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Virulence Strategies of the Dominant USA300 Lineage of Community Associated Methicillin Resistant *Staphylococcus aureus* (CA-MRSA)

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Abstract

Methicillin-Resistant *Staphylococcus aureus* (MRSA) poses a serious threat to worldwide health. Historically, MRSA clones have strictly been associated with hospital settings and most hospital-associated MRSA (HA-MRSA) disease resulted from a limited number of virulent clones. Recently, MRSA has spread into the community causing disease in otherwise healthy people with no discernible contact with healthcare environments. These community-associated (CA-MRSA) are phylogenetically distinct from traditional HA-MRSA clones and CA-MRSA strains seem to exhibit hyper virulence and more efficient host:host transmission. Consequently, CA-MRSA clones belonging to the USA300 lineage have become dominant sources of MRSA infections in North America. The rise of this successful USA300 lineage represents an important step in the evolution of emerging pathogens and a great deal of effort has been exerted to understand how these clones evolved. Here we review much of the recent literature aimed at illuminating the source of USA300 success and broadly categorize these findings into three main categories: newly acquired virulence genes, altered expression of common virulence determinants and alterations in protein sequence that increase fitness. We argue that none of these evolutionary events alone account for the success of USA300, but rather their combination may be responsible for the rise and spread of CA-MRSA.

Multidrug-resistance in *Staphylococcus aureus*: the rise of MRSA

The Gram-positive pathogen *Staphylococcus aureus* remains one of the most problematic and costly sources of bacterial infection worldwide[1]. Disease typically presents as mild skin/soft tissue infections but can also be the source of more serious bacteremia, endocarditis, osteomyelitis and necrotizing pneumonia[2]. *S. aureus* asymptomatically colonizes the skin, and more commonly, the anterior nasal passages of healthy people[3]. Nasal colonization is the most significant predictor of invasive disease, however, in some studies, nearly half of patients carrying *S. aureus* are strictly colonized extranasally[4]. Thus, estimates of *S. aureus* carriage at ~25% of the human population may be an underestimate of true colonization levels. Given the near ubiquity of *S. aureus* among the human population combined with its virulence potential, it is no wonder this organism has been recognized as a significant

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healthcare burden for over a century. *S. aureus* was first described by Alexander Ogston in 1881 as the sole microorganism within the fluid drained from a severe knee abscess[5]. Then, he noted that "once established the micrococci are hard to kill…" underscoring the recalcitrant nature of *S. aureus* towards antiseptic treatment[6]. During this time, Joseph Lister's influence on surgical procedures through the implementation of carbolic acid (phenol) to sterilize wounds and instruments, had greatly reduced the occurrence of post-operative infections[7]. However, it was subsequently shown that *S. aureus* was inherently resistant to phenol explaining its association with surgical infections despite good "sterile technique"[8]. Thus, *S. aureus* was recognized as an important hospital associated pathogen over 130 years ago in the pre-antibiotic era and little has changed to this day.

Perhaps because of its intimate association with hospitals and patients, *S. aureus* has always been among the first bacterial species reported to develop resistance to new antimicrobials, from sulfonamide-resistance in the early 1940s[9] to the identification of penicillinase in 1944[10] just months after US penicillin production reached full scale. Interestingly, these progenitor β-lactamase positive *S. aureus* clones were isolated from patients that had not even been treated with penicillin. Nonetheless, Penicillin-Resistant *S. aureus* (PRSA) was here to stay, and became pandemic in hospitals during the late 1950s and early 1960s[11]. Subsequently, a penicillinase-resistant β-lactam derivative, methicillin (Celbenin, Beecham Pharmaceuticals), was approved for use in the US in 1959. Less than two years later, the first report of methicillin-resistant *S. aureus* (MRSA) was published documenting the isolation of MRSA clones from a patient and hospital staff in the UK, again none of which were treated with methicillin[12]. It was immediately recognized that methicillin-resistance was mechanistically different than penicillin-resistance in that the MRSA phenotype did not involve direct inactivation of the drug. Rather, resistance was mediated through the acquisition of an alternative penicillin-binding protein (PBP2a) with lowered affinity for β-lactam antibiotics. Within 20 years after the first discovery of MRSA, it became a leading cause of hospital-acquired infections[13]. Currently, it can still be responsible for nearly 60% of skin/soft-tissue infections presenting to US emergency rooms[14].

The methicillin-resistance determining PBP2a is encoded by *mecA* harbored on a mobile genetic element, Staphylococcal Cassette Chromsome (SCC*mec*). A nearly identical homologue, now thought to be the ancestral *mecA*, was recently discovered in *Staphylococcus fleuretti*, an animal colonizing staphylococcal species[15]. Unlike a previously identified *mecA* homologue in *Staphylococcus sciuri* that does not confer methicillin-resistance[16], *S. fleuretti* is fully resistant to β-lactam antibiotics. Interestingly, the *S. fleuretti mecA* homologue is not found on a mobile SCC, but rather in the core chromosome between the mevalonate biosynthetic and xylose utilization operons, explaining the presence of *mva* and *xyl* gene fragments in some *S. aureus* SCC*mec* elements[15]. These mobile islands have diversified considerably over the 50-year history of MRSA such that there are currently eight distinct SCC*mec* types circulating among *S. aureus* as well as some species of coagulase negative staphylococci[17]. SCC*mec* elements can vary greatly in size and composition with the largest (SCC*mec* type II) spanning 52 kb and additionally encoding erythromycin-, spectinomycin- and tobramycin-resistance determinants[18]. Depending on the particular SCC*mec* type, these mobile islands peppered with IS elements, transposons and

integrated plasmids, can confer multidrug-resistance determinants that significantly diminish treatment options in a clinical setting. Thus, in addition to methicillin resistance, MRSA isolates have evolved multidrug-resistance leading to what the popular press refers to as an emerging superbug[19].

Paradigm-shift: The rise of CA-MRSA

After 1961, MRSA spread worldwide causing significant morbidity and mortality almost entirely as hospital-acquired infections. Advances in molecular epidemiology allowed for in depth analyses of MRSA spread and expansion at the evolutionary level. For instance, *spa*-typing (polymorphisms in Protein A coding sequence) and SCC*mec*-typing discriminated unrelated clones and identified clusters of related MRSA lineages responsible for disease[20,21]. Multi-Locus Sequence Typing (MLST) involves the sequencing of fragments from seven "housekeeping" genes (*arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi* and *yqiL*) yielding unique sequence types (STs)[22]. STs sharing identity at the majority of these loci are grouped into Clonal Complexes (CCs) encompassing related lineages of MRSA[23]. Another highly-discriminatory approach that can identify genomic rearrangements and insertions/deletions is Pulsed-Field Gel Electrophoresis (PFGE) whereby *Sma*I digested chromosomal DNA is separated and similarities in banding patterns reflect relatedness among lineages[24,25]. This allows for the classification of *S. aureus* strains into the now familiar PFGE-types USA100–1200. Employing these epidemiological approaches,researchers appreciated that most MRSA disease worldwide (nearly 70% of reported infections) was caused by five major CCs: CC5, CC8, CC22, CC30 and CC45[24,26] (Figure 1). CC5 includes clones belonging to the USA100 PFGE-type (*e.g.* SCC*mec*-II New York/Japan clone) the most common source of US hospital acquired MRSA as well as USA800 (SCC*mec*-IV Pediatric clone). CC8 includes the archaic, or original MRSA clones as well as the related Iberian clone, the SCC*mec*-III Brazilian/Hungarian clone and the SCC*mec*-IV USA500 clones. CC22 includes the EMRSA-15 clones that dominated hospital infections in the UK during the 1990s along with strains from CC30 encompassing EMRSA-16 as well as the USA200 PFGE type. Finally, CC45 consists of clones belonging to USA600 PFGE type (*e.g.* Berlin clone) that caused widespread MRSA hospital infections in northern Europe. In essence, after 30 years of investigation, the scientific community began to understand the population structure of the MRSA clones responsible for the majority of hospital-acquired disease. The source of high-virulence potential inherent to these five CCs was never fully appreciated before everything we knew about MRSA epidemiology changed at the turn of the century.

Initially reported in 1993, patients without any contact with healthcare settings contracted invasive MRSA infections in Kimberly Australia, a region in the northern part of Western Australia[27]. It was later discovered that simultaneously, strains related to these "community-acquired" MRSA (CA-MRSA) clones were causing serious and fatal respiratory infections in Chicago, again in patients without direct contact with hospital environments[28]. Prior to these reports, MRSA infections were exclusively associated with healthcare settings. These new clones belong to CC1 (USA400 PFGE type), a clonal complex unrelated to the five traditional Hospital-Associated MRSA (HA-MRSA) complexes[28]. CC1 clones spread quickly through Australia, the mid- and northwestern United States as well as Canada and Alaska where they still cause significant CA-MRSA disease[28–32]. Recent studies show that

USA400 can account for over 98% of MRSA infections in northern Canada[33] and has been implicated in isolated MRSA disease in southern Europe[34,35]. However, about 10 years ago a new source of CA-MRSA arose from one of the "traditional" virulent clonal complexes, CC8. Descending from a USA500 clone through acquisition of various mobile genetic elements (MGEs)[26,36], USA300 became the dominant CA-MRSA clone in US[14,37,38], effectively replacing USA400 clones in most regions[39,40], and has also been isolated from patients in Canada and Mexico[41,42]. The explosion of USA300 CA-MRSA across North America resulted from a very recent clonal expansion of a successful CA-MRSA clone as demonstrated by very low sequence divergence among geographically distinct USA300 isolates[43].

Given the occurrence of multiple CA-MRSA clones in the population, a formal definition was put forth by the Centers for Disease Control and Prevention for CA-MRSA disease as that which is contracted within 48 hours of hospital admission by patients not having recently undergone surgery, hemodialysis, prolonged hospitalization, catheterization or MRSA colonization[44]. Currently in the US, MRSA disease fitting these criteria is almost always caused by USA300 clones, followed by USA400 and occasionally USA1000 and USA1100[14]. To complicate matters further, USA300 clones have recently been implicated in causing significant HA-MRSA disease[38,45–47], blurring the lines between the two disease onset environments[38,45–47]. In some studies, USA300 accounted for at least half of hospital acquired MRSA infections[38,46]. Thus, USA300 represents a highly successful *S. aureus* clone that emerged in the community and quickly spread throughout the North American continent to become the leading cause of MRSA infection even in healthcare settings. For now, USA300 seems to be primarily limited to North America, while in Europe, South America and Asia CA-MRSA disease is dominated by divergent clones unrelated to CC8 (*e.g.* ST30, ST80 and ST59)[48]. Given the rapid and efficient transmissibility of USA300 in North America[49], it remains to be seen whether these clones will become the dominant source of MRSA disease worldwide.

USA300 Virulence

Animal models of *S. aureus* infection have repeatedly demonstrated the hypervirulence associated with USA300 compared to other MRSA strains[36,50–52]. USA300 strains exhibited enhanced production of dermonecrotic lesions in skin abscess models when compared to HA-MRSA clones[36,50,51] and USA300 was more lethal in a rat model of pneumonia compared with a USA400 isolate[52]. Furthermore, USA300 strains were more lethal in septic infections compared to archaic and Iberian clones as well as ST239 clones (Brazilian clones)[36]. When compared to other CA-MRSA clones, USA300 isolates generally exhibit increased virulence with the exception of ST80 and USA1000, which also possess enhanced virulence[51]. In contrast, nearly every clone of HA-MRSA tested was significantly less virulent than USA300 with the only exception being USA500 HA-MRSA[36,51]. This is of particular interest in that USA300 clones descended from USA500 via the acquisition of a prophage containing Panton-Valentine Leukotoxin (PVL), a mobile Arginine Catabolic Mobile Element (ACME) and enterotoxins K and Q (see below)[36]. Thus, the source of USA300 hypervirulence may have originally evolved in the HA-MRSA isolates belonging to USA500. However, for unknown reasons, despite exhibiting hypervirulence in animal

infection models, USA500 clones remain relegated to healthcare settings and do not cause significant CA-MRSA disease. Whether CA-MRSA USA300 clones exhibit hypervirulence in human disease has been difficult to directly discern, however, recent population based clinical data are beginning to corroborate conclusions drawn from laboratory animal model experiments.

In humans, USA300 *S. aureus* primarily causes skin infections of which, it can account for up to 98% of all MRSA presenting as skin/soft tissue infections to US emergency rooms[14]. In addition, USA300 can also cause more invasive disease such as bacteremia[53], endocarditis[54] and necrotizing fasciitis[55], a condition almost never associated with *S. aureus.* In particular, pulmonary infections caused by USA300 *S. aureus* can lead to aggressive and often fatal necrotizing pneumonia[56–58]. The populations most at risk for contracting USA300 CA-MRSA are military personnel[59], athletes[60–62], prisoners[63–65], African Americans[58,66], daycare attendees[67,68] and men who have sex with men[69]. Patients contracting CA-MRSA are, on average, younger than those with HA-MRSA and otherwise generally healthy[70,71]. Furthermore, CA-MRSA is often associated with worse clinical outcomes. For instance, USA300 infections were associated with increased in-hospital mortality and a higher occurrence of severe sepsis than HA-MRSA infections[66,72]. USA300-related strains were also more prone to spread from the initial infection site and caused more severe infections than HA-MRSA in patients suffering from pneumonia with pulmonary emboli[73,74]. However, other reports describe better clinical outcomes associated with USA300 infections[45,75]. Although some studies that reported more positive clinical outcomes with CA-MRSA also described hypervirulent CA-MRSA trends, such as increased risk of being admitted into intensive care, that merely lack full statistical significance (OR = 1.8, $p = 0.09$)[46]. Additionally, effective treatment, which is easier to achieve when treating CA-MRSA infections given their inherent susceptibility to clindamycin, tetracyclines, rifampicin and trimethoprim/sulfonamide, can reduce the severity of CA-MRSA disease outcomes in population-based studies[76]. Unfortunately, this trend of increased antibiotic susceptibility may be diminishing as new reports show increased antibiotic resistance among USA300 isolates, possibly through direct acquisition of resistance determinants from multidrug-resistant HA-MRSA strains[77]. Thus, the future clinical outlook appears grim with respect to USA300 infections given their increased prevalence in both hospital- and community-acquired infections, their propensity to acquire new antibiotic resistance determinants and the steady decline in positive clinical outcomes associated with USA300 infections.

Genetic Determinants Contributing to USA300 Success

Given the recent impact of USA300 on human health, significant research effort has been exerted to elucidate the source of USA300 success. Here we review these findings and broadly categorize them into three main classes: 1. Newly acquired genes that promote virulence and/or fitness, 2. Altered regulation of core genes resulting in elevated virulence and/or fitness and 3. Non-synonomous mutations in core genes that enhance virulence and/or fitness.

Newly Acquired Genes

Many different lineages of CA-MRSA (USA400, USA1000, and USA1100) cause outbreaks and invasive infections, but in North America, none are as prevalent as epidemic USA300. These clones have acquired many genes in the form of Mobile Genetic Elements (MGEs) that may confer a selective advantage over other CA-MRSA strains. Several groups have investigated many of these MGEs with the goal of elucidating factors (if any) that have contributed to the overwhelming success of USA300.

Enterotoxins K and Q

USA300 CA-MRSA isolates contain genes encoding enterotoxins K and Q (*sek*2 and *seq*2) in a unique pathogenicity island SaPI5 [78]. Sek2 and Seq2 are thought to contribute to pathogenesis by stimulating T-cells through binding of the Vβ chain of αβ T-cell receptors. Sek2 and Seq2 share 98% amino acid homology with enterotoxins (Sek and Seq) found on SaPI3 in *S. aureus* COL an archaic HA-MRSA clone belonging to ST250 that is less virulent than CA-MRSA isolates[79]. USA400 isolates (*e.g.* MW2) harbor νSA3, a pathogenicity island that shares similarity to SaPI3 of COL and SaPI5 of USA300, however, νSA3 does not contain the genes for Sek or Seq[78]. Thus, the acquisition of these toxins by USA300 and not US400 may potentially explain the differences in pathogenicity although direct demonstration of this has not been reported.

SCCmecIVa

The *mecA* gene encodes a penicillin-binding protein and is located on a mobile genetic element known as the Staphylococcal Cassette Chromosome *mec* (SCC*mec*). There are currently eight recognized SCC*mec* types (I VIII). SCC*mec* types I, II and III contain additional drug resistance determinants, whereas types IV, V, VI, and VII cause resistance only to β-lactams[80]. Initial sequence comparisons show that both USA400 and USA300 strains contain a nearly identical SSC*mec*IVa[78,81]. As it turns out, SCC*mec*IV is the most common form of SCC*mec* found across divergent *S. aureus* lineages in addition to ST8 (USA300) including ST1 (USA400), ST80, ST72 (USA700) and ST8 (USA500)[82,83]. It has been shown that SSC*mec*IV does not impose a fitness cost *in vitro* or *in vivo*, whereas acquisition of the SSC*mec* types I, II and III resulted in decreased *in vitro* growth rates[84–86]. Thus, it is thought that harboring SSC*mec*IV as opposed to other SCC*mec* types imparts CA-MRSA with an advantage in its ability to cause infection in healthy individuals. However, though SSC*mec*IV may provide a selective advantage to CA-MRSA over other SCC*mec* types, the fact that nearly all CA-MRSA isolates contain SSC*mec*IVa suggests that it is not a major contributing factor to the dominance of USA300 among CA-MRSA isolates.

Panton-Valentine Leukocidin (PVL)

The Panton-Valentine leukocidin (PVL) is a bicomponent pore-forming toxin that induces necrosis and apoptosis in leukocytes[87]. PVL is encoded by the genes *lukS-PV* and *lukF-PV* located on the prophage φSA2 *pvl*[78]. This phage is highly associated with CA-MRSA clones in that nearly all USA300, USA400 and USA1100 clinical isolates are positive for PVL as are many USA1000 strains[88,89]. Furthermore, epidemiological and clinical reports indicate a strong correlation between PVL production and severe skin/soft tissue infections, as well as

necrotizing pneumonia and fasciitis, suggesting PVL may be a major contributor to the virulence of CA-MRSA[90–92]. Moreover, PVL can be directly detected in human skin abscesses at levels known to result in rapid neutrophil lysis[93,94]. Thus, PVL is significantly correlated with invasive CA-MRSA disease, however, recent clinical studies demonstrate that CA-MRSA strains lacking PVL can still cause disease outbreaks[95–97].

Until recently, demonstrating a direct role for PVL in model disease has proven difficult. This likely stems from the host specificity of PVL in that it is rapidly leukocidal for rabbit and human neutrophils, but much less active against murine, rat or simian PMNs[98]. Consequently, a virulence effect of PVL in murine or rat pneumonia, sepsis and skin infection models has never been reproducibly defined[99–104]. Moreover, there was no demonstrable role for PVL in a pneumonia model involving nonhuman primates[105]. In contrast, using PVL susceptible rabbit models, isogenic USA300 strains lacking PVL were less virulent in pneumonia, osteomyelitis and skin abscess models[106–109]. However, the attenuation of mutants lacking PVL in rabbit skin lesions was not nearly as striking as a mutant lacking α-hemolysin or phenol-soluble modulin production underscoring the contributory nature of PVL towards *S. aureus* pathogenesis[108,110]. Furthermore, the nearly ubiquitous presence of PVL among CA-MRSA isolates clearly suggests that this toxin cannot explain the particular success of the USA300 lineage.

Arginine Catabolic Mobile Element (ACME)

Of all the genetic elements acquired by CA-MRSA isolates, only the arginine catabolic mobile element (ACME) is completely unique to USA300[78]. The type 1.02 ACME carried by USA300 is juxtaposed to the SCC*mec*IV island and was acquired from *S. epidermidis* through horizontal gene transfer via a mechanism likely involving the SCC*mec*-related CcrAB recombinases[78,84,111]. The physical linkage of ACME with SCC*mec*IVa is mirrored by an epidemiological linkage in that nearly all USA300 strains harboring SCC*mec*IVa also carry ACME, while USA300 clones with other SCC*mec* islands, with rare exceptions, do not[83,112]. The ACME of USA300 contains a complete arginine deaminase (*arc*) system that converts L-arginine to L-ornithine for both ATP and ammonia production. The island also encodes a putative oligopeptide permease, a zinc-containing alcohol dehydrogenase, and a spermine/spermidine acetlytransferase (SpeG) as well as several hypothetical proteins[78]. While a role for ACME in USA300 virulence was demonstrated in a rabbit sepsis model[84], no effect of ACME was observed in murine pneumonia or skin abscess models[113]. Thus, it has been proposed that ACME aids primarily in USA300 colonization, in part, through the Arc mediated ammonification of the acidic skin environment, though this has never been experimentally verified[84,114].

We have additionally observed a peculiar phenotype in *S. aureus* suggestive of a selective advantage afforded by the ACME cassette. Polyamines, including spermine, spermidine and putrescine are a group of polycationic compounds reportedly synthesized from L-arginine by all living organisms. Not only does *S. aureus* lack the ability to synthesize polyamines *de novo*, but spermine and spermidine are bactericidal to this organism at levels found within mammalian tissue[115,116]. Polyamine-sensitivity was apparent in all tested strains except those belonging to USA300, and in these isolates polyamine-resistance was dependent on

speG encoding a spermine/spermidine aceytltrasferase harbored on ACME. Could *speG* provide USA300 with a selective advantage by nullifying the staphylocidal effects of host polyamines? While no direct measure of host polyamine levels during *S. aureus* infections have been reported, several indirect lines of evidence may suggest that polyamines do affect the outcome of staphylococcal disease and/or colonization.

Upon wounding, the host response in the skin is proinflammatory and dominated by cytokines such as IL-1, INF-γ and TNF- α[117]. The resulting inflammation is mediated, among other effectors, by the production of reactive oxygen and nitrogen species, the latter of which, nitric oxide (NO·) is synthesized from L-arginine by the inducible NO·-synthase (iNOS, Figure 2). This enzyme competes for available L-arginine with host enzymes such as Arginase-1 (Figure 2) as well as with arginine-auxotrophic *S. aureus*[118]. Once tissue damage signals resulting from the primary inflammation outweigh pathogen-associated signals, the host response shifts away from proinflammatory mediators and initiates the profibrotic response[117]. This phase is dependent on the production of T_H2-like anti-inflammatory cytokines such as IL-4, IL-10, IL-13 and TGFβ and results in induction of host fibrotic response involving Arginase-1 expression. At this stage the L-ornithine produced by Arginase-1 can be converted to staphylocidal polyamines that will additionally promote fibroblast proliferation, collagen deposition and inhibition of inflammation (*e.g.* blocking iNOS translation)[119]. It therefore may be during this T_H2-dominant fibrotic phase that host polyamines exert their effects on invading *S. aureus* thereby selecting for ACME encoded SpeG. Indeed, inhibiting IL-4 signaling in mice increased organism burdens during *S. aureus* sepsis while INF-$\gamma^{-/-}$ mice (lacking robust inflammatory wound response) survived better than WT mice[120]. Thus, T_H2-dependent signaling, as opposed to an inflammatory T_H1 response, proved critical to the host's ability to control *S. aureus* infections. Recently, protection against chronic implant infections was also highly dependent on an effective T_H2/Treg response[121]. Furthermore, polymorphisms in the human IL-4 gene associated with reduced IL-4 production are significantly linked with increased *S. aureus* colonization[122]. These data are consistent with the T_H2 anti-inflammatory fibrotic response as being critical for controlling *S. aureus* infection. Whether this is directly due to the induction of polyamine synthesis has yet to be reported, but the acquisition of *speG*-encoding ACME would counter increased spermine levels in fibrotic tissue perhaps explaining the association of USA300 CA-MRSA with severe skin/soft tissue infections.

How do we reconcile a significant role for SpeG in *S. aureus* pathogenesis with the lack of a strong ACME phenotype in most model infections[84,113]? One explanation could be that the observed increase in α-hemolysin and Protein A expression upon ACME inactivation in USA300 could overcompensate for the resulting polyamine-sensitivity[84]. Another possibility is that the Arc operon on ACME actually drives excess polyamine production necessitating SpeG-mediated spermine detoxification. The Arc operon consists of genes that convert L-arginine to L-ornithine and CO2 while producing ATP and ammonia. The resulting L-ornithine is exchanged for extracellular L-arginine by the L-arginine/L-ornithine antiporter ArcD effectively converting extracellular L-arginine to L-ornithine. Thus, the Arc operon could skew the flux of host L-arginine away from iNOS towards polyamine synthesis rendering *speG* essential (Figure 2). Deleting all of ACME might allow the host to

partition available L-arginine towards NO·-production, an immune effector that *S. aureus* is known to effectively resist[123–125]. This is consistent with the presence of *speG* on ACME islands that harbor the auxiliary *arc* gene cluster (Figure 2). While this hypothesis could explain the modularity of ACME that results in Δ*speG* attenuation, it has several aspects that require experimental attention. First, all strains of *S. aureus* already encode an Arc operon on the core chromosome that could also result in excess host polyamine synthesis, yet SpeG is only associated with ACME-positive USA300 *S. aureus*. This could be explained by the fact that the chromosomal Arc operon is only expressed under conditions of low oxygen and low glucose and little is known about ACME Arc expression in *S. aureus*[126]. Second, a dominant MRSA clone of ST22 lineage in Irish hospitals harbors an ACME island with an *arc* gene cluster but appears to lack a *speG* homologue[112]. Another issue is that significant CA-MRSA disease in Latin America is caused by USA300 clones that lack ACME[127]. Thus, ACME may contribute to colonization and virulence, but it cannot fully explain the predominance of USA300 in CA-MRSA disease in North America.

Enhanced Virulence Gene Expression

S. aureus elaborates a wide variety of toxins and proteases that have proven critical for efficient dissemination, inflammation and disease progression[128–130]. For instance, α-toxin or α-hemolysin (Hla) is a potent heptameric pore-forming toxin known to be critical for virulence in nearly every tested disease model from skin lesions and endocarditis to murine mastitis[131–133]. Upon interacting with susceptible cells, which include leukocytes, keratinocytes, platelets and endothelial cells, it forms a 100 Å deep pore in the plasma membrane resulting in rapid cell lysis[134,135]. Recently, a number of reports have shown that Hla expression is highly elevated in USA300 clones compared with other *S. aureus* isolates[36,50–52]. Moreover, deletion of *hla* abrogates USA300 virulence in murine and rabbit skin lesion models as well as pneumonia[43,100,136]. However, it should be noted that *hla* mutants in almost any *S. aureus* background are attenuated[133,137–140], thus the loss of virulence in USA300 *hla* mutants is consistent with α-toxin in general being a critical pathogenicity factor to *S. aureus*. δ-toxin (encoded by *hld*) and related α-type phenol-soluble modulins (αPSMs) are amphipathic α-helical peptides with potent leukocidal and chemotactic properties[141]. They have been shown to be overproduced by CA-MRSA clones with respect to most HA-MRSA isolates[36,51,141]. Their abundant production is essential for full virulence in murine and rabbit skin models of infection as well as murine sepsis[108,141]. Interestingly, they have recently been shown to exert potent antimicrobial activity against multiple Gram-positive bacterial species[142]. This property may prove critical for efficient colonization of non-sterile sites such as skin and nasal passages, thereby providing CA-MRSA with a selective advantage during transmission. Finally, *S. aureus* expresses a number of secreted proteases that, while antagonistic to *in vitro* biofilm formation, likely mediate the breakdown of host fibrotic tissue synthesized to confine *S. aureus*-containing lesions thereby promoting bacterial dissemination and disease progression. As with α-toxin and αPSMs, USA300 clones are also known to excrete proteases in excess, potentially limiting the host's ability to control minor skin and soft-tissue infections[143]. Thus, several groups have consistently reported the robust expression of numerous virulence determinants in USA300 compared with other clinical isolates. It has therefore been hypothesized that this

over-production of toxins/proteases confers the selective advantage that explains the overwhelming success of USA300 clones. If true, the regulatory mechanisms explaining these virulence trait expression phenomena are poorly defined.

Agr Quorum Sensing System

S. aureus expresses a peptide-based quorum sensing system known as Agr for Accessory Gene Regulator[129,144]. Signaling is mediated through a peptide form of AgrD (processed by the combined activity of the AgrB endopeptidase and a type I signal peptidase, SpsB[145]) that stimulates the two-component system sensor kinase, AgrC. The resulting activation of the response regulator AgrA leads to induction of the *agrBDCA* operon as well as the divergently transcribed RNAIII. While RNAIII encodes δ-toxin, the RNA molecule itself mediates a significant proportion of Agr regulation by affecting the expression of α-toxin[146], protein A[147], repressor of toxins (Rot)[148] and others[149]. Active AgrA is also known to directly control the expression of other virulence determinants including the PSMs[150]. Thus, the reported overproduction of Hla, Hld and PSMs in USA300 clones may be explained by a hyperactive Agr system in these clones. Indeed, the RNAIII molecule was shown to be expressed to a higher level in USA300 clones than in other *S. aureus* isolates explaining the overabundance of δ-hemolysin production[51,52]. Additionally, the overactive USA300 Agr system was the source of excess PSM and protease production associated with these clones and was partially responsible for excessive Hla expression[50]. Consistent with these data, Δ*agr* mutants in USA300 are highly attenuated in murine sepsis, pneumonia and skin abscess models[50,108,151]. Though, given the importance of Agr in virulence gene regulation, it is not surprising that mutants exhibit such attenuation. Moreover, overproduction of PSMs was reported for USA400 CA-MRSA clones implying that the greater success of USA300 cannot be fully attributed to overactive Agr[51,141]. In fact, USA500 clones, thought to be ancestral to USA300 also exhibit phenotypes with hyperactive Agr as well as being highly virulent in murine model infections[36,51]. Thus, the high virulence potential of USA300, including high Agr-activity, likely evolved in the HA-MRSA clones belonging to USA500. Still, Δ*agr* mutants of USA300 are highly attenuated and exhibit no increased virulence relative to non-USA300 *agr* mutants underscoring its importance in the evolution of USA300[50].

SaeR Two-Component System

The *S. aureus* exoprotein expression (Sae) locus contains four genes, *saePQRS* the latter of which comprise a two-component regulatory system[152–154]. The response regulator/sensor kinase genes (*saeRS*) are preceded by genes encoding a membrane protein (SaeQ) and a lipoprotein (SaeP) of unknown function. All four genes are cotranscribed from a promoter that is strongly induced by active SaeR[155]. A second promoter drives the expression of *saeRS* alone and is modestly repressed by these regulatory gene products[155]. Activation of the Sae system seems to involve sensing changes in the overall integrity of the cell envelope and is highly stimulated by hydrogen peroxide and cationic peptides including α-defensins[155,156]. Active SaeR promotes the induction of a number of virulence genes in *S. aureus* through binding of a consensus sequence found upstream of promoters for *hla*, *sbi*, *efb*, *lukS*-PVL, *splA* and *saeP*[157]. Additionally, expression of β-hemolysin, fibrinogen-binding proteins, lactose catabolizing enzymes and the chromosomal arginine deiminase

operon are all highly affected by Sae[158]. It has been shown that SaeRS expression is higher in USA300 than in USA400 clones[52,155]_ENREF_52, which may be a result of overactive Agr system (see above) since RNAIII is known to positively regulate Sae expression[156]. Deletion of *saeRS* resulted in almost complete loss of Hla expression and a significant drop in PVL levels as well[151,157]. Moreover, Δ*sae* USA300 was attenuated in murine sepsis, peritonitis, dermonecrosis and pneumonia models[151,157–159]. This was surprising given that in USA400, Sae was only essential for sepsis and peritonitis and not for survival within skin abscesses[158,159]. However, USA400 clones do not induce the same level of dermonecrosis and do not express high levels of Hla as in USA300 infections[51,52]. Thus, it appears as though some of the hypervirulence attributed to USA300 clones in skin/soft tissue infections is likely due to Sae-mediated Hla overproduction. However, HA-MRSA USA500 clones also exhibit severe dermonecrosis during skin infections and overproduce Hla and PSMs yet have not disseminated as widely as USA300.

Source of overactive Agr

While it has not been directly tested, it is tempting to hypothesize that the overactive Agr system inherent to USA300 results in excessive PSMs and Sae expression, the latter of which leads to high Hla expression. However, the mechanism driving high Agr activity in USA300 is not defined. Agr activity can be modulated through the actions of a number of trans-acting regulators including SarA[160], Stk1[161], MgrA[162], SigB[143], CodY[163], CcpA[164], Sar-family proteins other than SarA[165–168], ArlRS[169], Rsr[161] and SrrAB[170]. Many of these regulators are presumed to affect Agr expression indirectly, however some (CodY[171], SrrA[172] and SarA[173]) have been shown to directly bind to the Agr locus. It is intriguing that many of these regulators are involved in modulating metabolic adaptation to various environments (CodY, CcpA, Rsr and SrrAB) given the apparent increase in fitness associated with USA300[174] (see below). Though any one of these or other unknown regulatory systems may be responsible for enhanced Agr activity in USA300, therefore investigations into strain-specific differences in activity among these regulators may prove enlightening. For instance, SarA positively affects Agr expression[160,175], and deletion of *sarA* in USA300 lead to drastic reductions in Hla and PSM levels[176,177]. However, recently it was demonstrated that the loss of cytolytic expoprotein expression in the Δ*sarA* mutant was attributed to the resulting overproduction of extracellular proteases and not due to altered exoprotein gene transcription[177].

While trans-acting regulators may prove to be major influences on USA300 Agr activity, cis-acting polymorphisms may also be involved. RNAIII transcripts among sequenced ST8 isolates are 100% conserved, but there is a single nucleotide polymorphism (SNP) 3 bp upstream of a known AgrA binding site within the RNAIII promoter that is only found among USA300 isolates. While this is the only SNP among ST8 and ST1 clones specific to USA300, other sites of variation exist when compared to USA100 and USA200 promoter sequences. SNPs in the Hla promoter were recently shown to drive its overexpression in bovine isolates by modulating SarZ binding[178]. It remains to be determined whether SNPs in the RNAIII promoter region of USA300 isolates affect expression leading to high Agr activity. Regardless of the mechanism behind hyperactive toxin production in USA300, it is important to remember that similar high-level expression is observed in the HA-MRSA

progenitor clone, USA500. Thus, while the high virulence potentials of USA300 and USA500 may result from overproduction of exoproteins, this phenomenon alone cannot fully explain the enormous success of USA300 in human disease.

Non-synonymous Mutations in Core Genes

The evolutionary forces that drive diversification in *S. aureus* have been recently examined, in part, due to the availability of more than 15 published *S. aureus* genome sequences. While a significant level of divergence is achieved through acquisition of mobile genetic elements (MGE), variability within the *S. aureus* core genome (~2000 orthologous genes shared among most *S. aureus* strains) is primarily generated through mutation[179,180]. The most common forms of mutation are single nucleotide polymorphisms (SNPs) or short insertion/deletions (indels) that have been estimated to be ~15-fold more attributable to *de novo* mutation than to recombination[179]. However, recent reports contend that the contribution of homologous recombination to core diversity in *S. aureus* may be underestimated[181]. Nevertheless, mutation is a significant driving force in *S. aureus* diversification allowing for evolutionary classification of strains into ST types (see above)[22]. Most SNPs are within coding regions reflecting the fact that ~80% of the core genome encodes protein[182]. Synonymous SNPs, those that do not result in amino acid changes, by far outweigh amino acid substituting non-synonymous SNPs in *S. aureus*[183–186]. This is likely because nonsynonymous mutations are more often detrimental and are therefore subject to evolutionary loss via purifying selection. Consequently, the relative ratio of nonsynonymous to synonymous substitution rate (dN/dS) among staphylococci is generally less than 1. In contrast, a recent report comparing the complete genome sequences of 10 newly isolated USA300 clones with the published FPRF3757 USA300 sequence revealed an unusually high ratio of nonsynonymous:synonymous SNPs (as high as 2.6:1, much higher than reported in comparisons of non-USA300 *S. aureus* lineages)[43]. This discrepancy can be rationalized by assuming a recent clonal expansion of the USA300 lineage such that new isolates still harbor nonsynonymous SNPs that have not yet undergone purifying selection[187]. To be sure, the unusually high dN/dS ratio of USA300 clones is inconsistent with evolutionary convergence among distantly related clones, an event that would only be consistent with normal to low dN/dS ratios if the converging progenitors were of sufficiently diverse origins[43].

It is important to note that overall low dN/dS ratios are not necessarily constant across all functional gene families. For instance, while housekeeping and metabolic genes generally exhibit low dN/dS ratios, genes encoding surface associated or secreted proteins can often have elevated dN/dS ratios[188,189]. This is indicative of forward selective pressures driving variability in these genes either to promote functional differences (*e.g.* an adhesin adapting to a host receptor molecule) or immune avoidance through changes in antigenicity. Indeed, comparisons among divergent *S. aureus* clones reveal higher dN/dS ratios for genes encoding components of the cell envelope and secreted proteins than genes encoding housekeeping or metabolic enzymes[182,184,185]. USA300 clones however seem to be an exception to this rule. A recent comparison of genome sequences from USA200, USA300 and a distantly related *S. aureus* strain revealed high dN/dS ratios indicative of forward selection in a large number of USA300 metabolic genes[190]. The largest subset of USA300

genes predicted to be under positive selection (45%) were involved with metabolism whereas only 7% encoded components of the cell envelope. This phenomenon cannot be explained by the fact that metabolic genes make up a large proportion of the core genome because this same study showed that in USA200, the most prominent class of genes undergoing positive selection were those encoding cell envelope components (a third of all genes with elevated dN/dS)[186,190]. An independent study verified that all of the metabolic genes in USA300 exhibiting forward selection were completely conserved among 10 sequenced USA300 genomes[43]. Moreover, data from this same study showed that, while relatively few SNPs were found among ten different USA300 genomes, genes encoding cell envelope proteins more commonly exhibited high dN/dS ratios (57% of all genes with multiple nonsynonymous substitutions)[43]. Thus, the peculiar overrepresentation of *S. aureus* metabolic genes among those undergoing positive selection is only evident when comparing USA300 with non-USA300 genomes implying that USA300 clones in general seem to be adapting to disproportionately high selective pressures at the metabolic level.

It is possible that the resulting adaptive mutations in the overall metabolism of USA300 directly contribute to the evolutionary success of this clone. For instance, it has been observed that USA300 clones simply grow faster than any other tested *S. aureus* isolate[174]. Taken together, it would appear that USA300 is more metabolically fit and/or adaptable than other *S. aureus* lineages. This may provide an advantage when competing for limiting nutrients with endogenous microflora as well as contribute to severe disease given a rapid growth rate within sterile sites of the body. Further inspection in our laboratory revealed that USA300 clones have growth advantages when metabolizing many different carbon sources (Table 1). In general, USA300 clones exhibited higher growth rates than other clones when cultivated on nutrients that are abundant in human sweat and skin[191], consistent with the high prevalence of skin/soft tissue infections associated with USA300 clones. But, can a relatively small set of amino acid changes in metabolic genes really account for such drastic growth differences? Laboratory adaptation of *E. coli* to growth on lactate resulted in strains that exhibited nearly twice the growth rate on lactate alone[192]. These adapted strains exhibited major alterations in metabolic flux capacity through gluconeogenic and pyruvate catabolic pathways, yet none of these changes were due to altered gene expression. This would be consistent with subtle changes in protein sequence (nonsynonymous SNPs) that alter enzyme activity or response to allosteric regulation. Furthermore, a laboratory adapted clone of *Caulobacter crescentus* exhibited a ~20% greater growth rate than its progenitor strain and this entire phenotype was explained by a single SNP altering the expression of glucose-6-phosphate dehydrogenase (*zwf*)[193]. This enzyme controls the primary flux between energy generating glycolysis and the precursor generating pentose-phosphate pathway (PPP). It was shown that lower flux through PPP with concomitant increased glycolytic activity lead to higher growth rates in lab-adapted *C. crescentus*[193]. Interestingly, one of the very genes exhibiting signs of positive selection in USA300 was *zwf* along with two glycolytic genes (*pgm* and *pfkA*) potentially linked to the USA300 growth advantage on numerous carbon sources[190]. Whether or not SNPs within these metabolic genes account for enhanced USA300 growth rates and whether that contributes to the success of this clone remain to be proven, however the unusual SNP distribution among metabolic genes in

USA300 combined with its enhanced growth rate suggest there may be more to USA300 virulence than newly acquired or overexpression of virulence genes.

Conclusions

The overwhelming success of USA300 in North America as the dominant source of CA-MRSA infections represents a fascinating example of a pathogenic variant emerging as a new threat to human health. The adaptations acquired by USA300 clones in the form of novel genetic components, altered gene regulation and sequence polymorphisms likely act in concert to provide these strains with a selective advantage. It appears as though USA300 hypervirulence, as assayed in animal models of infection, correlates with increases in virulence gene expression and is apparent in HA-MRSA progenitors as well as other unrelated CA-MRSA lineages. Whether this is due to hyperactive Agr resulting in elevated PSM production and Sae expression (which in turn could lead to excess Hla and other exoprotein excretion) remains to be proven. In contrast to overt virulence, traits that affect transmission and colonization efficiency are inherently difficult to model in the laboratory. It may prove, however that this aspect of USA300 biology is as critical to its success as is high virulence potential. It remains to be determined whether newly acquired genetic components (*e.g.* ACME) and/or sequence polymorphisms contribute to the rapid transmission and success of USA300 in the community. In the end, we may appreciate that none of the three evolutionary events (gene acquisitions, altered gene regulation, protein sequence divergence) outlined here can alone explain the success of USA 300. Rather, the amalgamation of all these events created the highly successful pathogen that we must contend with today.

Acknowledgments

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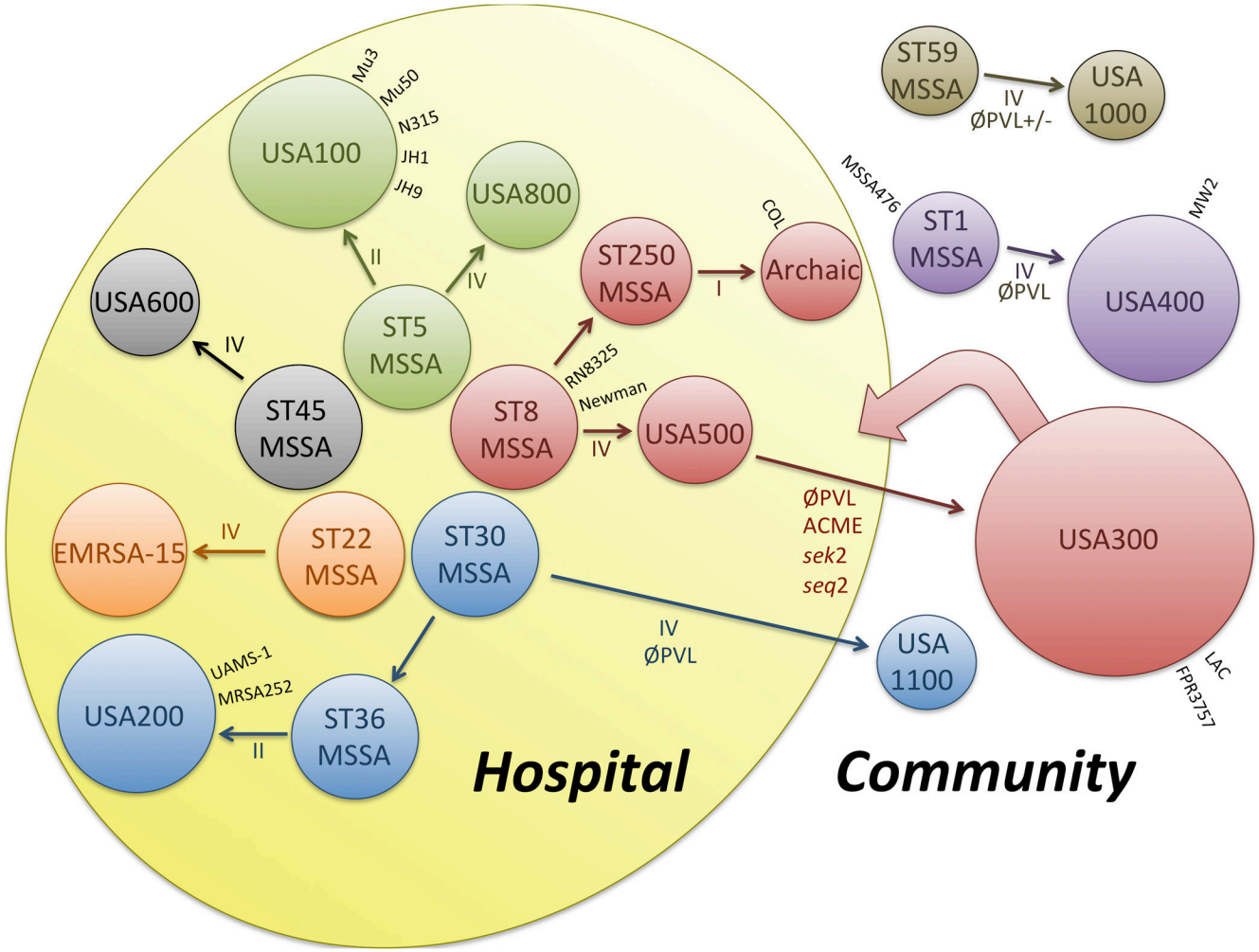

Figure 1. Schematic representation of the evolution of MRSA

Sequence Types (STs) belonging to established Clonal Complexes (CCs) are colored as follows: CC1, purple; CC5, green; CC8, red; CC22, orange; CC30, blue; CC45, black. ST59 has not been assigned to a CC. Roman numerals reflect acquired SCC*mec* type. Commonly used *S. aureus* strains are depicted around their relevant ST symbol.

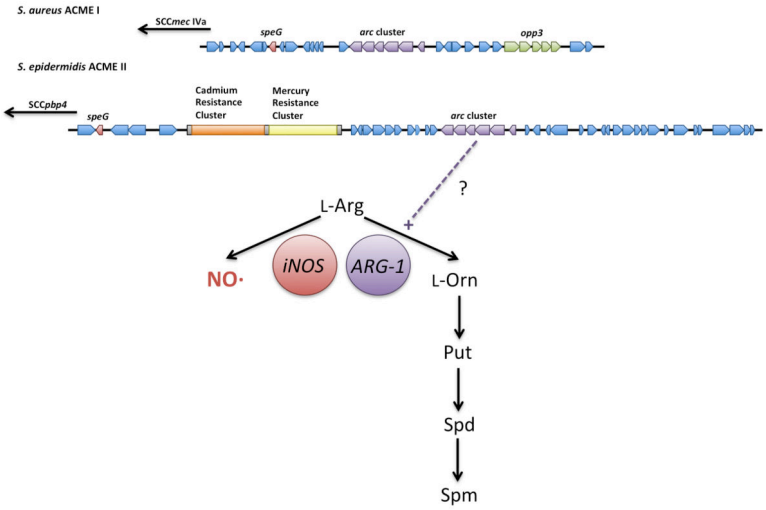

Figure 2. Association between *arc* gene cluster and *speG* in ACME. TOP

ACME type I, found in USA300 *S. aureus* and also found in many *S. epidermidis* isolates, and ACME type II, found primarily in *S. epidermidis*, both harbor *arc* gene clusters as well as *speG*. ACME type III (not shown) lacks an identifiable *arc* gene cluster but does contain an *opp*-3 locus. **BOTTOM:** Fate of host arginine depends on competition between iNOS and Arginase-1 enzyme activities. The net production of ornithine by Arc-expressing *S. aureus* may skew the fate of host arginine down the polyamine synthesis pathway thereby necessitating *speG*.

Table 1

Maximal Growth rates of *S. aureus* strains on various carbon sources

Rates (μ, h^{-1}) were calculated from at least 4 independent curves and mean ± S.D. are reported. Chemically defined medium[194] was used varying only the primary carbon source. Red font indicates rates significantly lower than those of USA300 strain SF8300 ($p \leq 0.05$, 2-tailed Students T-test).

	0.5% Glucose	1.0% Lactate	1.0% Pyruvate	1.0% Glycerol	1.0% Cas A.A.	1.0% Tryptone
SF8300 (USA300)	0.92 ± 0.02	0.60 ± 0.01	0.60 ± 0.01	0.90 ± 0.03	0.74 ± 0.02	0.65 ± 0.02
LAC (USA300)	0.89 ± 0.03	0.57 ± 0.02	0.57 ± 0.01	0.89 ± 0.03	0.72 ± 0.01	0.63 ± 0.02
Newman (ST8 MSSA)	**0.71 ± 0.01**	**0.43 ± 0.02**	**0.46 ± 0.02**	**0.73 ± 0.02**	**0.43 ± 0.02**	**0.36 ± 0.01**
MW2 (USA400)	0.85 ± 0.04	**0.41 ± 0.02**	**0.52 ± 0.02**	**0.76 ± 0.04**	0.75 ± 0.02	**0.51 ± 0.05**
UAMS-1 (USA200)	**0.73 ± 0.02**	**0.48 ± 0.01**	**0.52 ± 0.01**	**0.79 ± 0.01**	**0.57 ± 0.01**	**0.68 ± 0.02**