MAJOR ARTICLE

# Is Panton-Valentine Leukocidin the Major Virulence Determinant in Community-Associated Methicillin-Resistant *Staphylococcus aureus* Disease?

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**Methicillin-resistant *Staphylococcus aureus* (MRSA) remains a major problem in hospitals, and it is now spreading in the community. A single toxin, Panton-Valentine leukocidin (PVL), has been linked by epidemiological studies to community-associated MRSA (CA-MRSA) disease. However, the role that PVL plays in the pathogenesis of CA-MRSA has not been tested directly. To that end, we used mouse infection models to compare the virulence of PVL-positive with that of PVL-negative CA-MRSA representing the leading disease-causing strains. Unexpectedly, strains lacking PVL were as virulent in mouse sepsis and abscess models as those containing the leukotoxin. Isogenic PVL-negative (*lukS/F-PV* knockout) strains of USA300 and USA400 were as lethal as wild-type strains in a sepsis model, and they caused comparable skin disease. Moreover, lysis of human neutrophils and pathogen survival after phagocytosis were similar between wild-type and mutant strains. Although the toxin may be a highly linked epidemiological marker for CA-MRSA strains, we conclude that PVL is not the major virulence determinant of CA-MRSA.**

*Staphylococcus aureus* causes widespread human disease ranging from mild skin infections to fatal necrotizing pneumonia and sepsis [1]. For decades, the pathogen has become increasingly resistant to $\beta$-lactam antibiotics, and methicillin-resistant *S. aureus* (MRSA) is a leading cause of hospital-acquired infections [2]. Such infections typically occur in individuals with predisposing risk factors, including surgery [1]. By contrast, community-acquired (or -associated; CA) MRSA causes disease in healthy individuals [2–4], and there has been an alarming increase in the number CA-MRSA infections worldwide [2–7]. Recent studies have indicated that CA-MRSA strains are generally more virulent than hospital-acquired MRSA [8]—a finding consistent with the ability of CA-MRSA to cause disease in individuals without predisposing risk factors. Although the molecular basis for the enhanced virulence is not known, there is strong association between CA-MRSA infections and the presence of Panton-Valentine leukocidin (PVL) [4, 5, 7, 9–11].

The rapid emergence of CA-MRSA infections correlates with the increased incidence of strains containing PVL—for example, pulsed-field types USA300 and USA400 [6, 7, 12, 13]. Furthermore, unusually severe pathological signs have been associated with USA300 and USA400 infections, including necrotizing fasciitis, necrotizing pneumonia, and sepsis [10, 11, 14]. However, the role that PVL plays in CA-MRSA disease has not been tested directly. To that end, we evaluated the

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**Figure 1.** Generation and characterization of isogenic Panton-Valentine leukocidin (PVL)–knockout (Δ*pvl*) strains of MW2 and Los Angeles County clone (LAC).

virulence of PVL-positive ($PVL^+$) and PVL-negative ($PVL^-$) strains of the most common pulsed-field types in mouse models of sepsis and skin infection, and we generated isogenic $PVL^-$ strains (Δ*pvl*) of MW2 (USA400) and the Los Angeles County clone (LAC, USA300) with which to test directly the involvement of PVL in the pathogenesis of CA-MRSA.

## MATERIALS AND METHODS

***Bacterial strains and culture.*** *S. aureus* strains were selected on the basis of clinical relevance and genotype, including multilocus sequence type (MLST or ST), *spa* type, *agr* type, staphylococcal cassette chromosome (SCC) *mec* type, and the presence or absence of PVL (table 1). Unless specified, bacteria were grown in tryptic soy broth (Becton Dickinson) that contained 0.25% glucose. Overnight cultures were diluted 1:200 and incubated at 37°C with shaking (250 rpm). *S. aureus* were harvested at the midexponential phase of growth ($OD_{600}$, 0.75), washed in Dulbecco's PBS (DPBS; Sigma-Aldrich), and resuspended in RPMI 1640 medium (Invitrogen) buffered with 10 mmol/L HEPES (RPMI/H [pH 7.2]) or DPBS.

***Construction of isogenic* lukF/S-PV *deletion mutants.*** Standard molecular biological methods, such as the isolation of plasmid DNA and transformation into *Escherichia coli*, were performed as described elsewhere [15]. Staphylococcal plasmid DNA was isolated using a Qiafilter Plasmid Midi Kit (Qiagen) in accordance with the manufacturer's instructions, except that bacteria were incubated in P1 buffer (Qiagen) that contained 25 μg/mL lysostaphin for ~15 min at 37°C. To delete *lukF/S-PV* in *S. aureus* strains MW2 and LAC, polymerase chain reaction (PCR)–amplified regions flanking the *lukF/S-PV* locus were cloned into plasmid pBT2spec that contained a spectinomycin resistance cassette [16]. DNA fragments upstream (PCR fragment 1; 800 bp) and downstream (PCR fragment 2; 1900 bp) of *lukF/S-PV* were amplified with Ready-To-Go PCR beads (Amersham Biosciences) in accordance with the manufacturer's instructions (figure 1). PCR-amplified regions flanking the *lukF/S-PV* loci and a spectinomycin resistance cassette were cloned into plasmid pBT2 and verified by DNA sequencing (Applied Biosystems). The resulting plasmid, pBTΔ*lukF/S-PV,* was transformed sequentially into *E. coli, S. aureus* strain RN4220, and MW2 or LAC. The MW2 and LAC constructs were used for allelic replacement as described elsewhere [16]. The absence of *lukF/S-PV* genes and transcripts in *lukF/S-PV* isogenic MW2 (MW2Δ*pvl*) and LAC (LACΔ*pvl*) mutant strains were verified using PCR (figure 1) and TaqMan real-time PCR, respectively.

***Mouse infection models.*** Female CD1 Swiss and Crl:SKH1-hrBR hairless mice (outbred and immunocompetent) were purchased from Charles River Laboratories. Mice were housed in microisolator cages and received food and water ad libitum. All studies conformed to guidelines set forth by the National Institutes of Health and were reviewed and approved by the Animal Use Committee at Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases (NIAID).

*S. aureus* (table 1) were cultured to the midexponential phase of growth, washed twice with sterile DPBS, and resuspended in DPBS at $5 \times 10^7$ or $1 \times 10^8$ cfu/100 μL. For the mouse bacteremia studies, CD1 Swiss mice were inoculated with the indicated dose of *S. aureus* or sterile DPBS via tail-vein injection [8]. Criteria for determining morbidity or sickness in mice included hunched posture, decreased activity, ruffled fur, and

**Table 1.** ***Staphylococcus aureus*** **strains used in the study.**

| Strain | Place of isolation, description | MLST | *spa* | SCC*mec* | *agr* | PVL |
|---|---|---|---|---|---|---|
| MW2 (USA400) | North Dakota, prototype CA-MRSA | ST1 | 131 | IV | III | + |
| MW2Δ*pvl* (USA400) | Isogenic *lukF/S-PV* KO | ST1 | 131 | IV | III | − |
| MnCop (USA400) | Nebraska | ST1 | 35 | MSSA | III | + |
| BK6789 (USA400) | Ontario, Canada | ST1 | 35 | MSSA | III | − |
| LAC (USA300) | California, prominent CA-MRSA | ST8 | 1 | IV | I | + |
| LACΔ*pvl* (USA300) | Isogenic *lukF/S-PV* KO | ST8 | 1 | IV | I | − |
| BK11540 (USA300) | New Jersey | ST8 | 1 | IV | I | − |
| BK2394 (USA1000) | New York | ST59 | 17 | IV | I | + |
| BK648 (USA1000) | Georgia | ST59 | 17 | IV | I | − |
| BK9918 (USA400) | Nebraska | ST1[a] | 194 | IV | III | + |
| BK2370 (USA500) | New York | ST8 | 7 | IV | I | − |

**NOTE.** +, positive; −, negative; *agr, agr* type; CA, community acquired; HA, hospital acquired; KO, knockout; LAC, Los Angeles County clone; MLST, multilocus sequence type; MSSA, methicillin-susceptible *S. aureus*; PVL, Panton-Valentine leukocidin; *spa, spa* type; SSC, staphylococcal cassette chromosome; ST, sequence type.

[a] Similar to ST1.

**Table 2. TaqMan reverse transcription–polymerase chain reaction primer and probe sequences used in the study.**

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labored breathing. Mice were euthanized if they were unable to eat or drink or if they became immobile. All surviving mice were euthanized at 72 h. At the time of death, selected tissues were fixed in 10% neutral-buffered formalin, processed for 12 h in an Excelsior Tissue Processor (ThermoShandon), and dehydrated using a graded series of ethanol. Tissues were cleared with Histosolve (ThermoShandon) and infiltrated and embedded in paraffin. Blocks were sectioned at 5 $\mu$m, and slides were stained with hematoxylin-eosin. Samples were examined by a veterinary pathologist (D.J.G.).

We used the abscess model of Bunce et al. [17] to evaluate skin and soft-tissue infections. Crl:SKH1-hrBR mice were anesthetized with isoflurane and inoculated by subcutaneous injection in the right flank with 50 $\mu$L of DPBS that contained $1 \times 10^7$ *S. aureus*. The optimal dose of *S. aureus* for the abscess model was determined by preliminary studies in which small groups of mice ($n = 3$) were inoculated by subcutaneous injection with $1 \times 10^5$, $1 \times 10^6$, and $1 \times 10^7$ cfu of LAC (USA300; PVL$^+$) and BK11540 (USA300; PVL$^-$). Inoculation with $1 \times 10^7$ cfu produced consistent abscesses within 4 days that resolved 2 weeks after infection. In addition, the dose chosen for the experiments was at the threshold for inducing dermonecrosis for some of the strains. Mice were weighed before inoculation and at 24-h intervals for 14 days. The size of abscesses was calculated using the formula for a spherical ellipsoid [$v = (\pi/6) \times l \times w^2$], where $l$ is length and $w$ is width [17]. Area of abscesses with dermonecrosis was calculated using the formula $l \times w$.

Fifteen mice were used for each strain and/or control in bacteremia and abscess models, unless indicated otherwise, and the MW2 and LAC wild-type *(wt)* infections were repeated for the parallel infections with Δ*pvl* strains. One mouse infected subcutaneously with strain BK2394 developed unusual pathological signs and was not included in the data analysis.

***Human neutrophil assays.*** Polymorphonuclear leukocytes (PMNs or "neutrophils") were isolated from venous blood from healthy individuals in accordance with a protocol approved by the Institutional Review Board for Human Subjects, NIAID, as described elsewhere [8]. Informed consent was obtained from each subject who participated in the study. Killing of *S. aureus* by human PMNs was determined as described elsewhere [8]. PMNs ($1 \times 10^6$) were combined with *S. aureus* ($1 \times 10^7$) in 24-well tissue-culture plates and centrifuged at 380 *g* for 8 min to synchronize phagocytosis. Cells were incubated for up to 180 min at 37°C with 5% $CO_2$. At specified times, PMNs were lysed with 0.1% saponin (for 20 min on ice), plated onto tryptic soy agar, and incubated overnight at 37°C. The percentage of *S. aureus* survival was calculated using the equation ($cfu_{+PMN}$ at $t$/$cfu_{+PMN}$ at $t_0$) $\times$ 100. The assay measures the total number of viable ingested and uningested bacteria.

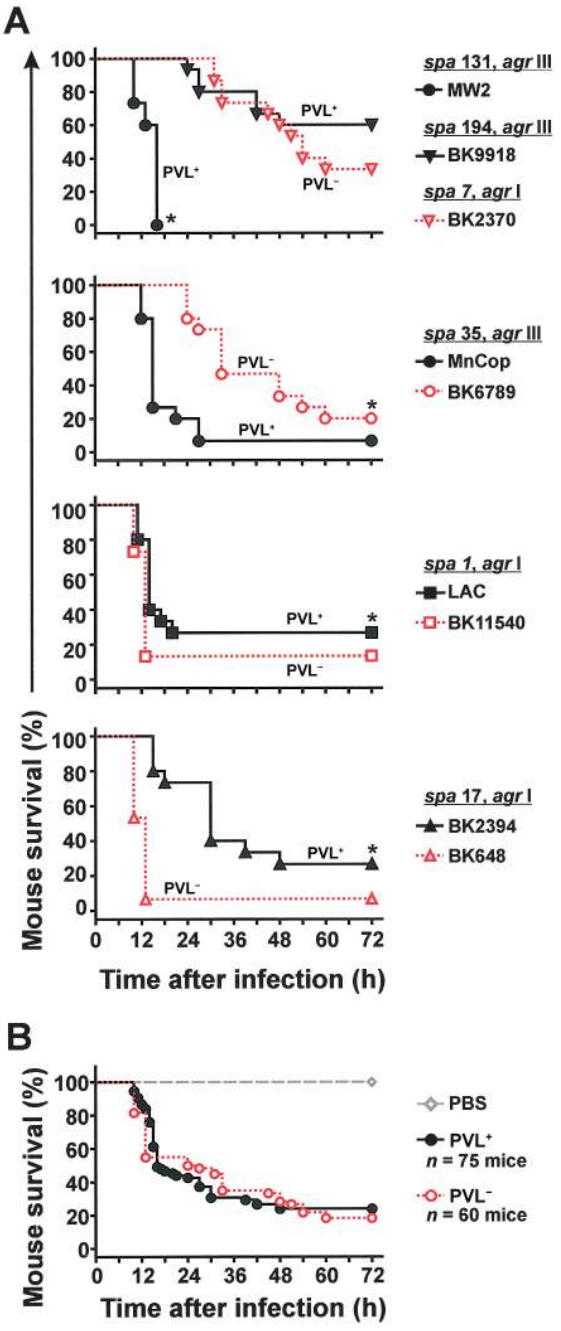


**Figure 2.** *Staphylococcus aureus* pathogenesis. *A,* Mouse survival. Mice were infected with $1 \times 10^8$ cfu of *S. aureus* by intravenous inoculation. Results are from 15 mice in each group. *Top panel,* $^*P < .0001$ vs. paired strain; *bottom panels,* $^*P < .05$ vs. paired strain. *B,* Mouse survival with all Panton-Valentine leukocidin (PVL)–positive (PVL$^+$) and PVL-negative (PVL$^-$) strains combined. LAC, Los Angeles County clone.

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**Figure 3.** Dissemination of community-associated *Staphylococcus aureus* (CA-MRSA).

Lysis of PMNs by *S. aureus* was determined as described elsewhere [8, 18]. Phagocytosis was synchronized, and cells were incubated for up to 9 h at 37°C. At the desired times, the lysis of PMNs was determined by the release of lactate dehydrogenase (LDH), using the Cytotoxicity Detection Kit (Roche Applied Sciences). Alternatively, *S. aureus* were grown in RPMI/H for 6 h at 37°C and centrifuged at 10,000 *g* for 20 min to remove bacteria. Clarified culture medium was sterilized by filtration and mixed with PMNs ($1 \times 10^6$) in 96-well tissue-culture plates, and PMN lysis was evaluated.

Damage to plasma membranes of human neutrophils (pore formation) was assessed by uptake of ethidium bromide (EtBr) as described by Gauduchon et al. [19]. Briefly, strains were cultured for 18 h at 37°C in 50 mL of YCP medium (3% [wt/vol] yeast extract, 2% Bacto-Casamino acids, 2% sodium pyruvate, 0.25% $Na_2HPO_4$, and 0.042% $KH_2PO_4$ [pH 7.0]) with vigorous shaking using a 500-mL flask (the flask's volume: culture volume ratio was 10:1). Bacteria were removed by centrifugation (2800 *g* for 10 min at 4°C), and culture medium was sterilized by filtration and stored at −80°C in aliquots until it was used. Culture medium was diluted in RPMI/H and mixed with human PMNs ($1 \times 10^6$) in the presence of 4 μmoL of EtBr, as described elsewhere [19]. At the desired times, PMNs were analyzed by flow cytometry (FACSCalibur; BD Biosciences). Alternatively, culture medium was tested for its ability to cause the eventual release of neutrophil LDH.

***TaqMan real-time reverse transcriptase–PCR.*** MW2 was opsonized in human serum for 30 min and used in PMN phagocytosis assays as described above. At specified times, RNA was isolated using a method published elsewhere [8]. Contaminating chromosomal DNA was removed by DNase treatment (Qiagen). Purified *S. aureus* RNA was subjected to One-Step TaqMan real-time PCR analysis using an ABI 7500 thermocycler (Applied Biosystems). The relative quantification of *S. aureus* genes was determined by the change in expression of target transcripts relative to *gyrB* (housekeeping or calibration gene), in accordance with the manufacturer's protocol (Applied Biosystems Relative Quantification Manual, available at: http://docs.appliedbiosystems.com/genindex.taf, document index number 4347824). Data are expressed as the change in *S. aureus* transcripts during or after neutrophil phagocytosis relative to *S. aureus* alone. The sequences of primers and probes for the TaqMan experiments are provided in table 2.

***SDS-PAGE and immunoblotting.*** Proteins present in YCP culture medium (prepared for use in the neutrophil pore formation assays as described above) were resolved by 12.5% SDS-PAGE (Bio-Rad) and transferred to nitrocellulose (Bio-Rad). Immunoblots were blocked overnight at 4°C with 10% normal goat serum in DPBS and then for 2 h at room temperature. Rabbit polyclonal antibodies specific for lukS-PV and lukF-PV (each at 1 μg/mL) were incubated with immunoblots for 1 h, followed by washing (3 times in DPBS that contained 1% Tween 20) and incubation with donkey anti–rabbit IgG conjugated to horseradish peroxidase (1:2500 dilution; BioRad) for 1 h. PVL subunits were visualized with enhanced chemiluminescence (SuperSignal West Pico) and Kodak X-Omat film (Eastman Kodak).

***Statistical analyses.*** Mouse survival statistics were performed with a log-rank test using 12–15 mice/group. Statistics for abscess volume or area of dermonecrosis were performed using a 1-way analysis of variance (ANOVA) and Bonferroni's post test for multiple comparisons. A Mann-Whitney rank sum test was used to compare the number of mice with dermonecrosis over the course of 14 days. Statistics for PMN bactericidal activity and the lysis of human neutrophils were determined using repeated-measures ANOVA and Bonferroni's post test. Results are expressed as means ± SEs, unless otherwise indicated. Analyses were done using GraphPad Prism software (version 4.0 for Windows; GraphPad).

## RESULTS

***PVL and CA-MRSA virulence.*** As a step toward understanding the molecular basis of the pathogenesis of CA-MRSA, we evaluated the virulence of selected CA-MRSA or CA–methicillin-susceptible *S. aureus* in a mouse sepsis model (figure 2*A*). The strains selected were among the most representative pulsed-field types associated with CA *S. aureus* infections in the United States [6, 7, 12, 13], including LAC (USA300), MW2 (USA400), and BK648 (USA1000) (table 1). Where possible, $PVL^+$ strains were paired with another of similar genetic background (similar *spa* and *agr* types and MLST) that lacked PVL or were of special clinical interest (table 1 and figure 2*A*). MW2 was the most lethal strain tested. This finding is consistent with the severity of infections often associated with this $PVL^+$ CA-MRSA strain [13, 14, 20]. On the other hand, 3 of 4 $PVL^-$ strains tested were either more virulent than or at least as lethal as a genetically similar $PVL^+$ strain in the sepsis model (figure 2*A*). For example, BK648, a $PVL^-$ strain, was significantly more virulent than BK2394 ($P < .001$, log-rank test) (figure 2*A*). With 1 exception, the survival of mice infected with BK648 was comparable to that of mice infected with MW2 (figure 2*A*). We also note that BK9918 produced 10-fold more *lukF-PV* transcript than MW2 in vitro [18] but was far less virulent in the mouse model (0/15 vs. 9/15 mice survived after infection with MW2 and BK9918, respectively) (figure 2*A*). Mortality after infection was mirrored closely with gross dissemination to major organs, including the heart and lungs, regardless of PVL

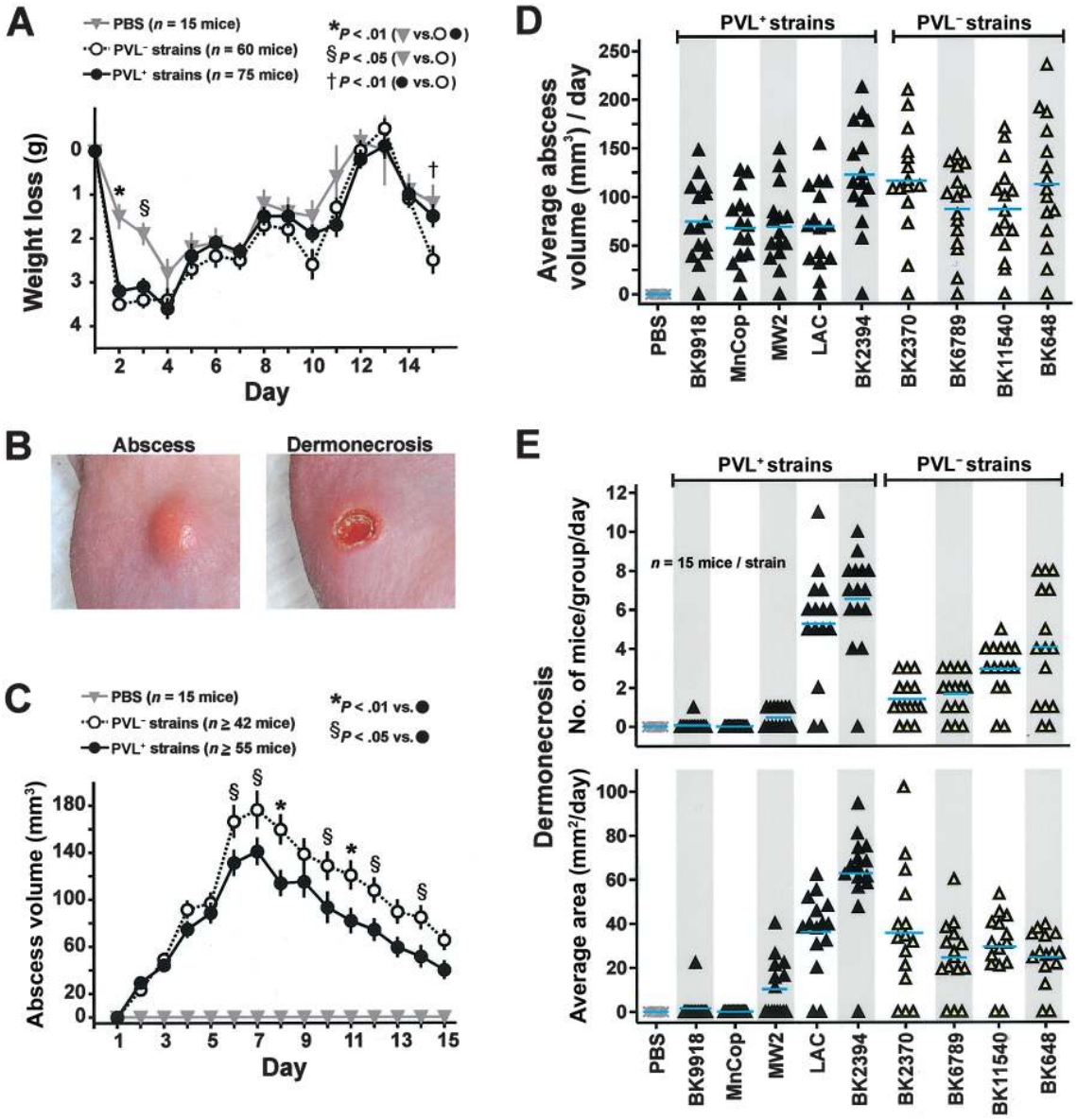


**Figure 4.** Panton-Valentine leukocidin (PVL) and abscesses. *A,* Weight loss. Results are the mean ± SE of 75 mice infected with PVL-positive (PVL⁺) strains and 60 mice infected with PVL-negative (PVL⁻) strains. *B, left,* Abscess; *right,* abscess with dermonecrosis. *C,* Skin abscess volumes. Results are the average abscess volume ± SE in mice infected with PVL⁺ and PVL⁻ strains. Although 15 mice were infected with each strain, abscesses with dermonecrosis were excluded from calculation of abscess volumes (see Materials and Methods). *D,* Strain-specific abscess volume. Each symbol represents abscess volume on a given day (15 days). *E,* Dermonecrosis caused by each strain. *Top,* Symbols represent the no. of mice with dermonecrosis on each day. *Bottom,* Symbols represent average area of abscesses with dermonecrosis on each day.

status (figure 3). In the aggregate, there was essentially identical mouse survival after bloodstream infection with PVL⁺ and PVL⁻ CA *S. aureus* strains (figure 2*B*).

CA-MRSA is known for its ability to cause skin and soft-tissue infections—manifestations typically associated with PVL [7, 9, 12, 21]. Therefore, we evaluated the ability of the CA *S. aureus* strains to cause abscesses and/or dermonecrosis in a mouse skin-infection model (figure 4). Unexpectedly, the average abscess volume in mice infected with PVL⁻ strains was slightly greater than that of mice infected with PVL⁺ strains ($P < .01$ on days 8 and 11) (figure 4*C*). Notably, MW2 and LAC, which together cause the greatest number of CA infections, produced, on average, slightly smaller abscesses than PVL⁻ strains (figure 4*D*). There were generally more mice with dermonecrotic abscesses after infection with LAC and BK2394, which are both PVL⁺ strains (figure 4*E*). However, this observation was offset by the striking finding that the other PVL⁺ strains, including MW2, caused little or no dermonecrosis (fig-

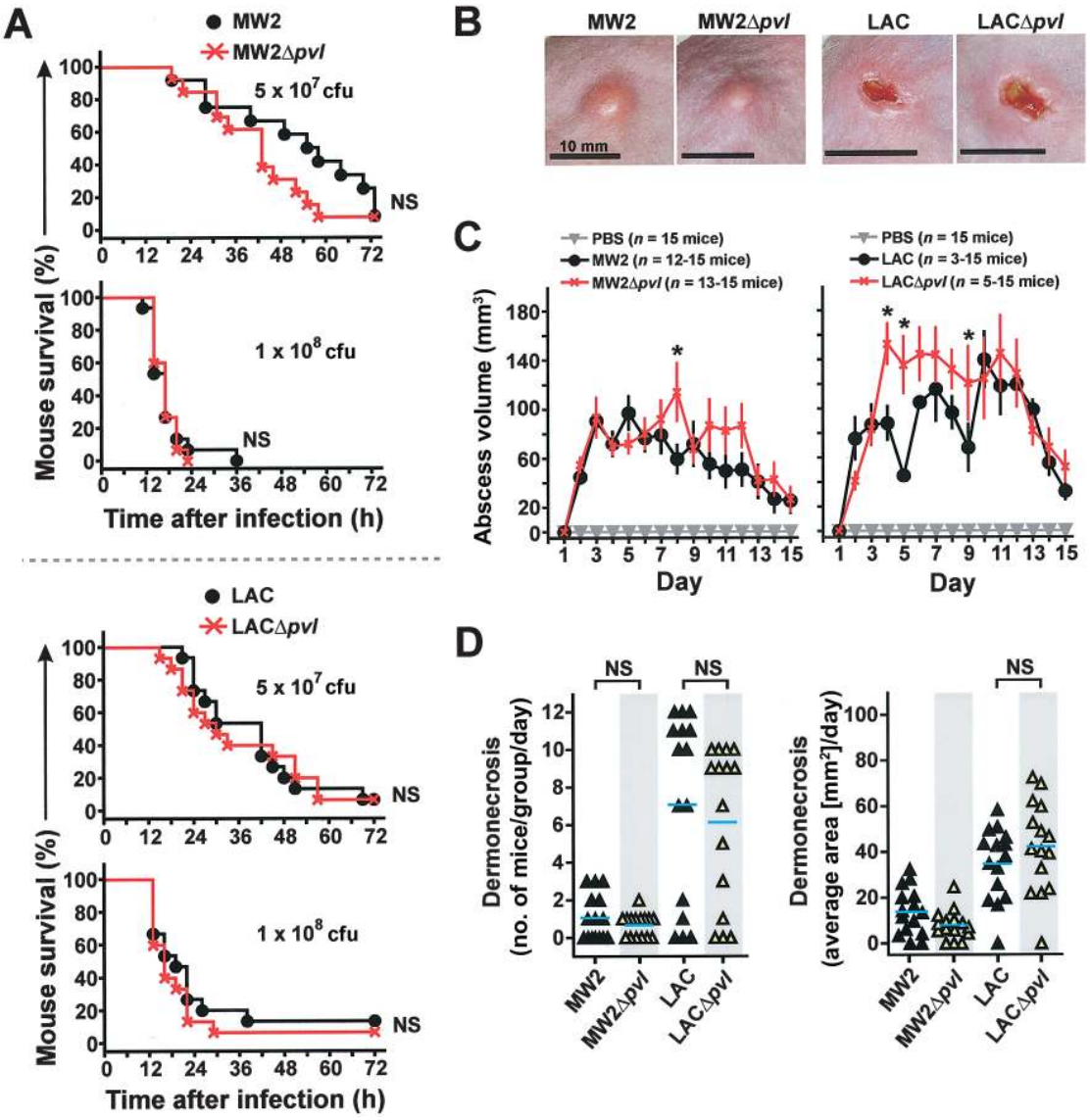


**Figure 5.** Disease in mice infected with MW2 and Los Angeles County clone (LAC) wild-type *(wt)* and isogenic Δ*pvl* strains. *A,* Mouse survival. Mice were infected with MW2 *(upper panels)* and LAC *(lower panels) wt* and isogenic Δ*pvl* strains by tail vein inoculation at the indicated dose. Results are from 12–15 mice in each group. *B, left,* Abscesses; *right,* abscesses with dermonecrosis. Bars, 10 mm. *C,* Abscess volume. Results are the mean ± SE. **P* < .05 vs. *wt,* analysis of variance with Bonferroni's post test. *D,* Dermonecrosis. NS, not significant.

ure 4*E*). In this regard, differences in the nature of abscesses caused by MW2 and LAC were dramatic (figure 5). Taken together, these data suggest that strain background, rather than the presence of PVL, is the underlying basis for the type and severity of CA-MRSA infections.

***Virulence of isogenic* lukS/F-PV *knockout strains* (Δpvl) *of MW2 and LAC.*** Given that genetic makeup of each strain appeared to be crucial to differences in pathogenesis in the mouse infection models, we generated isogenic *lukS/F-PV* knockout strains (Δ*pvl*) in LAC and MW2 to directly test the role that PVL plays in disease (figure 1). Consistent with the data from the genetically similar strains (figure 2*B*), mouse survival curves for those infected with each *wt* and Δ*pvl* strain pair were strikingly similar (figure 5*A*). That comparable levels of fatal sepsis were caused by MW2 and LAC *wt* and isogenic Δ*pvl* strain pairs underscores the importance of virulence factors other than PVL in pathogenesis.

We next compared the ability of *wt* and Δ*pvl* strains to cause abscesses and tissue necrosis directly (figure 5*B*–5*D*). As with the data for PVL$^+$ and PVL$^-$ strain pairs (figure 4), abscess volumes in *wt*- and Δ*pvl*-infected mice were similar (figure 5*C*), if not slightly larger in the Δ*pvl*-infected mice ($P < .05$). Furthermore, numbers of mice with dermonecrosis and areas of abscesses with dermonecrosis were comparable in each group

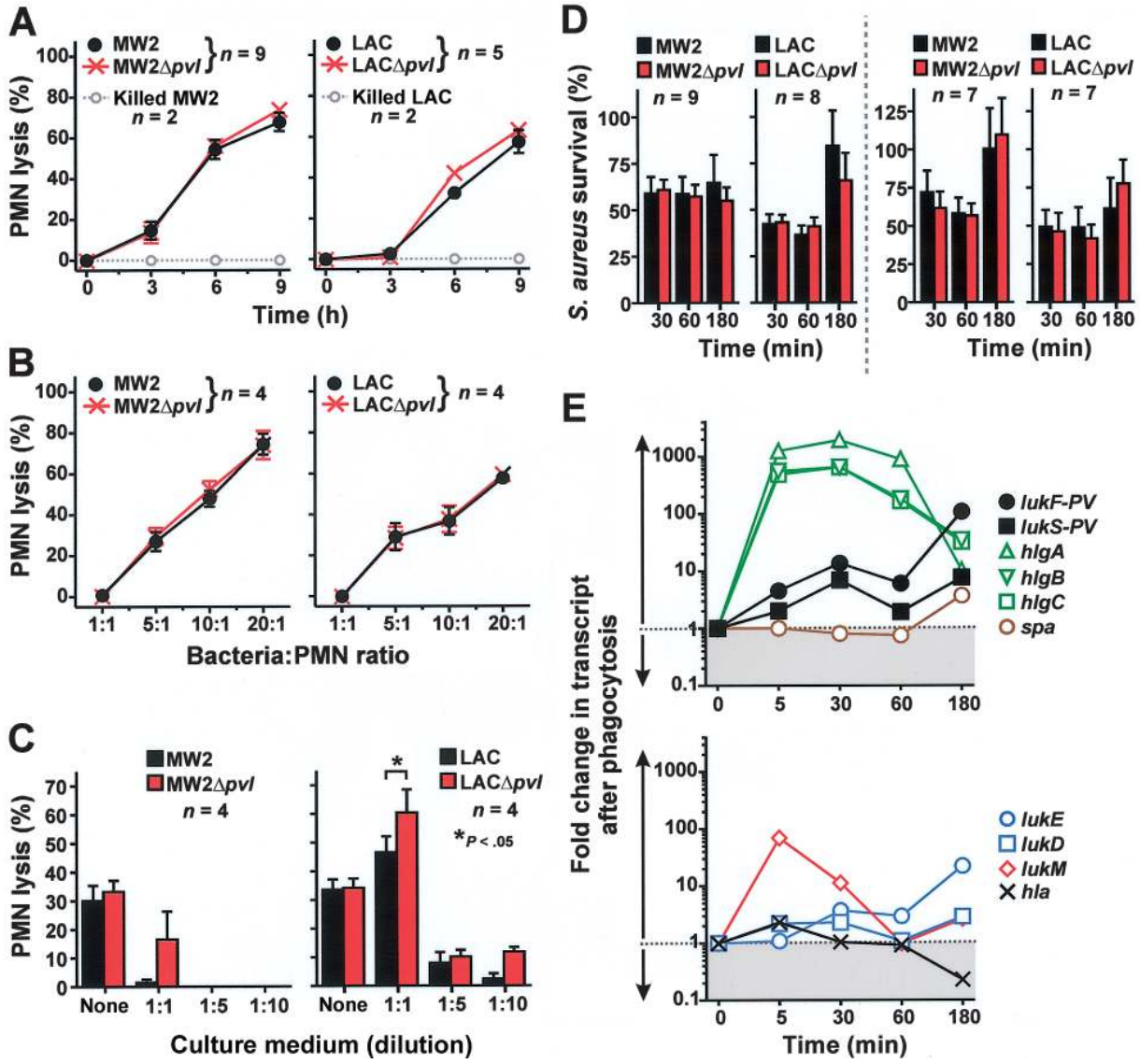


**Figure 6.** Polymorphonuclear leukocyte (PMN) lysis and bacterial survival after phagocytosis of wild-type *(wt)* and isogenic Δ*pvl* community-associated *Staphylococcus aureus* (CA-MRSA) strains. *A–C,* Neutrophil lysis. *A,* Time-dependent lysis with ~10 bacteria/neutrophil. *B,* Neutrophils cultured with varied nos. of bacteria for 6 h. *C,* Lysis of neutrophils with *S. aureus*–conditioned neutrophil culture medium (RPMI 1640 medium buffered with 10 mmol/L HEPES [RPMI/H]). PMNs were incubated for 3 h with conditioned RPMI/H. *$P < .05$ vs. *wt,* analysis of variance with Bonferroni's post test. *D,* Survival of *wt* and isogenic Δ*pvl* strains of MW2 and Los Angeles County clone (LAC) after phagocytosis by human PMNs. *Left,* Unopsonized bacteria; *right,* bacteria opsonized with human serum. For human neutrophil experiments, results are presented as the mean ± SE. *E,* TaqMan analysis of MW2 leukotoxins during phagocytosis by PMNs.

infected with *wt* and Δ*pvl* strain pairs (figure 5*D*). These observations are remarkable, given the accepted view that PVL is the underlying determinant of skin infections caused by CA-MRSA [7, 9, 12, 21].

***Destruction of human neutrophils by* Δpvl *strains of MW2 and LAC.*** PMNs are the key effector cells for defense against *S. aureus* infection. As such, lysis of human neutrophils by PVL has been proposed as a mechanism for the pathogenesis of CA-MRSA [19, 22]. To test the involvement of PVL in granulocyte destruction, we measured lysis of human PMNs in vitro after the phagocytosis of *wt* and isogenic Δ*pvl* strains of MW2 and LAC (figure 6*A* and 6*B*). In accordance with data from the mouse pathogenesis model, there was identical lysis of neutrophils by *wt* and isogenic Δ*pvl* strains (figure 6*A* and 6*B* and figure 7). Notably, PMN lysis was the same with *wt* and Δ*pvl* strains over time or with varied numbers of bacteria (figure 6*A* and 6*B*). These results were reflected by the ability of conditioned neutrophil assay medium (RPMI/H) from *wt* and isogenic Δ*pvl* strains to cause comparable neutrophil lysis (figure 6*C*). Conditioned neutrophil assay media from MW2 and LAC

The figure is available in its entirety in the online edition of the *Journal of Infectious Diseases*.

**Figure 7.** Ultrastructural analysis of human polymorphonuclear leukocyte lysis by wild-type *(wt)* and isogenic Δ*pvl* strains of Los Angeles County clone (LAC).

had varied capacities to cause host cell lysis, which provides support to the idea that factors other than PVL are key to the types of disease caused by each strain. This notion is expanded below (figure 8). Consistent with the neutrophil lysis data, there was similar survival of LAC and MW2 *wt* and isogenic Δ*pvl* strains after phagocytic interaction with human PMNs (figure 6*D*). The finding that *wt* and Δ*pvl* strains caused identical PMN lysis was not due to a lack of *lukS-PV* and *lukF-PV* transcripts under our assay conditions, given that there was rapid up-regulation of each within 5 min after exposure to human neutrophils (transcripts were up-regulated 2.0- and 4.6-fold for *lukS-PV* and *lukF-PV,* respectively) (figure 6*E*). Notably, genes encoding several other cytolytic toxins, including *hlgA, hlgB, hlgC, lukD,* and *lukE,* were up-regulated after phagocytosis by neutrophils (figure 6*E*).

Although the TaqMan data provide support to the idea that CA-MRSA produces PVL during phagocytosis, the data fail to demonstrate directly that the protein is made by MW2 or LAC *wt* strains under conditions that promote PMN lysis. To that end, we cultured each strain under conditions known to promote the synthesis of PVL and measured protein using immunoblot analysis (figure 8*A*). These results demonstrate that lukS-PV and lukF-PV are produced by each *wt* strain and are secreted into YCP culture medium. Because it is possible that slight differences in plasma membrane damage might not be revealed clearly by the PMN lysis assay, we evaluated the formation of neutrophil membrane pores at sublytic concentrations of culture medium using the method of Gauduchon et al. [19]. Consistent with the PMN lysis data (figure 6*A*–6*C*), the formation of pores in the plasma membrane of human neutrophils due to CA-MRSA culture medium was essentially identical between LAC and MW2 *wt* and isogenic Δ*pvl* strains (figure 8*B*). Moreover, damage to the plasma membrane was reflected by subsequent PMN lysis (figure 8*C*).

## DISCUSSION

The dramatic increase in the number of CA-MRSA infections over the past few years has triggered an intense search for the underlying factors. PVL emerged as the likely virulence determinant because of a strong association indicated by epidemiological studies [4, 5]. PVL is found in virtually all MRSA strains that cause CA infections—most commonly, pulsed-field types USA300 and USA400—but is almost never found in the prominent hospital-acquired MRSA that harbor SCC*mec* types I, II,

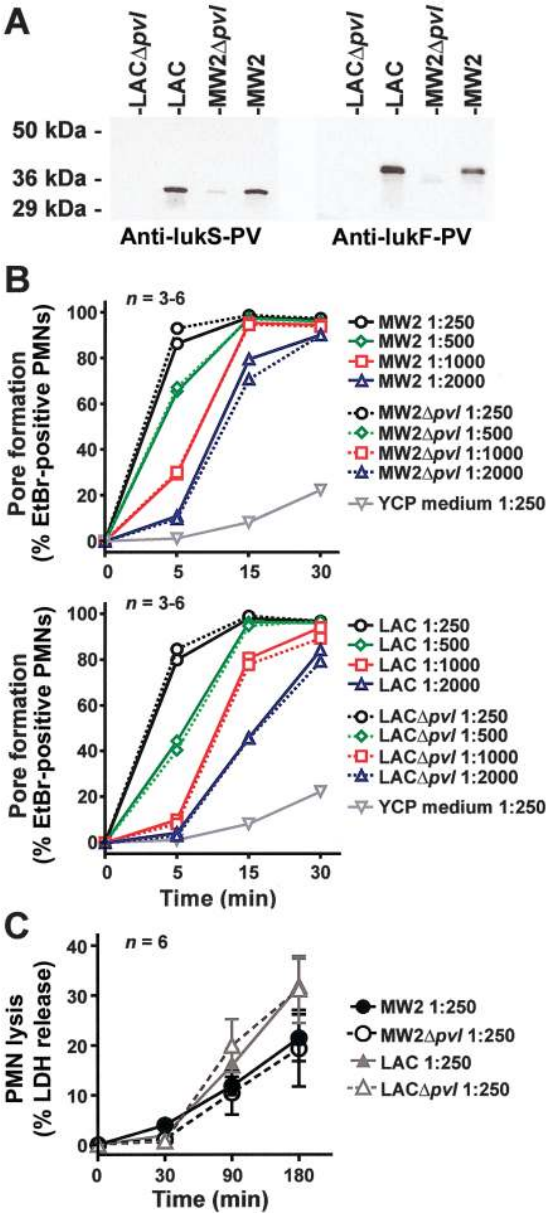


**Figure 8.** No relationship between the formation of membrane pores in human polymorphonuclear leukocytes (PMNs) and Panton-Valentine leukocidin (PVL). *A,* Immunoblot analysis of PVL produced by MW2 and Los Angeles County clone (LAC) wild-type strains. Strains were grown for 18 h in YCP medium as described in Materials and Methods, and the production of PVL in culture medium was determined using antibodies specific for lukS-PV and lukF-PV, as indicated. *B,* Formation of membrane pores in neutrophils was determined by uptake of ethidium bromide (EtBr), as described elsewhere [19]. Results are the mean of 3–6 experiments. Dilutions of YCP culture medium are indicated in the key. *C,* PMN lysis caused by MW2 or LAC culture medium. Release of lactate dehydrogenase (LDH) from human neutrophils was determined after incubation with a 1:250 dilution of 18 h YCP culture medium as indicated. Assays were performed with 2 sets of culture media generated on separate days. Results are the mean ± SE.

and III [5, 6, 23, 24]. The widespread increase in the severity of CA-MRSA disease correlates generally with infection by strains that contain PVL [3, 10, 11, 14]. As such, the toxin is strongly associated with necrotizing pneumonia and skin and soft-tissue infections, which account for the majority of CA-MRSA infections [7, 9, 11]. Notably, the ability of CA-MRSA strains to cause necrosis has been linked to the lysis of neutrophils, which are a primary target of PVL [8, 22]. Given that neutrophils are the main cellular defense against bacterial infections, the destruction of these host cells by CA-MRSA is likely a key component to disease. Recent studies by Voyich et al. [8] and Genestier et al. [22] have provided strong support to this idea. Genestier et al. further suggested that PVL is the primary cause of neutrophil destruction during human infections and thereby promotes disease such as necrotizing pneumonia [22]. Although evidence supporting the involvement of PVL in CA-MRSA infections has been circumstantial, the notion that PVL is critical for disease or is even the cause of these infections is widely accepted [4, 5, 9, 21, 25].

We evaluated virulence of the most prominent CA-MRSA strains and isogenic Δ*pvl* strains, to gain insight into the molecular basis of CA disease and to directly address the role that PVL plays in pathogenesis. The type and severity of disease caused by USA300 (ST8), USA400 (ST1), and USA1000 (ST59) varied significantly, regardless of PVL status. For instance, MW2 (USA400; ST1) was more virulent in the sepsis model than BK2394 (USA1000; ST59), whereas the latter caused far more dermonecrosis, and each is $PVL^+$ (compare figures 2*A* and 4*E*). The finding that USA1000 strains were highly virulent in the abscess model is consistent with the noted prevalence of these strains in skin infections among injection drug users [26]. There were also differences in virulence within pulsed-field types—for example, between BK648 and BK2394—in the sepsis model, and such variance was unrelated to presence or absence of PVL (figure 2*A*). The dramatic differences in disease noted in our studies indicates clearly that multiple factors in the overall genetic makeup of each strain, rather than any single determinant, such as PVL, promotes CA-MRSA infection. These observations may explain the results of Genestier et al. [22], who studied the involvement of PVL using a laboratory strain of *S. aureus* unrelated to CA-MRSA.

The present data, obtained using isogenic Δ*pvl* strains of USA300 and USA400, demonstrate directly that the toxin is not the major determinant of disease caused by these prominent CA-MRSA strains. These results were not unique to mouse models of disease, because we made parallel observations using human neutrophils (figures 6 and 8). Thus, although PVL is a useful marker for strains with the potential to cause severe *S. aureus* infections, its clinical significance has yet to be determined. It may be that PVL contributes to specific pathologic conditions such as necrotizing pneumonia, a disease not tested in our studies. Future efforts to understand the pathogenesis of CA-MRSA should focus on identifying the factors (other than PVL) directly responsible for the type and severity of disease caused by these emerging human pathogens.

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