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Looking toward basic science for potential drug discovery targets against community-associated MRSA

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Abstract

The difficulties to find a conventional vaccine against *S. aureus* and the increasing resistance of *S. aureus* to many antibiotics demand the exploration of novel therapeutic options, such as by targeting virulence determinants and using specific antibodies in an antitoxin-like approach. Community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) strains have recently emerged predominantly in the U.S., causing epidemic outbreaks of mostly skin and soft tissue infections, but also more dramatic and sometimes fatal diseases. CA-MRSA is now the most frequent cause of death by a single infectious agent in the U.S. The fact that at least in the U.S., CA-MRSA infections are almost entirely due to one sequence type, USA300, gives researchers a novel, unique chance to focus on one clone in their efforts to analyze pathogenesis in a clinically important *S. aureus*. While the molecular underpinnings of the exceptional virulence and transmissibility of USA300 are not yet well understood, recent findings indicate that increased expression of widespread virulence determinants and acquisition of mobile genetic elements have to be considered. Delineating the relative importance of virulence determinants in USA300 and other important clinical strains is a key endeavor needed to develop a potential antitoxin for CA-MRSA disease.

Keywords

Community-associated methicillin-resistant *Staphylococcus aureus*; antibiotic resistance; virulence

Introduction

S. aureus is an important pathogen. Treatment of *S. aureus* infections is complicated by antibiotic resistant strains, most notably those with resistance to penicillin and β -lactamase-resistant β -lactam antibiotics^{1–3}. The recent occurrence of community-associated methicillin-resistant *S. aureus* (CA-MRSA) in addition to the widespread problem of MRSA in hospitals, has underlined the high urgency to find novel treatment options for drug-resistant *S. aureus*^{4,5}. Drug development has extended far beyond simple screens for substances inhibiting in vitro pathogen growth. Modern development of drugs targeting virulence is based on extensive knowledge of bacterial virulence mechanisms⁶. Here, I will first describe the physiology, virulence mechanisms, and virulence determinants of *S. aureus*, MRSA, and CA-MRSA, and then explain how basic pathogenesis research helps to develop drugs against *S. aureus* and in particular, CA-MRSA.

S. aureus

Members of the genus *Staphylococcus* are frequent commensal microorganisms on the epithelial surfaces of animals and humans⁷. Staphylococci have their name from the Greek

word “staphylos”, meaning grape, and “kokkos”, meaning berry, describing their microscopic appearance in grape-like clusters that may vary in size from strain to strain. Many strains are opportunistic pathogens. In contrast, the most virulent species *S. aureus* may cause disease in otherwise healthy patients. It has the ability to coagulate blood using an enzyme called coagulase and on that basis, is systematically distinguished from most other, coagulase-negative staphylococcal species such as *S. epidermidis*⁸. The species designation “*aureus*” stems from the Latin word for “golden” and describes the frequently yellowish color of many clinical *S. aureus* isolates.

A colonizer of humans

S. aureus can be a permanent (10–20%) or transient asymptomatic colonizer of humans; and some individuals never carry *S. aureus* (~ 50%)^{9,10}. The reasons for these differences that suggest underlying host genetic factors are unknown. Most staphylococcal species that colonize humans have some specificity for the area of the body they colonize. *S. aureus* commonly resides in the nose, although it may also be found in other areas such as the intestine, axillae and groin. Most likely, genetic factors determine preference for the colonization sites, providing bacteria with mechanisms to better cope with dryness, changing temperature, mechanical stress, innate host defense mechanisms, and so on, all of which can vary dramatically between different areas of the human body. Additionally, it has been suggested that bacterial interference between staphylococcal species, based on secreted bacteriocins or other signals, may play a role in the differential colonization of body areas, but there is no direct evidence for this^{11–13}.

A pathogen

S. aureus is by far the most important pathogen among staphylococci. It is a very frequent cause of rather uncomplicated skin infections, but can cause serious diseases that may be fatal¹⁴. *S. aureus* invasive infection begins with the breach of the skin and the invasion of bacteria into the bloodstream. *S. aureus* bacteremia is a condition with a high rate of mortality (11 to 43 %) and may be further complicated by endocarditis, sepsis syndrome, or metastatic infection. *S. aureus* is one of the most common cause of endocarditis and the most frequent cause of nosocomial and prosthetic-valve endocarditis, diseases with an extremely high mortality rate. Some bacteremic conditions may progress to sepsis, with risk factors including age, immunosuppression, chemotherapy, and invasive procedures. Metastatic infections such as in the joints, kidneys, and lungs, may serve as a reservoir for recurrent infections.

S. aureus isolates differ significantly in their genetic composition with regard to mobile genetic elements that often carry genes encoding superantigenic and other toxins¹⁵. Many of these toxins may cause serious diseases, for example the toxic shock syndrome that is mediated by the superantigenic toxin, toxic shock syndrome toxin 1 (TSST-1)¹⁶. While mostly related to tampon use in the 1980s, the percentage of non-menstrual cases of *S. aureus* toxic shock syndrome has increased ever since. All pyrogenic superantigenic toxins function by binding to the invariant regions of MHC class II molecules, causing an extreme activation of T cells, an excessive expansion of the clonal T cell population, and rapid-onset disease characterized by high fever and multiorgan dysfunction¹⁷.

S. aureus and coagulase-negative staphylococci are by far the most frequent pathogens involved with infections of indwelling medical devices¹⁸. Contamination of such devices from the skin of the patient or health care workers is a very common source of *S. aureus* introduction into the human body¹⁹. It is largely due to these infections and the extraordinary capacity of staphylococci to colonize any type of surface of catheters,

prostheses, etc. that *S. aureus* is one of the most infamous and widespread nosocomial pathogens.

Attachment and biofilms

The ability of *S. aureus* to adhere to host tissue is an important prerequisite for the asymptomatic colonization of body surfaces as well as the establishment of an infection. In some strains, the formation of large, multi-layered clusters called biofilms further promotes persistence²⁰⁻²¹. *S. aureus* surface-bound proteins in charge of mediating attachment to host tissue have been coined MSCRAMMs (for Microbial surface components recognizing adhesive matrix molecules) and show extreme functional redundancy²²⁻²³, underscoring their importance for *S. aureus* physiology and pathogenesis. While attachment to the plastic or metal surfaces of indwelling medical devices may proceed directly, and in this case is most likely mediated by bacterial cell surface hydrophobicity, extracellular human matrix proteins cover the devices soon after insertion and thus, device colonization is believed to be mainly dependent on MSCRAMMs²¹.

After attachment is established, several *S. aureus* strains have the capacity to form biofilms using molecules with cell-cell adhesive properties. Biofilm formation represents an extreme advantage for the bacteria, because the efficacy of antibiotics and innate host defense mechanisms, such as antimicrobial peptides (AMPs) and leukocyte phagocytosis, is severely impaired against bacteria in biofilms²⁴⁻²⁵. Thus, biofilm-associated infections are extremely difficult to eradicate and represent one of the most challenging problems for drug development. Factors involved in cell-cell adhesion and formation of the extracellular biofilm matrix comprise a series of secreted molecules, such as protein, carbohydrate and nucleic acid polymers. Many of these may have primary functions in other processes, such as DNA that may be released to the extracellular milieu and aggregates cells owing to its polyanionic character²⁶. In contrast, the N-acetyl glucosamine polymer polysaccharide intercellular adhesion (PIA, or PNAG) is believed to represent a polymer specifically dedicated to cell-to-cell adherence²⁷⁻²⁸. It is important to stress that life in a biofilm represents the normal mode of growth for many bacteria, including biofilm-forming *S. aureus*. Biofilms have gene expression profiles vastly different from cells grown in planktonic mode²⁹⁻³⁰. On the other hand, the formation of biofilms is an enormously complex phenotype impacted by many subtle changes in bacterial physiology and gene expression.

Immune evasion

S. aureus produces a wide repertoire of molecules that undermine the efficiency of innate and acquired immune responses³¹. The chemotaxis inhibitory protein CHIPS inhibits chemotaxis and thus the attraction of immune cells by secreted bacterial products³². The surface protein Eap binds to ICAM-1 on the surface of endothelial cells, ultimately preventing leukocyte adhesion³³. Once professional phagocytes have found the bacteria despite these mechanisms, several strains are well-protected from phagocytosis by a capsular polysaccharide that is produced in different serotypes by many clinical *S. aureus* isolates³⁴. The biofilm exopolysaccharide PIA has a similar function in sheltering from recognition by immune defenses²⁸⁻³⁵. Conversely, there are mechanisms for both capsule and PIA to be recognized by immune cells, exemplifying the hide-and-seek interplay between bacteria and host during evolution³⁶. Protein A is an *S. aureus* surface protein with an even more sophisticated mechanism of hiding from phagocytosis. By interacting with the invariant part of IgG antibodies, protein A localization on the staphylococcal surface produces a coat of non-specific IgG around the bacteria that protects from specific immune recognition³⁷. In addition, *S. aureus* has a wide array of factors, such as SCIN and the

fibrinogen-binding protein Efb, that interfere with complement³⁸, a family of proteins that directly kill bacteria or regulate other effectors of the immune response. Finally, *S. aureus* may invade and persist in host cells such as macrophages³⁹. However, in contrast to well-studied intracellular pathogens, the role of intracellular survival for pathogenesis has not yet been sufficiently evaluated in the case of *S. aureus*.

AMPs are an evolutionarily old mechanism of innate host defense, whose importance for human immune defense has recently been recognized⁴⁰. AMPs contribute to our immune defense as parts of the bactericidal products released into neutrophil phagosomes⁴¹. Additionally, they represent likely the most crucial part of host defense on epithelial surfaces⁴². *S. aureus* has many mechanisms that protect from AMPs, including secreted proteases, alteration of the charge of the cell membrane or surface, and expulsion of AMPs from their frequent target, the cell membrane, by dedicated exporters⁴³. Interestingly, these mechanisms are inducible by binding of AMPs to an AMP sensor protein in the staphylococcal cytoplasmic membrane^{44,45}.

Toxins that lyse immune cells are believed to represent some of the most crucial weapons that *S. aureus* uses to undermine our immune defenses. *S. aureus* strains may produce a vast variety of toxins that attack red and white blood cells. Some are quite specific for a certain cell type while others lyse all leukocyte types or even a wider array of cells. The molecular underpinnings of target cell specificity and putative receptor-mediated interaction of these toxins are unknown. Many lytic toxins belong to the pore-forming β -barrel leukotoxins, which may be monomeric or composed of two different subunits⁴⁶. The archetype of the group is α -Toxin, which has long been recognized as a crucial virulence factor of *S. aureus*⁴⁷. It is a 33 kDa protein that integrates into the cytoplasmic membrane of several cell types including monocytes, platelets, and erythrocytes, to form large, heptameric, ionophoric pores. There is significant species specificity with regard to the lytic activity toward some cell types. Human erythrocytes are more resistant than for example those from rabbits. Importantly, human lymphocytes and neutrophils have a pronounced natural resistance to α -Toxin⁴⁸. However, α -Toxin causes adhesion of neutrophils to endothelial cells, a crucial step in inflammatory reactions⁴⁹. The pro-inflammatory activities of α -Toxin are manifold. For the most part, they are due to cytokine release from lysed cells, but also include activities at sub-lytic concentrations. Other members of the β -barrel leukotoxin family are the bicomponent toxins γ -toxin, Panton-Valentine leukocidin (PVL), leukocidin D/E and the PVL-like leukocidin M/F⁵⁰. There is significant amino acid sequence similarity between these toxins. Notably, whereas α -Toxin is present in virtually all *S. aureus*, and γ -Toxin in most, PVL and the other β -barrel leukotoxins are only found in a minority of *S. aureus*. All known leukotoxins except the phenol-soluble modulins (PSMs) described in the following are encoded on mobile genetic elements.

The recently discovered core-genome encoded PSMs represent a key mechanism of all *S. aureus* strains to circumvent elimination by neutrophils and likely other phagocytes⁵¹. PSMs occur as short ~ 20–25 amino acid peptides (α -type) or somewhat longer, ~ 45 amino acid peptides (β -type). Leukolytic activity is much more pronounced in the α -type peptides, which comprise 4 peptides encoded in the *psma* operon (PSM α 1 through α 4) and the δ -Toxin. PSM α 3 is by far the most potent leukolytic PSM. PSMs have been shown to lyse neutrophils and with somewhat lower efficiency, red blood cells. They have a pronounced amphipathic, α -helical structure, which is a common feature of small, pore-forming peptides that do not require receptor-mediated interaction for lytic activity. In fact, the δ -Toxin has been frequently used as a model pore forming toxin in artificial membranes⁵². In addition, PSMs have chemotactic function and elicit the secretion of cytokines in neutrophils⁵¹.

The role of colonization for disease

Why do only some people carry *S. aureus* in their nose? Why do only some carriers develop *S. aureus* infection? These are important questions and the fact that we are not able to answer them satisfactorily reflects that the role of host factors in *S. aureus* infection is almost completely unknown⁵³. More recent findings on host factors influencing nasal colonization gave first insight into host susceptibility, but many analyzed candidates showed no or only a very small impact on *S. aureus* colonization⁵⁴. However, we have some information as to how colonization impacts the chances to develop disease. Nasal carriage of *S. aureus* predisposes for blood-borne, surgical, and other nosocomial infections^{9,55}, while colonization is inversely correlated with more severe manifestations of *S. aureus* disease⁵⁶. In addition, we know more about bacterial genes that impact nasal colonization. These include the cell-wall associated protein clumping factor B (ClfB) that mediates attachment to epithelial cells and the surface exopolymer teichoic acids⁵⁷.

Antibiotic resistance

The development of antibiotic resistance in *S. aureus* is likely the best studied and most famous example of bacterial drug resistance. Penicillin was discovered by Alexander Fleming in 1929 and soon regarded as a wonder drug after its introduction into use in 1941. However, in the Hammersmith Hospital, London, penicillin resistance in *S. aureus* was at 13% in 1946, and had reached 59% in 1948⁵⁸. Clearly, already in the 1950s, one decade after it had first been used, penicillin was not an efficient drug for the treatment of *S. aureus* infections any longer. Nowadays, we experience similar timelines of resistance development after the introduction of an antibacterial substance, particularly if administered widely as a broad-spectrum antibiotic. *S. aureus* has acquired resistance to most antibiotics currently in use⁵⁹. This includes the glycopeptide vancomycin. Strains with slightly elevated levels of vancomycin resistance are quite frequent⁶⁰. In contrast, high-level resistance to this antibiotic of last resort, which is mediated by the *van* genes that *S. aureus* has likely acquired from enterococci, is very infrequent⁶¹⁻⁶². Most likely, presence of these resistance genes comes with a very high fitness cost to the bacteria, particularly in methicillin-resistant *S. aureus* (MRSA) strains owing to the molecular incompatibility of high-level methicillin and vancomycin resistance⁶³.

MRSA

In contrast to vancomycin-resistant *S. aureus*, strains with resistance to methicillin and other β -lactamase resistant β -lactam antibiotics are widespread. These antibiotics were developed after the spread of penicillin-resistant strains in the 1950s and introduced in 1959. Within a remarkable short time of only about a year, the first methicillin-resistant strains were detected in the United Kingdom⁶⁴. There was a first, large outbreak of MRSA in the U.S. in the 1960s and in the 1980s, MRSA was a global problem. Interestingly, most MRSA infections have been caused by a small subset of clones with apparent superior ability to spread and cause disease³. MRSA likely represents the most infamous nosocomial pathogen and the most important threat to public health systems in the U.S., Europe, Japan and other highly industrialized regions, costing more than \$10 billion / year in the U.S. alone. MRSA infection can be severe and occur in 31.3/100,000 people, 20% of which are fatal⁶⁵. The genetic information that confers methicillin resistance (*mecA* gene) and encodes a penicillin-binding protein with decreased affinity for β -lactam antibiotics is encoded on mobile genetic elements (SCC*mec* cassettes) that vary in length and composition⁶⁶. Often, these elements also carry other resistance genes, making antibiotic treatment even more difficult. Importantly, the fitness cost inferred by these elements – particularly the short SCC*mec* type IV – is relatively minor⁶⁷⁻⁶⁸, which is one reason for the rapid spread of MRSA. SCC*mec*

elements can be easily transferred between strains and have most likely originated from coagulase-negative staphylococci⁶⁹.

CA-MRSA

Traditionally, MRSA infections have occurred predominantly in hospitals. However, more recently there has been an increasing incidence of community-acquired (or community-associated) MRSA (CA-MRSA) infections⁷⁰. In 1999, 4 cases of fatal CA-MRSA infections were reported in children in Minnesota and North Dakota^{4,71}. Ever since, particularly the U.S. has experienced a large epidemic outbreak of CA-MRSA, which now represent the source for the majority of all skin and soft tissue infections being reported to U.S. emergency departments⁷². It has been calculated that MRSA strains cause more fatal invasive infections per year in the U.S. than HIV/AIDS⁶⁵. This is largely caused by the increase of CA-MRSA, adding to the number of hospital-acquired (HA-) MRSA infections, which have been relatively constant in number over the last years⁷³. CA-MRSA infections occur in healthy individuals and frequently are reported in risk groups that may be characterized as having close body contact paired with potentially low hygiene, including high school children, sports teams, prison inmates, army recruits, men who have sex with men, etc⁷⁴. Most CA-MRSA infections present as mild to moderate skin infections, but the number of serious invasive infections appears to be higher with CA-MRSA than hospital-associated MRSA (HA-MRSA) and includes necrotizing fasciitis in adults⁷⁵ and the Waterhouse-Friderichsen syndrome in children⁷⁶.

Although the U.S. has so far been hit most severely, CA-MRSA is a global problem⁷⁷. Interestingly, the clone that causes almost all CA-MRSA infections in the U.S., sequence type USA300 (ST8), is not (yet?) as prevalent in other countries. There, CA-MRSA infections are caused by different clones, such as ST80 in Europe, which are not closely related to USA300 and have not spread in a large epidemic. However, in Germany for example, USA300 already represents the second most frequent CA-MRSA clone⁷⁸, indicating that global CA-MRSA epidemiology might be changing toward a higher incidence of USA300 infections.

Transmissibility and virulence

The USA300 epidemic is characterized by two traits that distinguish this clone from many other MRSA, particularly because they are combined in one strain: the capacity to spread easily and sustainably in the population and that to cause exceptionally severe disease⁷⁷. We are only beginning to understand the molecular underpinnings of this extraordinary phenotype. There has not always been a clear distinction between transmissibility and virulence, which were often paired in the ambiguous term “success” (as a pathogen). We know that HA-MRSA strains have caused epidemic outbreaks⁷⁹, despite being incapable of infecting healthy individuals without predisposing risk factors, indicating that they are highly transmissible but not as virulent as the emerging CA-MRSA strains. Furthermore, there are extremely virulent strains – such as strong producers of certain toxins – that have not spread and caused epidemics comparable to that seen with USA300⁸⁰. To distinguish clearly between transmissibility and virulence may not be important in pathogens that rapidly cause disease. In contrast, in the case of *S. aureus*, which may colonize asymptomatically for extended periods before the outbreak of disease, this distinction is crucial for efforts to evaluate categorically different approaches to develop intervention measures. Namely, while development of drugs targeting virulence needs an evaluation of virulence factors that form the molecular basis for the exceptional disease severity in USA300 infections⁶, target-oriented decolonization strategies will have to target molecules

that make USA300 a good colonizer. Only in rare cases are the molecular factors responsible for virulence and colonization/transmissibility expected to be the same.

Pathogenesis of CA-MRSA

The pathogenic capacity of *S. aureus* is best reflected using animal infection models, and in vitro assays to assess immune evasion properties and the production of specific virulence determinants such as toxins. In animal infection models, CA-MRSA strains are more virulent than HA-MRSA strains. Voyich et al. for example compared the LAC (USA300) and MW2 (USA400) CA-MRSA strains in a mouse bacteremia model and found significantly higher morbidity and mortality compared to some common HA-MRSA strains, COL (USA500) and 252 (USA200)⁸¹. In the same study, it was shown that the capacity of the investigated CA-MRSA strains to lyse human neutrophils, the main cell type to eliminate invading *S. aureus*, is higher than in HA-MRSA strains. Furthermore, according to recent results achieved in my laboratory, USA300 causes significantly greater lesions in a mouse abscess model than most other MRSA strains of the same clonal complex, CC8, such as COL82. Moreover, USA300 appears to be distinguished from other MRSA by an exceptional expression of key virulence factors. The basis of the extraordinary pathogenic potential of USA300 and other CA-MRSA is a matter of much debate. In the following, I will therefore discuss what we currently know, with a focus on the best studied USA300.

Panton-Valentine Leukocidin (PVL)

The role of PVL in the pathogenesis of CA-MRSA is likely the most controversial and heavily discussed issue in CA-MRSA research. This is due to the fact that the striking epidemiological correlation of the PVL-encoding genes with CA-MRSA skin infections first suggested an important function of this toxin in CA-MRSA pathogenesis^{83,84}. However, animal and in vitro experiments did not confirm a significant impact of PVL on CA-MRSA disease^{77,85–89}.

Genetic deletion of the PVL-encoding genes in CA-MRSA strains and comparison with the corresponding isogenic wild-type strains in skin^{86,88}, bacteremia⁸⁶, and pneumonia mouse^{87,88}, and pneumonia rat⁸⁹ models did not result in any impact on CA-MRSA disease and thus, these experiments indicated that there is no significant role of PVL in CA-MRSA pathogenesis (Tab. 1). One study looked at different time points during the development of murine bacteremia and found that the effect of PVL was modest and only transient, but confirmed the absence of an effect at the endpoint of the study⁸⁵ (Tab. 1). Only one recent study is at variance with these reports, indicating a role for PVL in lung and skin and soft tissue infections⁹⁰.

While not directly addressing its role in CA-MRSA, Labandeira-Rey et al. investigated the contribution of PVL to pathogenesis in laboratory strains⁹¹. Although many comparisons in that study are hard to interpret, as they were not made between isogenic strains, one isogenic comparison indicated that PVL impacted the outcome of pneumonia in mice. In my laboratory, we were not able to reproduce these results and found that the effects attributed to PVL in that study were caused by using a strain harboring an unintended mutation in the global regulatory system *agr*⁹². This also includes the alleged gene regulatory effect of PVL that could not be confirmed using isogenic deletion constructs in CA-MRSA strains⁸⁵.

PVL is known to lyse human neutrophils⁹³. However, comparing *lukSF* (encoding PVL) gene deletion with isogenic wild-type strains for their capacity to lyse human neutrophils indicated that PVL does not significantly influence this major part of *S. aureus*' immune evasion strategy⁸⁶. It remains to be elucidated whether the strong inflammatory reaction seen when having mice inhale purified PVL toxin⁹¹ stems potentially from a secondary

immunological reaction rather than from a direct leukolytic effect. However, results from a recent rat pneumonia study indicate that there is no significant difference in the expression of host inflammatory genes after infection with a PVL+ versus an isogenic PVL– USA300 strain⁸⁹. In summary, one can conclude from recent investigation that the overall impact of PVL to CA-MRSA pathogenesis is minor.

α-Toxin

α-Toxin was shown to be a key virulence determinant in CA-MRSA-induced pneumonia in mice⁸⁷ (Tab. 1). Additionally, antibodies against α-toxin protected from mouse morbidity and mortality⁹⁴. Importantly, parallel investigation of PVL in the same model demonstrated that isogenic PVL-negative strains did not differ in the progression of pneumonia; nor did antibodies to PVL have an impact on disease development^{87,94}.

Phenol-soluble modulins (PSMs)

While most *S. aureus* strains produce PSMs, PSM production in CA-MRSA strains is higher than in HA-MRSA strains⁵¹. USA300 *psma* deletion strains have a dramatically diminished capacity to form skin abscesses in a mouse model, and a much lower mortality rate than wild-type USA400 in a bacteremia model⁵¹ (Tab. 1). Furthermore, PSMα expression in the standard HA-MRSA strain 252, which has a very low original capacity to lyse neutrophils, led to neutrophil-lytic capacity similar to that observed in USA300 and USA400⁵¹, further confirming the key role of PSMs in this major immune evasion property of *S. aureus*.

ACME

Analysis of the USA300 genome revealed the presence of a novel mobile genetic element of 30.9 kb that comprises an arginine deiminase (*arc*) and an oligopeptide permease (*opp*) gene cluster and is physically linked to an *SCCmec* element conferring methicillin resistance⁹⁵. The element is not found in other *S. aureus*, but appears to have been acquired from the coagulase-negative *S. epidermidis*, where it is frequent. Presence of the ACME element leads to selection for the wild-type strain in a competitive rabbit model of bacteremia when inoculated together with an isogenic ACME deletion strain⁹⁶ (Tab. 1). The mechanistic role of the ACME element in persistence is not clear. It has been speculated that the production of ammonia by the *arc* operon may balance the pH value on the skin and thus promote colonization^{77,95,96}. Alternatively or in addition, the *opp* operon may be involved in nutrient uptake or immune evasion.

Colonization/transmissibility factors

The fact that the USA300 clone has spread all over the U.S. and became the most frequent pathogen causing skin infections in less than a decade⁷², clearly demonstrates its extraordinary capacity to colonize humans and spread from one human being to another. Based on the investigation of disease outbreaks, we have to conclude that transmission may occur directly from human to human or via intermediate contamination of surfaces such as sports equipment, sauna banks, etc. Measures to control disease outbreaks therefore mainly include the sterilization of public places and educating people to increase personal hygiene and use effective disinfectants such as bleach⁹⁷. However, we do not know yet if these measures, even if rigorously applied, will prevent further spread of USA300 disease.

The molecular factors underlying efficient colonization of humans and abiotic fomites may represent a good target to stop spread of the pathogen. While much research has been performed on attachment molecules of *S. aureus* in general, it is yet unknown which of these factors mainly contribute to attachment of USA300. The colonization of surfaces in multi-

layered agglomerations is commonly referred to as biofilm formation⁹⁸. The formation of biofilms, which can to a certain degree withstand mechanic stresses and antibacterials²⁰, might contribute to prolonged survival on the skin and abiotic surfaces and thus cause high transmissibility. However, USA300 infections commonly do not include biofilm-associated infections. Nevertheless, they appear to have the capacity to do so, possibly linked to a deactivation of the *agr* system⁹⁹, a process that has been shown to occur frequently and increase biofilm formation in *S. aureus*¹⁰⁰⁻¹⁰¹. Overall, these observations indicate that an exceptional capacity to form biofilms is likely not the cause for the efficient spread of USA300 or contributes significantly to pathogenesis. Still, the potential capacity of USA300 to form biofilms under different environmental conditions and especially in vivo certainly warrants further experimental evaluation.

Role of host factors

As pointed out above, the role of host factors in *S. aureus* disease is not well understood. Similarly, for infections caused by CA-MRSA, host factors that play a significant role in predisposing for infection or determining disease severity have not been identified. Likely, skin infections as the most frequent type of infections caused by USA300 are not significantly impacted by the genetic background of the host. This can be surmised, because a wide variety of patients are infected and infection appears to spread without discriminating between hosts, such as found during the outbreak in the professional football team St. Louis Rams¹⁰².

Drug development

Most CA-MRSA infections present as abscesses and are best treated by incision and drainage, which works in 90% of cases⁹⁷. Post-procedure antibiotic treatment may be indicated if healing does not occur within a week⁹⁷. Antibiotic treatment is also indicated in patients having more severe manifestations of CA-MRSA disease such as sepsis syndrome or necrotizing fasciitis.

Antibiotics commonly used for CA-MRSA infections include trimethoprim-sulfamethoxazole, tetracycline, clindamycin, and vancomycin⁹⁷. Not all of these are approved for CA-MRSA infections and are thus used “off-label”, albeit rather successfully and regularly, such as trimethoprim-sulfamethoxazole for the treatment of skin infections by multi-drug-resistant CA-MRSA. Such multidrug-resistant USA300 strains have been detected in San Francisco where they initiated an outbreak of CA-MRSA skin infections among men who have sex with men¹⁰³. The strain responsible for the outbreak was resistant to tetracycline and clindamycin and appears genetically predisposed to acquire further antibiotic resistance. Spread of this clone has not yet been described, but is likely, leaving essentially only glycopeptides antibiotics and the novel antibiotics discussed later as efficient therapy for CA-MRSA, possibly creating a situation similar to that found in many HA-MRSA.

Therefore, the current CA-MRSA epidemic underlines the urgent need for novel therapeutic strategies against multi-drug resistant *S. aureus*. Three potential approaches can be distinguished: (i) the conventional approach to find antibiotics interfering with essential bacterial molecules or pathways, aiming at the development of a conventional bacteriostatic or bactericidal antibiotic; (ii) decolonization strategies to eliminate the pathogenic strains in risk groups or even the entire population; (iii) targeting bacterial virulence factors to diminish pathogenicity of the microbe without killing it. This approach is believed to reduce the development of resistant strains⁶.

Conventional antibiotics

Several novel antibiotics are being tested or have already been approved for use against MRSA infections. Approved antibiotics include linezolid, which belongs to a new class of antimicrobials called oxazolidinones¹⁰⁴⁻¹⁰⁵. Linezolid has efficacy against Gram-positive infections and works by inhibiting protein synthesis by interacting with the 50S ribosomal subunit. While not spread to a large degree, there are linezolid-resistant strains, with resistance caused mostly by a base mutation in the ribosomal RNA binding site for the drug. Furthermore, during longer use linezolid is sometimes associated with thrombocytopenia, neuropathy, and lactic acidosis.

Daptomycin is a cyclic lipopeptide that integrates into the cytoplasmic membrane¹⁰⁶. Several resistance mechanisms have been found that include reduced binding of daptomycin to its target. As daptomycin is a cationic peptide, resistance mechanisms may be similar to those identified to confer resistance to cationic AMPs, such as MprF, a ubiquitous enzyme responsible for integration of cationic lysyl-phosphatidylglycerol into the bacterial cytoplasmic membrane¹⁰⁷. The involvement of MprF in resistance to daptomycin has recently been demonstrated in *Bacillus subtilis*¹⁰⁸. Furthermore, intermediate resistance to vancomycin to some degree confers cross-resistance to daptomycin, likely owing to changes in cell wall structure¹⁰⁹.

Finally, tigecycline is a semisynthetic glycylcycline, member of a new class of tetracycline derivatives, with broad-spectrum antimicrobial activity. Tigecycline's mechanism of action is similar to that of tetracycline, but tigecycline is not subject to the two bacterial resistance mechanisms active against tetracycline, efflux (*tetR* gene) and target modification (*tetM* gene)¹¹⁰.

The glycopeptides dalbavancin and oritavancin are currently in clinical development¹¹¹ and telavancin has recently been approved by the FDA for skin and soft tissue infections. Ceftobiprole is a novel broad-spectrum cephalosporin that binds strongly to the penicillin-binding protein derivatives otherwise responsible for staphylococcal resistance - in addition to other penicillin-binding proteins. It has recently undergone phase 3 studies with promising results. However, Pfizer recently announced they would withdraw the marketing application being considered at FDA and the European Medicines Agency (EMA) for dalbavancin based on the agency's comments. Similarly, oritavancin, which is developed by Targanta Therapeutics, received rather negative reviews from the FDA.

Overall, there is no evidence to suggest that efficiencies of these new antibiotics against MRSA or CA-MRSA differ from those against other *S. aureus*, and thus they certainly represent important and promising treatment options for CA-MRSA. However, as with all antibiotics, the development of resistance has either already occurred or is to be expected. Given the narrow repertoire of treatment options and resulting frequent overuse of novel broad-spectrum antibiotics in clinical practice, the time frame for the spread of antibiotic resistant strains is likely short. As a consequence, alternative strategies to combat *S. aureus* and specifically, CA-MRSA infections, have recently gained increasing attention.

Conventional vaccines

Development of an efficient vaccine against *S. aureus* has been the "holy grail" among efforts to control *S. aureus* infections for decades¹¹². In addition to the fact that this has been unsuccessful, several lines of evidence suggest that a working vaccine for *S. aureus* might be very difficult or even impossible to find. First, *S. aureus* colonize humans and thus, most people have antibodies against *S. aureus* antigens¹¹³, which are however non-protective against infections. Furthermore, presence of anti-staphylococcal antibodies in

humans contrasts the immune status of animals in experimental protection models used to evaluate vaccines. Thus, only clinical trials ultimately tell whether an *S. aureus* vaccine works, which makes vaccine development against *S. aureus* costly and laborious. Underpinning the difficulty to find protective *S. aureus* vaccines, vaccine trials have failed so far¹¹². For example, Shinefield et al. reported on a failed trial of a capsular polysaccharide vaccine in hemodialysis patients¹¹⁴. Second, vaccines function by promoting the formation of bacteria-specific antibodies that enhance binding and uptake by neutrophils. However, *S. aureus* produces a series of toxins that eliminate neutrophils, thus undermining the enhanced neutrophil activation¹¹⁵. While the probability to achieve a working conventional vaccine against *S. aureus* therefore has to be very cautiously evaluated, approaches to find a vaccine against CA-MRSA may still be worthwhile owing to the immense potential benefit and advantages over antibiotics in terms of cost and possibility of prophylactic application.

Selecting the optimal antigen(s) for vaccine development is difficult. Optimally, a good antigen is surface-exposed for easy recognition, and essential for bacterial survival or virulence¹¹⁶. For CA-MRSA or any other *S. aureus*, selecting the best antigenic targets should therefore include assessment of surface protein composition, and essentiality for growth and virulence. More recently, Stranger-Jones et al. used a systematic approach to overcome the functional redundancy in *S. aureus* surface proteins by selecting a combination of *S. aureus* surface proteins as targets for vaccine development¹¹⁷. The 4-component antigenic preparation proved protective against many *S. aureus* strains in mouse protection models, but was not tested against USA300. Furthermore, it is often proposed to include toxins as antigens, as these frequently have a key role in *S. aureus* virulence. While the contribution of specific toxins to CA-MRSA virulence is not yet completely understood, and controversial in the case of the most frequently discussed PVL, secreted toxins may in general not represent the best target for conventional vaccines owing to the lack of surface exposition. They may however form a good target for an antitoxin-based approach.

Decolonization strategies

It has been suggested to eliminate colonization by USA300 in smaller risk group settings or even larger subsets of the population, using antibiotics or bactericidal agents¹¹⁸. However, data to support decolonization for patients infected with MRSA are controversial. In the Netherlands, rigorous search-and-destroy policies have kept MRSA infections at a very low rate across the entire country¹¹⁹. However, how such a policy would work in a country like the U.S. with already widespread CA-MRSA infections is hard to judge. On the other hand, intranasal application of the antibiotic mupirocin failed to decrease nasal colonization and incidence of infection in a large military study¹²⁰. Furthermore, long-term application of mupirocin led to the development of resistant strains¹²¹. Thus, the lack of an *S. aureus* vaccine and the reported failure to decolonize efficiently with antibiotics make decolonization approaches in larger settings and with approaches that exceed the use of simple bactericidal agents such as bleach rather unlikely to succeed. Moreover, the disappearance of clear risk groups in CA-MRSA infections would make large-scale prophylactic decolonization using a vaccine quite impractical, even if such a vaccine were available.

Targeting virulence and antitoxins – a valid alternative?

Using antibodies to sequester specific secreted virulence determinants, such as toxins, and thereby eliminating their pathogenic capacities, is a novel therapeutic strategy worth considering, especially given the problems encountered with conventional approaches¹²². To facilitate efficient production, such a strategy would have to be based on monoclonal

antibodies. Additionally, a potential antitoxin-based drug likely would have to simultaneously target several key virulence factors of *S. aureus*, owing to the functional redundancy of *S. aureus* virulence determinants. Probably this would be best achieved using a mixture of antibodies targeted against the most crucial virulence factors.

Obviously, this approach needs extensive basic research efforts. Most importantly, it would have to include the simultaneous, comparative analysis of virulence determinants in the same strain background. Due to the multitude of strains causing infections and the infrequent experimental use of the more difficult-to-handle clinical isolates, such parallel analyses are not commonly performed. However, the USA300 epidemic now gives us an almost unprecedented chance to focus on one overwhelmingly important clone. To use this novel, promising approach to develop a drug against CA-MRSA, it will thus be necessary to compare mutants containing deletions of several virulence factors in the USA300 strain background, and perform animal infection models mimicking the most important manifestations of CA-MRSA disease with these strains. While general knowledge about *S. aureus* virulence determinants would suggest including surface binding proteins, toxins, and secreted immune evasion factors in such an analysis, there is still insufficient information about virulence determinants in CA-MRSA, with only α -Toxin and α -PSMs having been identified as factors strongly impacting CA-MRSA virulence. In CA-MRSA, the only studies that have included parallel analysis of virulence determinants are the Bubeck Wardenburg et al. and Wang et al. studies comparing α -Toxin and PVL, and PSM α , PSM β , and δ -Toxin, respectively⁵¹⁻⁸⁷⁻⁹⁴. Thus, we will clearly need to strengthen our efforts to analyze virulence determinants in CA-MRSA strains.

To achieve broad applicability of a potential antitoxin mixture against infections by *S. aureus* including CA-MRSA, the targeted virulence determinants should optimally be crucial for virulence in a broad variety of *S. aureus* strains, as the epidemiology of MRSA infection might change. For example, the frequent absence of PVL from other virulent strains would argue against including PVL, or other virulence determinants limited almost entirely to CA-MRSA, as antitoxin targets in such a formula. However, as α -Toxin and the bicomponent leukotoxins share significant sequence similarity, one might be able to develop an antibody that eliminates activities of all members in this group.

Virulence regulators as drug targets?

Targeting regulators in charge of controlling virulence has frequently been proposed as a novel way to eliminate the production of several *S. aureus* virulence determinants simultaneously¹²³. The quorum-sensing regulator *agr* has been in the focus of such attempts. Synthetic peptides inhibiting *agr* that were developed based on the cross-inhibiting capacity of staphylococcal *agr*-activating peptides significantly decreased experimental skin infection in mice¹²⁴⁻¹²⁵. However, systemic application of these peptides is problematic owing to the labile thiolactone or lactone ring that is indispensable for their activity. Possibly, using antibodies against the peptides, which were recently found to work in animal infection models, presents a valuable alternative¹²⁶. Supporting the use of virulence regulators as targets against CA-MRSA, recent investigation indicates a crucial role of gene expression in CA-MRSA virulence⁵¹⁻⁸²⁻¹²⁷. Most notably, control of α -Toxin and PSMs by *agr* suggests that an *agr*-inhibiting drug may be efficient in treating CA-MRSA infection. On the other hand, inhibition of *agr* does not completely eliminate the pathogen's potential to cause infection. It rather appears to switch the pathogen's lifestyle from acute toxicity to chronic persistence. Accordingly, natural *agr* mutants are isolated frequently from biofilm-associated infections and show increased biofilm formation in vitro¹⁰⁰⁻¹⁰¹. Furthermore, glycopeptide resistance appears to be higher in *agr*-negative strains, possibly owing to the

broad effects of *agr* regulation on physiology and cell structure¹²⁸. The applicability of an *agr*-inhibiting drug would thus be limited to acute stages of CA-MRSA infection.

Conclusion and outlook

Rational development of drugs against CA-MRSA will not differ significantly from approaches already undertaken against *S. aureus* or MRSA. The CA-MRSA epidemic merely underpins the urgency to find novel therapeutics. For drugs specifically targeting virulence factors, a better understanding of the relative contribution of virulence determinants to pathogenesis may help us to fine-tune potential therapeutics, including specific antibodies, for use against CA-MRSA.

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Biography

Dr. Michael Otto received his masters degree in biochemistry from the University of Tuebingen, Germany in 1992, and his Ph.D. in microbiology at the same institution in 1998. After a 2-year postdoctoral fellowship at the department of Microbial Genetics of Tuebingen University, he accepted a tenure-track investigator position at the NIAID in Hamilton, Montana. He received tenure in 2007 and recently moved his laboratory to the NIH main campus in Bethesda, Maryland. Dr. Otto is an expert in staphylococcal pathogenesis, including coagulase-negative staphylococci and MRSA. He has published ~ 70 peer reviewed papers in this field.

Table 1
Virulence determinants of CA-MRSA evaluated in animal infection models using isogenic deletion mutants

Protein	Gene(s)	Model/animal	Bacterial strain	Result	Conclusion	Remarks	Ref.
α -toxin	<i>hla</i>	Pneumonia, mice (C57BL/6J)	LAC (USA300)	Strong reduction of mortality in <i>hla</i> mutant	α -toxin is a crucial virulence determinant in mouse pneumonia.	No effect for <i>lukSF</i> (PVL) deletion in same strain and model	87
α -toxin	<i>hla</i>	Pneumonia, mice (C57BL/6J)	LAC (USA300), MW2 (USA400)	Protection from pneumonia by anti-Hla antibodies	α -toxin is a crucial virulence determinant in mouse pneumonia.	No protection achieved using anti-PVL antisera	94
PSM α	<i>psma1</i> , <i>psma2</i> , <i>psma3</i> , <i>psma4</i>	Abscess, mice (SKH1-hrBR) bacteremia, mice (CD1 Swiss)	LAC (USA300); abscess MW2 (USA400); bacteremia	Strong reduction in mortality and abscess formation	α -PSMs are crucial virulence determinants in mouse skin infection and sepsis.	Results directly comparable to δ -toxin, PSM α	51
δ -toxin	<i>hld</i>	Abscess, mice (SKH1-hrBR) bacteremia, mice (CD1 Swiss)	LAC (USA300); abscess MW2 (USA400); bacteremia	Small (insignificant) reduction in mortality and abscess formation	δ -toxin is not a significant virulence determinants in mouse skin infection and sepsis.	Results directly comparable to PSM α , PSM β	51
PSM α	<i>psma1</i> , <i>psma2</i>	Abscess, mice (SKH1-hrBR) bacteremia, mice (CD1 Swiss)	LAC (USA300); abscess MW2 (USA400); bacteremia	No reduction in mortality and abscess formation	α -PSMs do not impact mouse skin infections and sepsis.	Results directly comparable to PSM α , δ -toxin	51
ACME	37 kb ACME fragment containing <i>arc</i> and <i>opp</i> loci	Bacteremia, rabbits	SF8300 (USA300)	Significantly more wild-type than ACME mutant bacteria at end of experiment	ACME is a significant contributor to rabbit sepsis.	Highly discriminative competition model	96
PVL	<i>lukS</i> , <i>lukF</i>	Abscess, mice (SKH1-hrBR) bacteremia, mice (CD1 Swiss)	LAC (USA300), MW2 (USA400)	No significant differences	PVL is not a virulence determinant in mouse sepsis or skin infections.		86
PVL	<i>lukS</i> , <i>lukF</i>	Pneumonia, mice (C57BL/6J)	LAC (USA300)	No reduction of mortality in <i>lukSF</i> mutant	PVL is not a virulence determinant in mouse pneumonia.	Strong effect for <i>hla</i> deletion in same strain and model	87
PVL	<i>lukS</i> , <i>lukF</i>	Pneumonia, mice (C57BL/6J)	LAC (USA300), MW2 (USA400)	No protection from pneumonia by anti-PVL antibodies	PVL is not a virulence determinant in mouse pneumonia.	Protection achieved using anti-Hla antisera in same model	87
PVL	<i>lukS</i> , <i>lukF</i>	Pneumonia, mice (BALB/cJ, BALB/cAnNHsd), mice (BALB/cJ, BALB/cAnNHsd)	LAC (USA300)	No significant effect of PVL	PVL is not a virulence determinant in mouse pneumonia or skin infection.		88
PVL	<i>lukS</i> , <i>lukF</i>	Bacteremia, rabbits	SF8300, LAC (USA300), MW2 (USA400)	Transiently significantly more wild-type than <i>lukSF</i> mutant bacteria, but not at end of experiment	PVL has a modest, transient effect on the progress of rabbit bacteremia.	Highly discriminative competition model	85
PVL	<i>lukS</i> , <i>lukF</i>	Necrotizing pneumonia, rats	LAC (USA300)	No significant effect of PVL	PVL is not a virulence determinant in rat pneumonia.	No significant effect of PVL on expression of host inflammatory genes	89

Protein	Gene(s)	Model/animal	Bacterial strain	Result	Conclusion	Remarks	Ref.
PVL	<i>lukS, lukF</i>	Necrotizing pneumonia and skin infection, mice (BALB/c)	LAC (USA300)	PVL impacts necrotizing pneumonia and skin infection in mice. Anti-PVL antibodies protect from infection.	PVL is a virulence determinant in mouse pneumonia and skin infection.	Only study showing a prolonged effect of PVL in experimental disease.	90