**Staphylococci: The evolving genome**

**Summary**

*S. aureus* causes an extensive variety of infections in a range of hosts which are described in many of the following chapters. The comprehensive analysis of the staphylococcal genome reveals details of its role in determining the organism’s biology, pathobiology and dissemination. Whole genome sequencing technologies have led to a quantum leap in our understanding of bacterial genomes. The recent reduction in cost of sequencing technologies has resulted in unprecedented volumes of genomic information about the staphylococci, one of the most sequenced bacterial species. Collecting, comparing and interpreting these data is challenging but fascinating insights have emerged. For example, it is becoming clearer which selective pressures are faced by staphylococci in their habitats, and which mechanisms allow adaptation, survival and spread. Staphylococci are constantly evolving as they alter their genomes, exchange DNA and adapt to new environments, leading to the emergence of increasingly successful, antibiotic resistant, immune-evading and host-adapted colonisers and pathogens. This chapter will introduce the structure of staphylococcal genomes, details how genomes vary between strains, outline the mechanisms of genetic variation and describe the features of successful clones.

**Introduction**

The genome of a bacterial cell encodes all of the potential functions of that cell and how they respond to environments (1). The first staphylococcal genomes to be sequenced in full were landmark achievements in 2001 (2), allowing the first overview and predictions of the metabolic, regulatory and virulence potential of *S. aureus*. As more genomes were sequenced in the next few years, different regions of the genomes were discovered that were core and conserved, often including essential genes, as well as regions that were variable, or non-essential, or mobile and allow the cell to adapt to new environments (3)(4) (5)(6) (7). Now that thousands of genomes are available, we understand the constant flux that staphylococcal genomes are under. Change occurs in small nucleotide variations (SNVs) or by horizontal gene transfer (HGT), and evolution occurs when these changes provide a selective advantage to the bacterial cell and become fixed in subpopulations. Particular variants adapt to evolving habitats, new environments and stresses, leading to the spread and expansion of successful clones that continue to evolve.

Staphylococci are colonisers and common opportunistic pathogens of mammals and birds. The pathogenic importance of staphylococci ensures that they are one of the most frequently sequenced genera. *S. aureus* is the species most studied due to its virulence and antimicrobial resistance (8) (9). *S. aureus* is a coloniser of humans, predominantly the nose, throat, axillae and groin of healthy humans. The colonisation rate of populations is approximately 25% but dependent on geography, age and risk factors (10). *S. aureus* colonisation is a risk factor for subsequent infection (11), and most infection is caused by the colonising isolate (12) (13). *S. aureus* is a common cause of minor skin infections that do not require antibiotic treatment due to a healthy innate immune response driven by neutrophils. In hospitalized or immunocompromised patients, particularly those with breaches in the protective skin barrier, infection risk is higher and treatment may require antibiotic therapy. *S. aureus* is one of the most common causes of hospital associated infection (8).

Acquisition of antimicrobial resistance (AMR) genes ensures *S. aureus* it is a formidable pathogen in healthcare. Penicillin resistance due to penicillinase enzymes that digest the -lactamase ring is almost ubiquitous in human isolates (14). The widespread introduction of second-generation -lactamase antibiotics such as methicillin selected for a new type of penicillin-binding protein mediated resistance encoded by the *mecA* gene. Methicillin-resistant *S. aureus* (MRSA) emerged in the 1960s, and spread globally and dramatically in the 1980s to 1990s (15); it is prevalent in healthcare clones of *S. aureus* at varying frequency across the world (16) and increases the overall burden of *S. aureus* infection. MRSA are often endemic in hospitals and resistant to a range of additional antimicrobials, drastically reducing the options available for prophylaxis and empirical treatment of *S. aureus* infection, leading to more infections, economic costs, and increased morbidity and mortality (17) (8). More recently, MRSA spreading amongst healthy community populations have dramatically impacted healthcare provision (CA-MRSA) (9), and livestock associated MRSA (LA-MRSA) clones have caused concern in high risk groups with exposure to animals (18).

Humans are also colonised by many coagulase-negative staphylococci (CoNS) species (19), so called because they are not *S. aureus* which is easily differentiated in the laboratory by its ability to clot plasma via coagulase production. CoNS are generally considered less pathogenic than *S. aureus*, yet are a common cause of infection in immunocompromised patients, particularly those with indwelling medical devices such as catheters and artificial joints and valves. The ability to produce extracellular polysaccharide biofilm, toxins and to carry multiple antimicrobial resistances contributes to their pathogenesis. The most prevalent species is *S. epidermidis*, while other important pathogens include *S. hominis,* *S. capitis, S. haemolyticus, S. simulans. S. xylosus, S. warneri, S. cohnii,* and *S. carnosus*. Uniquely, *S. saprophyticus* is a common cause of urinary tract infection, and encode proteins for binding to human urinary tract epithelial cells (20). Recently, several new coagulase positive or intermediate staphylococci have been identified that are capable of causing disease equivalent in humans to *S. aureus* but are much less prevalent, including *S. lugdunensis* (21) and *S. argenteus* (22).

*S. aureus* is also a common cause of infection in companion and livestock animals, in particular bovine mastitis in the dairy industry (14), and invasive infections in chickens (23). *S. pseudintermedius* is considered to be a major canine pathogen (24), while many other staphylococci have unique associations with particular hosts (25).

Adaptation of successful clones to evolving habitats such as host and antimicrobial pressure is increasingly studied using genomic tools. Analysis of whole genomes, genomic variability, epidemiology and evolution of staphylococci shed light on staphylococcal colonisation and interaction with host, pathogenic pathways, and adaptation and spread in response to selective pressures. Ultimately, these studies identify key features of genetic, microbiological and biological phenomena as well as being applicable to preventing and treating infection in humans and animals.

**Genomes**

A typical staphylococcal genome consists of a circular piece of DNA between 2.5 – 3.0 million base pairs, which may be supplemented by additional plasmids. This equates to around 2500 - 3000 putative open reading frames or predicted protein coding sequences (CDSs), as well as some tRNAs, rRNAs, non-coding RNAs and pseudogenes. An example genome is in figure 1.

Signatures in the DNA sequence allow automated prediction of the start and stop of each CDS, and the CDS can be compared to genes of known function in other bacteria to predict protein function. Signalling sequences and conserved domains can identify the active sites of proteins and their physical location in the cell. Promotors can be predicted, and estimates made of how genes are expressed, and which other genes are expressed at the same time. A holistic picture of the potential of the cell emerges (1).

The first staphylococcal genomes sequenced to completion belonged to the colonising and pathogenic species *S. aureus* (7). These early sequencing projects set the groundwork, identifying the key regions of the genome responsible for essential processes in growth, replication and survival and identifying novel genes and pathways.

Over the next few years, another 10 *S. aureus* genomes were sequenced to completion, predominantly methicillin-resistant isolates (1) and a laboratory reference strain (5). These projects began to identify the regions of the chromosome that were conserved amongst strains and those that were variable. Most of the variability was due to variation in genes associated with surface proteins and regulators, as well as the presence or absence of mobile genetic elements and variations in their genetic content.

At the time of writing, the public database GenBank holds 272 completed *S. aureus* genomes and whole genome shotgun genomes of another 9065 *S. aureus* isolates (<https://www.ncbi.nlm.nih.gov/genome/154>). By including those genomes in the NCBI Short Read Archive and the European Nucleotide Archive, this number is almost 50 000 (26). This enormous increase in the amount of sequencing data is driven by increasingly inexpensive technology, particularly the Illumina sequencing platforms, that generates shot gun genomes of short reads of sequence that are unable to be resolved into a single contig of DNA but can be aligned to a reference genome of the same lineage. Similarly, other species of staphylococci are increasingly being sequenced. Genbank lists whole genomes of at least 31 different staphylococcal species, including colonising and pathogenic coagulase-negative staphylococci (CoNS), animal colonisers and pathogens and food organisms . More than 200 staphylococci are deposited without a designated species, indicating the taxonomy is not completely resolved, although identifying and classifying species is increasingly accurate using whole genomes (27).

These databases are somewhat skewed by the choice of isolates for genome sequencing (26), and tend to favour those with clinical or industrial significance. Nevertheless the extraordinary volume of data available ensures an unprecedented resource for the study of trends in the evolution, selection and epidemiology of staphylococcal isolates.

The volume of information can be overwhelming to the non-specialist. There are increasingly tools to assist with the interpretation of genome information. For example, Aureowiki (aureowiki.med.uni-greifswalkd.de) is useful for searching for common genes across multiple *S. aureus* reference genomes with links to gene annotation and expression data (28). Staphopia assembles, maps and annotates raw genome sequence (26). Artemis Comparison Tool (ACT) is useful for aligning genomes and comparing where they vary (29). PanX can identify the distribution of accessory genes across multiple isolates (30). Regulatory RNAs can be identified using the SRD database (31). Mykrobe predictor reads a *S. aureus* rapid shot gun sequence result and identifies up to 12 antimicrobial resistance (AMR) markers in only 3 mins (32). The microreact project compares whole genome sequence (WGS) of isolates over time and geography to identify clones of public health importance (33). For laboratory researchers, an extremely useful tool is the Nebraska transposon library, identifying essential genes and actively distributing mutants deleted in specific genes in the reference strain JE2 (34) and tools to manipulate these strains (35).

The next generation of long-read sequence hardware technologies include Single Molecule Real-Time (SMRT) sequencers such as PacBio (Cooper 2017)$, and nanopore sequencing such as MinIon (Bayliss 2017)$. Long read technologies remove the need for alignment to reference genomes and are particularly useful for studying mobile genetic elements. Additional advantages include increased information about the epigenomes (methylation maps) of the DNA and enhanced portability and low cost will ensure new insights into staphylococcal genomes in the future.

**Core Genome variation**

The core genome is highly conserved amongst all isolates of a species, and typically has a conserved gene order. The core genome encodes all the essential genes as well as many non-essential genes, including those involved in metabolic, housekeeping, replication, structural, regulatory and some virulence genes (2008)$. We might consider the genome that is not composed of mobile genetic elements as the core genome.

Multiple colonies from the same host are likely to have identical core genomes or vary in only a few single nucleotide variants (SNVs) or single nucleotide polymorphisms (SNPs) compared to each other (36). This type of SNV variation may be due to a swap in a basepair, or an addition or deletion of a basepair or a very short sequence. From studying multiple colonies from the same host, it has been suggested that isolates are closely related if their core genomes contain 40 or fewer SNVs (39) (40). This level of variation within a colonising population in a host is called a ‘cloud of diversity’. Bacteria that spread between carriers or infected patients can have few SNV differences and therefore high levels of similarity, suggesting the transmission event was recent (41).

SNVs arise as errors in replication catalysed by the DNA polymerase enzyme as it rapidly unwinds and replicates the 3 million base pairs of staphylococcal genome in minutes during cell replication. Errors include incorporation of an incorrect base, deletion of a base, or insertion of an extra base. Bacteria have DNA error repair mechanisms, and in staphylococci these include the mismatch repair system. Occasionally a mutation in a gene such as *mutL* or *mutS l*eads to a ‘mutator’ phenotype where mutations accumulate at a higher frequency due to the lack of ability to repair them (37) (38).

From population studies of MRSA clonal types, the ‘molecular clock’ of *S. aureus* is estimated to result in one SNV per genome every 5-10 weeks (40) (36) (42). Based on this concept, it is presumed that patients with larger clouds of diversity have been colonised for longer periods of time (43). As bacteria spread to new hosts and across nations and continents, this molecular clock of variation can reveal the transmission pathway. This was first seen in a large-scale epidemiological study of MRSA ST239 isolates from across the globe (36) which confirmed expected transmission pathways and identified unexpected ones.

It is important to note that the Illumina whole genome sequence methodology used to compare bacterial genomes and identify SNVs for transmission studies restricts analysis to the conserved regions of the genome in any given dataset. This is typically the core genome only, and will vary between experiments. The analysis will be most discriminatory when related isolates are compared, such as those belonging to the same lineage. Variable regions of the chromosome, such as the MGEs, are usually excluded from analysis. Nevertheless, it is an extremely powerful method for analysis of the evolutionary relationship of isolates and for predicting how recently they shared a common ancestor.

***S. aureus* lineages**

The *S. aureus* species has a population structure that divides into lineages, which are often referred to as clonal complexes (CCs) (44). Some of these lineages are dominant (common) while most others are rare (45). Isolates from the same lineage share a core genome that is highly conserved and unique from other lineages. Each lineage is sufficiently different that whole genome sequencing is rarely used to compare isolates from different lineages as the variation is too great to infer epidemiological relationships. Lineage-specific variation includes thousands of SNVs throughout the genome, as well as insertions, deletions and variable regions. Isolates from the same lineage have extremely high levels of conservation in their core genomes across these SNVs and variable regions, indicating a close evolutionary relationship.

The naming of lineages is based on the original clonal complexes (CC) assigned by multi-locus sequencing type (MLST) typing system, where the sequence of seven housekeeping genes was compared. Isolates with seven identical sequences are grouped together into the same sequence type (ST) and those with five or six identical sequences are assigned to the same clonal complex (CC) (45). In contrast, two isolates that have four or less identical sequences typically belong to different CC. Subsequent analysis of the highly conserved core genome across the species by WGS analysis confirms the lineage structure and that assignment of isolates to CC by MLST or lineage by WGS correlate extremely tightly (26).

Microarray analysis across thousands of *S. aureus* genes and pan-genomes identified a broad range of genes that were present/absent or highly variable in *S. aureus* populations but were conserved within lineages. These included many genes encoding surface bound virulence factors and their regulators and immune evasion mechanisms (46)(47) and predicted to play a role in host-adaptation or virulence. In some cases the variation represents the acquisition/loss of an individual gene, such as collagen-binding protein. In other cases, one or more genes within an operon have been swapped with the corresponding genes from strains or other lineages, such as the four genes in the capsule locus that differentiate capsule type 5 from capsule type 8 or those determining the type VII secretion system . In some cases, a specific region within a gene varied, such as active sites within the fibronectin binding protein, or the region within the agr regulatory locus determining the different agr classes (1) (46).

*S. aureus* genomes typically contain two “genomic island” regions of 20-30kb called alpha and beta, containing strings of duplicated but variable genes (44) (48). These islands are highly variable between unrelated lineages, but very conserved within lineages. GI typically contains 7 – 11 variant copies of staphylococcal enterotoxin like genes that have superantigen-like (*ssl*) like properties involved in immune evasion, as well as multiple copies of related lipoprotein genes. GI carries multiple copies of serine proteins and different types of superantigens, as well as additional leukocidins or lantibiotic synthesis genes. Both GI and GI also carry a restriction modification system with a modification and restriction specificity that is lineage specific. Such a system allows DNA to be transferred more efficiently between isolates of the same lineage than between isolates of different lineages (49) (50). The Sau1 RM variant genes (*hsdS*) are potent markers of lineage (49) (51), and likely contribute to the formation and independent evolution of the lineages.

Despite these major differences in genomes between lineages, isolates from most lineages of *S. aureus* appear to cause the same types and incidence of disease, and lineages generally have similar ‘pathogenic potential’. (The exception are some specific toxin-mediated diseases such as toxic shock syndrome, scalded skin syndrome or food poisoning, where pathogenesis is dependent on the carriage of mobile genetic elements encoding relevant toxins.) Variation in the incidence of disease caused by each lineage correlates well with variation in the incidence of carriage, suggesting opportunistic infection (45)(44). Some lineages are more successfully carried in some hosts (for example, animal or human) compared to others (14), and carriage appears to be the main risk factor for subsequent infection (12,13). The importance of lineage specific genomic markers is colonisation and infection is still unclear, but redundancy of gene function is a common feature of staphylococcal genomes, suggesting that when one gene is missing or variable, others can compensate.

All *S. aureus* lineages share a common ancestor, but each lineage contains a unique combination of common variant genes indicating extensive recombination. Only some of these lineages have become prevalent including CC1, CC5, CC8, CC15, CC22, CC25, CC30, CC45 (45) (44). This evolutionary pattern of repeated recombination of surface and host-interacting genes with expansion of only a small number of successful combinations has led to some very stable lineages. There is a surprisingly close relationship between isolates from the same lineage that can have highly conserved lineage specific features despite very different epidemiological behaviour and geography. An example is the USA400 MRSA lineage of CA-MRSA represented by the MW2 isolate from the USA in 1998, which caused severe skin and soft tissue infection in children compared to the methicillin-sensitive isolate MSSA476 identified in a nasal carrier in Oxford in 1999 (52). The two isolates both belong to CC1 and have only 285 SNV differences in their core genome within functional coding regions. In contrast, they have many differences in mobile genetic elements (MGEs) (52). Similarly, CC8 isolates such as the laboratory strain 8325 (5) and MRSA USA300 FPR3757 (53) both belong to CC8 and have highly conserved core genomes despite extensive variation in MGEs and pathogenic and epidemiologic behaviour. Therefore, the success of some of these lineages appears to due to the emergence of specific clones that have acquired mobile genetic elements (MGEs) and resistances, allowing rapid adaptation to new habitats and environments.

**Mobile genetic elements (MGEs)**

MGEs are reviewed in depth in chapters X, Y & Z are are described briefly here. MGEs are discrete pieces of DNA that replicate on their own or integrate into the host chromosome. They are not essential for the host but have specific mechanisms ensuring their own ability to transfer between bacterial cells and survive. They include bacteriophages (54), *S. aureus* pathogenicity islands (55) (also known as phage-inducible chromosomal islands (56)), plasmids (57), transposons and the SCC elements. MGEs are important as they carry many virulence, host-adaptation and antimicrobial resistances, so their carriage and transferability can have a substantial impact on the success and spread of staphylococci.

MGEs are widely distributed in *S. aureus* and can make up to 10-20% of the genome (7). There is enormous variation in MGE gene carriage and distribution, even amongst related bacterial isolates. Variation and recombination between MGEs appears to be common (58) (57). Multiple MGEs per cell ensures a flexible bacterial genome that can acquire and lose a variety of virulence and resistance genes.

**Bacteriophages.** Virtually all natural isolates of *S. aureus* harbour one or more intact bacteriophage (or phage) genomes inserted into the chromosome. S. aureus phages are typically around 45kb, temperate, reside quiescently within the chromosomal DNA (where they are termed a prophage) and can be induced by DNA damage or stressful conditions to excise, replicate their DNA and produce phage proteins, including those that assemble into phage particles. Phage DNA is packaged into new phage particles, released on bacterial cell lysis, and the particles bind to and inject their DNA to new host bacteria. This results in either the lytic pathway or the lysogenic pathway. The lytic lifecycle induces further replication of phage DNA and protein production, lysis of the bacterial host and release of infectious particles. They lysogenic lifecycle results in the phage DNA integrating into the bacterial chromosome at the phage-specific conserved integration site. Some virulence factors such as Panton-Valentine leucocidin, enterotoxin A and exfoliative toxin A are encoded on lysogenic prophage where they may provide a benefit to the host bacterium during infection. Host-specific immune evasion pathways such as chips and scin and sak are also found on the 3 family of bacteriophage associated with human carriage and disease (58) (14).

**Pathogenicity islands, such as SaPIs**. S. aureus pathogenicity islands (SaPIs) are related to prophage but do not carry the genes for phage particles. They also excise, replicate and package their DNA into phage particles. The phage particles are supplied by helper phage which also regulate induction of the SaPIs and direct lysis of the bacterial cell. SaPIs carry toxic shock syndrome toxins and can also carry biofilm associated genes, iron uptake pathways and antibiotic resistance genes (55).

**SCC*mec***. In terms of clinical significance, the most important MGE may be the staphylococcal cassette chromosomes (SCC) that carry the *mecA* gene encoding methicillin resistance. SCC*mec* elements have been classified into at least 11 types, each with different combinations of *ccr* genes that catalyse excision and integration of the element into the chromosome (<http://www.sccmec.org>) (60). Integration only occurs at one chromosomal site, *orfX*, and each bacterial cell has either zero or one copy, ranging in size from 3 – 70kb (61). Early SCC*mec* tended to be larger and carry multiple resistance genes, while the smaller and streamlined SCC*mec* type IV is probably now the most successful and widely distributed type. While SCC*mec* regions can transfer between isolates, they appear to be more stable than other MGEs in clonal MRSA. The acquisition of an SCC*mec* by a sensitive *S. aureus* appears to be the first step in the evolution of MRSAs with unique epidemiology (36) (62) (63) (64) and the ability of MRSA clones to subsequently spread in specific environments, particularly healthcare, community and livestock, and will be discussed below (AMR). Successful MRSA clones tend to have stable SCC*mec* elements.

**Plasmids**. Staphylococcal plasmids are circular supercoiled DNA molecules ranging in size from 1 to >60kb. They replicate autonomously and regulate their own copy numbers. Smaller plasmids replicate by the unidirectional rolling circle mechanism, while larger ones utilize the bidirectional theta mechanism. Plasmids are common with most isolates harbouring one or more and strains with up to four are found. Plasmids carry a large proportion of antimicrobial resistance genes in *S. aureus* (59).

**Transposons**. Transposons are MGEs that encode transposases, capable of catalysing replication and/or excision of the transposon, followed by integration into a new insertion site on the genome. Insertions can be site-specific or random, and can be located on the chromosome, plasmid or another MGE. In staphylococci, transposons are particularly associated with antimicrobial gene carriage (59), especially penicillin, macrolides, tetracycline and vancomycin.

**Insertion sequences**. Insertion sequences (IS) encode only a transposase, but two IS can form a composite transposon and transfer the DNA between them, including resistance genes (73). IS such as IS256 can insert into coding or regulatory regions of the genome, altering gene function and expression. An example is IS256 that affects biofilm expression in S. epidermidis (74).

**Horizontal gene transfer (HGT)**

Horizontal gene transfer requires several steps for success. Firstly, DNA must transfer from one cell to another, it must find a way to replicate or integrate, evade host defence mechanisms, and not cause a fitness cost to the new cell (75). DNA can transfer between bacterial cells by transformation, conjugation or transduction. In *S. aureus* transformation is inefficient (76) and probably rare. While conjugation is functions effectively in staphylococci, the distribution of conjugative genes necessary for transfer is relatively constrained, suggesting conjugation is not the major driver of HGT in *S. aureus*. In contrast, bacteriophage are widespread in *S. aureus* genomes and HGT via transduction is efficient and widespread, probably the most common mechanism (75).

Transduction involves DNA transfer via bacteriophage packaging and delivery of DNA, such as transfer of phage DNA between cells. Generalized transduction occurs when host bacterial DNA is packaged into the phage particles, which are released by lysed cells and the non-infectious particles inject DNA into new bacterial hosts. Packaging can be dependent on pac-sites or cos-sites scattered through the genome (Chen Pathogenicity island directed transfer of unlinked chromosomal virulence genes) (Quiles-Puchalt 2014 Staphylococcal pathogenicity island DNA packaging system involving cos-site ), and packaging of host DNA is not accidental but dependent on environmental conditions such as antibiotic exposure (77). Some host DNA is packaged at higher frequency, particularly plasmids, which may be a reflection of copy number or concatemer formation (78). SaPIs are preferentially packaged into phage particles by targeting the terminase packaging proteins of the phage directly (79). Phage heads typically hold a maximum of 45kb of DNA, yet some larger MGEs appear to have been transferred by generalised transduction, perhaps by multiple transfer events (80).

Once DNA is delivered into the recipient cell, it needs to find a way to replicate in order to survive the next round of bacterial cell division. Plasmids replicate autonomously as circular DNA. Most other MGEs have a specific mechanism for integrating into the chromosome or a plasmid, using integrases or transposases. These may be site-specific or random integration events. In contrast, a chromosomal piece of DNA that enters a recipient cell without a mechanism of replication or integration relies on homologous recombination with the chromosome (81). In *S. aureus* this is an inefficient process.

Transduction of MGEs is highly efficient in colonising populations of bacteria. In a controlled evolution experiment in four gnotobiotic piglets, plasmids were shown to transfer between co-inoculated isolates within four hours (82). Multiple phage and plasmids transfers occurred continuously during 16 days of colonisation resulting in heterogeneous colonising populations that varied enormously in MGE content (82). Gene transfer appeared to localized to the bacterial populations within each of the four piglets, despite their close physical contact of the animals and opportunity for strains to spread (82). Similarly, in human MRSA carriers, free generalized transducing phages are detectable, and clonal colonising populations in individual hosts often vary in MGE and antimicrobial resistance gene content indicating frequent gene transfer (83). These studies also suggest loss of MGEs occur, although the mechanisms are not understood.

Since high level MGE transfer occurs within the host and MGEs are also lost within the host, it is expected that variation in MGE carriage is extensive in clonal populations isolates from multiple hosts. Since epidemiological studies typically analyze only one colony or isolate from each host, only a sampling of this variability is detected in studies investigating MGE distribution. This limits our ability to make predictions about the distribution and importance of virulence and AMR genes. However, genomic trees generated to investigate epidemiology of successful MRSA clones typically identify frequent acquisition and loss of the same or similar MGEs (44) (62) (84), suggesting they are widespread amongst the population and undergoing their own evolutionary adaptation.

There are several barriers to HGT that protect bacteria from phage DNA thats otherwise might lyse and destroy populations. Firstly, prophage produce regulators that prevent phage excision and also replication of incoming related phage and therefore protect the cell from lysis (58). The second barrier is restriction-modification (RM). The type I Sau1 system is widespread and lineage specific, ensuring that DNA transferred from one bacterial cell to another of the same lineage is recognised as ‘self’ due to specific modification and protection of target site sequences (49). Whereas DNA transferred from one bacterium to another of a different lineage is recognised as ‘foreign’ by its unprotected DNA target sites and digested with restriction enzymes (58) (49). Additional RM systems are also found, such as the type II, III and IV systems (75) and many of these genes are encoded on MGEs themselves, protecting host bacteria as well as protecting themselves from other MGEs. Clustered, regularly interspaced short palindromic repeat (CRISPR) systems were first described in S. epidermidis (85) but are very rare in *S. aureus* (86) (75). CRISPRs are an adaptive immunity system for bacteria, as they incorporate segments of invading DNA, which they recognize and target for digestion by CRISPR-associated (Cas) endonucleases on the next encounter (87).

Staphylococci co-evolve with some MGEs, with each providing advantages and disadvantages to each other. For example, resistance to phage infection by host receptor modification prevents lysis by phage (an advantage to the cell) but also HGT by that staphylococcal phage (a disadvantage to the cell)(88). The phage itself requires a host to replicate, driving adaptation to respond to altered host receptors. Plasmids and phage can also evolve to expand their host range. Plasmid immunity (incompatibility) occurs when two plasmids share the same replication mechanism ensuring that after several rounds of cell division, one plasmid tends to be lost (89). Plasmids have therefore recombined extensively so that related genes are carried on plasmids with different replication genes (57). Interestingly, some large conjugative plasmids have overcome type I RM barriers by removing restriction target sites from their genomes, freeing their transfer between certain lineages (50).

The high efficiency of MGE HGT, inefficiency of transformation and homologous recombination, and the distribution of barriers to gene transfer markedly shapes the genome of *S. aureus* and the population structure in comparison to other species. As a result of these mechanisms, the *S. aureus* lineages are distinct and stable, while the MGEs encode most of the adaptive traits and can be highly mobile and unstable, while those MGEs that maintain stability in successful clones are likely to be important and continuously selected.

**Selection and adaptation**

Evolution occurs when changes in the genome are selected for and variants outcompete established populations and may move into new niches. MGEs that encode host-adaptive or antimicrobial resistance markers are particularly involved in evolution of successful clones that can then expand and spread. Considering how rapidly MGEs can be transferred between isolates and lost, the stable presence of an MGE is taken as an indicator that it plays an important role in adaptation.

***Virulence and pathogenicity***

*S. aureus* produce dozens of known and putative virulence factors, including toxins, immune evasion proteins and adhesins. Regulation of these virulence processes is controlled by a multitude of gene regulators that respond to environmental conditions and ensure efficient expression at the appropriate time and place. Despite many virulence factors being encoded on MGEs, there are few cases where presence of a single virulence factor has been associated with evolution and adaptation. Since the normal niche of staphylococci is not the diseased host but the normal skin and mucous membranes, we can imagine the ability to cause disease may not neccessarily provide the bacteria with a selective advantage during colonisation. Early studies suggested that the presence or absence of virulence factor genes on MGEs was similar in both colonising and infecting isolates (44). For example, the role of the immune evasion cluster genes on 3 phage appears to be associated with successful human clones, but is also common in colonising isolates rather than being specifically selected in infection-causing isolates. Another interesting example is the Panton Valentine leukocidin, associated with severe skin and soft tissue infection, where the toxin may play a role in disease, but is also relatively stable in colonising populations (114) where perhaps it reduces clearance by neutrophils in the nose. A third example is the sasX gene which is carried on the SPb-like prophage in the successful ST239 HA-MRSA clone and encodes a surface protein for adherence to nasal epithelial cells (148) (36).

More recently, SNPs in genes associated with virulence were detected with a 3.1-fold enrichment in infecting versus nose-colonizing bacteria from the same host (149). This suggests these particular mutations arose and were beneficial to the bacteria during the pathogenic process. In particular, genes associated with the regulation of virulence were variable, such as the accessory gene regulator, agr (150) (149). While these regulators control virulence in mouse models of infection (Mayville 1999; Ram 2018 ), they are mutated in some human clinical isolates, while in others they may play a role in adaptation of colonising isolates as the infection develops (151) (152). In contrast, no differences were detected between isolates causing bacteremia versus endocarditis (153). Bacteremia isolates were shown to be less toxic to eukaryotic cells *in vitro* compared to non-bacteremia isolates, and genome wide association studies suggested that multiple loci could be involved (154).

Surprisingly, successful clones can maintain mutations in common virulence factors. The successful CA-MRSA USA300 do not produce capsular polysaccharide due to multiple mutations in the capsular operon (155). The HA-MRSA have mutations in the alpha hemolysin gene and agr (De Leo 2011). Extensive redundancy in the staphylococcal genomes, where multiple genes can encode for similar functions, may account for the lack of a virulence Achille’s heel. Unfortunately, this reduces options for therapeutics targeting a single virulence factor (156).

***Antimicrobial Resistance***.

Genomic analysis demonstrates that antimicrobial resistance is the most important evolutionary adaptation of staphylococcal populations. In particular, MRSA carry SCC*mec* elements which encode the genes necessary to resist nearly all types of -lactam antibiotics. MRSA cause an additional burden of disease, not just a replacement for S. aureus infection.

Since -lactam antibiotics are the most successful antibiotic class, effective, inexpensive, easy to tolerate and with limited side-effects, new -lactam antibiotic variants have continually been developed by pharmaceutical companies to evade bacterial resistance mechanisms (9). MRSA are only susceptible to ceftaroline and ceftobiprole, which dramatically reduces the types of antibiotics that can be effectively used for prophylaxis (prevention) and empirical therapy (treatment prior to diagnosis). The *mecA* gene carried on the SCC*mec* encodes an alternative penicillin binding protein PBP2A, allowing synthesis of cell wall peptidoglycan in the presence of -lactams that block a crucial enzyme PBP2 (103). B-lactams are widely used by humans and in agriculture, and hosts exposed to -lactams provide an advanteous niche for MRSA over other methicillin-susceptible colonising microbes. Once colonising successfully, MRSA acts as an opportunistic pathogen. Since there are only a relatively small number of successful MRSA clones and they cause significant concern in healthcare, they are often screened for, reported and their epidemiology and spread investigated. Therefore, we investigate the evolution of these clones more than any other staphylococci (47).

In epidemiological terms, there are three main types of MRSA, each evolving on multiple occasions and each with unique features of their genome (104). They are healthcare associated (HA-)MRSA, community associated (CA-)MRSA and livestock associated (LA-)MRSA. Each type has emerged in multiple lineages, demonstrating that lineage is not the major factor responsible for the unique epidemiology. Instead, there are common MGE types associated with each MRSA type, and these MGEs are relatively stable in the clonal population, indicating repeated selection for the MGEs for survival in the respective epidemiological niche (47). In each geographical location and niche, only one or two MRSA clones tend to dominate.

HA-MRSA have emerged in lineages CC5, CC8, CC22, CC30, CC45, CC59, CC80 and ST239. Each lineage carries an SCCmec type, usually type II, III or IV. Interestingly, ST239 evolved by a large recombination event between CC8 and CC30, where a large region of the genome containing the SCCmec region of HA-MRSA CC30 swapped into a CC8 lineage (105) (106). HA-MRSA clones also carry the 3 human host adapted bacteriophage, thought to be important for human colonisation. Successful HA-MRSA are often multi-drug resistant, and a particular association between HA-MRSA and fluoroquinolones has been identified. In the CC22 HA-MRSA clone, mutations in *gyrA* and *parC* genes seem to have been essential for the successful dissemination (62). In the UK, usage of fluoroquinolones appeared to select for HA-MRSA, and a decrease in usage correlated with a decrease in hospital infection incidence with HA-MRSA (107). This is consistent with exposure to fluoroquinolones being associated with increased HA-MRSA colonisation in Europe, Asia and the USA (108)(109) (110) (111). Resistance to aminoglycosides, macrolides, and others is also associated with HA-MRSA, but resistance rates are more variable within and between geographical locations (104), possibly due to different selective pressures.

CA-MRSA have evolved in lineages CC1, CC8, CC30 and CC59. CA-MRSA cause severe skin and soft tissue infection in healthy patients, often children, military, athletes, prisoners and those in close contact with others. Successful clones carry both an SCCmec and the 3 human adapted bacteriophage with genes for evading the host immune response. Uniquely, they also carry the 2 bacteriophage encoding Panton-Valentine leucocidin. While the importance of this toxin was initially controversial in models of skin infection, it is now clear that PVL is host specific and targets human and rabbit neutrophils (94). Successful clones include the USA400 (CC1), initially identified in the USA but now less prevalent, the successful USA300 (CC8) clone in the USA and spreading globally, the original Southwest-Pacific clone (CC30) and the Asian clone (CC59)(112)(and Australian clone (CC93) (42). Antimicrobial resistance levels appear to be increasing (113), and fluoroquinolone resistance may also play a role in spread (114).

LA-MRSA have evolved in lineages CC398 and CC9 and are associated with pig and veal-calf agriculture. The isolates do not cause significant disease in these animals, but can colonise the humans that come into contact with farms, and the organisms can cause subsequent opportunistic infection. Interestingly, the isolates do not spread in hospitals as effectively as HA-MRSA (115) and colonisation of farmers requires repeated exposure (116). CC398 isolates were originally considered untypable due to a modification of the SmaI restriction site that is lineage specific (117)(51). These successful clones are prevalent in mainland Europe and spreading to the USA and South America have acquired SCCmec elements but not 3 with human-host specific immune evasion genes. While the reasons for their adaptation to pigs is unclear, they typically encode resistance to tetracycline antibiotics (118). Tetracyclines are widely used in agriculture, but used much less frequently in hospitals and tetracycline resistance is relatively uncommon in hospital *S. aureus*. CC398 tetracycline resistance is due to genes carried on plasmids and/or transposons, and up to three distinct mechanisms in a single isolate identifed (118). CC9 isolates in Asia have been reported to be multi-drug resistant (119) (120). A recent increase in CC398 MRSA infection in humans with no contact to livestock has been attributed to higher carriage of this clone in the community and the acquisition of 3 host-adaptation genes (121).

Genomic analysis has also revealed that other resistances can be selective. For example, in New Zealand, high usage of fusidic acid in the community has driven the evolution and selection of three different MRSA clones that carry fusC resistance gene (122). However, not all resistances are selective. Vancomycin and other glycopeptides are widely used to prevent and treat MRSA infection, yet resistance is still rare. Only 14 cases of fully vancomycin-resistant MRSA have been described in the USA. They have evolved independently of each other due to the acquisition of the *vanA* operon on a transposon from vancomycin resistant enterococci (123). The VRSA are unfit compared to their MRSA parents, and are susceptible to both antibiotics in combination (124). VRSA have been reported in India, Brazil, Portugal, Turkey and Iran (125) (126) (127) (128) (129), but are still rare and there is little evidence of patient to patient spread. In contrast, intermediate-level vancomycin resistance in MRSA (VISA) are more widely reported, and these bacteria have thickened cell walls that absorb excess antibiotic, slightly reducing susceptibility. Populations are often hetero-resistant with only a small subpopulation showing the VISA phenotype (130). These isolates probably have limited ability to resist vancomycin in the clinic and associations between laboratory susceptibility and response to therapy are unclear (131). VISA does not appear to be a clonal trait associated with successful spreading clones. Instead, VISA are generally isolated from patients with chronic infection and exposed to long term antibiotic therapy, and they arise due to SNV variation (131)(130). It can be concluded that despite substantial selective pressure, *S. aureus* have not yet been able to develop resistance to vancomycin that allows maintenance of fitness in its normal habitats and spread to new hosts.

***Host***.

Genomic analysis of isolates from different hosts, particularly animal versus human, have identified key pathways that are associated with adaptation to these hosts. Evasion of host innate immune responses is central to survival of *S. aureus*, particularly escape from complement and neutrophil killing (48). Particular lineages are often associated with specific hosts, with each lineage carrying different combinations or variants of immune evasion genes particularly on the genomic islands, such as inhibitors of complement cascade or antibodies, neutrophil signalling, migration and recognition and cytolytic toxins (48).

In addition, host specificity can be strongly associated with MGEs. Human isolates typically carry the 3 prophage with at least two of three genes at one end that are associated with evasion of the human immune response, while animal isolates are usually missing the phage (90) (14). These genes are the staphylococcal complement inhibitor (*scin*) which blocks activation of the human complement system (91), chemotaxis inhibitory protein (*chips*) that prevents human neutrophil recruitment (92), and staphylokinase (*sak*) which activates human plasminogen and local spreading (93). Interestingly, the enterotoxin A (*sea*) gene, encoding the most common cause of staphylococcal food poisoning is also carried on 3 phage adjacent to the immune evasion genes, but is missing in many human isolates suggesting frequent recombination of this phage region and that *sea* is not required for host adaptation (14). The specific role of the 3 phage in selection is most likely to be a requirement for survival in the normal colonising habitat of the human nose, suggesting an active neutrophil response targeting *S. aureus* during colonisation. It is unclear why only about 25% of humans are continuously colonised with *S. aureus* while others are colonised intermittently or not at all.

Leukocidins are two component pore-forming toxins and function by binding to host-specific receptors on the surface of target cells (95) (96). One of these, Panton Valentine leucocidin in targets human neutrophils (94) and is carried on a 2 phage. While only a small proportion of human *S. aureus* carry PVL toxin, it has been implicated in rare lethal necrotizing pneumonitis (Gillet Etienne 2002), as well as severe skin and soft tissue infection and the success of CA-MRSA clones.

*S. aureus* are a common cause of bovine mastitis, causing economic losses in the dairy industry. Unique lineages are associated with bovine isolates (14). A novel SaPI found in bovine isolates coagulates ruminant plasma only (23 97). The leukocidin toxin LukMF is carried on a 1 phage in ruminant isolates only (48) and targets bovine neutrophils and immune cells (95).

In horses, a specific phage Saeq1 is associated, encoding a novel inhibitor of complement from horses, humans and pigs, eqSCIN (98). Isolates from chickens often carry avian adapted phage and plasmids, putatively involved in lysing chicken heterophils (99). In both wild mice and laboratory mice, *S. aureus* colonising isolates have lost 3 and immune evasion cluster genes (100).

A unique situation occurs in rabbit *S. aureus*, as host-specificity can be conferred by a single naturally occurring nucleotide mutation in the *dltB* gene altering teichoic acid and lipoteichoic acid charge (101), a receptor commonly used by bacteriophage and for HGT (102).

***Microbiome and competing bacteria***

The normal habitat of staphylococci is the skin and mucous membranes of animals and birds, and this is the reservoir of infecting isolates. During colonisation a variety of adapted bacteria compete for space and nutrients (132). Competition and co-evolution of these bacteria can result in direct inhibition, synergistic co-operation and/or gene exchange. Environments can also change in nutrient availability, humidity, immune responses, infection with viruses, or antibiotic exposure (132). The microbiome is constantly adapting, and the ability of staphylococci to adapt to these environments is vital for survival.

In the absence of a microbiome, such as a gnotobiotic animal model, *S. aureus* inoculated onto a single skin site can colonised the whole host within hours (82), presumably due to the lack of competing bacteria. However, once colonised, hosts in close contact exchange strains infrequently (82). Antimicrobials can also deplete microbiomes. In a mouse model of colonisation, those treated with streptomycin were more likely to be colonised and colonised with greater numbers of bacteria (133). Humans exposed to antimicrobials that *S. aureus* are resistant to are more likely to be colonised (134).

Nutrients are also important. *S. aureus* survive better on the nutrients available in the human nose than coagulase-negative staphylococci (135), accounting for their different niches.

During evolution, a staphylococcal cell that has altered its genome by SNV or MGE acquisition must compete with not only the microbiome, but with its peers that have not altered their genome. The relative fitness of an evolving isolate can be compared to its peers, but its ability to survive depends on environmental factors.

Competition between microbiome species can be for nutrients and space or can be targeted. *Staphylococcus lugdunensis* colonising the human nose produces lugdunin, a peptide antibiotic that inhibits *S. aureus* colonisation (136). Some *S. aureus* also produce lantibiotics that target other *S. aureus* isolates, staphylococcal species such as *S. epidermidis*, and lactococci (137)(138), and staphylococcal species and lactococci, in turn, are capable of producing inhibitory lantibiotics against *S. aureus* (139) (140). Corynebacteria spp., Haemophilus spp. and streptococci also inhibit *S. aureus* colonisation (141) (142) (143).

In the cystic fibrosis lung, *S. aureus* has a negative correlation with *Pseudomonas aeruginosa* which eventually displaces it. *P. aeruginosa* produce a range of antistaphylococcal factors, but most interestingly, may also activate the host to clear *S. aureus* specifically by inducing a phospholipase (144)(145). Within a *C. elegans* worm model, co-infection with *Enterococcus faecalis* caused *S. aureus* to evolve to produce less siderophore for iron scavenging (146), while *E. faecalis* evolved to produce more superoxide antimicrobial to combat *S. aureus* (147).

**Summary**

Staphylococci live on the skin and mucous membranes of mammals and birds and have adapted to this environment. They are formidable opportunistic pathogens, carrying multiple colonisation and virulence factors that are often variable, redundant and highly mobile. This genetic variability contributes to their ability to adapt to changing hosts, antimicrobial exposure and bacterial competitors, expanding their niches and ability to cause opportunistic infection. New and successful clones with unique epidemiology teach us about genetic adaptability and the selective pressures in different environments. As we improve our understanding, we improve our ability to predict the next wave of multi-drug resistant staphylococci in new host populations and identify opportunities to reduce their impact on health, wealth and welfare.

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**Figure 1.**

Fig 1 from <https://www.pnas.org/content/101/26/9786/tab-figures-data>.