

Intra-strain variability of methicillin-resistant *Staphylococcus aureus* strains ST228-MRSA-I and ST5-MRSA-II

S. Monecke · R. Ehricht · P. Slickers · N. Wiese ·
D. Jonas

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Abstract Isolates belonging to two major epidemic strains of methicillin-resistant *Staphylococcus aureus* (MRSA) from clonal complex 5 were characterised using diagnostic microarrays in order to detect and analyse intra-strain variability. Isolates were sampled from hospitals scattered all over Germany. The study included 56 isolates of ST228-MRSA-I, which is also known as the South German Epidemic Strain, and 40 isolates of ST5-MRSA-II (UK-EMRSA-3, Rhine-Hesse Epidemic Strain, New York/Japan Clone), as well as, for comparison, some control strains and overseas isolates of ST5-MRSA-II. Both strains showed a remarkable variability. This affected plasmid-borne resistance genes (*tetK*, *blaZ/R/I*, *aacA-aphD*, *qacA*), genes from SCC*mec* elements (*aadD*, *ermA*, *merA/B/R/T*), toxin gene clusters on pathogenicity islands (*sec/I*, *tstI*) or, probably, on plasmids, (*sed/j/r*), the

presence or absence of beta-haemolysin-converting phages (*sea*, *sea-N315*, *sak*, *chp*, *scn*), deletions of single chromosomal genes (*bbp*, *clfA*) or, occasionally, of rather large clusters of neighbouring genes (*seg*, *sei*, *sem*, *sen*, *seo*, *seu*, *lukD/E*). Both strains could be split into four major clusters each, based on the presence of a mercury resistance operon (*merA/B/R/T*) and *lukD/E* in ST228-MRSA-I or of *tstI* and enterotoxin genes *seD/J/R* in ST5-MRSA-II. The use of this variability for typing purposes as well as its phylogenetic significance are discussed.

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) poses a significant problem in healthcare. As therapy options for infections with such strains are limited, the need for infection control measures increases. The rapid typing of MRSA becomes necessary in order to trace and to interrupt chains of transmissions, as well as to handle litigation resulting from hospital-acquired infections. Typing methods are usually either pulsed-field gel electrophoresis (PFGE, [1]) or sequence-based approaches (*spa* [2], multilocus sequence typing (MLST) [3]), after phage typing has widely been abandoned because it proved to be rather labour-intensive. Using these methods, it was possible to split MRSA into a number of distinct strains [4]. Some of these strains were found in several countries, or even worldwide, while others have been restricted to certain regions or countries. Unfortunately, the definition of a strain is somewhat arbitrary. A strain, as defined by one method, could be split into several strains using another. For instance, the PFGE ‘strain’ USA600 comprises two strains with different SCC*mec* types (II and IV), or ‘ST8-MRSA-IV’ encompasses both Pantón–Valentine leukocidin (PVL)-

S. Monecke (✉)
Institute for Medical Microbiology and Hygiene,
Faculty of Medicine “Carl Gustav Carus”,
Technical University of Dresden,
Fetscherstraße 74,
01307 Dresden, Germany
e-mail: monecke@rocketmail.com

R. Ehricht · P. Slickers
CLONDIAG GmbH,
Löbstedter Straße 103–105,
07749 Jena, Germany

N. Wiese
MVZ Labor Dr. Fenner und Kollegen,
Bergstraße 14,
20095 Hamburg, Germany

D. Jonas
Department of Environmental Health Sciences,
Freiburg University Medical Centre,
Breisacher Straße 115 B,
79106 Freiburg, Germany

positive and -negative strains, which clearly differ in virulence. On the other hand, different designations and definitions might all refer to the same strain (e.g. UK-EMRSA-3, New York/Japan Clone, USA100, Rhine-Hesse Epidemic Strain, Irish AR7.3, AR7.4, AR7.11 and Canadian MRSA-2 are all ST5-MRSA-II). Another problem with these typing methods is a poor discriminatory power in a setting where only a few abundant strains predominate. If everybody carried the same strain, it would become problematic to prove, or to dismiss, a transmission from one individual patient to another.

Variations within strains could help to resolve this problem. Such variations affect *spa* repeats as shown previously (see *spa* database: <http://spaserver2.ridom.de/index.shtml>) and they also might affect the carriage of accessory resistance genes or toxins.

In order to assess intra-strain variability, we selected two major MRSA epidemic strains. Isolates of each strain were sampled from hospitals scattered all over Germany and characterised using diagnostic microarrays.

Targeted strains included a ST228-MRSA-I strain known as the South German Epidemic Strain [5] or Italian Clone [6]. This is a multiresistant, gentamicin-resistant *SCCmec* I strain from clonal complex (CC) 5.

The second strain was a ST5-MRSA-II strain which is locally known as the Rhine-Hesse Epidemic Strain. This is another multiresistant CC5 strain which, under different names (see above), recently achieved global distribution.

Materials and methods

Isolates

Isolates included into the present work were ST228-MRSA-I and ST5-MRSA-II, which were collected and typed within the framework of the “Surveillance of antimicrobial use and antimicrobial resistance in German intensive care units” (S.A. R.I.) study [7]. Primarily, 916 isolates collected in the period 2000–2004 were typed using PFGE as previously described [8]. One isolate per PFGE type and submitting intensive care unit (ICU) was further analysed by *spa* typing according to published protocols [2]. Six hundred and fifty-two isolates belonged to CC5 strains. Based upon a largest possible variety of *spa* types and geographic origin, strains were selected for microarray analysis. These isolates originated from the intensive care patients of different German university hospitals from the German Federal States Baden-Württemberg (in the tables and figures of the present paper coded as BWU), Bavaria (BAV), Berlin (BER), Bremen (BRE), Hesse (HES) and Thuringia (THU).

Additionally, isolates from various outbreak investigations at the University Hospital of Dresden and some other

cooperating hospitals within the State of Saxony (SAX) were included. Two further isolates came from hygiene surveillance in a hospital in the state of Saarland (SAA). These isolates were identified using diagnostic microarrays as described below [9, 10].

For comparison, one Australian (AUS) and four Irish (IRE) isolates of ST5-MRSA-II were included, as well as the USA100 molecular typing control strain NRS382 (USA100/626, from NARSA, Chantilly, Virginia, USA). Additionally, sequenced ST5-MRSA-II strains Mu50 (NC_002774/AP003367, [11]), N315 (NC_003140/AP003139, [11]), both obtained from the Collection Institut Pasteur, Paris, France, and Mu3 (AP009324, from NARSA) were used as controls, as well as for comparison with clinical isolates.

Sequenced ST5-MRSA-II strains JH1 (CP000736) and JH9 (CP000703) were not tested, but hybridisation profiles were deduced from their published chromosomal and plasmid sequences.

Array procedures

The DNA microarray (based on AT technology, by CLONDIAG, Jena, Germany) covered 334 targets. This corresponds to allelic variants of approximately 185 distinct genes, including species markers, resistance genes, exotoxins, genes encoding microbial surface components recognising adhesive matrix molecules of the host, as well as *SCCmec*, capsule and *agr* group typing markers [9, 10].

The protocols and evaluation procedures for the assay have been described previously in detail [9, 10]. Therefore, only a short overview shall be provided here. MRSA were grown overnight on Columbia blood agar. Cultures were harvested, digested using lysostaphin, lysozyme and ribonuclease A in a first step and Qiagen AL buffer plus proteinase K in a second step. Afterwards, DNA was purified using the Qiagen device EZ1 according to its tissue lysis protocol. All target genes of the assay were amplified simultaneously in a linear primer elongation. Within this reaction, biotin-16-dUTP was incorporated into the amplicons which were then subsequently hybridised to the array. This was followed by washing and blocking steps. Horseradish-peroxidase-streptavidin was conjugated to the array, followed again by incubation and washing. Finally, a precipitating dye (Seramun Green, Seramun, Heidesee, Germany) was added. After a short incubation, an image of the array was recorded and analysed using a designated reader and software (ATR01, IconoClust, by CLONDIAG).

SplitsTree analysis

In order to analyse similarities (not necessarily phylogenetic relations) between hybridisation patterns, the SplitsTree algorithm and software [12] was employed. Hybridisation

Table 1 Variable genes and variants in ST228-MRSA-I

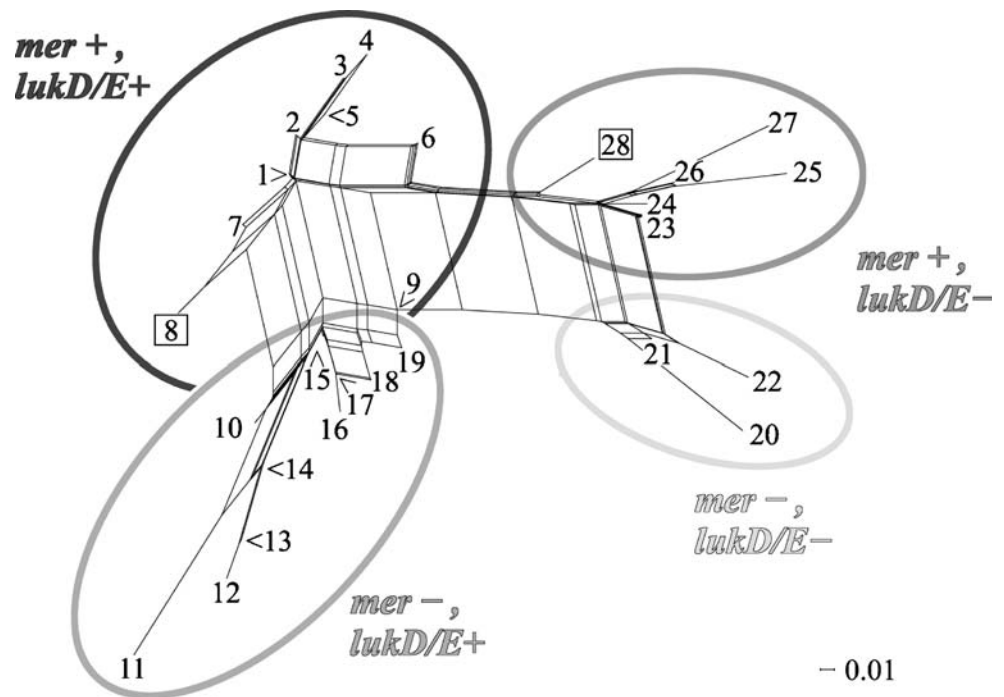
	spa type									Isolates																										
	001 (10), 023, 041, t110, t892 (1 each) 001 (6)									BAV (13), BWU (1) BAV (2), BER (1), SAX (5), THU (2) SAX (1) BER (1) SAX (1) n.a. 023 001 t143 062																										
										BAV (1) THU (1) BER (1) BER (1) BER (1) SAX (1) SAX (1, including 2 from an outbreak) SAX (1) SAX (1)									BAV (1) SAX (1) BWU (1)									SAX (1), BWU (2) BAV (1) HES (1) THU (1), BWU (1) HES (1) THU (1)								
<i>sdrD</i>	[Grid of black squares]																																			
<i>clfA</i>	[Grid of black squares]																																			
<i>bbp</i>	[Grid of black squares]																																			
<i>scn</i>	[Grid of black squares]																																			
<i>chp</i>	[Grid of black squares]																																			
<i>sak</i>	[Grid of black squares]																																			
<i>un-trunc. hlb</i>	[Grid of black squares]																																			
<i>lukY</i>	[Grid of black squares]																																			
<i>Q7A4X2</i>	[Grid of black squares]																																			
<i>lukD/E</i>	[Grid of black squares]																																			
<i>seu</i>	[Grid of black squares]																																			
<i>seo</i>	[Grid of black squares]																																			
<i>sei/sem</i>	[Grid of black squares]																																			
<i>seg/sen</i>	[Grid of black squares]																																			
<i>sea</i>	[Grid of black squares]																																			
<i>tstI</i>	[Grid of black squares]																																			
<i>qacC</i>	[Grid of black squares]																																			
<i>qacA</i>	[Grid of black squares]																																			
<i>mupR</i>	[Grid of black squares]																																			
<i>dfrA</i>	[Grid of black squares]																																			
<i>aphA3/sat</i>	[Grid of black squares]																																			
<i>aadD</i>	[Grid of black squares]																																			
<i>ermC</i>	[Grid of black squares]																																			
<i>ermA</i>	[Grid of black squares]																																			
<i>blaZ/I/R</i>	[Grid of black squares]																																			
<i>merA/B/R/T</i>	[Grid of black squares]																																			
Variant	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28								

results for relevant genes (see below) were converted into strings or ‘sequences’ using for each single gene in a predefined order an ‘A’ for ‘positive’ or a ‘T’ for ‘negative’ hybridisation results. Each of these strings, thus, contained the complete hybridisation profile of an isolate having always the same length. These ‘sequences’ were used for tree construction using SplitsTree 4.10 (characters transformation, uncorrected *P*; distance transformation, Neighbour-Net; and variance, ordinary least squares).

Results

All isolates shared characteristic features of CC5 [9, 10]. This included the affiliation to *agr* group II and capsule type 5, as well as the presence of the *egc* enterotoxin gene cluster (either complete or truncated) and of Mu50/N315-like alleles of *set/ssl* and MSCRAMM genes as previously described [9, 10]. ST228-MRSA-I carried *mecA*, *delta-mecR*, *ugpQ*, *ccrA-1*, *ccrB-1*, *plsSCC-COL* and *Q9XB68-dcs*, which is in

Fig. 1 SplitsTree similarity plot showing four major clusters of ST228-MRSA-I based on the presence of the mercury resistance operon and/or *lukD/E*. The strain numbers are as in Table 1. As an example for minor variations, the presence of *tst1* (strain numbers in boxes) is also shown



accordance to the presence of a SCCmec I element. ST5-MRSA-II harboured *mecA*, *delta-mecR*, *ugpQ*, *Q9XB68-dcs*, *mecI*, *mecR*, *xylR*, *ccrA-2*, *ccrB-2*, *kdpA-SCC*, *kdpB-SCC*, *kdpC-SCC*, *kdpD-SCC* and *kdpE-SCC*, thus, confirming SCCmec type II.

ST228-MRSA-I

Fifty-six isolates of the South German Epidemic Strain were tested. Their *spa* types were t001 (most common), t023, t041, t062, t110, t143, t811 and t892.

Variable genes (Table 1) were the mercury resistance operon (*merA*, *merB*, *merR*, *merT*), the beta-lactamase operon (*blaZ*, *blaI*, *blaR*), macrolide/lincosamide resistance genes (*ermA*, *ermC*), the tobramycin resistance gene (*aadD*), the neo-/kanamycin and streptothricin resistance genes (*aphA3*, *sat*), the trimethoprim resistance gene (*dfrA*), the mupirocin resistance gene (*mupR*), genes encoding unspecific efflux pumps (*qacA*, *qacC*), the genes encoding toxic shock syndrome toxin (*tst1*), enterotoxins (*sea*, *seg*, *sei*, *sem*, *sen*, *seo*, *seu*) or leukocidin homologues (*lukD*, *lukE*, *lukY1*), untruncated haemolysin beta, genes encoding staphylokinase (*sak*), chemotaxis inhibitory protein (*chp*), staphylococcal complement inhibitor (*scn*), bone-binding protein (*bbp*), clumping factor A (*clfA*), serine aspartate repeat protein D (*sdrD*) and Q7A4X2 (locus tags SAV1823 in Mu50, or SA1641 in N315, a hypothetical protein located next to *seg* and *lukD/E*).

A SplitsTree similarity plot allowed one to distinguish four major clusters (Fig. 1). One carried the mercury resistance operon (*merA*, *merB*, *merR*, *merT*), another

carried leukocidin homologues *lukD/E*, a third cluster harboured both and a fourth carried neither. The absence of *lukD/E* was usually associated with various deletions affecting neighbouring genes, including the *egc* enterotoxin gene cluster (*seg*, *sei*, *sem*, *sen*, *seo*, *seu*) and Q7A4X2.

ST5-MRSA-II

For this strain, several genome sequences are available. Sequenced strains N315, Mu50 and Mu3 were tested, and hybridisation experiments were in accordance to the prediction based on these sequences. The only difference was the detection of *aacA-aphD* and *qacA* in Mu3. In Mu50, these genes are plasmid-borne. In Table 2, the experimental data and predicted hybridisations based on *chromosomal* sequences of both strains are compared. It can be assumed that *aacA-aphD* and *qacC* were also plasmid-borne in Mu3, although sequence information on the plasmids of that strain is not yet available.

The *spa* types of ST5-MRSA-II were t002, t003, t045, t893 and t1290, with the former two being predominant.

Variable genes (Table 2) were *blaZ*, *blaI*, *blaR*, *mpbBM* (a macrolide resistance gene), *aacA-aphD*, *aadD*, *dfrA*, *tetK*, *tetM* (tetracycline resistance genes), *qacA*, *qacC*, *tst1*, enterotoxin genes *sea*, *sea-N315/sep*, *sec*, *sed*, *sej*, *sel* and *ser*, as well as *lukD*, *chp* and the staphylococcal exotoxin-like or superantigen-like gene *ssl4* (= *set9*). A SplitsTree analysis based on the carriage of the above-mentioned genes allowed one to discern four clusters within ST5-MRSA-II (Fig. 2).

Table 2 Variable genes and variants in ST5-MRSA-II. For Mu3 and Mu50, experimental findings as well as predicted hybridisation patterns (*) based on published chromosomal sequences (i.e. without

plasmids) are shown (see Results section). Sequenced strains JH1 and JH9 (**) are included based on the analysis of their genome sequences (including plasmids)

Variant or strain	Isolates	spa type
N315		t002
Mu3/Mu50		t002
Mu3/Mu50*		t002
1	BRE (1)	t002
2	HES (2), SAX (1)	t003 (2), t1290
3	SAX (1)	n.a.
4	SAX (3)	t003
5	SAX (1)	t003
JH1/JH9**		t002
6	AUS (1)	t002
7	BAV (1)	n.a.
8	BAV (4), SAA (1), SAX (1)	t002 (1), t003 (4), t893 (1)
NRS382		n.a.
9	SAA (1), SAX (1)	n.a.
10	SAX (3)	n.a.
11	BAV (1), BWU (6), HES (3), SAX (8)	t002 (7), t003 (5), t045 (1)
12	SAX (1)	n.a.
13	IRE (1)	n.a.
14	IRE (2)	n.a.
15	IRE (1)	n.a.

Gene	N315	Mu3/Mu50	Mu3/Mu50*	1	2	3	4	5	JH1/JH9**	6	7	8	9	10	11	12	13	14	15
<i>ssl4 (set9)</i>	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
<i>chp</i>	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
<i>lukD</i>	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
<i>ser</i>	■	■	■	■															
<i>sel</i>	■	■	■	■															
<i>sej</i>																			
<i>sed</i>																			
<i>sec</i>	■	■	■	■															
<i>sea-N315</i>	■	■	■	■															
<i>sea</i>	■	■	■	■															
<i>tst1</i>	■	■	■	■															
<i>qacC</i>																			
<i>qacA</i>		■	■	■															
<i>tetM</i>		■	■	■															
<i>tetK</i>																			
<i>dfrA</i>																			
<i>aadD</i>	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
<i>aacA-aphD</i>	■	■	■	■															
<i>mpbBM</i>																			
<i>blaR</i>	■	■	■	■															
<i>blaI</i>	■	■	■	■															
<i>blaZ</i>	■	■	■	■															

One cluster harboured *tst1* and enterotoxin genes *sed*, *sej* and *ser*; it comprised the Irish isolates. A second cluster carried *tst1* (and, additionally, *sec* and *sel*) but lacked *sed/j/r*. The sequenced strains N315, Mu3 and Mu50 from Japan, as well as the only *tst1*-positive German isolate of ST5-MRSA-II, belonged to this cluster. A third cluster was positive for enterotoxin genes *sed/j/r* but did not harbour *tst1*. Most German isolates and the control strain NRS382 belonged to it. Finally, a fourth cluster carried neither *sed/j/r* nor *tst1*.

Within all four clusters, there were both *sea-N315* (= *sep*)-positive and -negative isolates.

Discussion

Both strains showed quite a remarkable variability. This affected plasmid-borne resistance genes (*tetK*, *blaZ/R/I*, *aacA-aphD*, *qacA*), genes from SCCmec elements (*aadD*, *ermA*,

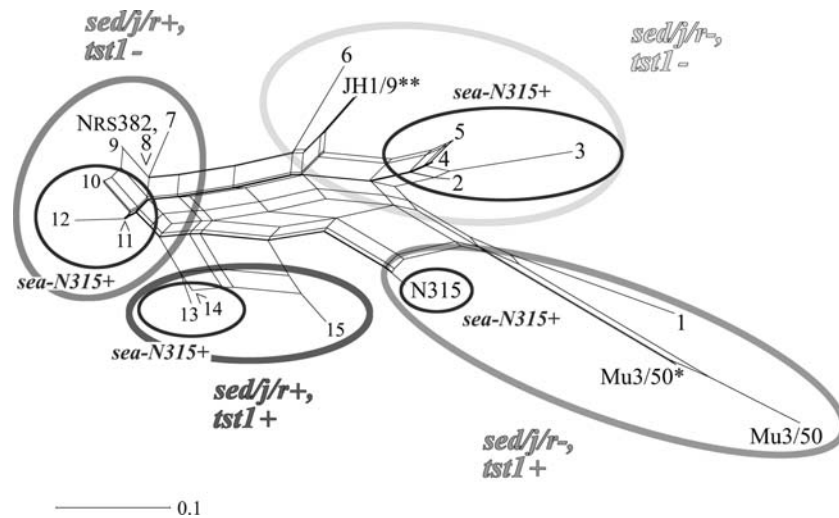


Fig. 2 SplitsTree similarity plot showing four major clusters of ST5-MRSA-II based on the presence of *sed/j/r* and/or *tst1*. The presence of *sea-N315*-positive isolates within these clusters is also indicated. For Mu3 and Mu50, experimental findings as well as predicted hybrid-

isation patterns (*) based on published chromosomal sequences (i.e. without plasmids) are shown. Sequenced strains JH1 and JH9 (**) are included based on the analysis of their genome sequences (including plasmids)

mer), toxin gene clusters on pathogenicity islands (*secI*, *tst1*) or, probably, on plasmids, (*sed/j/r*), the presence or absence of beta-haemolysin-converting phages (*sea*, *sea-N315*, *sak*, *chp*, *scn*), deletions of single chromosomal genes (*bbp*, *clfA*) or, occasionally, of rather large clusters of neighbouring genes (*seg*, *sei*, *sem*, *sen*, *seo*, *seu*, *lukD/E*, Q7A4X2).

These variations could be used to divide abundant, predominate, epidemic strains into smaller entities in order to facilitate epidemiological investigations. For instance, two isolates of ST228-MRSA-I (variant 17) were obtained from an epidemiologically proven outbreak in Saxony in 2006 which further affected seven other patients (data not shown). In addition to the ST228-MRSA-I (variant 23) isolate described herein, three others were found between 2005 and 2007, all of which were linked to one ward of a rehabilitation facility [9].

Even a small set of molecular (or even phenotypic) tests can be used to differentiate variants within the described strains once they have been identified using, e.g. using PFGE. For instance, two polymerase chain reaction (PCRs), one for *lukD* or *lukE* and one for the mercury resistance operon, could allow the assignment of isolates of the South German Epidemic Strain to one of its for major clusters. Three PCRs, e.g. for *tst1*, *sed* and *sea-N315*, could be used to identify eight subtypes within the Rhine-Hesse Epidemic Strain, and the detection of tobramycin resistance as a surrogate marker for *aadD* could even allow a higher degree of discrimination. Similar schemes for the easy and rapid subtyping of other abundant MRSA strains could be developed, e.g. ST22-MRSA-IV (Barnim Epidemic Strain, UK-EMRSA-15) can also be split into four clusters based on

test results for *ermC* and enterotoxins *secI* [9, 10]. Such PCRs could be run as multiplex reactions, and they might be helpful for infection control laboratories with limited access to molecular techniques. However, microarray technology allows the simultaneous screening of all relevant targets, resulting in a much higher resolution with not much more effort.

The analysis of intra-strain variation might give insight into the pattern of spread and distribution. The virtual absence of *tst1*-positive ST5-MRSA-II from Germany could be most plausibly attributed to a ‘population bottleneck’ or ‘founder effect’ upon importation, i.e. it could indicate that most German isolates originate from a very small imported population. This might provide a timeframe for the evolution of the observed diversity (about 15 years). Whether some variants truly were restricted to certain regions within Germany (such as, apparently, variant 1 of ST228-MRSA-I to south-western Germany, see Table 1) still needs to be substantiated with a higher number of isolates to be characterised.

The variability within these epidemic strains and within USA300 as described previously [13] raises the question as to what exactly a ‘strain’ is. A strain has been defined as a group of isolates sharing geno- or phenotypic traits distinguishing it from other strains of the same species [14, 15]. The selection of such traits is arbitrary and, with an increased number of interrogated markers, a number of variants within previously established ‘strains’ can be distinguished which could be referred to as variants, sub-strains or clones. A clone has been defined as a number of isolates being “indistinguishable by a variety of genetic tests” [14, 15]. However, the term ‘variety’ is not defined, and a “variety of tests” might refer to a multiplex PCR with

five to ten targets or to arrays covering from some hundreds of probes up to a fully sequenced genome. Thus, the definition of strains as well as of clones depends on the resolution of the technology used for typing. Using an approach with a high resolution, i.e. a relatively high number of variable genetic markers, an apparently homogeneous strain disintegrates into a cluster of related, but changeable genotypes differing by accidental gene deletions or acquisitions. This behaviour rather resembles the concept of a 'quasispecies' [16, 17] than of the traditional definition of a bacterial strain, although in this case, it affects whole genes rather than nucleotides. But the observation that the genome "cannot be described as a defined structure, but rather as a weighted average of a large number of individual sequences" [18] also applies. Thus, a strain definition can be based only on purely practical considerations. In the case of *S. aureus* and MRSA, such a definition might include MLST type/CC affiliation, SCCmec type (as proposed by Enright et al. [4]), plus the carriage of highly distinctive genes, such as PVL genes. Most enterotoxins and *tst1* are obviously affected by accidental variations to an extent which renders them unusable for a robust strain definition, but they might be helpful to differentiate variants within a strain.

Recently, the phylogeny of a worldwide collection of CC5 strains was scrutinised by the analysis of sequence polymorphisms distributed over 108 loci throughout the genome [19]. This demonstrated a regional-specific evolution of particular sublineages, indicating essentially a polyphyletic emergence of ST5-MRSA strains. Since we used a completely different approach, screened for another set of genetic markers and put more emphasis on mobile elements, the results cannot be directly compared. However, SCCmec elements could be regarded as just one class of mobile elements among others. Thus, it could also be assumed that gene transfers or losses resulting in, e.g. different toxin carriage might actually predate the acquisition of SCCmec elements. This issue still needs to be clarified.

Another question is about the biological significance of these variations. The acquisition of additional resistance genes is of obvious benefit for a hospital-acquired pathogen. However, the loss of adhesion factors could influence the ability to adhere to, e.g. intravenous lines or other synthetic implants, which was highly relevant for a hospital-acquired MRSA strain, and deletions of sets of enterotoxins could also adversely influence its pathogenic potential. In order to assess the role of individual virulence factors, it would be interesting to compare the fitness of these natural deletion variants. Given the high frequency with which such variants appear, it might well be that the redundancy of virulence factors in *S. aureus* was an adaptation to cope with accidental gene losses. However, the biological significance and modalities of this intra-strain variability still need to be clarified.

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