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# High diversity of Panton–Valentine leukocidin-positive, methicillin-susceptible isolates of *Staphylococcus aureus* and implications for the evolution of community-associated methicillin-resistant *S. aureus*

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## ABSTRACT

In total, 100 *Staphylococcus aureus* isolates from diverse cases of skin and soft-tissue infection at a university hospital in Saxony, Germany, were characterised using diagnostic microarrays. Virulence factors, including Panton–Valentine leukocidin (PVL), were detected and the isolates were assigned to clonal groups. Thirty isolates were positive for the genes encoding PVL. Only three PVL-positive methicillin-resistant *S. aureus* (MRSA) isolates were found, two of which belonged to European clone ST80-MRSA IV and one to USA300 strain ST8-MRSA IV. The remaining methicillin-susceptible PVL-positive isolates belonged to a variety of different multilocus sequence types. The predominant strains were *agr*I/ST22, *agr*II/CC5, *agr*III/CC30 and *agr*IV/ST121. In order to check for possible bias caused by regional or local outbreak strains, an additional 18 methicillin-susceptible, PVL-positive isolates from the UK were tested. Approximately two-thirds of the UK isolates belonged to types that also comprised approximately two-thirds of the isolates from Saxony. Some methicillin-susceptible PVL-positive isolates (*agr*I/ST152, *agr*III/ST80 and *agr*III/ST96) closely resembled known epidemic community-acquired MRSA (CaMRSA) strains. These findings indicate that the current rise in PVL-positive CaMRSA could be caused by the dissemination of novel SCC*mec* elements among pre-existing PVL-positive strains, rather than by the spread of PVL phages among MRSA strains.

**Keywords** Community-acquired methicillin-resistant *Staphylococcus aureus*, evolution, microarray screening, methicillin-resistant *Staphylococcus aureus*, Panton–Valentine leukocidin, *Staphylococcus aureus*

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## INTRODUCTION

*Staphylococcus aureus* is a common pathogen capable of causing a variety of infections in humans. These range from minor soft-tissue infections to life-threatening conditions, e.g., septicaemia, toxin-mediated toxic shock or scalded skin syndromes. One virulence factor of particular importance is Panton–Valentine leukocidin (PVL), which is composed of two separate proteins encoded by two adjacent genes [1]. Molecules of both proteins together form heptameric pores that selectively disrupt leukocyte membranes, leading to increased virulence. Thus, PVL-carrying strains are able to cause recurrent, chronic or particularly severe skin and soft-tissue infections [2–7], as well as rapidly fatal pneumonia [8–11], which occur notably among previously healthy, immunocompetent individuals. In the late 1990s, the first PVL-positive methicillin-resistant *S. aureus* (MRSA) isolates were observed [12], and such strains have spread globally in recent years [10,13]. Currently, there is a pandemic of PVL-positive MRSA that is associated with the spread of several distinguishable strains [3,7,13–19]. These MRSA strains are commonly called community-acquired or community-associated MRSA (CaMRSA), as

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they are not restricted to the healthcare environment. While PVL production is a common trait among CaMRSA, it is important to recognise that PVL-negative strains can also occur [20].

This is not the first occasion on which PVL-positive *S. aureus* strains have caused a pandemic. The PVL toxin was discovered in 1932 [21], although evidence of its existence was documented in the late 19th century [22]. The laboratory strains ATCC 25923 [13,23] and the 'Oxford *Staphylococcus*' strain [24], which are both used by many microbiology laboratories as drug-susceptible quality control strains, are PVL-positive isolates from the 1940s. The closely related phage type 80/81 strain acquired penicillinase and spread worldwide during the 1950s and 1960s. Later, this strain virtually disappeared, perhaps following widespread use of penicillinase-resistant penicillins and cephalosporins. One of the currently spreading PVL-positive CaMRSA clones (ST30, West Samoan phage pattern strain) is related to, and is thought to have evolved from, these pandemic clones [13,25]. However, a notable feature of the current epidemic of CaMRSA is the genetic diversity of PVL-positive MRSA strains [13,16], which derive from several different clonal complexes (CCs), as defined by multilocus sequence typing (MLST) [26]. Therefore, it can be assumed either that PVL-encoding genes have spread among diverse MRSA strains (which is compatible with their phage-borne nature [27,28]), or that SCC*mec* elements encoding methicillin resistance [29–31] have spread among pre-existing PVL-positive methicillin-susceptible *S. aureus* (MSSA).

In order to investigate the origin of the diversity of PVL-positive MRSA, the present study screened *S. aureus* isolates from skin and soft-tissue infections for PVL-encoding genes, and assigned them to specific CCs using a previously developed diagnostic DNA microarray [7,13,32]. It was hypothesised that this approach might help to identify ancestors of pandemic PVL-positive CaMRSA strains and allow a greater understanding of their success and worldwide emergence. Moreover, this study aimed to provide insights into the prevalence and epidemiology of PVL-positive strains in a setting in which CaMRSA strains are still thought to be rare (Saxony, Germany).

## MATERIALS AND METHODS

### Isolates

Isolates were obtained from routine diagnostic specimens submitted to the Institute for Hygiene and Medical Microbiology at the Technical University of Dresden, Germany. In total, 100 consecutive *S. aureus* isolates were collected, for which one of the following diagnoses was provided: abscess, abscessing mastitis, chronic soft-tissue infection, complicated acne, erysipelas, fistulating soft-tissue infection, furunculosis, phlegmon or paronychia. Subsequent isolates from the same patient, or isolates from screening swabs of contacts of MRSA-positive individuals, were excluded. To address possible sample bias associated with strains prevalent in Dresden, and to determine whether PVL-positive strains were locally restricted or widespread, 18 PVL-positive *S. aureus* isolates from England were studied in parallel. These 18 isolates were selected at random from isolates referred to the UK *Staphylococcus* Reference Laboratory (Centre for Infections, Health Protection Agency, London, UK) over a 2-year period (2004–2006). The isolates were recovered from individuals presenting with abscesses, and were epidemiologically unrelated. Reference strains used for array evaluation have been described previously [7,13].

All isolates were cultured in Columbia blood broth (Oxoid, Wesel, Germany) and incubated overnight at 37°C. Genomic DNA was prepared by resuspending a loopful of culture material in 100 μL of lysis solution containing 0.05 mg of lysostaphin (AMBI Products, Lawrence, NY, USA), 2 mg of lysozyme (Sigma, Steinheim, Germany), 2 mg of ribonuclease A (Sigma), 2 μL of 20 mM Tris-HCl, pH 8.0, 2 μL of 2 mM EDTA and 1 μL of Triton X-100. After incubation for 45 min at 37°C on a shaker (300 rpm), 10 μL of proteinase K and 100 μL of buffer AL (DNeasy kit; Qiagen, Hilden, Germany) were added. After a second incubation period (45 min at 56°C), the samples were processed using the Qiagen EZ1 device according to the tissue lysis protocol supplied by the manufacturer.

### Array procedures

DNA microarrays were manufactured and processed as described previously [7,13,32], using the ArrayTube system (AT) (CLONDIAG chip technologies, Jena, Germany). The hybridisation probes and amplification primers were directed towards species-specific markers of *S. aureus*, antibiotic resistance determinants and exotoxins. For typing purposes, allelic variants of the accessory gene regulator (*agr*) gene cluster and *set/ssl* genes were included. Targets and sequence data for probes and primers are listed in Table S1 (see Supplementary material). Probes were spotted in two-fold redundancy. Evaluation and trials of the assay have been described previously [7,13,32]. MRSA and MSSA isolates related to known epidemic MRSA strains (see below) were additionally tested using an expanded set of probes that encompassed markers carried by SCC*mec* cassettes (i.e., *ccrA*, *ccrB*, *dcs* region, Δ*mecR*, *mecR*, *mecI* and *ugpQ*).

Genomic target DNA was amplified in a thermally synchronised linear multiplex reaction, using one primer for each target sequence. This amplification was also used to incorporate biotin-16-dUTP (Roche, Penzberg, Germany) for internal labelling. Reaction conditions have been described in detail previously [13,32]. Some isolates were also examined using an

alternative protocol, based on a whole genome amplification with partially randomised primers [7], in order to detect allelic variants not covered by published sequence data.

Biotinylated single-stranded amplicons were then hybridised to the array. The hybridisation was visualised using streptavidin–horseradish peroxidase-mediated precipitation of the chromogenic substrate Seramun green (Seramun, Woizig, Germany). Data analysis was performed using the ATR01 reading device and the Iconoclust software package (CLONDIAG chip technologies). Data interpretation and threshold definition was performed as described previously [13].

#### Strain identification

*spa* typing was performed as described previously [33], and representative isolates of all strains (42 of 118 isolates) were typed using this method. Types were assigned using the nomenclature described on the Ridom SpaServer website (http:// www.spa.ridom.de) and SPATYPEMAPPER software (CLONDIAG chip technologies; freely downloadable at http://www.clondiag.com/technologies/download.php?file=spa).

Assignment of isolates to MLST groups was inferred on the basis of *spa* types and comparison of hybridisation patterns with strains of known MLST type. Some isolates that could not be assigned in this way were subjected to MLST typing as described previously [26].

## RESULTS

### Prevalence of PVL and methicillin resistance among the Saxony isolates

Affiliations and prevalence of the isolates are shown in Fig. 1 (PVL-positive isolates) and Fig. 2 (PVL-negative isolates). Thirty of the 100 isolates were positive for the genes encoding PVL. Of these, three were MRSA; two of these belonged to European clone ST80-MRSA IV [7,19,34] and one to the USA300 strain ST8-MRSA IV [35,36]. The remaining methicillin-susceptible, PVL-positive isolates belonged to a variety of different MLST types. The predominant strains were *agr*I/ST22, *agr*II/CC5, *agr*III/CC30 and *agr*IV/ST121.

Two methicillin-susceptible isolates resembled a ST152 CaMRSA strain from the Balkans [13] in terms of their hybridisation profiles and *spa* type (t355). Another isolate appeared to be a methicillin-susceptible variant or precursor of European clone ST80-MRSA IV that carried the same virulence factors [7], but no resistance genes except for *tetK*. This isolate belonged to *spa* type t044.

Among the 70 PVL-negative isolates, *agr*I/CC8 and *agr*III/CC30 were the most common strains. Four of these were MRSA; one belonged to *agr*I/ST8 and carried an atypical SCC*mec*IV element, two belonged to the Berlin epidemic MRSA (*agr*I/ST45) [37,38], and one belonged to the South German epidemic MRSA (*agr*II/CC5, ST228) [37,38].

### Comparison of PVL-positive MSSA from Saxony and UK

To address a possible regional bias, 18 PVL-positive MSSA strains from the UK were analysed in parallel with the 27 PVL-positive MSSA strains from Saxony. Approximately two-thirds of the UK isolates belonged to strains that also comprised approximately two-thirds of the isolates from Saxony (i.e., *agr*I/ST22, *agr*III/CC30 and *agr*IV/ST121) (Fig. 3). The remainder comprised a range of different strains.

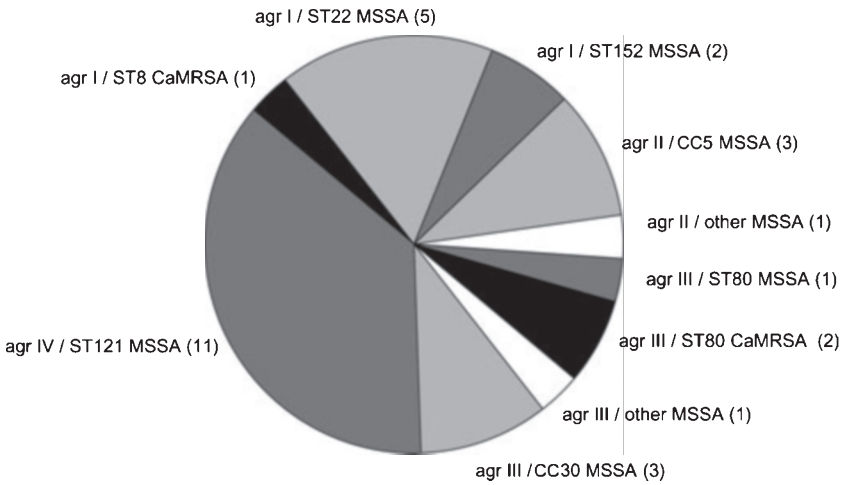


**Fig. 1.** Affiliations and prevalence of Panton–Valentine leukocidin-positive strains of *Staphylococcus aureus* from Saxony. Numbers of isolates are given in brackets. MSSA, methicillin-susceptible *S. aureus*; MRSA, methicillin-resistant *S. aureus*.

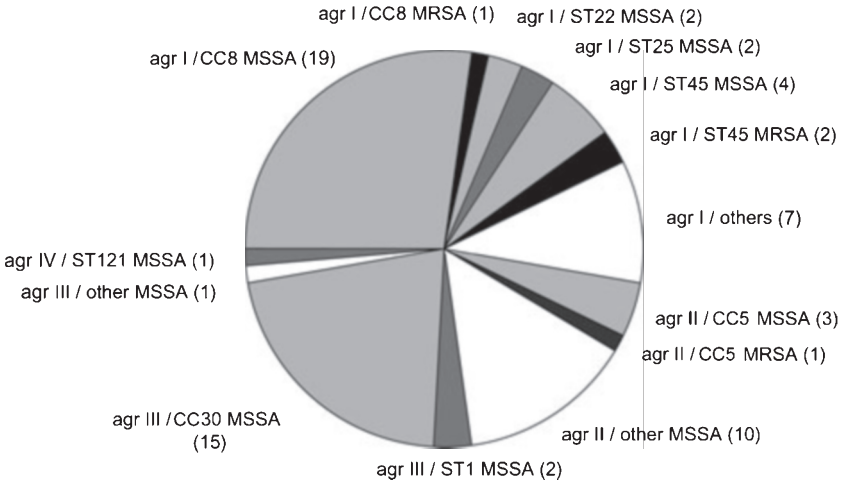


**Fig. 2.** Affiliations and prevalence of Panton–Valentive leukocidin-negative strains of *Staphylococcus aureus* from Saxony. Numbers of isolates are given in brackets. MSSA, methicillin-susceptible *S. aureus*; MRSA, methicillin-resistant *S. aureus*.

### Distribution of other virulence factors among the isolates from Saxony

Other important virulence factors detected included *tst-1* ($n$ = 15), *sea* ($n$ = 25), *seb* ($n$ = 7), *sec+l* ($n$ = 10), *sed+j+r* ($n$ = 16), the *egc* locus ($n$ = 53) and *sek+q* ($n$ = 4). The haemolysin-α gene *hla* was found in 96 isolates, the untruncated haemolysin-β gene (*hlb*) was detected in seven isolates, and all isolates were haemolysin-$\delta$ (*hld*)-positive. Haemolysin-$\gamma$ genes (*lukF/S*, *hlgA*) were detected in 92 isolates, although the existence of a deviant allelic variant can also be assumed for the remaining eight isolates (ST22 and ST152), based on positive results obtained using an alternative amplification and labelling technique [7]. Leukocidin genes (*lukD/E*) were found in 40% of isolates. Interestingly, deviant alleles of this locus might exist, as only one of two components was detectable in a number of study isolates (e.g., *lukE* in ST121 isolates). Exfoliative toxin A (*etA*) was found twice, and *etD* was found in five isolates. The latter occurred together with *edinB*, which was found in seven isolates.

### Strain characteristics

While particular virulence- or resistance-associated genes or gene complexes (e.g., complete pathogenicity islands or the *egc* enterotoxin clus-

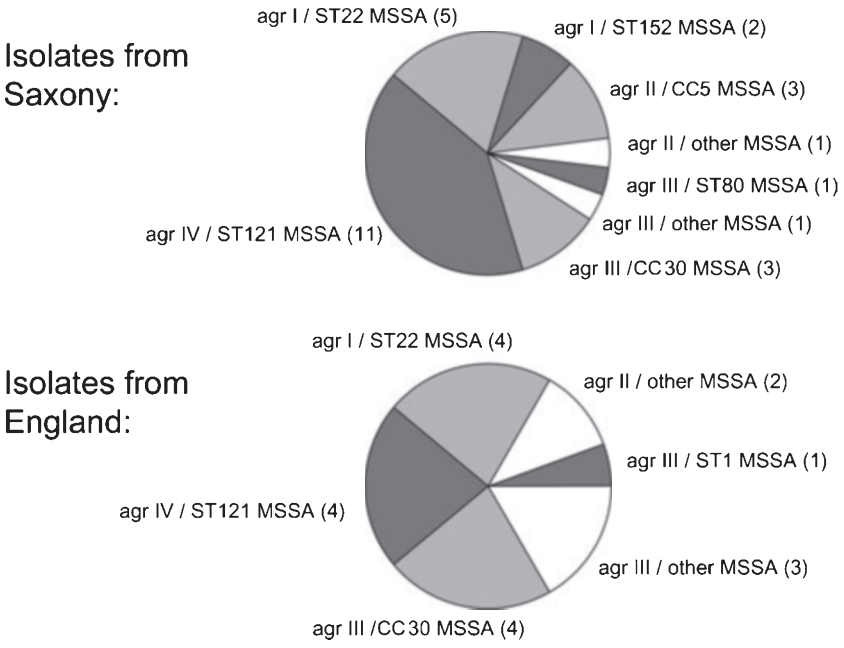


**Fig. 3.** Comparison of affiliations and prevalence of Panton–Valentine leukocidin-positive, methicillin-susceptible isolates of *Staphylococcus aureus* from Saxony and England. Numbers of isolates are given in brackets. MSSA, methicillin-susceptible *S. aureus*; MRSA, methicillin-resistant *S. aureus*.

ter) were not characteristic of particular strains or CCs, the complete hybridisation profile of a given isolate facilitated assessment of its relatedness to known strains or previously characterised CCs (Fig. S1, see Supplementary material). These assessments were in good agreement with the *spa*-typing data. The most important strains and isolates are described briefly below; complete profiles are shown in Fig. S1.

*agr* group I/CC8 strains formed a group with variable leukocidin and enterotoxin content, which otherwise resembled sequenced strains NCTC 8325 and COL. All 25 MSSA strains were PVL-negative. One isolate from this clonal group that carried a SCC*mec*IV element was found in a swab from an Ethiopian immigrant child with recurrent furunculosis. Another MRSA isolate belonged to the USA300 CaMRSA strain and carried a SCC*mec*IV element, as well as *msrA* (macrolide resistance), *aphA3* (neomycin/kanamycin resistance) and *sat* (streptothricin resistance) genes. This isolate was among the first isolates of that strain in the Dresden region of Saxony (isolated in May 2005).

*agr* group I/CC25 isolates had similar hybridisation profiles to CC8, but differed in *spa* type and by the presence of a pathogenicity island comprising *edinB* and *etD*. No PVL-positive clinical isolates belonging to this group were found in the present study, but one reference strain (NARSA 158) harboured PVL-encoding genes and belonged to this group.

*agr* group I/CC59 was represented by a single PVL-negative, methicillin-susceptible isolate belonging to *spa* type t1151.

CC22, CC45 and CC152 also belonged to *agr* group I, but produced deviant hybridisation patterns, especially with regard to the *set/ssl* genes.

*agr* group I/CC22 could be roughly divided into four strains, based on the presence of *mecA* and PVL-encoding genes. Both PVL-negative, *mecA*-negative and PVL-positive, *mecA*-negative isolates were detected. The latter occurred in the UK as well as in Saxony. In a previous study [13], *mecA*-positive, PVL-negative (Barnim epidemic MRSA, ST22-MRSA IV) [37,39] and *mecA*-positive, PVL-positive isolates have been characterised, but neither was found in the present study.

*agr* group I/CC45 comprised both MSSA and MRSA isolates. The latter belonged to the Berlin epidemic strain (ST45-MRSA IV) [37,38]. PVL-encoding genes were not detected among isolates from this group.

*agr* group I/CC152 (*spa* t355) comprised two PVL-positive, *mecA*-negative isolates that carried *edinB*, but not *etD*, and which had an unusual *set/ssl* pattern. A related PVL-positive MRSA strain has been characterised previously [13,40]; however, in contrast to that strain, the present isolate did not contain recombinase genes or *ugpQ*, both of which are associated with the SCC*mec*V element.

*agr* group II/CC5 was represented by a cluster of isolates that closely resembled the sequenced strains Mu50 and N315. One MRSA isolate belonged to the South German epidemic strain [37,38], which is an SCC*mec*I strain that is very common in Saxony. However, this isolate was atypical in having a truncated *egc* enterotoxin gene cluster (*seo*-positive, but negative for *seg*, *sel*, *sem*, *sen* and *seu*). Three MSSA isolates harboured PVL-encoding genes, and five were positive for *sed*, *sej* and *ser*.

Ten isolates belonging to *agr* group II had t160 (or related) *spa* sequences. One representative isolate was further analysed by MLST and was characterised as ST582/CC15. All isolates from this group were methicillin-susceptible, and two harboured *etA*. One isolate with similar hybridisation results carried PVL genes and belonged to *spa* t903.

Three isolates belonged to *agr* group III/ST1. One UK isolate from this lineage harboured PVL-encoding genes, but this virulence factor was not found among the German isolates studied. However, PVL-positive MSSA strains belonging to this group exist in this region as they have been found previously in a family outbreak in eastern Saxony (T. Juratzek, personal communication).

Three isolates from *agr* group III/ST80 were found. Two belonged to the PVL-positive European CaMRSA clone [7,19,34] and one was a PVL-positive, *mecA*-negative isolate that was related to the European CaMRSA clone on the basis of hybridisation pattern and *spa* type. Other targets from SCC*mec*IV cassettes (*ccrA2*, *ccrB2*, *dcs* region, Δ*mecR* and *ugpQ*) were not detected.

Isolates from a*gr* group III/CC30 showed a characteristic hybridisation pattern caused by the presence of Sanger MRSA252-like alleles of *set/ssl* genes. These alleles clearly set these isolates apart from other clonal groups, although ST22 and ST45

appear to be related. All except one isolate contained the *egc* enterotoxin gene cluster. Carriage of other enterotoxins and *tst1* was variable. PVL-encoding genes were detected in seven of 23 isolates, and these resembled the historic phage type 80/81 and ATCC 25923 strains. MRSA strains were not found among these isolates, although healthcare-associated UK EMRSA-16 and CaMRSA of the West Samoan phage pattern belong to this clonal group.

A PVL-positive isolate from the UK showed a hybridisation pattern, as well as *spa* type t202, which indicated similarity to a PVL-positive CaMRSA strain from Australia, i.e., Queensland clone ST93-MRSA IV [13,41]. In common with this strain, the UK isolate did not harbour any enterotoxin genes, gave unusually low hybridisation signals with *set/ssl* probes, and gave a signal with *splA*, but not with *splB*. In contrast to a representative of the Queensland clone, it did not contain any of the genes associated with a SCC*mec*IV element (*ccrA2*, *ccrB2*, *dcs* region, Δ*mecR* and *ugpQ*).

*agr* group IV/ST121 was a rather common and homogeneous group, occurring in the UK and in Saxony. This lineage may have a global distribution, as it also appears to be involved in most cases of tropical pyomyositis (W. Witte, personal communication). Most (15 of 16) isolates were PVL-positive. No MRSA strains belonging to this lineage were identified.

## DISCUSSION

The most striking finding of the present study was that 30% of routine *S. aureus* isolates in Saxony from skin and soft-tissue infections harbour PVL genes. Previous studies have indicated a prevalence of PVL among all *S. aureus* isolates of only *c.* 1%, but this prevalence is clearly much higher in the present collection of isolates from patients with pre-selected diagnoses known to be associated with PVL. It can be assumed that PVL-positive MSSA strains are rather common, but that they are usually overlooked, as no routine test for PVL is available. This reinforces the need to study the prevalence of PVL-encoding genes among MSSA isolates from other conditions, e.g., pneumonia.

Approximately one-third of the isolates from skin and soft-tissue infections in Saxony harboured PVL-encoding genes. This is a similar frequency to that indicated by recent epidemiological studies of PVL-positive MRSA isolates in the USA [42,43]. One possibility is that this proportion may define some kind of ecological niche which is occupied by strains carrying this virulence factor. The introduction of PVL-positive MRSA into a setting characterised by a high selective pressure caused by widespread use of β-lactam antibiotics is likely to lead to the replacement of highly diverse populations of methicillin-susceptible PVL-positive strains by a few different methicillin-resistant PVL-positive strains. The current epidemic of PVL-positive USA300 and USA400 strains in the USA could thus be interpreted as a replacement of MSSA by MRSA, rather than as spread of PVL-positive strains into ecological niches occupied previously by PVL-negative strains.

The present study highlights the genetic diversity of PVL-positive MSSA isolates. PVL-encoding genes were detected in strains representing all *agr* groups and belonging to most CCs (see above and [13]). The diversity of PVL-positive strains might be attributed to the fact that PVL genes are localised on phages [27,28], which facilitate spread of PVL genes through *S. aureus* populations. The current rise in PVL-positive MRSA could result either from MRSA acquiring a PVL phage, or from PVL-positive, methicillin-susceptible strains acquiring a SCC*mec* element. For ST1 and ST22 PVL-positive MRSA, both possibilities seem to be applicable, with the characterisation of ST1 and ST22 isolates containing neither *mecA* nor PVL, both determinants together, or either one of them (see above and [13]). ST80, ST93 and ST152 PVL-positive MRSA could have evolved by the second pathway, as PVL-positive, *mecA*-negative isolates have been identified rather than PVL-negative, *mecA*-positive precursors or relatives. A similar pathway for the evolution of ST30 PVL-positive MRSA has been described previously [25]. Since this development occurred simultaneously and independently within several unrelated clonal lines, it can be assumed that the novel SCC*mec*IV and SCC*mec*V elements are highly mobile, being able to spread through *S. aureus* populations in a similar way to phages.

These data provide evidence that the dissemination of novel SCC*mec* elements among pre-existing PVL-positive strains is likely to have played a major role in the current surge of PVL-positive CaMRSA, which was therefore not caused

solely by the epidemic spread of PVL phages within MRSA populations. This supports a hypothesis that the rise of PVL-positive CaMRSA is only the most conspicuous part of a general evolution towards β-lactam resistance in *S. aureus* mediated by the spread of these elements.

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## SUPPLEMENTARY MATERIAL

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

**Table S1.** Targets and sequence data for probes and primers used in this study.

**Fig. S1**. Strain characterisations obtained using diagnostic microarray hybridisation. For comparison purposes, some reference strains are included (hybridisation data taken from [13]), and these strains are labelled in italics. Black box, positive; grey box, variable or equivocal data, with numbers indicating the number of positive isolates.

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