Fatal *S. aureus* Hemorrhagic Pneumonia: Genetic Analysis of a Unique Clinical Isolate Producing both PVL and TSST-1

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Abstract

In 2008, an unusual strain of methicillin-sensitive *Staphylococcus aureus* (MSSA68111), producing both Panton-Valentine leukocidin (PVL) and toxic shock syndrome toxin-1 (TSST-1), was isolated from a fatal case of necrotizing pneumonia. Because PVL/TSST-1 co-production in *S. aureus* is rare, we characterized the molecular organization of these toxin genes in strain 68111. MSSA68111 carries the PVL genes within a novel temperate prophage we call ΦPVLv68111 that is most similar, though not identical, to phage ΦPV – a phage type that is relatively rare worldwide. The TSST-1 gene (*tst*) in MSSA68111 is carried on a unique staphylococcal pathogenicity island (SaPI) we call SaPl68111. Features of SaPl68111 suggest it likely arose through multiple major recombination events with other known SaPIs. Both ΦPVLv68111 and SaPl68111 are fully mobilizable and therefore transmissible to other strains. Taken together, these findings suggest that hypervirulent *S. aureus* have the potential to emerge worldwide.

Introduction

*Staphylococcus aureus* infections range in severity from superficial abscesses to complicated deep soft tissue infections and toxic shock syndrome (TSS) [1,2]. The pathogenesis of these diverse infections is attributed to multiple extracellular toxins including the Panton-Valentine leukocidin (PVL) and Toxic Shock Syndrome Toxin 1 (TSST-1). PVL has been epidemiologically linked to severe community-associated MRSA (CA-MRSA) infections [3,4] while TSST-1 clearly mediates shock and organ failure in staphylococcal TSS [5,6].

Historically, a single strain of *S. aureus* rarely produced both PVL and TSST-1. However, in 2005, one British report documented the TSST-1 gene in 4 of 30 PVL-positive isolates [7], one of which was associated with severe pneumonia [7]. Subsequently, twenty *S. aureus* isolates (15 MSSA; 5 MRSA) harboring both PVL and TSST-1 toxin genes were reported in the United Kingdom [8]. Of these, seventeen strains were from one of three clonal complex (CC) lineages: twelve (60%) belonged to lineage CC30 and five were either CC5 or CC22. The other three strains could not be assigned to any known clonal complex. Four of the 5 MRSA strains (80%) were multi-drug resistant. Eight of these isolates (40%) were associated with serious diseases including pneumonia, empyema, deep-seated abscesses and toxic shock. Nine patients presented with abscess or other skin infections [9].

One fatal case of necrotizing pneumonia was also reported in a 14-year-old child who presented initially with sore throat and pyrexia, and then deteriorated rapidly, developing hypotension and multiple organ failure [9].

In this report, we describe the genetic makeup of the hypervirulent TSST-1/PVL co-producing MSSA isolated from this fatal case (MSSA68111; CC30) [9]. Our results demonstrate that MSSA68111 produces both PVL and TSST-1 toxins. Further, its PVL-carrying phage and TSST-1-carrying pathogenicity island (SaPI) are both unique and not heretofore reported in *S. aureus*. Both novel genetic elements are fully mobilizable, suggesting that the emergence and dissemination of hypervirulent *S. aureus* may be forthcoming worldwide.

Materials and Methods

*S. aureus*

MSSA68111 was from a fatal case of necrotizing pneumonia in a 14-year-old child who presented initially with sore throat and pyrexia, and then deteriorated rapidly, developing hypotension and multiple organ failure [9].

It is penicillin-resistant but sensitive to erythromycin, tetracycline, vancomycin and linezolid [8]. It is of the MLST-CC30 lineage (sequence type ST776, a triple locus variant of ST30; spa type t399), harbors agr type 3 and is gene-positive for *sea* but negative for *sea*, *seb*, *sec*, *sed* and *sei* [8] (and unpublished data). TSST-1-positive MSSA strains were reported as being highly associated with the CC30 lineage, which is a very common genotype worldwide and is the most predominant lineage reported in the United Kingdom.
Ireland, and Switzerland [10–13]. Laboratory strain ATCC 49775 (American Type Culture Collection) harbors the PVL genes (herein referred to as luk-PV and luk-S-PV) and produces Luk-P/S-PV [14,15]. A clinical CA-MRSA isolate, strain 04-014, produces TSST-1 but not PVL [16]. Two clinical USA300 MRSA strains, 934814 and 09-301-0219, were from patients with septic arthritis and fatal post-influenza pneumonia, respectively. MSSA laboratory strain RN4220 is a restriction-deficient derivative of the 8325-4 strain [17].

MRSA strain MW2 (USA400) was originally isolated from a 16-month-old girl with fatal septicemia [18]. S. aureus were routinely cultured in Mueller-Hinton II broth; no differences in growth rates were observed. For analysis of toxin production, washed S. aureus from overnight cultures were diluted to 1–3 x 10^5 CFU/mL in fresh media and grown at 37°C in 5% CO2 with shaking (200 rpm) for 20 h (yields 1–4 x 10^8 CFU/mL). Culture supernatant fluids were filter sterilized and frozen at −70°C until assayed for toxins.

Toxin assays

TSST-1 and PVL were measured by ELISA [16,19]. Alpha-hemolysin activity was measured by lysis of rabbit erythrocytes [19]. All assays were run in duplicate or triplicate and included 2–3 biological replicates.

PVL induction and Phage DNA Purification

Exponential phase S. aureus (OD_{600} = 0.8) in CYPG (Casamino acids 10 g; yeast extract, 10 g; NaCl, 5 g; 0.06 M phosphoglycerate in 1 L) were treated with 1 μg/ml mitomycin C and cultured for 3.5 hrs at 32°C with slow shaking (80 rpm). The culture was centrifuged (8,000Xg, 20 min, 4°C) and the supernatant was dialyzed against 10 g; yeast extract, 10 g; NaCl, 5 g;0.5% glucose and 0.06 M g/ml mitomycin C, the DNA was re-suspended in CYPG-Phage Buffer (1:1) at a density of OD_{600} = 0.05 and infected with phage 11 or phage 80α at a multiplicity of 6:1 or 3:1. Cultures were grown at 32°C with slow shaking (80 rpm) for 1 hr. Samples (8 ml) were centrifuged and the bacterial pellet resuspended in 100 μl GET buffer (0.1 M glucose, 0.01 M EDTA and 0.05 M Tris-Cl, pH 8) and lysed with lysostaphin (0.5 mg/ml) in the presence of RNase A (0.5 mg/ml) at 37°C for 1 hr. The lysate was further digested with proteinase K (0.5 mg/ml) at 55°C for 3 hrs. Genomic DNA was then extracted with a commercial kit (Qiagen). The junction sequence of induced SaPI68111 was PCR-amplified using an inverse primer set targeting the sel (CTGGACCTGAT-CGCCGTTA) and SaPI-integrase (GTGTTGGATGAGCAAT-TACCAAG) genes. The chromosomal insertion site of SaPI68111 was further PCR-verified with the primer pair srf-up:TAGCGG-GAAAATCGTAAAGCAAG and integrase-down:GTGTTGGATGAGCAAT-TACCAAG.

Quantitative Real-Time PCR

DNase-treated total RNA (1 μg) was reverse transcribed using M-MuLV enzyme (New England Biolab, Ipswich, MA) and random hexamer primers and dNTPs (Invitrogen, Carlsbad, CA). cDNA was diluted fifty-fold in nuclease-free water and used for real-time PCR performed with a 7500 Fast Real-time PCR System (Applied Biosystems, Carlsbad, CA) using the RT^2 real-time SYBR green/Rox PCR master mix (SuperArray, Frederick, MD) and primer sets: (1) LukS-PV-up:TGTATCTCGTGCAGCTTTT-TTCA and LukS-PV-down: CAGACAAATGATACCCCATTTTTTCA and RecA-down:ACTCTAAATGGT-CCATT (Accession: YP_494079); (2) RecA-up:TCGACAGCAAGCCTAAACAGAGGA and RecA-down:ACTCTAAATGGT-CCATT (Accession: L25893); (3) gyrase-up:ATGTAGGTGCTACAGATTTTACCCCTGT and TSST-1-down:CGCCTACATTCAAGCCTTCCAGGTAA and gyrase-down:CAGAGTCCCTTT-GGTGCCACTT (Accession: YP_492727); (4) TSST-1-up:TGGCTAGATGATTTTTTACGCTTCACTACAGT and TSST-1-down:GTGTGGTATGCTTTTGACGTT (Accession: NC_002745). Relative gene expression was determined using the 2^[-ΔΔCt] method [20]. The fold change in expression of the gene of interest was normalized to the internal control gene (Gyrase B) and made relative to the calibrator sample (Time = 0).

Results

1. Toxin Production

In our initial studies, we compared by ELISA the levels of PVL and TSST-1 in 20-hr culture supernatant from S. aureus strain 68111 with known PVL- (ATCC49775) and a clinical MRSA isolate 934814) or TSST-1-producing (04-014) isolates (see details in Materials and Methods section) [14–16]. Our analysis showed that MSSA68111 produces 160.24 ng/mL PVL in 20-hr culture, which is significantly lower than that from the well-characterized PVL-producing MSSA strain ATCC49775 (Table 1), but comparable to that obtained from a clinical strain 934814 (USA300) (Table 1). Similarly, the production of TSST-1 in MSSA68111 is also relatively low, yielding 716.5 ng/mL, whereas the TSST-1 positive clinical isolate, strain 04-014, had a strongly positive ELISA result, producing 6739 ng/mL (Table 2).

Further, various hemolytic activities of MSSA68111 were assessed. When 20-hr culture supernatant of MSSA68111 was assayed for alpha-toxin with rabbit erythrocytes, a titer of 246 HU/ml was obtained (Table 3). With the same supernatant, no “hot-cold” hemolysis could be detected on sheep erythrocytes, indicating that staphylococcal beta-toxin is either absent or present only in a small amount in this strain (data not shown). Last, for the delta-hemolysin, we adapted a quick-screening methodology established by Herbert [21] and Traber [22] which takes
advantage of the fact that delta-hemolysin activity is enhanced by beta-hemolysin. Thus, by streaking test strains perpendicularly to strain RN4220, which produces only beta-hemolysin, one can visually identify delta-hemolysin production in test strains as an enhanced area of hemolysis where the two strains come in near to one another. As shown in Figure 1, MSSA68111 produces a significant amount of delta-hemolysin, indicated by the increased area of hemolytic activity when it nears strain RN4220. This same assay also can be used to detect alpha-hemolysin production in test strains since alpha-hemolysin activity is inhibited by beta-hemolysin. Using this technique, alpha-hemolysin production by MSSA68111 was also demonstrated (Figure 1). The moderate alpha- and delta-hemolysin activities, as well as production of TSST-1 (albeit in low amounts) suggested MSSA68111 carries an intact Agr/RNAIII operon. Having confirmed that MSSA68111 does indeed produce both TSST-1 and PVL – a phenomenon that is rare among S. aureus strains - we investigated the genetic organization of these genes in this unique clinical isolate.

2. Characterization of PVL-Carrying Phage in MSSA68111

2.1. PVL phage lineage and mobility. The genes for PVL are commonly carried by one of six lysogenic bacteriophages [18,23–27] having two distinct phage-head morphologies: icosahedral (ΦPVL and Φ108PVL) or elongated (ΦSlt, ΦSa2mw, ΦSa2958 and ΦSa2usa) [18,23–27]. Ma et al. recently reported a 2-step PCR-based scheme for characterizing S. aureus PVL-encoding phages [28]. The first round of PCR identifies the morphological group using primers specific to the gene lineage between lukS-PV and the tail gene. The second round of PCR establishes the phage type using primers recognizing phage-specific structures. With this approach, we determined that MSSA68111 carries an icosahedral-head type PVL-encoding phage, because PCR-1 (amplifying phage portal and tail genes) and PCR-3 (amplifying lukS-PV to group-specific tail gene) were positive, whereas PCRs-2 and -4 (amplifying genes specific for elongated-head type phage) were negative. Further, our PCR-5 result (targeting to phage specific structures) showed a PCR product of 1411 bps suggesting that the PVL-carrying phage in MSSA68111 belongs to the ΦPVL, but not Φ108PVL, lineage.

To assess the mobility of this PVL phage, we treated MSSA68111 with mitomycin C since it induces the lytic cycle of many temperate phages. After 2–4 hrs, the culture visibly cleared, suggestive of phage-mediated bacterial lysis. To confirm this, phage DNA was purified and amplified with an inverse primer set which targets the integrase and lukF-PV genes. The resultant PCR product (Figure 2A) suggested that the PVL-carrying phage in MSSA68111 did indeed become lytic following mitomycin C treatment. Commercial sequencing of the PCR product with subsequent GenBank BLAST searching demonstrated that the attP junction sequence of MSSA68111’s PVL-carrying phage was similar to that in other known PVL-phage lineages (Figures 2B, 2C), demonstrating that the nucleotide sequences of the integrase as well as the chromosomal integration site are highly conserved among different PVL-carrying phages.

We next compared the restriction profiles of the mitomycin C-elicted PVL-phage genomic DNA from MSSA68111 and ATCC49775 (carries ΦPVL [25]) by Southern blot analysis using a lukS-PV-specific probe. Identical hybridization patterns were seen following XbaI, but not SpeI, digestion (Figures 3A, 3B). Thus, the PVL-carrying bacteriophage in MSSA68111 has a similar, but not identical, restriction profile to ΦPVL. We propose the name ΦPVLv68111 for this ΦPVL variant. Last, sequence analysis of

![Figure 1. Alpha- and delta-hemolysin activities of S. aureus strain 68111.](image)

Table 1. PVL in 20-hr bacterial culture supernatants.

| Strain         | Concentration of LukF-PV (ng/mL) ± Std Dev |
|----------------|--------------------------------------------|
| ATCC 49775     | 852.7 ± 207.2                              |
| 934814 (USA300) | 113.74 ± 15.4                              |
| 68111          | 160.24 ± 28.8                              |

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Table 2. TSST-1 in 20-hr bacterial culture supernatants.

| Strain | Concentration of TSST-1 (ng/mL) ± Std Dev |
|--------|------------------------------------------|
| 04-014 | 6739 ± 533.2                             |
| 68111  | 716.5 ± 14.8                             |

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Table 3. alpha-hemolysin in 20-hr bacterial culture supernatants.

| Strain         | Alpha Hemolytic Activity (HU/mL) |
|----------------|----------------------------------|
| 09-301–02119 (USA300) | 310                              |
| ATCC 49775      | 98                               |
| 68111          | 246                              |

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sequences. The size of the PCR products and corresponding
performd using primers based on the known SaPIn1 and SaPIbov
organization of these toxin genes in MSSA68111, PCR was
respectively) which are commonly clustered in SaPI1 [32], SaPI2
SEL, SEC and TSST-1 [9] (coded for by
MSSA68111 was reported to be gene-positive for the exotoxins
exotoxin genes cluster within pathogenicity islands (SaPIs) [30,31].

The size of the PCR products and corresponding sequencing
data demonstrated that sel-sec-tst were clustered sequentially in MSSA68111. Like SaPIn1 or SaPIm1, the sec and
tst genes are approximately 2 kb apart and in opposite
orientations (data not shown).

3.3. Mobility of tst-carrying SaPI by helper phage. In the
excision-replication-packaging (ERP) cycle, temperate bacterio-
phages can induce excision and replication of SaPIs and can
package the ensuing DNA into special small phage-like particles
containing bacterial genomic DNA following helper phage
induction (data not shown), suggesting that this SaPI did not
undergo massive helper phage-mediated ERP.

To increase the sensitivity of detection of ERP, we used real-time
qPCR-based methods to quantify the copy number of tst-SaPI within
MSSA68111 host cells (Figure 5). Tst and gyr were the target and the
reference genes, respectively. Preliminary studies (data not shown)
demonstrated that S. aureus genome contained only one copy each of
tst and gyr and that the amplification efficiencies of these genes
(calculated from the slope of the standard curve generated with serial
dilutions of cDNA template for each gene) were equivalent (1.92 vs
1.90, respectively). Thus, the copy ratio of
tst
carrying SaPI by helper phage.

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| PVL phage            | attP sequence | accession number |
|----------------------|---------------|------------------|
| PVL-phage (strain68111) | AAATAAACATACATCATCAATAATGATGTT | AB009866         |
| φPVL                 | AAATAAACATACATCATCAATAATGATGTT | AP009363         |
| φ2958PVL             | AAATAAACATACATCATCAATAATGATGTT | AB243556         |
| φPV108               | AAATAAACATACATCATCAATAATGATGTT | BA000033         |
| φSa2MW               | AAATAAACATACATCATCAATAATGATGTT | BA045978         |
| φSLT                 | AAATAAACATACATCATCAATAATGATGTT |                  |

Figure 2. DNA sequence of the attP junction of MSSA68111 PVL phage. (A) PCR was performed with an inverse primer set using mitomycin
C-elicited phage genomic DNA from MSSA68111 as a template. PCR products were separated by agarose gel electrophoresis and stained with
ethidium bromide. ([Figure 2C]) Nucleotide sequence of the 68111 PCR product was determined. The sequence originating from PVL phage integrase is
printed in upper-case letters, and that derived from

2.2. Characterization of φPVLv68111 integration site. To investigate the chromosomal insertion site of φPVLv68111, we
performed PCR based on the known phage PVL insertion sites in
the other strains (e.g., ATCC49775, MW2) using primers that
hybridize to the lukF-PV gene and to a hypothetical gene
(Figures 4A, 4B). Commercial sequencing of the resultant PCR
product revealed a 29 nucleotide region of the

3.1. Gene organization of sel-sec-tst. Several S. aureus
exotoxin genes cluster within pathogenicity islands (SaPIs) [30,31].
MSSA68111 was reported to be gene-positive for the exotoxins
SEL, SEC and TSST-1 [9] (coded for by sel, sec and tst, respectively) which are commonly clustered in SaP1 [32], SaPI2
[30,33], SaP1n1/m1 [34] or SaP1bov [35,36]. To investigate the organization of these toxin genes in MSSA68111, PCR was
performed using primers based on the known SaP1n1 and SaP1bov
sequences. The size of the PCR products and corresponding sequencing data demonstrated that sel-sec-tst were clustered sequentially in MSSA68111. Like SaP1n1 or SaP1m1, the sec and
tst genes are approximately 2 kb apart and in opposite
orientations (data not shown).
either by electrophoretic analysis of sheared whole cell lysates or by the above qPCR method (data not shown). We conclude that the excision of $tst$-SaPI in MSSA68111 is highly phage 11-specific.

3.3. Characterization of the integrase of $tst$-carrying SaPI. To determine the integrase sequence and chromosomal integration site of the $tst$-carrying SaPI in MSSA68111, an outward-directed PCR on genomic DNA was conducted following helper phage 11 induction (Figure 6A). Sequencing of the PCR product showed a 15-bp direct repeat occurs at the junction between sel and integrase as indicated in Figure 6. The $att$ core (5'-TCCCGCCGTCTCCAT-3') matches the core $att$ site for several other SaPIs (e.g., SaPIm4, SaPImw2). Further comparison of the integrase sequence as well as the chromosomal integration site of the $tst$-carrying SaPI in MSSA 68111 with those from the previously characterized SaPIm4 and SaPImw2 showed 96% and 87% identity of intergrase genes, respectively, and the same chromosomal insertion site of all three, at 3' end of ssrP (Figure 6B).

In contrast, the organization of accessory genes in SaPI68111 including sel, sec, and $tst$ genes, is most closely related to that of SaPIn1. Because of these findings, a complete genome sequencing of the $tst$-carrying SaPI in MSSA68111 was conducted using a PCR and bi-directional primer walking-based approach. In all, the 16,422-bp SaPI68111 (Accession: JN689383) was determined to have 21 ORFs potentially encoding proteins over 50 amino acids in length, 3 of which encoded staphylococcal exotoxins (sel, sec, and $tst$), and many of which have homologs in SaPIm4, SaPIm1, SaPIbov1 and SaPI2 as indicated in Figure 7A, 7B and Table 4. These data suggest that the evolutionary history of SaPI68111 probably includes at least one major recombination event with these SaPIs or other similar elements. Also interestingly, we found that the predicted product of $stl$ in SaPI68111, a SaPI master repressor, shares 64% similarity (over 298 amino acids) to a hypothetical phage repressor protein of *Staphylococcus haemolyticus* bacteria (Accession: YP_254016; Figure 8). Particularly, the putative N-terminal HTH (helix-turn-helix) motif of SaPI68111-$stl$ repressor belongs to the HTH-XRE-family of proteins and shows a high degree of 80% similarity (as much as 65% identity) to the HTH motifs of repressor protein of *Staphylococcus haemolyticus* but not to HTH motifs of most *S. aureus* (Figure 8). These data have led to the supposition that this gene was acquired by *S. aureus* through horizontal transfer from *Staphylococcus haemolyticus* during co-colonization of human skin [38].

4. Transcription of PVL and TSST-1

Increased PVL transcription after mitomycin C treatment has been attributed to an increase of phage copy number following phage excision and replication [39]. Consistent with this, our quantification of lukS-PV mRNA and DNA in MSSA68111 by real-time PCR confirmed that mitomycin C treatment resulted in a marked increases of both PVL transcript (Figure 9A) and PVL-DNA copy number (Figure 9B). Mitomycin C also activates recA-mediated autocleavage of phage repressors and resumption of the lytic cycle [40,41]. Thus increased recA expression following DNA damage temporally coincides with increase phage-encoded gene transcription. Consistent with this notion, we observed a modest increase of recA expression in MSSA68111 following mitomycin C treatment (Figure 9A).

In contrast to PVL, mitomycin C treatment induced only a slight increase in TSST-1 mRNA and no excised form of $tst$-SaPI68111 was detected (Figures 9A, 9B). However, the abundance of both $tst$ transcripts as well as the $tst$ gene copy
number were markedly and dose-dependently increased by helper phage 11 (Figures 9C, 9D). These results were observed in the absence of a recA/SOS response (Figure 9C). Similarly, PVL genes at both the DNA and mRNA levels also remained unchanged by phage 11 treatment (Figures 9C, 9D). Thus, tst-SaPI68111 induction (excision and replication) is positively correlated with TSST-1 transcription and does not involve the recA/SOS response.

**Discussion**

Co-production of SEC, TSST-1 and PVL in MSSA68111 was proposed to contribute to the rapid demise of the young British patient with hemorraghic pneumonia [9]. Further, the recent report of severe infections due to three separate clonal lineages of S. aureus co-producing TSST-1 and PVL [8] portends a possible emergence of hypervirulent S. aureus worldwide.

In addition to its epidemiologic association with fatal pneumonia, MSSA68111 is also of considerable interest from a purely genetic point of view. For unknown reasons, certain combinations of staphylococcal toxins are rarely present in the same clinical isolate. This is true for TSST-1 with staphylococcal enterotoxin B (SEB) and for TSST-1 with PVL. Thus MSSA68111 is a significant deviation from the norm. As such, it provides a unique opportunity to investigate the mechanisms responsible for this phenomenon and to gain insight into likely emergence and dissemination of the next S. aureus “superbug”.

We confirmed that MSSA68111 produced both TSST-1 and PVL. PVL production by MSSA68111 was comparable to other S. aureus clinical isolates we have tested [16], however the level of TSST-1 was low. The relatively low level of TSST-1 in
MSSA68111 could not be explained by a non-functional Agr/RNAIII system since both alpha- and delta-hemolysins were produced. In addition, real-time RT-PCR showed that RNAIII transcripts in MSSA68111 were comparable to those from MRSA strains FPR3757 (USA300) and MW2 (USA400) (data not shown). Expression of RNAIII was inversely related to expression of spa gene in wild-type MSSA68111 but not in its isogenic RNAIII mutant (data not shown). The low levels of TSST-1 and PVL are also not an artifact of the ELISA-based detection system since their respective nucleotide sequences in MSSA68111 are highly similar to those of other strains (data not shown).

Although extensive studies have been conducted by others, the exact mechanism responsible for the wide variation in the amount of TSST-1 produced by different strains of S. aureus is still unknown. A study of 152 TSST-1-positive MRSA isolates by Nagao et al showed that differences in TSST-1 production (up to 170-fold) is not directly correlated with the allelic variations of the agr [42]. In addition, their sequencing of the promoter region of the tst gene, the entire sigma factor B and sar loci revealed no relevant nucleotide changes among these strains [42]. Thus it is likely that the low level of TSST-1 in MSSA68111 may not simply be explained by one mechanism, but rather multiple known or unknown complex interconnected regulatory systems are operative. In addition, the low level of TSST-1 production in MSSA68111 could be a consequence of co-production of both TSST-1 and PVL toxins, as other investigators have shown that some toxins function as autorepressors or negative regulators of other exoproteins [43].

Bacteriophages mediate dissemination of genes and hence bacterial diversity. We show that the PVL-carrying phage from MSSA68111 (F PVLv68111) is a variant of icosahedral-head type phage F PVL. The basis for this conclusion is that F PVL and F PVLv68111 share similar phage structural genes, identical XbaI restriction profiles and phage integration sites, but different SpeI restriction fragment profiles. Among clinical isolates that have been studied, F PVL is relatively rare in the United States but is more prevalent in Europe [28]. In contrast, the elongated-head type PVL-phage, represented by F Sa2usa, is currently dominant worldwide [44]. These findings suggest that F PVL and F PVLv68111 might have evolved from a common ancestor and that genetic drift may have occurred in one or both. Features unique to F PVLv68111 may have permitted MSSA68111 to acquire the genes for TSST-1 production.
Table 4. Relationships between SaPl68111 genes and those of other SaPIs.

| SaPI68111 | Mu50 | RF122 | RN3984 | MW2 | Annotation or Function |
|-----------|------|-------|--------|-----|------------------------|
| orf1      | int  | 96(98)|        | 87(100)| Integrase |
| orf2      | stl  |       | 88(91) | 88(91) | SaPI master repressor |
| orf3      | str  | 97(88)| 95(88) | 96(88) | Regulatory protein |
| orf4      | HP   | 82(92)| 83(91) |        | Hypothetical protein |
| orf5      | HP   | 96(100)| 96(100)| 90(100)| Hypothetical protein |
| orf6      | pri  | 96(100)| 93(69)| 93(69) | Similar to DNA primase |
| orf7      | rep  | 99(100)|        |        | Similar to replication initiation protein |
| orf8      | HP   | 99(100)|        |        | Hypothetical protein |
| orf9      | HP   | 99(100)|        |        | Hypothetical protein |
| orf10     | HP   | 99(100)|        |        | Hypothetical protein |
| orf11     | HP   | 96(100)| 96(100)| 95(100)| Hypothetical protein |
| orf12     | HP   | 97(100)| 97(100)| 96(100)| Hypothetical protein |
| orf13     | cp   | 98(100)| 94(100)| 98(100)| Capsid size determinant |
| orf14     | cp   | 99(100)| 96(100)| 100(100)| Capsid size determinant |
| orf15     | cp   | 96(100)| 97(100)| 95(100)| Capsid size determinant |
| orf16     | HP   | 96(100)| 98(100)| 96(100)| Hypothetical protein |
| orf17     | terS | 98(100)| 98(100)| 97(100)| 90(27) | Terminase small subunit |
| orf18     | tst  | 100(100)| 100(100)| 100(100)| Toxic shock syndrome toxin-1 |
| orf19     | HP   | 100(100)|        | 77(82) | Hypothetical protein |
| orf20     | sec3 | 99(100)| 95(100)| 97(100)| Staphylococcal enterotoxin type C3 |
| orf21     | sel  | 100(100)| 100(100)| 99(100)| Staphylococcal enterotoxin L |

**Note:** The similarities of SaPI68111 with other close-related SaPIs were determined with BLAST. Percentage of identity to corresponding gene in SaPI68111 and percentage of query coverage (in the bracket) were given in the table. Blank area indicated no corresponding genes.

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Figure 8. Amino acid alignment of predicted stl from MSSA68111 and hypothetical phage repressor protein SH2101 from Staphylococcus haemolyticus (Accession: YP_254016.1). The protein sequences were aligned by using the CLUSTAL alignment program. The Helix-turn-helix XRE-domain is highlighted in the black box. The asterisk denotes a position at which the two sequences have the same amino acid. Dots indicate the degree of homology when there is not complete sequence conservation.

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Phage-encoded toxin genes are not always transmissible often because integrated prophages are defective. However, \( \Phi \text{PVLv68111} \) is fully mobilized as part of a \( \text{recA} \)-mediated SOS response and its induction results in enhanced \( \text{lukS}-\text{PV} \) transcription. These findings indicate functional \( \Phi \text{PVLv68111} \) can be readily transmitted to other \( S. \text{aureus} \) strains.

Staphylococcal pathogenicity islands (SaPIs) are other mobile genetic elements responsible for inter- as well as intra-genic spread of exotoxin genes. MSSA68111 carries a unique SaPI which we have designated \( \text{SaPI68111} \). Unlike all known \( \text{tst} \)-carrying SaPIs, \( \text{SaPI68111} \) is inserted in an \( \text{att} \) site close to the \( \text{ssrP} \) gene with a 15-bp repeat. Further, the integrase gene in \( \text{SaPI68111} \) appears related to \( \text{SaPlmw2} \) and \( \text{SaPlm4} \), but not to \( \text{SaPln1/m1} \) or any other known \( \text{tst} \)-carrying SaPIs. Most interestingly, the predicted \( \text{stl} \) gene in \( \text{SaPI68111} \), a master regulator for SaPI EPR cycle, does not share any homology with any published sequence in \( S. \text{aureus} \), but has 64% similarity with a hypothetical phage repressor of \( \text{Staphylococcus haemolyticus} \). This unique combination of genetic characteristics suggests that \( \text{SaPI68111} \) probably arose following at least one major recombination event with several SaPIs or other similar elements, whereas the regulatory gene (e.g., \( \text{stl} \) gene) might be acquired by \( S. \text{aureus} \) through horizontal transfer from other species (e.g., \( \text{Staphylococcus haemolyticus} \)) during co-colonization or infections.

Induction of SaPIs excision/replication requires specific helper phages, and in MSSA68111, chromosomal excision of \( \text{tst} \)-\( \text{SaPI68111} \) was driven solely by helper phage 11. This finding is unique in that most SaPIs are specifically induced by phage 80\( \text{\alpha} \) alone or in combination with phage 11. To our knowledge, none is induced solely by phage 11. Like PVL, excision/replication of \( \text{tst} \)-\( \text{SaPI68111} \) was positively correlated with increased TSST-1 gene transcription. Whether features of \( \text{SaPI68111} \) contributed to the emergence of PVL/TSST-1 co-producing \( S. \text{aureus} \) remains to be determined.

Several hypotheses exist to explain the observed specific paired toxin exclusion in \( S. \text{aureus} \). First, exclusion results from competition for a single chromosome insertion site [45]. However, this cannot fully explain the mutual exclusion of TSST-1 and PVL in nearly all \( S. \text{aureus} \) since, as described above, the chromosomal location of PVL-carrying phages does not overlap with any currently reported \( S. \text{aureus} \) SaPIs including \( \text{SaPl68111} \) and induction of \( \text{tst} \)-carrying SaPIs requires specific helper phages that...
are genetically distinct from PVL-encoding phases. Second, unknown “exclusion factors” encoded by the first genetic element block acquisition of the second genetic element [46]. If such factors exist and mediate toxin exclusion, it would imply that MSSA68111 is exclusion factor-deficient. Third, one mobile genetic element commandeers a key bacterial resource essential for transduction. Fourth, membrane depolarization after infection by the first element prevents secondary infection [47,48]. Lastly, defects in the Sae1 restriction modification system may permit multiple transduction events as has been suggested in S. aureus RN4220 [49]. Our genetic characterization of MSSA68111 offers new tools to investigate these possibilities.

In summary, our genetic analysis of a hypervirulent TSST-1/ PVL co-producing CA-MSSA demonstrated that these potent virulence factors are each carried on unique and fully transmissible genetic elements. Together these findings portend a possible worldwide emergence of new hypervirulent S. aureus. Whether these elements are common among other recently described TSST-1/PVL co-producing S. aureus, and what their roles may be in overcoming mutual toxin exclusion, are under investigation.

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Author Contributions

Conceived and designed the experiments: ZL AEB DLS. Performed the experiments: ZL SMH TP. Analyzed the data: ZL AEB DLS. Contributed reagents/materials/analysis tools: AMK YM RWE. Wrote the paper: AEB ZL.

References

1. Archer GL (1998) Staphylococcus aureus: a well-armored pathogen. Clin Infect Dis 26: 1179–1183. 10.1093/clinids/26.5.1179.
2. Lowy FD (1998) Staphylococcus aureus infections. N Engl J Med 339: 520–532. 10.1056/NEJM199802020339006.
3. Kaneko J, Kamio Y (2004) Bacterial two-component and hetero-heptameric haemorrhagic pneumonia proves fatal in an immunocompetent child due to Staphylococcus aureus encoding both TSST-1 and PVL in the UK. Abstract of Infect Immun 72: 2002–2003. [pii];10.1128/IJMI.72.03-04.
4. Kearns AM, Smith IM, Ganner M, Perry C, Warner M, et al. (2008) In vitro production of panton-valentine leukocidin among strains of methicillin-resistant S. aureus. J Infect Dis 195: 209–211. 10.1086/582231.
5. Holmes A, Ganner M, McGuane S, Pitt TL, Cookson BD, et al. Staphylococcus aureus. J Bacteriol 191: 5577–5583. JB.00493-09 [pii];10.1128/JB.00493-09 [doi].
6. Holm ARE, Smales IM, Ebert T, Martin DR, Sherrard T, et al. (2007) Genetic analysis reveals genetic exchanges and intraspecific spread of SaPI2, a mobile genetic element, in community-associated methicillin-resistant S. aureus. J Infect Dis 197: 187–194. 10.1086/524684 [doi].
7. Herbert S, Ziebandt AK, Ohlsen K, Schafer T, Hecker M, et al. (2010) Repair modifiers of the immune system. Mol Med Today 6: 125–132. S1357-2725(09)00136-1 [pii];10.1016/j.mmet.2009.09.006 [pii].
8. Baba T, Takeuchi F, Kuroda M, Yuzawa H, Aoki K, et al. (2002) Genome and virulence determinants of a high virulence community-acquired MRSA. Lancet 359: 1819–1827. S0140-6736(02)08113-5 [pii];10.1016/S0140-6736(02)08113-5 [doi].
9. Stevens DL, Ma Y, Salmi DB, McIndoe E, Wallace RJ, et al. (2007) Impact of antibiotics on expression of virulence-associated exotoxin genes in methicillin-resistant and methicillin-sensitive Staphylococcus aureus. J Infect Dis 195: 202–211. 10.1086/501306.
10. Livak R, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT Method. Methods 25: 402–408. 10.1006/meth.2001.1262 [pii];S1046-2023(01)91262-9 [pii].
11. Fairweather N, Kennedy S, Foster TJ, Kehoe M, Dougan G (1983) Expression of a cloned Staphylococcus aureus alpha-hemolysin determinant in Bacillus subtilis and S. aureus. Infect Immun 41: 1112–1117.
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Author Contributions

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References

1. Hamilton SM, Bryant AE, Carroll KC, Lockary V, Ma Y, et al. (2007) New tools to investigate these possibilities. PLoS ONE 2: e27246.
35. Fitzgerald JR, Monday SR, Foster TJ, Bohach GA, Hartigan PJ, et al. (2001) Characterization of a putative pathogenicity island from bovine Staphylococcus aureus encoding multiple superantigens. J Bacteriol 183: 63–70. 10.1128/JB.183.1.63-70.2001 [doi].

36. Ubeda C, Tormo MA, Cucarella C, Trotonda P, Foster TJ, et al. (2003) Sip, an integrase protein with excision, circularization and integration activities, defines a new family of mobile Staphylococcus aureus pathogenicity islands. Mol Microbiol 49: 193–210. 3577 [pii].

37. Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29: e45.

38. Takeuchi F, Watanabe S, Baba T, Yuzawa H, Ito T, et al. (2005) Whole-genome sequencing of Staphylococcus haemolyticus uncovers the extreme plasticity of its genome and the evolution of human-colonizing staphylococcal species. J Bacteriol 187: 7292–7308. 187/21/7292 [pii];10.1128/JB.187.21.7292-7308.2005 [doi].

39. Wirtz C, Witte W, Wodz C, Goerke C (2009) Transcription of the phage-encoded Panton-Valentine leukocidin of Staphylococcus aureus is dependent on the phage life-cycle and on the host background. Microbiology 155: 3491–3499. mic.0.032466-0 [pii];10.1099/mic.0.032466-0 [doi].

40. Reaber JW, Hochlut B, Waldor MK (2004) SOS response promotes horizontal dissemination of antibiotic resistance genes. Nature 427: 72–74. 10.1038/nature02241 [doi];nature02241 [pii].

41. Galkin VE, Yu X, Belnitski J, Nolshka D, Bell CE, et al. (2009) Cleavage of bacteriophage lambda cl repressor involves the RecA C-terminal domain. J Mol Biol 385: 779–787. S0022-2836(08)01362-4 [pii];10.1016/j.jmb.2008.10.081 [doi].

42. Nagao M, Okamoto A, Yamada K, Haegawa T, Haegawa Y, et al. (2009) Variations in amount of TSST-1 produced by clinical methicillin resistant Staphylococcus aureus (MRSA) isolates and allelic variation in accessory gene regulator (agr) locus. BMC Microbiol 9: 52. 10.1186/1471-2180-9-52 [pii];10.1186/1471-2180-9-52 [doi].

43. Vojtov N, Ross HF, Novick RP (2002) Global repression of exotoxin synthesis by staphylococcal superantigens. Proc Natl Acad Sci U S A 99: 10102–10107. 10.1073/pnas.152152499 [doi];152152499 [pii].

44. Boakes E, Kearns AM, Gunner M, Perry C, Hill RL, et al. (2010) Distinct bacteriophages encoding PVL among international clones of PVL-MRSA. J Clin Microbiol [pii].10.1128/JCM.01917-10 [doi];01917-10 [pii].

45. De Boer ML, Chow AW (1994) Tox A: shock syndrome toxin 1-producing Staphylococcus aureus isolates contain the staphylococcal enterotoxin B genetic element but do not express staphylococcal enterotoxin B. J Infect Dis 170: 818–827.

46. Delbruck MA (1945) Interference between bacterial viruses; the mutual exclusion effect and the depressor effect. J Bacteriol 50: 151–170.

47. Parma DH, Snyder M, Sobolevski S, Nawroz M, Brody E, et al. (1992) The Rex system of bacteriophage lambda: tolerance and altruistic cell death. Genes Dev 6: 497–510.

48. Li BH, Beckrath R (1999) Photolyase-dimer-DNA complexes and exclusion stimulation in Escherichia coli: depolarization of the plasma membrane. Mol Gen Genet 260: 430–434.

49. Waldron DE, Lindsay JA (2006) Sau1: a novel lineage-specific type I restriction-modification system that blocks horizontal gene transfer into Staphylococcus aureus and between S. aureus isolates of different lineages. J Bacteriol 188: 5578–5585. 188/15/5578 [pii];10.1128/JB.00418-06 [doi].