Genomics of Natural Populations of *Staphylococcus aureus*

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Abstract

Staphylococcus aureus is a major human pathogen and an important cause of livestock infections. The first *S. aureus* genomes to be published, 15 years ago, provided the first view of genome structure and gene content. Since then, thousands of genomes from a wide array of strains from different sources have been sequenced. Comparison of these sequences has resulted in broad insights into population structure, bacterial evolution, clone emergence and expansion, and the molecular basis of niche adaptation. Furthermore, this information is now being applied clinically in outbreak investigations to inform infection control measures and to determine appropriate treatment regimens. In this review, we summarize some of the broad insights into *S. aureus* biology gained from the analysis of genomes and discuss future directions and opportunities in this dynamic field of research.

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STAPHYLOCOCCUS AUERUS: A VERSATILE AND WIDESPREAD PATHOGEN

Staphylococcus aureus is a notorious bacterial pathogen of humans and domestic animals. Because of its public health and economic importance, it is one of the most intensively studied microorganisms. *S. aureus* was first identified and characterized by Sir Alexander Ogston in 1880 in pus from contaminated surgical wounds (64). Another 80 species or subspecies belonging to the *Staphylococcus* genus have been described (as listed by the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures; **https://www.dsmz.de/bacterial-diversity/prokaryotic-nomenclature-up-to-date/prokariotic-nomenclature-up-to-date.html**), most having limited clinical importance. Although normally a commensal bacterium, inhabiting the nasopharynx, throat, and intestinal tract of approximately 30% of humans, *S. aureus* is the most clinically important staphylococcal species and is responsible for a wide spectrum of disease types and severities (89). Disease manifestations range from relatively benign skin infections such as boils and carbuncles to severe skin infections such as impetigo and staphylococcal scalded skin syndrome to life-threatening invasive infections including necrotizing pneumonia, osteomyelitis, and infective endocarditis (83).

A notable feature of *S. aureus* is its ability to cause disease in a range of physical settings. Clones of *S. aureus* have emerged in recent decades that cause infections in both health care and community settings, acquiring specific traits that facilitate colonization and infection in different environments with distinct selective pressures. Although health care and community clones have traditionally been viewed as niche specific and often been considered as discrete entities, the distinction is blurring with evidence for increasing transmission between these settings (75, 81).

Of particular importance is the capacity of *S. aureus* to acquire resistance to antimicrobials and antiseptics, resulting in infections that are refractory to treatment and that persist in hospitals and care centers (81).

In addition to setting-specific adaptation, another distinguishing feature of *S. aureus* is its capacity to colonize and cause opportunistic infections in an array of animal species beyond humans (68). In particular, livestock-associated *S. aureus* strains have emerged in recent decades that have a major economic impact on productivity in the agriculture industry (20). For example, *S. aureus* is a common cause of intramammary infection leading to bovine mastitis that results in substantial losses in the global dairy industry (6), and intramammary infections of lactating sheep and goats are of economic importance to cheese production (7). *S. aureus* is also a common cause of infection in broiler poultry, causing joint infections such as bacterial chondronecrosis with osteomyelitis leading to lameness that causes economic losses (96). Epidemics caused by *S. aureus* are a major blight on rabbit farming and may require a complete cull of all stock (28). *S. aureus* and its subspecies can also be isolated from companion animals such as cats and dogs (50, 51) and a wide array of wild animal species, including monkeys and bats (1, 72). Taken together, these findings make it clear that *S. aureus* is a tremendously versatile pathogen with the capacity to adapt to different environmental and anatomical niches in an array of host species.

In this review we describe the genome perspective of *S. aureus* that has emerged in recent years with the advent of high-throughput sequencing approaches. In particular we focus on the knowledge gained from sequencing large numbers of isolates, which has provided insights into the genetic variation and mechanism of evolution of natural populations of *S. aureus* in different hosts (**Figure 1**). In addition, the high-resolution differentiation of bacterial strains afforded by whole-genome sequencing (WGS) has allowed clinical applications with regard to the tracing of outbreaks and prediction of antibiotic sensitivity (**Figure 2**).

DIFFERENTIATING *S. AUREUS*

Traditionally, phenotypic methods such as phage typing and protein profiling were used to investigate the variation within populations of *S. aureus*, with rather limited success (82). Early *Staphylococcus* taxonomists including Meyer, Hajak, and Marsalek defined staphylococcal biotypes, which correlated loosely with their host species association based on several phenotypic markers, including coagulation of human and bovine plasma, production of fibrinolysin, crystal violet reaction type, beta hemolytic activity, and phage susceptibility (30, 58). These approaches demonstrated some success at providing discrimination and order across the staphylococcal genus, particularly with regard to host species associations. In 1980, Kloos (44) published a review article in *Annual Review of Microbiology* summarizing the state of understanding of staphylococcal diversity in the context of the known host species. This seminal article demonstrated remarkable insights that were inferred from very basic phenotypic testing, and many of the conclusions drawn have stood the test of time and the rigors of sophisticated modern sequence-based analyses (44).

In subsequent decades a series of molecular typing techniques were developed that have provided increasing resolution for distinguishing *S. aureus* isolates and understanding population structure. Multilocus enzyme electrophoresis (MLEE) allowed inference of allelic variation among *S. aureus* strains based on electrophoresis of housekeeping enzymes with varying charge (42, 61), before DNA-based molecular approaches such as multilocus sequence typing (MLST) allowed direct measurement of genetic diversity between strains of the same species (54). MLST involves sequencing fragments of seven housekeeping genes found in all strains of the species. The combination of allelic variants is used to assign a sequence type (ST), and STs that share alleles at ≥ 5 loci are considered to belong to the same clonal complex (CC). MLST has been instrumental in

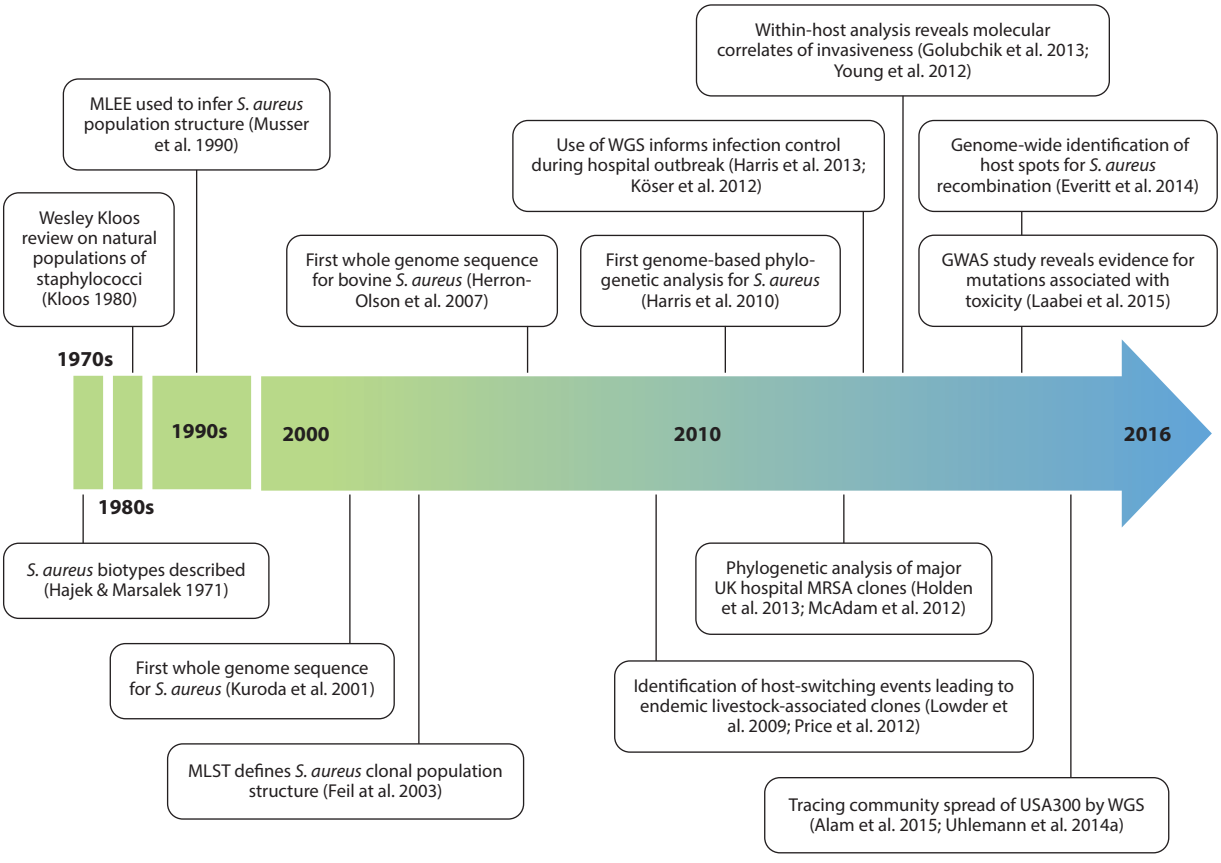

Figure 1

Chronology of discovery in *S. aureus* population biology. Abbreviations: GWAS, genome-wide association study; MLEE, multilocus enzyme electrophoresis; MLST, multilocus sequence typing; MRSA, methicillin-resistant *Staphylococcus aureus*; WGS, whole-genome sequencing.

revealing the population structure of *S. aureus* (15, 19) and has provided insights into the molecular genetic processes and events that have shaped the evolutionary history of the species (see section on Future Perspectives for Population Biology of *S. aureus*).

THE GENOME OF *S. AUREUS*

In this day and age of the $50-bacterial genome, we take for granted the genomic perspective of staphylococci that underpins our understanding of this organism. This is in part due to the intensity of *S. aureus* sequencing efforts, resulting in a wealth of sequences being deposited into public nucleotide sequence databases, and has revealed a rich genomic landscape.

The first staphylococcal genomes to be published appeared in a groundbreaking article by Kuroda et al. in 2001 (46), which described the complete genomes of two methicillin-resistant *S. aureus* (MRSA) strains. At the time this was a major achievement, revealing insights into the genome structure and content of *S. aureus* and making genomic comparisons between two strains sequenced in parallel. The comparative genomic analysis that was performed set a trend that

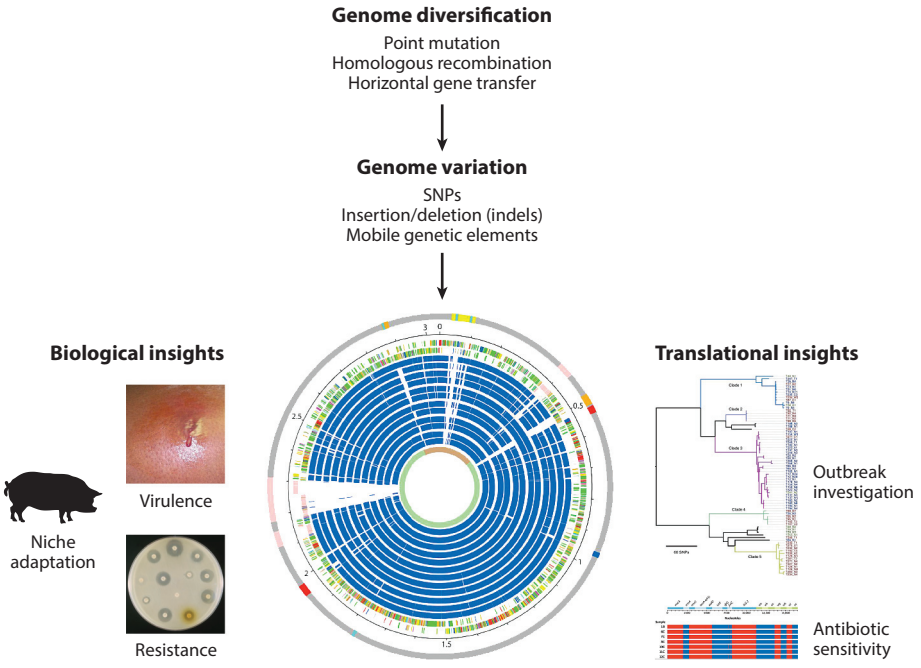

Figure 2

Comparison of *S. aureus* genomes has provided broad biological and translational insights. Images obtained with permission. Antibiotic sensitivity schematic diagram from *New England Journal of Medicine*: Köser CU, Holden MT, Ellington MJ, Cartwright EJ, Brown NM, Ogilvy-Stuart AL, Hsu LY, Chewapreecha C, Croucher NJ, Harris SR, Sanders M, Enright MC, Dougan G, Bentley SD, Parkhill J, Fraser LJ, Betley JR, Schulz-Trieglaff OB, Smith GP, Peacock SJ. Rapid Whole-Genome Sequencing for Investigation of a Neonatal MRSA Outbreak, Volume 366, Page 2272. Copyright © (2012) Massachusetts Medical Society. Reprinted with permission. Circular genome map from Reference 38. Outbreak investigation phylogenetic tree from Reference 84. Skin abscess and antibiotic sensitivity plate images courtesy of CDC, Bruno Coignard, and Jeff Hageman.

continues to this day, whereby a genome's content is placed in a wider context of comparison to other strains, thus providing insights into evolutionary events shaping genomes. This has proved to be a fruitful approach in staphylococci to investigate the genetic basis of host adaptation and the emergence of successful pathogenic and/or drug-resistant clones.

Kuroda et al. (46) compared the complete genomes of two clinical isolates that belonged to the same MLST clone, ST5. These health care–associated MRSA (HA-MRSA) isolates were N315, an isolate from a pharyngeal smear, and Mu50, a pus isolate from a surgical wound infection that had not responded to vancomycin treatment. Aside from the unprecedented view of the genomic inventory, the study revealed a remarkable arsenal of virulence determinants, and the contribution of lateral gene transfer in shaping the genomes. This latter observation was particularly pertinent for antibiotic resistance, given the ability of *S. aureus* to rapidly evolve resistance after the introduction of new antibiotics into clinical practice (43).

The general features and properties of the *S. aureus* genome first described by Kuroda et al. have proven to be remarkably consistent across the species as a whole. Subsequent sequencing has revealed that in a comparison of any two *S. aureus* isolates, most of the variation distinguishing them will be associated with mobile genetic elements (MGEs). *S. aureus* MGEs encompass diverse

types of elements, including staphylococcal cassette chromosome (SCC) elements, bacteriophages, transposons, integrative and conjugative elements (ICEs), insertion sequences (ISs), *S. aureus* pathogenicity islands (SaPIs), and plasmids (**Figure 2**). In addition, a limited number of loci in the chromosome contain variable sets of genes that do not have obvious origins in MGEs, based on the genes associated with them, but are predicted to have arisen by lateral gene transfer and recombination. These genomic regions typically contain arrays of genes that vary in complement in a strain-dependent manner and that encode exotoxins or other proteins involved in contingency functions (e.g., genomic islands νSaα and νSaβ) (21, 41, 46).

THE GENOMIC LANDSCAPE OF *S. AUREUS*

In the decade following the Kuroda et al. (46) study, additional complete *S. aureus* genome sequences were published, representing a diverse range of geographical, clinical, and host origins. The dominant group represented comprised human disease isolates from the major epidemic lineages, which in many cases were drug resistant. Consequently, much of the comparative genomic analysis focused on the genetic basis of pathogenicity and antibiotic resistance. Following the first genome publications, Baba et al. (5) published the genome of MW2, a high-virulence community-associated MRSA (CA-MRSA) originally isolated in the United States and belonging to CC1. In contrast to the HA-MRSAs N315 and Mu50, previously described by Kuroda et al. (46), MW2 exhibited resistance to fewer antibiotics. Comparative analysis revealed that MW2 contained an SCC element carrying the methicillin-resistance gene *mecA* (SCC*mec*), which was smaller than those found in the HA-MRSAs, and did not contain additional resistance determinants. Their analysis also demonstrated that MW2 contained an enhanced complement of toxins and virulence determinants found in genomic island regions or on MGEs. These included the prophage-located *lukF* and *lukS* genes, which encode Panton-Valentine leukocidin (PVL), a potent virulence factor linked to skin and soft tissue and infections (SSTIs) and severe disease. In addition, *ear*, *sel2*, and *sec4* were encoded on a SaPI, and the rest of the novel toxins were found in the νSaα and νSaβ genomic island regions. Baba et al. (5) noted that although MW2 contained an increased complement of virulence determinants, no single determinant was likely to be responsible for its increased virulence, which was due to the synthetic contribution of many virulence genes. Because many of these were carried on MGEs, the authors concluded that the pathogenic potential of each strain of *S. aureus* was not an intrinsic trait; instead, it was strongly influenced by lateral gene transfer between members of the same species (5).

Subsequent to these studies, Holden et al. (36) published the genomes of two clinical isolates: MSSA476, a methicillin-sensitive *S. aureus* (MSSA) representative of the community-associated lineage to which MW2 belongs; and MRSA252, an HA-MRSA that was representative of EMRSA-16, a successful epidemic clone in the United Kingdom. As previously, analysis of the genomes highlighted the role that MGEs play in accessorizing the genomes with novel determinants, but comparison of MSSA476 with MW2 revealed diversification over a relatively short time. Both MSS476 and MW2 belong to ST1, and an examination of their genomes revealed that they are distinguished by only 285 single-nucleotide polymorphisms (SNPs) within shared or core protein-coding regions (CDS). In contrast to the relatively small amount of SNP variation, there were a substantial number of differences in the MGE complement found in each isolate. At least five separate MGE acquisition or loss events distinguished these isolates and resulted in marked differences in key antibiotic resistance and virulence repertoires. In particular, MSSA476 was sensitive to methicillin but resistant to fusidic acid, whereas MW2 was resistant to methicillin but sensitive to fusidic acid; and MW2 contained the toxin genes *ear*, *sel2*, and *sec4* located on

SaPI3 and the PVL toxin genes *lukF* and *lukS* encoded on a ϕSa3 prophage, whereas MSSA476 lacked these toxins (36).

GENETIC VARIATION AND EVOLUTION OF THE *S. AUREUS* GENOME

Initial forays into the genomics of *S. aureus* led to the design of whole-genome microarrays that allowed the genome-scale interrogation of all genes in the reference strain represented on the microarray (22, 98) at a relatively low cost. These studies led to the first identification of core (conserved) and accessory (strain-dependent) components of the genome, with approximately 78% estimated to be shared among strains, and provided new insights into the genomic diversity and evolution of natural *S. aureus* populations (22). Since then, and particularly in the last five years, many thousands of *S. aureus* isolates have been sequenced using an array of different platforms designed for rapid, inexpensive, high-throughput sequencing, providing the opportunity for comparative genomics of large numbers of strains and high-resolution phylogenetic analysis. Such studies have provided broad new insights into the emergence and global spread of *S. aureus* epidemic clones; the source and routes of transmission of *S. aureus* during hospital outbreaks; and the molecular basis of *S. aureus* adaptation to different niches, including hospital and community settings and distinct host species.

MUTATION ON A MEASURABLE TIMESCALE

The first study to use next-generation sequencing (NGS) to investigate *S. aureus* diversity examined isolates from the blood of a patient undergoing treatment with the antibiotic vancomycin (62). Sequencing the first vancomycin-sensitive isolate and the last vancomycin-resistant isolate allowed Mwangi et al. (62) to identify 35 SNPs in 31 loci that distinguished the isolates. Sequencing additional intermittent isolates from the patient during chemotherapy revealed sequential mutations associated with increasing levels of resistance. One mutation associated with decreasing vancomycin susceptibility was in the *vraR* locus, which was also present in other vancomycin-resistant *S. aureus* strains. Importantly, this study demonstrated the within-host evolution of *S. aureus* in the face of strong selective pressure exerted by chemotherapy (62).

The capacity of high-throughput sequencing to investigate the population structure and transmission of a pandemic MRSA was demonstrated by Harris et al. (32), who examined a global collection of isolates belonging to ST239 using the Illumina (San Diego, California) sequencing platform and multiplex index tags for the sequencing libraries. Utilizing core genome SNPs and phylogenetic reconstruction, they were able to explore the evolutionary history of this successful pandemic clone and demonstrate its geographical structure and spread. The study provided the first insight into the genome-wide mutation rate, which was calculated as 3.3×10^{-6} substitutions per site per year, which equates to one SNP approximately every six weeks. Subsequently, mutation rates have been calculated for a number of other successful *S. aureus* lineages (37, 55, 63, 87). The rates range from 1.2×10^{-6} to 2.0×10^{-6} and indicate that ST239 is somewhat of an outlier in terms of its mutation rate. However, all of these mutation rates are in and around the range observed for other notable gram-positive bacterial pathogens, such as *Clostridium difficile* (between 1.47×10^{-7} and 5.33×10^{-7}) (18) and *Streptococcus pneumonia* (1.57×10^{-6}) (8). Significantly, this showed that *S. aureus* is evolving on a measurable timescale that can be captured by comparative genome sequence analysis. This has enabled the development of WGS for outbreak investigation, where the diversification of populations over relatively short timescales can be used to distinguish isolates and attribute transmission (18, 45, 63).

RECOMBINATION SHAPES THE *S. AUREUS* GENOME OVER LONG EVOLUTIONARY TIMESCALES

Another source of SNP variation in the *S. aureus* genome is homologous recombination. Studies with MLST have demonstrated that this species is clonal and that recombination is a minor contributor of genetic variation; estimates by Feil et al. (19) suggest that variation from point mutations was 15-fold more frequent than that from recombination. However, a number of genes and loci have been identified that exhibit variation proposed to arise by recombination (91, 92, 97). More recently, genome-wide studies have revealed evidence of recombination in the *S. aureus* genome, in particular, recombination driving change in regions of the genome associated with accessory elements (13, 17). Studying isolates from a global collection of a single clone of MRSA, ST239, Castillo-Ramírez et al. (8) demonstrated that the vast majority of recombination was restricted to accessory genes corresponding to MGEs and found rates of recombination in the core genome similar to those predicted from MLST data. This suggests that over relatively short periods (i.e., decades), during which sequence types diversify and spread, homologous recombination is not a major driver of genetic change. In contrast, with a population of *S. aureus* adequate to examine species-wide variation, Everitt et al. (17) were able to demonstrate considerable core genome recombination, suggesting a greater impact at the species versus the strain level. Additionally, they were able to show that there were hotspots of recombination near the insertion sites of MGEs and that there was a greater concentration of recombination nearer the origin of replication. Another feature of the *S. aureus* chromosome associated with recombination is the potential for large-scale DNA replacements leading to hybrid chromosomes in some strains. A number of sequence types have been identified as containing homologous recombination replacements of DNA fragments of several hundred kilobases. For example, in the case of ST239, the majority of the chromosome originates from an ST8 ancestor, but there is an ~600-kb region spanning the origin of replication that encompasses core and accessory regions and comes from an ST30 background (38, 71). Recently, Spoor et al. (80a) identified a bovine hybrid strain that demonstrates recombination-mediated alteration of its pathogenic properties, and Didelot & Wilson (12) described a 310-kb chromosomal replacement in ST582. The generation of these hybrid variants is thought to have arisen through single or multiple large-scale recombination events, although the precise mechanism remains unknown. Such events are considerably rare but may have dramatic effects on bacterial phenotype, leading to the emergence of new pathogenic clones.

GENOMICS AS A FORENSIC TOOL TO INVESTIGATE THE SPREAD AND TRANSMISSION OF *S. AUREUS*

Aside from the unprecedented view of the genetics of *S. aureus*, genomics has facilitated another revolution in understanding the biology of *S. aureus*—it has provided a fine-scale view of the transmission and global spread of this adaptable pathogen. WGS has been used as a high-resolution genotyping tool to investigate the epidemiology of infectious diseases and rule in, and also rule out, transmission events. The potential public health benefits of this approach have been seized upon, and the application of WGS as a clinical diagnostic and epidemiological tool for a range of pathogens is now well established, with much of the pioneering work focused on *S. aureus*.

The first study that highlighted the potential of WGS as a tool for tracing transmission in a hospital setting was that by Harris et al. (32). In their study of a global collection of ST239 isolates the authors included 20 isolates that originated from patients at a single hospital in northeast Thailand. These isolates were collected within seven months and were potentially linked via a chain of transmission. Phylogenetic analysis of the SNPs in the core genome resolved the

population structure not only at a global level, but also within hospital isolates; 5 of the isolates were differentiated by only 14 SNPs, and 4 of these isolates were obtained within 16 days. It was the combination of high-resolution genotyping with epidemiological linkage evidence that highlighted the potential of WGS as a powerful tool for transmission detection.

Translation of this potential was driven by technological developments in sequencing and the arrival of benchtop NGS machines, with their lower costs and smaller size. The first two studies to demonstrate the application of WGS for outbreak investigation were published by groups in Oxford and Cambridge (18, 45). In their study published in *BMJ Open*, Eyre et al. sequenced 24 isolates to investigate two MRSA clusters affecting patients in separate hospitals: Cluster 1 comprised eight MRSA from carriers and an associated bacteremia case in an intensive care unit (ICU), and cluster 2 included three wound site infections and two SSTIs from separate hospitals (18). In both clusters the isolates were indistinguishable by routine molecular strain typing (*spa*-typing and PFGE) but exhibited different antimicrobial sensitivity profiles, and within each cluster, the isolates were differentiated by a limited number of SNPs (four SNPs in the case of cluster 1, and one SNP in cluster 2), thus providing support for infections being caused by the same strain as part of the same outbreak in both clusters.

Hot on the heels of the Oxford group, Köser et al. (45) published an investigation of an MRSA outbreak that occurred on a neonatal intensive care unit (NICU) in Cambridge. In this retrospective study, isolates of a transmission cluster of epidemic MRSA-15 (EMRSA-15) from the NICU were sequenced; this included the index bacteremia case and carriage isolates from six other epidemiologically linked patients. In addition, seven other isolates were sequenced, including epidemiologically unlinked isolates from the NICU and contemporaneous MRSA isolates from elsewhere in the hospital. Phylogenetic analysis of the data provided support for the outbreak, which had previously been defined by routine microbiological and epidemiological surveillance, and also demonstrated that a hypermutator containing a mutation in the DNA-repair gene *mutS* could be considered part of the outbreak, despite the elevated number of SNPs that distinguished it from the other isolates (SNPs at 51 sites distinguished it from the rest of the outbreak clade, whereas the other 6 isolates were distinguished by SNPs at 41 sites). A cryptic transmission event was identified elsewhere in the hospital among the additional contemporaneous isolates that were sequenced; two isolates belonging to ST1 were 1 SNP different, and epidemiological investigation revealed that these were from two patients who were in adjacent beds for six days. Importantly, another significant benefit of WGS for clinical microbiology was demonstrated in this study, whereby the prediction of antibiotic sensitivities from WGS data was shown to be concordant with the phenotypic results generated in the laboratory (45). Subsequent studies have demonstrated >99% accuracy in in silico predicting antibiotic resistance (26, 37), highlighting the comprehensive nature of knowledge surrounding the genetic basis of antibiotic resistance in *S. aureus*, and also the potential of using WGS data for determining antibiotic sensitivities diagnostics.

The studies by Eyre et al. (18) and Köser et al. (45) heralded a powerful application of *S. aureus* genomics to successfully address previously intractable challenges of diagnosis and transmission in a hospital setting. The drivers for this have been the reduction in the cost associated with the sequencing, which makes WGS of an organism more economically viable (WGS now costs less than MLST), and the relative rapidity with which WGS can be generated (it is possible to go from colony to sequence data in less than two days, although five days is a more routine turnaround).

In the years following these landmark proof-of-principal studies, an increasing number of studies being published have used WGS to identify *S. aureus* outbreaks in a variety of settings and circumstances. These include both health care settings, where transmission was linked to

person-to-person contact (12, 13, 31, 52, 59, 63, 74, 84) or organ transplantation (3, 95); and community settings, where transmission occurred within households (2, 87, 88), during outbreaks in food (60), or between members of sporting teams (4). WGS has also been applied to reveal the propensity of *S. aureus* to spread between hosts, with the identification of zoonotic (34) and anthroponotic transmissions (66).

A study by Harris et al. (31) elegantly demonstrated how a strong synergy of genomic and epidemiological evidence could be used to unravel a complex outbreak and lead to clinical interventions to halt the outbreak. Originally established as a retrospective study of an MRSA outbreak on a special care baby unit (SCBU), it developed into a prospective study when new cases of MRSA carriage were detected on the ward after an MRSA-free period of more than two months. On the basis of the WGS data it was hypothesized that a member of staff may have continued to be colonized during the two-month period. As a result, over 150 members of staff were screened, and one had a positive test result for MRSA. WGS of colonies from this member of staff showed that they clustered among the outbreak isolates from the earlier and later outbreaks. Therefore, this member of staff received decolonization therapy, after which no new cases of MRSA related to this outbreak were detected on the ward. Another key finding of the study was that what started as MRSA transmission on the SCBU spread beyond the ward into the community, where the PVL-positive ST22 strain continued to cause SSTIs (31).

While the power of this technology to help identify transmission is often highlighted as its key public health benefit, it is arguable that its ability to rule out transmission is equally as important. In a study by Török et al. (85), a cluster of MRSA infections among patients on a hepatology ward were demonstrated to be unlinked. All of the patients were infected with *S. aureus* belonging to EMRSA-15, the most prevalent MRSA in the United Kingdom and in the rest of Europe (14, 27). The isolates from the patient were indistinguishable by routine typing techniques; therefore, these patients were deemed to have acquired the EMRSA-15 from transmission in the hepatology wards, based on epidemiological evidence of overlapping stays.

Most of the work published to date on the transmission of *S. aureus* is focused on person-to-person transmission; however, WGS has demonstrated transmission between hosts. Harrison et al. (34) examined two cases of MRSA in humans that were linked to transmission from farm animals. In these cases the MRSA contained the *mecC* determinant of methicillin resistance as opposed to the *mecA* variant, which is the most prevalent gene associated with MRSA. Lineages of *S. aureus* carrying *mecC* are predominantly livestock associated; therefore, it was thought likely that these infections arose from zoonotic transmission. In both cases the patients were smallholding farmers with livestock that tested positive for MRSA. Phylogenetic analysis of the human and animal isolates revealed two distinct clades that corresponded to the separate farms, and in both cases the human and animal isolates were closely related: The human isolate differed from the nearest animal strain by five and three SNPs, for the respective farms.

GENOMIC INSIGHTS INTO THE ASSOCIATION OF *S. AUREUS* WITH DIFFERENT HOST SPECIES

Because *S. aureus* is a major human pathogen, much research has focused on the pathogenesis and epidemiology of human strains. Our understanding of the diversity of livestock strains of *S. aureus* has traditionally lagged behind that of human *S. aureus*. However, in recent years the emergence of livestock-associated MRSA as a major zoonotic threat has given new impetus to studies aimed at investigating the evolution of animal strains, and their potential zoonotic capacity. As mentioned, early phenotypic biotyping studies were successful in distinguishing among *S. aureus* strains from distinct host ecological niches (44). Subsequently, population-genetic

analysis revealed that many animal strains belong to distinct clonal lineages, in contrast to human strains (78, 79), and phylogenetic analysis based on MLST loci sequences provided the first long-term picture of the evolution of human and animal strains (76, 94). The analysis revealed that *S. aureus* has likely coevolved with its human host for a long time in evolutionary terms but that the capacity to infect animals has evolved on multiple occasions via human-to-animal host jump events, leading to strains that are endemic in livestock (94).

The first animal strain of *S. aureus* whose genome was fully sequenced was RF122 (35). This study provided the first insights into the molecular processes that have contributed to ruminant host adaptation. In particular, the study demonstrated that pathways affecting iron uptake and host-pathogen interactions have diversified in bovine strains, presumably as a result of host-specific selective pressure. Subsequently, whole-genome sequences for *S. aureus* strains from poultry, sheep, and pigs were determined and proved invaluable for studies of host-specific evolution and pathogenesis (29, 53, 69, 73). Overall, these first genomes from animal strains have provided evidence for distinct genetic signatures linked to host adaptation involving acquisition of specific MGEs, allelic diversification, and loss of gene function. The observed gene decay often affects cell envelope determinants of host-pathogen interactions consistent with an ancestral *S. aureus* strain of human origin shifting into a novel animal host, followed by loss of function of genes not required for survival in the new host species.

HOST SWITCHING: *S. AUREUS* AND THE EMERGENCE OF LIVESTOCK-SPECIFIC CLONES

A major driving force for host-switching events may have been the development of agriculture in the Neolithic era and its industrialization and globalization in the last 30 years. A study by Lowder et al. (53) first highlighted the emergence of a major poultry-specific clone of *S. aureus* that is responsible for the majority of cases of bacterial chondronecrosis with osteomyelitis, an important cause of lameness in broiler poultry. The strain likely evolved via a human-to-poultry host jump event of an ST5 strain that occurred about 40 years ago, possibly promoted by increased interactions between humans and chickens after industrialization of the global broiler poultry industry, and was followed by acquisition of MGEs that were found to be widespread among other *S. aureus* clones colonizing and causing disease in birds (53). Importantly, the study demonstrated that genetic diversification of the ST5 clone since the host jump has led to enhanced resistance to innate immune cell killing by heterophils (avian neutrophils) (53). A subsequent study by Price et al. characterized a similar host-switch event in another *S. aureus* ST398 clone that led to the emergence of the major livestock-associated MRSA clone in pigs with capacity for zoonotic infection of humans. Of note, the strain later spread to other farm or domesticated animals such as turkeys, chickens, cattle, and horses (69). Importantly, the study indicated that resistance to beta-lactam antibiotics evolved multiple times independently since the host jump from humans, implicating the use of antibiotics for infection prophylaxis or growth promotion in pigs as the major driving force for emergence of resistance.

In addition to human-to-animal host jumps that have led to the emergence of livestock-endemic strains, there are host switches from animals back to humans leading to the emergence of new clones that are epidemic in human populations (94). For example, CC97 is one of the major global clones of *S. aureus* associated with bovine intramammary infections leading to mastitis (78). However, in recent years, there have been increased reports of CC97 isolates associated with human infections in addition to occasional CC97 infections of pigs (16, 57, 86). Of note, surveillance in Denmark indicated that CC97 infections of humans have increased sevenfold in recent years (80). Whole-genome-based phylogenetic analysis of CC97 isolates from cows,

humans, and pigs revealed the existence of two human subclades of CC97 that evolved through bovine-to-human host jumps followed by onward spread in global human populations, implying that cows represent a potential reservoir for the emergence of new human pandemic clones (80). Similar to the ST398 LA-MRSA pig clones, CC97 isolates from pigs have acquired multidrug resistance, but there was no evidence for the emergence of antibiotic resistance by CC97 isolates infecting dairy cows (80). Recently, Viana et al. traced the evolutionary history of the major rabbit epidemic clone of *S. aureus*, revealing a single mutation in the *dltB* gene that was required for infectivity (90). Overall these studies highlight the capacity for *S. aureus* strains to cross the species barrier and adapt to spread and cause disease in new host populations. In addition, the data suggest that different species of livestock or distinct agricultural production methods influence the emergence of antibiotic resistance. Another important discovery stemming from genomics was the identification of a novel allele of the methicillin-resistance determinant with 69% nucleotide identity to *mecA*, designated *mecC* (24). This existence of allelic variants of the gene encoding methicillin resistance is a potential confounder of effective diagnostics and genetic prediction of resistance. *mecC* is contained on a novel variant of the SCC*mec* island (SCC*mec* XI) that has been found in many countries in Europe in strains from human, livestock, wildlife, and companion animal sources, predominantly in ST425 and CC130 lineages (34, 65). While the origin of *mecC* is unclear, its prevalence among *S. aureus* lineages of animal origin suggests an animal reservoir (67). Furthermore, *mecC* has also been identified among other staphylococcal species colonizing animals (33).

GENOMIC INSIGHTS INTO THE DIVERSIFICATION OF *S. AUREUS* DURING COLONIZATION OR INFECTION

The capacity for deep sequencing of bacterial genomes has facilitated, for the first time, the opportunity to explore the diversity of bacterial populations within an infection. Such studies have important implications for our understanding of within-host dynamics, the emergence of antibiotic resistance, immune selection, and transmission between hosts (11). Until recently, single isolates of bacteria were considered representative of bacterial infections and were used to investigate epidemiology and to determine antibiotic sensitivity. However, it is now apparent that much greater infecting population diversity may exist than previously considered. For example, Young et al. (99) and Golubchik et al. (25) carried out longitudinal studies of *S. aureus* colonization of humans and subsequent transition into invasive disease. The studies revealed large fluctuations in the size of the colonizing populations over time, and the diversity of infecting populations varied between 6 and 40 SNPs, depending on the host (25, 99). Recombination was not detected within individuals, with limited evidence for positive selection, and purifying selection was the major selective force driving within-host evolution. The same group followed the transition from commensalism to invasive disease in an individual patient, providing the first insights into the genetic basis for a switch from commensalism to invasion (25, 99). The invasive population contained only eight mutations relative to the previously isolated commensal isolates, but several mutations were located in genes that are predicted to influence host-pathogen interactions, including truncating mutations in putative virulence determinants and a transcriptional regulator of virulence (AraC family) (25, 99). In a subsequent collaborative study with Massey and colleagues, the invasive isolates were intriguingly demonstrated to have lower toxicity than the commensal ancestors (48), a phenotype that appears to be broadly associated with bacteremia isolates, for as yet unknown reasons.

The within-host diversity of *S. aureus* has important consequences for the application of WGS for investigating transmissions. Typically, clinical microbiological diagnostics has utilized the

bacterial colony as the common currency of identification, with an assumption that a bacterial colony grown from a patient sample is representative of the strain causing an infection. In the initial studies using WGS to examine outbreaks and transmission in health care settings, single colonies were used. These demonstrated that, in conjunction with supporting epidemiological evidence, SNP variation provided strong evidence to rule in and to rule out isolates from transmission (18, 31, 45, 85). It was also observed that no two isolates were identical, suggesting that there was more diversity in the transmission population than was been captured by sampling a single colony. Harris et al. (31) provided an insight into the within-host diversity during a transmission study in their investigation of a SCBU MRSA. Sequencing 20 colonies from the staff member revealed a cloud of diversity that overlapped with the single isolates obtained from the affected babies on the SCBU.

One of the most comprehensive analyses of the diversification of bacteria within a single host involved an investigation into the source of an MRSA outbreak in a veterinary hospital. Paterson et al. (66) sequenced multiple isolates of MRSA from animal patients and veterinary staff at the hospital. Deep sequencing of up to 20 colonies from each MRSA-positive patient and staff member was carried out with the exception of the index case, a German shepherd dog who ultimately died of MRSA sepsis, for which 141 isolates were sequenced. Multiple subtypes (clades) of the major ST22 clone were identified among the individuals sampled, with a single clade seemingly responsible for the pathology observed (66). The investigators identified variation within individuals who were sometimes colonized with multiple strain genotypes, and the diversity of the colonizing population varied over time, with dynamic changes in the genetic complexity of the infecting bacteria observed. Importantly, the authors could utilize the richness of the data resulting from the deep-sampling approach to predict the likely transmission network for the outbreak and identify the likely sources for the two common circulating subtypes. They concluded that sequencing multiple colonies from each subject greatly enhanced the power for epidemiological resolution (66).

Other studies employing a comparative genomic approach to investigate within-host evolution have focused on the emergence of antibiotic resistance during treatment, an understanding of which is critically important to design improved treatment regimens. Longitudinal studies have revealed the evolutionary trajectory of the emergence of resistance within patients being treated for staphylococcal infections, particularly with respect to vancomycin resistance (40). For example, the evolution of a strain of MRSA that is also resistant to vancomycin was investigated by whole-genome sequencing, revealing the likely acquisition of a 57.9-kb plasmid containing the *vanA* determinant of vancomycin resistance from a coinfecting strain of *Enterococcus faecalis* (93). Subsequently, it was established using a phylogenomic approach that all cases of high-level vancomycin-resistant *S. aureus* are derived from the same clonal lineage, CC5, suggesting a possible selective advantage for ST5 strains in polymicrobial infections with other bacterial species, such as *E. faecalis* (93). As previously mentioned, in a seminal study, Mwangi et al. (62) identified mutations underpinning the emergence of multidrug resistance to rifampicin, β-lactams, and vancomycin within the same chronically infected endocarditis patient. Using this approach, mutations in the *vraR* locus were found to be associated with strains refractory to treatment with vancomycin. Importantly, the authors noted that resistance to daptomycin also developed in the infecting strain, even though the antibiotic had not been used to treat the patient (62). Mutations at loci encoding the two-component systems WalKR and GraSR have also been demonstrated to confer reduced sensitivity to vancomycin and daptomycin during infection (39). In addition, a similar genome-sequencing approach revealed mutations in the *relA* gene and in a gene specific for a ribosomal methyltransferase (*rlmN*) that were responsible for enhanced resistance to linezolid (23).

Resistance to antibiotics is attributable to not only mutations acquired in vivo, but also horizontal acquisition of MGEs encoding resistance determinants such as SCC*mec* (70). McCarthy et al. (56) recently demonstrated horizontal gene transfer of MGE among coinfecting strains of

ST398 *S. aureus* using a gnotobiotic pig model. The study revealed, for the first time, the capacity for efficient spread of MGEs among *S. aureus* bacteria in a colonizing population (56). Previous studies examining within-host diversity of *S. aureus* from clinical samples have identified the loss of MGEs encoding antibiotic-resistance determinants (31); deep sequencing a sample from a staff member who was linked to an outbreak of MRSA on a SCBU revealed that intermittent carriage of the *ermC* plasmid conferred the MRSA in this individual with a heterogeneous phenotypic response to erythromycin (31). The observation of the loss and gain of MGEs within a host highlights the rapidity with which new variants can evolve, and the diversity of mechanisms that can potentially drive adaptation.

FUTURE PERSPECTIVES FOR POPULATION BIOLOGY OF *S. AUREUS*

The explosion of sequencing data for *S. aureus* is transforming our understanding of the diversity of populations—from the level of individual infections to local, global, and cross-species transmission. The next few years promise to be a very exciting time both for our basic understanding of the population biology of *S. aureus* and for the application of this understanding to improve diagnostics, inform hospital infection control, and limit the emergence of multidrug-resistant pandemic clones.

Routine genome sequencing of pathogenic bacterial isolates in hospital settings to facilitate rapid decisions about treatment and infection control is becoming a reality. Implementation of this approach around the world would allow global surveillance of clinically relevant bacteria, leading to real-time identification of emergent clones and monitoring their spread, thereby revealing reservoirs of infection and routes of dissemination. A global surveillance system would offer tremendous benefits in limiting the emergence and spread of epidemic clones. However, our understanding of the factors influencing the global distribution of *S. aureus* clones is very limited, and studies are required to investigate the impact of interclone competition in addition to the role of host differences, including variation in the success of particular clones in different geographic locations. A nascent area of research on bacterial populations is the identification of niche adaptation using genome-wide association studies (GWAS). The primary application of GWAS has been to investigate markers of human disease or livestock breeding traits. More recently, however, new methods have been developed for bacterial populations and have identified genetic determinants of niche specificity (77), drug resistance (10), and virulence (47). Deep sequencing of large numbers of bacterial genomes (hundreds to thousands of sequences) will provide the statistical power to identify complex traits associated with particular ecological niches or phenotypes, providing new insights into mechanisms of adaptive evolution and pathogenesis (9).

Currently, we have very little understanding of the role of the *S. aureus* epigenome—in particular, the effect of DNA methylation on *S. aureus* pathogenesis during infection. Recent single-cell DNA sequencing approaches, such as single-molecule real-time (SMRT) sequencing, offer the potential for genome-scale identification of methylation sites that likely affect regulation of gene expression, thereby influencing host-pathogen interactions, in addition to restriction-modification systems mediating resistance to invasion by exogenous DNA elements. Population-scale analysis of the variation in DNA methylation sites across different strains in different ecological niches will very likely reveal previously unknown mechanisms of bacterial adaptation to different niches and host species.

CONCLUDING COMMENTS

In the 36 years since Wesley Kloos's landmark paper in *Annual Review of Microbiology*, our understanding of *S. aureus* populations has been greatly enhanced by the tremendous resolution

provided by WGS of large numbers of isolates. As we reflect on Kloos's original observations and the others he summarized in that review, it is remarkable how many of the conclusions and predictions about staphylococcal populations—based wholly on phenotypic characterization of strains—have been validated by genome-sequence-based analyses. The coming years will see continued progress in our understanding of the biology of *S. aureus* populations, and we anticipate this will in turn inform new strategies to limit the clinical impact of *S. aureus* in humans and in livestock species.

DISCLOSURE STATEMENT

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