

Differential Distribution and Expression of Pantan-Valentine Leucocidin among Community-Acquired Methicillin-Resistant *Staphylococcus aureus* Strains

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Community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) is an emerging threat world-wide. CA-MRSA strains differ from hospital-acquired MRSA strains in their antibiotic susceptibilities and genetic backgrounds. Using several genotyping methods, we clearly define CA-MRSA at the genetic level and demonstrate that the prototypic CA-MRSA strain, MW2, has spread as a homogeneous clonal strain family that is distinct from other CA-MRSA strains. The Pantan-Valentine leucocidin (PVL)-encoding genes, *lukF* and *lukS*, are prevalent among CA-MRSA strains and have previously been associated with CA-MRSA infections. To better elucidate the role of PVL in the pathogenesis of CA-MRSA, we first analyzed the distribution and expression of PVL among different CA-MRSA strains. Our data demonstrate that PVL genes are differentially distributed among CA-MRSA strains and, when they are present, are always transcribed, albeit with strain-to-strain variability of transcript levels. To directly test whether PVL is critical for the pathogenesis of CA-MRSA, we evaluated the lysis of human polymorphonuclear leukocytes (PMNs) during phagocytic interaction with PVL-positive and PVL-negative CA-MRSA strains. Unexpectedly, there was no correlation between PVL expression and PMN lysis, suggesting that additional virulence factors underlie leukotoxicity and, thus, the pathogenesis of CA-MRSA.

Staphylococcus aureus is an important human pathogen capable of causing diseases in the hospital and community settings (23). The increased incidence of multidrug-resistant *S. aureus* strains among nosocomial (or hospital-acquired [HA]) infections has added a challenging dimension to the *S. aureus* problem (5). These strains are typically labeled HA methicillin-resistant *Staphylococcus aureus* (MRSA) strains or simply MRSA strains. Several risk factors, such as recent hospitalization or exposure to a health care setting, residence in long-term-care facilities, invasive or surgical procedures, and injection drug use, predispose a patient to MRSA acquisition.

Methicillin resistance in *S. aureus* is conferred by the *mecA* gene, which encodes an altered penicillin binding protein (PBP 2') (22). The *mecA* gene is harbored in a large mobile genetic element (referred to as the staphylococcal chromosomal cassette *mec* [SCC*mec*]) that has a unique chromosomal integration locus (14). Sequence analyses defined three major SCC*mec* types (SCC*mec* types I, II, and III) among nosocomial MRSA strains. SCC*mec* types are distinguished on the basis of their sizes, which range from 26 and 67 kb, and genetic compositions, in which their genomes include recombinases and antibiotic resistance genes (16).

In the community, the majority of *S. aureus* infections, which include skin and soft tissue infections (6, 8, 13, 23), are caused by methicillin-susceptible *S. aureus* (MSSA) strains. However,

since 1991 there have been increasing reports of MRSA infections in the community and in patients with and without risk factors for MRSA infection (4, 5). These MRSA strains, commonly referred to as community-acquired MRSA (CA-MRSA) strains (7, 8, 11–13, 18, 20, 30), differ from nosocomial MRSA strains on the basis of their genetic backgrounds and antibiograms. CA-MRSA strains harbor the recently described SCC*mec* type IV element in their genomes. Compared with other SCC*mec* types, the SCC*mec* type IV element is distinguished by its small size, the presence of functional recombinases, and the absence of antibiotic resistance markers (25). This distinction is consistent with the observation that CA-MRSA strains are more susceptible to antibiotics than hospital-acquired MRSA strains. In fact, many CA-MRSA strains are highly susceptible and show resistance only to β -lactam antibiotics, an observation that clearly distinguishes them from the multidrug-resistant nosocomial MRSA strains.

The genome of prototypic CA-MRSA strain, MW2, was fully sequenced (1) and revealed the presence of the SCC*mec* type IV element described above (15). Additionally, the sequencing results for this strain revealed unique CA-MRSA-specific virulence factors. For example, MW2 harbors the Pantan-Valentine leucocidin (PVL), encoded by two contiguous and cotranscribed genes (*lukF* and *lukS*); staphylococcal enterotoxin H (*seh*); and staphylococcal enterotoxin C (*sec*). PVL, a bicomponent, secreted leucocidin found in <5% of *S. aureus* isolates (21), is cytotoxic to human and rabbit monocytes, macrophages, and human polymorphonuclear leukocytes (PMNs) (10). The protein forms nonspecific pores in

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leukocyte plasma membranes, which result in increased permeability and eventual host cell lysis (32). PVL has gained much attention as one of the key virulence determinants present in CA-MRSA strains. Lina and colleagues (21) found that 50 to 93% of the *S. aureus* strains causing primary skin infections produce PVL. Furthermore, a study by Gillet et al. (10) demonstrated a strong association between PVL and necrotizing pneumonia in healthy children and young adults. From these studies, the authors hypothesized that the propensity of many CA-MRSA strains to cause severe skin and soft tissue infections and, occasionally, necrotizing pneumonia is due to their ability to produce PVL (10, 21).

Previous studies have defined CA-MRSA strains as isolates recovered within 48 h after hospital admission and/or in patients without known risk factors for MRSA acquisition. Given the fluidity between community and health care settings, i.e., with hospital strains being transferred into the community and vice versa (29), we opted to utilize a genetic approach based on the presence of SCCmec type IV to define CA-MRSA. The presumed role of PVL in the pathogenesis of CA-MRSA led us to investigate the distribution of PVL among CA-MRSA strains in our collection and evaluate its expression. Furthermore, to gain insight into the role of PVL during infection, we assessed PMN lysis during phagocytic interaction with PVL-negative and PVL-positive *S. aureus* strains of closely related genetic backgrounds.

MATERIALS AND METHODS

Strain collection. One hundred twenty-one strains were grouped as CA-MRSA based on the presence of the SCCmec type IV element in their genomes. Eighty-three strains were sent to our laboratory by clinical institutions for genotyping. These strains were suspected of being CA-MRSA based on their reduced drug resistance profile; and SCCmec typing, indeed, classified them as SCCmec type IV (see below). These strains were susceptible to two or all three of the following antibiotics: fluoroquinolones, clindamycin, and erythromycin. As certain genetic backgrounds based on *spa* type (*spa* types 1, 7, and 17) appear to be prevalent among CA-MRSA strains, we selected from our database additional strains with these genotypes for SCCmec typing. Strains that were classified as SCCmec type IV were included in this study. In a previous study, we used several genotyping techniques, including a side-by-side pulsed-field gel electrophoresis (PFGE) comparison, to demonstrate that an MSSA strain known as MnCop was the parental strain of MW2 (8). The *spa* type 35 of MnCop is closely related to MW2 *spa* type 131, as they both share the same multilocus sequence typing (MLST) profile (data not shown). For this reason, we included several MSSA strains from our collection of *spa* type 35 strains in this study.

SCCmec typing. Multiplex PCR analysis was performed as described previously (26) to distinguish the four genetic elements for SCCmec, with one modification; that is, the *pls* gene was amplified by using the following primers from the sequence with GenBank accession number AF115379: primer PlsF (GGGG TGGTTAATGGTATGAATAAA) and primer PlsR (CGGAATGTTGCTCTT GGTGTGCGTTTTC).

***spa* typing.** The method of *spa* typing was developed in our laboratory, and its accuracy and discriminatory power for determination of the subspecies of *S. aureus* strains is superior to that of MLST analysis (19, 27). *Spa* typing is a DNA sequencing-based method that distinguishes strains based upon the makeup of the variable number of tandem repeats in the 3' region of the protein A gene, which is both unique and conserved among *S. aureus* isolates. Currently, the Public Health Research Institute database contains 625 different *spa* types among a collection of over 2,500 *S. aureus* isolates.

PFGE. PFGE was performed and the results were interpreted as described previously (33). Briefly, the organisms were embedded in agarose, and the intact chromosome was digested with SmaI. DNA fragments were resolved for 22 h with a Bio-Rad CHEF DR-II PFGE unit (Bio-Rad, Hercules, CA).

***agr* typing.** the *S. aureus* strains were analyzed for their *agr* types by the method of Jarraud et al. (17). The *agr* types were identified by PCR amplification of the

hypervariable domain of the *agr* locus by using oligonucleotide primers specific for each of the four major *agr* types, as described by Shoppin et al. (31).

Detection of PVL genes. The presence of genes encoding PVL was determined by Southern blot analysis, as described below, and by PCR with the following primers: primer LukS-PV (GGCCTTTCACATACAATATTGG) and primer LukF-PV (CCCAATCAACTTCATAAATTG). Thermal cycling was performed in a GeneAmp 9600 instrument (Perkin-Elmer Corporation, Applied Biosystems, Foster City, CA); and the parameters consisted of initial heating at 95°C for 5 min, followed by 35 cycles of denaturation (1 min at 94°C), annealing (30 s at 57°C), and extension (1 min at 72°C).

The presence of PVL was validated by molecular beacon analysis with the *lukF* component of *pvl*. The beacon experiment was carried out by using the following beacon and primers: primer *lukF* beacon [5'-6-FAM d(CGCGAAGAATTTAT TGGTGTCTATCTCGATCGCG)-DABCYL-3', where FAM is 6-carboxy-fluorescein and DABCYL is 4-(4'-dimethylaminophenylazo)benzoic acid], primer Luk F (5'-GCCAGTGTATCCAGAGG-3'), and primer Luk R (CT ATCCAGTTGAAGTTGATCC-3').

The quantitative real-time PCR mixture contained 1× I.Q. supermix (Bio-Rad), 0.1 μM of each molecular beacon, 0.5 μM of each primer, and DNA template. The thermal cycling program consisted of 10 min on a spectrofluorometric thermal cycler (iCycler; Bio-Rad) at 95°C, followed by 45 cycles of 30 s at 95°C, 30 s at 50°C, and 30 s at 72°C.

Southern blot analysis. Chromosomal DNA was digested with the ClaI restriction enzyme, and Southern blot hybridization was performed as described elsewhere (28).

RNA isolation. *S. aureus* strains were cultured to postexponential phase, and RNA isolation and detection were performed as described previously (28).

Reverse transcription-PCR with *lukF* beacon. The first-strand cDNA for the *lukF* gene was synthesized by using a Omniscript RT PCR kit (QIAGEN) with the *lukF* reverse primer described above. Samples incubated in the absence of reverse transcriptase served as controls. Quantitative real-time PCR was performed as described above. A molecular beacon probe for a *Staphylococcus* genome-specific region of the 16S rRNA gene (SG16S) was used as a control. The sequences of the SG16S forward and reverse primers, as well as the molecular beacon sequence, are as follows: primer SG16S-F, 5'-TGGAGCATGTGG TTTAATTCGA-3'; primer SG16S-R, 5'-TGCGGGACTTAACCCAACA-3'; and probe SG16S-MB, 5'-[HEX]-CGCTGACTTACCAAATCTTGACATCCT TCAGCG-[DABCYL]-3', where HEX is hexachlorofluorescein.

Standard curves were generated to calculate the copy numbers of each gene in the reaction. Briefly, this was accomplished by taking the cycle threshold (C_T) value for each sample and applying the following formula:

$$\text{copy number} = 10^{[(C_T - y \text{ intercept})/\text{slope}]}$$

where the slope and y-intercept values were calculated from the standard curve by using Stratagene Mx4000 software (Stratagene Corporation, La Jolla, CA). Each sample was assayed in triplicate, and the average number of *pvl* copies was divided by the average number of SG16S copies to obtain a normalization value. The average standard deviation in the cycle thresholds for both *pvl* and SG16S probes was less than 1 cycle.

Isolation of human PMNs and assay for PMN lysis (release of lactate dehydrogenase [LDH]). Human PMNs were isolated from the venous blood of healthy individuals, in accordance with a protocol approved by the Institutional Review Board for Human Subjects, National Institute of Allergy and Infectious Diseases. Human PMNs were purified by the method described by Boyum (3), but with modifications. Briefly, whole human blood was mixed 1:1 with 0.9% NaCl (Irrigation USP; Abbott Laboratories, North Chicago, Ill.) containing 3% dextran 500 (Amersham Biosciences, Piscataway, NJ) for 20 min to sediment the erythrocytes. The leukocyte-rich supernatant was transferred to a new tube and was centrifuged at $\sim 670 \times g$ for 10 min. The cells were resuspended in 35 ml 0.9% NaCl, and the suspension was underlaid with 10 ml Hypaque-Ficoll-Paque Plus (1.077 g/liter; Amersham). The cells were centrifuged at $350 \times g$ for 25 min at room temperature to separate the peripheral blood mononuclear cells (PBMCs) from the PMNs and erythrocytes. The PBMC layer was removed by aspiration, and the cell pellet containing the PMNs and erythrocytes was resuspended in water (Irrigation USP; Abbott Laboratories) for 15 to 30 s. Isotonicity was restored by adding an equal volume of 1.7% NaCl. PMNs were centrifuged again, resuspended in RPMI 1640 medium (Invitrogen Corporation, Carlsbad, CA) buffered with 10 mM HEPES (RPMI/H), and enumerated with a hemacytometer. The purities of the PMN preparations and cell viability were routinely assessed by flow cytometry (FACSCalibur; BD Biosciences, San Jose, CA). Cell preparations contained 98 to 99% PMNs, and all reagents used contained <25 pg/ml endotoxin.

Strains of *S. aureus* were cultured to mid-exponential phase of growth (optical

density at 600 nm = 0.75) and resuspended in RPMI/H. Bacteria (10^7) were combined on ice with human PMNs (10^6), and phagocytosis was synchronized by centrifugation at $350 \times g$ for 8 min at 4°C . The culture plates were incubated at 37°C with 5% CO_2 for the indicated times, and the release of cytosolic PMN LDH (cell lysis) was evaluated with a cytotoxicity detection kit (Roche Applied Sciences, Indianapolis, IN), according to the manufacturer's instructions. The assay is based on the conversion of lactate to pyruvate by LDH, whereby NAD^+ is reduced to NADH/H^+ . Diaphorase utilizes H/H^+ from NADH/H^+ to reduce 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride to formazan. Colorimetric detection of LDH activity was performed with triplicate wells by using a SpectraMax Plus384 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA) at 490 nm and a reference λ of 600 nm. The percent cell lysis induced by *S. aureus* was determined with the measured absorbance readings in the equation $\{[(\text{absorbance for } S. \text{ aureus and PMN mixture} - \text{absorbance for } S. \text{ aureus alone}) - \text{absorbance for time-matched, untreated human PMNs (spontaneous release of LDH)}]/[\text{absorbance for PMNs treated with 2\% Triton X-100 (maximum releasable LDH activity)} - \text{absorbance for time-matched, untreated human PMNs (spontaneous release of LDH)}]\} \times 100$.

RESULTS

Molecular characterization of CA-MRSA strains. (i) CA-MRSA *spa* types. One hundred twenty-one geographically diverse isolates in our strain collection (clustered based on *SCCmec* type IV) were genotyped by sequencing the polymorphic repeat region of the gene encoding protein A (*spa*) and by PFGE. From this analysis the CA-MRSA strains were divided into five major groups defined by their *spa* types (Table 1). The first group consisted of strains with the same *spa* type as prototypic CA-MRSA strain MW2 (*spa* type 131; MLST type 1-1-1-1-1-1). The second group included strains with *spa* type 1 isolated from human immunodeficiency virus (HIV)-positive as well as HIV-negative patients in Los Angeles and New York City. Group 3 consisted of strains of *spa* type 7. We note that these *spa* types differ by a single nucleotide and that their MLST types differ by a single allele; *spa* type 1 is MLST 3-3-1-1-4-4, and *spa* type 7 is MLST 3-3-1-1-4-16. The fourth group consisted of *spa* type 17 strains, and the fifth group had various *spa* types and is listed as miscellaneous (Table 1). These results suggest that although CA-MRSA strains share certain molecular characteristics, they are not a single clonal type but, rather, are derived from a number of genetic backgrounds.

(ii) CA-MRSA PFGE. Using a more discriminatory genotyping tool, PFGE, we were able to differentiate CA-MRSA strains of the same *spa* type. PFGE patterns were indistinguishable among *spa* type 131 isolates, indicating that *spa* type 131 strains define a clone (Fig. 1). The data for *spa* type 35 strains indicate that these *spa* types are closely related to each other (at least at the genetic level) and to *spa* type 131 strains. Furthermore, among the miscellaneous *spa* types, *spa* type 194 had a *spa* repeat pattern similar to that of *spa* type 131 (Table 1). Of interest, three *spa* type 194 strains in our collection were isolated from the Midwest (United States), as was our *spa* type 131 reference strain, MW2. These strains have subtle PFGE pattern differences and are thus closely related to each other and to *spa* type 131 (Fig. 1). On the other hand, strains of *spa* types 1, 7, and 17 had significant PFGE pattern differences and deviated significantly from *spa* type 131 strains. These data indicate that although these strains share the same *SCCmec* type, they are not clonal (Fig. 1) and their genetic background is quite distinct from that of the MW2 clone.

TABLE 1. Representatives of the CA-MRSA and parental MSSA strains used in this study^a

BK no.	SCCmec type	<i>spa</i> type	Location	PVL	<i>agr</i> type
10474	IV	1	New Jersey	Present	I
11490	IV	1	California	Present	I
11540	IV	1	New Jersey	Absent	I
11554	IV	1	New Jersey	Present	I
11588	IV	1	New York	Absent	I
11613	IV	1	New Jersey	Present	I
11627	IV	1	California	Present	I
11634	IV	1	New Jersey	Present	I
11628	IV	1	New York	Present	I
2370	IV	7	New York	Absent	I
2395	IV	7	New York	Absent	I
2533	IV	7	New York	Absent	I
6642	IV	7	Washington	Absent	I
8587	IV	7	Ontario, Canada	Absent	I
10654	IV	7	New York	Absent	I
11108	IV	7	New Jersey	Absent	I
11235	IV	7	Philadelphia	Absent	I
645	II	17	Georgia	Present	I
648	IV	17	Georgia	Absent	I
2394	IV	17	New York	Present	I
2402	IV	17	New York	Absent	I
5877	IV	17	California	Absent	I
6909	IV	17	Washington	Present	I
11495	IV	17	New York	Present	I
11558	IV	17	New Jersey	Present	I
6258	IV	131	Nebraska	Present	III
6614	IV	131	Washington	Present	III
9897	IV	131	North Dakota	Present	III
10370	IV	131	New Jersey	Present	III
10398	IV	131	New Jersey	Present	I/III ^b
11118	IV	131	New York	Present	III
11514	IV	131	New York	Present	III
11587	IV	131	New York	Present	III
11632	IV	131	New York	Present	III
3293	MSSA	35	United Kingdom	Absent	III
3485	MSSA	35	Texas	Present	III
3514	MSSA	35	Texas	Absent	III
3536	MSSA	35	Texas	Absent	III
4263	MSSA	35	New York	Present	III
6652	MSSA	35	Washington	Absent	III
6653	MSSA	35	Washington	Absent	III
6789	MSSA	35	Ontario	Absent	III
10254	MSSA	35	Nebraska	Present	III
9896	IV	194	Nebraska	Present	III
9918	IV	194	Nebraska	Present	III
10488	IV	267	New Jersey	Present	I
11633	IV	137	New York	Present	I
11635	IV	212	New Jersey	Absent	I
11636	IV	193	New Jersey	Absent	I

^a The genetic backgrounds, as defined by *spa* type and *agr* type, are listed along with the presence or absence of PVL. The following are the denominators for the different *spa* types represented in this table: *spa* type 1, 9 of 35; *spa* type 7, 8 of 26; *spa* type 17, 7 of 11; *spa* type 131, 9 of 25; *spa* 35, 9 of 13; and miscellaneous *spa* types, 6 of 16.

^b Based on the PCR results, BK 10398 was listed as both *agr* I and *agr* III, and for this reason a specific *agr* type could not be assigned to this strain.

(iii) CA-MRSA *agr* types. Four major *agr* types (*agr* types I, II, III, and IV) have been identified among *S. aureus* strains. Two recent studies demonstrated that CA-MRSA strains fall into the *agr* III group (24, 34). To further determine the genetic backgrounds of our CA-MRSA isolates, we assessed their

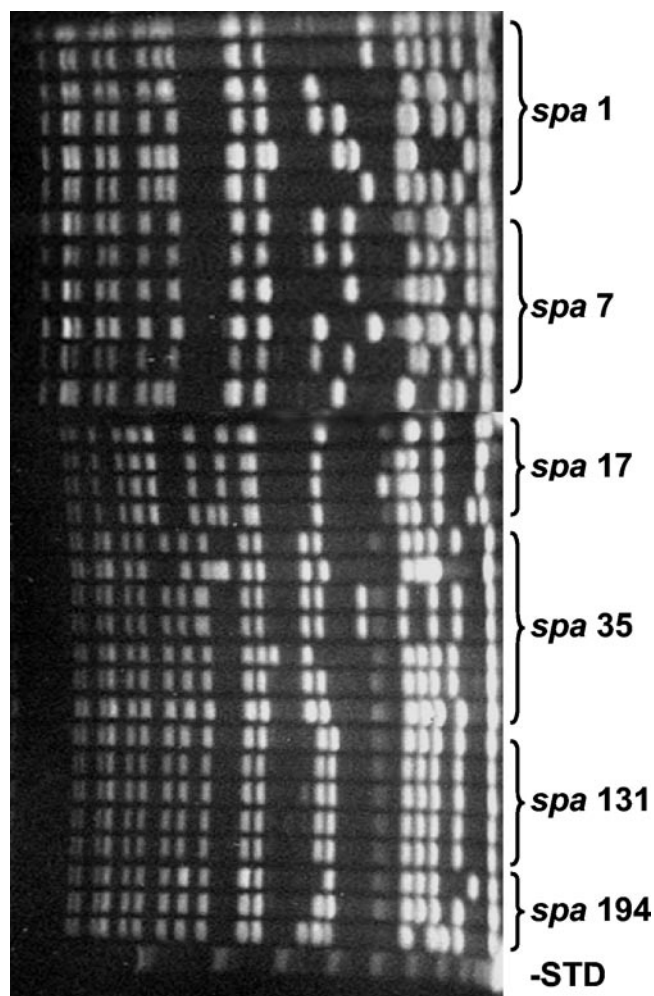


FIG. 1. PFGE of strains with different *spa* types. STD, molecular size standard.

agr types by PCR, as described previously (31). Consistent with the findings of previous studies (30, 31), CA-MRSA strains of the MW2 clone (*spa* type 131) and those of *spa* types 35 and 194 belong to *agr* type III. Thus, *agr* type III appears to be linked to strains genetically related to MW2. Interestingly, all of the other strains of the different *spa* types were *agr* type I. Taken together, our data from the different genotyping methods (*spa*, PFGE, and *agr* typing) demonstrate that CA-MRSA has multiple genetic backgrounds and that the MW2 clone is genetically distinct from the other CA-MRSA strains.

Distribution and expression of PVL in CA-MRSA. Previous studies suggest that PVL is a significant virulence determinant in the pathogenesis of CA-MRSA. Therefore, we analyzed the distribution of PVL among the different CA-MRSA strains using PCR coupled with Southern blot analysis and a *lukF*-specific beacon. We used additional techniques to validate the PCR results because the genes encoding PVL share high homologies to other leucocidins, such as γ -hemolysin, and in some instances, the primers can amplify these homologous genes. Our results indicate that the PVL genes are present among all *spa* type 131 isolates (MW2 clone), an observation that supports the idea that these strains are clonal (Table 1).

Furthermore, all *spa* type 194 strains analyzed possessed the PVL genes. On the other hand, none of the *spa* type 7 strains tested contained the genes encoding PVL. There was a differential distribution of PVL in *spa* types 1, 17, and 35; and only a subset of these strains contained the gene. Although not all CA-MRSA strains possessed PVL, we observed a significant increase in the number of PVL-positive strains (33 to 50%) among the CA-MRSA strains tested compared to that reported in the literature for other *S. aureus* strains (<5%) (21). This finding is in agreement with that of a recent study that demonstrated the increased prevalence of PVL among CA-MRSA strains compared to that among HA-MRSA strains (24).

Although a number of investigations have demonstrated an association of the PVL genes and CA-MRSA, their mRNA levels in different genetic backgrounds have not been analyzed. We first confirmed that CA-MRSA isolates containing the *lukF* and *lukS* genes expressed the corresponding messages. PVL transcripts were identified by real-time reverse transcription-PCR coupled with the use of a molecular beacon specific for *lukF*. CA-MRSA BK 9924, which lacks PVL genes, was used as a negative control. Notably, the PVL transcript was expressed in all strains harboring the PVL genes (Fig. 2). However, the levels of expression of genes encoding PVL varied from strain to strain, with isolate 9918 expressing nearly 10-fold more of the PVL message than any other isolate.

PVL- and CA-MRSA-induced PMN lysis. Previous investigators have suggested that PVL facilitates the pathogenesis of *S. aureus*, presumably by altering human leukocyte responses to infection (10, 32). To assess the impact of PVL in the pathogenesis of CA-MRSA, we determined the cytotoxicity (lysis) for human PMNs during phagocytic interaction with PVL-positive and PVL-negative CA-MRSA strains (Fig. 3A). Each pair of PVL-positive and PVL-negative isolates comprised strains of closely related genetic backgrounds (Fig. 3B). Unexpectedly, there was no significant correlation between strains that expressed PVL and human PMN cytotoxicity. For example, in some cases the PVL-negative strain caused higher levels of PMN lysis compared to the levels of lysis caused by the genetically matched PVL-positive strain. Finally, in the *spa* type 35 genetic background, the cytotoxicity for PMNs was similar for both PVL-negative and PVL-positive strains (Fig. 3A). Although there is a higher incidence of PVL among CA-MRSA strains, our in vitro data suggest that other factors produced by CA-MRSA strains facilitate leukocidal activity.

DISCUSSION

CA-MRSA is an emerging pathogen that can cause severe and, in some cases, fatal infections in the community. CA-MRSA strains share certain properties such as the presence of a genomic *SCCmec* type IV element and increased susceptibilities to a variety of antibiotics (compared to those of HA-MRSA strains). We and others demonstrated that CA-MRSA strains have multiple genetic backgrounds, and we have extended this analysis in this study to show how the clonal MW2 CA-MRSA strains are differentiated from other strain families. This is particularly important to understanding of the relationship between genetic backgrounds and CA-MRSA disease.

The *agr* data indicate that CA-MRSA can be subdivided into

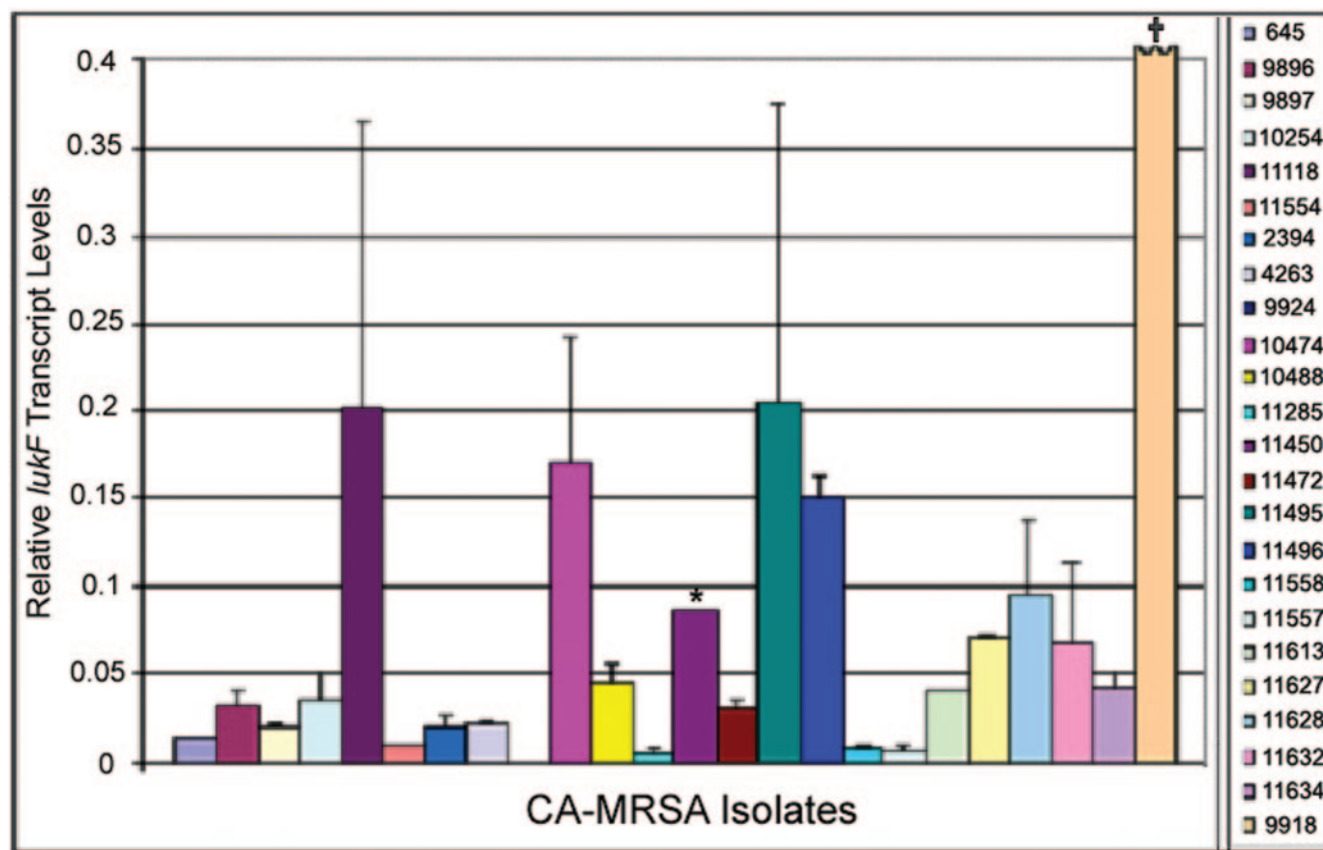


FIG. 2. Differential expression of the *lukF* gene of Pantone-Valentine leucocidin. The relative levels of *lukF* transcripts in CA-MRSA strains were determined by real-time PCR with a molecular beacon probe specific for the *lukF* gene. A molecular beacon directed against a *Staphylococcus*-specific region of 16S rRNA was used for normalization. †, Strain 9918 had greater than fivefold higher relative *lukF* transcript levels (1.29) than any of the other strains tested; *, the standard error for strain 11450 could not be reliably calculated due to the presence of an outlier.

two groups. The first group consists of the “true” CA-MRSA strains, i.e., MW2 and related strains (*spa* types 131 and 194). These strains tend to be susceptible to a wide array of non-beta-lactam antibiotics and have the *agr* III subtype (Table 1). The prevalence of *agr* type III among CA-MRSA strains has been reported previously (24, 34), and our results are in agreement with the findings from those studies. The second family consists of strains of *spa* types 1, 7, 17, and other miscellaneous *spa* types. In addition to the beta-lactams, this group is also resistant to erythromycin and, sometimes, fluoroquinolones. Unlike the MW2 clone, the second group belongs primarily to the *agr* I subtype. The grouping of CA-MRSA strains into two *agr* subgroups is in agreement with our previous study that classified CA-MRSA strains into two epidemiological groups, i.e., CA-MRSA strains with and without risk factors (29). Interestingly, CA-MRSA strains without risk factors correspond to the *agr* type III subgroup, and CA-MRSA strains with risk factors correspond to the *agr* type I subgroup.

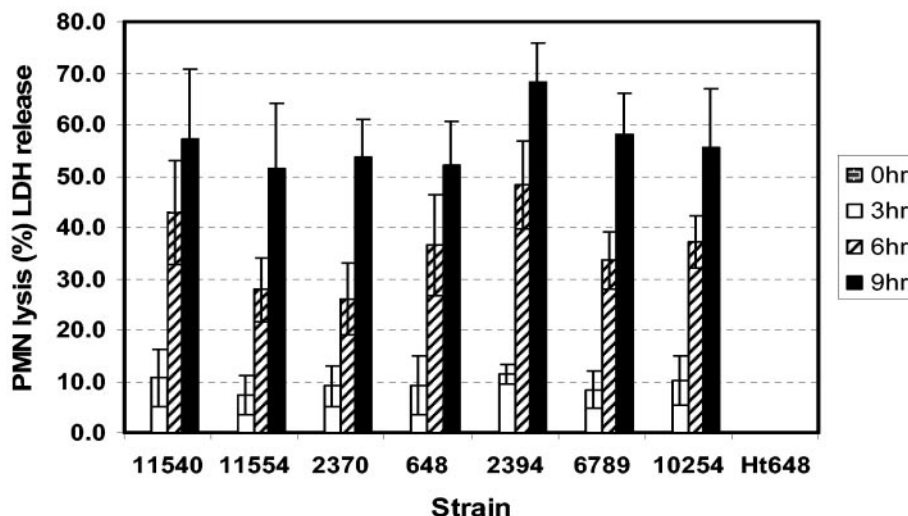
Although the genes encoding PVL are rarely present in *S. aureus* strains (<5%) (21), they are highly represented among CA-MRSA strains. All *spa* type 131 and 194 strains tested contain *lukF* and *lukS*, consistent with their clonality, as determined by PFGE analysis (Fig. 1). On the other hand, only a subset of the other *spa* types has these genes, again, in concordance with their heterogeneous pulsotypes, as determined

by PFGE. These results are in contrast to those of a recent study that reported on the presence of PVL in all CA-MRSA strains analyzed (34). Although the study by Vandenesch and colleagues (34) analyzed a collection of diverse CA-MRSA strains, they can be categorized as CA-MRSA strains without risk factors (34). In addition, 97% of the isolates tested were of *agr* type III. The discrepancy between the two studies may be explained by the difference in the two collections. For instance, the collection used by Vandenesch and colleagues (34) would exclude the *agr* type I CA-MRSA strains previously described in the Los Angeles population of men who have sex with men (6). As our collection is more inclusive (based on *agr* types) and consists of CA-MRSA strains both with and without risk factors, we report an unequal distribution of the genes encoding PVL among our strains.

PVL has been demonstrated to be cytotoxic to human PMNs, which are essential for the innate host defense against invading microorganisms (23, 32). Therefore, we used PMN lysis as a proxy for CA-MRSA virulence and pathogenesis. However, our results demonstrate that the presence (or absence) of PVL failed to correlate with the degree of PMN lysis, suggesting that additional factors are involved in leukotoxicity and the pathogenesis of CA-MRSA.

The conflicting results of the role of PVL in the pathogenesis of CA-MRSA are similar to the results reported on the role of

A.



B.

BK #	Mec type	Spa	Comments	PVL genes	PVL expression	% PMN lysis (9h)
11540	IV	1	New Jersey	NEG	NEG	57.3
11554	IV	1	New Jersey	POS	POS	51.3
2370	IV	7	New York	NEG	NEG	53.7
648	IV	17	Georgia	NEG	NEG	51.7
2394	IV	17	New York	POS	POS	68.2
6789	MSSA	35	Toronto, ON	NEG	NEG	58.2
10254	MSSA	35	Nebraska	POS	POS	55.6

FIG. 3. PMN lysis during phagocytic interaction with PVL-negative (NEG) and PVL-positive (POS) *S. aureus* strains of closely related genetic backgrounds. (A) Percentage of PMN lysis over a period of 9 h. Ht648, heat-killed strain 648. (B) Genetic characteristics of the pairs used in the PMN assay.

the *Salmonella* plasmid virulence (*spv*) genes (9). One study demonstrated that *spv* genes are important virulence determinants, as an *spvR* mutant of *Salmonella enterica* serovar Dublin was attenuated in both enteric and systemic diseases. Conversely, another study reported that an *spvR* mutant of *S. enterica* serovar Typhimurium causes lethal enteric infection similar to that caused by the wild-type strain. Taken together, these results reiterate the multifactorial nature of bacterial pathogenesis. To better elucidate the role of PVL in the pathogenesis of CA-MRSA, comparative analyses with isogenic strains (PVL positive and negative) both in vitro and in vivo are needed. Deciphering of the pathogenesis of CA-MRSA strains will require multipronged strategies aimed at both the microbial level and the host level (2).

In summary, this study demonstrates that prototypic CA-MRSA strain MW2 and its related strains form a subclone different from other CA-MRSA strains at the genotypic and phenotypic levels. The presence and expression of the PVL-encoding genes are not uniform among CA-MRSA strains. Finally, these data do not support a clear correlation between

the presence of PVL and the ability of the strain to cause PMN cytotoxicity.

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