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USA300 and USA500 Clonal Lineages of *Staphylococcus aureus* Do Not Produce a Capsular Polysaccharide Due to Conserved Mutations in the *cap5* Locus

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**ABSTRACT**

The surface capsular polysaccharide (CP) is a virulence factor that has been used as an antigen in several successful vaccines against bacterial pathogens. A vaccine has not yet been licensed against *Staphylococcus aureus*, although two multicomponent vaccines that contain CP antigens are in clinical trials. In this study, we evaluated CP production in USA300 methicillin-resistant *S. aureus* (MRSA) isolates that have become the predominant community-associated MRSA clones in the United States. We found that all 167 USA300 MRSA and 50 USA300 methicillin-susceptible *S. aureus* (MSSA) isolates were CP negative (CP−). Moreover, all 16 USA300 isolates, which have been postulated to be the progenitor lineage of USA300, were also CP−. Whole-genome sequence analysis of 146 CP− USA300 MRSA isolates revealed they all carry a *cap5* locus with 4 conserved mutations compared with strain Newman. Genetic complementation experiments revealed that three of these mutations (in the *cap5* promoter, *cap5D* nucleotide 994, and *cap5E* nucleotide 223) ablated CP production in USA300 and that Cap5E75 Asp, located in the coenzyme-binding domain, is essential for capsule production. All but three USA300 MSSA isolates had the same four *cap5* mutations found in USA300 MRSA isolates. Most isolates with a USA500 pulsotype carried three of these four USA300-specific mutations, suggesting the fourth mutation occurred in the USA300 lineage. Phylogenetic analysis of the *cap* loci of our USA300 isolates as well as publicly available genomes from 41 other sequence types revealed that the USA300-specific *cap5* mutations arose sequentially in *S. aureus* in a common ancestor of USA300 and USA500 isolates.

**IMPORTANCE**

The USA300 MRSA clone emerged as a community-associated pathogen in the United States nearly 20 years ago. Since then, it has rapidly disseminated and now causes health care-associated infections. This study shows that the CP-negative (CP−) phenotype has persisted among USA300 isolates and is a universal and characteristic trait of this highly successful MRSA lineage. It is important to note that a vaccine consisting solely of CP antigens would not likely demonstrate high efficacy in the U.S. population, where about half of MRSA isolates comprise USA300. Moreover, conversion of a USA300 strain to a CP-positive (CP+) phenotype is unlikely *in vivo* or *in vitro* since it would require the reversion of 3 mutations. We have also established that USA300 MSSA isolates and USA500 isolates are CP− and provide new insight into the evolution of the USA300 and USA500 lineages.

*S. aureus* is an important pathogen that causes a wide range of infections in health care and community settings. Methicillin-resistant *S. aureus* (MRSA) isolates in particular, which have become increasingly prevalent in the last decade, are resistant to nearly all β-lactams and are often multiply resistant to several classes of antibiotics. A vaccine that could protect against *S. aureus* infection would be important for public health, although development of an effective vaccine has remained elusive (1, 2).

Capsular polysaccharides (CPs) envelope the surface of many bacterial pathogens and have been the primary or sole protective antigen used in vaccines that are effective against certain serotypes of *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Neisseria meningitidis* (serotypes A, CW, and Y) (3). Among encapsu-
lated *S. aureus* isolates, serotypes 5 and 8 prevail (4–9). Capsular polysaccharides 5 (CP5) and 8 (CP8) have similar trisaccharide repeating units but differ in the linkages between the sugars and the sites of O-acetylation of the monosaccharide residues (10). Previous reports have indicated that ~20% of *S. aureus* isolates fail to produce CP5 or CP8 (4–9).

The *S. aureus* CP protects the bacterium from host phagocytes (2); however, this protection can be overcome by CP-specific antibodies that enhance opsonophagocytic killing by human neutrophils (10, 11). Vaccines that contained CP5 and CP8 antigens conjugated to *Pseudomonas aeruginosa* exotoxoid A were tested for efficacy in patients with end-stage renal disease. In phase III clinical trials, these CP-based vaccines failed to protect against *S. aureus* bacteremia (12–14).

In the United States, the community-associated (CA)-MRSA clonal type USA300 has been the most frequently isolated *S. aureus* genotype from all body sites, including blood, surpassing the USA100 health care-associated MRSA strain type as a cause of nosocomial infection in some locales (15). USA300 MRSA strains are most often associated with CA skin and soft tissue infection (SSTI) (16) and belong to multilocus sequence type 8 (ST8). USA300 MRSA isolates characteristically contain the SCCmec type IV element (17), a phage carrying the genes encoding Panton-Valentine leukocidin (PVL) (18) and the arginine catabolic mobile genetic element (ACME) carrying the arginine deiminase cluster and the gene encoding the spermidine resistance factor, SpeG, which promotes skin colonization (19–21). Although they usually lack SCCmec IV, ACME, and PVL, USA500 MRSA isolates are the closest relative of USA300 among the members of the ST8 lineage and have been postulated to be the progenitor of USA300 (21).

We previously demonstrated that neither CP5 nor CP8 was produced by several USA300 MRSA clinical isolates obtained in 2004 and 2005 from our center in Chicago, IL (22). A subsequent study of isolates obtained during the same period (2004 to 2005) from Washington, DC, also reported CP-negative (CP-) USA300 MRSA strains (23). These studies suggested that the failure to produce a CP was a common trait among USA300 MRSA isolates circulating in 2004 through 2005, but this was not investigated subsequently.

A decade has passed since the CP- USA300 MRSA isolates were described. Since then, USA300 MRSA strains have widely disseminated and increased in prevalence in the United States (15). It is not known if there are CP5-positive (CP5+) USA300 variants in circulation or if the CP- USA300 MRSA isolates identified in 2004 have become the sole or dominant USA300 clone.

Because CP5 and CP8 are components in two *S. aureus* vaccines under development (1, 24, 25), the aim of this study was to determine if currently circulating USA300 MRSA isolates produce a CP. Further, we evaluated whether the CP- phenotype was shared by USA300 methicillin-susceptible *S. aureus* (MSSA) isolates and the closely related USA500 lineage (21, 26). Since we found that all USA300 and USA500 isolates were uniformly CP-, we analyzed the evolutionary relationship between the *cap5* mutations in USA300 and USA500 isolates.

(This work was presented in part at the International Symposium on Staphylococci and Staphylococcal Infections, Chicago, IL, 2014.)

### RESULTS

**CP serotyping.** We chose for CP serotyping 233 clinical *S. aureus* isolates from our collection that were identified as either USA300 MRSA, USA300 MSSA, or USA500. Isolates were obtained from either colonized or infected body sites from subjects in Chicago, IL, Los Angeles, CA, San Francisco, CA, or Connecticut between 1995 and 2011, as described in Table 1. Clonal types consisted of (i) 167 USA300 MRSA isolates, (ii) 50 USA300 MSSA isolates, (iii) 14 USA500 MRSA isolates, and (iv) 2 USA500 MSSA isolates. The USA300 sample included 16 ACME *arcA*-negative USA300 MRSA isolates and 38 ACME *arcA*-negative USA300 MSSA isolates (as detailed in Table S1 in the supplemental material), since the absence of ACME *arcA* is atypical compared with other USA300 strains, and these isolates might exhibit altered CP phenotypes. Table S1 provides the typing characteristics of USA300 MSSA isolates, USA500 isolates, and ACME *arcA*-negative USA300 isolates.

**USA300 MRSA and MSSA isolates.** Capsule production was evaluated in 167 USA300 MRSA isolates. Although all USA300 MRSA strains tested carried a *cap5* locus, all were nonreactive to CP5 polyclonal antiserum, whereas the CP5+ control strains Reynolds and Newman produced a signal (Fig. 1). Figure 1 also shows the nonreactivity to CP5 antiserum of the CP8+ strains, ST80 and MN8, as well as the CP- strains NCTC 8325-4 (27) and USA300 strain LAC. All 9 MRSA isolates that had a pulsotype consistent with USA300 but lacked ACME *arcA* were also CP-.

USA300 MRSA isolates were nonreactive with CP8 polyclonal antiserum, as expected, since they do not encode a CP8. USA300 MRSA and MSSA isolates failed to produce CP7 or CP18 (4–9).

**Determining USA300 MRSA and MSSA isolates.** USA300 MRSA and MSSA isolates fail to produce CP. Further, we evaluated whether the CP- phenotype was shared by USA300 methicillin-susceptible *S. aureus* (MSSA) isolates and the closely related USA500 lineage (21, 26). Since we found that all USA300 and USA500 isolates were uniformly CP-, we analyzed the evolutionary relationship between the *cap5* mutations in USA300 and USA500 isolates.

(This work was presented in part at the International Symposium on Staphylococci and Staphylococcal Infections, Chicago, IL, 2014.)

### Table 1: Demographics of USA300 and USA500 clinical isolates

<table>
<thead>
<tr>
<th>Parameter</th>
<th>USA300</th>
<th>USA500</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colonization vs infection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colonization</td>
<td>134</td>
<td>0</td>
</tr>
<tr>
<td>Infection</td>
<td>33</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>167</td>
<td>0</td>
</tr>
<tr>
<td>Geographic source</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chicago</td>
<td>83</td>
<td>12</td>
</tr>
<tr>
<td>Los Angeles</td>
<td>84</td>
<td>0</td>
</tr>
<tr>
<td>San Francisco</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Connecticut</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>167</td>
<td>0</td>
</tr>
<tr>
<td>Yr of isolation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1995</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>1996</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1997</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2004</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>2008</td>
<td>37</td>
<td>0</td>
</tr>
<tr>
<td>2009</td>
<td>92</td>
<td>0</td>
</tr>
<tr>
<td>2010</td>
<td>33</td>
<td>0</td>
</tr>
<tr>
<td>2011</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Unknown</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>167</td>
<td>16</td>
</tr>
</tbody>
</table>

* This isolate is the USA500 reference strain NRS385.
killed acapsular S. aureus cells (see Fig. S1 in the supplemental material). The control CP5+/H11001 strain Newman produced CP5 as visualized by the blue-staining Alexa Fluor 660-tagged secondary antibodies reacting with CP5 antibodies. In contrast, neither the inoculum of USA300 MRSA strain CDC3 nor the CDC3 bacteria isolated from mouse blood 6 h after bacterial challenge showed detectable reactivity with the CP5 antibodies. Similar results were obtained with samples of blood from mice challenged with USA300 strain LAC. Thus, our results show no evidence that USA300 MRSA isolates produce CP5\(^+\) in vivo.

**USA500 isolates.** Due to the close phylogenetic relationship between USA500 and USA300 isolates (21, 30), we hypothesized that USA500 isolates might also be CP5\(^-\). Figure 2 documents the pulsed-field gel electrophoresis (PFGE) patterns and relevant typing characteristics of the 16 USA500 isolates we tested for CP production. The USA500 isolates belonged to ST8, carried SCCmec type IV, and lacked ACME arcA (26, 30, 31) (see Table S1 in the supplemental material). However, most of these USA500 isolates carried pvl genes, which is atypical for USA500. The USA500 isolates belonged to three spa types: t064 (11 of 16 isolates), t008 (\(n = 3\)), or t211 (\(n = 2\)) (see Table S1). All 16 USA500 isolates were CP\(^-\) as represented in a panel in Fig. 1.

**DNA sequence analysis of cap loci of USA300 MRSA isolates.** To evaluate the genetic basis for the CP5\(^+\) phenotype, we evaluated the cap loci from assembled whole-genome shotgun data from 146 CP\(^+\)/H11000 USA300 MRSA isolates. Each strain carried an intact cap5 locus but had 4 identical point mutations relative to the CP5\(^+\) reference strain Newman (Fig. 3A).

The cap5 promoter had a T\(\rightarrow\)C replacement 73 bp upstream (−73) from the ATG translation start codon of cap5A (P\(_{\text{cap5}}\)−73). This was located in the cap5 promoter in an inverted repeat (Fig. 3) known to be essential for transcription of capABCDEF (27, 32). We also identified a frameshift mutation within a polyadenine (AA) tract that begins at nucleotide (nt) 1006 of cap5D in strain Newman. This corresponds to nt 994 in cap5D of the USA300 reference strain because the annotations for the start codon of strain Newman and USA300 differ by four codons. There was also a G\(\rightarrow\)T mutation at position 223 of cap5E (cap5E−223), which converts Asp to Tyr in the encoded protein at codon 75 (Asp75Tyr) and a T\(\rightarrow\)C mutation in cap5G−478, resulting in a Phe160Leu conversion in the encoded protein. Additional non-conserved mutations were found in various cap5 genes among USA300 isolates (data not shown) (33). The mutations in USA300 at cap5E−223 and cap5G−478 have not been previously recognized in USA300 isolates, whereas the mutations in P\(_{\text{cap5}}\) and cap5D

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**FIG 1** CP immunoblots of USA300 and USA500 clinical isolates. Capsule serotyping was performed by a colony immunoblot method with the use of CP5-specific antibodies as described previously (43). The first row shows the results from the CP5-positive (CP5\(^+\)) control strains Reynolds and Newman, the CP5- and CP8-negative (CP\(^-\)) control strains LAC and 8325, and the CP8\(^+\) control strains ST80 and MN8. The second and third rows include six representative USA300 and USA500 clinical isolates, respectively.

**FIG 2** Pulsed-field gel electrophoresis (PFGE) documenting the SmaI digestion patterns of USA500 isolates relative to the USA500 reference strain NRS385 (USA500ref) and relevant typing characteristics. The USA300 reference strain (USA300ref) has PFGE pattern USA300-0114 and was obtained from the Network for Antimicrobial Resistance in Staphylococcus aureus (NARSA). “Cap5/8” refers to capsule polysaccharide types 5 and 8, “CapE 223” refers to the nucleotide at position 223 in cap5E, and “CapG 478” refers to the nucleotide at position 478 in cap5G.
were identified previously in three USA300 MRSA isolates from 2004 to 2005 (22).

cap5 loci in publicly available USA300 S. aureus genome sequences. To confirm that the USA300-specific mutations were not confined to the Chicago and Los Angeles locales, we also analyzed 319 previously published USA300 isolate sequences from San Diego, CA (*n*/H11005 35), and New York (*n*/H11005 277). (Annotations of the included isolates are outlined in Table S2 in the supplemental material.) All but one isolate had all 4 cap5 mutations identical to those in our USA300 collection.

cap5 mutations among USA300 arcA-negative MRSA and USA300 MSSA isolates. We performed Sanger sequencing of PCR products amplified from the cap5 locus to determine if the four cap5 mutations identified in USA300 MRSA isolates were also conserved among 9 atypical USA300 MRSA isolates that were ACME arcA-negative and 19 USA300 MSSA isolates (Table 2). All USA300-specific mutations were conserved among 8 of 9 (89%) ACME arcA-negative USA300 MRSA isolates tested (Table 2). One USA300 MRSA outlier (isolate 111395) had only 2 of the 4 cap mutations (Pcap5/H11002 73 and cap5D-994). However, this isolate

FIG 3 USA300 cap5 locus showing universal mutations in USA300 relative to the cap5 reference strain Newman. Orange arrows depict open reading frames (ORFs). “Pcap5” refers to the cap5 promoter region. The inverted repeat sequence, as reported by Ouyang et al. (32), is shown as opposing arrows beneath Pcap5 indicating the T→C mutation in strain USA300 relative to strain Newman. The yellow line indicates a disruption of the cap5D ORF due to insertion of an A at nt 994 in USA300 (994AA). The cap5E G→T mutation at nt 223 (G223T) and the cap5G T→C mutation at nt 478 (T478C) are indicated beneath each ORF. (B) Lines beneath the map indicate the cap5 regions from strain Newman that are cloned in the complementation plasmids pKOR19 (cap5ABC plus truncated cap5D), pKOR18 (cap5ABCD plus a portion of cap5E), pCap16 (cap5ABCDE), and pCap17 (which contains cap5ABCDEF). (C) CP5 serotyping results from USA300 MRSA strain 923 transformants harboring the cap5 complementation plasmids depicted in panel B.

**TABLE 2** Summary of conserved mutations in cap5 loci detected in USA300 and USA500 isolates in our collection

<table>
<thead>
<tr>
<th>Gene</th>
<th>nt position</th>
<th>Strain Newman</th>
<th>USA300 Strain Newman</th>
<th>USA300 Strain USA300</th>
<th>Codon position</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pcap5</td>
<td>−73&lt;sup&gt;d&lt;/sup&gt;</td>
<td>T</td>
<td>C</td>
<td>NA'</td>
<td>NA</td>
</tr>
<tr>
<td>cap5D</td>
<td>994&lt;sup&gt;e&lt;/sup&gt;</td>
<td>A</td>
<td>AA</td>
<td>AAA (Lys)</td>
<td>STOP</td>
</tr>
<tr>
<td>cap5E</td>
<td>223</td>
<td>G</td>
<td>T</td>
<td>GAT (Asp)</td>
<td>TAT (Tyr)</td>
</tr>
<tr>
<td>cap5G</td>
<td>478</td>
<td>T</td>
<td>C</td>
<td>TTC (Phe)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>USA300</th>
<th>MRSA ACME arcA&lt;sup&gt;a&lt;/sup&gt; (n = 155)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>MRSA ACME arcA&lt;sup&gt;c&lt;/sup&gt; (n = 9)&lt;sup&gt;c&lt;/sup&gt;</th>
<th>MSSA (n = 19)&lt;sup&gt;d&lt;/sup&gt;</th>
<th>MRSA (n = 2)&lt;sup&gt;e&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>C (155/155)</td>
<td>C (9/9)</td>
<td>C (19/19)</td>
<td>C (14/14)</td>
<td></td>
</tr>
<tr>
<td>AA (155/155)</td>
<td>AA (9/9)</td>
<td>AA (19/19)</td>
<td>AA (14/14)</td>
<td></td>
</tr>
<tr>
<td>T (155/155)</td>
<td>T (8/9)</td>
<td>T (16/19)</td>
<td>AA (2/2)</td>
<td></td>
</tr>
<tr>
<td>G (19/19)</td>
<td>G (3/19)</td>
<td>G (14/14)</td>
<td>G (2/2)</td>
<td></td>
</tr>
<tr>
<td>C (155/155)</td>
<td>C (8/9)</td>
<td>C (10/19)</td>
<td>C (11/14)</td>
<td></td>
</tr>
<tr>
<td>T (1/9)</td>
<td>T (3/14)</td>
<td>T (2/2)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> SNPs of clinical isolates subjected to whole-genome sequencing or Sanger sequencing of PCR products as described in the text.

<sup>b</sup> USA300 ACME arcA MRSA isolates were characterized by PFGE or by molecular typing as described in Materials and Methods.

<sup>c</sup> Details of the molecular typing characteristics are provided in Table S1 in the supplemental material.

<sup>d</sup> Pcap5 promoter region of the cap5 operon.

<sup>e</sup> Seventy-three nucleotides upstream of the cap5A ATG translation initiation codon.

<sup>f</sup> NA, not applicable since the polymorphism is in an intergenic region.

<sup>g</sup> cap5D nt 994 in the USA300 reference strain corresponds to cap5D nt 1006 in strain Newman.
was obtained in 1995 and is not typical of recent USA300 MRSA isolates. Although isolate 111395F had a Smal digestion pattern similar to that of a USA300 isolate, it had features consistent with USA500 isolates, including spa type t064, ACME arcA, and PVL. However, strain 111395F also had features atypical for USA500, including carriage of SCCmec II.

Among the USA300 MSSA isolates tested, 16 of 19 (84.2%) had all 4 USA300-specific mutations in P$_{cap5}$, cap5D-994, cap5E-223, and cap5G-478 (Table 2; see Table S1 in the supplemental material). Three outlier MSSA isolates had just three of the USA300-specific mutations (P$_{cap5}$, cap5D-994, and cap5G-478).

**USA500 isolates.** Among USA500 isolates, the four USA300 cap5 mutations described above were common but not universal. By Sanger sequencing, we found that 11 of the 14 (78%) USA500 MRSA isolates had 3 of the 4 USA300-specific mutations (P$_{cap5}$ –73, cap5D-994, and cap5G-478), and none had the G→T mutation at cap5E-223. Three outlier USA500 MSSA isolates and both USA500 MSSA isolates that had only 2 of the 4 mutations (P$_{cap5}$ –73 and cap5D-993 ) (Table 2), but these are each sufficient to yield a CP$^-$ phenotype (27).

**Analysis of USA300-specific cap5 mutations in S. aureus phylogenies.** To evaluate the appearance of the cap5 mutations on the core phylogeny of **S. aureus**, we analyzed the USA300-specific cap5 mutations in 319 publicly available USA300 S. aureus genome sequences, 146 USA300 MRSA genomes in this study, and 90 non-USA300 genomes from 41 STs (Fig. 4). This allowed us to propose that the USA300 cap5 mutations evolved in a stepwise fashion. Moreover, these data suggest that USA300 and USA500 appear to have emerged from a common ancestor, and the P$_{cap5}$ T→C mutation likely occurred in the last common ancestor (LCA) of all USA300 and USA500 strains. The cap5D insertion may also have occurred in the LCA of both USA300 and USA500 but may have reverted back to wt in a recently emerged branch within USA500 (exemplified by strains GA231 and GA27). The cap5G T→C mutation had its origins in an ancestor of all USA300 strains that was also a progenitor of a clade of strains falling between the classical USA300 and USA500 patterns.

Our analysis also suggests that the isolates with ST609 and ST623 are intermediate between USA300 and USA500 and might have a common ancestry. The conserved cap5 mutations that we describe were not present in any of the other 20 clonal complexes and were not in all ST8 strains. Thus, these mutations are unique to USA300 and its close relatives USA500, ST609, and ST623.

**Complementation studies to test the phenotypic effect of cap5 gene mutations.** To determine the phenotypic relevance of the cap5 single nucleotide polymorphisms (SNPs) in strain USA300, we tested whether expression of wild-type cap5 genes in trans on a complementation plasmid could restore CP5 production to USA300 MRSA strain 923. The cap5 genes included in each complementation plasmid are depicted in Fig. 3B. Transformation of USA300 strain 923 with pKOR1 (expressing wt cap5ABC) or pKOR1A (expressing wt cap5ABCD) was not sufficient to restore the CP5$^+$ phenotype (Fig. 3C). This was not due to mutations in the cloned cap5 promoter or cap5D, as confirmed by DNA sequence analysis. In contrast, transformation of USA300 strain 923 with pCap16 (expressing wt cap5ABCDE) or pCap17 (wt cap5ABCDEDEF genes) restored CP5 production (Fig. 3C). These data indicate that the mutations in cap5D and P$_{cap5}$ in USA300 and USA500 strains ablate CP production. This data also reveal a vital role for the Asp→Tyr substitution at Cap5E-75 in ablating CP production. In contrast, Cap5G tolerates the Phe160Leu substitution in USA300 because complementation of CP5 production in a USA300 strain was achieved by a plasmid lacking cap5G.

**DISCUSSION**

The fact that CP antigens have been used successfully in vaccines against several bacterial pathogens has encouraged a similar vaccine strategy for **S. aureus**. Several vaccines that have been designed for use against **S. aureus** have included CP5 and CP8 because they are the most common CP types produced by **S. aureus** clinical isolates. However, this study shows that USA300 MRSA isolates, as well as USA300 MSSA and closely related USA500 isolates, are universally CP$^-$ negative. We also showed no evidence that USA300 isolates produce CP5 in vivo during infection in mice. In contrast, Timofeyeva et al. demonstrated that CP5 was detected in vivo on USA300 MRSA after a 6-h infection (28). We cannot explain the discrepancy between the two studies. However, it is unlikely that a strain could revert all 3 mutations that would be required for USA300 to revert to a CP$^+$ phenotype.

USA300 isolates have increased in prevalence in the United States to become one of the most common **S. aureus** genotypes (15), especially among SSTIs, where they have accounted for 98% of MRSA isolates (16). Because USA300 isolates comprise a substantial portion of the **S. aureus** disease burden in the United States, a vaccine based solely on CP would likely demonstrate low efficacy in a U.S. population.

These results extend the observations reported a decade ago when USA300 MRSA isolates were first reported to be acapsular (22, 23). We now show that the CP$^-$ phenotype has persisted among USA300 MRSA isolates and is a universal and characteristic trait of this highly successful MRSA lineage. Moreover, we have now established that USA300 MSSA isolates and USA500 isolates are also CP$^-$.

Our prior understanding of the genetic basis of the CP$^-$ phenotype among USA300 isolates was limited to our identification of two mutations in the cap5 locus in three USA300 isolates (22) that had been shown previously to ablate CP production in other **S. aureus** strains (27, 32). The comprehensive genetic analyses of USA300 isolates in this study reveals that the 4 cap5 mutations are present in all but 1 USA300 MRSA isolate and in all but 3 USA300 MSSA isolates. Importantly, 3 of those 4 mutations are alone sufficient to ablate CP production. It would therefore require reversion of all 3 mutations in USA300 MRSA to produce a strain that produces a CP. Also, 3 of the 4 same mutations are common in the USA500 lineage. It has been shown previously that the mutations in P$_{cap5}$ –73 and the frameshift mutation in cap5D ablate CP production in **S. aureus** (22, 27). Whereas the promoter mutation attenuates expression of capABCD, the cap5D and cap5E mutations ablate production or activity of enzymes responsible for producing key soluble precursors UDP-d-FucNac and UDP-1-L-FucNac, respectively, that are needed for the biosynthesis of CP (2, 34). This is the first report to show that Asp75 is a critical residue in Cap5E, which is an essential enzyme for CP5 production (35). This enzyme converts the precursor (UDP-GlcNAc) to a keto-intermediate that is further reduced (by Cap5F) and epimerized at C-2 (by Cap5G) to yield UDP-2-acetyl-L-fucosamine (36). Thus, USA300 is unable to produce UDP-2-acetyl-L-fucosamine, which is required for CP5 and CP8 production. When the Cap5E Asp75Tyr conversion is overlaid on the Cap5E three-dimensional crystal struc-
A maximum likelihood phylogeny based on whole-genome alignments of publicly available \textit{S. aureus} genomes. (A) Phylogenetic analysis of 104 \textit{S. aureus} strains from 41 STs showing the distribution of conserved mutations in \textit{cap5} in USA300 (Table 2). There are 14 USA300 strains in the tree that are representative of 146 strains from our study and 319 publicly available USA300 genomes. The remaining 90 genomes are from non-USA300 isolates. Strain names are shown as either the common name or the accession number followed by the sequence type (ST) in parentheses. Strains with red and blue text, respectively, are previously confirmed USA300 and USA500 isolates. The clade in orange is clonal complex 8 (CC8). Other common CGs are also highlighted. For simplicity, only a representative strain from each ST with the same pattern of \textit{cap5} mutations was included. Carriage of PVL and ACME is indicated with black and red diamonds, respectively, due to their association with the USA300 subclone. \textit{cap5} mutations are shown as solid or open circles as indicated. (B) High-resolution phylogeny of CC8 strains (a subset from panel A). USA300 and USA500 strains are annotated as in panel A.
ture (37), it lies within the coenzyme-binding domain. This information could be useful in developing inhibitors of CP biosynthesis.

Our analysis of S. aureus whole-genome sequences (WGSs) in the public databases from 41 STs also provides insight into the evolution of the USA500 and USA300 S. aureus lineages not previously appreciated. All USA500 isolates that we evaluated had at least 2 of the 4 cap5 mutations found in USA300 MRSA (in P_cap5 and in cap5D—both essential for CP production [36]). Moreover, most USA500 MRSA isolates had the cap5G→478 T→C mutation. Notably, because none of the USA500 isolates had the cap5E→223 G→T mutation, this is a feature in the core genome that might be used to distinguish USA500 from USA300 isolates in future epidemiological studies. By overlaying the USA300 mutations on the phylogeny of S. aureus genomes (Fig. 4), it is apparent that the 4 USA300 cap5-specific mutations only exist in clonal cluster 8, and they arose sequentially, starting with P_cap5, in a common ancestor of USA300 and USA500 isolates. Importantly, this is the first study to reveal that USA300 and USA500 lineages inherited the cap muta-
tions from a common ancestor rather than USA300 inheriting them from USA500, as suggested previously (21).

MRSA isolates arise by horizontal acquisition of a mobile ge-
netic element called SCCmec that carries the methicillin resistance gene, mec (18). Interestingly, only a limited number of genetic backgrounds have become successful MRSA clones (18). Despite the close genetic similarity between the USA300 and USA500 lineages (21, 26, 30), USA300 has disseminated more widely in the United States. Comparisons of virulence factors produced by USA500 versus USA300 may provide insight into their relative successes. USA500 and USA300 MRSA isolates both exhibit high-
level virulence in animal infection (22, 26, 30), and both express high levels of global regulators and exotoxins encoded in the core genome (22, 26). CP is another core virulence determinant that we have ruled out as a key player in the relative success of USA300 over USA500, because CP is not expressed in either subclone. The success of USA300 may lie in the fact that it carries ACME, which is thought to enhance transmissibility and competitiveness of USA300, although not its virulence (19, 20, 22).

Our findings shed light on the roles of CP5 and CP8 in staph-
ylococcal virulence of USA300 and USA500 strains. CP has been shown to protect S. aureus from opsonophagocytic killing by hu-
man neutrophils (11, 29) and thus protects S. aureus from host immune killing. Moreover, the capsule has been shown to en-
hance virulence in animal models of bacteremia, subcutaneous and renal abscess formation, surgical wound infection, septic ar-
thritis, and lethality (2). Yet its absence in USA300 and USA500 strains underscores the fact that a CP is not necessary for virulence of these strains. This is consistent with studies that have shown that CP production can attenuate staphylococcal virulence in sit-
uations in which bacterial adherence is critical, as in endocarditis and murine mastitis, because it masks the adhesins on the cell surface (2). USA300 is best known for causing SSTIs, so we can speculate that perhaps this niche favors a CP− phenotype by ex-
posing adhesins on the surface of the bacteria. Because the patho-
genesis of staphylococcal infections is multifactorial, it is likely that other virulence factors produced by USA300 and USA500 compensate for lack of CP during infection. Overexpression of agr, sae, α-hemolysin, and a variety of leukocidins (22), may even have been selected for in the absence of a CP.

Our study has limitations. Our sample of isolates was obtained mainly from patients in Chicago and Los Angeles. This was offset by analyzing public genome sequences, which supported and ex-
tended the findings from our isolates.

We conclude that a vaccine designed for a U.S. population should not be solely based on CP antigens because USA300 and USA500 CP− isolates cause a large proportion of S. aureus infections.

MATERIALS AND METHODS

Determination of USA300 and USA500 genetic backgrounds. S. aureus isolates were confirmed with a catalase test and by agglutination using Staphaurex Plus (Remel) and underwent genotyping by multilocus sequence typing (MLST) (38), Ridom spa typing (39), SCCmec typing (40), and PCR detection of meca, the ACME-borne mecA gene (ACME mecA) (19), and genes encoding PVL (41) as described previously (39). USA300 MRSA isolates were classified by Smal digestion patterns using pulsed-field gel electrophoresis (PFGE) as described previously (39). In the ab-
sence of PFGE data, the USA300 genotype was inferred in MRSA isolates that belonged to ST8 and carried SCCmec type IV and PVL genes (39). All USA500 isolates and USA300 MSSA isolates were identified solely by PFGE using Smal digestion patterns. PFGE patterns were assigned to a given clonal group by comparison to a reference strain using an 80% similarity cutoff using the Dice coefficient in BioNumerics software (Ap-
plied Maths, TX) (42). Isolates were obtained from consenting patients as approved by the Institutional Review Board of the participating institu-
tions.

CP5 and CP8 serotyping. Capsule serotyping was performed by a colony immuno blot method with the use of CP5- and CP8-specific poly-
clonal antibodies as described (43). Briefly, tryptic soy agar plates were spot inoculated in a grid pattern with up to 60 S. aureus isolates and incubated overnight at 37°C. The colonies were blotted onto nitrocellu-
lose filter membranes (diameter, 8.25 mm) for 5 min at ambient tempera-
ture. Adherent colonies were fixed to the membranes by being heated at 60°C for 15 min. After being washed twice in phosphate-buffered saline (PBS) to remove excess cells, the filters were immersed in a solution of trypsin (1 mg/ml) for 60 min at 37°C to remove protein A. After two washes in PBS, the filters were blocked with 0.05% skim milk for 1 h and washed in PBS containing 0.05% Tween 20 (PBST). Capsule type-specific polyclonal antiserum (diluted 1:1,000) was incubated with each filter at 37°C for 1 h. After being washed in PBST, horseradish peroxidase-
conjugated goat anti-rabbit immunoglobulin was incubated with each

USA300 strains subjected to whole-genome sequencing. To determine the basis for the CP− phenotype, we analyzed the entire cap5 locus from the WG8 assemblies we produced (33) from 146 USA300 MRSA isolates that were included in the sample tested for CP production. These isolates were obtained between 2008 to 2010 from the University of Chi-
icago Medical Center (n = 75) and Harbor-UCLA Medical Center in Los Angeles (n = 71) (44).

WG5 and SNP calling. Genomic DNA was sequenced on an Illumina HiSeq 2000 (Illumina Inc., San Diego, CA) at ~215× the median depth of coverage per strain as described in reference 33 and the supplemental material. Mutations in the cap5 locus were retrieved from the assemblies by comparison to the cap5 locus of the CP+ reference strain Newman using Jalview (45). All nucleotide positions are reported relative to the USA300 reference strain TCH1516.

Confirmation of cap5 mutations. To evaluate whether the conserved mutations we identified in the WGS analysis were present in S. aureus isolates of interest, the regions surrounding the mutations were PCR am-

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plified from the indicated strains using primers listed in Table 3. The amplification products were subjected to Sanger sequencing in the DNA sequencing and genotyping facility (University of Chicago) using the amplification primers. Mutations were identified using Vector NTI software (Invitrogen) by alignment to the cap5 locus of the CP5 reference strain Newman.

**Table 3** Sequences of the primers used for PCR amplification and sequencing of the cap5 gene fragments

<table>
<thead>
<tr>
<th>Primer (expected size [bp])</th>
<th>Cap5A promoter, P&lt;sub&gt;cap5&lt;/sub&gt;</th>
<th>Cap5D (803)</th>
<th>Cap5E (381)</th>
<th>Cap5G (543)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward</td>
<td><code>5′-GAATCTAGTTTACGTTCTCAG-3′</code></td>
<td><code>5′-GCACCTTATTGTTGATTTCA-3′</code></td>
<td><code>5′-GCAACGTATTCGGTAAT-3′</code></td>
<td><code>5′-TGGACGCGGATAATGATGC-3′</code></td>
</tr>
<tr>
<td>Reverse</td>
<td><code>5′-ATGGAATGACAGTCTCCT-3′</code></td>
<td></td>
<td><code>5′-CTTTTGAAATACCATAGCA-3′</code></td>
<td><code>5′-GGACGACAGGGAAGCTTCAA-3′</code></td>
</tr>
</tbody>
</table>

* Forward primers have a sense orientation, and reverse primers have an antisense orientation.

**SUPPLEMENTAL MATERIAL:**

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.02585-14/-/DCSupplemental.

Text S1, DOCX file, 0.1 MB.
Figure S1, TIF file, 0.5 MB.
Table S1, PDF file, 0.1 MB.
Table S2, PDF file, 0.1 MB.

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