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Understanding the rise of the superbug: investigation of the evolution and genomic variation of *Staphylococcus aureus*

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Abstract The bacterium *Staphylococcus aureus* is a common cause of human infection, and it is becoming increasingly virulent and resistant to antibiotics. Our understanding of the evolution of this species has been greatly enhanced by the recent sequencing of the genomes of seven strains of *S. aureus*. Comparative genomic analysis allows us to identify variation in the chromosomes and understand the mechanisms by which this versatile bacterium has accumulated diversity within its genome structure.

Keywords *Staphylococcus aureus* · Mobile genetic elements · Resistance · Virulence

Bacterial genetics and evolution

The increasing levels of antibiotic resistance and the emergence of epidemic strains of bacterial pathogens over the last decade (Enright et al. 2002; Livermore 2003) highlight the adaptability of bacteria and the remarkable speed of bacterial evolution. In the face of constant environmental challenges, the ability of bacteria to generate genetic variation is crucial for their survival. Bacterial genomes tend to evolve through several routes: mutation to existing genes, DNA loss or rearrangement or horizontal transfer of gene/s from one bacterium to another (Ziebuhr et al. 1999). Selective pressures exerted by environmental conditions promote the survival of cells that possess advantageous genes (Preston et al. 1998). The haploid nature of a bacterial

genome means that there is a direct link between allele and phenotype. The speed of bacterial growth and division, coupled with their asexual reproduction, ensures that a successful allele can become prevalent in a clonal population very quickly. Adaptation via mutation tends to be a slow process, whereas horizontal gene transfer allows a bacterium to adapt to a new niche very rapidly (Hacker and Carniel 2001).

Recent years have seen a bonanza of bacterial genomes, with the publication of more than 200 complete genomes (for a comprehensive list of the publicly available genomes, see <http://www.ebi.ac.uk/genomes/bacteria.html>). For several species, there are now sequences for more than one strain available (Bolotin et al. 2004; Cazalet et al. 2004; Fouts et al. 2005; Tettelin et al. 2005). As a result, we are now able to examine genetic variation on a genomic scale and map the evolutionary changes that define closely related bacteria. This has proved to be a very valuable approach in understanding the mechanisms of evolution of bacterial pathogens. In this review, we are going to focus on the versatile pathogen *Staphylococcus aureus*. Six genome sequences have been published for this organism (Baba et al. 2002; Gill et al. 2005; Holden et al. 2004; Kuroda et al. 2001). By focusing on the differences in the genomes, we aim to illustrate the mechanisms that generate change in *S. aureus* and how these shape its virulence and drug resistance.

S. aureus as a pathogen

S. aureus is a bacterium that has gained notoriety over recent years due to its ability to evolve new virulent and drug-resistant variants (Chambers 2005; Pearson 2002). In particular, the spread of *S. aureus* in hospitals has placed an increased burden on health care systems (Kim et al. 2001; Nathwani 2003). *S. aureus* is the most common cause of hospital-acquired infection and is estimated to cause clinical disease in 2% of all patient admissions (Emmerson et al. 1996; Jones 2003). Accompanying the spread of this bacterium has been an increase in the resistance to antibiotics. In parts of Europe, the United States and Japan, 40–

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60% of all hospital *S. aureus* infections are now resistant to the β -lactam antibiotic methicillin (Fluit et al. 2001; Tiemersma et al. 2004). Methicillin-resistant *S. aureus* (MRSA) strains were first described in the 1960s; however, MRSA did not spread rapidly until the late 1980s to mid-1990s, such that they are now endemic in hospitals and cannot be eradicated (Enright et al. 2002). MRSA infections are treated with vancomycin, but the first high-level vancomycin-resistant isolates (VRSA) were recently described (Anonymous 2002a,b; Chang et al. 2003). There are few reliable antibiotics on the market for treating MRSA infections, and fewer are expected to be developed in the near future (Projan and Shlaes 2004).

S. aureus is a persistent resident of the human nose in 20% of the population and intermittently carried by another 60% (Kluytmans et al. 1997). For most healthy individuals, colonization is not a problem; however, if *S. aureus* contaminates a breach in the skin or mucous membranes, it can go on to infect any tissue of the body. Patients in hospital are often particularly prone to *S. aureus* infection due to compromised immune systems and frequent catheter insertions and injections. Infections range from the trivial, such as minor skin infections, including boils and carbuncles, to more serious and life-threatening conditions, such as bacteremia, endocarditis and haemolytic pneumonia. Hospitalized patients with *S. aureus* bacteremia have a mortality rate of at least 20%, and those with endocarditis and haemolytic pneumonia have a mortality rate of more than 20% (Cosgrove et al. 2003).

Whilst the *S. aureus* infection rates are lower outside the hospital, there is an emerging problem of community-acquired severe invasive infections caused by *S. aureus* (Chambers 2005). In recent years, increasingly virulent strains of *S. aureus* have emerged in the community, causing severe skin and soft tissue infections and a lethal form of haemolytic pneumonia in children (Dufour et al. 2002a; Gillet et al. 2002; Herold et al. 1998). These new strains are associated with a toxin called Panton–Valentine leucocidin, and some are also resistant to methicillin and flucloxacillin, the first-line antibiotics for community infections. In comparison to hospital-acquired strains, the drug resistance of community-acquired strains tends to be lower. However, the last decade has seen an increase in the number of community-acquired MRSA strains reported (Zetola et al. 2005).

***S. aureus* genome sequencing**

Currently, there are six published *S. aureus* genome sequences from strains isolated from different clinical settings and with varied antibiotic resistances: N315 and Mu50 are hospital-acquired MRSA strains, the latter of which has decreased susceptibility to the antibiotic vancomycin (VISA) (Kuroda et al. 2001); MW2 is a community-acquired MRSA (Baba et al. 2002); MRSA252 is a hospital-acquired MRSA strain (Holden et al. 2004) and representative of the highly successful epidemic EMRSA-16 clonal group responsible for 50% of UK MRSA

infections and one of the major MRSA clones found in the USA (USA200) (Johnson et al. 2001; McDougal et al. 2003); MSSA476 is a community-acquired methicillin-susceptible strain (Holden et al. 2004); and COL is an early MRSA strain originally isolated in the 1960s (Gill et al. 2005). In addition, the complete genome of NCTC8325, a genetically tractable laboratory strain, is also freely available (<http://www.genome.ou.edu/staph.html>).

The sequenced *S. aureus* genomes range in size from 2.813 to 2.903 Mb and comprise a single circular chromosome, and in some cases a plasmid, and are predicted to contain between 2,592 and 2,748 genes. The overall structure of the *S. aureus* chromosomes is well conserved, with the majority of genes exhibiting high levels of DNA identity (>97%) and organized in the same order, interrupted by small regions of difference (Fig. 1). If the comparison is widened to include related bacteria from the same genus (*Staphylococcus epidermidis*) (Gill et al. 2005) and order (*Bacillus subtilis*) (Kunst et al. 1997), the level of gene order conservation falls away with the taxonomic diversity (Fig. 2a). However, the detectable blocks of gene conservation in the most distant comparison represent the conservation of chromosomal structure maintained over millions of years since these bacteria diverged from a common ancestor (Battistuzzi et al. 2004). Many of these regions are concerned with central metabolism and house-keeping functions. Taxonomic diversity is also reflected in the number of orthologous genes, i.e. those genes that are derived from a common ancestor and whose products are predicted to perform the same function or role; the closer the relatedness, the higher the number of orthologues (Fig. 2b).

To understand the genetic basis for the marked differences in the biology and pathology of *S. aureus* strains, we must look in more details at the genomic inventories of the sequenced strains. From Fig. 1, it is apparent that the *S. aureus* genome is clearly made up of two major components: the conserved core genome, which is present in all isolates, and the accessory genome, which is unique or is only found in some of the strains.

***S. aureus* core genome**

Approximately 75% of a *S. aureus* genome comprises a core component consisting of genes present in all of the strains. Apart from those genes that are essential for growth and survival, there are genes with common species-associated functions, including virulence genes not carried by other staphylococcal species, such as cell surface binding proteins, toxins, exoenzymes and the capsule biosynthetic genes (Lindsay and Holden 2004). Whilst this component would appear to be a stable constituent of the *S. aureus* genome, the contribution of the genes contained within it to *S. aureus* strain differences should not be overlooked. Small-scale sequence variation can have marked effects on gene expression and protein function. Genetic diversity in the core genome can therefore account for some of the important phenotypic differences between strains.

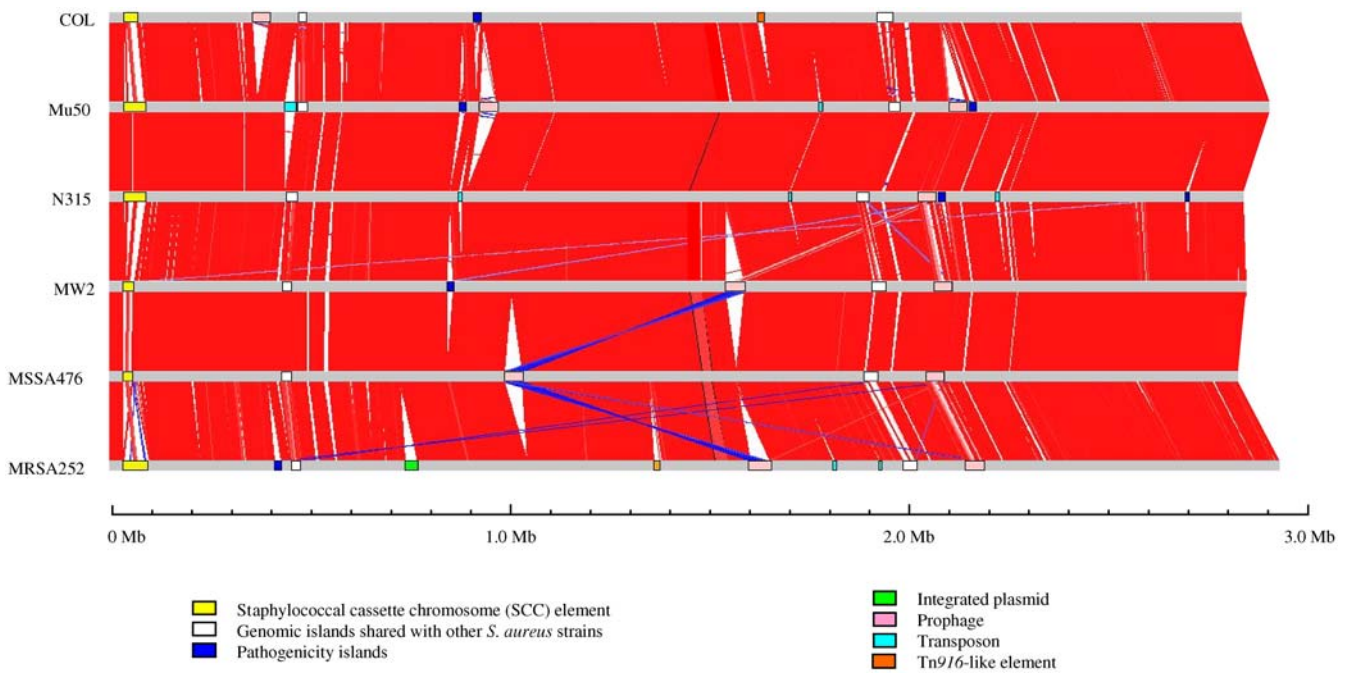


Fig. 1 Comparison of the chromosomes of six sequenced *Staphylococcus aureus* strains. Pairwise comparisons of the *S. aureus* COL, Mu50, N315, MW2, MSSA476 and MRSA252 chromosomes displayed using the Artemis Comparison Tool (ACT) (Carver et al. 2005). The sequences have been aligned from the predicted replication origins (*oriC*; right), with the terminus of replication in the centre. The coloured bars separating each genome (red and

blue) represent orthologous matches identified by reciprocal FASTA analysis (Pearson and Lipman 1988). Red lines link orthologues in the same orientation, blue lines link orthologues in the reverse orientation. Variable regions of the chromosomes containing MGEs such as prophages, plasmids, transposons, SaPI and other genomic islands are marked as coloured boxes (see figure for key)

Single nucleotide polymorphisms

The DNA encoding much of the *S. aureus* core genome is highly similar. The average level of nucleotide identity between all orthologous gene pairs was 99.8% when MW2 and MSSA476 were compared, 98.65% for MSSA476 and N315 and 97.7% for MRSA252 and N315 (Ed Feil, personal communication). Much of the sequence divergence in the core genome is due to single nucleotide polymorphisms (SNPs). The functional effect of a polymorphism depends on its nature and position. Many of the SNPs within coding regions will be phenotypically silent, as the nucleotide change introduced will not result in a change in the amino acid encoded (synonymous substitution). Non-synonymous substitutions (substitutions that result in a change in the encoded amino acid) may however contribute to differences in the expression and function of genes, thus creating a mutant allele. SNPs are more likely to have functional effects if they occur in the first or second base pair of a codon. Redundancy in the amino acid coding system means that the nucleotide in the third base position of a codon can often be changed without affecting the amino acid it encodes. The functional effect of the allele may be positive or negative depending on the selective conditions.

The routine use of antibiotics to treat *S. aureus* infections has exerted a sustained and strong selective pressure on clinical strains. Spontaneous antibiotic resistance mutants have appeared for many commonly used therapeutic

agents. Fusidic acid is a potent antibiotic that blocks bacterial protein synthesis by inhibiting the function of elongation factor G (EF-G). Point mutations within the gene encoding EF-G (*fusA*), result in amino acid changes (non-synonymous substitutions) that confer fusidic acid resistance. Analysis of fusidic acid-resistant clinical strains and mutants isolated under fusidic acid selective pressure in vitro has identified 13 amino acid residues within EF-G, which are targets for amino acid exchanges to generate fusidic acid resistance in *S. aureus* (Besier et al. 2003). Interestingly, the evolution of fusidic acid resistance is not as straightforward as acquiring a single point mutation in the target protein. Recent studies have shown that point mutations in EF-G also have a fitness cost which may limit their long-term selective advantage (Besier et al. 2005). It has been speculated that secondary compensatory mutations in EF-G may help reduce the detrimental effect of the primary resistance-mediating mutation (Besier et al. 2005).

Using comparative analysis, it is possible to identify mutations that will disrupt the translation of a gene (e.g. nonsense and frameshift mutations), as well as mutations that disrupt the function of the protein (e.g. larger-scale insertions and deletions that alter the number of amino acids in a protein). In *S. aureus*, the percentage of the genome comprising pseudogenes (MRSA252, 2.5%; MSSA476, 0.8%; COL, 36 1.3%; Mu50, 42 1.5%; N315, 1.1%; MW2, 1.0%) (Gill et al. 2005; Holden et al. 2004; Lerat and Ochman 2005) is similar to the level found in most other sequenced bacterial genomes (Liu et al. 2004).

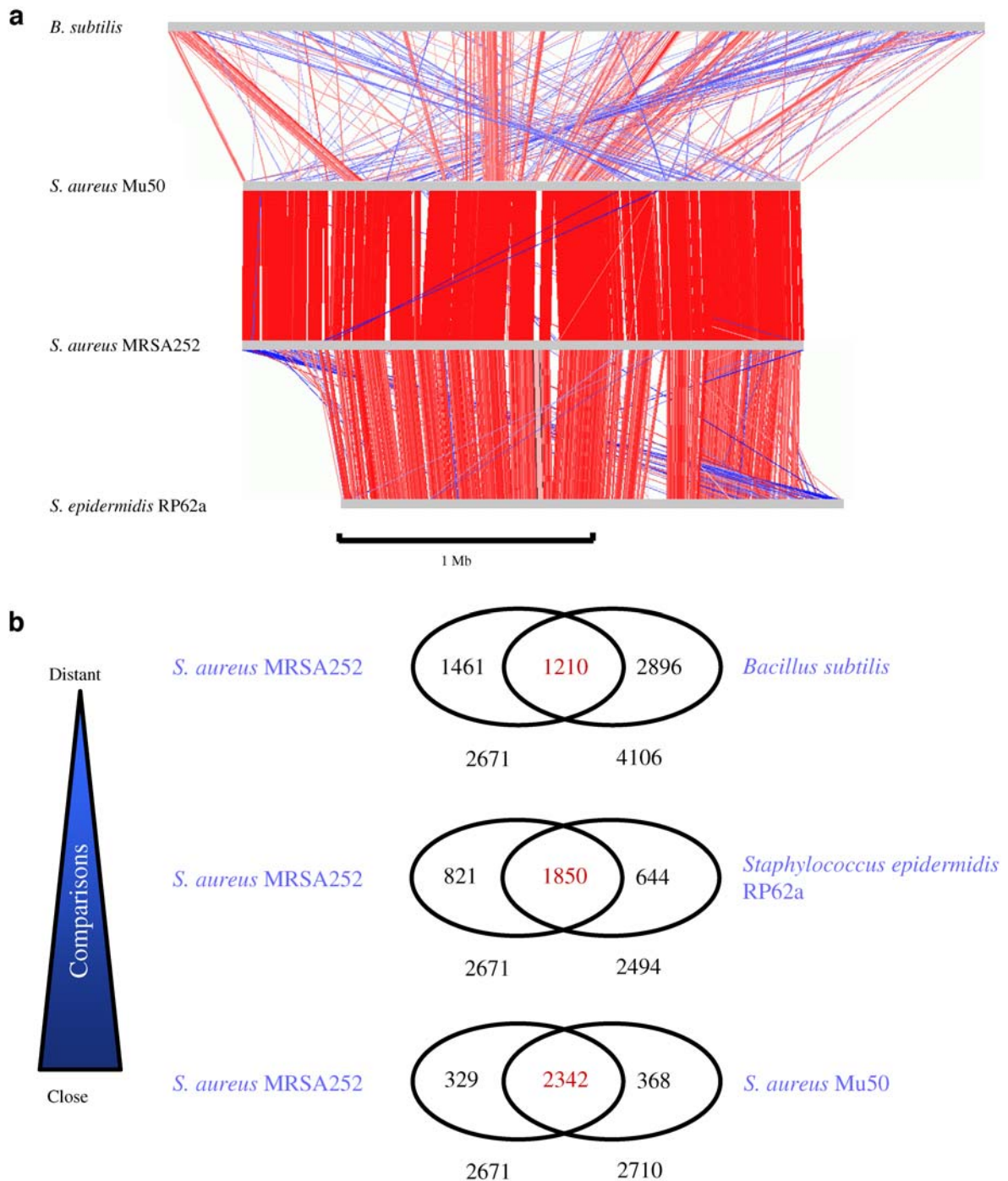


Fig. 2 Orthologue comparisons of the chromosomes of *S. aureus* and related bacteria. Orthologues were identified by reciprocal FASTA analysis (Pearson and Lipman 1988), with an identity cut-off of 30% and a length of match cut-off of 80%. The sequences used for the comparisons were as follows: *S. aureus* strains MRSA252 (accession number BX571856; Holden et al. 2004) and Mu50 (accession number BA000017; Kuroda et al. 2001), *Staphylococcus epidermidis* strain RP62a (accession number CP000029; Gill et al. 2005) and *Bacillus subtilis* strain (accession number AL009126; Kunst et al. 1997). **a** Pairwise orthologue comparisons of the *B. subtilis*, *S. aureus* Mu50, *S. aureus* MRSA252 and *S.*

epidermidis RP62a chromosomes displayed using the ACT (Carver et al. 2005). The sequences have been aligned from the predicted replication origins (*oriC*; right), with the terminus of replication in the centre. The coloured bars separating each genome (red and blue) represent orthologous matches identified by reciprocal FASTA analysis (Pearson and Lipman 1988). Red lines link orthologues in the same orientation, blue lines link orthologues in the reverse orientation. **b** Distribution of orthologues in *S. aureus*, *S. epidermidis* RP62a and *B. subtilis*. Orthologues were identified as described above

In some bacteria, the accumulation of a large number of pseudogenes is indicative of recent evolutionary change

(Cole et al. 2001; Parkhill et al. 2003). In *S. aureus*, there is no indication from the level of pseudogenes that niche

change or host specialization has recently occurred. The pseudogenes identified in the sequenced genomes are more likely to be the product of recent mutational events and therefore may provide clues as to the selective pressures experienced by the strain.

SNPs have also been exploited by multilocus sequence typing (MLST) to investigate the phylogenetic relationships of bacterial strains. MLST is a molecular typing technique exploiting allelic variation in the core genome. The technique is based on the sequence fragments of seven core housekeeping genes (*arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi* and *yqiL*) (Maiden et al. 1998). The stable nature of the core genome and its vertical inheritance mean that allelic variation can be used to measure genetic relatedness. For each *S. aureus* isolate, these sequences are compared with existing alleles in more than 1,300 *S. aureus* isolates in the MLST database (<http://saureus.mlst.net>), generating a numerical allelic profile or sequence type (ST) (Enright et al. 2000). Isolates with STs that have five or more identical alleles out of seven can be assigned to specific clonal complexes (CCs), inferring that these isolates are related and are descendants of a recent clonal ancestor. The method is highly discriminatory, reliable and reproducible, and the data generated have allowed a comprehensive understanding of the diversity of *S. aureus* populations (Feil et al. 2003). Using MLST, we can compare the sequenced strains to each other and to the wider population and therefore add valuable context to the genomes (see later section for discussion).

Diversity within genes and operons

Genetic diversity in the core genome can extend beyond SNPs, to encompass larger regions of DNA diversity. These can range in size from a few nucleotides within a gene to larger stretches of several kilobase pairs that include complete or partial genes within operons. Sequence comparisons of these regions reveal blocks of divergent sequence, giving the impression of a mosaic genetic structure. The introduction of diversity at this level can have a marked impact on gene function. The apparently mosaic structure of these larger regions, where divergent sequence is flanked by homologous DNA, has led to the suggestion that these regions have arisen by some form of genetic exchange and homologous recombination.

An example of where the replacement of genes within a conserved set of genes can lead to a change in biological properties can be found in the capsule cluster (Sau et al. 1997). *S. aureus* produces a capsular polysaccharide (CP) that enhances its resistance to host innate immune defences (Thakker et al. 1998). Most clinical isolates of *S. aureus* are encapsulated, and the serotypes CP5 and CP8 are the most prevalent (O’Riordan and Lee 2004). Significantly, CP5 and CP8 differ in a number of biological properties which are thought to contribute to the relative virulence of serotype CP5 and CP8 *S. aureus* in vivo (Watts et al. 2005). The gene clusters responsible for the biosynthesis of CP5 and CP8 contain the same number

of genes in a single operon and are located at the same site on the chromosome (Sau et al. 1997). However, the two clusters differ in an internal region that contains four capsule type-specific genes (*cap5HIJK* and *cap8HIJK*) that exhibit little sequence similarity. Capsules CP5 and CP8 comprise the same trisaccharide repeating units and differ only in the linkages between the sugars and sites of O acetylation (Fournier et al. 1984; Moreau et al. 1990). The capsule-specific genes of the CP5 and CP8 clusters are thought to be responsible for the differing linkages and acetylation of the carbohydrates.

Within the *S. aureus* genome, there are also examples of apparently chimeric genes, which contain internal regions of variation. The *agr* cluster contains four genes that encode components of a quorum-sensing system involved in the regulation of virulence (Novick et al. 1995). AgrA and AgrC comprise a two-component signal transduction system (Lina et al. 1998), and AgrB and AgrD are required to produce an autoinducing peptide (AIP) that activates the quorum-sensing system (Ji et al. 1995). AIP is a modified peptide derived from AgrD, which is processed by AgrB (Ji et al. 1997). Four groups of *agr* systems have been identified on the basis of the ability of their AIP to cross-activate or cross-inhibit *agr* quorum sensing (Ji et al. 1997; McDowell et al. 2001). The AIP of each group has a different structure. Sequence comparison of the *agr* loci of these four groups reveals an internal variable region including the C terminus of *agrB*, the whole of *agrD* and the N-terminal half of *agrC* (Dufour et al. 2002b). The covariation of the AIP structural gene and domains of the processing and sensing proteins maintain the functional integrity of each *agr* system. Replacement of this signal-specific cassette provides a potential means by which strains could swap the specificity of their quorum signals and maintain the induction activity.

Repeat variation

Repeated sequences are widespread in the *S. aureus* genome. Several studies have identified families of perfect repeats (Baba et al. 2004; Cramton et al. 2000) and tandem repeats (Hardy et al. 2004) predominantly found within intergenic regions. In addition, there are many examples of polymeric tracts and imperfect repeat regions within protein-coding sequences (McDevitt and Foster 1995; Roche et al. 2003). Comparisons of these repeat regions in the different strains show that they can vary in size from a single nucleotide to several hundreds.

An example of how small-scale repeat variation may influence the expression of virulence genes is to be found in the gene encoding a major histocompatibility complex analogue protein (MapW). In six of the seven sequenced strains (N315, MW2, Mu50, COL, NCTC8325 and MSSA476), this gene is truncated due to a frameshift mutation occurring at a poly(A) tract. The repeat in these strains contains either 9 or 10 adenine residues, which introduces a stop codon immediately after it. If the tract contains 8 or 11 residues, the correct frame is maintained

and the full-length gene exists. In a random selection of 45 *S. aureus* strains, the number of adenines in this tract was shown to be varied, with 80% of the strains containing repeats resulting in frameshifts (Buckling et al. 2005). The other 20% of the strains contain either 8 or 11 residues in the tract, thus resulting in a full-length product gene. Protein analysis of strains containing the full-length and truncated forms of the *mapW* detected both long and short forms of the protein (Buckling et al. 2005). Interestingly, the number of adenines in the repeat can vary in closely related strains, suggesting that phase variation may be occurring. In a clonal bacterial population, phase variation, in which individual cells either express the phase-variable protein or not, results in a heterogenic phenotype (for a review on phase variation, see van der Woude and Baumber 2004). Mechanisms such as slip-strand mispairing (Moxon et al. 1994) may have a role to play in the regulation of this locus.

One group of proteins that contain larger variable repeats is the surface-associated proteins. These proteins play an important role in modulating the interaction between the bacterium and the host and are likely to be immunogenic (Mazmanian et al. 2001; Schneewind et al. 1992). Fibrinogen-binding protein ClfA and collagen-binding protein Cna both contain repetitive protein motifs that have been shown to be important for functional surface expression (Hartford et al. 1997; Hartford et al. 1999). It has been postulated that the repeat regions span the cell wall peptidoglycan and act as a 'stalk' to allow the ligand-binding domain of the protein to be displayed in a flexible manner some distance away from the cell surface (Hartford et al. 1997). Many of the other surface-associated proteins also contain repeat regions (e.g. SasA, SasC, SasI) (Roche et al. 2003). The sizes of the repeat regions of some of these proteins have shown to vary between different strains (McDevitt and Foster 1995; Roche et al. 2003). For example, ClfA possesses a dipeptide repeat region composed predominantly of aspartic acid and serine residues. Analysis of this region in several strains showed that the size of the *clfA* repeat varied from 580 to 1320 bp (McDevitt and Foster 1995). Variation generated by DNA recombination in the regions encoding the repeat regions may represent a means by which *S. aureus* can evade the host immune system during a long-term infection (Jarraud et al. 2001).

Recombination between repeat regions may also play a role in shaping the inventory of surface proteins in a genome. For example, the locus encoding the bone sialoprotein-binding protein (*bbp*) and the serine-aspartate repeat proteins *sdrD* and *sdrC* contains large regions of repetitive DNA sequence (Roche et al. 2003). In some strains such as MSSA476, all three genes are present, but in MRSA252, *sdrD* is missing. If the MSSA476 and MRSA252 loci are compared, it is possible to identify homologous DNA sequences in adjacent genes (Fig. 3). For example, the C-terminal region of *sdrC* is similar to the C-terminal regions of *sdrD* and *bbp*. It is therefore possible that the genes in this locus may have originally evolved by gene duplication. In MRSA252, the lack of *sdrD* is possibly the result of recent

gene deletion via homologous recombination between these repeat sequences and the C-terminal regions of *sdrD* and *sdrC* (marked in yellow in Fig. 3), resulting in a gene fusion, thus leaving two genes at this locus.

Accessory genome

The variable, or accessory, component of the *S. aureus* genome contains genes that encode a diverse array of non-essential functions, ranging from virulence, drug and metal resistance to substrate utilization and miscellaneous metabolism. Many of the regions that make up the accessory genome are mobile genetic elements (MGEs) that can be transferred horizontally between bacteria, including bacteriophage, *S. aureus* pathogenicity islands (SaPI), staphylococcal cassette chromosomes (SCC), plasmids and transposons (Lindsay and Holden 2004). Interestingly, virulence genes tend to be found on phages and SaPI (Table 1), whilst resistance genes rely on SCC, plasmids and transposons for transfer (Table 2). In the next section of this review, we will focus on the different groups of MGEs found in *S. aureus* and on the genes carried that are important for transfer and function.

All horizontal gene transfer in bacteria is via three mechanisms: transformation, conjugation and transduction (Thomas and Nielsen 2005). However, *S. aureus* lack the genes necessary for transformation, and conjugative transfer of elements between *S. aureus* does not appear to be common. Therefore, most horizontal transfer by *S. aureus* is probably dependent on transduction by bacteriophage, which therefore plays a pivotal role in the evolution of this pathogen.

Bacteriophage

The horizontal transfer of virulence factors by bacteriophage occurs in two ways: first, by transfer and integration of a bacteriophage that encodes a virulence gene on its genome (phage conversion); second, by generalized transduction.

Many *S. aureus* bacteriophage genomes contain virulence genes, such as *sak* (staphylokinase A thrombolytic enzyme; Collen 1998), *chp* (chemotaxis inhibitory protein and inhibitor of leucocyte migration; de Haas et al. 2004), *sea* (enterotoxin A, a common cause of food poisoning; Betley and Mekalanos 1985), *eta* (exfoliative toxin A, a cause of scalded skin syndrome; Yamaguchi et al. 2000) and *lukSF-PV* (Panton-Valentine leucocidin implicated in haemolytic pneumonia and severe skin and soft tissue infections; Kaneko et al. 1998; Narita et al. 2001). The transfer of *S. aureus* bacteriophage into and out of isolates probably occurs with significant frequency in nature, including during the course of colonization or infection of patients (Goerke et al. 2004; Moore and Lindsay 2001).

In *S. aureus*, phages integrated into the chromosome (prophage) are common, with most strains carrying between one and three (Table 1) (Iandolo et al. 2002). These lysogenic phages are "dormant" and are replicated as

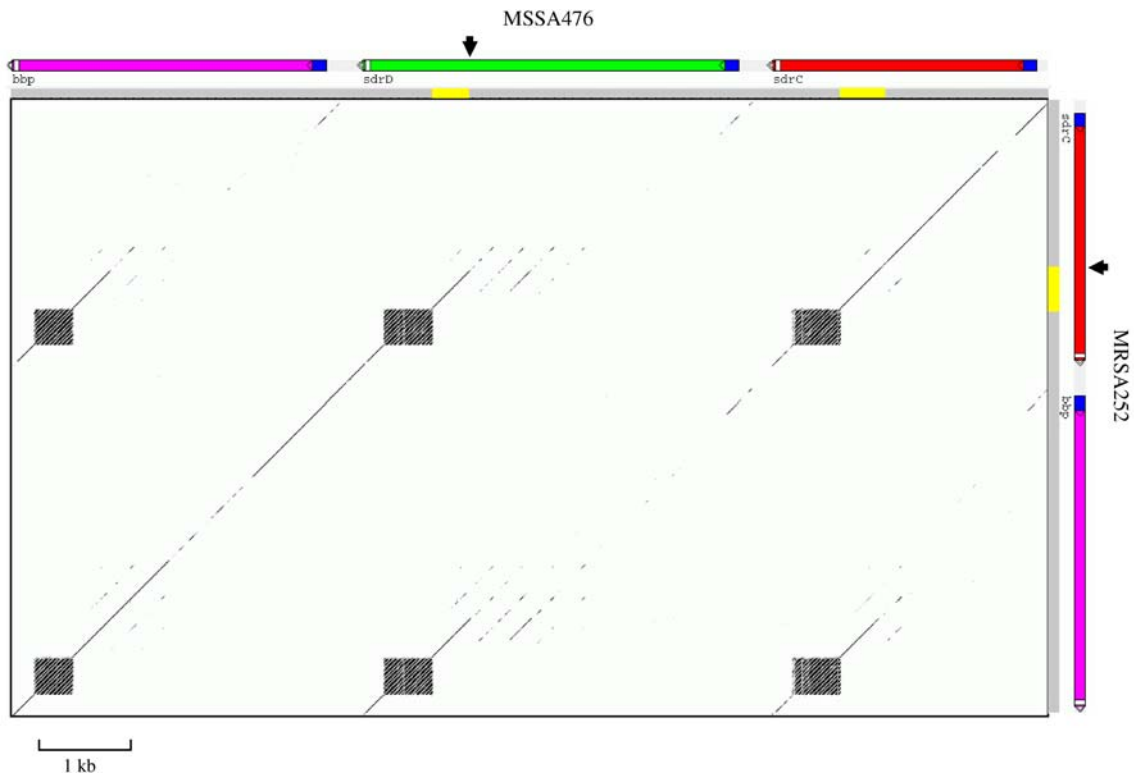


Fig. 3 DNA repeat variation in *S. aureus* surface-associated proteins. Dotplot comparison of the *sdr* and *bbp* surface protein loci of MRSA252 and MSSA476. The centre of the figure shows the DNA–DNA similarities between the *sdrC* and *bbp* genes of MRSA252 and *sdrC*, *sdrD* and *bbp* genes of MSSA476, with similar sequences represented by dots or lines. The genes are colour-coded to identify the orthologous genes; the N-terminal signal sequence (blue box) and C-terminal transmembrane regions (white box) are marked on the genes. Regions of sequence identity between

the orthologous genes are displayed as lines. The serine–aspartate rich repeat regions in the C termini of the surface proteins can be seen as hatched region; the rectangular shape of some of the boxes indicates the variation in the number of repeats between orthologues. The C termini of MSSA476 *sdrC* and *sdrD* and MRSA252 *sdrC* share repeat regions (marked below the genes as yellow boxes). Recombination between the repeats in *sdrC* and *sdrD* may be the cause of deletion of *sdrD* in MRSA252

part of the entire bacterial chromosome and passed to daughter cells during cell division (vertical transfer). Prophages are induced by stress, such as UV light, DNA-damaging agents and some antibiotics, most likely via the bacterial SOS response (Lindsay et al. 1998; Ubeda et al. 2005). During induction, the phage is excised from the chromosome and begins replicating. As phage genomes multiply, the proteins necessary for phage head and tail structures are produced, and the phage genomes are packaged. Eventually, the bacterial cell will lyse, releasing infectious phage particles. These phages then bind to recipient *S. aureus* cells via an unknown receptor, delivering the phage genome to the cell. The phage may then enter a lytic cycle and lead to the production of more infectious phage or enter a lysogenic cycle and integrate its genome into the bacterial chromosome. An interesting consequence of the induction of phage and phage genome replication is the increased copy number of any phage-carried toxin genes, leading to enhanced toxin production (Sumbly and Waldor 2003).

The *S. aureus* phage genomes contain an integrase gene carried at one end of the genome, and some also have a putative excisionase gene. All have an integrase promoter region similar to the lambda phage model (Carroll et

al. 1995), which is therefore predicted to control the lytic/lysogenic switch. It also suggests that a bacterial cell carrying a phage (lysogen) will be immune to infection with a similar phage, but this remains to be proven. *S. aureus* phage also appear to integrate at only a single site, and this is due to specificity of the integrase and the phage genome's left and right junctions (Lee and Iandolo 1986a). At least two *S. aureus* phage integration sites are within known virulence genes, β -haemolysin (*hlyB*) and lipase (*geh*), and thus, phage conversion leads to loss of these gene products (Coleman et al. 1989; Lee and Iandolo 1986b).

Generalized transduction in *S. aureus* is thought to be widespread and is the only feasible mechanism for the horizontal transfer of many non-phage MGEs. Only one naturally occurring generalized transducing phage (ϕ 11) (Novick 1963) has been well studied, although how it “accidentally” packages up to 45 kb of bacterial DNA instead of its own phage genome is not clear. The resulting phage heads are able to deliver this DNA to other *S. aureus* cells. Once delivered, the DNA will only survive if it can replicate or integrate into a replicon such as chromosome or a plasmid. For normal chromosomal DNA with no encoded replication mechanism, the only chance of survival is to recombine with host chromosome via homologous recom-

Table 1 Summary of the major mobile genetic elements (MGEs) in sequenced *S. aureus* strains associated with virulence

	N315	Mu50	MW2	MSSA476	MRSA252	COL
Bacteriophage						
φSa1	–	NI	–	–	–	–
φSa2	–	–	<i>lukS</i> -PV, <i>lukF</i> -PV	–	NI	–
φSa3	<i>sak, sep, chp</i>	<i>sea, sak</i>	<i>sea, sak, seg2,</i> <i>sek2</i>	<i>sea, sak, seg2,</i> <i>sek2</i>	<i>sea, sak, chp</i>	–
φSa4	–	–	–	NI	–	–
φCOL (L54a)	–	–	–	–	–	NI
Genomic islands						
νSaα	<i>set</i> (10), <i>lpl</i> (9)	<i>set</i> (9), <i>lpl</i> (9)	<i>set</i> (11), <i>lpl</i> (5)	<i>set</i> (11), <i>lpl</i> (5)	<i>set</i> (9), <i>lpl</i> (7)	<i>set</i> (7), <i>lpl</i> (6)
νSaβ	<i>spl</i> (5), <i>lukDE</i> enterotoxin (6)	<i>spl</i> (5), <i>lukDE</i> enterotoxin (6)	<i>spl</i> (4), <i>lukDE,</i> <i>bsa</i>	<i>spl</i> (4), <i>lukDE,</i> <i>bsa</i>	<i>hysA, spl</i> (5), enterotoxin (6)	<i>spl</i> (6), <i>lukDE,</i> <i>bsa</i>
Pathogenicity						
Islands						
SaPI1	–	–	–	–	–	<i>seb, ear, seq,</i> <i>sek</i>
SaPI2	<i>sel, sec3, tst</i>	<i>sel, sec3, tst</i>	–	–	–	–
SaPI3	–	<i>fhuD</i>	<i>ear, sel2, sec4</i>	–	–	–
SaPI4	–	–	–	–	NI	–

The presence of MGEs and genomic islands is indicated by text that lists the genes involved in virulence; in the absence of genes proven to play a role in virulence, the presence of an element is indicated by NI (virulence or drug resistance genes not identified at present). The absence of a MGE or genomic island is indicated by –. Figures in brackets indicate the number of homologues in that region. Bacteriophages and SaPI families are classified according to integrase gene homology and insertion site, as described before (Lindsay and Holden 2004). Genomic island regions are consistent with those previously defined (Baba et al. 2002)

bination. This process is very inefficient in *S. aureus*. Nevertheless, in the laboratory setting, generalized transduction is the most efficient mechanism for moving DNA between *S. aureus* isolates.

S. aureus pathogenicity islands

S. aureus pathogenicity islands (SaPI) encode a number of virulence genes, including the superantigens, toxic shock

Table 2 Summary of the MGEs in sequenced *S. aureus* strains associated with drug resistance

	N315	Mu50	MW2	MSSA476	MRSA252	COL
SCC						
SCC <i>mec</i> type I	–	–	–	–	–	<i>mecA</i>
SCC <i>mec</i> type II	<i>mecA</i>	<i>mecA</i>	–	–	<i>mecA</i>	–
SCC <i>mec</i> type IV	–	–	<i>mecA</i>	–	–	–
SCC <i>far</i>	–	–	–	<i>far</i>	–	–
Plasmids						
Class I	<i>ble, kan</i> (Integrated)	<i>ble, kan</i> (Integrated)	–	–	<i>ble, kan</i> (Integrated)	<i>tet</i> (pT181)
Class II	<i>cadDX, arsBC</i> (pN315B)	–	<i>blaZ, cadD</i> (pMW2)	<i>blaZ, cadD</i> (pSAS)	<i>cadAC, arsBC</i> (Integrated)	–
Class III	–	<i>aacA-aphD, qacA</i> (VRSAP)	–	–	–	–
Transposons						
Tn554	<i>ermA, spc</i> (5)	<i>ermA, spc</i> (2)	–	–	<i>ermA, spc</i> (2)	–
Tn552	–	–	–	–	<i>blaZ</i>	–
Tn5801	–	<i>tetM</i>	–	–	–	–
Tn916-like	–	–	–	–	NI	NI

The presence of MGEs is indicated by text that lists the genes involved in drug resistance; in the absence of genes proven to play a role in drug resistance, the presence of an element is indicated by NI (resistance genes not identified at present). The absence of a MGE or genomic island is indicated by –. Text in brackets for the plasmids indicates the name of the plasmid when free or “Integrated” when it is integrated into the chromosome. The number of copies of a transposon in the genome is indicated in brackets

syndrome toxin-1 (*tst*) and enterotoxins B and C (*seb*, *sec*) (Table 1). Superantigens are so called as they can elicit a non-specific T-cell response, leading to massive cell expansion and shock. However, superantigen genes are widespread in *S. aureus*, and shock is a rare complication of *S. aureus* colonization or infection. In the sequenced *S. aureus* strains, six SaPI have been identified, four of which carry superantigen genes (Table 1).

SaPI transfer horizontally in the presence of ‘helper’ phage (Lindsay et al. 1998). SaPI themselves are approximately 15 kb and encode an integrase at one end, similar to the bacteriophage integrases. The integrase promoter region is also similar to that of phage, and SaPI also appear to integrate site specifically (Lindsay et al. 1998). However, they do not encode other genes for the actual transfer process and rely on phage genes for induction. The process can be triggered by infection of the bacterial cell with the helper phage or induction of a helper prophage using stress (Lindsay et al. 1998). The SaPI is excised and packaged into miniature phage-like particles with heads and tails at the same time that normal-sized infectious phage particles are produced (Ruzin et al. 2001). The SaPI particles deliver the SaPI DNA to new *S. aureus* recipient cells, and the SaPI DNA integrates into the recipient chromosome. This process is extremely efficient because the SaPI-infected cells are not infected with a viable bacteriophage that often causes cell lysis (Lindsay et al. 1998).

Staphylococcal cassette chromosomes

Staphylococcal cassette chromosomes (SCC) always insert into a specific region of the *S. aureus* chromosome, approximately 25 kb from the origin of replication. SCC often encode antibiotic resistance genes, such as the *mec* operon for methicillin resistance (SCC*mec*) (Ito et al. 2001) and *far* for fusidic acid resistance (SCC*far*) (Holden et al. 2004). At least five different versions of SCC*mec* are found in *S. aureus*, SCC*mec* types I to V (Ito et al. 2001, 2004; Ma et al. 2002), three of which are found in the sequenced strains (Table 2). SCC frequently include transposons and integrated plasmids which carry other resistances such as kanamycin and erythromycin (Hiramatsu et al. 2001). Genes responsible for a particularly mucoid version of the *S. aureus* capsule have also been reported on SCC (Luong et al. 2002).

SCC vary substantially in length and look like a mosaic structure of integrations and recombinations with various genetic fragments (Ito et al. 2001; Ito et al. 2004; Ma et al. 2002). Typically, SCC carry one (*ccrC*) (Ito et al. 2004) or two *ccr* genes (*ccrA* and *ccrB*) (Katayama et al. 2000) that encode site-specific recombinases, which catalyse the excision and integration of a circular version of the SCC element. How the element replicates and then transfers horizontally between bacteria is not clear. Since the original SCC are too large to fit into a generalized transducing phage head, this may account for why SCC*mec* spread at relatively low frequency. However, the new SCC*mec* type IVs appear to spread much more easily be-

tween isolates (Ma et al. 2002; Robinson and Enright 2003); this could be because of their smaller size, which allows them to be transduced.

Plasmids

There are three families of *S. aureus* plasmid based on size and ability to conjugate (Paulsen et al. 1997). Class I are the smallest and have the highest copy number, whilst only class III are conjugative. Class I are usually less than 5 kb, found at high copy number and encode only one or two resistance genes (Table 2), such as pT181 encoding tetracycline resistance in COL (Gill et al. 2005; Khan and Novick 1983). In some cases, class I plasmids can also be integrated into the chromosome; pUB110 carrying kanamycin and bleomycin resistance is integrated into the type II SCC*mec* elements found in three of the sequenced strains (Holden et al. 2004; Ito et al. 2001; Kuroda et al. 2001) (Table 2). Class I plasmids can be transferred between *S. aureus* by generalized transduction at approximately 100 times the frequency of chromosomal markers (Ubelaker and Rosenblum 1978). This could be due to plasmid concatemers that preferentially package into phage heads (Dyer et al. 1985). They can also be mobilized by some class III conjugative plasmids at low frequency (Projan and Archer 1989).

Class II plasmids are up to 40 kb and typically carry resistance to one or more of the β -lactams (*bla*), heavy metals (*ars*, *cad*, *mer*), antiseptics (*qac*) or aminoglycosides (*aacA-aphD*) (Table 2). The resistance genes are often on transposons that have integrated into the plasmid, and plasmid variants are often due to rearrangements of these elements. It is likely that class II plasmids are transferred between *S. aureus* by generalized transduction.

Class III plasmids are similar to the class II plasmids, but also encode transfer (*tra*) genes that allow conjugative transfer of the plasmid between bacterial isolates at low frequency on solid surfaces (Thomas and Archer 1989) (Table 2). Since some class III plasmids are up to 60 kb, they can be too large to be transferred by transduction.

Transposons

S. aureus transposons often encode resistance genes. For example, Tn554 encodes resistance to erythromycin (Phillips and Novick 1979) and is often associated with type II SCC*mec*. Once a strain is positive for this transposon, multiple copies can become integrated at various sites around the chromosome (Table 2). Tn552 encodes β -lactamase resistance and is often associated with class II and III plasmids, but can also be found in the chromosome (Rowland and Dyke 1989). Tn5801 is a large transposon integrated into the Mu50 genome (Table 2) and encodes tetracycline resistance (*tetM*) (Kuroda et al. 2001). Many other transposons have also been described.

All the transposons encode a transposase gene, and the product of this gene catalyses excision and/or replication of

the element, as well as integration. Horizontal transfer of transposons to other *S. aureus* cells is presumably by “piggybacking” onto another MGE that is transferred, most likely a plasmid. The plasmid can then be transferred by transduction or conjugation. We are unaware of any reports of a transposon integrating into a phage or SaPI.

Conjugative transposons have been described in *S. aureus*; they are transposons that include *tra* genes, but they cannot replicate autonomously. Examples are Tn916 and Tn918, originally from streptococci (Clewell et al. 1985). However, it is not clear if native conjugative transposons are found in *S. aureus*. Interestingly, the genomes of MRSA252 and COL contain novel regions containing genes that have weak similarity to Tn916, which could be a related element (Table 2).

The first fully vancomycin-resistant strains of *S. aureus* have now been described (Anonymous 2002a,b, 2004), and if these isolates were to spread in hospitals, they would cause a serious treatment dilemma. The gene for resistance, *vanA*, is encoded on a transposon derived from enterococci (Weigel et al. 2003). The likely mechanism of transfer was that the transposon in the enterococcus jumped onto a pheromone-responsive conjugative plasmid, which then transferred via conjugation to *S. aureus*. The plasmid was unable to replicate in *S. aureus*, but the transposon had the opportunity to jump into the *S. aureus* chromosome and/or a resident *S. aureus* plasmid (Clewell et al. 1985; Weigel et al. 2003).

Genomic islands and islets

In addition to the previously characterized MGEs, there are regions of the *S. aureus* chromosome that have been designated as genomic islands, which are thought to have arisen by horizontal gene transfer. The origins and modes of transfer remain unclear; however, the compositions of these regions vary between strains and often contain genes associated with pathogenicity. Two such regions (Kuroda et al. 2001) are ν Sa α , encoding multiple exotoxins (*set*) (Jarraud et al. 2001; Williams et al. 2000) and lipoproteins (*lpl*), and ν Sa β , encoding multiple *set* and serine protease homologues (*spl*) (Reed et al. 2001). Both these genomic islands seem extremely stable as they are found in all sequenced isolates (Table 1) in the same location but exhibit allelic variation (Lindsay and Holden 2004). For example, in ν Sa α , the number of *set* and *lpl* varies between strains: MRSA252 contains nine *set* and seven putative *lpl*, one of which is a pseudogene; MSS476 contains eleven *set* and five putative *lpl*. ν Sa β is more diverse than ν Sa α : MRSA252 contains five *spl*, two of which are pseudogenes, and six *set*; MSSA476 contains four *spl*, two leucotoxin homologues (*lukDE*) (Gravet et al. 1998) and a lantibiotic biosynthesis cluster (*bsa*). In addition, the MRSA252 island also contains a hyaluronate lysase paralogue (*hysA*), with 75% identity at the amino acid level to a hyaluronate lysase found elsewhere on the chromosome (SAR2292) (Farrell et al. 1995).

Horizontal gene transfer in other bacteria

The diversity of the MGEs in *S. aureus* suggests that it has catholic taste, utilizing different classes of elements to augment the virulence and drug resistance components of its genome. This raises the question as to what drives the species to be so varied and accommodating in its evolution. For some bacterial pathogens, strain diversity can be reliant on a particular type of MGE or mechanism of genetic exchange. For example, *Streptococcus pyogenes* (alternatively referred to as group A *Streptococcus*) is responsible for a diverse number of diseases in humans, including pharyngitis, acute rheumatic fever (ARF), toxic shock syndrome (TSS), impetigo and scarlet fever. The genomes of five different *S. pyogenes* strains have been sequenced, and the main source of diversity between the genomes is mainly due to the presence of different prophages. Each strain contains between four and six prophages or prophage-like elements, and each of these MGEs contains characterized or putative virulence factors (Banks et al. 2004; Beres et al. 2004; Ferretti et al. 2001; Nakagawa et al. 2003; Smoot et al. 2002). Like *S. aureus*, *S. pyogenes* has a close association with the human host and also has the potential to evolve new variants with increased virulence and drug resistance. Why, therefore, has *S. pyogenes* taken the route of poly-lysogeny to be the main source of strain variation, whereas *S. aureus* is more eclectic? The answer to this question perhaps lies in acquiring a greater understanding of the diversity of the accessory genome of a species.

Are six *S. aureus* genomes enough?

Whilst the increasing multiplicity of genome sequences provides an insight into the sets of genes carried by individual species and the diversity of MGEs, it can also mislead. The apparent genetic make-up of a species can also be distorted by the choice of strains sequenced. Often, they are strains chosen that are deemed to be important for some biological or political reason, for example, epidemic strains, laboratory strains and strains associated with a distinct pathology. However, the strains chosen may not be typical of the species as a whole. This is illustrated by the *S. aureus* strains thus far sequenced. *S. aureus* is a commensal bacterium that is carried by a large proportion of the population. For the vast majority of individuals, carriage will not cause any problems and they will be asymptomatic. The population of *S. aureus* is not only confined to humans; *S. aureus* are carried by, and can cause disease in, animals (van Duijkeren et al. 2004; Vautor et al. 2005). However, our knowledge of the *S. aureus* genome is shaped by the sequence of five human disease-causing isolates (N315, Mu50, MW2, MRSA252 and MSSA476), an MRSA relic (COL) and a laboratory strain (NCTC8325). This therefore raises the question of how representative the genomes are in comparison to carriage strains and the wider population of *S. aureus*.

Using MLST, it is possible to investigate evolutionary relationships of the genome sequence strains in relation to each other and representatives of the wider population. Figure 4 shows a population snapshot of STs in the MLST database generated using eBURST (Feil et al. 2004). *S. aureus* has a clonal population structure (Enright et al. 2002; Feil et al. 2003). In a study of 334 isolates, including carriage and clinical strains, 87% of *S. aureus* in hospitals and the community belong to any one of 11 CCs (Fig. 4) (Feil et al. 2003). The seven sequenced strains belong to four of these CCs: both the hospital-acquired strains N315 and Mu50 and community-acquired strains MW2 and MSSA476 belong to identical but separate STs (ST5 and ST1) in different CCs. COL and NCTC8325 belong to closely related STs (ST250 and ST8) but in the same CC. MRSA252 belongs to CC30 and appears to be the most divergent of the sequenced strains (ST36). Comparative genomic analysis revealed that about 6% of the genome is novel in comparison to other sequenced strains (Holden et al. 2004). Using the population framework as a guide, it is possible to see that the apparent diversity of MRSA252 is due to the relatedness of the other sequenced genomes rather than any true divergence. If a fuller picture of the *S. aureus* genome is to be gained,

strains belonging to the other CCs and non-CCs should be sequenced.

Future evolution of pathogenic and drug-resistant strains

Many MGEs encode genes that contribute to *S. aureus* virulence and pathogenesis, and they can be transferred between *S. aureus* isolates. This transfer leads to the evolution of *S. aureus* causing novel clinical challenges. For example, SCCmec encoding methicillin resistance are now found in *S. aureus* that are particularly adapted to surviving and spreading in hospitals (Moore and Lindsay 2002). Other examples are the community-acquired MRSA (positive for SCCmec) that also carry *lukSF*-PV on a bacteriophage, which seem to spread and cause skin and soft tissue infection in otherwise healthy people (Dufour et al. 2002a). A further example is the recent emergence of three isolates of vancomycin-resistant MRSA, which are positive for SCCmec and the *vanA* transposon (Weigel et al. 2003).

It therefore seems likely that these MGEs will be capable of transferring further, potentially generating strains of *S. aureus* that are resistant to methicillin and vancomycin and

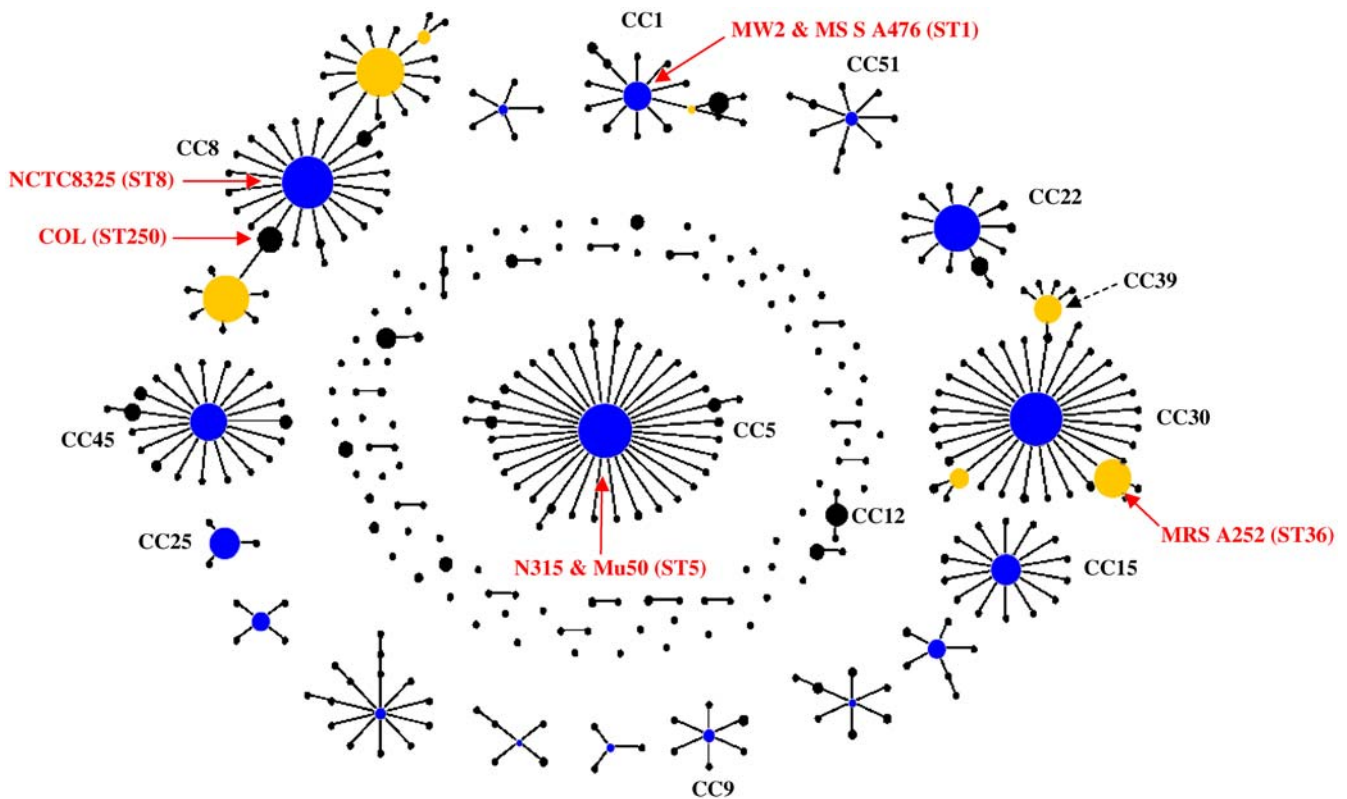


Fig. 4 Population snapshot of *S. aureus*. Clusters of related sequence types (STs) and individual unlinked STs within the entire *S. aureus* multilocus sequence typing (MLST) database (<http://saureus.mlst.net/>) are displayed as a single eBURST (Feil et al. 2004) diagram by setting the group definition to zero of seven shared alleles. The figure was generated from 1,360 isolates, corresponding to 372 STs, in the *S. aureus* MLST database. Clusters of linked

isolates correspond to CCs. Primary founders (blue) are positioned centrally in the cluster, and subgroup founders are shown in yellow. The eight major and three minor CCs described by Feil et al. (2003) are indicated; the ST labels have been removed for clarity. The position of the seven sequenced strains are indicated in red, with their ST indicated in brackets

capable of spreading in hospitals and causing disease in healthy patients.

Despite the widespread distribution of *S. aureus* and serious antibiotic selective pressure, such strains have not been identified as yet. A likely explanation is that there are barriers to horizontal transfer and that these are efficient in nature. For example, *S. aureus* have restriction–modification (RM) pathways (Iordanescu and Surdeanu 1976), which probably inhibit horizontal transfer. Restriction enzymes digest DNA at specific sequences, and modification enzymes modify those sequences in the bacterium’s own DNA, preventing restriction. Thus, RM should prevent the horizontal transfer of MGE from “foreign” bacteria. The genome sequencing projects identify at least two *S. aureus*-encoded type I RM genes on the chromosome and genomic islands (Holden et al. 2004; Kuroda et al. 2001), and gene variation between sequenced strains occurs. More recently, Dempsey et al. (2005) have identified RM genes in a *S. aureus* bacteriophage, which is capable of conferring resistance to lysis by other bacteriophage.

Other barriers to horizontal transfer may also be important. It is thought that an isolate lysogenic for a bacteriophage is resistant to infection with a related bacteriophage (bacteriophage immunity), and a similar mechanism may be functioning in SaPI. Plasmids can be classed into different “incompatibility” groups, such that two plasmids with the same replication mechanism cannot survive in the same cell (Novick 1987). A further explanation is that much horizontal transfer is dependent on generalized transduction by bacteriophage, but the distribution and frequency of such phages are not known. Further investigation of horizontal transfer of MGE is essential to truly understand *S. aureus* evolution and the potential for more virulent and resistant strains to emerge.

In addition to the barriers associated with the mode of transport of MGEs, there is growing evidence that the genetic background of a recipient can also contribute to the success of a transfer. The distribution of methicillin resistance conferring SCC*mec* elements is not uniform in the population; not all the major CCs of *S. aureus* contain a high frequency of MRSA strains (Enright et al. 2002). It has been hypothesized that some genetic backgrounds may be restrictive of the methicillin resistance gene (*mecA*) and its expression, which could account for the restricted clonal distribution of SCC*mec* in nature (Katayama et al. 2005). When *mecA* was introduced on a low-copy-number plasmid into methicillin-susceptible *S. aureus* clinical isolates, the transformability and stability of the *mecA* plasmid were greater in strains from the CCs that contain higher numbers of MRSA strains (Katayama et al. 2005). As far as SCC*mec* is concerned, it would therefore appear that all *S. aureus* are not equal; some innate genetic or biochemical property of the strains influences the success of SCC*mec*.

In summary, *S. aureus* is a bacterium that typically lives in the human nose and has evolved with us. Approximately 11 clonal lineages dominate, and it remains to be seen what

gene combinations and variations make these lineages so successful. In addition, MGEs move in and out of *S. aureus* isolates with significant frequency. The accumulation of MGEs in some isolates makes them increasingly virulent and resistant to antibiotics and able to cause new clinical challenges. Our current understanding suggests that we may have seen only “the tip of the iceberg” and that *S. aureus* will continue to evolve, potentially generating isolates that can create havoc in our hospitals as well as in healthy patients outside of the healthcare setting.

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