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Comparative genomics and DNA array-based genotyping of pandemic *Staphylococcus aureus* strains encoding Panton-Valentine leukocidin

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ABSTRACT

Within the last few years, methicillin-resistant *Staphylococcus aureus* (MRSA) strains encoding Panton-Valentine leukocidin (PVL) have emerged and spread worldwide. This epidemic can be attributed to a small number of distinct clones. The present study used a novel assay, based on multiplex linear DNA amplification and subsequent microarray hybridisation, to simultaneously detect all relevant exotoxins, antimicrobial resistance determinants and the allelic variants of *agr*. The genes of the staphylococcal exotoxin-like (*set*) locus were also included for typing purposes. This assay, together with multilocus sequence typing (MLST) and *spa* typing, was applied to 56 clinical isolates and reference strains representing all major pandemic PVL-MRSA lineages, as well as to phylogenetically-related strains and putative ancestors. Array hybridisation results allowed the assignment of isolates to clonal groups, which were in accordance with MLST and *spa* typing data. Ten distinct clonal groups of PVL-MRSA (ST1, ST5, ST8, ST22, ST30, ST59/359, ST80/583, ST88, ST93 and ST152), including 12 MLST types, were identified and analysed with regard to resistance determinants and genes coding for exotoxins. The array hybridisation data confirmed that pandemic PVL-positive strains originate from very diverse genetic backgrounds, and provided insights into the evolution of some lineages. The DNA microarray technique provides a valuable epidemiological tool for the detailed characterisation of clinical isolates and comparison of strains at a global level.

Keywords Community-associated MRSA, DNA microarray, evolution, exotoxins, methicillin-resistant *Staphylococcus aureus*, Panton-Valentine leukocidin

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INTRODUCTION

One of the most important clinical pathogens is *Staphylococcus aureus*. This bacterium causes diverse infections, such as wound infections, abscesses and septicaemia. Some strains can also cause food poisoning, Ritter's disease and toxic shock syndrome (TSS) [1,2]. *S. aureus* has evolved to exploit ecological niches created by modern medicine and intensive care. It frequently infects intravenous lines and synthetic implants [2], and expresses a complex system of resistance determinants [3–5] that cause major therapeutic problems.

Panton-Valentine leukocidin (PVL) is a well-characterised virulence factor of *S. aureus*. PVL is composed of two distinct protein components, which together form heptameric pores in

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leukocytes [6], leading to increased virulence. PVL-positive strains are able to cause chronic or recurrent skin and soft-tissue infections in immunocompetent individuals [7–11]. Such strains have also been reported to cause necrotising pneumonia, with extremely high mortality, either in previously healthy patients, or as a complication of viral respiratory tract infection [12–16]. In addition, PVL-positive *S. aureus* have been associated with necrotising fasciitis [17], purpura fulminans [18] and Waterhouse–Friderichsen syndrome [19].

PVL was described initially in 1932 [20]. During the 1950s/1960s, the pandemic spread of a PVL-positive, β-lactamase producing strain ('phage type 80/81') was observed, but this strain receded after the introduction of penicillinase-resistant β-lactam agents such as oxacillin [21]. Currently, global spread of new PVL-positive strains carrying *mecA* is occurring, which exhibit resistance to all β-lactam antibiotics. This development has been paralleled by the emergence of two new staphylococcal chromosome cassette *mec* (SCC*mec*) types (IV and V), which, in contrast to the 'older' SCC*mec* types (I–III) found in healthcare-associated methicillin-resistant *S. aureus* (HA-MRSA), do not affect the growth rate of the bacteria adversely [22,23]. Strains carrying these older types were restricted mainly to hospitals, where they had a selective advantage over susceptible wild-type strains. In contrast, strains carrying SCC*mec* types IV and V have spread outside hospitals as easily as the wild-type, and are therefore referred to as community-associated MRSA (CA-MRSA).

Some SCC*mec* type IV strains also carry the genes for PVL; such strains were first described in the 1990s [24] and are now distributed worldwide. They have caused occasional large-scale outbreaks in communities in which individuals are in close contact, or share sanitary facilities, e.g., military units, sports teams or isolated rural communities [25–29]. Hospital-acquired infections have also been reported, sometimes reaching epidemic proportions [30]. Furthermore, these developments have been observed in countries that do not have major problems with healthcare-associated MRSA infections, e.g., Denmark [31] and The Netherlands [32].

It is known that there are distinct epidemic clones of PVL-positive MRSA, which can be defined by multilocus sequence typing (MLST) or *spa* typing [33,34]. These strains also differ in resistance to antibiotics other than β-lactams, and in the presence of virulence determinants other than PVL [11,35].

The present study established and evaluated a novel assay for the simultaneous detection of all relevant exotoxins and antimicrobial resistance determinants. The technique is based on multiplex linear DNA amplification and subsequent diagnostic microarray hybridisation. The assay was applied to PVL-positive *S. aureus* isolates from various countries in order to characterise them with regard to resistance determinants and exotoxins.

MATERIALS AND METHODS

Bacterial isolates

Isolates of *S. aureus* were cultured on Columbia blood agar and incubated overnight at 37°C. Single colonies were selected and subcultured. Tests for clumping factor and coagulase were performed using commercial kits. Antimicrobial susceptibility tests, including detection of methicillin resistance, were performed according to the standards of the country of origin. The present study investigated 56 isolates selected because they had been found to be either PVL-positive or genetically similar to PVL-positive isolates. The sequenced *S. aureus* strains MW2, Sanger MRSA252 and Sanger MSSA476 were also included, and other sequenced strains (COL, N315, Mu50 and NCTC 8325) were used for array evaluation. Sequenced strains RF122 and USA300 (whose sequences were released during the course of this study) were not available for testing. Reference strains ATCC 23925, ATCC 43300 and NARSA247 were included because of their similarity to study isolates. The strains included in the study are described in detail in Table S1 (see Supplementary material).

Preparation of genomic DNA

Two inoculation loops of culture material from Columbia blood agar were suspended in 100 µL of a lysis solution containing 0.05 mg lysostaphin (Sigma, Steinheim, Germany), 2 mg lysozyme (Sigma), 2 mg ribonuclease A (Sigma), 2 µL 20 mM Tris/HCl pH 8.0, 2 µL 2 mM EDTA and 1 µL Triton X-100. The suspension was incubated with shaking (300 rpm) for 45 min at 37°C, after which 10 µL proteinase K and 100 µL buffer AL (DNeasy kit; QIAgen, Hilden, Germany) were added. After a further incubation for 45 min at 56°C, the sample was processed using the QIAgen EZ1 device according to the manufacturer's tissue lysis protocol.

Plasmid preparation

Plasmid preparation was performed using buffers P1 to P3 from a Plasmid Midi Kit (QIAgen). Four inoculation loops of culture material from Columbia blood agar were suspended in 500 µL of buffer P1 containing 0.05 mg lysostaphin, 2 mg lysozyme and 2 mg ribonuclease A. This suspension was incubated with shaking (300 rpm) for 45 min at 37°C, after which 500 µL of buffer P2 was added. After incubation for

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10 min at room temperature, 500 µL of ice-cold buffer P3 were added. The suspension was then cooled on ice for 15 min and centrifuged (15 000 *g*) for 35 min at 4°C. Two subsequent phenol-chloroform precipitations were performed. The remaining aqueous phase was purified using columns from a DNaesy Tissue Kit (QIAgen) after being mixed with equal volumes of buffer AL (DNaesy Tissue Kit) and ethanol. Plasmid DNA was resuspended in 50 µL distilled water and visualised by non-denaturing gel electrophoresis. Finally, bands were cut from the gel and the DNA was resuspended using a Zymo Gel Recovery kit (HiSS Diagnostics, Freiburg, Germany). Plasmid preparations were used for microarray-based genotyping in the same way as genomic DNA.

Microarray design

Table S2 (see Supplementary material) shows the target genes, sources of the sequence data (accession numbers in GenBank), and the locations of the probe and primer sequences. Probe sequences were derived from published sequences using the Array Design software package (CLONDIAG, Jena, Germany). All published sequences for specific targets were used for sequence alignment. Adjacent consensus regions in the alignments of all available sequences of each target were selected for the design of probes and primers. The resulting oligonucleotide sequences were designed to be specific for each target, to be free of self-hybridising sequences, and to have a similar length, GC content and melting temperature, in order to provide comparable binding efficiency. The nomenclature used for this study is based on the alignments. For that reason (for example) enterotoxin '*seP*' was regarded as synonymous with *seA*. Finally, probe and primer sequences were again analysed against the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/) to exclude false-positive reactions resulting from possible cross-reactivities. Database entries were also reviewed in order to identify sequence variations that might result in false-negative reactions. A local database was established that covered all probe and primer sequences, as well as all available *S. aureus* genome sequences. Match tables were designed using the Array Design Software Package (CLONDIAG), which listed all theoretically possible hybridisations of the oligonucleotides to the genomes, allowing up to five mismatches per oligonucleotide, although measurable signals can only be produced with up to two mismatches. This facilitated a prediction of hybridisation patterns for the sequenced strains. These strains were used for experimental evaluation, and the prediction was compared with the hybridisation data obtained.

Microarray-based genotyping

Samples were tested using diagnostic DNA microarrays based on the ArrayTube platform (CLONDIAG) as described previously [5,10,36–40]. Probes directed towards species markers, identification of *agr* types, antibiotic resistance determinants and exotoxins of *S. aureus* have been described partially and evaluated previously [5,10]. Targets are listed in Figs 1–4, and a complete list of targets and probes is shown in Table S2 (see Supplementary material).

The protocol used for amplification and labelling of target DNA is based on a linear PCR-like approach that uses only one primer per target (site-specific amplification and labelling), thereby facilitating an extremely high degree of multiplexing, in which 128 primers were used simultaneously (Table S2). The amplification protocol has been described previously [5].

For at least one representative strain from every clonal group, an alternative protocol for random-primed genome labelling was used [10,37,38]. In brief, arbitrary extension products were generated from genomic DNA in a sequenase-driven primer extension using primers consisting of a randomised octamer and a conserved sequence. In a second round, the extension products were re-amplified using a PCR with a biotinylated primer targeting the conserved sequence. The resulting amplicons were used for hybridisation.

Hybridisation procedures were identical for both approaches and have been described previously [5]. After staining for 10–30 min with horseradish-peroxidase-based tetramethylbenzidine precipitation staining, the resulting pattern was recorded and analysed using an ATR01 reader (CLONDIAG) and IconoClust software (CLONDIAG) according to the manufacturer's instructions. The signal intensity and local background of each spot (i.e., probe position) on the array were measured by transmission. For the interpretation of raw data, the normalised intensity (NI) was first calculated, in which NI = 1 – (M/BG), where M is the average intensity of a spot, and BG is the intensity of the local background, giving results between 0 (weak signal/negative) and 1.0 (strong signal/positive). The average values for control spots and species markers (ribosomal probes, *femA*, *gapA*, *katA*, *coa*, *spa*, *sak* and *sbi*) were calculated and used for assessing the test validity and for defining breakpoint values. If this average value was below a predefined threshold (0.4), the test was regarded as invalid. If the normalised intensity for a given probe was < 33% of the average value of the control spots and species markers, the corresponding target was regarded as absent/negative.

An exception concerned the probes for the *set1*, *set4* and SACOL1180/SAR1141 genes. These genes each have two or three allelic variants, which differ only by single nucleotide polymorphisms. For these targets, the probe with the strongest signal was regarded as positive, while the probes that yielded weaker signals were considered to be negative, regardless of their actual normalised intensities. For *set6*, four probes recognising two distinct binding sites were designed (Table S2), and determination of a *set6* allele resulted from the combination of positive results.

Typing methods

The isolates were also typed using several different approaches.

agr groups are based on a signalling pathway involving auto-inducing peptides. Peptides produced by some strains inhibit the expression of *agr* in other strains, resulting in bacterial interference and exclusion of the other strains. This incompatibility has resulted in the formation of four mutually inhibitory groups that are associated loosely with clinical disease [41,42]. The *agr* groups were determined by microarray hybridisation using the probes listed in Table S2.

spa typing, i.e., typing based on sequence polymorphisms of repeating units within the staphylococcal protein A gene (*spa*), was performed as described previously [33]. Types were assigned using the Ridom SpaServer database (http://www.spa.ridom.de) and SPA Type Mapper software (http://www.clondiag.com/technologies/download.php?file=spa).

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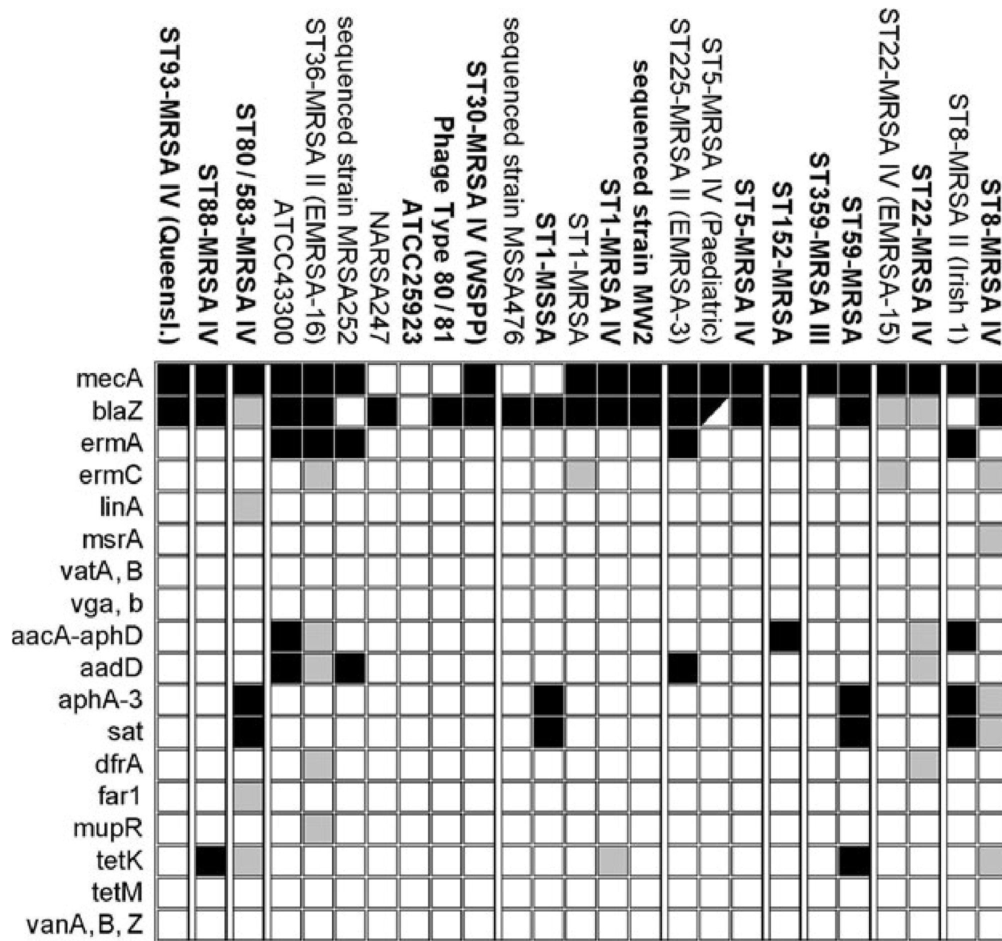

Fig. 1 Antibiotic resistance genes in the strains studied. Panton-Valentine leukocidin (PVL)-positive strains are indicated in bold letters. Black boxes, positive; white boxes, negative; divided boxes, divergent results using the two labelling approaches; shaded boxes, variable.

Multilocus sequence typing (MLST), a sequence-based approach targeting polymorphisms among several housekeeping genes, was performed as described by Enright *et al.* [34]. PCR products were purified using a PCR Purification Kit (QIAgen) and both strands were sequenced on the CEQ 8000 Genetic Analysis System (Beckman Coulter, High Wycombe, UK). BioNumerics software (Applied Maths, St-Martens-Latem, Belgium) was used to align and analyse the sequences. Allelic profiles and sequence type (ST) designations were assigned using the MLST database (http://www.mlst.net).

SCC*mec* elements (I–IV) were identified as described previously [43].

RESULTS

Array evaluation

Experimental procedures and data processing were evaluated by performing array experiments, using both labelling and amplification procedures, on DNA samples from *S. aureus* strains sequenced previously, i.e., MW2, Mu50, N315, MRSA 252, MSSA 476, NCTC 8325 and COL. The hybridisation patterns for these strains were in full agreement with the published data [44–47] and the match tables described above. For targets that were not present in these strains, e.g., *etA*, *etB* and *edinC*, other reference strains (CIP107093, NARSA266) were used as controls (for additional strains, see [5]). Results for the strains MRSA252, MSSA476 and MW2 are shown in Figs 1–4. For targets of high clinical significance (i.e., genes encoding antibiotic resistance genes, PVL, toxic shock syndrome toxin, enterotoxins A, B and C, and exfoliative toxins A and B), PCR products were generated from a reference strain that included both probe and primer-binding sites of the array-based assay. Amplicons were cloned into plasmids and used in microarray experi-

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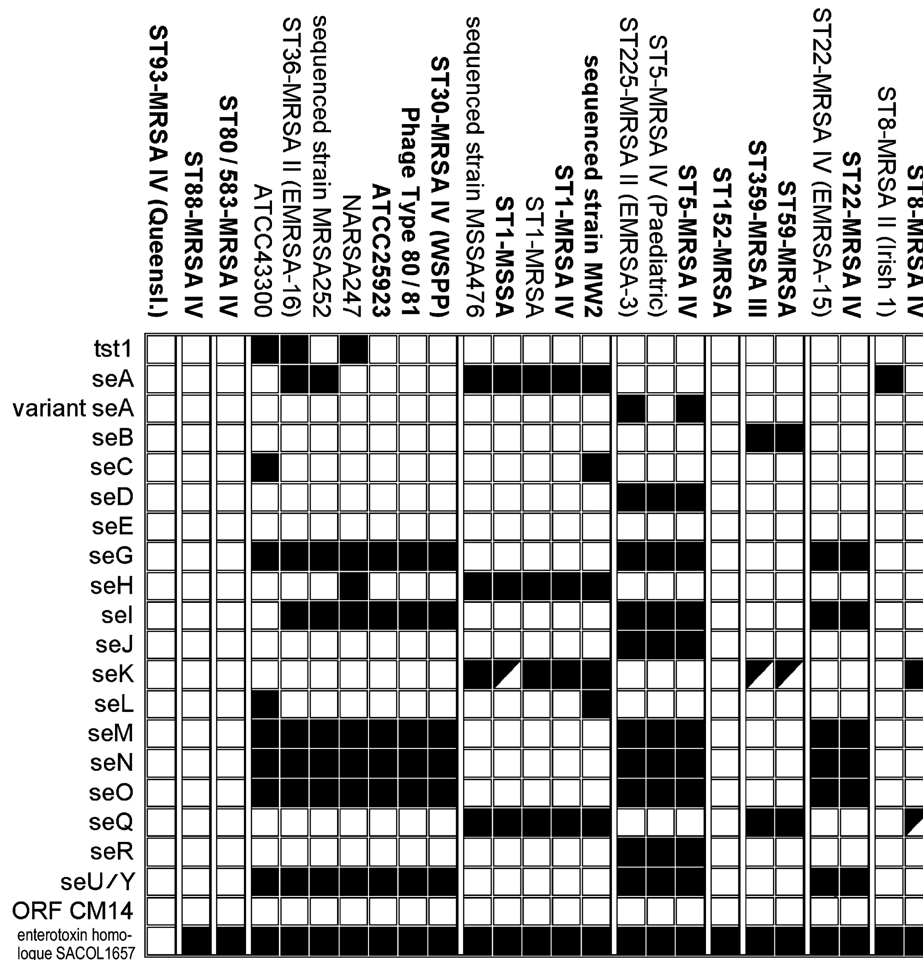

Fig. 2 Genes for superantigenic toxins in the strains studied. Panton-Valentine leukocidin (PVL)-positive strains are indicated in bold letters. Black boxes, positive; white boxes, negative; divided boxes, divergent results using the two labelling approaches.

ments instead of genomic DNA, using an individual array for each individual target [5]. As each of these amplicons hybridised only with the corresponding probe on the array, cross-reactivities were ruled out experimentally.

Array hybridisation results

Thirty-nine PVL-positive isolates of *S. aureus* were tested. A further 17 strains were included for comparison because their hybridisation patterns and/or MLST and *spa* types suggested phylogenetic relationships to PVL-positive strains. Strain characteristics and typing results are listed in Table S1. Hybridisation patterns were characteristic for clonal groups as defined by MLST. Complete array hybridisation data are presented in Figs 1–4, which show, respectively, the distribution of antibiotic resistance genes, superantigenic toxins, leukocidins and other virulence factors, as well as *set* genes. Brief descriptions of the different clonal groups (listed by *agr* group and MLST type) are given below.

agr group I, ST8

Array hybridisation data for ST8 clinical isolates were generally in agreement with the published sequence of strain USA300 (GenBank NC_007793 [48]), although these isolates were obtained from geographically diverse locations. All isolates carried *lukS/F*-PVL, *mecA* and *blaZ*, enterotoxin genes *seK* and *seQ* and the ubiquitous enterotoxin homologue (GenBank entries SAUSA300–1559,

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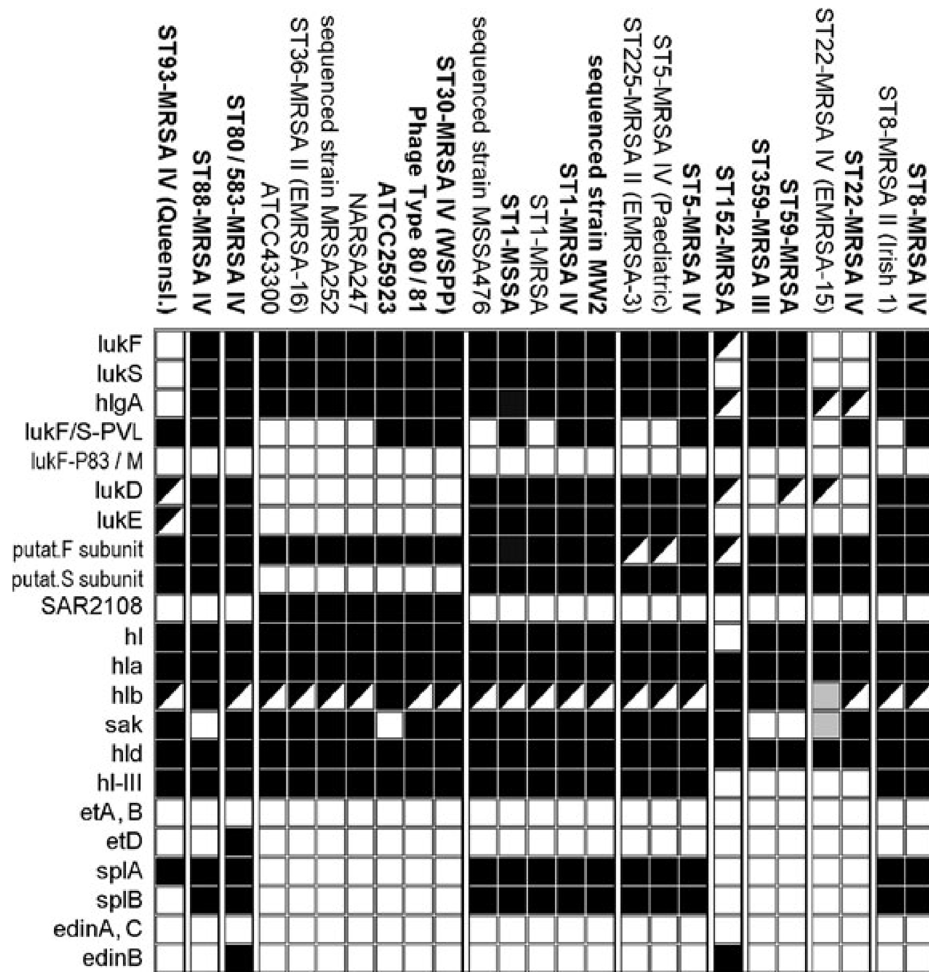

Fig. 3 Genes for leukocidins, haemolysins and other virulence factors in the strains studied. Panton-Valentine leukocidin (PVL)-positive strains are indicated in bold letters. Black boxes, positive; white boxes, negative; divided boxes, divergent results using the two labelling approaches; shaded boxes, variable.

SAV1601, SA1429, MW1552, SAR1679, SAS1538 and SACOL1657). Genes *seQ* and *set21* were detectable only by random-primed genome labelling, which is consistent with the genome sequence data showing divergent sequences within the primer-binding sites of these genes. The isolates also harboured the *lukD/E* and *hlg* genes (*lukS/*F, *hlgA*). The β-haemolysin gene was truncated, as shown by positive results with the random-primed genome labelling and negative results with site-specific amplification and labelling, as the primer and probe were designed to produce no signal in case of a phage insertion into *hlb* (see Discussion). While chromosomal genes were conserved among the isolates and the sequenced strain USA300, the plasmid content proved to be highly variable. Plasmid preparations of all the ST8 isolates tested yielded several plasmids. Resistance genes *ermC* and *tetK* were shown to be plasmid-borne, but some plasmids carried genes that were not represented on the array.

The ST8-MRSA II strain (also known as Irish-1 EMRSA) appeared to be related to this PVL-negative MRSA, but differed in *spa* type, SCC*mec* type (an *aadD*-negative variant of SCC*mec*II), absence of PVL, and in harbouring *seA* instead of *seK* and *seQ*.

agr group I, ST22

Two PVL-positive isolates, one outbreak isolate from Bavaria and one from the UK, were analysed. The Bavarian isolate contained *mecA* and

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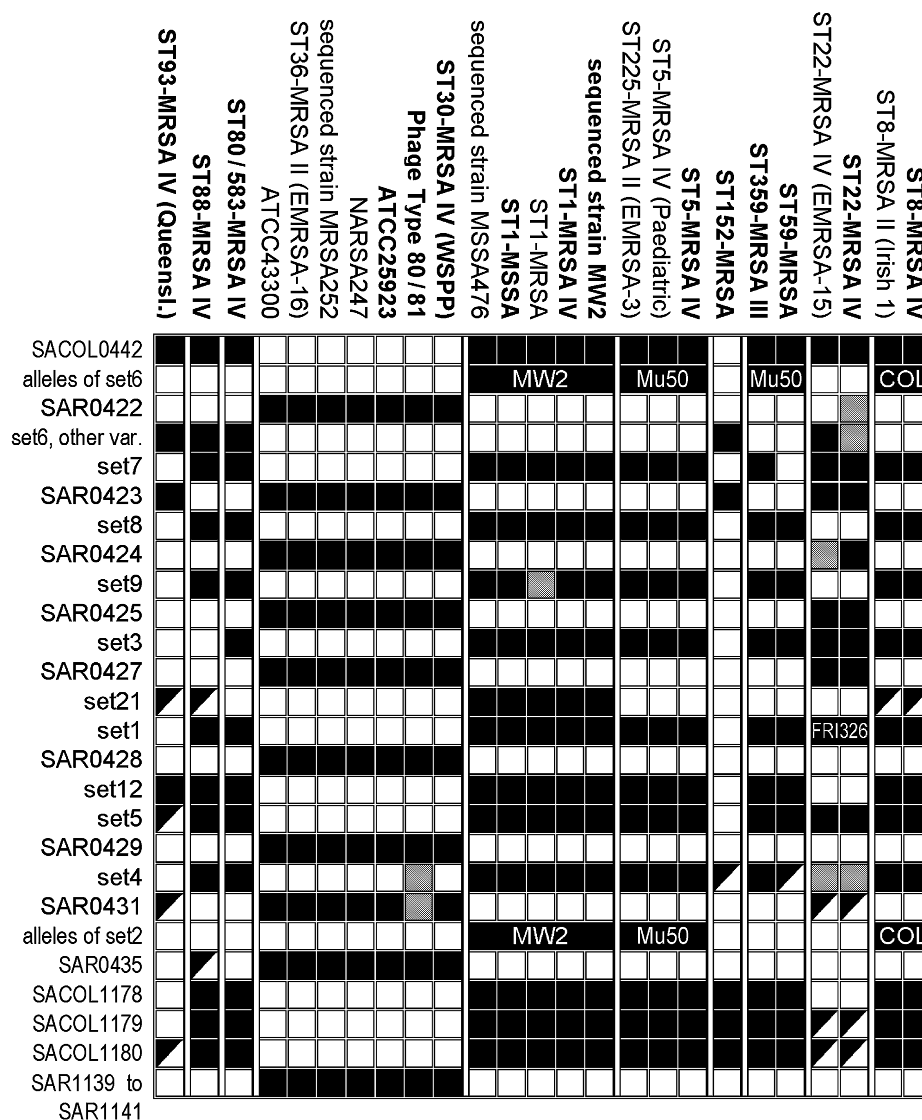

Fig. 4 Staphylococcal exotoxin-like (*set*) genes in the strains studied. Panton-Valentine leukocidin (PVL)-positive strains are indicated in bold letters. Black boxes, positive; white boxes, negative; divided boxes, divergent results using the two labelling approaches; shaded boxes, variable. The *set* genes are listed in the succession in which they occur in the sequenced strains (for nomenclature and definitions of allelic variants, see Table S2).

blaZ, and the UK isolate contained *mecA*, *aacA-aphD*, *aadD* and *dfrA*. In both isolates, enterotoxin genes *seG*, *seI*, *seM*, *seN*, *seO*, *seU/seY*, as well as the ubiquitous enterotoxin homologue, were found. The isolates were positive for the *lukS/F*-PVL genes, but the *hlg* genes and *lukD/E* were not present. Haemolysin genes *hla* and *hld* were detected. *set* carriage was remarkable as isolates from this group showed a strong cross-reactivity between *set3* and SAR0427, as well as between *set7* and SAR0423, which was not observed in any isolates from the other groups.

These isolates showed great similarity to EMRSA-15 (two isolates from Germany (the Barnim epidemic strain) and two from the UK), which differed in being *lukS/F*-PVL-negative, but which were usually *ermC*-positive.

agr group I, ST59/359

Two isolates were tested. One isolate from Australia was MLST type 59 and *spa* type t437, while the other (from the UK) belonged to MLST type 359 (a single locus variant of ST59) and *spa* type

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t316. Both harboured *mecA*, but differed with regard to other resistance genes. The Australian ST59 isolate contained *blaZ*, *aphA3*, *sat* and *tetK*. Both isolates were positive for *seB*, *seK*, *seQ* and the ubiquitous enterotoxin homologue; they contained *lukS/F*-PVL and the *hlg* genes, but not *lukD/E*. The β-haemolysin gene was intact (i.e., detectable by site-specific amplification and labelling), and *sak* (staphylokinase gene) was not present.

agr group I, ST152

The description of this unusual type belonging to *agr* group I, MLST type 152 and *spa* type t355 was based on an isolate obtained from an immigrant child from Macedonia. It was positive for *mecA* and *blaZ*, and harboured the gentamicin resistance gene *aacA-aphD*, which is rare among CA-MRSA. It contained the ubiquitous enterotoxin homologue, but none of the other enterotoxin genes. It was positive for *lukS/F*-PVL; *lukD/E* was not detectable by site-specific amplification and labelling, but was detected using the random-primed genome labelling approach. Faint signals were obtained for *lukF* and *hlgA* using the former approach, but both were clearly positive using the latter approach. This might indicate a deviant sequence. This isolate was the only one tested that contained both an untruncated *hlb* gene and *sak*. The *edinB* gene was detected, but *etD* (exfoliative toxin D) was absent. This contrasts with other isolates (ST80) in which these genes occurred together.

agr group II, ST5

One ST5 *spa* type t002 isolate from the UK was remarkable for being the only *lukS/F*-PVL-positive *agr* group II isolate found. It carried *mecA*, *blaZ* and several enterotoxin genes (variant *seA*, *seD*, *seG*, *seI*, *seJ*, *seM*, *seN*, *seO*, *seR*, *seU*, *seY* and the enterotoxin homologue). Furthermore, it harboured *hlg* genes and *lukD/E*. The β-haemolysin gene *hlb* seemed to be truncated and *sak* was present.

Related *lukS/F*-PVL-negative specimens included a SCC*mec*IV, *spa* type t548 isolate (the 'paediatric MRSA clone') and two *spa* type t003 isolates belonging to the Rhine-Hessen epidemic strain (ST225). The latter also differed in being *ermA*- and *aadD*-positive. This was consistent with the presence of a SCC*mec*II element [43].

agr group III, ST1

The ST1 clonal group included the archetypal CA-MRSA strain MW2 (GenBank NC_003923) and the PVL-negative MSSA476 (GenBank NC_002953), both of which have been fully sequenced. Array-based data for strains MW2 and MSSA476 were in full agreement with the genome sequences published previously. Essentially, these strains were identical, except for the presence of *mecA*, enterotoxin genes *seC* and *seL*, and *lukS/F*-PVL in MW2.

Two PVL-positive clinical MRSA isolates (from Australia and the UK) and one PVL-positive *mecA*-negative isolate (from Germany) were assigned to ST1. Two related, but *lukS/F*-PVL-negative, MRSA isolates (from Germany and the UK) were also included. All isolates were highly similar to each other and to the sequenced strains. The carriage of *set* genes was identical to MW2 and MSSA476. Variability occurred with respect to the presence of PVL, enterotoxin genes *seC* and *seL* (see above) and resistance determinants. The PVL-positive *mecA*-negative MSSA from Germany contained *blaZ*, *aphA* and *sat*. One PVL-negative MRSA from the UK contained a plasmid-borne *ermC* gene, and the *lukS/F*-PVL-positive isolate from the UK harboured *tetK*.

agr group III, ST30/36

The analysis of the ST30 clonal group ('South-west Pacific clone' or WSPP, 'West Samoan Phage Pattern') was based on four isolates, two from Switzerland, one from the UK, and one from Australia. For comparison, putative *mecA*-negative precursors of this strain, namely ATCC 25923 (Seattle, USA, 1945) and phage group 80/81 isolates (from the UK, 1955, and from Australia, late 1960s) were included. Additionally, a *mecA*-negative isolate (from a patient with long-standing furunculosis), which was unique in carrying both *lukS/F*-PVL and *tst1*, belonged to this group (not shown in Figs 1–4). ATCC 25923 and the latter isolate harboured neither *blaZ* nor *mecA*, the phage group 80/81 isolates were positive for *blaZ*, and all 'South-west Pacific clone' MRSA isolates carried both *mecA* and *blaZ*. Enterotoxin genes *seG*, *seI*, *seM*, *seN*, *seO*, *seU/seY* and the ubiquitous enterotoxin homologue, as well as *lukS/F*-PVL and the *hlg* genes, were detected in all isolates. In contrast to the other isolates, ATCC 25923 did not contain *sak*, and its *hlb* locus was intact.

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All strains in this group carried a unique complex of *set* genes, which is represented in sequenced strain MRSA252 (SAR0422-025, SAR027-029, SAR031, SAR035 and SAR1139-1141), and which sets ST30/36 clearly apart from other lineages (Fig. 4).

Two clinical isolates of EMRSA-16, MRSA252 and ATCC 43300, as well as NARSA247, were found to be related and produced similar hybridisation patterns, especially with regard to *set* genes. These strains were *lukS/F*-PVL-negative. The *tst1* gene was found in NARSA247, the two clinical EMRSA-16 isolates and ATCC 43300, but not in MRSA252. Other differences included the carriage of resistance genes (*mecA*, *blaZ*, *aadD*, *acaA-aphD*, *ermA*, *mupR*, *dfrA*) and enterotoxin genes (Figs 1 and 2).

agr group III, ST80

The ST80 clonal group has previously been described in detail, based on outbreak isolates from Germany [10,49]. Additional isolates from Australia (WA-MRSA-17), the UK and Switzerland were tested in the present study. ST80 isolates belonged to *agr* group III, MLST type 80 or 583 (a single locus variant of ST80 [50]), and *spa* types t044, t131 or t416. All contained *mecA*, *aphA3* and *sat*, and most isolates contained the plasmid-borne *blaZ*, *tetK* and *far1* genes [10]. Two isolates carried *linA* on a second plasmid. ST80 isolates were invariably positive for *lukD/E*, *lukS/F*-PVL, *edinB* and *etD.* Interestingly, isolates from this group did not harbour any enterotoxin genes, except for the ubiquitous enterotoxin homologue. The carriage of *set* genes was similar to MW2 and MSSA476. Differences included a mutation/deletion in *set6* (one of two probes gave a signal) and the absence of *set21.*

agr group III, ST88

This description is based on one isolate, from a patient with a history of travel from Bangladesh to the UK, which belonged to *agr* group III, MLST type 88 and *spa* type 690. Based on an analysis of *set* genes (Fig. 4) and *spa* typing, this isolate was related to ST1 and ST80; *spa* type t690 has the repeat sequence 07-12-21-17-13-13-34-34-34–33–34, while the other two clones usually have *spa* type t044 (07-23-12-34-34-33–34), t127 (07-23-21-16-34-33–13) or t131 (07-23-12-34-33-34). The resistance genes *mecA*, *blaZ* and *tetK* were detected, as well as *lukS/F*-PVL, *hlg* genes and *lukD/E*, but this isolate did not contain *sak*. There was a positive signal for *hlb* when using the site-specific amplification and labelling approach, indicating that this gene was intact.

agr group III, ST93

One isolate of ST93 (also known as 'WA-MRSA-7' or 'Queensland Clone') was tested and found to be positive for *mecA* and *blaZ*. This isolate did not harbour *tst1* or any enterotoxin genes, including the ubiquitous enterotoxin homologue. The γ-haemolysin genes were absent; *lukS/F*-PVL was detected, and haemolysins α, δ and III were present. The isolate was unique in carrying *splA* but not *splB*. The leukocidin genes *lukD* and *lukE* were detectable by random-primed genome labelling, but only *lukD* was detected using site-specific labelling. The carriage of *set* genes by this strain was distinctive, and it was observed that several of these genes were detectable only by random-primed genome labelling (Fig. 4).

DISCUSSION

Clinical isolates of *S. aureus* from Australia, Germany, Switzerland and the UK, as well as reference strains, were analysed using a novel DNA microarray approach. To the best of our knowledge, the array covered all relevant exotoxin genes and antimicrobial resistance determinants. There was a good correlation with established typing methods, resulting in rather uniform hybridisation patterns for isolates that belonged to a given *spa* or MLST type. This finding is remarkable, as the strategies used for typing were completely different. MLST and *spa* typing are based on random changes in one or several ubiquitous genes. Assuming that the environmental pressure on these mutations is limited, analysis was thought to reveal phylogenetic relationships. In the microarray-based approach, a comparatively high number of targets (144 genes or distinct allelic variants thereof) were monitored; these are likely to be subject to high selective pressure as they are involved in antibiotic resistance or interactions with the host, with many being encoded on mobile genetic elements. None of these genes is specific for a clone, but parallel analysis of a high number of genes

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resulted in a stable lineage profile that was generally in agreement with MLST and *spa* typing data. Occasional minor differences were observed; thus, the array was not able to discriminate between ST80 and ST583, with the latter being a single locus variant of ST80 [50], or between *spa* types t44 and t416, which both belong to ST80. Nevertheless, the array provided comprehensive strain characterisation within a single experiment, including data concerning antimicrobial resistance and toxin gene carriage, thereby allowing an assessment of virulence.

At a practical level, the DNA microarray technique provided more clinically useful information than (e.g.) MLST, which fails to differentiate between coexisting PVL-positive and PVL-negative ST22 strains, or between oxacillin-susceptible and oxacillin-resistant ST1 isolates. The DNA microarray had a high speed and throughput, and proved to be a powerful tool for inter-strain analyses in order to facilitate epidemiological monitoring and assignment of clinical isolates to epidemic strains. In general, the random-primed genome labelling approach was suitable for detecting truncated genes or unknown allelic variants, because it required only the presence of a relatively conserved probe-binding site. For site-specific amplification and labelling, the adjacent presence of two binding sites (for probe and primer) was needed. This resulted in higher specificity, but deletions or divergent sequences affecting the primer binding site (e.g., *entQ* and *set21* in ST8), as well as large insertions between the binding sites (see below), caused negative results. Therefore, unknown or aberrant strains should be examined using random-primed genome labelling, but site-specific amplification and labelling is more suited for routine tasks. This protocol required *c*. 1.5 h for DNA preparation, 2–3 h for amplification (site-specific labelling), and 3 h for hybridisation, staining and analysis, enabling one technician to test up to 20 isolates a day.

Data from this study, as well as results published previously [30,35,51], show that completely unrelated strains from different *agr* groups may evolve into epidemic MRSA clones. The diversity of genetic backgrounds and the detection of PVL-negative, but otherwise related, strains indicated frequent and independent acquisition of *lukS/F*-PVL by *S. aureus*. This is in agreement with sequence data and observations showing that *lukS/F*-PVL is carried and transmitted by phages [52–54]. In evolutionary terms, these phages represent a transition from parasitism to cooperation, as they cause no harm to their host, and actually enhance its fitness. However, without testing a large number of chronological isolates, it is not possible to determine whether a strain started to spread following acquisition of PVL, or whether an already widespread strain had a higher probability of being transformed, simply by being more common and more likely to encounter a PVL-carrying phage. The first possibility could have occurred with the European ST80 clone, which has spread throughout Europe and the Middle East [10,35,51,55] after it was first observed in 1993 in Denmark [56] and 1998 in Greece [57], but for which no PVL-negative relatives or putative ancestors have yet been identified. The second alternative is more likely for ST5 and ST22. The PVL-negative ST5 ('paediatric clone') and ST22 (EMRSA-15/'Barnim epidemic strain') clones were already widespread and common for many years before outbreaks caused by PVL-positive descendants were first observed (ST5 in Slovenia [28] and ST22 in Bavaria [30]).

The *sak* staphylokinase gene is also a phage-borne virulence factor, and is transferred by bacteriophages that integrate into *hlb* [58]. This explains the divergent results of the two different amplification and labelling approaches for *hlb* in most strains. In the random-primed genome labelling approach, *hlb* was always detected because the phage does not affect the probe-binding site. With site-specific amplification, the phage integration renders *hlb* undetectable by increasing the distance between primer and probe binding sites from 17 or 18 bp to nearly 43 000 bp (phage phi13 provirus genome, GenBank NC_004617 [58]). Strains that carry the phage do not produce β-haemolysin; thus, it is impossible to perform a CAMP test [59] with *Streptococcus agalactiae*. The majority of study isolates contained *sak* and truncated *hlb*. The ST152 isolate was unique as it carried *sak* and an intact *hlb*. Some isolates (from ST22, ST59/359 and ST88; Fig. 3), as well as the ancient strain ATCC 25923, harboured an intact *hlb*, but not *sak*.

This comparative analysis of a large number of *S. aureus* isolates showed that mobile genetic elements and horizontal gene transfer are of great clinical importance. For instance, resistance deter-

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minants are commonly situated on transposons (*aphA3* plus *sat* [60]) or plasmids [10,48,61]. Using the DNA microarray, it was possible to compare related isolates in order to identify variable features. Plasmid preparations were then obtained from isolates with such traits, amplified and hybridised. This experimental approach proved to be very efficient for the detection of plasmids, including plasmids coding for *ermC*, *tetK*, *far1*, *blaZ* and *linA* in the study strains. Many virulence factors were found to occur in clusters corresponding to so-called pathogenicity islands (*seD* plus *seJ* plus *seR*; *etD* plus *edinB*; *tst1* plus *seC* plus *seL*). These clusters were observed in unrelated strains, which is consistent with their location on mobile genetic elements. Large, multigene clusters even occurred in unrelated *S. aureus* strains belonging to different *agr* groups. Thus, the *egc* cluster (*seG*, *seI*, *seM*, *seN*, *seO*, *seU/seY)* was detected in *agr* group I (ST22), *agr* group II (ST5) and *agr* group III (ST30/36). In contrast, strains belonging to a single *agr* group might carry different multigene clusters, and (e.g.) several different *set* clusters were found in *agr* group III strains (Fig. 4). The occurrence of a *set* or enterotoxin gene cluster within different *agr* groups, and the presence of different clusters within a single *agr* group, could indicate occasional replacements of rather large portions of the chromosome. Thus, Robinson and Enright [62] suggested that ST239 was a mosaic form derived from ST8 and ST30.

The different forms of inter-strain transfer of genetic elements facilitate evolutionary changes resulting in the emergence and spread of more successful and/or virulent strains, e.g., those associated with the epidemic of TSS in the 1970s/1980s, or recurrent pandemics of PVL-positive *S. aureus*. The evolution of the especially successful clonal group ST30/36, which comprises the ancient clone 80/81, the majority of TSS isolates, the 'South-west Pacific PVL-MRSA', and the widespread EMRSA-16, has been described previously [21]. ATCC 25923, which was found recently to be PVL-positive [63], belongs, according to the present hybridisation data, to the same group. As this strain was isolated in 1945, the evolution of the ST30/36 lineage can be traced back for 60 years. Compared with ATCC 25923, phage group 80/81 isolates from the 1950/1960s acquired β-lactamase and a *sak*-carrying phage. After a further 30 years, the 'South-west Pacific clone' emerged following acquisition of a SCC *mec*IV element. The ST30/36 lineage also includes EMRSA-16 and NARSA247 (as a representative for TSS strains), which were highly similar with respect to most markers, including the MRSA252-like alleles of *set* genes. The evolution of this clonal group is especially interesting as it includes multiple independent acquisitions of *mecA*. Thus, the HA EMRSA-16 strain harbours SCC*mec* type II, while the CA 'South-west Pacific clone' contains SCC*mec*IV. Evidence was also found for parallel developments in ST5 and ST8. A PVL-positive ST5 isolate and a related PVL-negative epidemic strain ('paediatric clone') differed from another strain (ST5/ST225-MRSA II, 'Rhine-Hessen epidemic strain', or EMRSA-3) in having SCC*mec*IV. ST8-MRSA may harbour SCC*mec*II ('Irish 1' strain) while others contain an SCC*mec*IV element as well as PVL ('USA300' and ST8-MRSA study isolates).

Several isolates of ST1 were characterised that represent different steps in the evolution of this lineage. MSSA476 could be regarded as the *lukS/F*-PVL-negative, methicillin-susceptible precursor of this clonal line (Table S1; Figs 1 and 3). Two of the isolates described above had acquired *mecA*, but not *lukS/F*-PVL. Another isolate was positive for *lukS/F*-PVL, but not for *mecA*. Given that MW2 and two isolates eventually contained *lukS/F*-PVL and *mecA*, the evolution of this clonal line probably involved multiple acquisitions and/or deletions of these genes. Two other types, ST80 and ST88, appeared to be related to ST1 because of similar or identical *spa* types, and a similarly structured *set* locus. Whether these lineages diverged before or after acquiring PVL and/or *mecA* cannot be determined as no 'missing links' have been identified to date.

ST88 and ST152 represent distinctive strains from patients with a history of travel (Bangladesh and Macedonia, respectively). ST152 has been found recently in Slovenia [28], which together with the Macedonian background of the study isolate might indicate a distribution in the Balkans. There are currently no detailed data concerning the distribution or phylogenetic background of these strains, although strains related to ST152 have been described in western Europe and Australia [64]. It is possible that these and other, as yet undescribed, clones of PVL-MRSA evolve and circulate largely unmonitored. The well-known ST1, ST8, ST30 and ST80 strains are

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prevalent in regions where there are laboratories collecting and characterising PVL-MRSA (mainly Australia, Japan, western Europe and North America). Even within these regions, it is likely that a high proportion of PVL-MRSA infections remains undiagnosed [10], and the evolution of *S. aureus* outside these regions is largely unmonitored. Given the high plasticity of the *S. aureus* genome, its multitude of resistance and virulence factors, and the relatively high frequency of gene transfers, it is highly probable that further emergence and spread of new epidemic strains will occur. For this reason, continued surveillance is imperative to elucidate the global epidemiology of these strains and to guide effective therapeutic and control strategies.

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SUPPLEMENTARY MATERIAL

The following supplementary material is available for this article online at http://www.blackwell-synergy.com:

Table S1 *Staphylococcus aureus* strains used for this study.

Table S2 Targets, probes and primers.

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