Comparative Analysis of Virulence and Toxin Expression of Global Community-Associated Methicillin-Resistant Staphylococcus aureus Strains

Min Li, Gordon Y. C. Cheung, Jinhui Hu, Decheng Wang, Hwang-Soo Joo, Frank R. DeLeo, and Michael Otto

1Department of Laboratory Medicine, Huashan Hospital and Key Laboratory of Medical Molecular Virology, Institutes of Medical Microbiology and Biomedical Sciences, Shanghai Medical College, Fudan University, Shanghai, China; 2National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland; 3National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, Montana

The current pandemic of community-associated methicillin-resistant Staphylococcus aureus (CA-MRSA) skin infections is caused by several genetically unrelated clones. Here, we analyzed virulence of globally occurring CA-MRSA strains in a rabbit skin infection model. We used rabbits because neutrophils from this animal species have relatively high sensitivity to Panton-Valentine leukocidin (PVL), a toxin epidemiologically correlated with many CA-MRSA infections. Virulence in the rabbit model correlated with in vitro neutrophil lysis and transcript levels of phenol-soluble modulin α and α-toxin, but not PVL genes. Furthermore, abscesses caused by USA300 and its PVL-negative progenitor USA500 were comparatively large and similar in size, suggesting that PVL has played a limited role in the evolution of USA300 virulence in the context of skin infections. Our study indicates a major but not exclusive impact of virulence on the epidemiological success of USA300 and other CA-MRSA strains and emphasizes the importance of core genome-encoded toxins in CA-MRSA skin infections.

Staphylococcus aureus is a human pathogen that may cause serious infections, ranging from moderately severe skin infections to toxic shock and scalded skin syndromes, endocarditis, osteomyelitis, and sepsis [1]. Frequent resistance to antibiotics considerably complicates treatment of S. aureus infections [2]. In particular, many strains of S. aureus are resistant to methicillin and other beta-lactam antibiotics [3]. These methicillin-resistant S. aureus (MRSA) have become so abundant among nosocomial isolates of S. aureus that methicillin is no longer a drug of first choice for the treatment of S. aureus hospital-associated (HA) infections.

Although MRSA infections were traditionally limited to hospitals, community-associated cases of MRSA (CA-MRSA) were reported starting in the late 1990s [4]. The epidemiological success of CA-MRSA strains is believed to stem from the combination of antibiotic resistance at low fitness cost [5, 6] with extraordinary virulence, allowing these strains to infect otherwise healthy individuals and spread sustainably in the population [7]. Within the last decade, CA-MRSA strains have caused a pandemic of mostly skin and soft tissue infections. A particularly pronounced epidemic is seen in the United States, where CA-MRSA is the most frequent cause of skin and soft tissue infections being reported to emergency departments [8]. Importantly, almost all CA-MRSA infections in the United States are caused by closely related clones belonging to pulsed-field type USA300 [9].
CA-MRSA infections are also being reported at increasing numbers in other parts of the world [4, 10, 11]. Interestingly, these global CA-MRSA strains do not commonly belong to pulsed-field type USA300 but cover virtually the entire diversity of *S. aureus* as a species. However, research on CA-MRSA virulence has been performed largely with strains belonging to the USA300 lineage, whereas our knowledge of virulence of other, globally occurring CA-MRSA, is comparatively limited. Thus, to better understand the virulence of global CA-MRSA, we here analyzed isolates belonging to major CA-MRSA lineages found worldwide and compared them to the most abundant HA-MRSA strains. For this purpose, we used a rabbit model of skin infection. We chose this rabbit model because skin infections are the predominant manifestation of CA-MRSA disease [4]. Furthermore, it has recently been demonstrated that rabbit neutrophils have high sensitivity in vitro to the cytolytic activity of Panton-Valentine leukocidin (PVL), a leukocyte toxin epidemiologically correlated with many CA-MRSA infections [12, 13]. Thus, assuming that neutrophils also are a primary target of PVL in vivo, a rabbit infection model should allow appropriate analysis of the relative contribution of PVL to CA-MRSA virulence. In addition, to investigate whether in vitro analyses allow predictions on the relative virulence of CA-MRSA strains, we analyzed in vitro expression of virulence factors shown or proposed to be associated with CA-MRSA virulence.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** MRSA isolates SF8300 (pulsed-field type USA300, sequence type [ST] 8), BD02–25 (USA500, ST8), SF1497 (USA1100, ST30), SF1208 (USA200, ST36), SF1681 (USA1000, ST59), and SF2561 (USA100, ST5) were isolated from patients at San Francisco hospitals. Strain MW2 (USA400, ST1) was obtained from the Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA). Strain 07–02662 (ST80) was isolated from a patient in the Seoul area, South Korea, and kindly provided by Dr J. Kim, Hallym University College of Medicine, Republic of Korea. Bacteria were grown in tryptic soy broth (TSB) (Oxoid) or casein hydrolysate and yeast-extract containing medium (CCY) [14] at 37°C, with shaking in an orbital shaker at 180 revolutions per minute (rpm).

**Rabbit skin abscess model.** New Zealand white female rabbits (12 rabbits per strain) were used for the abscess model. All rabbits weighed between 2.0 and 2.3 kg at the time of use. Before the experiments, animals were shaved around the site of injection. *S. aureus* strains were grown to mid-exponential growth phase, washed, and resuspended in sterile phosphate-buffered saline (PBS) at 5 × 10⁶ colony-forming units (CFUs)/100 μL. Rabbits were anesthetized with isoflurane and inoculated with 100 μL PBS containing 5 × 10⁷ live *S. aureus* or with PBS alone in the right dorsum by intradermal injection. Abscess dimensions were measured daily with a caliper for a total of 14 days. Length (L) and width (W) values were used to calculate the area of lesions using the formula L × W. A 0.5-mL blood sample was collected daily from the marginal ear vein of each rabbit for quantitative blood culture and measurement of cytokine concentrations, which were performed with rabbit-specific enzyme-linked immunosorbent assay (ELISA) kits (Ever Systems Biology Laboratory, Inc) according to the manufacturer’s instructions. On the fourth day after infection, 6 rabbits per strain were killed, the abscess regions were excised, and part of the excised tissue material was fixed in 10% formalin (Sigma). Paraffin embedding and hematoxylin & eosin (H&E) staining were performed as described elsewhere [15]. Abscess material samples of 0.2 g were homogenized in 1 mL PBS for the measurement of cytokines. Samples from lungs, spleens, and kidneys (0.2–0.3 g) were processed for quantitative bacterial cultures. All animals were killed after completion of the entire procedure. All animal work was approved by the ethics committee of Fudan University, Shanghai, People’s Republic of China.

**Neutrophil lysis assays.** Human neutrophils were isolated from heparinized venous blood of healthy individuals with a standard method [16] in accordance with a protocol approved by the ethics committee of Huashan Hospital, Fudan University, Shanghai. All individuals gave informed consent prior to donating blood. Lysis after phagocytosis was measured using a lactate dehydrogenase (LDH) cytotoxicity detection kit according to the manufacturer’s protocol (Roche) as described elsewhere [17]. Bacteria grown to midlogarithmic growth phase and neutrophils were used at a 10:1 ratio, and samples were diluted 1:50 for the assays.

**Quantitative reverse-transcription polymerase chain reaction.** For RNA isolation, overnight cultures were diluted 1:100 into 50 mL TSB and incubated at 37°C with shaking at 180 rpm until grown to stationary growth phase (8 h). Complementary DNA was synthesized from total RNA using the QuantiTect reverse transcription system (Qiagen) according to the manufacturer’s instructions. Oligonucleotide primers were designed using Primer Express. The primers used were gyrB-F, CAAAATGATCACAGCATTTGGTACAG, gYR-R, CGGGATCATGTCATAATGACGAT; Hla-F, AATAACTGTAAGGGAATGCCTTGTTGAAA, HLA-R, GCAGCAGATAACTTC-CTTGATCT; RNAIII-F, ATAGCACTGAGTCCAAGGAATAAACT, RNAIII-R, GCCATCCCCAACTTTAAACCCTAGT; PSMα-F, TATCAAAAGCTTAAATCGAACAATT, PSMα-R, CCCCTTCATATAAGATGTCTCATT; PSMβ-F, CGAATGAAA- TCTCAGGATGTGCGTACT, PSMβ-R, GCTCAAGACAAAAACCTTTAATGC. Primers to detect PVL hybridize to the *lukS*-PV and those to detect *psmα* to the *psma1* and *psma2*.
Table 1. Strains Used in This Study

<table>
<thead>
<tr>
<th>Strain name</th>
<th>Pulsed-field type</th>
<th>Sequence type</th>
<th>CA-MRSA or HA-MRSA</th>
<th>lukSF-PV Region of isolation</th>
<th>Reference</th>
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<td>[41]</td>
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NOTE. CA-MRSA, community-associated methicillin-resistant Staphylococcus aureus; HA, hospital-associated methicillin-resistant Staphylococcus aureus.

Table 1: Strains Used in This Study

A summary of the strains used in the study, including their pulsed-field type, sequence type, whether they are CA-MRSA or HA-MRSA, the lukSF-PV region of isolation, and the reference.

- SF8300: USA300, 8, CA, Positive San Francisco
- MW2: USA400, 1, CA, Positive Midwest U.S.
- 07–02662: USA400, 80, CA, Positive Germany
- SF1681: USA1000, 59, CA, Positive San Francisco
- SF1497: USA1100, 30, CA, Positive San Francisco
- CN1: USA1000, 72, CA, Negative South Korea
- SF2561: USA100, 5, HA, Negative San Francisco
- SF1208: USA200, 36, HA, Negative San Francisco
- BD02–25: USA650, 8, HA, Negative San Francisco

NOTE. CA-MRSA, community-associated methicillin-resistant Staphylococcus aureus; HA, hospital-associated methicillin-resistant Staphylococcus aureus.

genes within the psmα operon. The resulting complementary DNA and negative control samples were amplified using the QuantiTect SYBR green PCR kit (Qiagen). Reactions were performed in a MicroAmp Optical 96-well reaction plate using a 7900 Sequence Detector (Applied Biosystems). Standard curves were determined for each gene, using purified chromosomal DNA at concentrations of 0.005–50 ng/mL. All quantitative reverse-transcription polymerase chain reaction (qRT-PCR) experiments were performed in duplicate, with gyrB as control.

**Determination of minimum inhibitory concentrations of different antimicrobial peptides.** Minimum inhibitory concentrations (MICs) were determined essentially as described by Wu and Hancock [18] with the following modifications. S. aureus cells were grown to midlogarithmic growth phase; cultures were harvested, washed twice with 10 mmol/L sodium phosphate buffer (pH 6.5) with 100 mmol/L NaCl, and resuspended in Luria-Bertani (LB) media. The cells were diluted in LB to a final concentration of 10^6 cells/mL in each sample and exposed to a range of antimicrobial peptide (AMP) concentrations at 37°C with shaking at 180 rpm for at least 12 h, after which optical density value at 600 nm (OD 600) was measured. MIC was defined as the concentration at which the OD 600 was reduced by 50%.

**Western blot analysis.** Protein bands were transferred from 12.5% bis/acylamide SDS-PAGE gels onto nitrocellulose membranes using the iBlot Dry Blotting System (Invitrogen). Western blotting was performed using rabbit anti-Hla (1:5000), anti-LukS-PV (1:400), and anti-LukF-PV (1:2200) antisera [14]. Primary antibodies were detected using an IRDye-conjugated anti-rabbit 700DX antibody (1:5,000) and the Odyssey Infrared Imaging System (Licor Biosciences). Membranes were washed in between incubations with Tris-buffered saline (KD medical) containing 0.1% Tween-20 (Sigma). All antibodies were diluted into Odyssey blocking buffer (Licor biosciences). Band intensities were measured using ImageJ software (version 1.43).

**Measurement of phenol-soluble modulin.** Phenol-soluble modulin (PSM) concentrations were determined by reversed-phase high-pressure liquid chromatography/electrospray ionization mass spectrometry (RP-HPLC/ESI-MS) of S. aureus culture filtrates as described elsewhere [19]. Samples were taken from cultures inoculated 1:100 from pre-cultures and grown for 8 or 24 h.

**Statistics.** Statistical analysis was performed using GraphPad Prism, version 5.0 (GraphPad).

**RESULTS**

**Rabbit skin infection model.** To examine virulence and virulence factor expression of global CA-MRSA strains, we used 6 strains representing different lineages of globally occurring CA-MRSA [4], of which 5 were positive for the lukSF-PV genes encoding PVL, and 3 strains that were representative of the major HA-MRSA clones, USA100, USA200, and USA500 [20] (see Table 1). USA500 is the direct progenitor of the predominant US CA-MRSA clone USA300 [8, 21].

We first determined virulence in a rabbit abscess model. Disease outcome as measured by the size of abscesses varied significantly between the strains (Fig. 1A and 1B). There was one highly virulent group of strains, consisting of USA300, USA500, and ST80, which produced large abscesses ranging from 5 to 7 cm in diameter, a group of strains that produced moderate abscesses of 2 to 4 cm in diameter (USA400, USA1000, ST72, USA100), and a group of strains that caused almost no abscess formation (USA200, USA1100) (Fig. 1A and 1B). At day 4 after infection, when maximal abscess formation was observed, abscess sizes in rabbits infected with the USA300 strain were significantly different from those infected with the USA100, USA200, USA400, USA1100, and ST72 strains, but not those infected with the USA500, USA1000, and ST80.
Figure 1. Rabbit abscess model. A, Abscess sizes (length × width) over the time of the experiment in global community-associated methicillin-resistant Staphylococcus aureus (CA-MRSA) and hospital-associated methicillin-resistant Staphylococcus aureus (HA-MRSA) strains. Each rabbit (12 rabbits per bacterial strain) received 5 × 10^6 colony-forming units (CFUs) at the right dorsum by intradermal injection. Filled symbols represent Panton-Valentine leukocidin (PVL)–positive, open symbols PVL-negative strains. Black symbols represent CA-MRSA strains, and gray symbols represent HA-MRSA strains. Error bars show means ± standard error of the mean. B, Pictures of abscesses that developed in mice infected with the USA500, USA400, and USA200 strains. C, Histological examination of abscess tissue in mice infected with the USA500, USA400, and USA200 strains. B, C, Abscesses and degrees of neutrophil infiltration in strains ST80 and USA300 were similar to those observed in the USA500 strain. In strains USA1000, ST72, and USA100 these parameters were similar to those observed in USA400, and in strain USA1100 to those in strain USA200.
strains. In addition, we analyzed abscess material at day 4 for leukocyte infiltration by histopathology (Fig. 1C) and concentration of the inflammatory cytokines tumor necrosis factor α (TNF-α) and interleukin 8 (IL-8) (Fig. 2). The degree of leukocyte infiltration was correlated with abscess formation. No obvious differences in the histopathological presentation were detected in the muscles underlying the abscesses formed by the different *S. aureus* strains. Levels of IL-8 and TNF-α in abscess tissue and blood correlated strongly with abscess size (Fig. 2, Fig. 3A). We also determined cytokine levels at days 1 and 10. Although less pronounced, relative differences among strains at days 1 and 10 were similar to those observed at day 4 (data not shown). In summary, the most noticeable results from the animal model were that (1) signs of inflammation were clearly correlated with abscess sizes, (2) virulence varied considerably among global CA-MRSA strains, and (3) virulence of the PVL-positive USA300 strain was not significantly different from that of its PVL-negative progenitor, USA500.

**Resistance to innate host defense mechanisms.** The outcome of *S. aureus* disease is determined to a considerable extent by the interaction of *S. aureus* with human neutrophils [22]. The predominant CA-MRSA strains in the United States, USA300 and USA400, were shown to have higher cytolytic capacity toward human neutrophils than did common HA-MRSA strains [23], which may explain in part the high virulence potential of those strains. Thus, to determine whether this phenomenon in vitro correlates with MRSA virulence in skin infections, we evaluated the capacity of global CA-MRSA and HA-MRSA strains to lyse human neutrophils after phagocytosis. The degree of neutrophil lysis caused by USA300 was significantly higher than that of all other strains, with the noticeable exception of strain USA500 (Fig. 4). Overall, the capacity to lyse human neutrophils was correlated strongly with abscess size in the rabbit skin infection model (Fig. 3A and 3D), consistent with the idea that neutrophil lysis is a determinant of virulence in MRSA skin infections.

Furthermore, we determined MICs to several cationic and anionic AMPs. MICs did only differ marginally, by a factor of 2 or less, and in one case by a factor of 2–4 (nisin) (data not shown). MICs were not correlated with any parameter measured in this study, indicating that resistance to AMPs does not have a major impact on the difference in virulence potential of the investigated strains. This observation is consistent with results of a previous study that determined MICs of granule

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**Figure 2.** Cytokine concentrations in abscess tissue and blood. Concentrations of the cytokines TNF-α and interleukin 8 (IL-8) were measured by enzyme-linked immunosorbent assay (ELISA) in abscess and blood samples at day 4 after injection. Filled bars represent Panton-Valentine leukocidin (PVL)-positive, and open bars represent PVL-negative strains. Black bars represent community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA), and gray bars represent hospital-associated methicillin-resistant *Staphylococcus aureus* strain. *P < .05; **P < .01; ***P < .001 (1-way analysis of variance [ANOVA], Dunnett’s multiple comparison test versus USA300).
Figure 3. Correlation analyses. A, Correlation table showing 2-tailed P values calculated from raw data of each parameter using Pearson correlation analysis. If several measurements were available, mean values were calculated before correlation analysis. Cells are colored corresponding to the statistical significance values determined: , yellow; , orange; , red. 1, Correlation analysis for Panton-Valentine leukocidin (PVL) in tryptic soy broth (TSB) at 8 h was not possible, because no PVL was detectable in any sample. B, C, D, Graphical presentation of most important correlation analyses: B, Transcript levels versus neutrophil lysis. C, Transcript levels versus abscess size. D, Neutrophil lysis versus abscess size.

Proteins against several CA- and HA-MRSA strains [24]. Remarkably, MIC values were exactly the same for strains USA300 and USA500, further underlining the close evolutionary relationship of these 2 strains.

In vitro virulence factor expression. The *S. aureus* toxins α-toxin, PSMε peptides, and PVL have a demonstrated impact on experimental disease caused by CA-MRSA [19, 25, 26], although so far, only PSMα peptides have been shown to sig-
with our previous results [21], expression of RNAIII-encoded toxins are regulated by the quorum-sensing system [19, 27, 28]. PVL and PSM peptides, a, the 8-h time point was selected because all 3 toxins are potent cytolysins and regulated by the agr quorum-sensing system [19, 27, 28]. PVL and PSM peptides are cytolytic toward human neutrophils [19, 29].

First, we analyzed presence of the respective gene loci by analytical PCR. All strains were positive for the psmA and hla (α-toxin) genes, and for RNAIII (agr), as expected from the core genome location of psmA, hla, and agr. Only the USA300, USA400, USA1000, USA1100, and ST80 strains were positive for lukS-PV (PVL) (data not shown).

Importantly, mere presence of gene products does not allow conclusions on the contribution of core genome-encoded virulence determinants, such as α-toxin and PSMs, to virulence. Furthermore, expression of the mobile genetic element (MGE)—encoded PVL may differ significantly between strains [30]. Therefore, we examined whether expression of PVL, PSMa peptides, α-toxin, and agr is correlated with CA-MRSA virulence in the rabbit skin abscess model. We first measured transcript levels of the lukS-PV, psmA, and hla genes, and of RNAIII (agr) by qRT-PCR (Fig. 5). Preliminary studies had shown that agr is maximally expressed at ~8 h of growth; and because all 3 toxins are regulated by agr, the 8-h time point was selected for qRT-PCR analysis. Expression of the examined genes differed significantly among the strains. However, in accordance with our previous results [21], expression of hla, psmA, and RNAIII, was almost exactly the same, and not significantly different, between USA300 and USA500 (Fig. 5). Notably, these analyses demonstrated a significant correlation between the expression of psmA, hla, and agr, but not lukS-PV, with the capacity to form abscesses, cause cytokine release in vivo, and lyse neutrophils in vitro (Fig. 5; Fig. 3A–3C).

In addition, we determined production of α-toxin and PVL by Western blotting (Fig. 6A and 6B), and of PSM peptides by RP-HPLC/ESI-MS (Fig. 6C), to analyze whether concentrations of these toxins in in vitro cultures correlate with abscess size and other disease parameters in the CA- and HA-MRSA strains. We measured protein concentrations also in CCY medium, because much research on PVL has been performed using that medium, in which PVL is strongly overproduced [14]. In vitro toxin concentration differed considerably between strains. We observed dramatic differences in toxin production when different growth media (TSB and CCY) and different culture harvest times (8 and 24 h) were used, as described previously for PVL [14]. In the case of PVL, there was no significant correlation between toxin concentrations in vitro and in vivo abscess size, neutrophil lysis, or cytokine concentrations (Fig. 6B; Fig. 3A). For α-toxin, a significant correlation with abscess size, neutrophil lysis, and almost all readouts of cytokine concentrations was observed when α-toxin levels were measured in cultures grown for 24 h in TSB media, but not at 8 h, and not when cultures were grown in CCY media (Fig. 6A; Fig. 3A).

PSMα3 is by far the most potent cytolytic PSM [19], which is why we analyzed the obtained RP-HPLC/ESI-MS data focusing on that PSM peptide. PSM peptides are secreted with the N-terminal methionine N-formylated, but may be deformylated depending on strain background and growth conditions [19, 31]. Amounts of N-formylated PSMα3 peptide in 8-h cultures, but not 24-h cultures, correlated with neutrophil lysis and only barely failed to reach statistical significance when analyzed for correlation with abscess size and cytokine levels (Fig. 3A). There was no significant correlation when the N-deformylated form of PSMα3 was included in the computation, which may be due to the lower cytolytic capacity of N-deformylated PSMα3 (unpublished data), or when cultures were grown in CCY instead of TSB media.

Altogether, these data indicate that in vitro growth conditions influence toxin concentrations in culture supernatants to a degree that only allows a very limited prediction of the virulence potential in skin infections. Likely, strongly varying proteolytic activity in culture supernatants under the different conditions has a strong impact on toxin concentrations. In contrast, our results show that psmA, hla, and RNAIII transcript levels are correlated with disease severity and key virulence mechanisms in skin infection.

**DISCUSSION**

In this study, we found that the virulence potential in a rabbit skin infection model varied significantly among CA- and HA-MRSA strains. The epidemiologically successful CA-MRSA clone, USA300, was among the most virulent strains and elicited the most pronounced neutrophil infiltration and cytokine release. Furthermore, USA300 had significantly higher potential...
Figure 5. In vitro toxin gene expression and agr activity. Expression of the *hla* (*α*-toxin), *psmA*, and *lukS* (Panton-Valentine leukocidin [PVL]) genes were measured by qRT-PCR of cultures grown to stationary growth phase (8 h) in tryptic soy broth (TSB). Activity of the agr system was measured by qRT-PCR of RNAIII under the same conditions. Black bars represent community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) strain, and gray bars represent hospital-associated methicillin-resistant *Staphylococcus aureus* strain. *; **; *** (1-way analysis of variance [ANOVA], Dunnett’s multiple comparison test versus USA300).

to kill neutrophils in vitro than other CA-MRSA strains tested. Our investigation lends support to the idea that the capacity of strain USA300 to cause infection in otherwise healthy individuals is due to its high virulence capacity. It is possible that the extraordinary virulence capacity of USA300 also promotes epidemicity, thereby providing an explanation for the high abundance of USA300 compared with other CA-MRSA strains [8]. On the other hand, the ST80 strain used here was comparable in virulence to USA300 in the rabbit skin infection model, but ST80 strains cause far fewer human infections than USA300 [32]. This observation provides support to the notion that the epidemiological success of CA-MRSA as pathogens is not merely determined by the virulence potential but may be influenced by other factors such as the potential to colonize human epithelial surfaces [6, 10]. In addition, it needs to be stressed that, although epidemic USA300 is a very coherent clone [9], other STs such as USA200 may comprise strains that considerably differ in virulence from the example strains investigated in the present study.

The molecular basis of CA-MRSA virulence is controversial, particularly regarding the importance of PVL. It has been suggested that studies of PVL-positive CA-MRSA skin infection performed in mice [17, 21, 33–35] allow only limited conclusions about the contribution of PVL to disease, because mouse neutrophils have limited sensitivity to the cytolytic activity of PVL in vitro [12, 13]. Here, we confirmed in the rabbit—an animal species whose neutrophils are highly PVL-sensitive—our previous observation [21] demonstrating that the virulence potential of USA300 in skin infection is not significantly different from that of its HA-MRSA progenitor USA500. Therefore, although presence of the *lukSF-PV* genes is associated with certain types of necrotizing skin infections (eg, furunculosis and carbuncles [36]), our findings suggest that acquisition of the PVL-encoding phage had little or no impact on the evolution of virulence of USA300 in the context of skin infections despite high expression of PVL in that strain. This idea is supported by recent studies of humans with complicated *S. aureus* skin infections, in which it was shown that clinical outcomes of PVL-positive skin infections were not worse than those of PVL-negative infections [37].

Furthermore, we analyzed whether in vivo and in vitro parameters of pathogenesis that were examined in our experiments were correlated with each other. Importantly, these analyses confirmed the notion that neutrophil lysis plays a key role in MRSA skin infection [23]. However, in particular with regard to the roles played by specific virulence determinants, conclu-
Figure 6. In vitro toxin expression (protein level). A, Analysis of α-toxin and Panton-Valentine leukocidin (PVL) levels in cultures grown for 8 and 24 h in tryptic soy broth (TSB) and casein hydrolysate and yeast-extract containing medium (CCY). Samples were analyzed by SDS-PAGE on 10% Tris/glycine gels and immunoblotting. Signal intensities were determined by densitometry. LukS-PV and LukF-PV were detected separately using specific antisera and signals achieved for LukS-PV and LukF-PV were added. B, Analysis of phenol-soluble modulin (PSM) production in TSB at 8 h of growth by RP-HPLC/ESI-MS. Values for selected PSMs (PSMα3, PSMβ1, δ-toxin, and the SCCmec-encoded PSMmec) are shown. PSM concentrations were also determined at 24 h of growth and in CCY (not shown). PSM concentrations were lower in CCY than in TSB, by a factor of ∼2, but relative PSM compositions were similar to those found in TSB. N-formylated and N-deformylated forms of PSMs were determined and are shown as clear and striped bar portions, respectively.

sions from correlation analyses should be drawn with caution. For example, in a recent study, a correlation between in vitro PVL expression and virulence in mice was reported, which may be interpreted as evidence for a role of PVL in murine skin infection [38]. This conclusion would be at odds with the relative insensitivity of mouse neutrophils to PVL [12, 13] and previous reports that indicated PVL has a limited or no role in murine skin infection [17, 34, 35]. Given that PSMα peptides strongly impact skin infection in mice [19], it is likely that the correlation observed in that study is caused by a correlation between the expression of PVL and PSMα peptides that is due to agr control of both these toxins [19, 27, 39], rather than a contribution of PVL to pathogenesis. Similarly, the correlation of hla expression with neutrophil lysis that was observed here and contrasts the lack of cytolytic activity of α-toxin toward human neutrophils [40] also likely stems from regulation of both hla and psmα by agr. Thus, although such correlation analyses of only a single virulence factor may be misleading, a comparative correlation analysis as performed here should allow careful conclusions on the relative importance of the in-
vestigated factors compared to each other. In that regard, results of our cooperation analyses are consistent with the notion that PSMα peptides have a more pronounced impact on CA-MRSA skin infection than on PVL [19] and suggest significant contributions of α-toxin and agr to that type of disease. Nevertheless, a detailed analysis of the relative contribution of staphylococcal toxins to CA-MRSA virulence will need to be performed using isogenic gene deletion strains.

In summary, our study indicates that virulence potential, cytolytic capacity, and expression of core genome-encoded toxins play an important role in CA-MRSA skin infections. Furthermore, our results suggest that measurement of in vitro expression of those toxins at the transcript level may be used to evaluate the potential of CA-MRSA strains to cause severe disease. However, determinants other than those involved in virulence sensu strictu may also determine the epidemiological success of CA-MRSA strains such as USA300 as pathogens.

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References


