Re-emergence of methicillin susceptibility in a resistant lineage of *Staphylococcus aureus*

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Abstract

Objectives

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a leading cause of hospital-associated infection. Acquired resistance is encoded by the *mecA* gene or its homologue *mecC* but little is known about the evolutionary dynamics involved in gain and loss of resistance. The objective of this study was to obtain an expanded understanding of *S. aureus* methicilin resistance microevolution *in vivo*, by focusing on a single lineage.

Methods

We compared the whole genome sequences of 231 isolates from a single epidemic lineage (clonal complex CC30 and *spa*-type t018) of *S. aureus* that caused an epidemic in the United Kingdom.

Results

We show that resistance to methicillin in this single lineage was gained on at least two separate occasions, one of which led to a clonal expansion around 1995 presumably caused by a selective advantage. Resistance was however subsequently lost *in vivo* by nine strains isolated between 2008 and 2012. We describe the genetic mechanisms involved in this loss of resistance and the imperfect relationship between genotypic and phenotypic resistance.

Conclusions

The recent re-emergence of methicillin susceptibility in this epidemic lineage suggests a significant fitness cost of resistance and reduced selective advantage following the introduction in the mid 2000s of MRSA hospital control measures throughout the United Kingdom.

Introduction

Staphylococcus aureus is a commensal bacterium frequently colonising the nose and skin, but also a potential pathogen, causing diseases ranging from mild skin infections to septicaemia. Worldwide *S. aureus* is a leading cause of hospital-associated infections, exacerbated by strains resistant to commonly used antibiotics. Methicillin-resistant *S. aureus* (MRSA) is resistant to most beta-lactam antibiotics, including penicillins and cephalosporins [1]. MRSA genomes are typically distinguishable from methicillin-sensitive *S. aureus* (MSSA) by the presence of the *mecA* gene or its homologue *mecC*. In the United Kingdom healthcare-associated MRSA came to the fore in the 1990s mostly in the form of the two epidemic clones EMRSA-15 and EMRSA-16, which declined after 2005 [2]. Genome sequence analysis to detect *mecA* allows prediction of resistance phenotype with high, although imperfect, accuracy [3,4]. The *mecA* gene is part of the SCC*mec* cassette that can be inserted into the staphylococcal chromosome and inherited vertically or transferred between lineages via horizontal gene transfer [5]. Most MRSA lineages evolved from MSSA ancestors after gaining SCC*mec*, providing a selective advantage, which likely contributed to worldwide spread. However, little is known about the fitness cost of resistance and the dynamics of SCC*mec* acquisition and re-emergence of genomic and phenotypic susceptibility. Additionally, there are reports of phenotypic resistance in the absence of *mecA* and conversely of phenotypic susceptibility in the presence of apparently functional *mecA* [3], although the underlying mechanisms are poorly understood.

In order to shed new light on these important issues, we compared whole-genome sequences of 231 isolates (197 MRSA, 34 MSSA) sampled from across England between 1997 and 2013. All isolates belonged to the clinically important clonal complex 30 (CC30) and to *spa*-type t018. This collection includes the successful healthcare-associated MRSA clone known as EMRSA-16 (ST36-SCC*mec*II).

Materials and Methods

Isolates

We selected 231 isolates (Supplementary Table 1) obtained from clinical specimens (one isolate per patient), which all belonged to both *spa*-type t018 (as determined using *spa*-typing) and CC30 (as determined based on genome sequences). 48 isolates were from

carriage screening swabs and 183 from diagnostic samples, including 167 from blood cultures. Isolates originated from Brighton (131), Oxford (47), London (19), elsewhere in southern England (21), the Midlands and northern England (13). 39 isolates were obtained from material archived at the PHE reference laboratory, Colindale. 10 isolates had been collected by the UK Clinical Infection Research Group (UKIRG). Sequence types represented were: ST36 (213), ST30 (15), ST34 (2) and ST38 (1). The methicillin susceptibility of the isolates was assessed phenotypically on primary testing as part of routine diagnostic laboratory procedures. Methicillin susceptibility was subsequently reassessed by disc diffusion (cefoxitin) and Etest (oxacillin). Isolates were stored, cultured, identified and sequenced as described elsewhere [3,6].

Bioinformatics methods

The sequenced reads were assembled both *de novo* and by reference-based mapping against MRSA252 [7] using a previously described bioinformatics pipeline [8]. Sequence types were determined *in silico* based on the *de novo* assemblies. The phylogeny was built using PhyML [9], corrected for the effect of recombination using ClonalFrameML [10] and dated using previously described methodology [11]. The dating process relied on the sampling date of each sample and on a mutation rate which was assumed to be 8.4 mutations per year per genome [12–14].

Ethics statement

Isolate storage and data collection was approved in Brighton by the BSUH Research and Development office as a service evaluation, involving anonymized data from patient records and not requiring formal ethical review. Isolates were collected for epidemiological studies covered by Statutory Instrument Regulations 2002 No. 1438, section (iii) 'Communicable disease and other risks to public health (Health Service Control of Patient Information)' of Section 60 of the Health and Social Care Act and therefore did not require research ethics committee approval.

Results

Phylogenetic distribution of resistance

A dated phylogeny was constructed using the genome sequences of all isolates (Figure 1). As expected, the samples cluster in accordance with multilocus sequence type (MLST) as

determined *in silico*. The most recent common ancestor for the entire lineage dates to 1978, with divergence thereafter of branches leading to ST30, ST34 and ST36. Most isolates belong to ST36 (EMRSA-16) whose most recent common ancestor was dated to 1993. This is more recent than a previous estimate of 1975 [14], but our result is in good agreement with the timing of the first observations in the UK of ST36 [2]. The unique ST38 sequence nests within the ST36 clade indicating its direct derivation from ST36. Both available ST34 isolates were MSSA. Most ST30 isolates were methicillin-susceptible although two were methicillin-resistant following the acquisition of SCC*mec*IV. Most ST36 isolates were methicillin-resistant, with many branches diverging close to the most recent common ancestor, suggesting rapid clonal expansion associated with a fitness advantage conferred by the loss of sensitivity. Resistance acquisition by ST30 and ST36 cannot be dated more accurately than between 1980 and 1995 as both events occurred on long branches. Surprisingly, within the predominantly resistant ST36 lineage were 19 MSSA isolates. Ten of these could be explained by loss of resistance during storage [15], because the isolates had been found to be resistant in susceptibility tests performed directly after isolation. In contrast, the remaining nine isolates had been identified as MSSA at the time of primary culture (Figure 1).

Discrepancies between resistance phenotype and genotype

The *mecA* gene, encoding resistance to beta-lactam antibiotics [16], is located in the SCC*mec* cassette which represents a hotspot of recombination [8]. Many different alleles of SCC*mec* have been described, differing in the number and type of genes present [5]. In our dataset we found two different SCC*mec* types, each paired to a different ST type: ST36 harbours a type II cassette, with *mecR1*, *mecI*, *ccrA*, *ccrB* and *mecA* [1], ST30 harbours a type IV cassette, lacking the *mecI* gene and having a partial *mecR1*. In general we found concordance between resistance phenotype and genotype (Table 1). SCC*mec* does not have to be complete to be functional [17]. We found partial SCC*mec* in 7 isolates. In one case only the *ccrA/B* genes were missing and the strain was phenotypically resistant, in all other cases only the *ccrA/B* genes were present and the strain was phenotypically susceptible.

Five isolates were methicillin-sensitive despite the presence of the *mecA* gene, and all of them had lost methicillin resistance during storage (labelled 1 to 5 in Figure 1). An ST30 isolate (labelled 1 in Figure 1) had the gene but lacked the rest of the operon, which might explain its susceptibility. The entire operon was present in the other four discrepant isolates. One isolate (labelled 2 in Figure 1) shows deletion of a single base-pair in *mecA* resulting in a

frameshift, premature stop codon and gene inactivation. In the remaining three discrepant isolates (labelled 3, 4 and 5 in Figure 1) the *mecA* gene is identical to functional *mecA* genes present in resistant isolates. Other SCC*mec* genes in these discrepant isolates do not exhibit any particular differences from resistant isolates in the collection. Analysis of genes previously described as interacting with SCC*mec* (*blaZ*, *blaI*, *blaR1*, *femA*, *femB*) yielded no conclusive result. Similarly, analysis of polymorphic sites known to be associated with resistance yielded no significant result.

Re-emergence of susceptibility

The distribution of susceptible isolates within the timed tree shows that the ancestral resistant phenotype was lost *in vivo* in nine strains isolated in Brighton (n=7) and London (n=2) between 2008 and 2012. Three of these formed a genetic cluster whilst the others were genetically distant, with their nearest neighbours being MRSA. Methicillin-susceptibility therefore re-emerged independently on at least seven separate occasions within the ancestrally resistant ST36, and this was confirmed using ancestral state reconstruction. Within ST36, the dates of the nine MSSA isolates were significantly more recent than the dates of the MRSA isolates (p-value <0.01, Kolmogorov Smirnov test), suggesting that re-emergence of susceptibility was linked with MRSA specific control measures introduced in the UK in the mid-2000s [2]. Interestingly we found several different molecular mechanisms that led to the loss of the resistant phenotype *in vivo* or in storage. The most frequent genetic background for the susceptible phenotype (nine genomes out of the total 19) was loss of the entire SCC*mec* cassette. In six of the susceptible samples we were able to detect only part of the cassette, but no resistance-associated *mec* genes (*mec*A, *mec*I or *mec*R1). In one genome (labelled 2 in Figure 1) the entire SCC*mec* cassette was present, but the susceptibility can be explained by a deletion causing a frameshift and loss of function in the *mec*A gene. Finally, there remain three cases (labelled by 3, 4 and 5 in Figure 1) for which we were unable to find a genetic explanation for the phenotypic loss of resistance, as described above.

Discussion

By comparing 197 MRSA and 34 MSSA genomes, representing a single epidemic lineage (CC30) of *S. aureus*, we show that ST36 (corresponding to EMRSA-16) gained SCC*mec* before the mid-1990s and subsequently underwent clonal expansion (Figure 1). Loss of methicillin resistance during the storage retrieval process is well documented [15] and we found

ten examples of this in our study. More surprisingly, we also demonstrate many examples of loss of methicillin resistance *in vivo* affecting multiple sublineages within ST36 and occurring after 2008, at a time when MRSA control measures were being implemented in UK hospitals. These observations suggest that methicillin resistance originally provided a selective advantage to ST36 compared with other members of CC30, including the putative methicillin-susceptible ST36 ancestor, which does not feature in our dataset. However, resistance may impart a fitness cost [18] which has apparently not been overcome by compensatory mutations. When the fitness cost exceeds the selective advantage of resistance, susceptible strains are expected to re-emerge. Recent initiatives to limit beta-lactam usage, including restricted prescribing of cephalosporins, may partly explain our observations [19]. Further work will be needed to determine to what extant our observation is unique to the lineage ST36 we studied, or whether similar dynamics of resistance loss occur for all MRSA, which would for example explain why Swedish MSSA outbreak isolates contained remnants of SCC*mec* [20].

We demonstrate multiple disparate mechanisms to explain reversion from MRSA to MSSA and our detection of a cluster of three susceptible genetically related isolates suggests that such strains are transmissible and have the potential to spread. As we have shown, the prediction of phenotypic resistance from genomic sequence data has yet to be perfected, although increasing interest in this subject suggests that it will improve rapidly [3,4]. More accurate resistance prediction, combined with reductions in sequencing costs and turnaround times may allow more targeted use of antibiotics and facilitate antibiotic stewardship. Our findings represent an encouraging observation for MRSA control efforts and more generally for the control of antibiotic resistant pathogens.

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Transparency declarations

We declare no competing interests.

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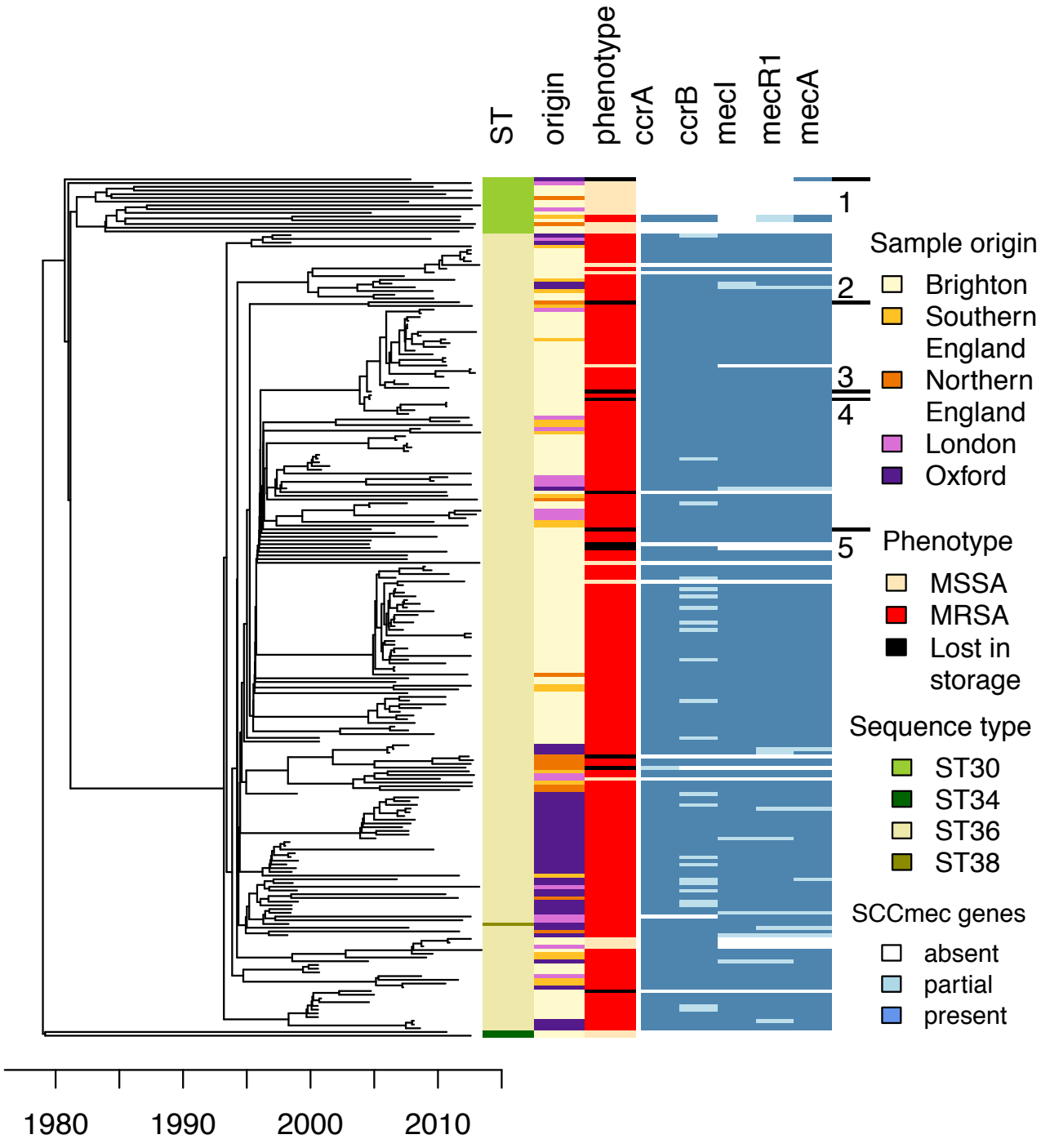
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Genotype		Phenotype	
		Resistant	Susceptible
ST36	SCC*mec* (Type II)	191	5
	Partial SCC*mec* (Type II)	1	5
	No SCC*mec*	0	9
ST30	SCC*mec* (Type IV)	2	0
	Partial SCC*mec* (Type IV)	0	1
	No SCC*mec*	0	14
ST34	SCC*mec*	0	0
	Partial SCC*mec*	0	0
	No SCC*mec*	0	2
ST38	SCC*mec* (Type II)	1	0
	Partial SCC*mec*	0	0
	No SCC*mec*	0	0

285
286 **Table 1:** Summary of genotypic and phenotypic methicillin resistance status for all 231
287 isolates described in this study.
288

Figure Legend

Figure 1: Dated phylogenetic tree showing the relationship between all 231 *Staphylococcus aureus* genomes. The panel on the right shows a number of properties of the genomes, namely (from left to right) the MLST sequence type (ST), geographical location of origin (origin), phenotypic resistance status (MRSA, MSSA or loss of resistance during storage), and presence/absence of five genes typically present in SCC*mec* type II (*ccrA*, *ccrB*, *mecI*, *mecR1*, *mecA*). The five genomes for which phenotypic and genotypic resistance data were discrepant are labelled 1 to 5.


ST
origin
phenotype
ccrA
ccrB
mecI
mecR1
mecA
1
2
3
4
5
Sample origin
Brighton
Southern England
Northern England
London
Oxford
Phenotype
MSSA
MRSA
Lost in storage
Sequence type
ST30
ST34
ST36
ST38
SCCmec genes
absent
partial
present
1980
1990
2000
2010