All animals survived throughout the standardisation of this model until their sacrifice day. At each time point chosen (1, 3, 5, 7 and 14 d), the colonisation status was determined by sacrificing one mice group from which the nasopharyngeal tissue was aseptically removed. The recovered nasopharynx parts were smashed and mixed with PBS, resulting in nasal material that was cultured and enumerated by plating 60 μL of serial dilutions on mannitol salt agar that was incubated for 24-48 h at 37 °C. The homogenates were then stored at -80 °C so they could be further analysed at a later date.

After colony counting of the mannitol agar plates, it was evident that *S. aureus* USA300 LAC JE2 successfully colonised the mouse nasopharynx for up to 14 d

(Fig 3.1). *S. aureus* USA300 LAC JE2 experienced a noticeable cell number drop in the first three days of nasopharyngeal colonisation followed by increased survival level as per CFU values. From day 5 onwards a slight variation in *S. aureus* population number is observed as the strain seems to reach a steady colonising status. The observed colonisation over 14 d represents a time-frame suitable for the requirements of this study.

The bacterial load can determine a successful nasal colonisation. A study demonstrated association between the inoculum dose (10^4 to 10^8 CFU) and nasal colonisation status in a mice model using three *S. aureus* strains (Reynolds, Newman and DAK). Lower doses resulted in absence or reduced levels of colonisation (Kismer *et al.*, 1999). The current model uses a low dose inoculum (5 x 10^4 CFU) of *S. aureus* USA300 LAC JE2 relative to inocula used in previous mouse colonisation experiments. This cell number was used because one of the goals was not to induce a strong immune response from the mice.

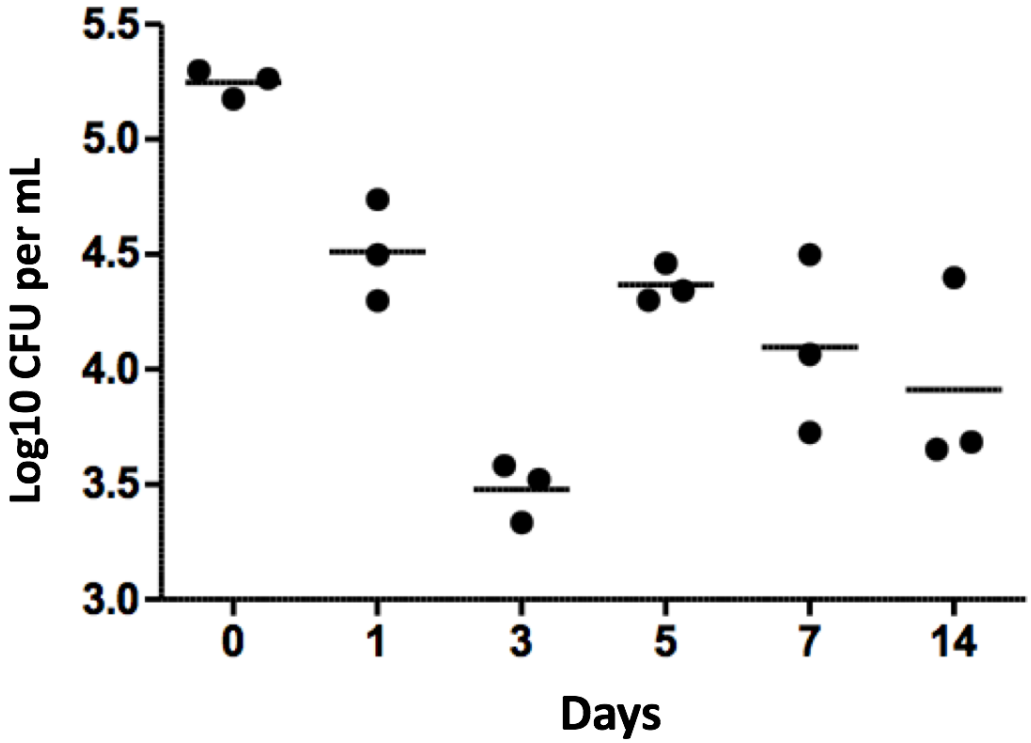

Figure 3.1 Nasopharyngeal colonisation of mice by *S. aureus* USA300 LAC JE2.
Inoculation was initiated with a 5 x 10^4 CFU dose. At each time point the nasopharynx of 3 mice were recovered and cultured for *S. aureus*. Bars represent mean values of the mice in each time group.

3.3.2 Serial passages of *S. aureus*

Once the model was established, a total of three serial passages of *S. aureus* USA300 LAC JE2 were performed with each one comprising 7 days. Nasopharynx removal was performed as described in the procedure performed in the model standardisation: the nasopharynx was aseptically homogenised with PBS resulting in a nasal wash. For the serial passages no intermediate quantitative analysis of the colonisation was determined. The nasopharynx homogenate was spread onto mannitol agar plates and bacteria cultured overnight. From the resulting clones on the agar plates, ten from each mouse were randomly picked and used for the next passage which was established with identical load. DNA from all the isolates was extracted so they could be screened by *spa* gene typing.

In the first attempt to complete all three consecutive colonisation experiments, the first two passages were performed without problems. All three mice from both passages survived over the incubation period and clones of *S. aureus* USA300 LAC JE2 were recovered. PCR of the *spa* gene was performed as previously described (Shopsin *et al*., 1999) for all the clones and the wild type and electrophoretic separation of the fragments revealed they were of similar size (Fig 3.2). The resulting fragments were sequenced and from BLASTn output it was determined that the clones were identical and each was matching with USA300 LAC JE2 (passaged strains and wild type) *spa* sequence (Fig 3.3). Subsequently, from the third passage a different outcome occurred. Again all mice survived from inoculation until the sacrifice day, however the *S. aureus*

isolates recovered from the nasopharynx were sequence identified as being a *S. aureus* strain that was not a match with the inoculum. PCR of the *spa* gene of isolates from all the three mice from the third passage revealed fragments that were a different size compared with the inoculated clone pools (Fig 3.2). Using BLASTn once more the sequence of the amplified *spa* fragments revealed a different *S. aureus* strain was recovered (Fig 3.3). Analysis of the *spa* gene repeat region using the Ridom StaphType software (Harmsen *et al*., 2003) assigned the *S. aureus* strain to t024 (USA300 belongs to t008).

The source of this strain was not identified and an initial explanation was that it could be a laboratory-derived contamination either within the separate microbiology or animal facilities. This unexpected result led to two further attempts to repeat the inoculations and third serial passage. When the same outcome occurred twice more over a several month period this led to the most likely explanation that the mice were nasally pre-colonised by *S. aureus*. Consequently, the project was put on hold for an extended period of months before attempting to complete the third and final passage. After this long break the third passage was finally and successfully performed with clones from *S. aureus* USA300 LAC JE2 recovered from the nasopharyngeal tissue concluding the designed *in vivo* experiment.

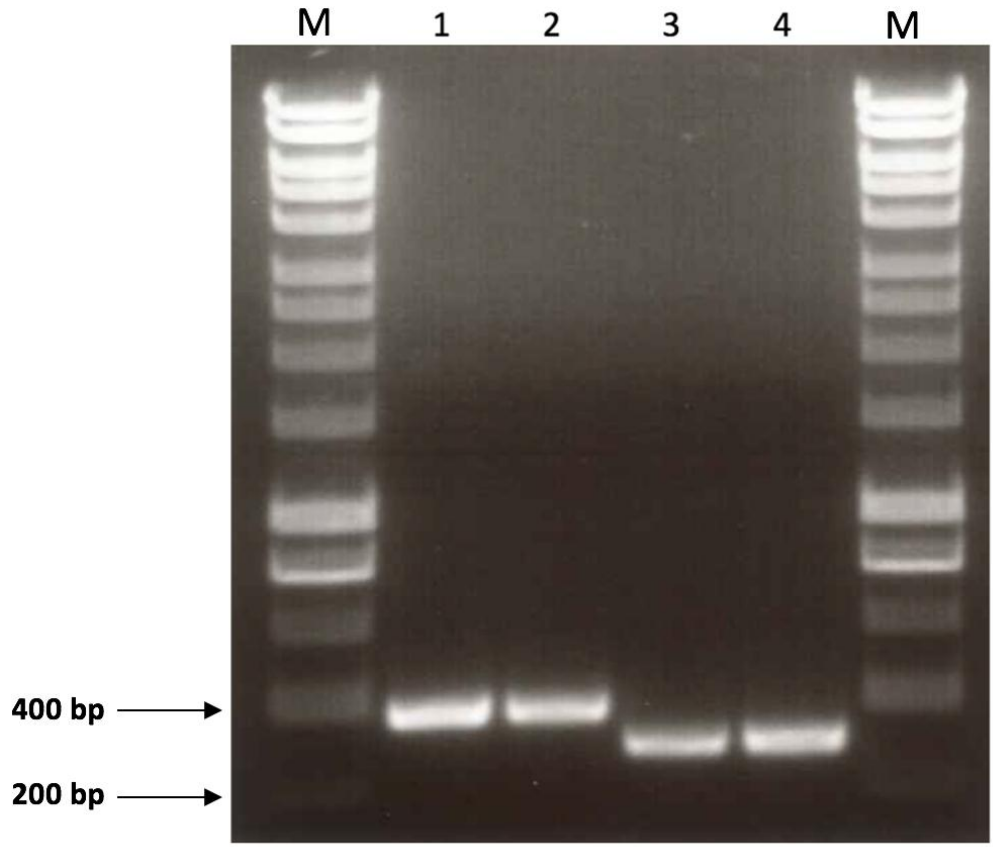

Figure 3.2 PCR products of *S. aureus spa* amplified from serial passage clones.
Agarose gel showing different sizes of *spa* gene PCR fragments for *S. aureus* USA300 JE2 LAC (Lane 1), an *S. aureus* isolate from the successful second passage (Lane 2) and isolates from the two failed third passages (Lanes 3 and 4). M: Molecular weight marker (Hyperladder 1 kb, Bioline).

Description	Max score	Total score	Query cover	E value	Ident	Accession
Staphylococcus aureus strain C2406, complete genome	563	1093	100%	7e-157	100%	CP019590.1
Staphylococcus aureus strain JE2, complete genome	563	1093	100%	7e-157	100%	CP020619.1
Staphylococcus aureus strain USA300-SUR24, complete genome	563	1093	100%	7e-157	100%	CP014444.1
Staphylococcus aureus strain USA300-SUR23, complete genome	563	1093	100%	7e-157	100%	CP014441.1
Staphylococcus aureus strain USA300-SUR22, complete genome	563	1093	100%	7e-157	100%	CP014438.1
Staphylococcus aureus strain USA300-SUR21, complete genome	563	1093	100%	7e-157	100%	CP014435.1
Staphylococcus aureus strain USA300-SUR20, complete genome	563	1093	100%	7e-157	100%	CP014432.1
Staphylococcus aureus strain USA300-SUR19, complete genome	563	1093	100%	7e-157	100%	CP014429.1
Staphylococcus aureus strain USA300-SUR18	563	1093	100%	7e-157	100%	CP014426.1
Staphylococcus aureus strain USA300-SUR17, complete genome	563	1093	100%	7e-157	100%	CP014423.1

Description	Max score	Total score	Query cover	E value	Ident	Accession
Staphylococcus aureus strain C2406, complete genome	562	1092	99%	3e-156	100%	CP019590.1
Staphylococcus aureus strain JE2, complete genome	562	1092	99%	3e-156	100%	CP020619.1
Staphylococcus aureus strain USA300-SUR24, complete genome	562	1092	99%	3e-156	100%	CP014444.1
Staphylococcus aureus strain USA300-SUR23, complete genome	562	1092	99%	3e-156	100%	CP014441.1
Staphylococcus aureus strain USA300-SUR22, complete genome	562	1092	99%	3e-156	100%	CP014438.1
Staphylococcus aureus strain USA300-SUR21, complete genome	562	1092	99%	3e-156	100%	CP014435.1
Staphylococcus aureus strain USA300-SUR20, complete genome	562	1092	99%	3e-156	100%	CP014432.1
Staphylococcus aureus strain USA300-SUR19, complete genome	562	1092	99%	3e-156	100%	CP014429.1
Staphylococcus aureus strain USA300-SUR18	562	1092	99%	3e-156	100%	CP014426.1
Staphylococcus aureus strain USA300-SUR17, complete genome	562	1092	99%	3e-156	100%	CP014423.1

Description	Max score	Total score	Query cover	E value	Ident	Accession
Staphylococcus aureus strain FORC_045, complete genome	430	958	100%	8e-117	100%	CP017115.1
Staphylococcus aureus DNA, almost complete genome, strain: No.10	430	958	100%	8e-117	100%	AP015012.1
Staphylococcus aureus strain FORC_026, complete genome	430	958	100%	8e-117	100%	CP013132.1
Staphylococcus aureus strain C22 protein A (spa) gene, partial cds	430	958	100%	8e-117	100%	EF203494.1
Staphylococcus aureus strain PC8 protein A (spa) gene, partial cds	430	958	100%	8e-117	100%	EF094507.1
Staphylococcus aureus partial spa gene for immunoglobulin G binding protein A, isolate H9779	425	953	100%	3e-115	99%	AM407390.1
Staphylococcus aureus partial spa gene for immunoglobulin G binding protein A precursor, strain H9779	425	953	100%	3e-115	99%	AM076311.1
Staphylococcus aureus strain NT937 protein A (spa) gene, partial cds	423	951	98%	1e-114	100%	FJ491258.1
Staphylococcus aureus subsp. aureus strain GR2, complete genome	407	932	100%	9e-110	98%	CP010402.1
Staphylococcus aureus genome assembly NCTC13435, chromosome : 1	407	932	100%	9e-110	98%	LN831036.1

Description	Max score	Total score	Query cover	E value	Ident	Accession
Staphylococcus aureus strain FORC_045, complete genome	443	971	98%	1e-120	99%	CP017115.1
Staphylococcus aureus DNA, almost complete genome, strain: No.10	443	971	98%	1e-120	99%	AP015012.1
Staphylococcus aureus strain FORC_026, complete genome	443	971	98%	1e-120	99%	CP013132.1
Staphylococcus aureus strain C22 protein A (spa) gene, partial cds	443	971	98%	1e-120	99%	EF203494.1
Staphylococcus aureus strain PC8 protein A (spa) gene, partial cds	443	971	98%	1e-120	99%	EF094507.1
Staphylococcus aureus partial spa gene for immunoglobulin G binding protein A, isolate H9779	437	965	98%	6e-119	99%	AM407390.1
Staphylococcus aureus partial spa gene for immunoglobulin G binding protein A precursor, strain H9779	437	965	98%	6e-119	99%	AM076311.1
Staphylococcus aureus strain NT937 protein A (spa) gene, partial cds	430	958	97%	8e-117	99%	FJ491258.1
Staphylococcus aureus subsp. aureus strain GR2, complete genome	419	988	98%	2e-113	98%	CP010402.1
Staphylococcus aureus genome assembly NCTC13435, chromosome : 1	419	988	98%	2e-113	98%	LN831036.1

Figure 3.3 BLAST hit list of *spa* gene sequence for different *S. aureus* strains.

Comparison of the sequence match lists returned by BLASTn for *S. aureus* USA300 JE2 LAC (A), an *S. aureus* isolate from the successful second passage (B) and isolates from the two failed third passages (C and D).

With the successful completion of a set of serial colonisation passages, the outcome was that the experimental evolution was achieved with the following set of isolates stored frozen, ready to be accordingly further assessed:

- Control: Wild Type (*S. aureus* USA300 LAC JE2 – 1 isolate);
- First Passage: Mouse A1 (10 isolates), Mouse B1 (10 isolates), Mouse C1 (10 isolates);
- Second Passage: Mouse A2 (10 isolates), Mouse B2 (10 isolates), Mouse C2 (10 isolates);
- Third Passage: Mouse A3 (10 isolates), Mouse B3 (10 isolates), Mouse C3 (10 isolates).

Schematically the experiment and the clones obtained are shown below (Fig 3.4).

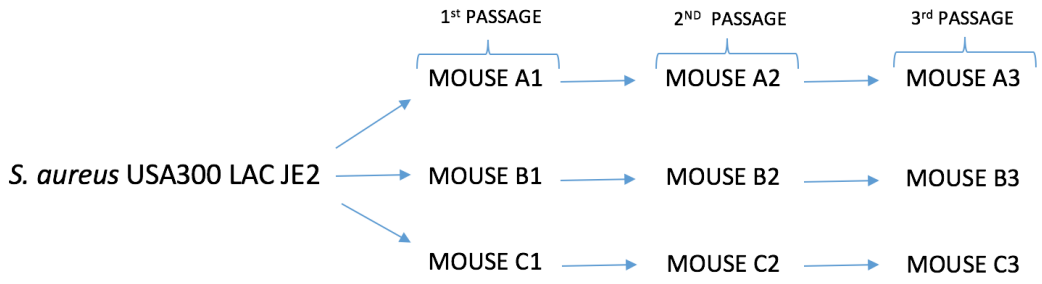

Figure 3.4 Experimental evolution of *S. aureus* in a murine nasopharyngeal model. Representation of three serial passages of *S. aureus* USA300 LAC JE2 through the nasopharynx of mice where each colonisation passage lasted 7 d. For every mouse used in this work, 10 isolates were recovered from the nasopharynx to be genome sequenced and for passages 1 and 2 to serve as inoculum for the subsequent passage.

3.4 DISCUSSION

In this chapter, a long-term *S. aureus* nasopharyngeal colonisation murine model was established and was determined to be sufficiently robust for it to be used to perform an experimental evolution using serial passages. The colonisation model was successfully standardised for *S. aureus* USA300 LAC JE2 strain as judged by the relatively stable maintenance of the bacteria with isolates recovered from the nasopharynx up to 14 d post-inoculation. This interval represented sufficient time for the purposes of this study but given the difference in numbers between 7 and 14 days, the lesser time was chosen with the aim of removing as much potential for variability at the extreme of the colonisation timeframe. Kieser *et al.* (1999) reported one of the first murine models to study *S. aureus* nasal colonisation where carriage was observed for more than 20 days depending on the treatment that the mice were exposed to. Prior to their investigation only a few studies were performed with animal models to assess the features of *S. aureus* nasal carriage (Sanford & Ramsay, 1987; Sanford *et al*., 1989). Later, Kokai-Kun and co-workers (2003) established a model for cotton rats where the authors observed *S. aureus* nasal carriage for more than 40 days of study.

The model described here uses a relatively low inoculum of *S. aureus*: 5×10^4 CFU. This cell number was selected from prior work looking at 7 days of colonisation that evaluated a range of cell numbers in the inoculum to identify the minimum threshold that generated reproducibility (Dr. Elaine Waters, personal communication). Using this previous data there was a desired attempt

to establish the model such that it simulated as much as possible a realistic scenario of colonisation. Studies of the human nasophrynx have identified that this tissue is typically colonised with numbers of *S. aureus* ranging from 10^1 to 10^4 CFU (Xu *et al*., 2015). In contrast, natural nasopharyngeal colonisation by *S. aureus* in mice was not described at the outset of this study. Most of the nasal colonisation models described for *S. aureus* have used higher doses of bacteria in the inoculum (Schulz *et al*., 2017).

Mulcahy *et al.* (2012) developed a murine model to assess the relevance of adhesins in *S. aureus* nasal colonisation. The model used a dose of 10^8 CFU and the mice were pre-treated with streptomycin one day before inoculation. Kiser and co-workers (1999) in their model also used a 10^8 CFU inoculum. However, the authors observed colonisation with lower levels of bacteria (10^5 to 10^7 CFU) but only after streptomycin treatment and with *S. aureus* Reynolds strain. The model that is standardised here presents some advantages because it not only uses a low level inoculum, but it also does not require pre-treatment with antibiotics, which could change the dynamics and relevance of *S. aureus* nasal colonisation and inter-species competition.

Considering the dose size used in the inoculum for the model proposed in this work the number of *S. aureus* consistently decreases until day 3 and after day 5 it reaches a steady state (Fig 3.1). Margolis *et al*. (2010) assessed the dynamics of different populations (including *S. aureus*) colonising the nasal passages from neonatal rats and the authors concluded that the bacterial density in this niche is well controlled, regardless of the cell numbers in the inoculum. Thus the

model proposed in this current study can be useful to assess the nasopharyngeal colonisation features by *S. aureus* especially from day 5 until day 14. A cotton rat model proposed previously with 7 to 10 days of incubation for analysis of nasal colonisation (Kokai-Kun, 2008) supports the model standardised here.

With the model established, a series of three consecutive passages were performed with each lasting for 7 d. The main challenges for this experiment were to make sure that the low dose was enough to colonise the mice and that the clones recovered after the incubation period belonged to the same strain used in the inoculum. Initially two passages were successfully completed. However, a difficulty emerged in completion of the third passage that was a repeated issue and initially did not allow the conclusion of the experiment for several months. This problem caused an intermission for the third passage as it could not be successfully completed.

With two attempts, even though the mice were colonised by *S. aureus*, *spa* gene typing revealed that the isolates recovered from the nasopharynx were different from that included in the inoculum (Fig 3.2 and 3.3). The most likely conclusion made at the time was that the mice were nasally pre-colonised. This hypothesis matched with the later findings from Schulz *et al*. (2017) where the authors reported that laboratory mice supplied by several European and North American breeders tested are frequently colonised by *S. aureus,* with nasal carriage rates as high as 20%.

Although it is well accepted by some researchers that mice are not a natural host for *S. aureus* (McCarthy *et al*., 2012; Mulcahy *et al*., 2012) several studies support the use of mouse models as a good representation for *S. aureus* nasal carriage assessment (Gonzales-Zorn, 2005; Mulcahy *et al*., 2012). Here, it was observed that mice can serve as a valuable model system to evaluate *S. aureus* nasopharyngeal asymptomatic colonisation over a course of 1-2 weeks, but in this case extra care is needed regarding pre-colonisation by another *S. aureus* strain.

It is possible that higher doses or pre-treatment of the mice with streptomycin would solve the pre-colonisation issue found in this work, but as mentioned above such actions would not reflect what happens in a typical scenario where the established flora will provide a competitive exclusion barrier. Particularly for studies looking at the ecology of *S. aureus* nasal colonisation with a view to determining competitive fitness the removal of the existing flora will alter the interpretations.

In this respect, Margolis and colleagues (2010) detailed several interactions between competitors in the nasal niche using neonatal rats. The authors concluded that pre-colonisation by *S. aureus* prevents the establishment of an invasive *S. aureus* strain in the nasal passages, supporting the classical ecological theory of competition (Hardin, 1960). Whilst for the first two passages in the establishment of the current model no problem was observed, two sets of mice from different periods used for the third passage were pre-colonised and such issue seemed to persist over a reasonably long time period

as the third passage was only successfully completed almost one year after the isolates were recovered from the second passage.

Since the source of the *S. aureus* strain blocking the colonisation by USA300 LAC JE2 was unknown and also the fact that such an event occurred twice in a relatively long time frame, a pause in carrying out the third passage was taken. The long break in attempting to perform the third passage were due to ethical and economic reasons because it was not justifiable from a scientific and ethical perspective to perform further attempts with mice likely to be pre-colonised by a possibly more adapted *S. aureus*.

S. aureus animal–adapted strains have been reported as the cause of disease with bovine and ovine mastitis (Guinane *et al*., 2010), as well as disease in chickens (Lowder *et al*., 2009) and pigs (de Neeling *et al*., 2007). Holfreter *et al*. (2013) characterised for the first time a mouse-adapted *S. aureus* strain which was responsible for an abscess outbreak in male mice at the animal breeding facility at the University of Auckland. The authors found that the identified outbreak strain was still persistently colonising the mouse colony for 2.5 years after the outbreak declined and it showed better fitness to colonise the nasal passages of mice in a murine model compared with other strains.

It would be worthwhile to perform characterisation of the strain colonising the mice that would be expected to be better adapted to the nasopharynx than *S. aureus* USA300 LAC JE2 that it inhibited from colonisation. Known ecological

principles could simply explain the lack of invasion but a comparative study of mouse isolates and experimentally-adapted strains would provide insights.

S. aureus USA300 LAC JE2 strain derives from the parental strain *S. aureus* USA300 LAC which was originally isolated from skin and soft tissue infection at the Los Angeles County Jail (LAC). The parental strain harbours two plasmids (p01 and p03) and JE2 was generated after curing the strain for removal of the two plasmids (Fey *et al*., 2013). JE2 strain was chosen for this model since USA300 is one of the main clones responsible for Community-Acquired MRSA infections (Diep *et al*., 2006) and also because there is transposon-based mutant library specifically constructed for this strain, Nebraska Transposon Mutant Library (Fey *et al*., 2013). The fact that JE2 is not mouse-adapted may explain why the resident strain was possibly more adapted to the mice and resisted invasion.

Holfreter *et al*. (2013) reported remarkable differences in the virulence repertoire of mice-adapted and human-related strains. The authors described that the sequence type associated to the mouse-adapted strain is uncommon in humans and it lacked an *hlb* phage responsible for encoding human-specific immune evasion factors. JE2 was recently assessed in a murine infection model and it was found that the major *S. aureus* autolysin – Atl - is not required for the establishment of device-related infections (McCarthy *et al*., 2016). Such reports would suggest a parallel need for murine models with strains adapted to the animal. Given that the intention of this study is to mimic the human situation, JE2 was selected for the establishment of the model to provide insights into a

human disease strain with respect to colonisation. The methods standardised here show that it was successfully passaged through the nasopharynx of the mice.

To date there is no report regarding *in vivo* serial passages of *S. aureus* as an experimental evolution approach to assess nasopharyngeal colonisation features. As described in the introduction of this thesis, the murine models currently available in the literature are used mainly to assess specific genes and their role in the nasal colonisation by comparing carriage rates between wild type and mutant strains. Serial passages as a methodology have been extensively used for *in vitro* assays to discover mutations with potential relevance in antimicrobial resistance.

Other microorganisms – especially viruses – have been evaluated in terms of host-adaptation by serial passages using *in vivo* models. Qiu *et al*. (2014) passaged a virulent strain of Marburg virus (MARV/Ang) 24 times in a mouse model and the authors observed that the evolved clones became adapted and capable to cause disease in mice while the wild type remained unable to cause infection. Years later, sequencing of the passaged strains revealed several mutations in the evolution course relevant for the virus adaptation (Wei *et al*., 2017). Similar works for other microorganisms like the H7N9 influenza virus (Zhao *et al*., 2016), Sendai virus (Pena *et al*., 2016) and *Corynebacterium pseudotuberculosis* (Silva *et al*., 2017) have been recently reported in murine models and a ferret model (Angel *et al*., 2013).

In summary, this study represents an important and relevant approach to understand aspects of nasopharyngeal colonisation by *S. aureus* by using serial passages as an experimental evolution model to compare with the many *in vitro* models.

Chapter 4 Tracking *in vivo* evolution of *S. aureus* during nasopharyngeal colonisation using whole genome sequencing (WGS)

4.1 Introduction

4.1.1 Insights regarding *S. aureus* evolution

S. aureus is an important human pathogen and a commensal commonly found in the normal flora of the nasopharynx. How *S. aureus* colonises the human nasopharynx asymptomatically or establishes an infection event is presumed to rely on its evolved ability to sense discrete environmental signals that direct gene expression appropriate to diverse host sites. In this way, *S. aureus* is versatile in the range of anatomical tissues where it persists, reflecting its high genomic and phenotypic diversity. The major events that lead to variability in the *S. aureus* genome are the acquisition of mobile genetic elements (MGEs), mutations in the bacterial genome and occasionally homologous recombination (Fitzgerald, 2014).

MGEs are fragments of DNA that encode the ability to move between and within genomes. MGEs such as bacteriophages, plasmids, transposons and staphylococcal cassette chromosomes are known to promote horizontal genetic transfer frequently in *S. aureus* (Lindsay, 2010). For example, the pandemic MRSA strain (ST239) was founded after a single chromosomal replacement

occurred that accounted for a considerable portion of the bacterial genome (Robinson & Enright, 2004).

Besides the relevance of MGE and recombination to *S. aureus* evolution, the high mutation rate of *S. aureus* is proposed to be an important promoter of adaptation under diverse selection pressures. Lopez-Collazo *et al*. (2015) tracked the evolution of a single clinical MRSA clone over a period of 13 years. The study of evolution used whole genome sequencing of isolates collected in the first and last year of the timeframe to reveal a loss of 66 genes with an overall reduction in genome size. The authors also observed several SNPs, of which three were proposed to be important for attenuation of virulence in the evolved clone, potentially resulting in improved capability of *S. aureus* to persist in host tissue. In a separate study, ten CA-MRSA (USA300) isolates from patients of distinct geographic regions were analysed by comparative genomics to investigate the evolutionary changes of the strain responsible for a rapid emergence of invasive infections. Sequence diversity showed that evolution of USA300 was due to clonal expansion followed by diversification and that the clinical isolates harboured between 11 to 408 SNPs (Kennedy *et al*., 2008).

Genetic variation from mutations are mostly due to either SNPs or short INDELs (insertion/deletion). SNPs present in coding regions can either result in an amino acid change (non-synonymous SNP) or no change (synonymous SNP). SNPs present in intergenic regions may affect the promoter and leader of operons under its regulation or change the sequence of regulatory small RNAs (sRNA). The consequence of indels can be more drastic where these occur in

coding regions and lead to a frame shift mutation. Tracking the occurrence of such variants is a valuable approach to identify contributory genes involved in *S. aureus* adaptation to specific selective pressures over an evolutionary time-frame, such as those studies described by Kennedy *et al*. (2008) and Lopez-Collazo *et al*. (2015).

4.1.2 WGS of *S. aureus*

Advances in WGS enable the rapid genetic discrimination of bacterial strains with resolution to a single base pair. The sequencer platforms available are under constant improvement, leading to their more frequent usage and greater, more accurate coverage. As a research instrument for *S. aureus*, WGS has enabled studies of their population structure, lineage expansion and spread, antimicrobial resistance and virulence (Price *et al*., 2012). Harris *et al*. (2010) used WGS to analyse the evolution of one MRSA lineage over four decades that provided insights about mutation rate and intercontinental transmission, as well as transmission between hospital patients. Their data revealed insights of the expansion and dominance of clones in new geographical regions, and the discrimination of isolates recovered from the same healthcare setting.

WGS was also used by Young *et al*. (2012) to ascribe genetic differences that were related to both colonisation and infection events. Their study evaluated the transition from nasal carriage to bloodstream infection status by *S. aureus* using WGS, which detected the mutation rate (one mutation per genome every seven weeks). The bloodstream isolate differed from the colonising isolate by

only 8 mutations that were mainly incorporation of stop codons in regulatory proteins, suggesting potential relevant mechanisms relating to *S. aureus* pathogenesis.

The advent of desktop sequencers has promoted investigations to assess if the application of rapid whole genome sequencing can be implemented in routine clinical practice. Eyre *et al*. (2012) used sequencing with the benchtop Illumina platform to investigate transmission features during both *S. aureus* and *Clostridium difficile* outbreaks. The study successfully characterised within 5 days those isolates related to the outbreak using their similarity at the core genome level.

Collectively these studies highlight the resource and capability of the current whole genome sequencing platforms to study characteristics of *S. aureus* isolates that pertain to colonisation of its host. A greater knowledge of the features that are important and contribute to nasopharyngeal colonisation will help in the prevention of carriage and diseases caused by this pathogen.

4.2 Aims

S. aureus exhibits genetic variability that is critical in its adaptation to overcome diverse selective pressures and persist in a variety of environments such as host tissues, whether colonising or infecting them. Interrogation of mutations that occur during an evolutionary timeframe of persistence during nasopharyngeal colonisation could give indications of contributing components, pathways and mechanisms that contribute to *S. aureus* survival.

This chapter aims to capture the genetic changes occurring during *S. aureus* colonisation of the murine nasopharynx by using experimental evolution experiments, based on serial passages of this tissue. The hypothesis is that serial passages of *S. aureus* in this niche will select genetic variants with improved fitness for colonisation, which will provide novel insights of host/pathogen interactions. The dataset that is generated could also highlight relevant features of *S. aureus* nasopharyngeal colonisation that will aid prevention and control of human colonisation and transmission.

4.3 Results

4.3.1 Sequencing QC and alignment statistics

Genome sequencing was performed to determine whether repeated *S. aureus* colonisation selected for SNPs that promote nasopharyngeal colonisation in mice. Genetic changes from selection might confer upon *S. aureus* benefits to overcome different challenges for persistence in the nasopharynx, such as antimicrobial resistance, nutrient acquisition and surface adherence. Sequencing was performed for the control strain (USA300 JE2 LAC) and 90 isolates randomly picked from the pool of bacteria isolated from the nasopharynx of three mice colonised in each of the three passages (see Chapter 3).

Prior to sequencing, the quality, yield and concentration of the DNA extracts were checked to ensure these were suitable for sequencing. According to both 260/230 and 260/280 ratios analysed using Nanodrop, all the purified samples of DNA were of sufficient quality with respect to low levels of salt, solvent and protein contaminants (Table 4.1). Measurement using Qubit indicated that the yield of DNA was sufficient and gel electrophoresis showed that the integrity of each was appropriate. Equivalent amounts of the DNA of ten isolates from each mouse was pooled prior sequencing. This approach of pooled DNA was used to maximise capture of sequence diversity across the chosen isolates. DNA libraries were prepared using Illumina TruSeq PCR-free kits (350 bp inserts) and sequenced on MiSeq platform (Illumina) to an average genomic depth of 62 according to the manufacturer's instructions.

Table 4.1: Quality control analysis of *S. aureus* DNA samples pooled for sequencing.

DNA quality data of chosen isolates prior to sequencing. DNA concentration measurements were determined using Qubit and absorbance ratios at 260/280 and 260/230 using Nanodrop.

Isolate	DNA concentration (ng/μL)	Absorbance 260/280	Absorbance 260/230
S. aureus USA300 LAC JE2	80.1	1.8	2.1
A1_1	139.3	1.8	1.9
A1_2	98.8	2.0	2.3
A1_3	70.0	1.9	2.1
A1_4	85.9	1.8	2.3
A1_5	40.7	1.8	2.2
A1_6	98.9	1.8	2.2
A1_7	143.3	1.9	2.3
A1_8	87.4	1.9	2.1
A1_9	56.7	1.8	2.1
A1_10	110.8	2.0	2.1
B1_1	47.4	1.8	2.3
B1_2	85.5	2.1	2.2
B1_3	78.8	2.0	2.3
B1_4	90.1	1.9	2.4
B1_5	80.3	1.9	2.1
B1_6	75.5	2.1	2.1
B1_7	65.0	1.8	2.3
B1_8	123.8	1.9	2.3
B1_9	65.9	2.0	2.3
B1_10	78.8	2.0	2.2
C1_1	80.3	1.8	2.3
C1_2	120.3	1.8	2.1
C1_3	107.8	1.9	2.1
C1_4	90.7	1.8	1.9
C1_5	50.3	1.9	2.3
C1_6	48.9	1.9	2.1
C1_7	45.4	2.1	2.2
C1_8	130.4	1.8	2.3
C1_9	103.4	2.0	2.1
C1_10	90.4	1.9	2.3

Isolate	DNA concentration (ng/μL)	Absorbance 260/280	Absorbance 260/230
A2_1	38.7	1.8	2.2
A2_2	49.9	1.8	2.3
A2_3	51.2	1.8	2.1
A2_4	62.3	2.0	2.2
A2_5	73.4	2.0	2.3
A2_6	64.5	1.8	2.3
A2_7	83.9	2.1	2.3
A2_8	90.1	1.8	2.2
A2_9	48.9	1.9	2.3
A2_10	74.5	1.9	2.1
B2_1	129.9	1.8	2.1
B2_2	110.0	1.8	2.2
B2_3	98.4	2.0	2.3
B2_4	76.0	1.9	2.2
B2_5	45.9	2.0	2.1
B2_6	43.4	2.1	2.2
B2_7	63.3	1.8	2.3
B2_8	98.0	1.9	2.3
B2_9	104.1	2.0	2.1
B2_10	120.3	1.8	2.1
C2_1	62.3	1.8	2.1
C2_2	74.3	1.8	2.2
C2_3	53.2	1.9	2.3
C2_4	89.4	1.9	2.2
C2_5	74.2	1.8	2.1
C2_6	75.8	1.9	2.2
C2_7	82.3	2.0	2.3
C2_8	64.5	1.8	2.3
C2_9	74.4	1.9	2.1
C2_10	63.9	1.8	2.2

Isolate	DNA concentration (ng/μL)	Absorbance 260/280	Absorbance 260/230
A3_1	73.9	1.8	2.3
A3_2	56.0	1.8	2.2
A3_3	45.0	2.0	2.1
A3_4	128.8	2.0	2.1
A3_5	43.7	1.8	2.3
A3_6	89.7	1.8	2.2
A3_7	70.1	1.9	2.1
A3_8	60.2	1.9	2.3
A3_9	62.4	2.0	2.3
A3_10	78.5	1.8	2.3
B3_1	111.4	1.9	2.2
B3_2	120.9	1.9	2.2
B3_3	54.7	2.0	2.1
B3_4	83.4	1.9	2.3
B3_5	93.5	2.0	2.3
B3_6	72.3	1.8	1.9
B3_7	56.3	2.1	2.1
B3_8	45.8	1.9	2.4
B3_9	115.8	1.9	2.3
B3_10	121.2	2.0	2.3
C3_1	89.7	1.8	2.2
C3_2	75.7	1.8	2.1
C3_3	74.3	1.8	2.3
C3_4	69.8	1.9	2.2
C3_5	75.8	1.9	2.1
C3_6	98.3	2.0	2.1
C3_7	110.3	1.8	2.3
C3_8	135.4	1.9	2.2
C3_9	38.4	1.8	2.1
C3_10	41.7	1.9	2.3

A sequence comparison pipeline was established to identify variation across the read data that was generated from DNA sequencing using Illumina. Firstly, the sequence reads of both control and pool outputs were aligned to the USA300 LAC JE2 reference genome (NC_007793.1) using Burrows-Wheeler aligner (BWA). To increase the reliability of these alignments and to minimise false-positive base calls observed in the final dataset, two mapping algorithms were used: BWA-aln and BWA-mem. Next, mapped reads from both algorithms were filtered for removal of secondary alignments, PCR duplicates and poor quality reads according to Samtools. Subsequently, BCFtools was used to call the variants in the aligned sequences and generate an output dataset (Fig 4.1).

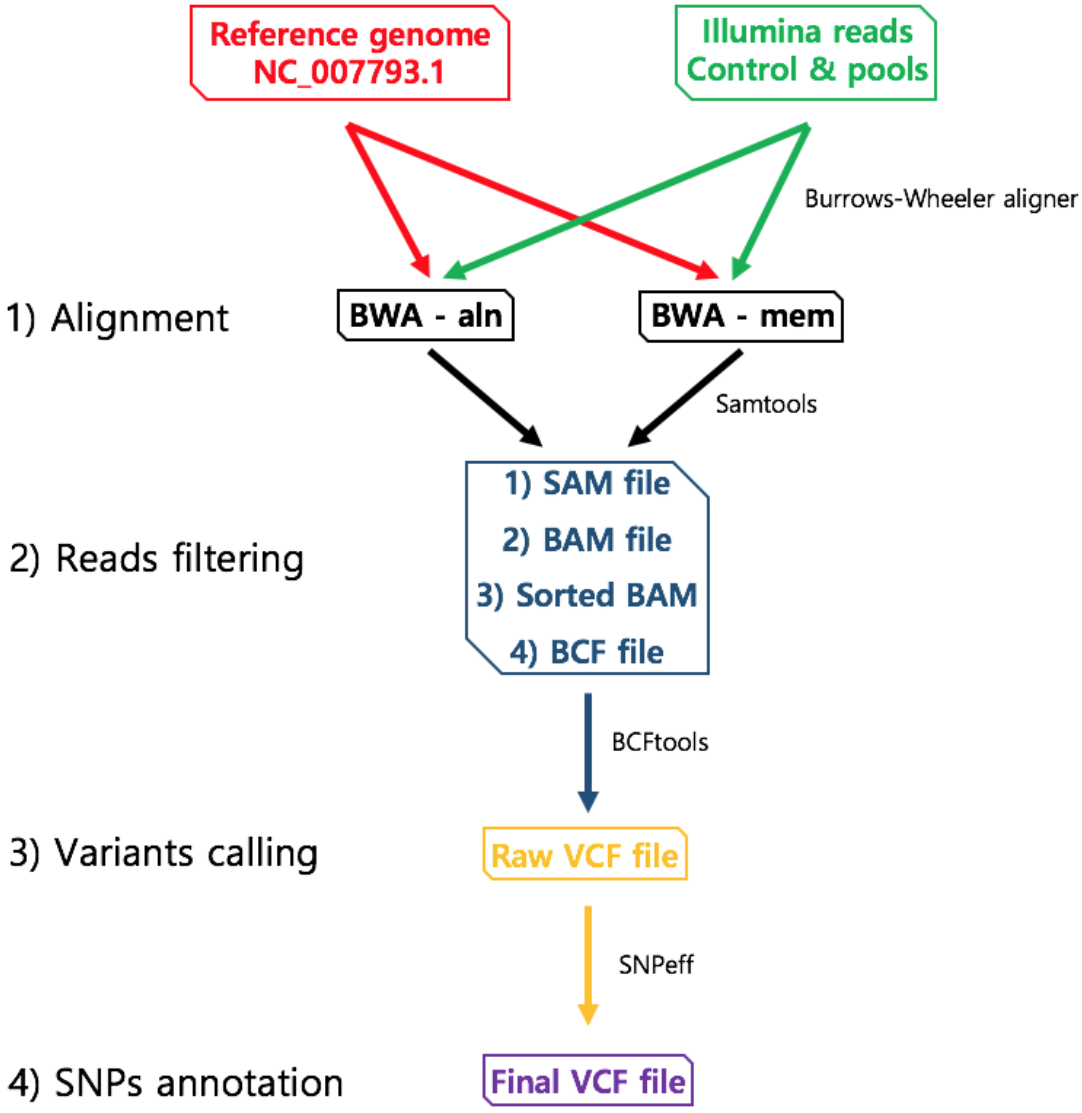

Figure 4.1 Schematic representation of the pipeline used for sequence variants identification.

Burrows-Wheeler aligner (BWA) was used for alignment IIllumina reads against *S. aureus* USA300 reference genome. Samtools was then undertaken to convert the resulting alignment file to SAM format which, after bad quality reads filtering, was converted to a BCF file. BCFtools called the SNPs followed by annotation using SNPeff.

4.3.2 Sequence variant calling of nasopharyngeal colonisation isolates

The SNPeff algorithm was used to annotate the polymorphisms in the VCF files from control sequence of USA300 JE2 LAC and the sequences of pooled clones from the nasopharyngeal passages (Fig 4.1). A bespoke perl script was used that would filter sequence variation that was present in the reads from the serial passage clones and in the control passage relative to the reference USA300 JE2 LAC genome (NC_007793.1). In this way, identified sequence variants are expected to be consequential of selection during the experimental nasopharyngeal colonisation. Since the sequence pools represent the genomes of ten isolates, a second bespoke perl script was used to assess the frequency of reads containing polymorphisms to quantify the contribution of each polymorphism that was present in the total read pool. A polymorphism frequency ~20% would indicate selection of the DNA sequence variant was present in more than one pool member isolate, while a frequency approaching 100% would represent genetic variation was uniform across each isolate in the pool. For the purposes of this study, a threshold of 9.5% was used to ensure that variants present in at least one isolate would be captured from the read data. Subsequently, a dataset was curated that described all of the sequence variation present in the genomes (intergenic and intragenic variants, synonymous and non-synonymous variants).

4.3.3 A single nasopharyngeal passage reveals very few intragenic sequence variants

After a single passage of seven days within the nasopharynx using three mice, bacteria were harvested and 10 isolates of *S. aureus* USA300 LAC JE2 from each mouse were sequenced as three separate pools: mouse A1, B1, C1. Comparison of these data revealed that the intragenic sequence variation across the 30 isolates was low (Table 4.2).

While the genome sequence pool from mouse B1 did not show any intragenic SNP, the pool of isolates sequenced from mice A1 and C1 presented the same SNP in gene SAUSA300_RS00145 producing protein variant L190F. SAUSA300_RS00145 encodes a transposase from the IS*6* family involved in mobilisation of the staphylococcal cassette chromosome mec (SCCmec), a genomic island that carries determinants for beta-lactam resistance, including *mecA* gene.

In addition, the sequence pool from mouse A1 revealed an intragenic SNP in SAUSA300_RS02185 (A110T) which encodes one of the 11 staphylococcal superantigen-like exoproteins (SSL1-11) located in SaPIn2, a pathogenic island from *S. aureus* (Fig 4.2). SSL11 binds glycoproteins of myeloid cells and inhibits neutrophil attachment. Polymorphism in SSL11 has been associated with complete loss of the binding ability by the exoprotein. A single mutation (T168P) introduced into an allele of SSL11 completely abolished carbohydrate binding, indicating failure in neutrophil attachment (Chung et al., 2007). Although a different SNP was found in this work, both variants occur in

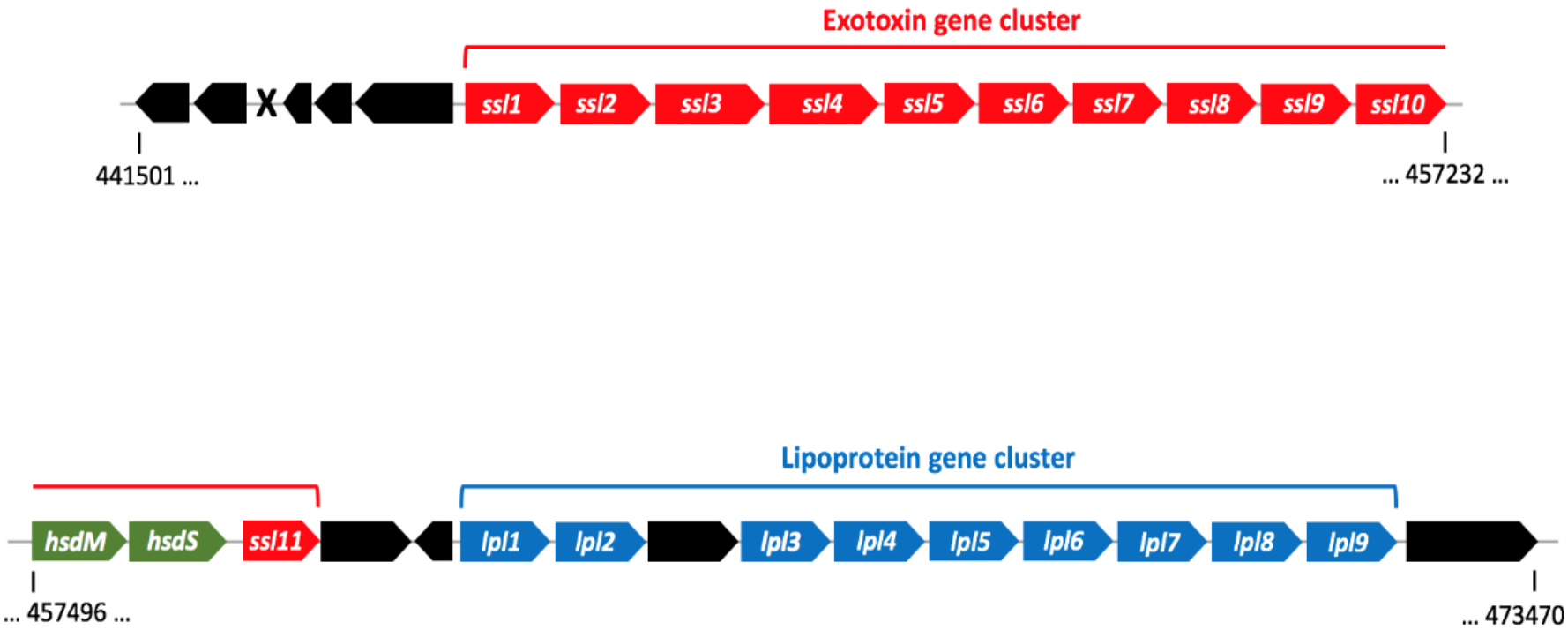

Figure 4.2: Map of the SaPIn2 pathogenicity island from *S. aureus* USA300 strain.
The *ssl11* gene is located adjacent to the restriction modification genes *hsdSM* and a lipoprotein gene cluster

neighbouring genomic positions, making it appealing to suggest that RS02185. A110T is possibly involved in prevention of internalisation by myeloid cells.

A further non-synonymous intragenic SNP was found in the sequence pool from mouse C1. The mutation was observed in *valS* (K800R) that encodes valyl-tRNA-synthetase responsible for catalysing the attachment of valine to tRNA. All of the non-synonymous intragenic SNPs identified in isolates from the first nasopharyngeal passage showed frequency around 20%, suggesting that at least two of the ten isolates forming the genomic pool carried the mutation. The observation that one sequenced pool did not bear any SNP and that the genic mutations observed in the other pools were found in a relatively low percentage of reads suggests that there was not a pronounced or uniform selection for intragenic change in *S. aureus* to persist for the seven days of the first nasopharyngeal colonisation.

Table 4.2 Nasopharyngeal colonisation isolates have multiple non -synonymous intragenic SNPs after 7 days.

S. aureus SNPs observed in pooled genomic DNA of ten isolates after the first passage of murine nasopharyngeal colonisation and sampling. Mouse isolate pools in which the SNPs were detected are indicated by A1, B1 and C1. Frequency in pool data shows the percentage of reads from each pool that harbours the SNP.

Gene symbol	Function	Genome change	Codon change	Position in protein	Mouse Pool	Frequency in pool
SAUSA300_ RS00145	Putative transposase in SCCmec element	35982 T/C	L/F	190 aa	A1	19.20%
					C1	21.51%
SAUSA300_ RS02185	Staphylococcal superantigen-like 11 (SSL11)	460966 G/A	A/T	110 aa	A1	21.45%
valS	Valyl-tRNA synthetase	1762705 T/C	K/R	800 aa	C1	23.88%

4.3.4 A single nasopharyngeal passage reveals very few intergenic sequence variants

The intergenic sequence variation observed in the pools from the first nasopharyngeal passage was determined to be minimal, like the intragenic polymorphism. One polymorphism was detected in the DNA sequence pool recovered from mouse C1. This mutation showed a read frequency (24.92%) similar to the intragenic SNPs, with just two of the ten randomly picked isolates bearing the altered sequence compared with wild-type (Table 4.3). Positionally, both SAUSA300_RS05520 and SAUSA300_RS05515 transcriptional start site are 200 bp upstream of this SNP. Therefore, it is possible that the mutation affects the expression levels of one or more of these genes as it can alter the genes promoter activity. While SAUSA300_RS05520 is involved in cell division, the role SAUSA300_RS05515 is possibly implicated in DNA repair due the enzyme activity encoded by this locus.

Table 4.3 Nasopharyngeal colonisation isolates have intergenic SNPs after 7 days.
S. aureus SNPs observed in pooled genomic DNA of ten isolates after the first passage of murine nasopharyngeal colonisation and sampling. Mouse isolate pools in which the SNPs were detected are indicated by B1 and C1. Frequency in pool data shows the percentage of reads from each pool that harbours the SNP.

Genome change	Distance from coding region	Gene function	Mouse	Frequency in pool
1121360 T/A	-27 bp / SAUSA300_RS05520 (+) -100 bp / SAUSA300_RS05515 (-)	DNA-binding protein Nucleotidyl transferase	C1	24.92%
1997607* A/G	Synonymous intragenic SNP (*rrfC*)	5S rRNA	B1	17.12%

*Variant annotated by SNPEff as intergenic SNP but located in rRNA gene.

A second SNP was annotated as intergenic by the SNPeff algorithm in the DNA sequence pool of isolates recovered from mouse B1. Annotation and mapping to the *S. aureus* USA300 LAC JE2 genome revealed that this polymorphism was a synonymous intragenic SNP in *rrfC* (Table. 4.3) that is a gene for 5S rRNA. Overall, the number of SNPs (including other synonymous intragenic SNPs; data not shown) in each of the three sequence pools ranged from 2 to 5, with a ratio of 0.6:1 non-synonymous/synonymous SNPs.

4.3.5 A second nasopharyngeal passage reveals increased intragenic sequence variants

The ten isolates that were collectively sequenced as a pool from three mice each after the first passage were cultured separately and then pooled in equal numbers. The mixed *S. aureus* clones were then introduced into mouse nasopharynx (A2, B2 and C2) as a second passage, where these isolates were left to colonise for seven days, as a repeat of the first passage. After this time period for the second passage, bacteria were recovered from the nasopharynx of each mouse and 10 isolates were selected randomly. Genome DNA was purified, pooled in equal amounts for each input strain and sequenced as before.

Curation and annotation of the DNA sequence data from the second nasopharyngeal passage revealed a greater number of intragenic variants in the passaged *S. aureus* clones than for clones sequenced after the first seven days of nasopharyngeal colonisation (1st passage). Across the genomic DNA pools from

the second passage, polymorphisms ranged from 108 to 112 SNPs, with a rather different 1.7:1 non-synonymous/synonymous ratio, compared with the first passage.

A higher number of intragenic SNPs was detected in the three sequence pools from the second passage with 43 (Table 4.4) and none of these mutations matched those intragenic SNPs detected after the first nasopharyngeal passage. Notably, a distinct non-synonymous intragenic SNP was found in the gene SAUSA300_RS00145 (A194V). Although two distinct SNPs were localised to this gene after both the first and second nasopharyngeal passages, this occurred at different base pair positions that are adjacent in the genome. The cognate amino acid changes for both (L190F, A194V) in the same protein sequence region resulted in relatively conservative amino acid changes: all hydrophobic amino acids, though leucine is replaced by the hydrophobic, aromatic amino acid, phenylalanine.

A notable difference between the variants from the first and second passages is the higher frequency of reads for each variant in the latter. The sequence pools from mice B2 and C2 revealed that most of the intragenic SNPs were present in at least 90% of the reads, so present in almost all input *S. aureus* isolates. The pool of isolates from mouse A2 had lower frequency of the identified variants, where most of the SNPs were detected in at least 60% of the reads, albeit substantially higher than the variant frequencies in the first passage (Table 4.4). These observations do not explain the greater extent of SNP variants that emerged after the first passage compared with the subsequent passage.

Further, the high number of mutations found in all three sequence pools was not expected, but gives an indication there is a strong selection for allelic variants of *S. aureus* USA300 LAC JE2 due nasopharynx selection pressure.

Mutations were observed in a wide range of cell function categories. Regarding loci with DNA replication, recombination and repair activity, non-synonymous SNPs were observed in genes encoding GyrA (L84S) and GyrB (I183T), which are topoisomerase II DNA-gyrase subunit A and B, respectively, and play an important role in DNA replication. A SNP was also detected in the gene encoding topoisomerase IV: ParC (T80S). RecF is responsible for nucleotide binding during DNA replication and repair and a SNP was present in the gene, producing variant RecF (P358A). A SNP was also detected in the gene for the DNA translocase relevant cell division event protein FtsK (A180G). A second protein from the FtsK/SpoIIIE family also showed polymorphism in its sequence: SAUSA300_RS09220 (G1142D). Finally, the gene of subunit C of the exonuclease SbcCD, which is enrolled in DNA repair also showed variation in its sequence. The relative high number of genes associated with DNA activity harbouring SNPs could be related to increased mutability by *S. aureus* as an adaptation mechanism. Weakened DNA replication control including DNA repair by *S. aureus* would promote allelic variability, which would be further exposed to selection according to the environmental pressures in the nasopharynx, benefiting *S. aureus* adaptation. The sequential and temporal occurrence of the SNPs that were detected is unclear.

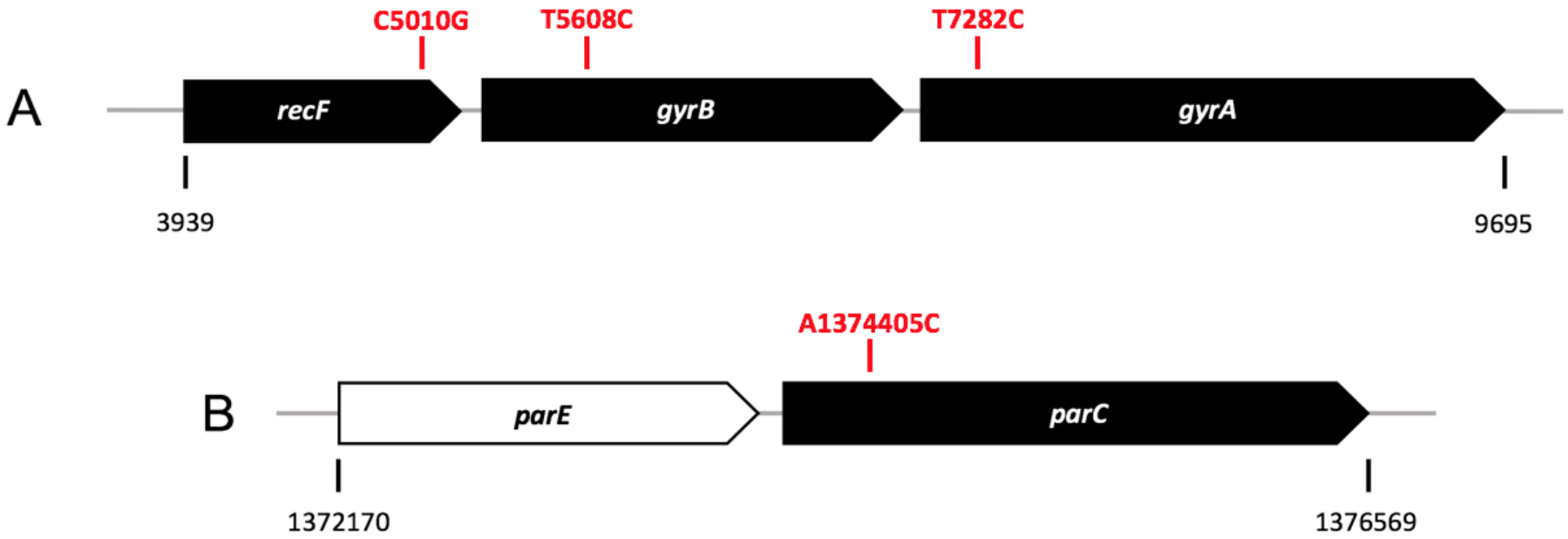

Figure 4.3. *S. aureus* genome map of genes encoding topisomerases II (*gyrA* and *gyrB*) and IV (*parC* and *parR*) and RecF protein (*recF*).
Black lines indicate genome position of genes and red lines indicate genome position of non-synonymous intragenic SNPs.

Mutations in genes associated with RNA pathway activity were present in the sequence pools from the second passage (Table 4.4). Three non-synonymous SNPs were observed for genes involved in RNA modification: Sun (Y306H; 16S rRNA small subunit methyltransferase which is responsible for incorporation of modifications into RNAs), SAUSA300_RS04890 (S150F; pseudouridine synthase from the RluA family associated to post-transcriptional isomeration of uridine to pseudouridine of a variety of cellular RNAs) and SAUSA300_RS09885 (V174I; epoxyqueuousine reductase which catalyses the conversion of epoxyqueuosine to the hypermodified base queuosine in tRNAs). Another SNP associated with RNA activity was found in SAUSA300_RS06375 (D28E) which encodes an endoribonuclease implicated in RNA decay.

With respect to SNPs associated with metabolism, a non-synonymous SNP was found within *mtlA* (E56G), which encodes the PTS system mannitol-specific EIICB component for mannitol transport. D-Mannitol is a sugar alcohol and is one of the many carbohydrate sources used by *S. aureus*. Expanding this functional category, *gutB* encodes a dehydrogenase with catalytic activity for glucitol/sorbitol (sugar alcohols) and had a SNP in its sequence: T341P. A mutation was present in SAUSA300_RS11285, which encodes a TenA/Thi-4 family protein associated with thyaminase activity. This aminohydrolase is involved in thiamine (vitamin B) biosynthesis, an important co-factor for several key enzymes in carbohydrate metabolism. A further gene involved in carbohydrate metabolism also revealed a SNP in its sequence for all the genomic pools of the second passage: SAUSA300_RS09425 (I6V). The protein encoded by this gene is a transaldolase of the pentose phosphate pathway

(PPP) that has versatile roles for cellular metabolism. A SNP was located within the SAUSA300_RS07925 (E196G) gene that encodes an oxidoreductase from the short chain dehydrogenase/reductase (SDR) family which have wide specificity for sugar, alcohol, and aromatic compounds as substrates.

Further potential contributions to altered *S. aureus* metabolism were identified with a non-synonymous SNPs in gene enrolled in fatty acid metabolism: SAUSA300_RS01200 (T601A). This gene encodes the catabolic 3-hydroxyacyl-CoA dehydrogenase involved in fatty acid oxidation. A non-synonymous SNP (K207A) was located within the gene encoding AmpA (Table 4.4), an aminopeptidase involved in processing of intracellular proteins via amino acid removal. Further SNPs in loci with a role in *S. aureus* metabolism include SAUSA300_RS01715 (Oye family flavin oxidoreductase).

Sequence variants were identified within the genome sequence pools associated with oxidative stress resistance (Table 4.4). The gene SAUSA300_RS08410 (*hemN*) that encodes coproporphyrinogen III oxidase contained a SNP (Y145A). The enzyme has a role in haem biosynthesis important for catalase and cytochrome biosynthesis. Potential selection for altered responses to a variety of stresses were attributed to mutations in genes that contribute to *S. aureus* anaerobic metabolism through nitrite transport protein NarK (A241V), peroxidase AhpF (G108D) and general stress tolerance protein ClpL (SAUSA300_RS13805.S249A).

Genes associated with the bacterial cell envelope appeared to be subject to selection during *S. aureus* adaptation to the nasopharynx. Non-synonymous SNPs were found in genes associated with capsular polysaccharide biosynthesis protein Cap5I (L253V), peptidoglycan metabolism enzyme undecaprenyl-diphosphatase DacA, SAUSA300_RS03590 (R69H) and cell membrane lipid components biosynthesis/mevalonate pathway/isoprenoid and isopentenyl-PP biosynthesis, SAUSA300_RS01310 (G119D).

In addition, SNPs were found in membrane-anchored lipoproteins: SAUSA300_RS01070 (N358K) (peptide/nickel transport) and SAUSA300_RS02220 (E209D) (tandem lipoprotein from SaPIn2 pathogenic island; Fig 4.2) adjacent to the *ssl* gene which bears a SNP. SNPs were also identified in genes encoding hypothetical proteins, with special note to SAUSA300_RS09205 which presented 7 allelic variants across the genome sequence pools.

Table 4.4 Nasopharyngeal colonisation isolates after two passages have non-synonymous intragenic SNPs.

S. aureus SNPs observed in pooled genomic DNA of ten isolates after the second passage of murine nasopharyngeal colonisation and sampling. Mouse isolate pools in which the SNPs were detected are indicated by A2, B2 and C2, reflecting three independent mice. Frequency in pool data shows the percentage of reads from each pool that harbours the SNP.

Gene symbol	Function	Genome change	Codon change	Position in protein	Mouse Pool	Frequency in pool
recF	DNA recombination and repair	5010 C/G	P/A	358 aa	A2 B2 C2	66.08% 99.50% 89.47%
gyrB	DNA Topoisomerase II subunit B	5608 T/C	I/T	183 aa	A2 B2 C2	66.62% 99.87% 91.74%
gyrA	DNA Topoisomerase II subunit A	7282 T/C	L/S	84 aa	A2 B2 C2	67.60% 100% 92.43%
SAUSA300_RS00145[1]	Putative transposase in SCC*mec* element	36124 G/A	A/V	194 aa	A2 B2 C2	35.68% 53.86% 51.40%
SAUSA300_RS00265	Hypothetical protein	61025 G/A	L/F	55 aa	A2 B2 C2	63.37% 99.34% 93.45%
cap5I	Capsular polysaccharide CP5 synthesis	182746 T/G	L/V	253 aa	A1 B2 C2	66.75% 99.88% 92.15%
SAUSA300_RS01070	RGD-containing lipoprotein	240358 T/A	N/K	358 aa	A2 B2 C2	66.15% 100% 92.37%
SAUSA300_RS01200	Hydroxyacyl Coenzyme A dehydrogenase	270510 T/C	T/A	601 aa	A2 B2 C3	62.76% 97.07% 90.58%
gutB	Sorbitol dehydrogenase	292738 A/C	T/P	341 aa	A2 B2 C2	64.99% 99.64% 91.64%
SAUSA300_RS01310	Cell wall and capsule metabolism	294913 G/A	G/D	119 aa	A2 B2 C2	64.14% 99.77% 92.05%
SAUSA300_RS01715	Oye family flavin oxidoreductase	376057 C/T	D/N	258 aa	A2 B2 C2	65.77% 99.86% 92.24%

SAUSA300_ RS01715	Oye family flavin oxidoreductase	376231 T/G	S/R	200 aa	A2 B2 C2	63.97% 99.73% 92.89%
ahpF	Alkyl hydroperoxide reductase	429073 C/T	G/D	108 aa	A2 B2 C2	64.37% 100% 91.73%
SAUSA300_ RS02220	Tandem-type lipoprotein Lpl4	467549 A/T	E/D	209 aa	A2 B2 C2	68.29% 99.26% 89.27%
SAUSA300_ RS03590	Undecaprenyl diphosphatase	742477 C/T	R/H	69 aa	A2 B2 C2	69.23% 99.58% 90.45%
SAUSA300_ RS04050	Sporulation Regulator WhiA	837108 G/T	E/D	17 aa	A2 B2 C2	67.52% 100% 93.17%
ampA	Cytosol aminopeptidase	923896 G/T	K/A	207 aa	A2 B2 C2	64.95% 99.72% 91.94%
SAUSA300_ RS04890	Rlu family pseudouridine synthase	996224 C/T	S/F	150 aa	A2 B2 C2	65.68% 100% 91.67%
SAUSA300_ RS05275	Hypothetical protein	1074815 G/A	D/N	71 aa	A2 B2 C2	64.97% 99.75% 92.06%
sun	16S rRNA methyltransferase	1215913 T/C	Y/H	306 aa	A2 B2 C2	64.19% 99.87% 90.09%
ftsK	DNA translocase	1286986 A/G	K/R	180 aa	A2 B2 C2	65.14% 100% 91.76%
SAUSA300_ RS06375	RNase Y	1298198 T/G	D/E	28 aa	A2 B2 C2	68.71% 99.85% 93.01%
sbcC	DNA repair: exonuclease	1363207 A/T	I/F	761 aa	A2 B2 C2	66.24% 99.84% 91.75%
parC	DNA replication: Topoisomerase IV subunit A	1374405 A/C	T/S	80 aa	A2 B2 C2	63.75% 99.85% 90.89%
SAUSA300_ RS07265	Hypothetical protein	1496455 T/A	I/L	663 aa	A2 B2 C2	65.96% 99.86% 93.06%

SAUSA300_ RS07925	SDR family Oxidoreductase	1604107 T/C	E/G	196 aa	A2 B2 C2	61.10% 100% 92.77%
SAUSA300_ RS08410	Copro-porphyrinogen III oxidase	1693739 T/C	Y/A	145 aa	A2 B2 C2	67.43% 100% 91.83%
SAUSA300_ RS09205	Hypothetical transmembrane protein	1857089 C/T	V/I	179 aa	B2 C2	23.98% 20.28%
SAUSA300_ RS09205	Hypothetical transmembrane protein	1857109 A/G	I/T	172 aa	B2 C2	29.15% 24.40%
SAUSA300_ RS09205	Hypothetical transmembrane protein	1857182 G/A	P/S	148 aa	A2 B2 C2	31.10% 44.48% 40.93%
SAUSA300_ RS09205	Hypothetical transmembrane protein	1857188 C/T	E/K	146 aa	A2 B2 C2	31/83% 47.30% 42.20%
SAUSA300_ RS09205	Hypothetical transmembrane protein	1857202 G/A	A/V	141 aa	A2 B2 C2	31.97% 42.25% 41.06%
SAUSA300_ RS09205	Hypothetical transmembrane protein	1857215 T/C	T/A	137 aa	A2 B2 C2	35.55% 51.15% 46.47%
SAUSA300_ RS09205	Hypothetical transmembrane protein	1857232 T/C	K/R	131 aa	A2 B2 C2	39.91% 56.41% 52.01%
SAUSA300_ RS09220	FtsK/SpoIIIE Family protein	1859999 C/T	G/D	1142 aa	A2 B2 C2	65.07% 99.86% 90.30%
SAUSA300_ RS09290	Hypothetical flavoprotein	1876812 A/T	D/V	97 aa	A2 B2 C2	65.61% 100% 91.97%
SAUSA300_ RS09425	Transaldolase	1908169 T/C	I/V	6 aa	A2 B2 C2	67.73% 99.87% 90.37%
SAUSA300_ RS09885	Epoxyqueuosine reductase	1988983 C/T	V/I	174 aa	A2 B2 C2	66.57% 99.73% 90.73%
SAUSA300_ RS10590	Phage tail tape measure protein	2100157 A/C	I/S	384 aa	A2 B2 C2	74.75% 100% 92.87%

SAUSA300_ RS10650	Phage terminase protein	2109096 G/T	A/D	84 aa	A2 B2 C2	74.13% 99.73% 91.26%
SAUSA300_ RS11285	Thiaminase II	2214645 C/T	G/R	224 aa	A2 B2 C2	63.53% 100% 91.84%
mtlA	PTS system Mannitol transporter	2276564 A/G	E/G	56 aa	A2 B2 C2	65.21% 99.75% 89.40%
narK	Nitrite extrusion protein	2508817 G/A	A/V	241 aa	A2 B2 C2	66.90% 100% 90.41%
SAUSA300_ RS13805	Clp protease ATP-binding subunit	2685939 T/G	S/A	249 aa	A2 B2 C2	66.21% 100% 90.81%
SAUSA300_ RS13825	Antibiotic transport-like protein	2690087 T/G	K/G	795 aa	A2 B2 C2	63.58% 99.66% 94.32%
hisG	ATP phosphoribosyl-transferase	2839280 C/A	G/C	140 aa	A2 B2	22.62% 31.12%

1: Locus also harbouring a SNP in sequence pools from first nasopharyngeal passage.

4.3.6 A second nasopharyngeal passage reveals increased intergenic sequence variants

Intergenic SNPs increased in *S. aureus* genomic pools after two serial nasopharyngeal passages, following the same pattern observed for the non-synonymous intragenic SNPs. While only one SNP could be detected in the intergenic region from the first passage sequence pools, two repeated passages of *S. aureus* in the nasopharynx revealed 26 variants in non-coding regions. Similar to the intragenic mutation frequency, the proportion of intergenic variants reads were greater than 90% for DNA pools from mice B2 and C2, and 60% for mouse A2, indicating almost all nasopharynx isolates randomly picked possess the SNPs. Similarly, the mouse A2 pool presented a similar frequency of variant reads to that of the intragenic SNPs (around 60%). Thirty variants mapped in all three genomic pools were annotated as intergenic SNPs by the SNPeff algorithm. Further curation of these SNPs relative to the *S. aureus* USA300 LAC JE2 genome revealed that five were synonymous intragenic SNPs (Table. 4.5).

Seven intergenic SNPs were located within 200 bp upstream of coding sequences, suggesting that such variants could impact on their transcription levels. Two of these intergenic SNPs are separated by 8 bp from each other and both are within 20 bp upstream of the coding sequences of the pseudogene SAUSA300_RS15240 and SAUSA300_RS15245 encoding a hypothetical protein.

Two intergenic variants with possible impact on transcription of genes involved in arginine utilisation were detected: *arcA* and *argF*. Both *arcA* and *argF* encode enzymes that direct formation of citruline (Fig 4.4), the former as part of the arginine deiminase pathway which serves as energy source for *S. aureus* in anaerobic conditions, and the latter within arginine synthesis from glutamate. A further copy of *arcA* is present on the arginine catabolic mobile element that increases versatility of USA300 clones and their ability to persist on skin (Diep *et al*., 2006; Pi *et al*., 2009). Combined with an intragenic SNP in *narK,* encoding a nitrite respiration transporter in the second passage, it is plausible that these SNPs help counteract environmental conditions in the nasopharynx to modify respiration.

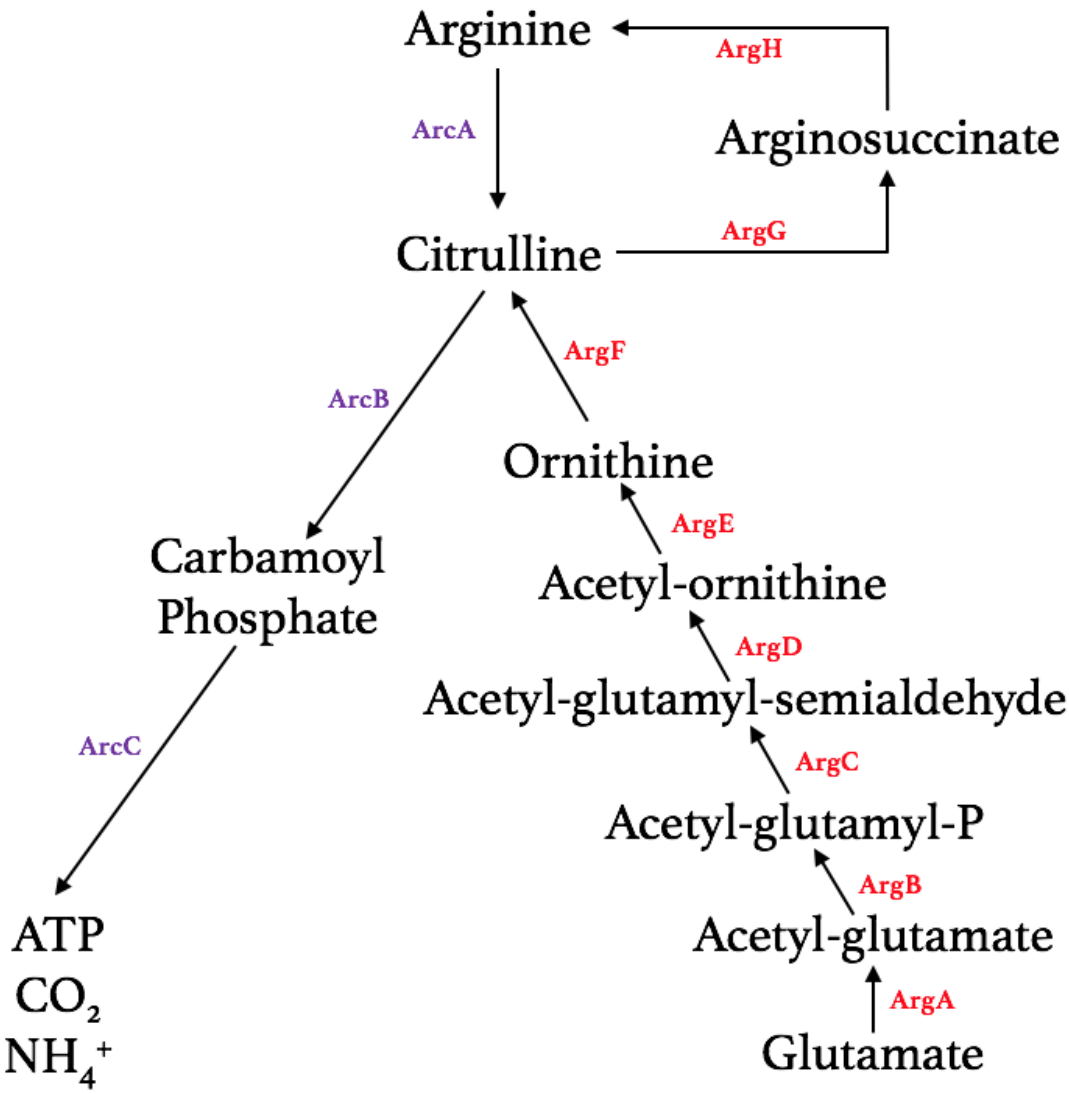

Figure 4.4. Arginine metabolism pathways in *S. aureus* USA300.

Enzymes associated with the arginine deiminase pathway are coloured purple and the arginine biosynthetic pathway from glutamate in red.

Intergenic SNPs were identified within 200 bp upstream of the genes for ribosomal protein S1 (*rpsA*) and RNA polymerase β subunit (*rpoB*) concerned with transcription and translation. The intergenic base changes close to their coding sequence selected over the evolution of *S. aureus* in the nasopharynx might alter their transcription levels.

Whilst most of the SNPs located in non-protein coding regions were dispersed around the genome, 13 intergenic SNPs were found in a 151 bp region (1929046-1929196) of which five are within 200 bp of the open reading frame of transposase SAUSA300_RS09560. This transposase is located in the pathogenic island SaPIn3, a variable mobile element that comprises virulence genes encoding, most commonly, proteins: serine protease-like; enterotoxins, ferrichrome uptake. Eight of the 13 SNPs are located in the regulatory sRNA (SprA1) sequence, according to Staphylococcal Regulatory RNAs database (Sassi *et al*., 2015). The frequency of these SNPs was around 50% across the three pools from mice A2, B2, C2 indicating that in each case the variants could be variably present across the ten members of the pool. A further SNP within this set (genome position: 35817) is within the the sRNA Teg5as. These regulatory non-coding, short (50-550 nt) molecules can interact with mRNAs and affect the translation or stability of their targets. The remaining intergenic SNPs were located outwith coding sequence regions and may not influence transcription of genes.

Table 4.5 Nasopharyngeal colonisation isolates after two passages have intergenic SNPs. *S. aureus* genome sequence variants (SNPs) in non-coding regions observed for isolates after the second passage performed in the experimental nasopharyngeal evolution by. Mouse column relates to the pool of isolates (A2, B2 and C2) which the SNPs were detected. Frequency shows the percentage of reads from the pools harbouring the SNP.

Genome change	Distance from coding region	Gene function	Mouse	Frequency of reads
35817			A2	45.98%
	sRNA sequence (Teg5as)	-	B2	56.94%
A/T			C2	57.29%
74496	-1069 bp / SAUSA300_RS00340 (+)	Universal stress protein	A2	62.18%
			B2	99.77%
T/G	-148 bp (arcA) (-)	Arginine deiminase	C2	92.38%
260357	-222 bp / *pflB* (+)	Formate acetyltransferase	A2	67.00%
			B2	100%
C/T	-366 bp / *hptA* (-)	Iron-binding protein	C2	91.01%
349622		Conserved	A2	62.02%
	-517 bp / SAUSA300_RS01590 (+)	Hypothetical	B2	99.79%
T/C		Protein	C2	91.61%
386867*			A2	68.30%
	Intragenic SNP	Pseudogene	B2	100%
T/A	SAUSA300_RS15055		C2	91.35%
585363			A1	67.96%
	-191 bp / *rpoB* (+)	DNA-directed RNA	B2	100%
G/A		polymerase	C2	91.28%
841852*			A2	36.26%
	Intragenic SNP	Pseudogene	B2	49.54%
C/T	(SAUSA300_RS15200)		C2	45.78%
841853*			A2	36.03%
	Intragenic SNP	Pseudogene	B2	49.14%
G/C	(SAUSA300_RS15200)		C3	45.27%
906507*			A2	64.83%
	Intragenic SNP	Hypothetical protein	B2	99.58%
A/C	(SAUSA300_RS04455)		C2	91.19%
940710	-9 bp / SAUSA300_RS15240 (+)	Pseudogene	A2	54.30%
			B2	99.73%
A/G	-18 bp / SAUSA300_RS15245 (-)	Hypothetical protein	C2	85.03%
940718	-17 bp / SAUSA300_RS15240 (+)	Pseudogene	A2	56.38%
A/G			B2	100%
	-10 bp / SAUSA300_RS15245 (-)	Hypothetical protein	C2	85.80%

1161534	-44 bp / *argF* (+)	Amino acid metabolism	A2	68.70%
			B2	100%
C/T	-394 bp / SAUSA300_RS05750 (-)	Superantigen-like protein	C2	93.17%
1533312	-237 / SAUSA300_RS07455 (+)	Hypothetical protein	A2	66.52%
A/T			B2	99.86%
	-165 bp / *rpsA* (-)	30S ribosomal protein S1	C2	91.07%
1929046	-289 bp / SAUSA300_RS09565 (+)	Hypothetical protein	A2	45.10%
T/C			B2	54.13%
	-132 bp / SAUSA300_RS09560 (-)	Transposase	C2	57.68%
1929047	-288 bp / SAUSA300_RS09565 (+)	Hypothetical protein	A2	45.04%
G/A			B2	54.30%
	-133 bp / SAUSA300_RS09560 (-)	Transposase	C2	57.55%
1929049	-286 bp / SAUSA300_RS09565 (+)	Hypothetical protein	A2	45.08%
C/A			B2	54.10%
	-135 bp / SAUSA300_RS09560 (-)	Transposase	C2	57.32%
1929054	-280 bp / SAUSA300_RS09565 (+)	Hypothetical protein	A2	45.72%
A/G			B2	54.78%
	-141 bp / SAUSA300_RS09560 (-)	Transposase	C2	58.31%
1929062	-272 bp / SAUSA300_RS09565 (+)	Hypothetical protein	A2	47.73%
			B2	56.39%
G/A	-149 bp / SAUSA300_RS09560 (-)	Transposase	C2	59.75%
1929098			A2	46.37%
	sRNA sequence (SprA1)	-	B2	55.89%
C/A			C2	58.84%
1929167			A2	45.53%
	sRNA sequence (SprA1)	-	B2	55.43%
C/T			C2	57.57%
1929168			A2	45.52%
	sRNA sequence (SprA1)	-	B2	55.48%
A/G			C2	57.50%
1929175			A2	45.64%
	sRNA sequence (SprA1)	-	B2	56.03%
T/A			C2	57.39%
1929178			A2	45.74%
	sRNA sequence (SprA1)	-	B2	56.01%
T/A			C2	57.18%
1929180			A2	46.14%
	sRNA sequence (SprA1)	-	B2	56.09%
T/A			C2	57.43%
1929191			A2	44.82%
	sRNA sequence (SprA1)	-	B2	55.04%
A/T			C2	56.90%
1929196			A2	45.36%

	sRNA sequence (SprA1)	-	B2	54.93%
G/A			C2	57.04%
1944161*			A2	66.66%
	Intragenic SNP	Hypothetical protein	B2	99.87%
A/G	(SAUSA300_RS09625)		C2	92.24%
1957938	-100 bp / SAUSA300_RS09690 (+)	Hypothetical protein	A2	65.46%
			B2	99.88%
C/T	-699 bp / *lukE* (-)	Leukotoxin	C2	92.30%
1959504				66.49%
	-313 bp / SAUSA300_RS15420 (+)	Pseudogene	B2	100%
T/A			C2	90.40%
2322700			A2	67.73%
	-290 bp / *opuD2* (-)	ABC transporter permease	B2	100%
T/C			C2	91.06%

*Variant annotated by SNPEff as intergenic SNP located in a pseudogene.

4.3.7 A third nasopharyngeal passage reveals refinement of intragenic sequence variants

A pronounced expansion of *S. aureus* sequence variants was evident after a second nasopharyngeal passage. Consequently, a third passage was performed under the same conditions and this revealed there was no continued large-scale expansion of sequence variants. In contrast a lesser extent of sequence variation was identified across the genome pools of the three mice, each with ten isolate pools. The numbers of intragenic variant SNPs in each of the three sequence pools from the third passage ranged from 36-39 mutations, with a ratio of 2:1 for non-synonymous/synonymous. Of these, 19 of 20 non-synonymous intragenic SNPs were present in each of the three sequence pools. This increase was mirrored with an increased frequency of variant reads shared across the three sequence pools, with at least 90% variant sequence reads, compared with the previous passages (apart from SNPs detected in the hypothetical transmembrane protein SAUSA300_RS09205). This higher percentage of reads harbouring SNPs suggests a clonal selection for certain variants from the inoculation pool.

Among the 20 non-synonymous SNPs detected in the sequence pools of the third passage, 15 were identified after the second passage. Moreover, a SNP found in one sequence pool from the first nasopharyngeal passage (A1), which was not subsequently detected in the second passage isolates, reappeared in all three genome sequence pools after the third passage.

Sequence variants unique to the third nasopharyngeal passage include intragenic SNPs in genes for the stress response chaperone DnaJ and the BioA biotin co-factor biosynthesis. The former gene adds to those genes with variants in the same pathway from the first passage. The remaining non-synonymous intragenic SNPs unique to the third passage are located in hypothetical unknown function proteins.

Table 4.6 Non-synonymous intragenic SNPs of nasopharyngeal colonisation isolates after three passages.

S. aureus genome sequence variants (SNPs) in non-coding regions observed for isolates after the third passage in an experimental nasopharyngeal model. Mouse column relates to the pool of isolates (A3, B3 and C3) which the SNPs were detected. Frequency shows the percentage of reads from the pools harbouring the SNP.

Gene symbol	Gene function	Genome change	Codon change	Position in protein	Mouse pool	Frequency of reads
recF[2]	DNA replication and repair	5010 C/G	P/A	358 aa	A3 B3 C3	100% 100% 100%
SAUSA300_RS00265[2]	Hypothetical protein	61025 G/A	L/F	55 aa	A3 B3 C3	99.97% 100% 99.98%
SAUSA300_RS01070[2]	RGD-containing lipoprotein	240358 T/A	N/K	358 aa	A3 B3 C3	100% 100% 100%
SAUSA300_RS01200[2]	Hydroxyacyl Coenzyme A dehydrogenase	270510 T/C	T/A	601 aa	A3 B3 C3	99.98% 100% 100%
gutB[2]	Sorbitol dehydrogenase	292738 A/C	T/P	341 aa	A3 B3 C3	99.98% 100% 100%
SAUSA300_RS01715[2]	Oye family flavin oxireductase	376057 C/T	D/N	258 aa	A3 B3 C3	99.98% 99.93% 99.97%
SAUSA300_RS01715[2]	Oye family flavin oxireductase	376231 T/G	S/R	200 aa	A3 B3 C3	99.97% 100% 100%
SAUSA300_RS02185[1]	Staphylococcal superantigen-like 11 (SSL11)	460966 G/A	A/T	110aa	A3 B3 C3	100% 92.30% 95.83%
ftsK[2]	DNA translocase	1286986 A/G	K/R	180 aa	A3 B3 C3	100% 100% 100%
dnaJ	Chaperone protein	1688528 C/T	A/T	250aa	A3 B3 C3	99.97% 100% 99.95%
SAUSA300_RS09205[2]	Hypothetical transmembrane protein	1857089 C/T	V/I	179 aa	B3	13.10%

SAUSA300_ RS09205[2]	Hypothetical transmembrane protein	1857109 A/G	I/T	172 aa	A3 B3 C3	17.90% 24.42% 24.67%
SAUSA300_ RS09205[2]	Hypothetical transmembrane protein	1857182 G/A	P/S	148 aa	A3 B3 C3	38.13% 41.51% 41.87%
SAUSA300_ RS09205[2]	Hypothetical transmembrane protein	1857188 C/T	E/K	146 aa	A3 B3 C3	41.28% 43.10% 43.85%
SAUSA300_ RS09205[2]	Hypothetical transmembrane protein	1857202 G/A	A/V	141 aa	A3 B3 C3	40.21% 40.75% 40.88%
SAUSA300_ RS09205[2]	Hypothetical transmembrane protein	1857215 T/C	T/A	137 aa	A3 B3 C3	46.58% 45.50% 45.44%
SAUSA300_ RS09205[2]	Hypothetical transmembrane protein	1857232 T/C	K/R	131 aa	A3 B3 C3	50.28% 49.51% 50%
SAUSA300_ RS15385	Hypothetical protein	1872844 T/C	Y/C	28 aa	A3 B3 C3	99.97% 99.95% 99.94%
SAUSA300_ RS10080	Hypothetical lipoprotein	2007223 G/T	R/L	48 aa	A3 B3 C3	100% 99.97% 99.96%
bioA	Amino-Transferase	2549677 C/T	R/H	189 aa	A3 B3 C3	99.96% 99.93% 99.98%

1: SNP also observed in the sequence pools from first passage.

2: SNP also observed in the sequence pools from second passage.

4.3.8 Three serial nasopharyngeal passages reveal refinement of intergenic sequence variants

Matching the reduction of the intragenic SNPs detected in the sequence pools after the third passage, was a reduction of intergenic SNPs. Specifically, genomic pools from the second passage showed 26 intergenic SNPs, while the three sequence pools from the third passage presented only 6. Three of these allelic variants were identified in the previous nasopharyngeal passage and the frequency of reads harbouring these SNPs supports that every isolate of the third nasopharyngeal passage contains these sequence variants.

Considering the 3 SNPs uniquely detected in the isolates from the third nasopharyngeal passage, one was located in the Teg16 sRNA sequence. The other two sequence variants are situated within 200 bp upstream of genes: *lctP2* encoding a lactate transporter associated with anaerobic respiration and *pnhD* encoding an ABC transporter with phosphate/phosphite/phosphonate uptake specificity. A hypothetical phage-associated protein with glycerol transport activity involved in acid teichoic acid biosynthesis is possibly affected by an intergenic SNP, however, the frequency of reads for this SNP was lower across the genome sequence pools.

Table 4.7: Non-synonymous intergenic SNPs of nasopharyngeal colonisation isolates after three passages.

S. aureus genome sequence variants (SNPs) in non-coding regions observed for isolates after the third passage in an experimental nasopharyngeal model. Mouse pool column relates to the pool of isolates (A3, B3 and C3) which the SNPs were detected. Frequency shows the percentage of reads from the pools harbouring the SNP.

Genome change	Distance from coding region	Gene function	Mouse	Frequency of reads
164912	-97 bp / SAUSA300_RS00765 (+)	Hypothetical protein	A3	45.98%
			B3	56.94%
		Phosphate/phosphite/Phos	C3	57.29%
G/A	-132 bp / *phnD* (-)	phonate ABC transporter		
1533312[1]	-237 / SAUSA300_RS07455 (+)	Hypothetical protein	A3	99.98%
			B3	99.94%
A/T	-165 bp / *rpsA* (-)	30S ribosomal protein S1	C3	99.94%
1957938[1]	-100 bp / SAUSA300_RS09690 (+)	Hypothetical protein	A3	99.97%
			B3	100%
C/T	-699 bp / *lukE* (-)	Leukotoxin	C3	99.98%
2164596			A3	62.18%
	sRNA sequence (Teg16)	-	B3	99.77%
A/G			C3	92.38%
2322700[1]			A3	100%
	-290 bp / *opuD2* (-)	ABC transporter permease	B3	100%
A/G			C3	99.98%
2487639	-189 bp / SAUSA300_RS12785 (+)	Glycerol transferase	A3	67.00%
			B3	100%
A/G	-128 bp / *lctP2* (-)	Lactate permease	C3	91.01%

1: SNP also observed in the sequence pools from second passage.

Table 4.8 Non-synonymous intragenic SNPs of nasopharyngeal colonisation isolates that persisted between passages.

S. aureus genome sequence variants (SNPs) in coding regions observed for isolates from repeated passages in an experimental nasopharyngeal model.

Gene symbol	Gene function	Genome change	Codon change	Position in protein	Mouse pool	Frequency of reads
recF[2]	DNA replication and repair	5010 C/G	P/A	358 aa	A3 B3 C3	100% 100% 100%
SAUSA300_RS00265[2]	Hypothetical protein	61025 G/A	L/F	55 aa	A3 B3 C3	99.97% 100% 99.98%
SAUSA300_RS01070[2]	RGD-containing lipoprotein	240358 T/A	N/K	358 aa	A3 B3 C3	100% 100% 100%
SAUSA300_RS01200[2]	Hydroxyacyl Coenzyme A dehydrogenase	270510 T/C	T/A	601 aa	A3 B3 C3	99.98% 100% 100%
gutB[2]	Sorbitol dehydrogenase	292738 A/C	T/P	341 aa	A3 B3 C3	99.98% 100% 100%
SAUSA300_RS01715[2]	Oye family flavin oxireductase	376057 C/T	D/N	258 aa	A3 B3 C3	99.98% 99.93% 99.97%
SAUSA300_RS01715[2]	Oye family flavin oxireductase	376231 T/G	S/R	200 aa	A3 B3 C3	99.97% 100% 100%
SAUSA300_RS02185[1]	Staphylococcal superantigen-like 11 (SSL11)	460966 G/A	A/T	110aa	A3 B3 C3	100% 92.30% 95.83%
ftsK[2]	DNA translocase	1286986 A/G	K/R	180 aa	A3 B3 C3	100% 100% 100%
SAUSA300_RS09205[2]	Hypothetical transmembrane protein	1857089 C/T	V/I	179 aa	B3	13.10%
SAUSA300_RS09205[2]	Hypothetical transmembrane protein	1857109 A/G	I/T	172 aa	A3 B3 C3	17.90% 24.42% 24.67%
SAUSA300_RS09205[2]	Hypothetical transmembrane	1857182	P/S	148 aa	A3 B3	38.13% 41.51%

	protein	G/A			C3	41.87%
SAUSA300_ RS09205[2]	Hypothetical transmembrane protein	1857188 C/T	E/K	146 aa	A3 B3 C3	41.28% 43.10% 43.85%
SAUSA300_ RS09205[2]	Hypothetical transmembrane protein	1857202 G/A	A/V	141 aa	A3 B3 C3	40.21% 40.75% 40.88%
SAUSA300_ RS09205[2]	Hypothetical transmembrane protein	1857215 T/C	T/A	137 aa	A3 B3 C3	46.58% 45.50% 45.44%
SAUSA300_ RS09205[2]	Hypothetical transmembrane protein	1857232 T/C	K/R	131 aa	A3 B3 C3	50.28% 49.51% 50%

1: SNP also observed in the sequence pools from first passage.

2: SNP also observed in the sequence pools from second passage.

Table 4.9 Non-synonymous intergenic SNPs of nasopharyngeal colonisation isolates that persisted between passages.

S. aureus genome sequence variants (SNPs) in non-coding regions observed for isolates from repeated passages in an experimental nasopharyngeal model.

Genome change	Distance from coding region	Gene function	Mouse	Frequency of reads
1533312[1] A/T	-237 / SAUSA300_RS07455 (+) -165 bp / *rpsA* (-)	Hypothetical protein 30S ribosomal protein S1	A3 B3 C3	99.98% 99.94% 99.94%
1957938[1] C/T	-100 bp / SAUSA300_RS09690 (+) -699 bp / *lukE* (-)	Hypothetical protein Leukotoxin	A3 B3 C3	99.97% 100% 99.98%
2322700[1] A/G	-290 bp / *opuD2* (-)	ABC transporter permease	A3 B3 C3	100% 100% 99.98%

1: SNP also observed in the sequence pools from second passage.

4.4 DISCUSSION

In this chapter, whole genome sequencing of *S. aureus* isolates from consecutive passages of a nasopharynx colonisation experimental evolution model was performed. The sequencing experiment was successful overall in the aim to detect variants that were possibly selected due to pressures of colonising the nasopharynx of the mice in this model that was standardised in Chapter 3. The dataset from this chapter reveals a wide range of loci that harbour sequence variation and these are involved in multiple pathways and mechanisms with potential contributions in the establishment and maintenance of nasopharyngeal colonisation by *S. aureus*. Since a large number of mutations was detected in this work, an extended future study to assess the actual contribution of each mutation is necessary. First, a few questions arise based on the results of this chapter: are the mutations detected here a direct consequence of *S. aureus* adaptation according to the selective pressures during nasopharyngeal colonisation? Do these genetic changes provide better fitness for *S. aureus* to persist in the nasopharynx? Why and how does the number of SNPs change substantially depending on the passage of *S. aureus* through the nasopharynx? The following chapter is therefore aimed at beginning to elucidate some of these questions.

The genetic changes in *S. aureus* USA300 identified by sequencing make it tempting to suggest that *S. aureus* evolved to become more adapted to the nasopharynx through mutations that increase fitness for the anatomic site. Several of the intragenic SNPs found in this work associated to diverse cell

functions (GyrA.L84S, RS08410.Y145A, RS09425.I6V and, ParC.Y80S,) were also identified by a study that assessed the evolution of USA300 clinical isolates in the United States (Uhlemann *et al*., 2012). This overlap might indicate that the mutations in this study contribute to adaptation of *S. aureus* through shared drivers of selection, however their relevance to the murine model used here is unclear.

Moreover, the mutations observed in the clones might give indications of components directly affected in *S. aureus* by the selective pressure in the murine nasopharynx. By introducing *S. aureus* into the mouse nasopharynx, the bacterial cell must defend itself against multiple host stress factors such that prolonged successful colonisation could only be achieved if *S. aureus* overcomes these challenges. *S. aureus* and its weaponry can naturally defeat host and environmental factors that impose barriers for *S. aureus* survival and their refinement through selected mutations might be the observed outcome across the consecutive passages in this study. Certain components or pathways might require a shift in their structure, activity and expression to improve *S. aureus* fitness to the murine nasopharynx with this transition the result of the identified mutations.

Foster (2007) states that one of the bacterial responses to stress is to induce genetic variability, originating with the promotion of mechanisms that favour mutagenesis. Such a response is critical for the adaptive evolution and this theory would support the outcomes of this work if *S. aureus* directly responded to the nasopharynx pressure by increasing mutations that could be selected

during the evolutionary time-course. Because the sequence variation captured from the clones in this work is represented by pools of isolates randomly sampled from the nasal washes, an important feature to be considered is the frequency of the reads harbouring the nucleotide variation.

Sequence variation from the pools of isolates after seven days of colonisation showed that only a few SNPs appeared in the *S. aureus* genome. The only locus harbouring a SNP after the first nasopharyngeal passage that also revealed a SNP in at least one of the second passage genomic DNA sequence pools is SAUSA300_RS00145, which encodes a transposase. This enzyme can bind DNA and catalyse a "cut and paste" reaction of a DNA fragment (e.g. transposon) enabling it to move either between genomes, or for some fragments, within the genome. Transposons are one type of mobile genetic element and as part of the accessory genome they play an important role in the survival of *S. aureus* in different ecological niches; certain genes of transposons encode proteins for antimicrobial resistance and virulence factors (Rouch *et al*., 1987; Ito *et al*., 2003; Olsen *et al*., 2006).

The staphylococcal cassette chromosome *mec* (SCC*mec*) transposase in this work is associated with the IS*6* family of insertion sequences, which are usually found flanking resistance genes (Mahillon and Chandler, 1998). More specifically, the transposase is part of the IS*431* insertion sequence elements. Widely found in *S. aureus* and other staphylococci like *S. epidermidis* and *S. haemolyticus*, this transposon has relevance in the mobilisation of genes responsible for the resistance to methicillin (Kobayashi *et al*., 2001). Altered

activity by this transposase might impact the mobilisation of the *mecA* gene, which confers resistance to beta-lactams. But since the isolates were not screened for excision of the gene, the specific contribution of the SNP cannot be concluded; regulation of mobile genetic elements excision by the SOS response has been suggested (Simmons *et al*., 2008; Zgur-Bertok, 2013). Intergenic SNPs near to the transcriptional start site of a transposase that belongs to SaPIn3 pathogenic island were detected from the sequence pools of the second passage. This variant might alter the transcription levels of the transposase, though it is unclear if this altered its regulation, how mobilisation benefits nasopharyngeal colonisation by *S. aureus*.

A non-synonymous intragenic SNP after the first passage was also detected for SAUSA300_RS02185 which encodes an exotoxin, one of the many weapons from the *S. aureus* virulence machinery. This SNP could not be detected in sequence pools from the second passage, but was detected again in the third passage. Despite the critical relevance of virulence factors in the establishment of an infection process by *S. aureus*, such components are thought not to play a critical role for the persistence of *S. aureus* during nasal colonisation (Chavez-Moreno *et al*., 2016). Recent work has evaluated differences in the gene expression of virulence determinants during colonisation and disease by *S. aureus* in rat models. It was demonstrated that toxin genes have lower expression compared with other virulence components during nasal colonisation and that the expression level increased after 1 hour of induced bacteremia (Jenkins *et al*., 2015). Schlievert *et al*. (2007) demonstrated that haemoglobin inhibits the production of of exotoxins. Since it was proposed that

haemoglobin in nasal secretions promotes nasal colonisation (Pynnonen *et al*., 2011), it is possible that exotoxins are not required for the nasal colonisation of *S. aureus*. Further studies could examine whether the SNP found in the exotoxin gene affects exotoxin production or activity to identify if it promotes *S. aureus* adaptation to the nasopharynx.

With respect to the second passage dataset, mutations were found in diverse cell function genes. DNA metabolism-related genes presented non-synonymous SNPs within loci encoding DNA topoisomerases: *gyrA*, *gyrB* and *parC*. Mutations in these genes of *S. aureus* and other microorganisms have been extensively studied over the last two decades (Heisig, 2009; Redgrave *et al*., 2014). GyrA and GyrB constitute the two subunits of topoisomerase II that acts as a DNA gyrase. Topoisomerase IV has two subunits: ParC and ParE. Both enzymes are involved in DNA packaging and segregation, with DNA gyrase involved in the maintenance of the negative supercoiling of the bacterial chromosome during replication, whilst topoisomerase IV decatenates the bacterial chromosomes allowing further segregation.

These genes are part of the highly conserved Quinolone Resistance-Determining Region (QRDR). Both topoisomerases II and IV act by binding to DNA and inducing double-strand breaks with posterior ligation of the DNA strands. When fluoroquinolone is present, it leads to a drug-enzyme-cleaved DNA structure formation that will consequently result in cell death.
A few bacteria are able to survive with only one gyrase although most of them have both enzymes. With *S. aureus* and other Gram-positive bacteria,

topoisomerase IV is more susceptible to quinolones and therefore ParC it is the primary target for the antibiotic. Additional mutations in *gyrA* confers high-resistance to *S. aureus*. These mutations are usually found in hotspots identical to the SNPs observed in this work for *gyrA* and *parC*. An evolution study regarding USA300 clinical and colonising strains detected identical SNPs for both *gyrA* (GyrA.L84S) and *parC* (ParC.Y80S) in two out of eight strains (Uhlemann *et al*., 2012). GyrA.L84S was also detected in 15 out of 21 CA-MRSA USA300 clinical samples from the Austrian national reference laboratory (Lepuschitz *et al*., 2018). This supports the potential role of topoisomerases and the mutations present in their respective genes for *S. aureus* nasopharyngeal colonisation.

In addition to the genes for topoisomerases II and IV, mutations in DNA repair function gene *recF* was identified. RecF is involved mainly in DNA damage repair that occurs during the replication via homologous recombination, by binding single-strand DNA filamentous (Pages, 2016). The *recF* gene is immediately upstream of *gyrB* and and *gyrA,* in the same open reading frame (Alonso & Fisher, 1995). Although *recF* has been characterised in *S. aureus* and its role was identified more than 20 years ago, little is known about the protein and its relevance for *S. aureus* colonisation or pathogenesis. The SNP found in *recF* in this work was one of the few identified in both the second to the third nasopharyngeal passage, though how this gene might be relevant is unclear. The same SNP (RecF.P358F) was found in 9 out of 10 *S. aureus* USA300 clones assessed in an evolution work (Kennedy *et al*., 2008), reinforcing the potential for the *recF* SNP to increase *S. aureus* fitness or versatility for nasopharyngeal

colonisation. Future experiments could determine whether the mutation rates are altered by this SNP and what the colonisation outcomes are with engineered *recF* SNP variant strains, if such a strain could be constructed as an isogenic variant.

A further locus with roles in DNA repair that harboured a SNP after the second passage was *sbcC*. This gene encodes the subunit C of the exonuclease SbcCD, where SbcC is a protein with ATPase activity and SbcD is responsible for the nuclease activity. Exonucleases are important enzymes involved in the hydrolysation of phosphodiester bonds from the end of a double-stranded DNA by exhibiting 3'→5' activity. Few studies have examined the roles of this gene in *S. aureus*, with most studies performed in *E. coli* (Lovett, 2011). Chen *et al*. (2007) proposed a role for SbcCD in *S. aureus*, when they speculated, based on homology, that both SbcC and SbcD contribute to DNA repair. They found that SbcCD increases survival under exposure to UV irradiation, where sbcCD is part of the SOS regulon (set of genes enrolled in response to DNA damage). Considering the role of SbcC protein is possible to hypothesise, the identified mutation might affect DNA repair. Reduced activity might promote population variation by increasing mutations. This could be tested in future.

A non-synonymous SNP was identified in *ftsK*; a locus that encodes a DNA translocase. Proteins from this family facilitate chromosome segregation in the final stages of cell division by recruiting DNA from the division site before cytokinesis is completed (Kaimer & Graumann, 2011). *S. aureus* encodes two DNA translocases: FtsK and SpoIIIE. A recent study of DNA translocases in *S.*

aureus concluded that both enzymes might have similar roles and that at least one of the DNA translocases is required for normal chromosome segregation (Veiga & Pinho, 2017). Based on their function, the appearance of mutations in these loci might increase population fitness. The mutation found in all three DNA sequence pools from the second passage (FtsK, K180R) is one of two variants detected in genes with DNA repair activity that persisted to the third nasopharyngeal passage. A separate study revealed a non-synonymous SNP in a FtsK/SpoIIIE family protein in a clinical isolate assessed for evolution during asymptomatic carriage (Golubichik *et al*., 2013), which hints that the SNP found in this work contributes for the *S. aureus* nasopharyngeal colonisation.
Analysis of sequence variants after nasopharyngeal passage revealed several SNPs in RNA-associated pathways. SAUSA300_RS06375 is annotated with RNase Y activity. RNaseY is an endoribonuclease involved in RNA decay, a critical response mechanism of the bacteria to changes in the microenvironment to inhibit the production of unnecessary proteins (Khemici *et al*., 2015). RNaseY also indirectly regulates the expression of virulence genes (Marincola *et al*., 2012). Based on the RNaseY role in *S. aureus*, a SNP that alters enzyme activity might promote adaptation to new environments can be expected. Since the SNP is a conservative replacement (D28E), however, it is possible that no functional change occurred in protein activity.

A second locus with RNA activity that presented a SNP in all the sequence pools from the second nasopharyngeal passage is SAUSA300_RS04890. This locus encodes a pseudouridine synthase from the RluA family involved in the site-specific isomerisation of tRNA uridine into tRNA pseudouridine (Hamma &

Ferré-D'Amaré, 2006). Pseudouridine modification is the most abundant post-transcriptional reaction in cellular RNAs and its biochemical functions are still to be clearly elicited. Pseudouridine confers a protective effect for the RNA tertiary structure as the pseudouridine form associates with an additional hydrogen bond that increases the stability and rigidity of RNAs (Spenkuch *et al*., 2014). In *E. coli*, pseudouridine seems to influence mRNA translation (Kariko *et al*., 2008). Although the biological role of pseudouridine remains unclear, it has been suggested that pseudouridine synthase is relevant, not only for the RNA isomerisation but as an important component for other cell functions. Whether pseudouridine synthase is critical only for uridine modification or plays a bigger role in *S. aureus* metabolism, the activity of this enzyme is relevant for the maintenance of the cell viability. The deletion of two pseudouridine genes in *E. coli* results in growth deficiency which can be reverted by a point mutation in the enzymatically inert proteins (Gutgsell *et al*., 2000; Gutgsell *et al*., 2001).

Similar to SAUSA300_RS04890, another SNP was found in a gene involved in RNA maturation. Gene *sun* encodes a methyltransferase responsible for nucleotide modifications in a site-specific manner in the 16S rRNA small subunit. Although the relevance of methylation of individual nucleotides is unclear, these modifications seem to increase the ribosomal stability and consequently affect the synthesis of proteins (Decatur & Fournier, 2002). A link between the SNP in *sun* from this work and post-transcriptional modification of RNAs relevant to the success of *S. aureus* in establishing a nasopharyngeal colonisation is not clear.

In the context of genes with roles in metabolism, several non-synonymous SNPs were found in loci of carbohydrate metabolism pathways after the second passage only. Bacteria have the ability to use diverse carbohydrates as sources of carbon and energy. Two SNPs associated with sugar metabolism were found in loci after the second passage: *mtlA* and *gutB*. Mannitol and sorbitol, are polyols (sugar alcohols), that are widely abundant hexitols utilised as a carbon source. Sorbitol is an isomer of mannitol and is transported by the mannitol (Mtl) uptake system in *S. aureus*, (Kenny *et al*., 2013). Mannitol fermentation is used as a species signature for laboratory identification that discriminates it from most other staphylococci. MtlA forms part of the phosphotransferase system (PTS) that is the main transport mechanism responsible for acquisition of this carbohydrate. Uptake leads to initial phosphorylation of mannitol to mannitol-1-P that is further oxidised into fructose-6-P for use in the glycolytic pathways for energy production. Mannitol is a relevant carbon source for *S. aureus*, and its use as aa resource by *S. aureus* could aid survival in deprived-nutrient microenvironments like the nasopharynx (Krismer *et al*., 2014). A SNP in the gene for an SDR oxidoreductase (Table 4.4) could be linked to sugar metabolism based on pathway analysis, potentially associated with polyol interconversion.

A SNP in a further carbohydrate metabolism gene that encodes a transaldolase was detected in all the three sequence pools in the second passage: SAUSA300_RS09425 (I6V). Transaldolase provides a link between the glycolytic and pentose-phosphate-pathway (PPP) by catalysing a reversible reaction involving fructose-6-P (Richardson *et al*., 2015). The same SAUSA300_RS09425

SNP identified in this work for was observed in a recent study that assessed the evolution of clinical *S. aureus* USA300 isolates across the United States (Jamrozy *et al*., 2016).

Two intragenic SNPs in SAUSA300_RS01715 that encodes a gene ascribed a role in carbohydrate metabolism. These were identified after the second passage and could be detected after the third passage as the only carbohydrate metabolism-related gene. The reason for the lack of maintenance of certain SNPs across passages is unclear. Two possibilities exist, either purifying selection promotes clones with greater success (fitness) or the mutations are reversed, where the former explanation appears most likely.

A SNP identified in the passages was located in SAUSA300_RS11285. This gene encodes an enzyme of thiamine (vitamin B_1) diphosphate biosynthesis that hydrolysis of amino-pyrimidine of thiamine compounds with a potential role in hydrolytic cleavage of thiamine. Thiamine pyrophosphate (TPP, active form of thiamine) is a critical co-factor of some key enzymes in carbohydrate and amino acid biosynthesis (Begum *et al*., 2013). Thiamine biosynthesis is strongly regulated by TPP at transcriptional and enzymatic levels (Muller *et al*., 2009). If the SNP found in this work modifies thiamine levels it might modify utilisation of carbohydrate sources in the nasopharyngeal microenvironment, where low levels of carbon and amino acid source are reported (Krismer *et al*., 2014).

A mutation was identified in gene SAUSA300_RS03590 (R69H) that encodes enzyme UppP, an undecaprenol diphosphatase. The SNP might contribute to

altered peptidoglycan synthesis since this enzyme is essential for its biosynthesis. Peptidoglycan is a linear polymer constituted by repeats of linked N-acetylglucosamine and N-acetylmuramic acid that has many roles including helping *S. aureus* resist osmotic pressure and maintain shape (El Ghachi *et al*., 2018).

As a facultative anaerobe, *S. aureus* can grow in low-oxygen environments by fermentation or nitrate respiration. NarK is a nitrate/nitrite transporter involved in respiration with alternative electron acceptors and a missense SNP was found in its gene. Under anaerobic conditions *narK* is induced in *S. aureus* (Fuchs *et al*., 2007). In *E. coli*, Schlag *et al*. (2008) showed that *narK* is positively regulated by the NreBC system and in response to low oxygen availability with very low activity during aerobic growth. Although *S. aureus* can survive in a completely anaerobic environment like abscesses, oxygen availability is not known to be reduced in the nasopharynx unless the bacteria grow in a biofilm or restricted niche. Still *S. aureus* has the ability to change its energy metabolism pathway which represents an advantage for survival. The SNP found in *narK* might alter *S. aureus* efficiency in incorporating nitrate, thereby increasing fitness in the model. Linked with this respiration, intergenic SNPs that might impact the transcription levels of genes involved in arginine metabolism e.g. *arcA,* could help *S. aureus* survival under anaerobic conditions.

In contrast to this apparent potential anaerobic metabolism link, oxidative stress is another environmental factor that *S. aureus* needs to overcome to survive within a host. Reactive oxygen species (ROS) are produced by *S. aureus*

and by the host immune defences. Under aerobic respiration, reduction of oxygen by *S. aureus* eventually leads to the production of superoxide anions and hydrogen peroxide. These ROS are also generated by macrophages, monocytes and neutrophils due the presence of NADPH oxidase (NOX) in these cells. The oxidative damage of ROS is due their potential to oxidise several molecules of the bacterial cell including lipids, DNA and proteins and one of the ways that *S. aureus* protects itself from ROS is by producing antioxidants, including peroxiredoxins that catalyse the reduction of alkyl hydroperoxides into their respective alcohols (Gaupp *et al*., 2012). AhpC, acting together with AhpF, is one of the major peroxireoxins and it was shown in *S. aureus* to confer resistance to ROS (Cosgrove *et al*., 2007). AhpF enhances the catalytic efficiency of AhpC and a SNP found in the sequence pools from the second nasopharyngeal passage for *ahpF* could alter AhpC catalysis, with consequent effects on ROS defence. The study of Cosgrove *et al*., (2007) showed that AhpC is important for nasal colonisation in a cotton rat model.

A further SNP in a locus involved in stress resistance was observed in SAUSA300_RS13805 encoding ClpL, a protein conserved in most bacterial species with a substrate-specific chaperone activity. Clp ATPases can associate with ClpP forming a proteolytic system that responds to stress by degrading accumulated and misfolded proteins (Frees *et al*., 2004). Studies have shown the relevance of Clp protease activity for *S. aureus* in the response to nutrient limitation (Farand *et al*., 2013), degradation of *S. aureus* antitoxins (Donegan *et al*., 2010), oxidative and heat stress (Chatterjee *et al*., 2005) and biofilm formation (Frees *et al*., 2004). Given the critical role of Clp protease and

ATPases in several aspects of the cell survival and maintenance, mutations that increase fitness for colonisation are not unexpected. The missense SNP in this work needs detailed characterisation since a distinct single mutation was reported to result in gain of function by ClpP protease to become nonspecific and capable of degrading more than 500 proteins, inhibiting *S. aureus* growth (Ni *et al*., 2016).

From all the mutations observed in loci from the second passage pools, only one is linked to a virulence factor. Cap5I is a protein involved in the biosynthesis of *S. aureus* capsule polysaccharide 5 (CP5), a virulence factor that confers resistance for the bacterial cell against clearance by the host immune defences. *S. aureus* clinical strains mainly produces either serotype CP5 or CP8 from all the 13 different serotypes already characterised. Both are constituted by trisaccharide repeating units (N-acetyl mannosaminuronic acid, N-acetyl-L-fucosamine and N-acetyl-D-fucosamine) with distinct connections between the sugars in the acetylation sites. These CPs act by conferring an escape route to the bacterial cells from phagocytic killing (Nanra *et al*., 2013) and it was shown that encapsulated *S. aureus* strains have more pathogenic potential than acapsular strains in models of bacteremia (Thakker *et al*., 1998), septic arthritis (Nilsson *et al*., 1997) and abscess formation (Portoles *et al*., 2001). The relevance of this SNP in *cap5I* is unclear since USA300 clones have a CP-negative phenotype due to multiple mutations (in the cap5 promoter, *cap5D* nt 994, and *cap5E* nt 223; Boyle-Vavra *et al*., 2015). The lack of CP expression makes it difficult to predict the significance of the SNP to colonisation.

Other intragenic SNPs worthy of further study include a hypothetical transmembrane protein (SAUSA300_RS09205) with multiple non-synonymous SNPs. This protein has a conserved domain that indicates membrane-located protease activity that is linked to a wide range of protein partner targets. Further work is needed to identify the contribution of the protein to cell function and colonisation of hosts.

Most of the SNPs from the second nasopharyngeal passage were not identified after the third passage. Only 8 loci carried the same SNP in both second and third passages comprising genes with DNA or RNA activity (*recF*, *ftsK* and SAUSA300_RS00265), metabolism (SAUSA300_RS01070, SAUSA300_RS12485, *gutB* and SAUS300_RS01715) and nitrate respiration (SAUSA300_RS09205). There are several possibilities to explain the lower number and altered target of mutations observed from nasopharyngeal passage two to three. First, there could be a refinement in the genome variability repertoire of *S. aureus* during nasopharyngeal colonisation. This would require that some mutations were dominant in their effects or could alternatively overcome the same reproduction limitation. Nasopharyngeal colonisation could require that the bacterial load reaches a threshold and the selection is distinct each time the clones are inoculated into the nasopharynx. This would account for both different mutations emerging, purifying selection and reversion of mutations. Such a scenario could be due to different competitor microbiota, variation in genetics and differences in the pre-existing mutations present in the inoculum on each occasion. There may also be multiple routes through selection of mutations to combinatorially achieve the same broad aims. Such answers

depend not only on mutation detection through sequencing but an assessment of the functional outcome of the mutations.

Regardless, genetic modification of genes involved in certain pathways appears to be relevant for *S. aureus* fitness when colonising the nasopharynx, since each passage revealed SNPs unique to that passage. The final passage revealed a suite of mutations with some overlaps at the pathway level. According to the findings, mutations in genes for responses to diverse types of stress could contribute to survival in the nasopharynx colonisation by *S. aureus*. Although a SNP in *clpL* observed in the second passage was not detected in the third passage, a mutation in *dnaJ* was revealed in the third passage. DnaJ belongs to the heat shock protein (Hsp) family and similarly to Cpl protease contributes to stress tolerance by preventing misfolding and aggregation of proteins under abnormal conditions. *dnaJ* is part of an operon consisting of 5 genes, including *dnaK*. DnaK was shown to be critically relevant for *S. aureus* response to heat, oxidative and antibiotic stress (Singh *et al*., 2007), biofilm formation and adherence to eukaryotic cells (Singh *et al*., 2012). DnaJ presents unfolded proteins to DnaK, and it is tempting to speculate that the SNP found in *dnaJ* might increase performance from the DnaJ/DnaK system and the response to external stress factors.

A second gene harbouring a SNP unique to the third nasopharyngeal passage was present in *bioA*. This locus encodes an aminotransferase in the biotin synthesis pathway. Biotin is a co-factor for carboxylases, which catalyse essential reactions in metabolic pathways (da Costa *et al*., 2012). The

identification of a non-synonymous SNP in SAUSA300_RS11285 (thiaminase; vitamin B_1 metabolism) from the second passage and *bioA* from the third passage supports that these co-factors and the reactions in which they are involved are relevant for *S. aureus* adaptation to the nasopharynx.

In summary, this chapter gives indications of potential components and pathways that might contribute to the success of nasopharyngeal colonisation by *S. aureus*. While this fingerprinting approach identifies potential candidates during growth and survival, it also raises the requirement for subsequent functional genomics approaches to discern individual roles of genes, SNPs, proteins and pathways to this host-pathogen interaction.

Chapter 5 Assessing *in vivo* nasopharyngeal adaptation of *S. aureus*

5.1 Introduction

5.1.1 Understanding *S. aureus* adaptation

S. aureus' considerable ability to sense environmental factors and selection changes to its genome in a colonisation model were extensively discussed in Chapter 4. This chapter focuses on the consequence of mutations due to selective pressures in an evolutionary-time frame, specifically adaptation of *S. aureus* resulting in evolved clones exhibiting enhanced fitness to survive and persist in a host.

Two-component systems (TCS) are the primary apparatus of *S. aureus* sensorial response mechanisms. These conduits perceive environmental cues and signal a response to modulate several cell functions (Villanueva *et al*., 2018). A study that included *S. aureus* early and late isolates from cystic fibrosis patients colonised for at least 5 years described adaptive changes in capsule production, biofilm formation, haemolysis, antibiotic susceptibility and cytotoxicity that were proposed to contribute for *S. aureus* persistence in the patients' airways (Hirschhausen *et al*., 2013). A different study showed that mutations within the *agr* locus, which led to less virulent phenotype, promoted *S. aureus* adaptation during chronic osteomyelitis over a period of 13 months (Suligoy *et al*., 2018). These studies highlight that efficient phenotypic outcomes arise in *S. aureus*

clones adapted to a specific tissue that relieve important environmental limitations, including temperature, oxygen tension, cell population density, pH and nutrient starvation (Dastgheyb & Otto, 2015; Majerczyk *et al*., 2010).

Molecular oxygen (O_2) is a critical component for *S. aureus* growth and its presence in humans ranges from anaerobic conditions (e.g. intestines) to oxygen-enriched organs (e.g. kidneys and liver). The *S. aureus* response to changes in O_2 tension is regulated by the SrrAB (Staphylococcal respiration response) TCS, which is activated during a shift from aerobic to anaerobic conditions and regulates the respiration switch (Yarwood *et al*., 2001; Kinkel *et al*., 2013). Additionally, *S. aureus* harbours other O_2 regulated TCS (NreBC and AirSR/YhcSR) and regulator proteins (Rex and AgrA) that interact to overcome low oxygen stress in the host tissue (Pagels *et al*., 2010; Schlag *et al*., 2008; Sun *et al*., 2012; Yan *et al*., 2011).

Moreover, *S. aureus* is the major pathogen of biofilm-associated infections with low oxygen availability, like osteomyelitis (Pendleton & Kocher, 2015), and it was shown that *S. aureus* induces hypoxia in tissues with high levels of oxygen, such as the kidneys and liver, in the formation of abscesses (Vitko *et al*., 2015). Fuchs and co-workers (2007) identified that under anaerobic conditions *S. aureus* promotes glycolysis, fermentation and represses the Krebs cycle. Therefore, *S. aureus* clones better adapted for promotion of hypoxia and also for an aerobic to anaerobic respiration transition could be more likely to persist in the host tissues.

In a similar manner to oxygen tension changes, shifts in environmental pH also requires appropriate responses from *S. aureus*. Human tissues provide a wide pH range varying from mildly acidic conditions (pH 5.5) on skin to near neutral values (pH 7.35-7.45) in the blood. Recently, Flannagan *et al*. (2018) described GraXRS as the major regulatory system associated with *S. aureus* survival within highly acidified macrophage phagolysosomes and that optimal growth in these microbicidal organelles is reached after acidic pH exposure. In addition, low pH values, as found in the urogenital tract, lung, mouth and abscesses, were associated with up-regulation of *S. aureus* virulence genes by Weinrick *et al*. (2004) and their microarray-based study observed that the main *S. aureus* acid defences are increased urease activity, proton excretion and macromolecule-repair mechanism after the bacteria were exposed for 10 min to acid conditions (Bore *et al*., 2007). The ability of *S aureus* to sense, adapt and negate acid stress by employing diverse responses was demonstrated in a transcriptomic study in which the bacteria were able to increase the medium pH mainly through ammonium accumulation and acid groups removal (Rode *et al*., 2010).

Diverse conditions also induce *S. aureus* responses to grow according to nutritient availability. Carbon sources, including glucose, are the major energy source for the majority of organisms and glucose is the most abundant free carbohydrate in human serum (Balasubramanian *et al*., 2017). Studies implicated that *S. aureus* is metabolically adapted to increase its carbohydrate uptake capacity and promote glucose fermentation for growth under anaerobic conditions (Vitko *et al*., 2016). When glucose and other primary carbon sources are abundant, a signal cascade through the conserved transcription factor CcpA

is activated resulting in up-regulation of RNAIII expression which leads to virulence gene expression modulation (Seidl *et al*., 2006). In addition to carbohydrate availability, *S. aureus* responds to scarcity of amino acids by increasing the activity of metabolic biosynthesis pathways through derepression of target genes regulated by CodY metabolic regulator (Majerczyk *et al*., 2010).

Iron is another vital nutrient across all organisms and it requires adequate responses from *S. aureus* when the bacteria finds itself immersed in environments with low concentrations of iron. Host extracellular iron concentration is held extremely low and *S. aureus* is adapted mainly to acquire iron via scavenger siderophores called staphyloferrin A and B (Hammer & Skaar, 2011). *S. aureus* possesses multiple extracellular iron acquisition mechanisms, which involve haemolysins, cytotoxins and the iron-regulated surface determinant (Isd) system (Haley & Skaar, 2012; Torres *et al*., 2006). Both staphyloferrin operons (*sfa* and *sfb*) and *isd* locus are under Fur control (Ferric Uptake Regulator) (Dale *et al*., 2004; Cheung *et al*., 2009; Torres *et al*., 2010). Adaptive responses from *S. aureus* were described for other environmental stresses, like temperature (Cebrian *et al*., 2009) and cell density (Geisinger *et al*., 2012).

5.1.2 *S. aureus* nasal adaptation mechanisms

The human nose is the main ecological niche for *S. aureus* in humans and it was shown that nasal secretions contain limited nutrients. When grown in a synthetic nasal medium (SNM) designed to resemble similar conditions to human nasal secretions, global gene analysis revealed high dependence of *S. aureus* on methionine biosynthesis for bacterial growth reinforcing the ability of *S. aureus* to overcome nutrient limitation and adapt to human nasal passages (Krismer *et al*., 2014). In terms of regulatory networks, the WalKR TCS has an essential role in maintenance of *S. aureus* colonisation (Burian *et al*., 2010b). The WalKR regulator, also known as YycGF, is an essential signal transduction pathway for cell wall metabolism. Certain SNPs in WalKR are linked with increased vancomicin resistance due to wall thickness changes among other alterations revealing that variation in this locus can contribute to *S. aureus* adaptation (Howden *et al*., 2011). With respect to WalKR relevance to *S. aureus* nasal colonisation, an *in vivo* study involving swabs from asymptomatic *S. aureus* carriers showed by qRT-PCR no activation of prominent regulators such as *agr*, SaeRS, SigB, and GraRS; WalKR being the only exception (Burian *et al*., 2010b). A second *in vitro* study using qRT-PCR showed no increase in transcript levels of RNAIII during *S. aureus* growth in SMN synthetic nasal medium (Krismer *et al*., 2014).

The absence of *S. aureus agr* system activity during nasal colonisation supports the notion of repressed biofilm formation that favours a dispersed mode of growth, since the referred mechanism is usually activated by dense biofilm

populations (Krismer & Peschel, 2011). Low numbers of bacteria isolated from nasal swabs (Nouwen *et al*., 2004), histological studies (Burian *et al*., 2010a; ten Broeke-Smits *et al*., 2010) and routine eradication of *S. aureus* by community antibiotics in the nasal niche (Ammerlaan *et al*., 2009; Van Rijen *et al*., 2008) reinforce *S. aureus'* preference for a dispersed mode of growth phenotype in the nose. Although *S. aureus* has been extensively studied, many host-microbe determinants of *S. aureus* carriage are yet to be characterised and their understanding will elucidate *S. aureus'* adaption ability to colonise the human nasopharynx (Sollid *et al*., 2014).

5.2 Aims

A key factor that determines versatility of *S. aureus* to colonise and invade diverse host tissues is its ability to sense growth-limiting environmental conditions followed by the employment of modulated responses accordingly. The extensive repertoire of metabolic pathways tightly controlled by regulator systems ensures *S. aureus* adapts expression after exposure to environmental cues thereby improving its fitness.

The current chapter focuses on the contribution of genetic changes selected in an experimental nasopharyngeal model for *S. aureus* nasopharyngeal colonisation. The hypothesis is that *S. aureus* genetic variants selected after serial passages in the nasopharynx point a finger at adaptation in this niche, leading to improved phenotypic responses against environmental conditions.

5.3 Results

5.3.1 Single *S. aureus* nasopharyngeal isolates posses niche adaptation

In the previous chapter, an experimental evolution model comprising three serial passages of *S. aureus* pools of isolates in the nasopharynx of mice was performed and SNPs were curated. To assess whether these genetic changes captured in the sequence pools of *S. aureus* after repeated nasopharyngeal passages account for bacterial adaptation in this niche, a single clone was randomly selected from each pool of isolates from both second (C2) and third passages (C3) (see section 3.3.2), named C12_7 and C14_7 respectively. Since few sequence variants were detected in the genomic pools from the first nasopharyngeal passage, no clone from this evolutional stage was included in subsequent experiments.

5.3.1.1 Variation in the colonisation of single *S. aureus* isolates from distinct nasopharyngeal evolutionary periods

Colonisation ability from nasopharyngeal clones was compared with the control isolate (*S. aureus* USA300 LAC JE2) using the murine model established in Chapter 3. Due to technical problems described for the conclusion of the serial nasopharyngeal passages (section 3.3.2), in this experiment differences in the colonisation level was assessed successfully only between the control isolate and the C12_7 clone selected from the second nasopharyngeal passage. Inocula of each strain were identically prepared (~ 5 x 10^4 cfu) and then groups of ten mice were inoculated in their nose (one group per bacterial isolate). The

success of colonisation was analysed over a period of 14 d where two mice for each strain were sacrificed at successive time points (1, 2, 3, 7 and 14 d). The nasopharynx was aseptically removed and homogenised followed by colony counting on mannitol salt agar (incubated for 24-48 h at 37°C).

As shown in Figure 5.1, the single isolate collected after two serial passages in the nasopharynx of mouse showed better colonisation status when compared with the control isolate. Whilst after one day of inoculation both isolates were present at similar colonisation levels, by day 2 the nasopharyngeal passaged clone showed increased colonisation of the nasopharynx compared with the control strain that decreased in frequency within this niche. From day 3 onwards a slight variation in the colonisation patterns is observed, but overall both strains reach a steady colonising status with the C12_7 clone showing a trend of greater frequency that is not statistically significant.

Similar to the colonisation levels of *S. aureus* observed during the long-term murine model development, cell numbers of both isolates drop markedly in the first 24 h. After the next 24 h, C12_7 demonstrates increased survival levels as per greater counts in opposition to the control isolate that experienced a progressive reduction of its CFU values. Although after 14 d both clones are not significantly distinct regarding their colonisation capability, these data indicate a trend for *S. aureus* nasopharyngeal adaptation after two serial passages in this niche.

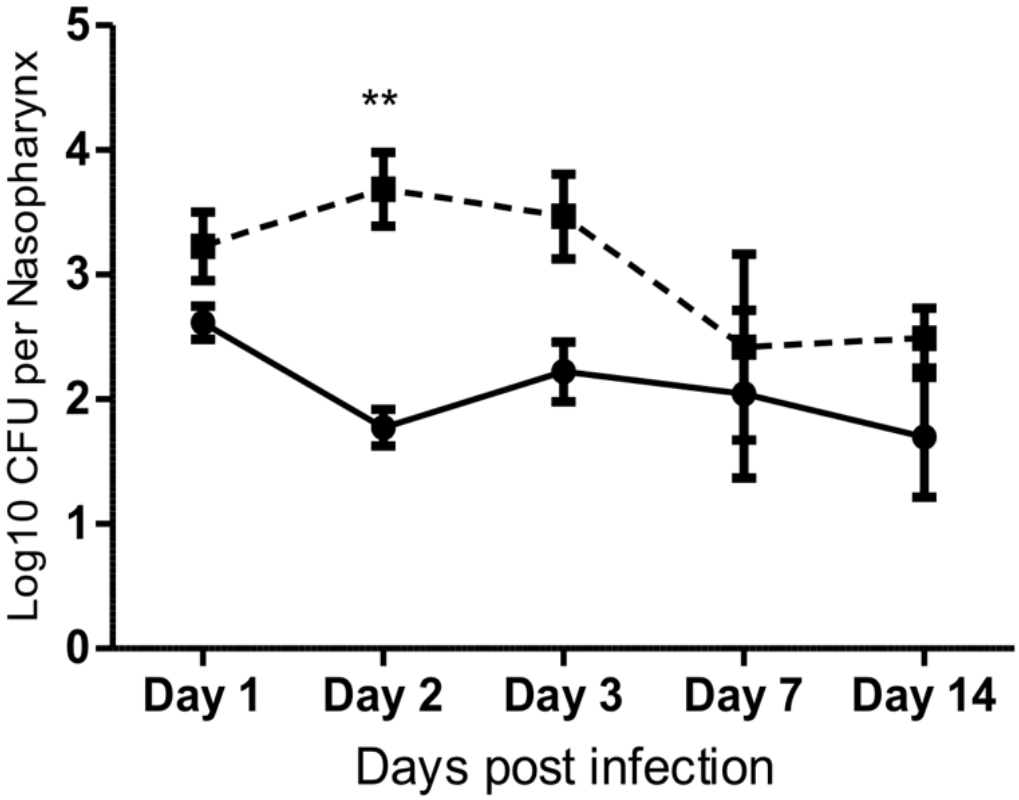

Figure 5.1 Nasopharyngeal colonisation of mice by *S. aureus* USA300 LAC JE2 and *S. aureus* C12_7.

Inoculation was initiated with a 5 x 10^4 CFU dose of both *S. aureus* USA300 LAC JE2 (control; circle) and C12_7 (clone collected after two serial passages; square). At each time point, the nasopharynxes of 2 mice were recovered and cultured for *S. aureus*.

To gain further insight into fitness of the clones selected from the serial passages the experiment was repeated with both clones C12_7 and C14_7. The murine model was used once again to detect variation in *S. aureus'* ability to colonise the mouse nasopharynx by comparing the colonisation success of the three isolates: WT control, C12_7 and C14_7, over a period of 14 d. On this occasion, after day one the mean bacterial load in the nasopharynx was decreased substantially (Fig 5.2) compared with the previous experiments (c.f. with Fig 5.1) considering that the inoculum was ~ 5 x 10^4 CFU, an outcome that was not previously observed when only the control and C12_7 were analysed regarding their colonisation status. Amongst the three clones analysed, C14_7 showed the greatest overall colonisation numbers. Although C12_7 exhibited higher population numbers at 24 h, C14_7 showed a trend for increased nasopharyngeal colonisation fitness, as judged by numbers, from day 2 onwards. The control isolate mostly displayed a pattern the lowest numbers of bacteria recovered from the nasopharynx over the course of this experiment.

Unexpected outcomes like the apparent clearance of the control isolate from day 3 onwards and of C12_7 after 7 d with reappearance after 14 d were determined in this experiment. These observations coupled with pronounced CFU values reduction after 24 h might suggest various scenarios: that the mice were under inoculated, which ultimately led to clearance of some isolates or presence of them in the nasopharynx at below detectable levels; there is greater batch variation in the mice for these experiments. However, even considering such conditions, the clone from the third nasopharyngeal serial passage showed a trend for being better adapted to this anatomic site compared to other isolates

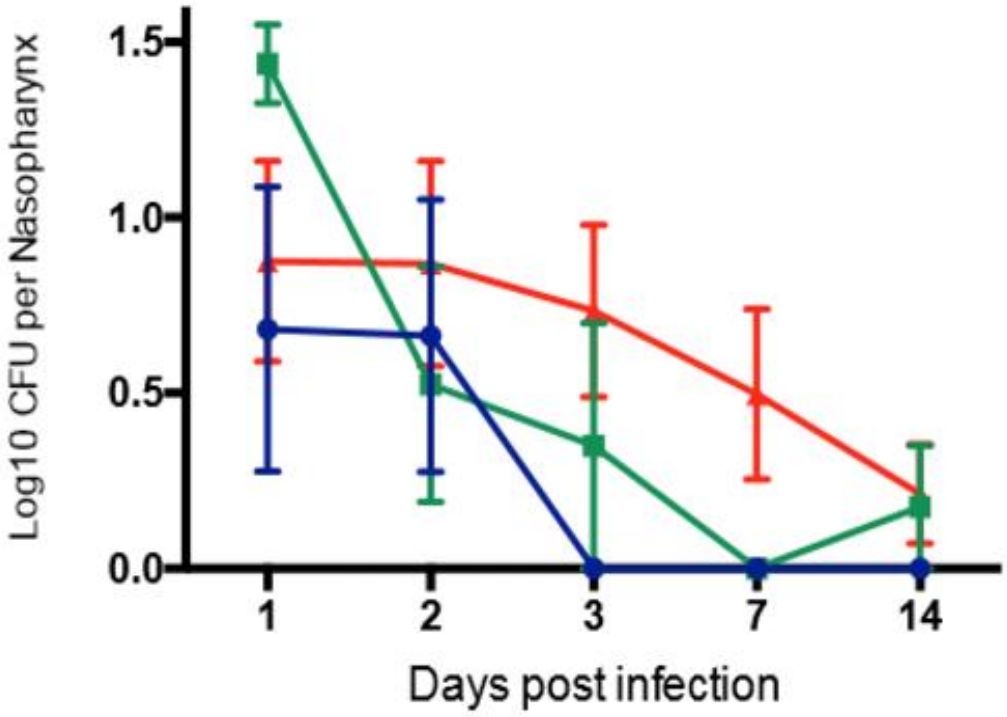

Figure 5.2 Nasopharyngeal colonisation of mice by *S. aureus* USA300 LAC JE2 and clones C12_7 and *S. aureus* C14_7.

Inoculation was initiated with a 5 x 10^4 CFU dose for both *S. aureus* USA300 LAC JE2 (control; blue circle), C12_7 (clone collected after two serial passages; green square) and C14_7 (clone collected after three serial passages; red triangle). At each time point the nasopharynxes of 2 mice were recovered and cultured for *S. aureus*.

and with use of greater numbers of mice, which were not originally deemed justifiable, could have provided better statistical support for any actual differences in colonisation ability.

5.3.1.2 Sequence variation of single *S. aureus* nasopharyngeal colonisation isolates

Taking into account the variation in colonisation levels from the three isolates (control, C12_7 and C14_7), albeit largely not significant differences, genome sequencing was performed followed by SNPs analysis to determine which sequence variants from the genomic pools were present in the passaged single clones. DNA extraction, quality check and sequencing for both C12_7 and C14_7 was performed as described in section 4.3.1. Genome sequencing analysis of the control (USA300 JE2 LAC) was previously performed (see Chapter 4).

Output from the SNPeff algorithm revealed a strong overlap between the SNPs dataset of both clones and their corresponding genomic pools, supporting the effectiveness and reproducibility of the methodology used in Chapter 4 to capture sequence variation over the nasopharyngeal experimental evolution experiment. The isolate from the second nasopharyngeal passage (C12_7) showed 92 SNPs overall of which 46 were intragenic non-synonymous sequence variants (Table 5.1) and 15 were variants located in intergenic regions (Table 5.2).

Considering firstly the intragenic non-synonymous SNPs, 97.8% (45/46) of the variants detected in the second passage sequence pools were present in clone C12_7 (Table 5.1). The single intragenic non-synonymous SNP absent in C12_7 was V179I located in the hypothetical transmembrane protein SAUSA300_RS09205. In the sequence pool, this locus had six further intragenic non-synonymous SNPs and all these variants were detected in C12_7. Given that SAUSA300_RS09205 (V179I) had the lowest percentage frequency in C2 pool (20.28%) makes the absence of this SNP unsurprising.

Regarding intergenic SNPs, the overlap of sequence variants between the pool of isolates and clone C12_7 was high with 11 out of 13 intergenic SNPs confirmed (Table 5.2). Two absent from C12_7 were located in a 151 bp region and potentially affected either or both SprA1 (regulatory sRNA) and SAUSA300_RS09560 (transposase from the pathogenic island SaPIn3).

Table 5.1 *S. aureus* nasopharyngeal clone clone C12_7 non-synonymous intragenic SNPs.
S. aureus SNPs observed in genomic DNA of a single clone (C12_7) isolated after the second passage of murine nasopharyngeal colonisation.

Gene symbol	Function	Genome change	Codon change	Position in protein
recF	DNA recombination and repair	5010 C/G	P/A	358 aa
gyrB	DNA Topoisomerase II subunit B	5608 T/C	I/T	183 aa
gyrA	DNA Topoisomerase II subunit A	7282 T/C	L/S	84 aa
SAUSA300_ RS00145[1]	Putative transposase in SCC*mec* element	36124 G/A	A/V	143 aa
SAUSA300_ RS00265	Hypothetical protein	61025 G/A	L/F	55 aa
cap5I	Capsular polysaccharide CP5 synthesis	182746 T/G	L/V	253 aa
SAUSA300_ RS01070	RGD-containing lipoprotein	240358 T/A	N/K	358 aa
SAUSA300_ RS01200	Hydroxyacyl Coenzyme A dehydrogenase	270510 T/C	T/A	601 aa
gutB	Sorbitol dehydrogenase	292738 A/C	T/P	341 aa
SAUSA300_ RS01310	Cell wall and capsule metabolism	294913 G/A	G/D	119 aa
SAUSA300_ RS01715	Oye family flavin oxidoreductase	376057 C/T	D/N	258 aa
SAUSA300_ RS01715	Oye family flavin oxidoreductase	376231 T/G	S/R	200 aa
ahpF	Alkyl hydroperoxide reductase	429073 C/T	G/D	108 aa
SAUSA300_ RS02220	Tandem-type lipoprotein Lpl4	467549 A/T	E/D	209 aa

SAUSA300_ RS03590	Undecaprenyl diphosphatase	742477 C/T	R/H	69 aa
SAUSA300_ RS04050	Sporulation Regulator WhiA	837108 G/T	E/D	17 aa
ampA	Cytosol aminopeptidase	923896 G/T	K/A	207 aa
SAUSA300_ RS04890	Rlu family pseudouridine synthase	996224 C/T	S/F	150 aa
SAUSA300_ RS05275	Hypothetical protein	1074815 G/A	D/N	71 aa
sun	16S rRNA methyltransferase	1215913 T/C	Y/H	306 aa
ftsK	DNA translocase	1286986 A/G	K/R	180 aa
SAUSA300_ RS06375	RNase Y	1298198 T/G	D/E	203 aa
sbcC	DNA repair: exonuclease	1363207 A/T	I/F	761 aa
parC	DNA replication: Topoisomerase IV subunit A	1374405 A/C	T/S	80 aa
SAUSA300_ RS07265	Hypothetical protein	1496455 T/A	I/L	663 aa
SAUSA300_ RS07925	SDR family Oxidoreductase	1604107 T/C	E/G	196 aa
SAUSA300_ RS08410	Copro-porphyrinogen III oxidase	1693739 T/C	Y/A	145 aa
SAUSA300_ RS09205	Hypothetical transmembrane protein	1857109 A/G	I/T	172 aa
SAUSA300_ RS09205	Hypothetical transmembrane protein	1857182 G/A	P/S	148 aa
SAUSA300_ RS09205	Hypothetical transmembrane protein	1857188 C/T	E/K	146 aa
SAUSA300_ RS09205	Hypothetical transmembrane protein	1857202 G/A	A/V	141 aa
SAUSA300_	Hypothetical	1857215		

RS09205	transmembrane protein	T/C	T/A	137 aa
SAUSA300_ RS09205	Hypothetical transmembrane protein	1857232 T/C	K/R	131 aa
SAUSA300_ RS09220	FtsK/SpoIIIE Family protein	1859999 C/T	G/D	1142 aa
SAUSA300_ RS09290	Hypothetical flavoprotein	1876812 A/T	D/V	97 aa
SAUSA300_ RS09425	Transaldolase	1908169 T/C	I/V	6 aa
SAUSA300_ RS09885	Epoxyqueuosine reductase	1988983 C/T	V/I	174 aa
SAUSA300_ RS10590	Phage tail tape measure protein	2100157 A/C	I/S	384 aa
SAUSA300_ RS10650	Phage terminase protein	2109096 G/T	A/D	84 aa
SAUSA300_ RS11285	Thiaminase II	2214645 C/T	G/R	224 aa
mtlA	PTS system Mannitol transporter	2276564 A/G	E/G	56 aa
narK	Nitrite extrusion protein	2508817 G/A	A/V	241 aa
SAUSA300_ RS13805	Clp protease ATP-binding subunit	2685939 T/G	S/A	249 aa
SAUSA300_ RS13825	Antibiotic transport-like protein	2690087 T/G	K/G	795 aa

Table 5.2 *S. aureus* nasopharyngeal single isolate after two repeated passages have intergenic SNPs.

S. aureus genome sequence variants (SNPs) in non-coding regions observed for C12_7 isolate after two serial passages performed in the experimental nasopharyngeal colonisation model.

Genome change	Distance from coding region	Gene function
35817 A/T	sRNA sequence (Teg5as)	-
74496 T/G	-1069 bp / SAUSA300_RS00340 (+) -148 bp (arcA) (-)	Universal stress protein Arginine deiminase
260357 C/T	-222 bp / *pflB* (+) -366 bp / *hptA* (-)	Formate acetyltransferase Iron-binding protein
349622 T/C	-517 bp / SAUSA300_RS01590 (+)	Conserved Hypothetical Protein
585363 G/A	-191 bp / *rpoB* (+)	DNA-directed RNA polymerase
940710 A/G	-9 bp / SAUSA300_RS15240 (+) -18 bp / SAUSA300_RS15245 (-)	Pseudogene Hypothetical protein
940718 A/G	-17 bp / SAUSA300_RS15240 (+) -10 bp / SAUSA300_RS15245 (-)	Pseudogene Hypothetical protein
1161534 C/T	-44 bp / *argF* (+) -394 bp / SAUSA300_RS05750 (-)	Amino acid metabolism Superantigen-like protein
1533312 A/T	-237 / SAUSA300_RS07455 (+) -165 bp / *rpsA* (-)	Hypothetical protein 30S ribosomal protein S1
1929054 A/G	-280 bp / SAUSA300_RS09565 (+) -141 bp / SAUSA300_RS09560 (-)	Hypothetical protein Transposase
1929062 G/A	-272 bp / SAUSA300_RS09565 (+) -149 bp / SAUSA300_RS09560 (-)	Hypothetical protein Transposase
1957938 C/T	-100 bp / SAUSA300_RS09690 (+) -699 bp / *lukE* (-)	Hypothetical protein Leukotoxin

1959504 T/A	-313 bp / SAUSA300_RS15420 (+)	Pseudogene
2322700 T/C	-290 bp / *opuD2* (-)	ABC transporter permease

The sequenced C14_7 clone from the third nasopharyngeal passage showed even overall higher concordance with its genomic pool counterpart. A total of 19 non-synonymous intragenic SNPs were detected in clone C14_7 matching with the same 19 variants in coding regions observed in the C3 sequence pool (Table 5.3). Considering intergenic SNPs, clone C14_7 revealed five out of six variants observed in the C3 genomic pool (Table 5.4). The sequence variant SAUSA300_RS00765 (G164912A) was not detected in C14_7 and it might be involved in the regulation of *pnhD* (encoding a phosphate/phosphite/ phosphonate transporter). This SNP was the only intergenic variant detected in less than 90% of C3 sequence reads suggesting that other isolates from the C3 pool, excluding C14_7 possess this particular SNP.

The high level of sequence agreement between single clones and isolate pools reinforces the decision to group evolved nasopharynx isolates to maximise the SNPs detection. It also validates the use of single clones randomly chosen for adaptation studies since most of the C2 and C3 sequence pool variants were also present in selected clones C12_7 and C14_7, respectively.

Table 5.3 *S. aureus* nasopharyngeal clone C14_7 non-synonymous intragenic SNPs.
S. aureus SNPs observed in genomic DNA of the clone C14_7 after three serial passages of murine nasopharyngeal colonisation and sampling.

Gene symbol	Gene function	Genome change	Codon change	Position in protein
recF	DNA replication and repair	5010 C/G	P/A	358 aa
SAUSA300_RS00265	Hypothetical protein	61025 G/A	L/F	55 aa
SAUSA300_RS01070	RGD-containing lipoprotein	240358 T/A	N/K	358 aa
SAUSA300_RS01200	Hydroxyacyl Coenzyme A dehydrogenase	270510 T/C	T/A	601 aa
gutB	Sorbitol dehydrogenase	292738 A/C	T/P	341 aa
SAUSA300_RS01715	Oye family flavin oxireductase	376057 C/T	D/N	258 aa
SAUSA300_RS01715	Oye family flavin oxireductase	376231 T/G	S/R	200 aa
SAUSA300_RS02185	Staphylococcal superantigen-like 11 (SSL11)	460966 G/A	A/T	110aa
ftsK	DNA translocase	1286986 A/G	K/R	180 aa
dnaJ	Chaperone protein	1688528 C/T	A/T	250aa
SAUSA300_RS09205	Hypothetical transmembrane protein	1857109 A/G	I/T	172 aa
SAUSA300_RS09205	Hypothetical transmembrane protein	1857182 G/A	P/S	148 aa

SAUSA300_ RS09205	Hypothetical transmembrane protein	1857188 C/T	E/K	146 aa
SAUSA300_ RS09205	Hypothetical transmembrane protein	1857202 G/A	A/V	141 aa
SAUSA300_ RS09205	Hypothetical transmembrane protein	1857215 T/C	T/A	137 aa
SAUSA300_ RS09205	Hypothetical transmembrane protein	1857232 T/C	K/R	131 aa
SAUSA300_ RS15385	Hypothetical protein	1872844 T/C	Y/C	28 aa
SAUSA300_ RS10080	Hypothetical lipoprotein	2007223 G/T	R/L	48 aa
bioA	Amino-Transferase	2549677 C/T	R/H	189 aa

Table 5.4 *S. aureus* nasopharyngeal clone C14_7 intergenic SNPs.

S. aureus genome sequence variants (SNPs) in non-coding regions observed for C14_7 isolate after three passages in the experimental nasopharyngeal colonisation model.

Genome change	Distance from coding region	Gene function
1533312[1] A/T	-237 / SAUSA300_RS07455 (+) -165 bp / *rpsA* (-)	Hypothetical protein 30S ribosomal protein S1
1957938[1] C/T	-100 bp / SAUSA300_RS09690 (+) -699 bp / *lukE* (-)	Hypothetical protein Leukotoxin
2164596 A/G	sRNA sequence (Teg16)	-
2322700[1] A/G	-290 bp / *opuD2* (-)	ABC transporter permease
2487639 A/G	-189 bp / SAUSA300_RS12785 (+) -128 bp / *lctP2* (-)	Glycerol transferase Lactate permease

5.3.2 *S. aureus* intra-species competition in the nasopharynx niche

Although it was previously suggested that *S. aureus* are not natural colonisers of mice (McCarthy *et al*., 2012; Mulcahy *et al*., 2012), recent findings demonstrate there is frequent colonisation of laboratory mice by *S. aureus* (Schulz *et al*., 2017). Chapter 3 includes description of recurrent failures in performing the third nasopharyngeal passage due to prior colonisation of mice by *S. aureus*. Since it was described that colonisation by *S. aureus* prevents the invasion of *S. aureus* strains (Margolis *et al*., 2010), a hypothesis that nasopharynx-adapted *S. aureus* strains obstruct the experimental colonisation was proposed. Therewith, genome sequencing was performed followed by variant analysis with the aim of characterising the presumed mouse-adapted strain (named SA_MOU) to provide insights of possible genome sequence variation (alleles) that might match with the experimental evolution changes from repeated colonisation of the nasopharynx.

Ten *S. aureus* isolates were randomly picked from mannitol salt agar after growth from washed nasopharynx of mice. DNA extraction was performed for all isolates and the quality, yield and concentration were checked prior to sequencing (Table 5.5). DNA from the isolates was pooled in equivalent amounts and both DNA library preparation and sequencing were performed as described in section 4.3.1. The resulting raw sequencing reads were quality filtered using Cutadapt and Sickle for removal of adapter sequences and reads shorter than 10 bp, resulting in four files (two forward and two reverse reads) (Table 5.6).

Table 5.5: Quality control analysis of DNA samples pooled for sequencing from nasopharynx *S. aureus* SA_MOU isolates.

DNA quality data of 10 SA_MOU isolates prior to sequencing. DNA concentration measurements were determined using Qubit and absorbance ratios at 260/280 and 260/230 using Nanodrop.

Isolate	DNA concentration (ng/μL)	Absorbance 260/280	Absorbance 260/230
SA_MOU_1	95.4	1.9	1.9
SA_MOU_2	90.9	1.8	2.1
SA_MOU_3	104.8	1.9	2.2
SA_MOU_4	151.2	1.8	2.2
SA_MOU_5	72.4	1.8	2.2
SA_MOU_6	76.6	2.0	2.1
SA_MOU_7	92.6	1.9	1.9
SA_MOU_8	97.5	2.0	2.3
SA_MOU_9	98.2	1.8	2.3
SA_MOU_10	120.1	1.8	2.1

Table 5.6: Sequencing statistics of Illumina libraries after sequence adapter removal and quality filtering.

Raw data from SA_MOU sequencing reads generated by Illumina TruSeq PCR-free kits and sequenced on MiSeq platform.

	SA_MOU_F1	**SA_MOU_F2**	**SA_MOU_R1**	**SA_MOU_R2**
Number of reads	2.495.866	2.470.449	2.495.866	2.470.449
Mean read length (bp)	246.5	242.3	246.6	242.4
Mean Base Quality Score	39.5	39.4	38.7	38.7
A content (%)	33.8	33.8	34.3	34.3
C content (%)	16.2	16.2	15.8	15.8
G content (%)	15.9	15.9	16.1	16.2
T content (%)	34.1	34.1	33.8	33.8
N content (%)	0.0	0.0	0.0	0.0

Briefly, the four data files showed Base Quality Score (error rate) above 38, GC% content ranging from 31.9% to 32.1% and absence of ambiguous bases (N content). At least 2,470,449 reads of 242.3-246.6 bp in size were generated per file. These quality filtered paired-end reads were used for the assembly stage.

Due the lack of information about the adapted pre-colonising *S. aureus* strain, a pipeline was established for genome assembly without a reference genome (Fig 5.3). Therefore, the trimmed data generated by Illumina sequencing was constructed with the SPAdes assembler (Bankevich *et al*., 2012) for *de novo* assembly using K-mer sizes of 21, 33, 55, 77, 99 and 127. Scaffolding and alignment of contigs was performed using SSPACE-BASIC (Baseclear). Subsequently, Prokka (Seemann, 2014) was used for genome annotation. Genome visualisation of the resulting annotated assembly was performed by both Geneious (Kearse *et al*., 2012) and Blast Ring Image Generator (BRIG) (Alikhan *et al*., 2011).

SPAdes was run according to the manufacturer's instructions for Illumina paired-end reads (2x250 bp) including the use of options aimed at minimising the number of mismatches in the final contigs. Scaffolding of pre-assembled contigs was then performed using SSPACE script.

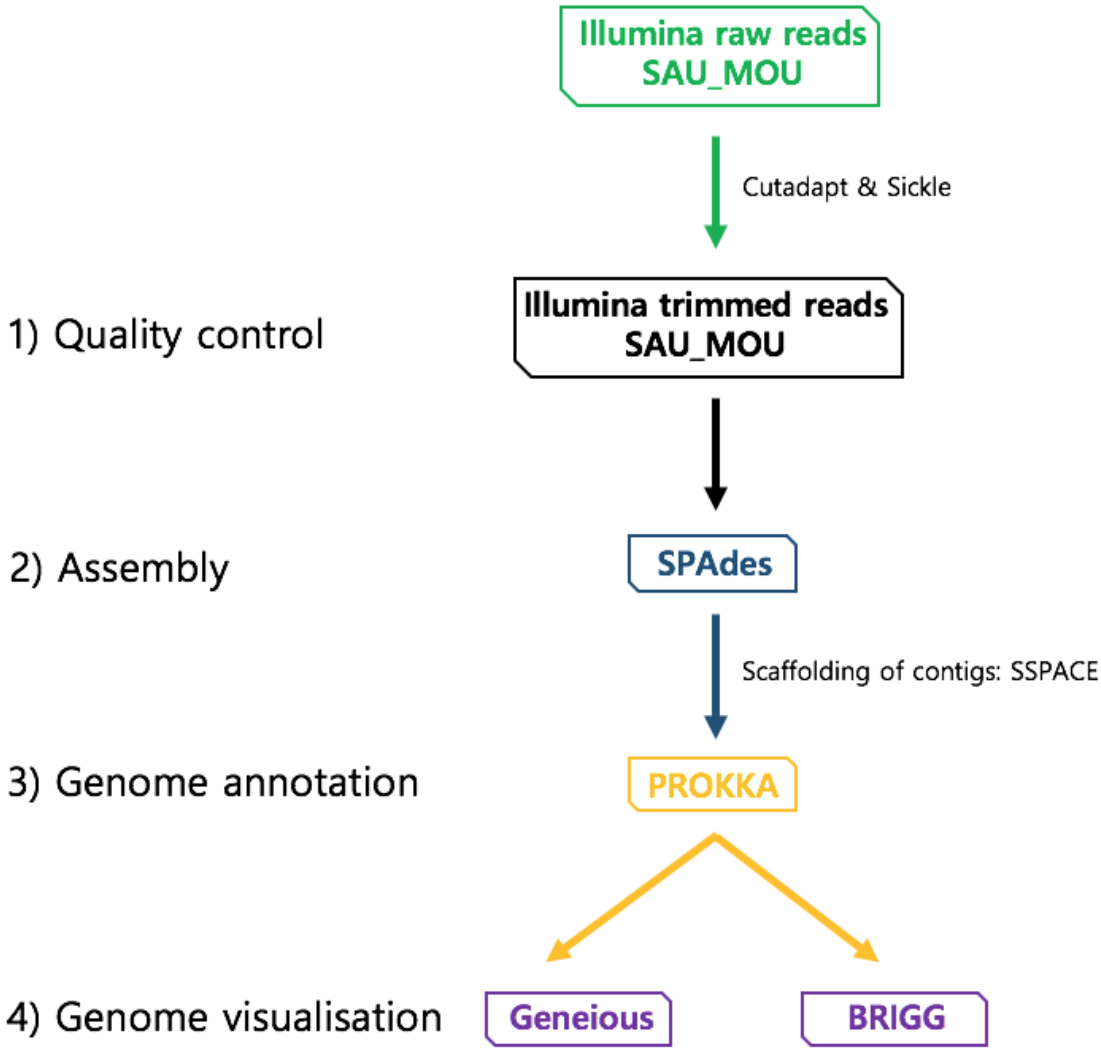

Figure 5.3 Schematic representation of the pipeline used for *de novo* genome assembly of *S. aureus* SA_MOU nasopharynx strain.

Raw reads of SA_MOU genome sequencing generated by Illumina sequencer were quality filtered using Cutadapt and Sickle. Trimmed reads were assembled into contigs by SPAdes. Scaffolding of pre-assembled contigs was performed SSPACE. PROKKA software was used to annotate the resulting assembly which was then visualised by Geneious and BRIGG.

The resulting scaffolds were annotated by Prokka, an annotation software tool specialised in bacterial, archeal and viral annotation. Prokka was used so open reading frames (ORFs) could be detected in the assembled file for the ultimate purpose of strain characterisation based on house-keeping genes analysis. Prokka output revealed 2869 CDS, 57 tRNA, 15 rRNA and one tmRNA.

Having the ORFs identified, a multilocus sequence typing technique (MLST) was used to gain insights of the *S. aureus* strain colonising the mouse nasopharynx. As required by the MLST server (www.mlst.net), fragments of approximately 400-520 bp from multiple loci (Table 5.7) were extracted from Prokka annotated files prior to allelic profile matching against a sequence type (ST) database. The *S. aureus* MLST database is constituted of 3302 STs including the major MRSA clonal lineages circulating in the UK. The search showed that the *S. aureus* strain pre-colonising the mice belongs to ST8, a highly relevant ST since it includes CA-MRSA USA300. A recent study demonstrated that the genetic variation within ST8 clones is rather substantial considering space and time (Strauß *et al*., 2017). Molecular evolution observations showed that a less virulent ST8 ancestor from Central Europe gradually acquired USA300 virulent factors after expansion to North America followed by a broad geographic spread (Strauß *et al*., 2017). ST8 possesses clonal heterogeneity in Europe, where it co-exists with the dominant ST80 and other clones like ST22 (Otter & French, 2010). Natural colonisation and infection of animals by ST8 is rather unusual, however it has been demonstrated in horses (Cuny *et al*., 2008) and dogs (Haenni *et al*., 2012).

Table 5.7: MLST profile of *S. aureus* SA_MOU.

List of house-keeping genes and their functions used for MLST typing. Internal fragments of seven loci were matched against a database constituted by hundreds of unique alleles for each gene. Combination of the allelic assignments produces a sequence type profile (ST8 for *S. aureus* SA_MOU).

Gene symbol	Gene function	Fragment Size (bp)	Number of unique alleles in MLST database	SA_MOU allelic profile
arc	Carbamate Kinase	456	357	3
aroE	Shikimate Dehydrogenase	456	476	3
glpF	Glycerol Uptake Facilitator Protein	465	435	1
*gmk_*	Guanylate Kinase	417	248	1
*pta_*	Phosphate Acetyltransferase	474	380	4
*tpi_*	Triosephosphate Isomerase	402	360	4
yqil	Acetyl-CoA Acetyltransferase	516	415	3

The only mouse *S. aureus* strain characterised to date belongs to ST88 (Holtfreter *et al*., 2013). However, the wide genetic variability between ST8 clones and its vast global presence within microbiology research facilities due to its clinical relevance make it tempting to indicate that nasopharyngeal colonisation by ST8 occurred in the originating animal handling facility.

Within the described research programme in this thesis, there were multiple occasions where *S. aureus* strains colonising the nasopharynx prevented the experimental introduction of the experimental strain, most problematically the conclusion of the third serial passage with *S. aureus* USA300 LAC JE2 (section 3.2.2). Since isolates from this experimental evolution stage showed a trend for better colonisation (Fig 5.2) and since it also belongs to ST8, subsequent investigations were performed based on whole genome comparison and non-synonymous intragenic SNPs captured in the passaged *S. aureus* isolates to determine if there were allelic overlaps between the species.

Prior to SNP analysis, investigations on the genome of the SA_MOU nasopharyngeal coloniser strain were made. Firstly, the assembled genome from SA_MOU was visualised using Geneious where the resulting assembly was aligned by LASTZ to the USA300 reference genome (USA300_FPR3757; NCBI Ref Seq: NC_007793.1) and two additional reference genomes representing ST22 (HO 5096 0412; NCBI Ref Seq: NC_017763.1) and ST80 (NCTC 8325; NCBI Ref Seq: NC_007795.1), dominant STs in Europe. LASTZ is a program designed for pairwise alignment and it is a useful tool for finding similar regions between genomic sequences millions of nucleotides in length. Alignment showed fewer

gaps with the SA_MOU assembled contig matched against the USA300 reference genome, supported by a pairwise identify of 99.5% across 94.5% coverage as the highest values among the three genome alignments (Fig 5.4). Alignment of SA_MOU against ST22 and ST80 genomes revealed high pairwise identify (97.9% and 99.4%, respectively) but with considerably lower coverage (88.0% and 90.9%, respectively).

Secondly, whole genome comparison from SA_MOU sequenced in this study and USA300, ST22 and ST80 publicly available reference sequences was performed using Blast Ring Image Generator (BRIG) software. The comparison was done with SA_MOU serving as the reference sequence and it clearly shows higher similarity with USA_300 (Fig 5.5). Sequence comparison from both ST22 and ST80 against SA_MOU identified far more apparent gaps, suggesting sequence variation. Of note, SA_MOU also presented gaps in its genome, reflecting that scaffolding and alignment of contigs could not be performed in certain regions.

These observations reflect the fact that both SA_MOU and USA300 LAC JE2 are closely related strains which belong to the same ST but also present a degree of dissimilarity. Slight differences in the genome composition may include presence/absence of sequence variants responsible for the differential nasopharyngeal adaptation status observed for SA_MOU and USA300 LAC JE2.

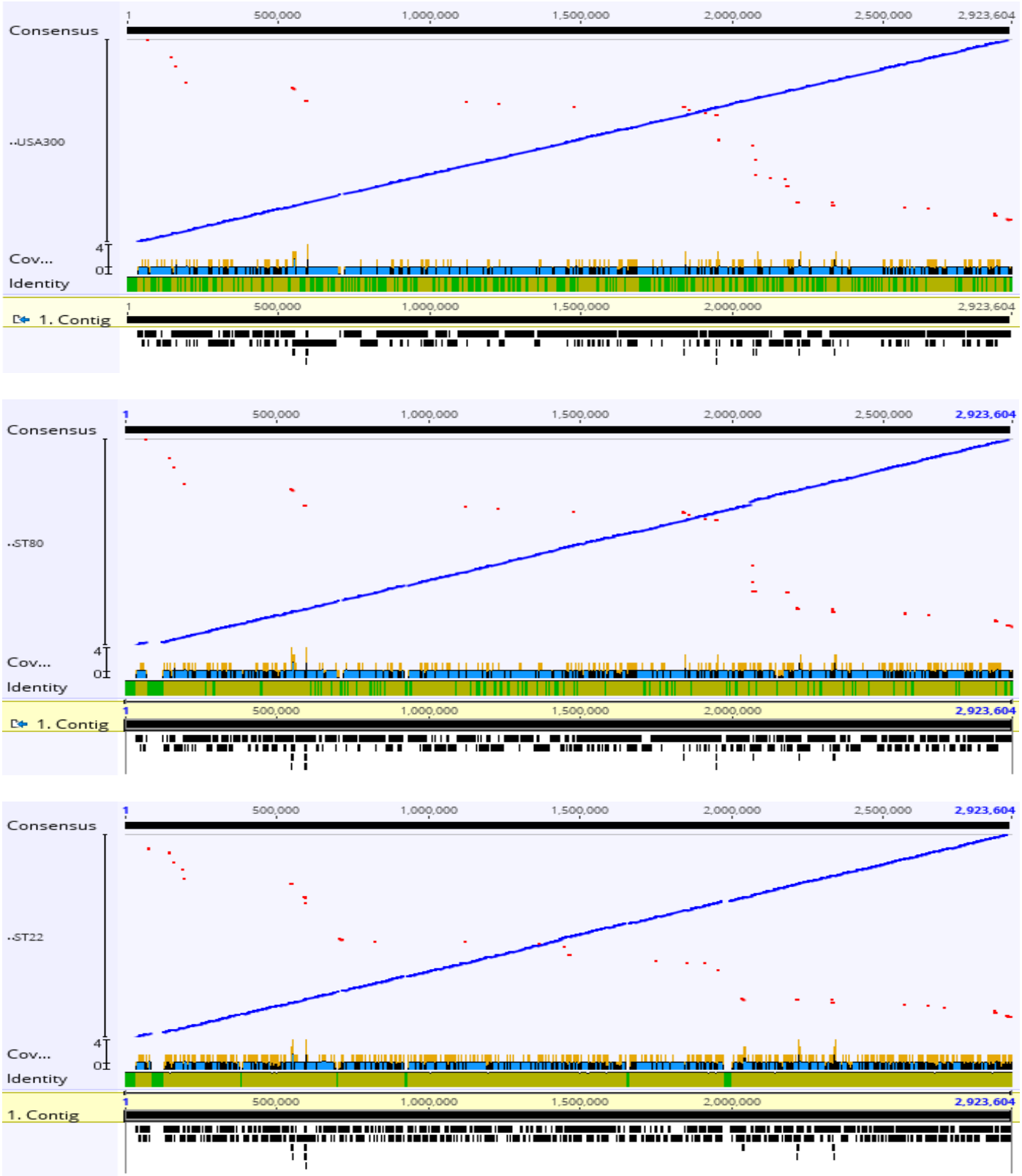

Figure 5.4. LASTZ pairwise alignment of *S. aureus* genomes.

S. aureus SA_MOU (ST8 mouse nasopharynx isolate) assembled genome aligned to reference genomes of ST8 USA300 (top), ST80 (middle) and ST22 (bottom) with default parameters.

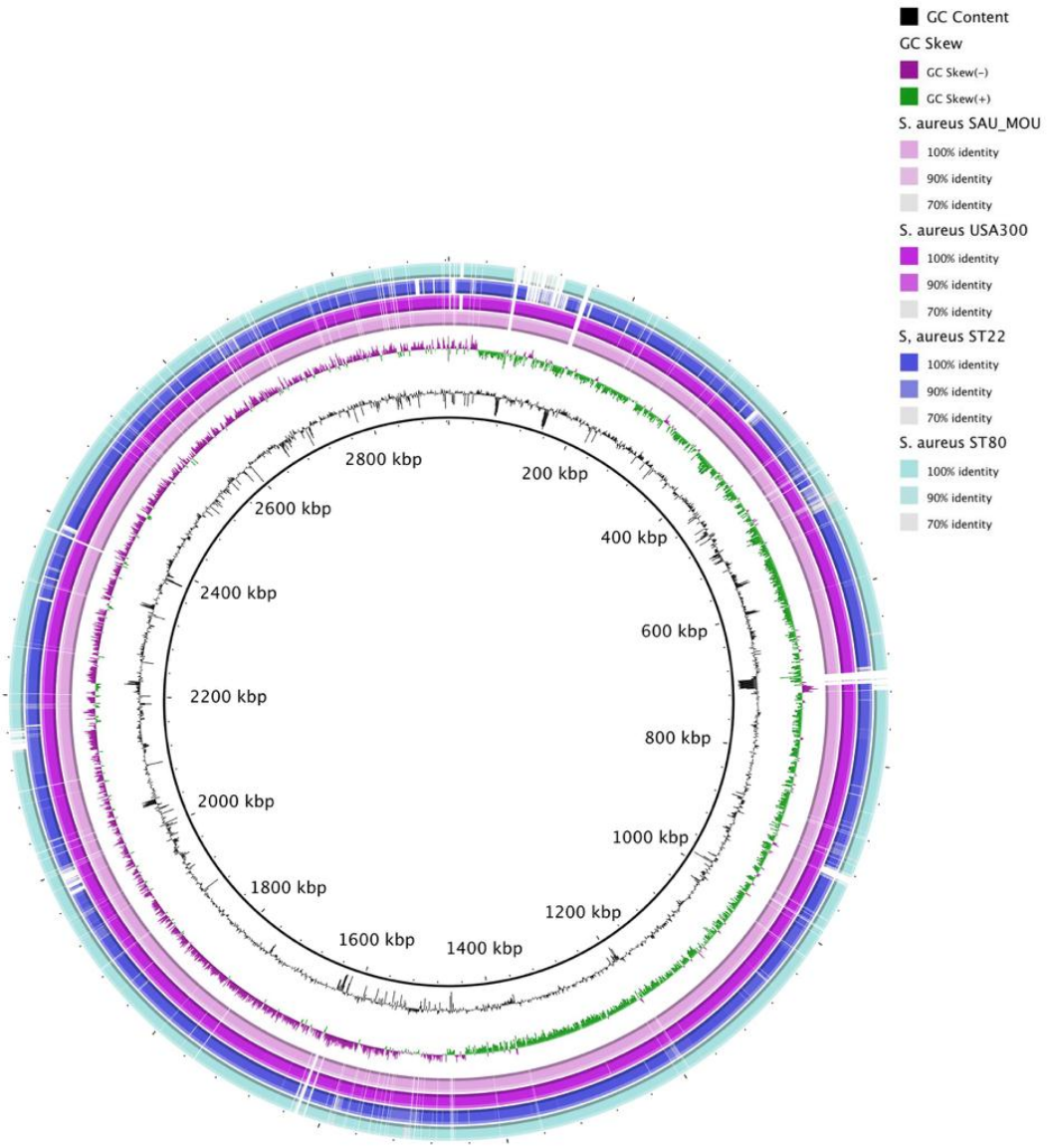

Figure 5.5 BRIG analysis of *S. aureus* genome sequences.

BRIG comparison of whole genome sequences of four *S. aureus* strains: *S. aureus* SA_MOU and three from NCBI database (USA300, ST22 and ST80). SA_MOU was the chosen reference genome.

Subsequent to the genome observations, interrogation of serial passage mutations from the C14_7 clone in respective SA_MOU genes was performed. Firstly, a search for sequences of 13 loci that harbour non-synonymous intragenic SNPs in C14_7 (Table 5.3) was performed for SA_MOU annotated genome. The hypothesis was that a certain level of concordance between SNPs found in C14_7 and alleles of SA_MOU might be expected if they represent adaptation to the mouse nasopharynx.

Sequences for nine of the thirteen genes were identified in SA_MOU genome (Table 5.8) with four loci not found in the assembled genome, including *gutB* (sorbitol dehydrogenase), SAUSA300_RS02185 (superantigen-like exoprotein), SAUSA300_RS09205 (hypothetical transmembrane protein) and SAUSA300_RS15385 (hypothetical protein). The protein sequences detected in SA_MOU were then matched against the USA300 reference genome (NC_007793.1) using BLASTp to check coverage and identity and also reveal if equivalent SNPs identified in C14_7 were present in SA_MOU. Of the nine genes, SA_MOU sequences showed 100% identity and coverage, with the exception of *ftsK* and *recF*. Although FtsK protein sequence showed 100% identity between both strains, SA_MOU *ftsK* is 42 bp longer.

With respect to SNPs correlation outcomes between C14_7 and SA_MOU, only two proteins showed matching sequence variation: RecF and FtsK. This indicates that there is potential for SA_MOU adaptation to the nasopharynx to be mediated through different mechanisms than C14_7, even though the two clones are closely related. It also raises the possibility that *S. aureus* genes *ftsK*

and *recF* that encode proteins with DNA recombination pathway activity are under constant selective pressure and contribute to adaptation during bacterial nasopharyngeal colonisation. The allelic variants found in this work for *ftsK* and *recF* were detected in other *S. aureus* ST8 strains in previous studies (Kennedy *et al*., 2008). This raises the hypothesis that the SNPs correspondence between SA_MOU and C14_7 was based on conserved mutations among ST8 clones.

Table 5.8 Non-synonymous intragenic SNPs comparison between two ST8 *S. aureus* nasopharyngeal single isolates

Table shows SNPs observed in genomic DNA of C14_7 and their presence/absence in SA_MOU.

***S. aureus* C14_7**		***S. aureus* SA_MOU**	
Gene symbol	**Codon change**	**Gene symbol**	**Codon change**
recF	358 aa (P/A)	FNBPBLMC_ 00036	358 aa (P/A)
SAUSA300_ RS00265	55 aa (L/F)	FNBPBLMC_ 00083	-
SAUSA300_ RS01070	358 aa (N/K)	FNBPBLMC_ 00236	-
SAUSA300_ RS01200	601 aa (T/A)	FNBPBLMC_ 00260	-
gutB	341 aa (T/P)	-	-
SAUSA300_ RS01715	258 aa (D/N)	FNBPBLMC_ 00376	-
SAUSA300_ RS01715	200 aa (S/R)	FNBPBLMC_ 00376	-
SAUSA300_ RS02185	110 aa (A/T)	-	-
ftsK	180 aa (K/R)	FNBPBLMC_ 01253	180 aa (K/R)
dnaJ	250aa (A/T)	FNBPBLMC_ 01670	-
SAUSA300_ RS09205	172 aa (I/T)	FNBPBLMC_ 01821	-
SAUSA300_ RS09205	148 aa (P/S)	FNBPBLMC_ 01821	-
SAUSA300_ RS09205	146 aa (E/K)	FNBPBLMC_ 01821	-
SAUSA300_ RS09205	141 aa (A/V)	FNBPBLMC_ 01821	-
SAUSA300_ RS09205	137 aa (T/A)	FNBPBLMC_ 01821	-
SAUSA300_ RS09205	131 aa (K/R)	FNBPBLMC_ 01821	-
SAUSA300_ RS15385	28 aa (Y/C)	-	-
SAUSA300_ RS10080	48 aa (R/L)	-	-
bioA	189 aa (R/H)	FNBPBLMC_ 02616	-

5.4 Discussion

This chapter has evidenced *S. aureus* nasopharynx adaptation after serial passages in this niche. In addition, the study revealed the complicating factor of *S. aureus* intra-species competition for nasopharyngeal colonisation. This study presents a potential cornerstone for future studies aimed at elucidating details of contributing loci during *S. aureus* nasal colonisation and adaptation with respect to how they contribute to bacterial persistence. *S. aureus* colonisation data from animal models and comparative genomics analyses can give indications of components and processes involved in adaptive responses of *S. aureus* in the nasopharynx. This part of the study helped answer several questions raised in Chapter 4, however some interrogations like the contribution of certain variants for nasopharyngeal adaptation remains unknown. Construction of defined allelic variant strains coupled with transcriptomics and/or metabolomics studies and phenotypic assays would elucidate further particular characteristics of the *S. aureus* modulated response against stress conditions found in the nasopharynx. The discussion that follows is aimed at the major mechanisms and pathways that might act to increase *S. aureus* nasopharyngeal fitness. *S. aureus* genetic variants under selection over a nasopharyngeal evolution time-frame were discussed in Chapter 4.

Putting together genetic changes tracked over an evolution time-course with evaluation of their contribution for bacterial fitness is a valuable approach to identify mechanisms controlled by microorganisms during adaptation. Lopez-Collazo *et al*. (2015) investigated microbial variation of a single MRSA clone

over a 13-year timeframe of bronchopulmonary colonisation in a patient with cystic fibrosis and the authors suggested a correlation between the genetic differences observed in the in the *S. aureus* long-term evolution and the bacterial adapted response against the host innate immune components. In this context, considering the genetic differences and colonisation rate variation between isolates from distinct nasopharyngeal serial passages, it was shown here that *S. aureus* adapts to the nasopharynx niche by regulating a number of factors involved in multiple cell pathways and mechanisms resulting in improved colonisation status. Similarly, extended bacterial persistence within the host tissue has been linked to adaptive changes (Hirschhausen *et al*., 2013; Hoboth *et al*., 2009; Kennedy *et al*., 2008). Conversely, a study demonstrated limited bacterial diversity in the transition from nasal carriage to fatal bloodstream infection caused by a MSSA strain (Young *et al*., 2012), a finding that reflects and reinforces the need to address the real contribution of genetic variants for adaptation in general.

The data from this chapter showed the potential connection between *S. aureus* nasopharyngeal adaptation and fluctuation of selective genetic changes. A substantial increase in the number of SNPs across the passages may explain the trend for better colonisation numbers observed for the *S. aureus* isolate from the second nasopharyngeal passage compared with the control, while the refinement of such variants would further improve *S. aureus* fitness for the nasopharynx. However, it is pertinent to mention that adaptive responses by *S. aureus* in this work were due to designed experimental evolution, in contrast to natural adaptation during a lifecycle to distinct niches during invasion and

colonisation/infection. Additionally, it is plausible to suggest that the *S. aureus* nasopharyngeal adaptation demonstrated here over three serial passages of 7 days each might possesses different aspects than clinical adapted strains isolated from patients colonised for years as previously discussed. Still, the finding that *S. aureus* potentially improved its fitness to persist in the murine nasopharynx and that this phenomenon was accompanied by genetic variation similar to other evolutionary studies endorses the methodology used in this study (serial nasopharyngeal passages of pooled *S. aureus* isolates) producing the outcomes from this work.

Although a definitive statement cannot be reached in this study regarding the actual contribution of each mutation detected in the evolved isolate genome sequences towards *S. aureus* adaptation in the nasopharynx, indications of pathways modulating *S. aureus* fitness for nasopharyngeal colonisation can be made. The majority of the genetic changes detected here relate to metabolism rather than virulence factors. In a separate study, gene expression profiles demonstrated up-regulation of *S. aureus* virulence components upon transition from nasal colonisation to bacteraemia (Jenkins *et al*., 2015). Additionally, evolved clones of *S. aureus* isolated from cystic fibrosis patients provoked attenuated immune response by human monocytes (Lopez-Collazo *et al*., 2015). These studies reflect a bacterial colonisation strategy focused primarily in regulation of persistence components instead of virulence factor expression, which contributes to the long-term colonisation. This is in accordance with the minimal number of virulence factors and their regulators with mutations during nasopharyngeal adaptation found in this study. In a similar fashion, metal

cation acquisition might be largely unaffected by the adaptive changes in *S. aureus* during nasopharynx colonisation as no cytotoxins, haemolysins or other acquisition determinants involved in iron transport, for example, showed mutations in their sequences.

Data from the nasopharyngeal passaged single isolates suggests a contribution of co-factors in *S. aureus* nasopharyngeal adaptation. Chaffin *et al*. (2012) assessed adaptation features of *S. aureus* to the lung using transcriptomics assays and the authors reported increased *in vivo* transcription of co-factors genes associated with changes in the energy source and higher growth rate. This study detected genetic variation in genes for co-factors (biotin and thiamine) linked to amino acid and carbohydrate metabolism. The improved fitness suggested here for nasopharyngeal passaged clones might reflect a high level of involvement between the co-factors and the adaptive response of *S. aureus* to the nasopharyngeal growth limiting conditions.

Aligned with *S. aureus* metabolism, data from evolved clones also suggests that carbohydrate metabolism could be implicated in *S. aureus* adaptation to the nasopharynx. Several mutations located in genes related to carbohydrate biosynthesis and transport (mannitol, sorbitol and fructose-6-P metabolism) shown in this chapter might contribute for persistence of *S. aureus* in the nasal passages. Human nasal secretions contain low concentration of glucose (Krismer *et al*., 2014) which ultimately leads *S. aureus* to find alternative ways to acquire and utilise other molecules as carbon sources. In terms of carbohydrate catabolism, *S. aureus* possesses three major central carbon

metabolism pathways: glycolytic, PPP and tricarboxylic acid (TCA) cycle (Fuchs *et al*., 2012).

The ability of *S. aureus* to sense environmental changes with subsequent regulation of its multiple carbon pathways reflects its potential to grow under aerobic and anaerobic conditions and also to use oxygen, nitrite or nitrate as the final electron acceptor during respiration. For instance, oxygen deprivation and glucose-enriched conditions drive TCA repression by *S. aureus* while bacterial up-regulation of genes involved in glycolysis can be observed (Fuchs *et al*., 2007). A study using gene expression analysis showed adaptive responses of *S. aureus* in a mouse model of pneumonia where the bacteria down-regulates both glycolytic and TCA central metabolic pathways (Chaffin *et al*., 2012). Additionally, Ferreira *et al*. (2013) showed that in environmental conditions similar to the nasal cavities niche (low concentration of free monosaccharides and high oxygen availability), *S. aureus* can use glucose and lactose simultaneously for growth. The lactate oxidation therefore confers *S. aureus* competitive advantage and better fitness for nasopharyngeal colonisation since lactate is the end product of many fermentative commensal bacteria. These findings support the capability of *S. aureus* to modulate an appropriate response against environmental limiting condition, albeit if that is also selected by the niche in addition to conserved encoded regulatory control.

While it remains unclear which specific aspects of carbohydrate metabolism were affected during the *S. aureus* adaptation to the nasopharynx observed in this study, potential components can be highlighted based on selected genetic

changes and it is tempting to suggest that they provide increased fitness based on improved carbohydrate metabolism of alternative sugar sources which could also be combined with an adaptive response to oxygen tension variation as the nasal cavities displays high O_2 concentration (Ferreira *et al*., 2013). Future studies should clarify the influence of adapted responses by *S. aureus*.

As well as carbohydrate central metabolism, another *S. aureus* factor that seems to come under selective pressure within the nasopharynx is stress resistance. The ability of *S. aureus* to cope with diverse types of stress was mostly addressed by the bacterial response via signal transduction and regulation of gene expression (Morikawa *et al*., 2012). The classical *S. aureus* stress response comprises multiple components such as sigma factors (e.g. SigB, enrolled in general response to stress) (van Schaik & Abbe, 2005), two-component systems (e.g. *agrAC*, involved in the regulation of virulence gene expression) (Bronner *et al*., 2004), SarA (staphylococcal accessory regulator) (Cheung *et al*., 2008) and specific regulatory systems (e.g. HrcA and CtsR, implicated in response to heat shock responses) (Frees *et al*, 2004). These global regulators/transcription factors act to provide *S. aureus* with a modulated and adapted response according to changing environmental conditions. In this study, some genes under control of regulatory components mentioned above showed genetic variation during the nasopharyngeal experimental evolution, which might explain the trend for better fitness observed with evolved clones for this niche.

In consonance with stress resistance, the response to oxidative stress could be of particular relevance in the adaptation of *S. aureus* to the nasopharynx. There

are many relevant stressors with reactive oxygen species (ROS), including free radicals (superoxide anion, hydroxyl radical and hydrogen peroxide) and reactive nitrogen species (nitric oxide, peroxynitrite anion). These oxidants are formed via diverse reactions from both bacterial aerobic growth and host oxidative killing mechanisms and the imbalance between their presence in the cell and ROS detoxification results in oxidative damage to a range of macromolecules such as proteins, DNA and lipids (Groves & Lucana, 2010). *S. aureus* displays multiple defensive molecules that act in the battle against ROS (Painter *et al*., 2015) and adaptive bacterial responses in the nasopharynx leading to quicker and more efficient actions against oxidative stress provides *S. aureus* improved fitness for the referred niche.

Characterization of *S. aureus* adaptation to the airways of a CF patient (Treffon *et al*., 2018), the intracellular niche of human bronchial epithelial cells (Michalik *et al*., 2017) and to the lung (Chaffin *et al*., 2012) showed increased abundance of proteins associated with oxidative stress response. Chavez-Moreno *et al*. (2016) showed high levels of *in vivo* expression for *S. aureus* components involved in protection from oxidative stress in the anterior nares, indicating that *S. aureus* experiences oxidative stress in its natural niche. These data indicate that the differential colonisation potential observed for nasopharyngeal passaged clones is partially correlated with adaptive oxidative stress response due genetic variation in stress resistance genes (*hemN*, *ahpF* and *clpL*) found among these isolates.

Adaptive responses against oxidative stress are not only mediate by defensive molecules. Exposure to ROS leads to increased mutation rates via activation of the SOS response (Painter *et al*., 2015). Higher mutation rates increase the probability of selection for adapted variants, which ultimately enhance the bacterial survival from a survival bottleneck. This study detected *S. aureus* variants that were potentially selected due to the stress conditions of the nasopharynx related to both oxidative stress and DNA repair machinery. Therefore, it is feasible to suggest that exposure to ROS in the nasopharynx was a driver of selection of adaptive responses from both oxidative stress and SOS responses in *S. aureus,* which act in a coordinated manner to improve the bacterial fitness for the nasopharynx, a phenotypic trend proposed in this chapter.

Additionally, the data presented here showed intra-species competition for the nasopharynx niche colonisation. Pre-colonised mice prevented the third serial nasopharyngeal passage by *S. aureus* USA300 LAC JE2 for almost one year, a phenomenon observed in distinct sets of mice. Clones from the naturally colonising strain were named *S. aureus* SA_MOU and further analysis showed that they belong to the same USA300 LAC JE2 sequence type - ST8 – even though they do not share the same *spa* type (USA300 and SAU_MOU belong to t008 and t024 respectively). ST8 is a highly important clonal group as it includes the American epidemic CA-MRSA USA300 clone, a genotype characterised by high virulence expression and the unique linkage between SCC*mec*IVa and arginine catabolic mobile element (ACME), which provides the

bacteria both drug resistance and pronounced virulence factor expression during colonisation/infection (Diep *et al*., 2008).

Genetic diversity within ST8 is reasonably high and according to the MLST server used for typing SA_MOU, at least 16 *spa* types are associated with ST8, including t008 (CA-MRSA USA300 *spa* type) and t024 (SA_MOU *spa* type). Iwao *et al*. (2012) showed that a ST8-derived CA-MRSA genotype has become the major clone associated with skin, soft tissue and invasive infections. The authors revealed variation regarding the *spa* types and although it possesses high genomic similarity to USA300, a reasonable level of variation with the accessory genes was detected, including a novel pathogenic island (SaPIj50). Genetic diversity within ST8 MRSA clone collections has also been demonstrated in other studies (Glaser *et al*., 2016; Kawaguchiya *et al*., 2013; Von Dach *et al*., 2016).

These observations answer several questions regarding the intra-species competition observed in this chapter. Although USA300 LAC JE2 and SA_MOU are closely related (both share identical ST), genetic differences between both strains might account for SA_MOU ability to naturally colonise the mouse nasopharynx and persist in this niche or for SAU300 LAC JE2 inability to invade a pre-colonised nasopharyngeal tissue and establish a colonisation. It was suggested that ST8 isolates in Europe are formed by a genetically diverse population comprised of both ancestral representatives and strains with multiple introductions (Strauß *et al*., 2017). In this study, SA_MOU assembled contigs covered 94.5% of USA300 LAC JE2 genome with 99.5% of identity

which clearly reflects a degree of genetic dissimilarity between the two genomes.

Extended assessment of variation between USA300 LAC JE2 and SA_MOU in terms of genetic characters such as PVL, ACME, SCC*mec* and core/accessory genome was not investigated in detail due to time limitation, but it unclear what would indicate determinants for successful colonisation by SA_MOU or competition and colonisation determinants of murine nasopharyngeal tissue with SAU300 LAC JE2 best studied for its assets that contribute to human pathogenesis and particularly skin and soft tissue infections. A study compared an emerging ST8 MRSA lineage which became the major clone in Japan with USA300 and it was demonstrated that the genome from both strains were 97.4% homologous (Iwao *et al*., 2012). This finding is in line with the data from this study: genetic variation, even at limited levels, between closely related *S. aureus* ST8 strains could be responsible for distinct adaptive responses that play a major role in intra-species competition in the context of nasopharyngeal colonisation.

Bacterial competition in the nasal cavities is a consequence of diverse mechanisms including a dispute for adhesion site and nutrients and direct (bacteriocins release) or indirect (host defence stimulation) interference (Krismer *et al*., 2017). Whilst most of the competition studies to date address the relationship between *S. aureus* and other nasal commensals, including coagulase-negative *Staphylococcus* species, it is likely that the intra-species competition observed in this study is due to an antagonistic battle for host

ligands and nutrients, since the production of antimicrobials would be likely to be ineffective against other *S. aureus* strains and the stimulation of the host immune system would probably affect any genetically similar *S. aureus* lineages.

In respect of bacterial adhesion, *S. aureus* expresses a vast range of adhesins enrolled in microbial adherence such as WTA, ClfB, IsdA, and SdrD and their host binding partners have been identified: SREC1 receptor, loricrin and cytokeratin matrix proteins and epithelial desmoglein 1 (Askarian *et al*., 2016; Baur *et al*., 2014; Clarke et al., 2009; Mulcahy *et al*., 2012). Because attachment site availability in the nasal epithelia is limited and bacterium-host adhesion occurs via specific and strong mechanisms (Schade & Weidenmaier, 2016), the experimental colonisation of mouse nasopharynx by USA300 LAC JE2 was potentially limited due to the lack of host ligands. SA_MOU however, successfully established a colonisation using attachment sites for *S. aureus* prior to inoculation with USA300 LAC JE2.

Similarly, the ability of SA_MOU to colonise the nasopharynx with subsequent prevention of USA300_LAC_JE2 establishment could be accounted for by its competence in acquiring nutrients within the nasal epithelia. Nasal secretions display scarce nutritional conditions (Krismer *et al*., 2014; Vanthanouvong & Roomans, 2004), and bacteria from the nasal microbiota need to overcome such growth factor limitation to grow in this niche. The strain SA_MOU may be adapted to coexist with other member of the murine nasal flora to obtain certain nutrients (Yan *et al*., 2013). Colonisation of the mouse nasopharynx by SA_MOU could be highly favoured for persistence in contrast to the naïve

experimental USA300 LAC JE2 colonisation, since SA_MOU presumably has had more time to modulate adaptive responses for survival and to obtain nutrients from the host. Therefore, SA_MOU persisted in the nasopharynx even when challenged by the serially passaged evolved clone C12_7.

In the light of intra-species competition, it is possible that SA_MOU was adapted for the stress conditions in the mouse nasopharynx, which differs to USA300 LAC JE2. SA_MOU revealed only two variants that matched with the non-synonymous intragenic SNPs from USA300 LAC JE2 C14_7 (nasopharyngeal clone evolved after three serial passages), the isolate better adapted to the nasopharynx from the experimental evolution performed here. The SNPs found in both strains are located in genes with DNA metabolism (*recF* and *ftsK*). RecF and FtsK have been associated with the SOS response, a defensive mechanism activated upon DNA damage (Simmons et al., 2008) which increases the mutation rate in the bacterial cell (Vestergaard et al., 2015). Therefore, increased mutagenesis could be an important event during *S. aureus* nasopharyngeal adaptation that leads to distinct adaptive responses from different lineages.

Chapter 6 General discussion

The multifactorial drivers of *S. aureus* nasal colonisation makes its establishment and persistence difficult to be elucidated (Mulcahy & McLoughlin, 2016). There are suggestions of adaptive responses modulated by *S. aureus* in the nasal cavities (Burian *et al*., 2010b; Krismer *et al*., 2014), which reflects the bacterium's ability to interact bidirectionally with the host and this will indubitably select variants under these pressures. It was hypothesised in this research project that an experimental nasopharyngeal evolution of *S. aureus* would enable the detection of genetic sequence variants to be naturally selected through this host-pathogen interaction, providing the bacteria with improved fitness to colonise and persist in the nasopharynx. In this study, a robust genetic variation within *S. aureus* nasopharyngeal isolates was found after serial passage to impose selection and the identity of the variants may explain the trend for adaptation and increased frequency that was observed in experimentally evolved clones, using the long-term colonisation murine model established here.

The established murine model that was developed in Chapter 3 was effective for two different colonisation approaches of *S. aureus*: serial passages of 7 d each and long-term colonisation of 14 d. While the model is animal based and human isolates of *S. aureus* were used, the methodology addressed one concern that plagues the reliability of interpreting *in vitro* studies. Lijek *et al*. (2012) used a murine model to demonstrate that antibodies produced after

pneumococcal colonisation cross-react with *S. aureus* and inhibits nasal colonisation of the latter demonstrating the complex interplay between colonising species and host immunity. Meanwhile, *in vitro* assays were interpreted to suggest inhibition of *S. aureus* colonisation due to hydrogen peroxide produced by *S. pneumoniae* (Park *et al*., 2008; Regev-Yochay *et al*., 2006). Subsequent findings from *in vivo* studies did not support this *in vitro* data (Margolis, 2009, Lijek, *et al*., 2012). Murine models have successfully demonstrated the association between various host immune system components and both *S. aureus* infection (Planet *et al*., 2016) and colonisation (Xu *et al*., 2015). The model developed here made it possible in this study to gain insights about *S. aureus* evolution and adaptation in the nasopharynx to add to previous studies.

While *in vivo* models are clearly superior to *in vitro* studies, they are not without complications. Over the course of the murine model establishment, two main factors challenged its standardisation and robustness: inoculum size (~ 5 x 10^4 cfu) and issues of prior nasopharyngeal colonisation of the mice. The inoculum size chosen was lower than other studies with murine models of *S. aureus* colonisation and infection (Archer *et al*., 2016; Satorres *et al*., 2009) and this size aimed to not only mimic low bacterial density found during natural nasal colonisation by *S. aureus* (Krismer & Peschel, 2011) but also to avoid a strong immune response from the host. The model was successfully established with this inoculum size although one event of bacterial clearance could be observed in the subsequent experiments (Section 5.3.1.1), which could be due to problems with instilling the inoculum or animal behaviour.

Initially, no preexisting colonisation of the nasopharynx by *S. aureus* was detected during the murine model establishment, making it possible to inoculate and recover the USA300 LAC JE2 strain used in this study. However, over time subsequent use of the model for serial passages of *S. aureus* (Section 3.3.2) showed that another *S. aureus* strain was colonising the nasopharynx and this apparently blocked further colonisation by the strain used in this work. Although mice are not considered natural hosts of *S. aureus*, high rates of colonisation were reported in these rodents from commercial vendors (Schulz *et al*., 2017), which supports interpretation of the problem encountered in this study. Despite the frustrating change in usefulness of the purchased mice for primary colonisation, the model developed here demonstrated it to be an effective experimental tool for future investigations of *S. aureus* in the nasopharynx. With careful adherence to the inoculum preparation and instillation protocols developed in this study together with surveillance of the nasal microbiota, the *in vivo* model can be used to interrogate various aspects of *S. aureus* nasopharyngeal colonisation including invasion, establishment, persistence and host immunity.

Further improvement of the mouse model developed here could be achieved using humanised mice, which with characteristics of the human immune system, and this may enable better interpretation relative to the various human specific immune evasion components, for example. Rodents and humans possess different nasal anatomic structures, distinct metabolic features, altered cell lipid composition, potentially microbiome ROS generation and inflammatory responses (Seok *et al*., 2013; Xiao *et al*., 2015; Warren *et al*.,

2015). These substantial differences were associated with failures in the development of anti-staphylococcal vaccines, since antibodies designed from mice studies proved to be ineffective against *S. aureus* infections in humans (Parker *et al*., 2017). By utilising the model developed in this study with humanised mice our understanding of host-pathogen interactions during *S. aureus* nasopharyngeal colonisation could be maximised. Humanised mice models were used in several *S. aureus* infection studies where they showed remarkably increased susceptibility to infection compared with non-humanised control mice (Knop *et al*., 2015; Prince *et al*., 2016; Tseng *et al*., 2015).

Repeated passages of *S. aureus* in the nasopharynx of mice revealed DNA sequence variation as the passages were performed (Section 4.3.3 – 4.3.8). This approach exposed clear evidence of natural selection of mutants in the *S. aureus* population during nasopharyngeal colonisation over the experimental evolutionary timescale. Published studies identified genomic mutations in *S. aureus* evolved clinical isolates primarily associated with infections (Kennedy *et al*., 2008; Lopez-Collazo *et al*., 2015). The identification of several non-synonymous intragenic SNPs in this study shared with those identified in previous *S. aureus* evolutionary studies (Section 4.4) emphasizes the strength of the methodology used here to investigate relevant features of *S. aureus* nasopharyngeal colonisation. Furthermore, collation and curation of such studies could lead to subsequent investigation of common alleles that are selected in these models to determine the key pathways subject to evolutionary pressure.

Future studies using the murine model developed here coupled with additional *S. aureus* strains will help to clarify the major bacterial components and pathways involved in *S. aureus* nasal carriage. Repeated passages using closely and distantly related strains to USA300 would provide more consistent insight of genetic variation outcomes, which will reveal both strain-specific and general components under selective pressure in the genome during *S. aureus* nasopharyngeal colonisation. Additionally, the replication of three serial passages of *S. aureus* USA300 LAC JE2 using a distinct batch or strains of mice would answer the question whether the *S. aureus* evolution captured here was a unique event or a typical consequence of variants that can be selected under environmental pressure in the nasopharynx. Attempts were made to reinforce the data presented here by repeating the 1st and 2nd passages, however the experiments were frustrated due to pre-colonisation of the mice (data not shown). Finally, performing additional serial passages and using different *S. aureus* strains could help to identify novel *S. aureus* components under selective pressure in the nasopharynx and potentially indicate sequence variants relevant throughout more persistent nasal colonisation. Here, an expansion in the number of SNPs found in pooled genomic DNA from the second passage was followed by refinement of these mutations after the third passage. Therefore, carrying out a fourth or even a fifth repeated passage would reveal the pattern of *S. aureus* genome modulation for longer colonisation periods (e.g. stabilisation, re-expansion or further refinement of genomic sequence variants). However, such an experiment could also produce evolutionary bottlenecks determined by early mutations, so a more broad study would also be required to reduce this possibility.

The genetic changes captured during *S. aureus* murine nasopharyngeal colonisation in this study was accompanied by a trend of bacterial adaptation to this anatomic site with some weak statistical support (Section 5.3.1). Adaptive responses of *S. aureus* evolved clones after exposure to stress conditions have been described many times and some responses are well understood (Chaffin *et al*., 2012; Ferreira *et al*., 2013; Chavez-Moreno *et al*., 2016). When *S. aureus* is introduced into the mouse nasopharynx, the bacteria must overcome diverse growth-limiting conditions, such as low nutrients availability, competition for host ligands, modulated epithelial inflammation and competing microbiota that can secrete antimicrobials, making it logical to hypothesise that genetic changes that promote improved bacterial fitness will be selected over the evolutionary timescale and are likely to represent the sum total of the pressures. The individual contribution of the numerous intragenic and intergenic SNPs to *S. aureus* nasopharyngeal adaption captured in this study are yet to be addressed raising the need for future studies. Several methodologies could be used to investigate and elucidate the involvement of an individual SNP in *S. aureus* nasal carriage. However, with many SNPs identified in this study and only partial, weak statistical support for improved fitness the study of single alleles could be challenging without more *in silico* study.

In addition, work should be undertaken to endorse the contribution of DNA sequence variants by confirmation of the SNPs identified in the sequence pools and single isolates found in this study through PCR amplification and Sanger DNA sequencing. Once the variants are attested, differences in the nasopharyngeal colonisation status from sets of strains for each SNP (control

and mutants: a strain containing a single SNP, mutant strain lacking the gene with the SNP and a complemented or knock-in strain) could be investigated. Distinct survival rates among these different strains relative to a single SNP would indicate that the sequence variant contributes to *S. aureus* persistence in the nasopharynx.

Future analyses should also include global gene expression analysis of evolved nasopharyngeal isolates from this study. Comparisons with the control strain could reveal the consequences of genetic variation in up/down-regulation of *S. aureus* genes during growth and identify pathways or individual components contributing to nasopharyngeal colonisation. Trancriptomics approaches were performed to investigate *S. aureus* adaptation using a mouse pneumonia model (Chaffin *et al*., 2012) and separately a synthetic nasal medium – SNM - that simulates the nutritional conditions of the human nose (Krismer *et al*., 2014). Both studies indicated metabolic pathways strongly regulated during *S. aureus* adaptation. Therewith, analysing gene expression profiles of adapted nasopharyngeal isolates from either early/late colonisation stages using the murine model established here together with different time-points after growth in SNM could collectively help to understand *S. aureus* niche adaptation.

Intra-species competition in the nasopharynx was also addressed in this study. In some occasions, the mice used in the murine model were pre-colonised by another *S. aureus* ST8 strain (SA_MOU), which blocked further colonisation by USA300 LAC JE2 and prevented the third experimental serial passage to be performed. Genome investigations revealed that both strains are closely related

with high rate of similarity when aligned against each other. However, SNPs analysis showed that SA_MOU nasopharyngeal adaptation is likely to have occurred through distinct mechanisms compared with USA300 LAC JE2, since only two identical sequence variants were identified in both SA_MOU and C14_7 (USA300 LAC JE2 clone from the third serial nasopharyngeal passage). However, the SA_MOU could represent a strain that has been subjected to a very long evolutionary timescale of selection relative to the inoculated USA300 LAC JE2, making comparison of limited value. The *S. aureus* competition observed in this study is in accordance with the competitive exclusion principle (Hardin, 1960) which states that two species competing for similar resources cannot co-exist in the same environment since one will always have advantage over the other, leading to extinction of the second competitor. SA_MOU most likely prevented USA300 LAC JE2 invasion through these principles and its occupation of host ligands for colonization.

It is possible that SA_MOU possess a better fitness than USA300 LAC JE2 due to improved metabolic responses against stress conditions in the nasopharynx. Future studies competing the SA_MOU strain with USA300 LAC JE2 and the evolved variants could provide insights regarding relative fitness for the nasopharynx. Transcriptomics/metabolomics studies coupled with phenotypic assays would reinforce any difference in the cell metabolism between both strains. In addition to these investigations, future studies could simultaneously explore competitive exclusion due to limited host ligands. Inducing the colonisation with USA300 LAC JE2 first, followed by a challenging colonisation with SA_MOU would give highlight the nature of the competition between both

strains. If only USA300 LAC JE2 parent and evolved isolates are differentially present in the nasal wash after the nasopharynx is challenged with SA_MOU, it would support that early adhesion to the nasal epithelium is a critical factor in *S. aureus* carriage.

In summary, this study showed that *S. aureus* DNA sequence variants selected during nasopharyngeal colonisation could indicate loci contributing to better fitness in this niche. The outcomes of this study raises questions on a broad range of components with potential role in *S. aureus* nasal carriage. The individual understanding of their relevance will serve as basis for future studies focused on prevention/elimination of *S. aureus* nasal colonisation, a high-risk factor of transmission and infections caused by this opportunistic pathogen.

Chapter 7 References

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