

REVIEW

Multilocus sequence typing and the evolution of methicillin-resistant *Staphylococcus aureus*

D. A. Robinson and M. C. Enright

Department of Biology and Biochemistry, University of Bath, Claverton Down, Bath, UK

ABSTRACT

The prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) in many countries is increasing and, in hospitals in some areas, more than half of all *S. aureus* disease isolates are MRSA. MRSA strains are becoming increasingly multiresistant, and have recently developed resistance to vancomycin, used successfully to treat MRSA for more than 30 years. This review summarises recent studies that have elucidated the evolutionary history of MRSA. The first MRSA isolate evolved from a sensitive, epidemic strain prevalent in Europe, and its progeny—the first MRSA clone—quickly spread to other continents. Analyses of epidemic MRSA isolates from hospitals in different countries by molecular methods, including multilocus sequence typing (MLST) and DNA microarray analysis, reveal that MRSA strains have evolved separately within five distinct epidemic, sensitive lineages. However, resistance has been transferred to *S. aureus* on many more than five occasions, as some lineages have acquired different structural types of the element carrying the methicillin resistance gene. The emergence of MRSA as a community pathogen has been noted in several countries, and MLST and SCC*mec* typing have been used to demonstrate that community-acquired MRSA strains are typically related only distantly to hospital MRSA strains, and thus represent novel acquisitions of SCC*mec*.

Keywords Evolution, MLST, MRSA, multilocus sequence typing, *Staphylococcus aureus*

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BACKGROUND

Nosocomial methicillin-resistant *Staphylococcus aureus* (MRSA) infections represent a major challenge to hospital microbiologists because of the emergence and spread of clones with decreased susceptibility to many antibiotic classes. Since the mid to late 1990s, hospital MRSA isolates have increased in prevalence in Europe, the USA and elsewhere [1,2]. In one European study of 25 university hospitals [3], one-quarter of 3051 *S. aureus* isolates collected were MRSA, with a geographical bias towards higher rates in southern countries such as Italy (50.5%) and Portugal (54%), and lower rates in northern European countries, including The Netherlands (2%), Austria (9%) and Switzerland (2%). MRSA infections

are associated with increased morbidity, mortality and length of hospital stay, and represent a major financial burden on healthcare services [4,5].

The first strain of MRSA was isolated in 1961 [6], 2 years after the introduction of methicillin; this strain rapidly spread to other countries throughout the 1960s, and became a problem in the USA in the 1970s. The antibiotic of choice for treating MRSA infections is the glycopeptide vancomycin, but reports of vancomycin intermediately susceptible *S. aureus* (VISA), first isolated in Japan in 1997 [7,8], caused widespread alarm among physicians fearful of an era of untreatable MRSA infections. Reports of VISA isolates with an MIC ≥ 8 mg/L have so far been very rare, but two recent reports of fully vancomycin-resistant *S. aureus* (VRSA) from Michigan [9] and Pennsylvania [10] (MICs of 32 and 128 mg/L, respectively) in the USA have again caused alarm, and it is as yet unclear whether either VISA or VRSA isolates will become epidemic, leading to an exacerbation of the global MRSA problem.

Corresponding author and reprint requests: M. C. Enright, Department of Biology and Biochemistry, University of Bath, Claverton Down, Bath, BA2 7AY, UK
Tel: +44 122 538 6871
Fax: +44 122 538 6779
E-mail: m.c.enright@bath.ac.uk

In a climate of increasing *S. aureus* antibiotic resistance, the study of MRSA epidemiology has assumed new importance, because any strategies to contain the spread of MRSA at the local (hospital), national or international level require knowledge of how strains are spread and how MRSA epidemics occur. Epidemiological studies can be used to provide basic knowledge of the population biology of MRSA, and can help to answer fundamental questions such as: (1) how strains spread; (2) the number of major MRSA, VISA and VRSA clones circulating globally, and their relatedness to each other and to susceptible isolates; and (3) the ancestry of modern MRSA, VISA and VRSA strains. The answers to these questions have, until recently, been unclear, but several recent studies employing modern molecular typing technologies have now significantly increased our knowledge in these areas.

MODELS OF MRSA EVOLUTION

Two models of MRSA evolution were initially proposed in the early 1990s, based on studies using different typing techniques. The simplest model, described by Kreiswirth *et al.* [11], was based on analysis of restriction fragment length polymorphisms generated by *Cla*I digestion of chromosomal DNA, followed by hybridisation with Tn554 and *mecA* probes. The limited number of patterns observed in a geographical and temporally diverse sample of MRSA isolates was taken as evidence that *S. aureus* had acquired the methicillin resistance gene (*mecA*) on only one occasion, and the authors therefore hypothesised that all extant MRSA clones were recent descendants of this prototypical isolate.

Several months before the publication of this study, Musser and Kapur [12] described MRSA as being polyclonal in a multilocus enzyme electrophoresis analysis of 254 MRSA isolates. The association of *mecA* with divergent genetic backgrounds was taken as strong evidence that the gene is transferred horizontally between *S. aureus* isolates. The only alternative explanation for these results was that MRSA isolates had diversified so rapidly in the 31 years between 1961 and 1992 that they had lost any genetic similarity. The existence of modern MRSA lineages that are unrelated to the first MRSA strain by molecular typing methodologies supports the theory of Musser and Kapur, and this is further streng-

thened by evidence from microarray analysis [13] and multilocus sequence typing (MLST) [14] that conclusively demonstrate horizontal movement of the *mecA* gene.

TOOLS FOR INVESTIGATING CLONAL SPREAD

The recovery of isolates with identical bacteriophage types from different hospitals within and between countries was described in the 1950s in seminal studies by Rountree and Freeman [15] and Rountree and Beard [16], in which the existence of *S. aureus* types with increased epidemicity was demonstrated. However, bacteriophage typing has fallen out of favour as a means of characterising *S. aureus*, because of difficulties in typeability and reproducibility, as well as the cryptic genetic basis upon which characterisation relies [17,18].

Molecular typing techniques are commonly used to study the epidemiology of *S. aureus*. The international spread of epidemic clones, such as the Iberian [19], UK epidemic [20,21], New York–Japanese [22,23], Viennese [24] and German MRSA [24], has been investigated with a variety of techniques, the most popular of which has been pulsed-field gel electrophoresis (PFGE) [25,26]. PFGE is suitable for MRSA outbreak investigation because of the high level of discrimination attainable, which allows outbreak isolates to be separated from unrelated isolates. The main drawback of such simple and widely used ‘band-based’ technologies in studying longer-term, national or global epidemiology is the difficulty faced in making inter-laboratory comparisons of PFGE data. Standardisation of reagents and electrophoresis conditions increases the portability of PFGE, but subjective decisions about DNA banding pattern similarity still constitute a major barrier to establishing PFGE as a satisfactory method for characterising *S. aureus* clones.

MLST

MLST [27] involves sequencing DNA fragments (typically *c.* 500 bp) of seven housekeeping genes. The sequences of these genes are compared to known alleles at each locus via the MLST website (<http://www.mlst.net>), where every isolate is described by a seven-integer allelic profile that defines a sequence type (ST). For example, isolates

of the Iberian clone have the MLST profile 3-3-1-12-4-4-16, which defines ST247. MLST was first applied to *S. aureus* in a study published in 2000 [28], in which 155 invasive *S. aureus* isolates were typed. In addition to validating the method against PFGE, the study showed how epidemic clones of MRSA and methicillin-susceptible *S. aureus* (MSSA) could be unambiguously defined by their ST.

SCCMEC TYPING

The methicillin resistance structural gene *mecA* is a small (2007 bp) part of a much larger genetic element which is inserted precisely into the *S. aureus* chromosome. This staphylococcal chromosomal cassette *mec* (SCC*mec*) varies in size from *c.* 20 to 68 kb, but always contains *mecA* and at least part of a regulatory gene *mecR1* and chromosomal cassette recombinase genes (*ccr*). Four main types of SCC*mec* have been described [29,30], and although the same types are often associated with divergent lineages, particular MRSA clones are associated with single SCC*mec* elements. For example, all epidemic MRSA (EMRSA) clone 16 (ST36) isolates from the UK have SCC*mec* type II (<http://www.mlst.net>).

MRSA NOMENCLATURE

A report on 912 MRSA and MSSA isolates from 20 different countries [14] contained a proposal that MRSA clones be named according to their MLST and SCC*mec* types in the form ST-resistance phenotype (i.e., MRSA, MSSA, VISA or VRSA)-SCC*mec* type (i.e., I, II, III or IV). For example, the Iberian clone would be known as ST247-MRSA-I. This was agreed by a subcommittee of the International Union of Microbiology Societies in Tokyo, 2002. It is hoped that this nomenclature will replace, or at least supplement, existing arbitrary designations of MRSA clones, based on geographical location or other less satisfactory typing methods, since MLST is systematic and objective, and provides a key for investigators to search for clones in the MLST website databases.

ORIGINS OF THE FIRST MRSA

The first study to examine the ancestry of the original MRSA isolates used MLST to compare MRSA isolates from different countries with a

collection of MSSA isolates from the 1950s [31]. The study showed that the first MRSA clone belongs to ST250 (allelic profile 3-3-1-1-4-4-16), which is a genotype shared with epidemic MSSA isolates common in Denmark in the 1950s. This 'archaic' clone was found to be ancestral to the Iberian clone [19], as it shares six of seven housekeeping gene alleles. The Iberian clone is a modern pandemic MRSA that shares the high epidemicity of the 'archaic' clone, a genotype that spread extremely rapidly after its first emergence in 1961 and was isolated as recently as 1993 (<http://www.mlst.net>).

The origins of early MRSA isolates were further clarified in the larger-scale MLST study described above [14]. In this work, the authors used MLST and SCC*mec* typing to show the complexity of evolutionary events leading to the emergence of the first MRSA isolate and modern epidemic MRSA and MSSA clones from an ancestral isolate of ST8 (MSSA) (Fig. 1). The designation of ST8 as the ancestral genotype was achieved using the BURST algorithm (based upon related sequence types; http://155.198.40.150/new/data_analysis/burst/burst.htm), which separates MLST data sets into groups called clonal complexes (CCs). These share at least five housekeeping gene alleles in common with at least one other member. For each ST in the group, the ancestor is assumed to be the genotype with the largest number of variants differing at only one gene. The assignment of ST8-MSSA as the ancestor of ST250-MSSA is supported by the finding that ST8 and ST250 differ at a single locus whose alleles are identical except for a point mutation in *yqiL*, unique to ST250 and its descendants [14]. Further support for this hypothesis is the finding that all isolates of ST250 and its descendants are MRSA with SCC*mec* class I. Fig. 1 shows an evolutionary scenario that could have led to the emergence of major clones such as ST239-MRSA-III (named variously as UK EMRSA-1, UK EMRSA-4, Viennese clone and the Portuguese/Brazilian clone [32]) and ST247-MRSA-I (the Iberian clone, UK EMRSA-5 and UK EMRSA-17 [33]), among others.

HOW MANY MRSA CLONES ARE THERE?

Analysis of a collection of 36 *S. aureus* isolates by DNA microarray analysis [13] showed that the *mecA* gene was associated with five genetically

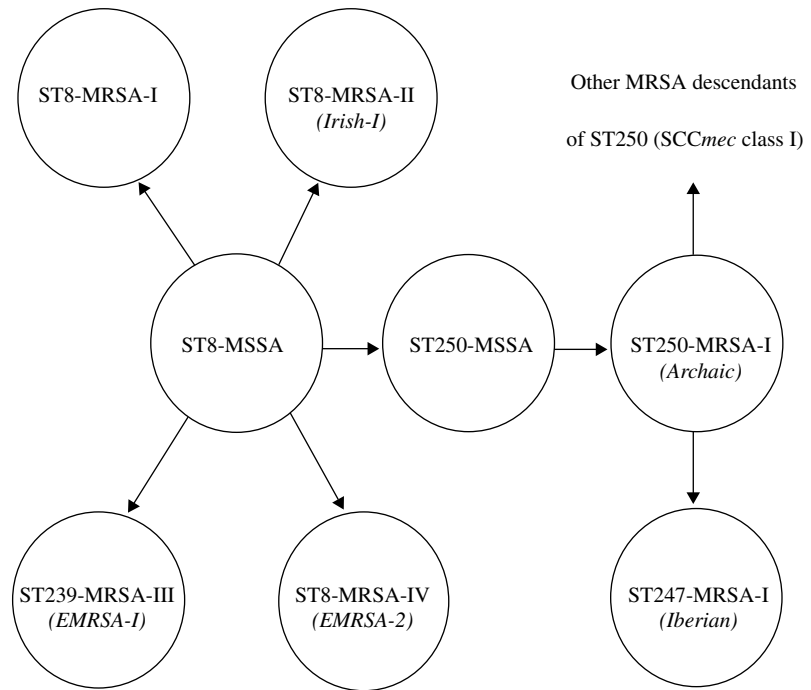


Fig. 1. Evolutionary origins of notable CC8 genotypes. Arrows indicate either (1) acquisition of *SCCmec*, or (2) change of ST. For some epidemic MRSA clones, the name used previously in the literature is included in parentheses.

divergent groups of isolates, indicating that the gene for methicillin resistance has been horizontally transferred at least five times in *S. aureus*. This finding was confirmed by an MLST study [14] which showed that all epidemic hospital MRSA isolates that have been found in more than one country belong to five CCs. These CCs are named according to the ST of their proposed ancestor, and include CC8 (archaic MRSA), CC5, which contains most of the VISA isolates studied to date, and three clonal complexes (CC45, CC30 and CC22) that contain recently emerged international MRSA clones such as UK EMRSA-16 (CC30) [34], Berlin epidemic MRSA (CC45) [35], and UK EMRSA-15 or Barnim epidemic MRSA [36].

Major MRSA clones have emerged from some CCs on multiple occasions, resulting in isolates with the same MLST type that differ in *SCCmec* type. Enright *et al.* [14] described 11 major epidemic MRSA clones, defined as genotypes (same MLST and *SCCmec* type), found in more than one country and represented by at least ten isolates. This arbitrary designation contains all the epidemic MRSA isolates described in the literature, but the isolates examined are over-representative of European countries. What is clear from MLST studies is that a small number of ecologically successful genetic backgrounds can acquire the methicillin resistance gene and retain a high level

of epidemicity. This is demonstrated by the fact that in each of the five MRSA-containing CCs, the ancestral genotype is represented by recent disease-causing MSSA isolates (<http://www.mlst.net>).

MRSA strains are emerging as a cause of community-acquired disease in some countries but, until recently, the genetic relatedness of such strains to each other and to hospital MRSA isolates was not known. Analysis of 47 community MRSA isolates from Australia and the USA [37] by MLST and *SCCmec* typing showed that some types are closely related to hospital MRSA, but that most isolates characterised had STs not found in studies of hospital MRSA to date. Isolates of ST1, the most common genotype in this study, and ST30 have been reported as common causes of community-acquired MSSA disease in the past [28], but the acquisition of *SCCmec* IV by these successful epidemic genotypes is worrying, especially as in this study isolates of these clones expressed Pantón–Valentine leukocidin, which in one study was found to be associated with necrotising pneumonia in patients with a much younger average age than is usual for pneumonia [38]. This expansion of the host range of isolates causing MRSA disease to the community and to younger age groups is troubling at a time when the therapeutic options

for the treatment of MRSA are becoming increasingly limited.

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