Community-associated and healthcare-associated methicillin-resistant *Staphylococcus aureus* virulence toward *Caenorhabditis elegans* compared

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Community-associated (CA) methicillin-resistant *Staphylococcus aureus* (MRSA) strains have emerged as major human pathogens. CA-MRSA virulence appears to be distinct from healthcare-associated (HA) MRSA with several factors [α-hemolysin (Hla), Panton-Valentine leukocidin (PVL), α-type phenol soluble modulins (PSMα) and SCCmec IV] postulated to enhance virulence or fitness. Using the *Caenorhabditis elegans* infection model, we compared the virulence of clinical and laboratory isolates of CA-MRSA and HA-MRSA and explored the contribution of CA-MRSA associated virulence factors to nematode killing. All CA-MRSA strains were highly pathogenic to nematodes, while HA-MRSA strains demonstrated variable nematode killing. Nematode killing by isogenic mutants of *hla* or the loci for PVL, PSMα, PSMβ, PSMδ or SCCmec IV was not different than the parental strains. These results demonstrate that CA-MRSA is highly virulent, shows some strains of HA-MRSA are equally virulent toward nematodes and suggests CA-MRSA virulence in *C. elegans* is not linked to a single virulence factor.

**Introduction**

Community-associated (CA) methicillin-resistant *Staphylococcus aureus* (MRSA) strains have emerged as major human pathogens, capable of causing infection in otherwise healthy individuals. These organisms are now responsible for the majority of skin and soft tissue infections seen in many emergency departments in the United States and have been shown to cause unusually severe invasive infections such as sepsis, necrotizing pneumonia, necrotizing fasciitis and brain abscesses.1,4 Two principle strains of CA-MRSA have been identified in the United States, USA300 and USA400, with USA300 causing the majority of infections.1 The virulence of CA-MRSA appears to be distinct from traditional healthcare-associated (HA) strains, but the etiology of this difference is incompletely understood. Several factors have been hypothesized to play a role in CA-MRSA virulence and fitness, including α-hemolysin (Hla), Panton-Valentine leukocidin (PVL), α-type phenol soluble modulins (PSMα), the arginine catabolic mobile element (ACME) and the staphylococcal cassette chromosome mec (SCCmec) specific to CA-MRSA, named SCCmec type IV.4

The nematode *Caenorhabditis elegans* has previously been established as a model host to study staphylococcal pathogenesis and host-pathogen interaction.3,5-8 Many factors important for *S. aureus* pathogenesis in vertebrates are similarly important for nematode killing, including *hla*, *agr* and *sarA* (global virulence regulators of *S. aureus*) and V8 protease.5-8 Other factors that are important during nematode infection but with unclear roles in virulence determination in vertebrates include alternative sigma factor σB, *saeRS* and *srrAB* (two-component regulators) as well as genes involved in capsule biosynthesis and intermediary metabolism.6-8 Recently, Wu and colleagues reported that CA-MRSA strains are more virulent in *C. elegans* than HA-MRSA strains; however the genetic reason for the difference in virulence was not explored.7 In this study, we compare the virulence of a collection of laboratory and clinical isolates of CA-MRSA and HA-MRSA and explore the contribution of putative CA-MRSA associated virulence determinants to CA-MRSA virulence in the *C. elegans* infection model.

**Results**

We evaluated the relative virulence of clinical isolates and laboratory strains of MRSA toward *C. elegans* using the nematode killing assay.6 The CA-MRSA USA400 strain MW2, USA300 strain LAC, and low passage USA300 clinical isolates CAV1001 and CAV1039 were as virulent in the *C. elegans* infection model as the MSSA strains NCTC 8325 and Newman, which have previously been shown to be highly virulent toward nematodes (Fig. 1A and B).6 By contrast, HA-MRSA specimens were
associated with greater strain-to-strain variability in survival. Focusing on laboratory MRSA strains, the epidemic MRSA strains EMRSA-1 and EMRSA-4 caused markedly less nematode killing than the CA-MRSA strains (all log rank p values < 0.0001) (Fig. 1). However, the archaic MRSA strain COL was as virulent as the CA-MRSA clones (Fig. 1). Next, we examined 10 low-passage HA-MRSA clinical isolates obtained from patients at our institution over the decade between 1988 and 1998 (Table 1). Nematode survival was followed for 2–6 d and compared by log rank analysis to LAC as a prototypic USA300 CA-MRSA strain; population death at 42 h is shown in Figure 1A, as it correlated well with overall nematocidal activity. Four HA-MRSA clinical isolates (1245, 9562, 9811 and 9856) showed markedly less nematode killing compared with all CA-MRSA strains (log rank p value range, < 0.0001–0.01) (Fig. 1). However, six HA-MRSA clinical isolates (1052, 1065, 1455, 9573, 9608 and 9614) had a rate of killing that was indistinguishable from the CA-MRSA strains. Note that the nematocidal activities of isolates 9608 and 9614, which appear to be at an intermediate level at 42 h, were not significantly different than LAC when measured over the course of the experiment. Figure 1B shows representative complete survival curves for several isolates (LAC, NCTC 8325, EMRSA-4, 1052, 9562 and 9856) with a range of virulence potentials in the infection model. Characteristic of S. aureus–C. elegans infection model, the intestinal tract of the worm was colonized by all strains of S. aureus, regardless of nematocidal activity, after 24 to 36 h of exposure. Slowed locomotion, reduced pharyngeal pumping and loss of intestinal cell volume and architecture preceded worm death. These results demonstrate that while USA300 and USA400 lineages of CA-MRSA are highly virulent toward nematodes, so are a subset of HA-MRSA.

Next, we explored the contribution of specific putative CA-MRSA virulence determinants to nematode killing. There was no significant difference in nematode killing by S. aureus between the bha mutant and the parental strain LAC (Fig. 2A). This contrasted with our findings for the MSSA laboratory strain RN6390, where the bha deletion mutant was markedly attenuated in worm killing (p < 0.0001) (Fig. 2A). The consequence of loss of PVL on nematode killing was investigated in two USA300 backgrounds, LAC and SF8300. In all cases, the Δpol mutants were as virulent as the parental strains, as shown in Figure 2B (data not shown for SF8300). Furthermore, deletion of the genes for α- or β-type PSMs or mutation of bld that inactivates δ-toxin function but does not impact agr quorum sensing did not affect killing in either the USA300 strain LAC (Fig. 2C) or the USA400 strain MW2 (Fig. 2D). Deletion of ACME did not change nematode survival tested in the USA300 strain SF8300 (data not shown). Finally, deletion of SCCmec IV in the USA300 strain SF8300 similarly did not impact nematode survival (Fig. 2E).

**Discussion**

The emergence of MRSA as an important community-associated pathogen in North America reflects two overlapping epidemic waves of genotypically distinct clones of S. aureus—USA400, a multilocus sequence type (ST) 1 strain that first appeared in the US upper midwest and central Canada in the 1990s and USA300, an ST8 strain unrelated to USA400 that emerged in the early 2000s and quickly supplanted USA400 as the leading cause of CA-MRSA in North America. The association of CA-MRSA with widespread and occasionally severe infections in otherwise healthy individuals has led to the speculation that these clones are more virulent and/or more adept at evading host immune defenses than traditional HA-MRSA. Indeed, USA300 and USA400 have been shown to be much more virulent than HA-MRSA in animal models of infection and in vitro virulence assays.

Wu and colleagues have similarly reported that CA-MRSA strains are more pathogenic than HA-MRSA strains in the C. elegans model. However, they only examined the nematocidal activity of a single pulsotype of invasive HA-MRSA, termed CMRSA6, and found that it was much less virulent toward worms than isolates of the USA300, USA400 and CMRSA2 pulsotypes. A local nasal-colonizing MRSA isolate, M92, which shares the same multilocus sequence type as CMRSA6 (ST239), was also found to be avirulent toward worms. However, it is uncertain whether CMRSA6 is representative of all HA-MRSA strains, as it comprised a small minority (~2%) of all invasive isolates recovered in the Canadian province between 2000 and 2006. In addition, the authors also considered CMRSA2 to be a CA-MRSA rather than an HA-MRSA genotype, because it had been associated with both community- and healthcare-associated disease in the province. However, epidemiological studies have traditionally classified CMRSA2 as an HA-MRSA genotype, since the clone (which goes by the designation USA100:ST5 in the US) has been the most common healthcare-associated strain in Canada and the US for the last two decades.

In order to determine whether CA-MRSA strains were more virulent that HA-MRSA strains, we compared several USA300 and USA400 clones with a collection of laboratory and clinical HA-MRSA strains. Four independent isolates of USA300 and the first recognized isolate of CA-MRSA, MW2/USA400, were evaluated. Confirming the findings of Wu and colleagues, we found that CA-MRSA clinical isolates and characterized strains of the USA300 and USA400 lineages were highly virulent in the C. elegans infection model, capable of killing nematodes as rapidly as the most virulent MSSA strains examined to date. However, we failed to demonstrate that HA-MRSA clones were uniformly less virulent toward worms. Rather, the well-studied HA-MRSA strain COL and the majority of HA-MRSA isolates collected over a decade prior to the emergence of CA-MRSA were virulent toward worms. Indeed, virulence of COL and four of ten clinical isolates tested were indistinguishable from the USA300 and USA400 strains. These results are similar to what we have previously observed for MSSA, where strain-to-strain differences in nematocidal activity exist, but the reason for the differences was not clear.

It is interesting to note that EMRSA-1 and EMRSA-4, examined in this report, and CMRSA6 and M92, tested by Wu et al., are all of the ST239 lineage. ST239 is a hospital-transmitted, internationally disseminated HA-MRSA strain variant that emerged in Brazil in the early 1990s and rapidly spread to Portugal, Eastern Europe and Asia. While these ST239
clones were avirulent in the *C. elegans* infection model, ST239 strains are clearly capable of causing invasive disease in man. One recent report suggests that an ST239 strain (TW) may in fact be more virulent than non-ST239 strains, as it had an increased ability to cause catheter-related bacteremia.\(^\text{17}\) Whether these strains lack of a specific virulence gene (or complement of virulence genes) that contributes to worm killing or share a microbial attribute conserved among ST239 strains that leads to avirulence is unclear.

While USA300 and USA400 originate from different lineages of *S. aureus*, they share several genetic attributes that suggest possible roles in pathogenesis. Among these determinants, PVL, a phage-encoded bi-component toxin, has been epidemiologically linked to CA-MRSA infections caused not only by USA300 and USA400 but also by the Australian CA-MRSA strain ST93 (the “Queensland clone”), the Southwest Pacific/Oceania CA-MRSA strain ST30, the Pacific strain ST59, the South American CA-MRSA strain ST5 and the European CA-MRSA strain ST80.\(^\text{1,18}\) While PVL is a \(\beta\)-barrel toxin that can lyse human myeloid lineage cells in vitro, the role of PVL in CA-MRSA disease is less clear. Studies primarily using murine-disease models have shown conflicting results regarding the importance of PVL to virulence.\(^\text{19-21}\) In this study, we were unable to identify a role for PVL in the pathogenicity of CA-MRSA in the *C. elegans* infection model. The lack of killing attenuation seen in nematodes with loss of PVL is predicable, as the leukolytic activity of the toxin is highly species-specific, preferentially targeting human neutrophils over neutrophils from other mammals.\(^\text{22}\) Let alone a target in a phylogenetically distant host. Lack of killing attenuation in the PVL mutants is also consistent with studies that have shown that PVL had no direct impact on other virulence gene expression.\(^\text{24}\)

Increased production of other cytolytic toxins has also been proposed to account for the heightened virulence potential of CA-MRSA. Hla is a core-genome encoded, \(\beta\)-channel pore-forming cytotoxin that is produced at high levels in USA300 CA-MRSA and implicated in the increased pathogenicity of these clones.\(^\text{19,23}\) While loss of *hla* expression in *S. aureus* RN6390 led to significant attenuation in the *C. elegans* model, we found that an *hla* deletion mutant in USA300 strain LAC was as virulent as the parental strain. Furthermore, recent work by Irazoqui et al.\(^\text{5}\) showed that the lifespan of sterile nematodes were equivalent when exposed to wild-type *S. aureus* and isogenic *hla* mutants. Together, these data suggest that the influence of Hla on nematode lifespan is strain dependent and may reflect a feature of the host-pathogen relationship that does not extend beyond matricide. Further work is needed to understand the nature of this relationship.

PSMs are amphipathic, \(\alpha\)-helical peptides found in several species of staphylococci.\(^\text{24}\) *S. aureus* contains three different classes of PSM and related peptides—PSM\(\alpha\), PSM\(\beta\) and \(\delta\)-hemolysin. These surfactant-like molecules can recruit, activate and (in the case of PSM\(\alpha\) and \(\delta\)-hemolysin) lyse human neutrophils.\(^\text{24}\) Like Hla, PSMs are encoded in the core genome of *S. aureus* but are produced at higher levels by USA300 and USA400 CA-MRSA strains than by HA-MRSA strains.\(^\text{24}\) While less virulent in murine infections models, USA300 and USA400 deletion mutants of the PSM\(\alpha\) and PSM\(\beta\) loci and loss-of-function \(\delta\)-hemolysin mutants were not virulence attenuated in nematodes.

Finally, we evaluated whether the CA-MRSA associated elements SCCmecIV or ACME alter virulence toward nematodes. While not a virulence factor per se, SCCmecIV appears to impose
a smaller fitness cost in vitro and in vivo than HA-MRSA SCC\textit{mec} elements, and thereby may have helped facilitate the epidemic spread of CA-MRSA.\textsuperscript{25} The absence of any attenuation in nematode killing due to the presence of SCC\textit{mec}IV lends support to the hypothesis that SCC\textit{mec}IV does not impart a significant fitness cost. However, we did not evaluate whether the SCC\textit{mec} elements of HA-MRSA isolates influence virulence fitness in nematodes. ACME, which is located adjacent to SCC\textit{mec}IV in Table 1.

Table 1. Strains used in this study

| Strain | Description | Reference and/or source |
|--------|-------------|-------------------------|
| Staphylococcus aureus | Clinical HA-MRSA, isolated 1988 | Lab collection; UVAHS, Charlottesville |
| 1052   | Clinical HA-MRSA, isolated 1988 | Lab collection; UVAHS, Charlottesville |
| 1065   | Clinical HA-MRSA, isolated 1991 | Lab collection; UVAHS, Charlottesville |
| 1245   | Clinical HA-MRSA, isolated 1991 | Lab collection; UVAHS, Charlottesville |
| 1455   | Clinical HA-MRSA, isolated 1998 | Lab collection; UVAHS, Charlottesville |
| 9573   | Clinical HA-MRSA, isolated 1998 | Lab collection; UVAHS, Charlottesville |
| 9562   | Clinical HA-MRSA, isolated 1998 | Lab collection; UVAHS, Charlottesville |
| 9608   | Clinical HA-MRSA, isolated 1998 | Lab collection; UVAHS, Charlottesville |
| 9614   | Clinical HA-MRSA, isolated 1998 | Lab collection; UVAHS, Charlottesville |
| 9811   | Clinical HA-MRSA, isolated 1998 | Lab collection; UVAHS, Charlottesville |
| 9856   | Clinical HA-MRSA, isolated 1998 | Lab collection; UVAHS, Charlottesville |
| CAV1001| USA300 CA-MRSA, isolated 2006 from a brain abscess, ACME-3 | UVAHS, Charlottesville |
| CAV1029| USA300 CA-MRSA, isolated 2008 from necrotizing pneumonia | Lab collection; UVAHS, Charlottesville |
| Newman| MSSA lab strain | 28 |
| NCTC 8325| MSSA lab strain | 29 |
| COL | Archaic HA-MRSA strain | 11 |
| EMRSA-1 | HA-MRSA strain | 10 |
| EMRSA-4 | HA-MRSA strain | 10 |
| SF8300 | USA300 CA-MRSA strain | 19; Henry Chambers |
| SF8300 \(\Delta pv\)l | SF8300 \(\text{lukF/S-PV}\):spc PVL- Sp\(^{\prime}\) | 19; Henry Chambers |
| SF8300ax | SF8300 \(\Delta\text{ACME}\) | 21; Henry Chambers |
| SF8300ex | SF8300 \(\Delta\text{SCCmec}\) | 21; Henry Chambers |
| LAC | USA300 CA-MRSA strain | 19; Michael Otto |
| LAC \(\Delta pv\)l | LAC \(\text{lukF/S-PV}\):spc PVL- Sp\(^{\prime}\) | 19; Michael Otto |
| LAC \(hla:erm\) | LAC \(hla:erm\) Hla- Em\(^{\prime}\) | 20; Olaf Schneewind |
| LAC \(\Delta PSM\alpha\) | LAC \(psm\alpha 1-4\):spc PM\(\alpha\)- Sp\(^{\prime}\) | 24; Michael Otto |
| LAC \(\Delta PSM\beta\) | LAC \(psm\beta 1-2\):spc PM\(\beta\)- Sp\(^{\prime}\) | 24; Michael Otto |
| LAC \(\Delta hld\) | LAC \(hld\) point mutant Hld- agr\(^{+}\) | 24; Michael Otto |
| MW2 | USA400 CA-MRSA strain | 24; Michael Otto |
| MW2 \(\Delta PSM\alpha\) | MW2 \(psm\alpha 1-4\):spc PM\(\alpha\)- Sp\(^{\prime}\) | 24; Michael Otto |
| MW2 \(\Delta PSM\beta\) | MW2 \(psm\beta 1-2\):spc PM\(\beta\)- Sp\(^{\prime}\) | 24; Michael Otto |
| MW2 \(\Delta hld\) | MW2 \(hld\) point mutant Hld- agr\(^{+}\) | 24; Michael Otto |
| RN6390 | MSSA laboratory strain, prophage-cured derivative of NCTC 8325 | 30 |
| ALC837 | RN6390 \(hla:erm\)C Hla- Em\(^{\prime}\) | 30 |
| Escherichia coli | Uracil auxotrophy | 27; CGC |
| OP50 | Uracil auxotrophy | 27; CGC |
| Caenorhabditis elegans | Wild-type isolate from Bristol, England | 27; CGC |

HA-MRSA, healthcare-associated methicillin-resistant \textit{Staphylococcus aureus}; CA-MRSA, community-associated methicillin-resistant \textit{Staphylococcus aureus}; MSSA, methicillin-susceptible \textit{Staphylococcus aureus}; Em\(^{\prime}\) erythromycin resistant; Sp\(^{\prime}\) spectinomycin resistant; UVAHS, University of Virginia Health System; CGC, Caenorhabditis Genetics Center

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USA300 and carries several putative virulence genes, may confer increased pathogenicity and fitness. However, our study demonstrated ACME did not influence virulence of USA300 SF8300 in the *C. elegans* model.

There are several limitations to this study that should be considered. First, we did not evaluate the contribution of several putative CA-MRSA virulence factors to virulence in nematodes, most notably the *S. aureus* superantigens SEB, SEC, SE-l Q and TSST-1. However, it would be very surprising for mitogenic T-cell superantigens to influence pathogenesis in an animal that lacks adaptive immunity. Second, the contribution of each putative virulence trait was examined in isolation, so whether additive effects exist when multiple virulence factors are eliminated was not determined. Finally, we only examined four isolates of USA300 and one isolate of USA400; it is possible that

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**Figure 2.** Kaplan-Meier survival plots of N2 *C. elegans* fed *S. aureus*. (A) LAC (wild type; solid black square/solid black line), LAC hla::erm (open black square/dashed black line), RN6390 (wild type, solid red circle/solid red line) and ALC837 (RN6390 hla::erm, open red circle/dashed red line). Pairwise comparisons by strain: RN6390 vs. ALC837, p < 0.0001; LAC vs. LAC hla::erm, p = not significant (NS). (B) LAC (solid black square/solid black line) vs. LAC Δpvl (open green square/dashed green line), p = NS. (C) LAC (solid black square/solid black line) vs. LAC ΔPSMα (open red circle/dashed red line), LAC ΔPSMβ (open blue triangle/dashed blue line) and LAC Δhld (open inverted green triangle/dashed green line), all p = NS. (D) MW2 (wild type, solid black square/solid black line) vs. MW2 ΔPSMα (open red circle/dashed red line), MW2 ΔPSMβ (open blue triangle/dashed blue line) and MW2 Δhld (open inverted green triangle/dashed green line); all p = NS. (E) p SF8300 (wild type, solid black diamond/solid black line) vs. SF8300ex (open red diamond/dashed red line), p = NS. All comparisons by the log rank test.
examination of additional isolates of CA-MRSA may have demonstrated more variation in nematode killing.

In conclusion, our study confirms that CA-MRSA strains are highly virulent in the nematode infection model. However, in contrast to the findings of Wu and colleagues, we demonstrate that some strains of HA-MRSA are as virulent to nematodes as prototypic strains of CA-MRSA. Furthermore, we found no association between the presence of CA-MRSA specific virulence determinants and disease in the C. elegans model. Together, these finding suggest that C. elegans is not likely to be an informative host for the study of pathogenic mechanisms that may be distinct to CA-MRSA. However, the observation that ST239 strains may, in general, be less virulent toward nematodes than other genotypes of S. aureus suggests that the relationship between S. aureus lineage and virulence in C. elegans is a potential area of future exploration.

Materials and Methods

Bacterial strains and growth conditions. Bacterial strains used in the study are listed in Table 1. All strains of bacteria were maintained at -75°C in tryptic soy (TS) broth or Luria-Bertani (LB) medium with 15% glycerol. S. aureus strains were grown at 37°C with aeration in TS broth. The medium was supplemented with antibiotics when indicated (erythromycin 10 µg/ml or 100 µg/ml, spectinomycin 150 µg/ml or 250 µg/ml). Escherichia coli strains were grown in LB broth supplemented with streptomycin 100 µg/ml when appropriate. Solid medium was made with the addition of 15 g of Bacto Agar (Difco Laboratories, 214010) per liter. Isolates were designated as being low passage if they have been passaged ≤3 times.

Caenorhabditis elegans. Bristol N2 C. elegans nematodes (Table 1) were maintained at 15°C on nematode growth medium plates with the OP50 strain of E. coli used as a food source. Nematodes were manipulated using standard techniques.

Nematode survival assays. S. aureus strains and isolates were streaked out from frozen stocks on TS agar plates (supplemented with antibiotics as appropriate) and grown overnight at 37°C. Strains and isolates were also streaked out on TSA with 5% sheep blood (Remel, R01200) to ensure they retained their hemolysin phenotype during freezing. Single colonies were incubated with aeration at 37°C overnight in TS broth supplemented with erythromycin or spectinomycin as indicated. The overnight cultures were diluted 1:10 in TSB and 10 µl was placed on 35 mm tissue culture plates (Falcon, 353001) containing TSA with nalidixic acid 5 µg/ml. Plates were then incubated four hours at 37°C, cooled to room temperature and 30 to 40 L4 stage nematodes were placed per plate. The plates are then incubated at 25°C and counted for live and dead worms at least every 24 h (unless otherwise specified). A worm was considered dead if it did not respond when the plate is tapped or it is gently touched. Nematodes that die on the side of the plate were censored. Each strain was tested in triplicate and the data shown is representative of these experiments.

Statistical analysis. Nematode survival rates were calculated by the Kaplan-Meier method and tested for significance using the log rank test (Graph Pad Prism, Version 4.0; GraphPad Software, Inc.). p value < 0.05 was considered statistically significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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