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Review

Current concepts on the virulence mechanisms of meticillin-resistant *Staphylococcus aureus*

Richard R. Watkins,[1] Michael Z. David[2] and Robert A. Salata[3]

Correspondence
Richard R. Watkins
rwatkins@agmc.org

[1]Division of Infectious Diseases, Akron General Medical Center, Akron, OH 44302, USA

[2]Departments of Medicine and Health Studies, The University of Chicago, Chicago, IL, USA

[3]Division of Infectious Diseases and HIV Medicine, University Hospitals Case Medical Center, Cleveland, OH, USA

Meticillin-resistant *Staphylococcus aureus* (MRSA) strains are prevalent bacterial pathogens that cause both health care and community-associated infections. Increasing resistance to commonly prescribed antibiotics has made MRSA a serious threat to public health throughout the world. The USA300 strain of MRSA has been responsible for an epidemic of community-associated infections in the US, mostly involving skin and soft tissue but also more serious invasive syndromes such as pneumonia, severe sepsis and endocarditis. MRSA strains are particularly serious and potentially lethal pathogens that possess virulence mechanisms including toxins, adhesins, enzymes and immunomodulators. One of these is Panton–Valentine leukocidin (PVL), a toxin associated with abscess formation and severe necrotizing pneumonia. Earlier studies suggested that PVL was a major virulence factor in community-associated MRSA infections. However, some recent data have not supported this association while others have, leading to controversy. Therefore, investigators continue to search for additional mechanisms of pathogenesis. In this review, we summarize the current understanding of the biological basis of MRSA virulence and explore future directions for research, including potential vaccines and antivirulence therapies under development that might allow clinicians to more successfully treat and prevent MRSA infections.

Introduction

Ever since it was first discovered by Sir Alexander Ogston in 1880, *Staphylococcus aureus* has been regarded as a serious threat to human health, capable of causing a multitude of infections. The rise of antibiotic-resistant strains in the 1960s and 1970s, particularly meticillin-resistant *S. aureus* (MRSA), has created additional therapeutic challenges. Currently, MRSA strains account for >50 % of all *S. aureus* isolates causing clinical disease in the US (Drago *et al.*, 2007). This is a much higher percentage compared to other countries, such as France at 14.5 % (Lamy *et al.*, 2012) and the Netherlands at 3.1 % (Wassenberg *et al.*, 2012). In a review of 31 observational studies from Western Europe, the authors found that the percentage of MRSA among *S. aureus* clinical isolates ranged between 5 % and 54 %, but was limited by the different methodologies used in the studies (Dulon *et al.*, 2011). Initially, MRSA strains afflicted hospitalized patients and those with chronic illnesses. The 1990s saw the emergence of community-associated MRSA (CA-MRSA) strains that primarily caused skin and soft tissue infections (SSTIs) in otherwise healthy individuals, often children. These strains quickly led to an epidemic of CA-MRSA infections including some with severe consequences, for example, community-acquired pneumonia with high mortality rates (Francis *et al.*, 2005). The high prevalence of CA-MRSA among infecting MRSA strains in the US is mostly due to the Panton–Valentine leukocidin (PVL)-positive USA300 clone, while in Europe the predominant strain of CA-MRSA is a PVL-positive ST80 clone (Otter & French, 2010). A mathematical model predicted that CA-MRSA will become the dominant MRSA strain in hospitals because of the expanding community reservoir, CA-MRSA strains are more fit (higher replicative capacity) than hospital-associated types and CA-MRSA infections will become increasingly severe (D'Agata *et al.*, 2009).

With the emergence of MRSA, there has been debate over its relative impact on overall *S. aureus* morbidity and mortality compared with meticillin-susceptible *S. aureus* (MSSA). A meta-analysis found that, in invasive infections, patients with MRSA had a significantly higher mortality than those with MSSA [odds ratio (OR) 1.93, $P<0.001$] (Cosgrove *et al.*, 2003). These investigators suggested that delay in administration of an appropriate antibiotic and the inferiority of vancomycin to other antistaphylococcal antibiotics may be important reasons why outcomes in MRSA bacteraemia are worse. MRSA infections have also been associated with longer hospital stays and increased costs

to the health care system than MSSA infections (Cosgrove *et al.*, 2005).

Compared to heath care-associated MRSA (HA-MRSA) strains, CA-MRSA has several distinctive features. First, genomic analyses have shown that the chromosomal elements for meticillin resistance in community-associated strains are chromosome cassette *mec* (SCC*mec*) types IV or V, which are smaller and more mobile than those typically found in hospital-acquired MRSA (SCC*mec* types I–III) (David & Daum, 2010; Cameron *et al.*, 2011). The larger gene elements in health care-associated strains are associated with reduced bacterial fitness as well as decreased toxin production (Collins *et al.*, 2010). Second, the PVL toxin is more common in CA-MRSA than in MSSA. Third, there is an increased expression of certain virulence determinants in CA-MRSA that may contribute to more severe disease, such as phenol-soluble modulins (PSMs) (Wang *et al.*, 2007). Finally, while all strains of *S. aureus* have a proclivity to form biofilms, emerging data suggest differences in biofilm matrix in CA-MRSA compared to other strains, in particular the USA300 lineage (Kiedrowski *et al.*, 2011). However, there remains insufficient evidence in the literature that MRSA of any strain type has a greater capacity to cause invasive infection than MSSA strains.

These wide-ranging and diverse virulence determinants in MRSA have important clinical implications. It is estimated that the annual death rate due to MRSA in the US is the highest for any infectious agent (Klevens *et al.*, 2007). The aim of this review is to explore the biological basis of the virulence mechanisms that MRSA uses to overcome host defences. While many of the virulence factors discussed have been described in both MRSA and MSSA strains, the focus of this review is on virulence factors particularly associated with MRSA strains. Better understanding of these complex pathogen–host interactions may lead to advances in therapeutic strategies, including novel antibiotics, anti-virulence agents and an effective MRSA vaccine, with the goal of improving patient outcomes and preventing disease.

Infections caused by MRSA

CA-MRSA and HA-MRSA strains cause distinct clinical syndromes and affect different patient populations. HA-MRSA has most commonly been associated with pneumonia, bacteraemia and other invasive infections in patients exposed to health care settings, who often have co-morbid illnesses. In contrast, CA-MRSA usually causes SSTIs in otherwise healthy individuals. More severe manifestations can include necrotizing pneumonia (Kreienbuehl *et al.*, 2011), pyomyositis (Burdette *et al.*, 2012), sepsis (Bassetti *et al.*, 2011), osteomyelitis (Kechrid *et al.*, 2011) and necrotizing fasciitis (Changchien *et al.*, 2011). The host factors that predispose certain people to develop severe manifestations compared to the more common SSTIs are not understood. However, CA-MRSA strains, and USA300 in the US, are still the most common cause of invasive *S. aureus* infections in patients without risk factors for health care exposure. Outbreaks of CA-MRSA have occurred in a wide range of groups, including professional football players (Kazakova *et al.*, 2005), soldiers (Ellis *et al.*, 2009) and incarcerated populations (Malcolm, 2011). Close body contact and poor personal hygiene were the likely catalysts for infections in these groups (Turabelidze *et al.*, 2006). CA-MRSA infections have also become common among children, disadvantaged urban populations and emergency department patients with SSTIs, among others (David & Daum, 2010). Humans have reportedly acquired MRSA infections from asymptomatic pet dogs (Rankin *et al.*, 2005) and cats (Sing *et al.*, 2008), although there are limited data to support this association.

The distinction between CA-MRSA and HA-MRSA has become blurred from an epidemiological and clinical point of view. The CDC has defined a CA-MRSA infection as any MRSA infection diagnosed for an outpatient or within 48 h of hospitalization if the patient lacks the following HA-MRSA risk factors: haemodialysis, surgery, residency in a long-term care facility or hospitalization within the preceding year or the presence of an indwelling catheter at the time of culture (Morrison *et al.*, 2006). Other criteria that have been utilized to differentiate between HA-MRSA and CA-MRSA strains include differences in antibiotic susceptibility patterns, fragment patterns of DNA on pulsed-field electrophoresis, protein A gene (*spa*) typing, multilocus sequence typing, carriage of PVL genes and the type of SCC*mec* element carried (David & Daum, 2010). Some CA-MRSA infections are actually due to HA-MRSA strains, possibly as a result of pressure (i.e. cost-related) to treat patients outside of acute care settings, at home and elsewhere (Lescure *et al.*, 2006). Moreover, CA-MRSA strains have been increasingly isolated from health care settings. For example, the USA300 strain of CA-MRSA was responsible for 20 % of nosocomial bloodstream infections at a hospital in Detroit, MI, between 2005 and 2007 (Chua *et al.*, 2008). The association between livestock-associated strains of MRSA and human acquisition is an emerging area of research. A recent report from Germany found that 24 % of farmers with occupational exposures to poultry and pigs were colonized with the animal MRSA strain ST398 (Bisdorff *et al.*, 2011). While human infections from animal strains of MRSA are rare, little is currently known about the virulence factors of these strains and the mechanisms of transmission from animals to humans.

PVL

PVL is a bi-component exotoxin transmitted by bacteriophages that is encoded by two genes, *lukF-PV* and *lukS-PV*. PVL genes are carried by nearly every CA-MRSA strain as well as a small proportion of clinical MSSA strains. This suggests that PVL has an important role in fitness, transmissibility and virulence, but the role of PVL in the pathogenesis of CA-MRSA infections is controversial. See Table 1 for recent major studies on the role of PVL in the pathogenesis of CA-MRSA infection.

Table 1. Studies demonstrating the pathogenic mechanisms and potential of Panton–Valentine leukocidin

Study	Major finding(s)
Genestier *et al.* (2005)	PVL may inactivate mitochondria, leading to apoptosis
Voyich *et al.* (2006)	In mouse model of SSTI and sepsis, PVL in an MRSA strain did not affect strain virulence
Bubeck Wardenburg *et al.* (2007)	In mouse model of pneumonia, PVL in *S. aureus* did not affect mortality; use of isogenic PVL knockouts of USA300 and USA400 did not alter cytopathic effect *in vitro* in alveolar epithelial cells
Labandeira-Rey *et al.* (2007)	In mouse model both purified PVL protein alone and an *S. aureus* strain overexpressing PVL caused necrotizing pneumonia
Wolter *et al.* (2007)	Allelic variation exists in PVL genes from various *S. aureus* isolates; 7 nt substitutions were identified in 28 isolates collected over a long period of time at different geographical sites around the world
Bubeck Wardenburg *et al.* (2008)	In rodent models of SSTI and pneumonia, no differences noted in USA300 and an isogenic PVL knockout
Montgomery *et al.* (2008)	In rat model of pneumonia, wild-type and isogenic PVL knockout strains of USA300 and USA400 caused similar disease
Diep *et al.* (2008)	(1) USA300 and isogenic PVL knockout did not differ in their proteomes or in their global gene expression profiles; (2) in rabbit sepsis model, competition assay showed that at 24 and 48 h, wild-type USA300 was present in a higher concentration in the kidney tissue than was its isogenic PVL knockout; PVL may provide survival benefit to USA300 early in infection
O'Hara *et al.* (2008)	Two major allelic variants of PVL were identified in a large international collection of clinical *S. aureus* isolates; the R variant was found in USA300 and USA400 while the H variant was in other genetic backgrounds
Dumitrescu *et al.* (2008)	In a large collection of clinical isolates, the R variant of PVL was identified in USA300, USA400 and in a CC93 isolate from Australia; the H variant was in many other lineages and dominated in MSSA and MRSA strain backgrounds from outside of North America
Tristan *et al.* (2009)	PVL may be a factor in the adhesion of *S. aureus* to mucous membranes
Bae *et al.* (2009)	Human SSTIs caused by a PVL+ MRSA strain were not associated with a worse outcome than those caused by PVL-negative MRSA strains
Tseng *et al.* (2009)	In mouse model of myositis, wild-type USA300 caused greater tissue damage in young (and not older) mice than its isogenic PVL knockout
Hongo *et al.* (2009)	PVL did not lyse neutrophils from mice but did lyse human neutrophils; this lysis was prevented in the presence of monoclonal anti-PVL antibodies
Montgomery *et al.* (2009)	In a rat pneumonia model, USA300 and its isogenic PVL knockout did not differ in the initial, rapid massive inflammatory cytokine response as measured by RT-PCR
Hermos *et al.* (2010)	Children with PVL+ MRSA infections have high serum titres of anti-PVL antibodies that strongly inhibit PVL-mediated lysis of human neutrophils; even in children with a high titre of these protective antibodies PVL+ MRSA strains can cause skin infections
Löffler *et al.* (2010)	PVL lysed human and rabbit neutrophils, but did not lyse neutrophils from mice or monkeys
Varshney *et al.* (2010)	PVL production by *S. aureus* strains varies; in a mouse SSTI model, MSSA and MRSA strains with greater PVL production caused larger lesions with a greater number of bacteria recovered from them than a strain with lower PVL production
Dumitrescu *et al.* (2011)	In CA-MRSA isolates *in vitro* and in a mouse pneumonia model, PVL transcription was increased in the presence of imipenem and oxacillin (but not β-lactams that are PBP2, -3 or -4 selective); this was likely mediated by PBP1 binding and induction of the global virulence regulator *sarA* and reduction in the transcription of *rot*
Malachowa *et al.* (2011)	PVL gene expression by microarray analysis increased in a USA300 strain in human blood compared with the strain in TSB, along with several other virulence factors
Zivkovic *et al.* (2011)	In a mouse model, lung inflammatory response to PVL is mediated via NF-κB in alveolar macrophages by direct binding of PVL to CD14/TLR2
Kobayashi *et al.* (2011)	In a rabbit SSTI model, the virulence of USA300 and its isogenic PVL knockout strain did not differ until >11 days into the infection, when the PVL knockout lesions were larger than those caused by wild-type USA300
Ma *et al.* (2012)	In a rabbit pneumonia model, PVL protein led to NF-κB-mediated release of inflammatory cytokines derived from neutrophils; the injury resulting from this cytokine release was mild in neutropenic rabbits, suggesting the central role of neutrophils mediation in the pathogenic effect of PVL in the lung

PVL forms pores in the membranes of leukocytes, causing their lysis. Among a sample of 1055 *S. aureus* isolates from the US, 36 % were positive for *lukSF-PV* genes (Brown *et al.*, 2012). PVL is highly prevalent among CA-MRSA strains worldwide (Monecke *et al.*, 2011). For example, researchers from Australia reported that 98 % of CA-MRSA isolates were positive for the PVL gene (Costello & Huygens, 2011). The majority of CA-MRSA infections in the US are caused by the USA300 strain, which secretes PVL (Tenover & Goering, 2009). The mechanism by which PVL genes spread among *S. aureus* strains appears to be a combination of clonal expansion and horizontal transfer (O'Hara *et al.*, 2008). Among CA-MRSA strains, there are lineage-specific relationships among the type of PVL phage lysogenized in the genome, the sequence of the genes that encode the toxin, and the position in which the phage inserts into the host chromosome (Boakes *et al.*, 2011). The observation that PVL-bearing MSSA strains may form a reservoir for PVL in MRSA strains suggests that the frequent transfer of virulence factors to MRSA enhances the public health threat from this pathogen (Rasigade *et al.*, 2010).

PVL was first associated with SSTIs in 1932 by Panton and Valentine. Experimental and clinical evidence shows that PVL-producing strains are associated with severe, necrotizing skin infections and pneumonia (Lina *et al.*, 1999; Gillet *et al.*, 2002; Labandeira-Rey *et al.*, 2007). However, not all studies have reached this conclusion and controversy has emerged on the pathogenic role of PVL. For example, one study reported that strains of CA-MRSA lacking PVL were as virulent in mouse sepsis and abscess models as those with the toxin (Voyich *et al.*, 2006). Another study that used the USA300 and USA400 CA-MRSA strains concluded that α-haemolysin and not PVL was responsible for mortality in a murine pneumonia model (Bubeck Wardenburg *et al.*, 2007). The diversity of the animal models used in the different studies may explain these discordant results. Additional evidence has called into question the comparability of murine models of PVL-associated disease and human infections. One study used neutrophils from mice, humans, rabbits and monkeys to test the cytotoxic effect of PVL and to elucidate differences among species (Löffler *et al.*, 2010). Murine neutrophils were insensitive to the effects of PVL, suggesting that models using mice do not correctly replicate PVL-bearing *S. aureus* disease in humans. Rabbit compared to murine neutrophils were much more susceptible to PVL, indicating a closer approximation to human disease. Moreover, low concentrations of PVL that correlated with amounts produced by CA-MRSA strains during an infection were sufficient to kill human neutrophils. These data support the potential importance of PVL in the pathogenesis of human CA-MRSA infections because neutrophils are major components of the response of the immune system to this bacterial infection (Rigby & DeLeo, 2012). Their premature destruction likely leads to increased local tissue destruction through the release of neutrophil components such as oxygen radicals.

Animal models, despite their limitations, have provided useful data on the role of PVL. Using a rabbit model, researchers demonstrated that PVL expression in the CA-MRSA USA300 strain is associated with more severe SSTIs compared with non-PVL strains (Lipinska *et al.*, 2011). These findings differ from another report that showed a PVL-negative *S. aureus* strain (USA500) produced skin lesions similar to the PVL-positive strain, leading the authors to conclude that PVL does not contribute to the formation of skin lesions (Li *et al.*, 2010). However, other strain differences between USA500 and the PVL-bearing strain may account for the observed result. Also, differences in study design may explain the conflicting results between the two studies. For example, the study by Li *et al.* (2010) did not detect PVL mRNA by qRT-PCR in the abscesses of PVL-positive strains. The disparate results may also be due to different amounts of PVL produced by individual strains. However, a study of CA-MRSA USA300 strain in a rabbit model of SSTIs failed to detect a role for PVL (Kobayashi *et al.*, 2011). Indeed, the controversy over the role of PVL in the pathogenesis of the current CA-MRSA epidemic remains unsettled.

Production of PVL is increased *in vitro* by β-lactam antibiotics through transcriptional activation (Dumitrescu *et al.*, 2007; Stevens *et al.*, 2007). Conversely, antibiotics that inhibit protein synthesis, like clindamycin and linezolid, decrease the production of PVL, suggesting a role for these antibiotic agents in the early therapy of severe CA-MRSA infections (Dumitrescu *et al.*, 2007; Bernardo *et al.*, 2004). A recent study demonstrated that some β-lactam antibiotics promote PVL production while others do not (Dumitrescu *et al.*, 2011). These researchers found that oxacillin and imipenem increased PVL production in four separate CA-MRSA strains, while cefotaxime, cefaclor and cefoxitin had no effect. Increased PVL production, however, may not universally result in increased virulence. For example, an increase in PVL production did not lead to higher mortality among mice used in an experimental lung infection model (Bubeck Wardenburg *et al.*, 2008). As previously mentioned, mouse neutrophils are relatively resistant to the effects of PVL, which may explain this finding. Investigators developed a rabbit model of necrotizing pneumonia that compared the virulence of a USA300 strain with that of an isogenic PVL-deletion mutant (Diep *et al.*, 2010). They discovered that PVL enhanced the capacity of the USA300 strain to cause lung necrosis, pulmonary oedema, alveolar haemorrhage, haemoptysis and death. Purified PVL injected directly into the lung caused lung injury through the recruitment and lysing of neutrophils, which damage lung tissue by releasing cytotoxic granules. Additional studies using rabbit neutrophils, which more approximate those of humans, should be performed to determine whether increased PVL production caused by β-lactam antibiotics has an effect on mortality.

As noted in a recent review, most of the evidence from rabbit models of infection to date suggests that PVL contributes to the virulence of CA-MRSA, but it is not the

sole factor contributing to the CA-MRSA epidemic (Otto, 2010). Moreover, a large, multinational study found that PVL was not associated with better or worse outcome in complicated SSTIs (Bae *et al.*, 2009). In patients with hospital-acquired pneumonia (HAP) caused by MRSA, the presence of PVL was not associated with either higher risk for clinical failure or mortality (Sharma-Kuinkel *et al.*, 2012). Similar findings were reported from a multicentre observational study of patients with HAP and ventilator-associated pneumonia caused by MRSA (Peyrani *et al.*, 2011). Hence, additional or alternative virulence factors are likely to play an important role in the pathogenesis of both HA-MRSA and CA-MRSA infections.

α-Toxin

Another pore-forming leukocyte toxin, α-toxin, has been well described as a virulence factor in many *S. aureus* strains (Kielian *et al.*, 2001). Unlike PVL, α-toxin does not lyse neutrophils but instead lyses other immune cells such as macrophages and lymphocytes. α-Toxin also alters platelet morphology, which may contribute to increased thrombotic events associated with *S. aureus* sepsis (Schubert *et al.*, 2011). In a murine model of pneumonia, α-toxin significantly worsened disease caused by CA-MRSA strains USA300 and USA400 (Bubeck Wardenburg *et al.*, 2007). The USA300 strain in particular is known to produce significant levels of α-toxin due its high-level expression of the accessory gene regulator *agr*, a 'master switch' operon that regulates many virulence factors (Kobayashi & DeLeo, 2009). When the staphylococcal accessory regulator (*sarA*) is inactivated by mutation in the USA300 strain, its ability to produce α-toxin is reduced, leading to fewer skin lesions in a murine model (Weiss *et al.*, 2009). Recently, investigators reported that the mutation of *sarA* in the USA300 strain limits accumulation of α-toxin and PSMs through the increased production of extracellular proteases rather than from transcription of the *hla* or *agr* genes (Zielinska *et al.*, 2011).

Studies of α-toxin have suggested potential new strategies for antimicrobial therapy of human SSTIs. In a mouse model of dermatonecrosis, a group of researchers used monoclonal antibodies (mAbs) against α-toxin from three strains of *S. aureus*, including CA-MRSA USA300 (Tkaczyk *et al.*, 2012). The size of the lesion was significantly reduced in the mice given these mAbs compared with controls. These data are consistent with a prior study that demonstrated that mice given α-toxin-specific antiserum or actively immunized with a non-toxigenic form of α-toxin had significantly reduced size of skin lesions caused by USA300 and dermonecrosis was prevented (Kennedy *et al.*, 2010). These results may lead to new treatment options for human skin infections from MRSA, and further research is warranted. Using mice whose lung epithelial tissue was deficient in the receptor ADAM10, researchers found them to be resistant to an otherwise lethal *S. aureus* pneumonia (Inoshima *et al.*, 2011). This suggests that ADAM10 is the receptor for α-toxin and blockade may be a potential therapeutic strategy for MRSA pneumonia.

PSMs

PSMs are a class of secreted α-helical peptides produced by several species of staphylococci. Genes for PSMs are found in all *S. aureus* and do not significantly differ among strains (Otto, 2010). PSMs are able to recruit, activate and lyse human neutrophils and are generated at high concentrations by standard CA-MRSA strains (Wang *et al.*, 2007). The human formyl peptide receptor 2 (FPR2/ALX) senses PSMs at nanomolar concentrations and initiates proinflammatory neutrophil responses to CA-MRSA. Blocking this receptor markedly diminished the ability of neutrophils to detect CA-MRSA (Kretschmer *et al.*, 2010). The highly cytolytic PSMs of the α type produced by CA-MRSA strains are encoded by the *psm*α operon, which is located in the core genome. Mutant strains with a deletion in this operon have a reduced capacity to cause SSTIs and bacteraemia in animal models (Wang *et al.*, 2007). Another recently described PSM gene, *psm-mec*, is the first to be localized within an SCC*mec* mobile genetic element (MGE) (Queck *et al.*, 2009). MGEs often carry antibiotic resistance genes and this study established a possible link between virulence mechanisms and antibiotic resistance in staphylococci. These authors did not find PSM peptides other than PSM-mec in a survey of a large strain collection, indicating that PSM-mec is likely the only MGE-encoded staphylococcal PSM. Moreover, PSM-mec peptide production was considerable in two strains of HA-MRSA: USA100 and USA200. Subsequent work by this group determined that the *psm-mec* gene is linked to the class A *mec* gene complex present in SCC*mec* types II, III and VIII, with a conserved location next to the *mecI* gene (Chatterjee *et al.*, 2011). They showed that the absence of *psm-mec* in four clinical strains either did not alter virulence in a mouse skin infection model or decreased virulence.

Another group demonstrated that the *psm-mec* locus has a regulatory function through RNA and has an impact on virulence counter to the effect of the PSM-mec peptide (Kaito *et al.*, 2011). The transcription and translation products of an open reading frame (ORF) encoding 70 amino acids (F region) suppressed PSMα production and promoted biofilm formation. The authors hypothesized that the absence of this F region in CA-MRSA strains is the reason for their high production of PSM. Hence, their conclusion that the *psm-mec* ORF had a negative effect on virulence seems to contradict what Queck *et al.* (2009) reported. The explanation for the discordant findings may be that Kaito *et al.* (2011) studied plasmid-based overexpression in strains not naturally harbouring the *psm-mec* gene (Chatterjee *et al.*, 2011).

Strains of MRSA have the ability to colonize human epithelia (Quinn & Cole, 2007). This feature may be due to the activity of derivatives of PSM peptides PSMα1 and PSMα2 (Joo *et al.*, 2011). In this study, the authors demonstrated that processed PSMα1 and PSMα2 exerted considerable activity against *Streptococcus pyogenes*, a pathogen that competes with MRSA for colonization of the human

body. Thus, in addition to acting as a virulence factor, PSMs may confer a competitive advantage over other species for strains of MRSA in disseminating among human populations.

Chromosomal genetic elements

Resistance to meticillin occurs in *S. aureus* through the acquisition of the *mecA* gene, located within the large chromosomal element known as the SCC*mec*. HA-MRSA strains typically have SCC*mec* types I, II and III while CA-MRSA carry types IV, V or VII. In the US, sequence type 8 (ST8), SCC*mec* IV (USA300 by PFGE) is the predominant CA-MRSA clone, while in Europe, ST80, SCC*mec* IV predominates (Gould *et al.*, 2012). Recently, SCC*mec* types IX and X have been described in a strain of MRSA, along with type V(5C2&5) subtype C and type IVa (Li *et al.*, 2011). Regions of type V(5C2&5), IX and X SCC*mec* elements carried genes related to detoxification of heavy metals, and the majority of type V(5C2&5) also contained the tetracycline resistance gene *tet*(K). It remains to be elucidated how metal and antibiotic resistance genes have evolved with the SCC*mec* elements in the current epidemic MRSA strains, but the source may be animal agriculture since antibiotics and metals are used to promote animal growth.

The type IV SCC*mec* was originally associated with MRSA infections in patients with no HA-MRSA risk factors (Ma *et al.*, 2002). However, recent data have shown that SCC*mec* IV is now common in hospitalized patients in the US (Tenover *et al.*, 2012). In this study, 299 nares and 194 blood isolates of MRSA were collected between 2009 and 2010 from 23 US hospitals. SCC*mec* type II-bearing strains (e.g. USA100) were the most common among nasal isolates, while SCC*mec* type IV-bearing strains (e.g. USA300) were the most common among the blood isolates. This finding differs from a nationwide survey study of MRSA strains from Japan conducted between 2008 and 2009 (Yanagihara *et al.*, 2012). There the most common SCC*mec* types were type II (73.6 %), type IV (20 %) and type 1 (6 %). The SCC*mec* type IV isolates were significantly more common in outpatients than among those who were hospitalized, and only 2.3 % of the type IV isolates were PVL-positive. The spread of CA-MRSA with SCC*mec* type IV in Japan may be enhanced by a unique 1604 amino acid cell-wall anchored surface protein (CWASP/J), encoded within SCC*mec* by the *spj* gene (Iwao *et al.*, 2011). The investigators designated this new SCC*mec* SCC*mec* IVI. PCR testing for the *spj* gene and SCC*mec* IV found that ST8/SCC*mec* IVI MRSA is widespread in Japan. Further investigation is needed to determine whether CWASP/J contributes to the community spread of MRSA strains.

Gentamicin-susceptible MRSA strains have been replacing gentamicin-resistant strains in European countries over the last few years, and most of the gentamicin-susceptible strains possess SCC*mec* type IV (De Angelis *et al.*, 2011). In this study, home nursing care (OR 8.1) and high Charlson scores (OR 7.1) were associated with strain replacement. Children may be at higher risk of infection from SCC*mec* type IV-containing MRSA strains than adults (David & Daum, 2010). In a cohort of paediatric patients from Columbia, virulence genes were more diverse and frequent in MSSA strains compared with MRSA strains and SCC*mec* type IVc was the most common SCC*mec* type among MRSA isolates (Jiménez *et al.*, 2011). The lower diversity of virulence factors among the MRSA strains may be due to the fitness cost associated with meticillin resistance. Indeed, the large SCC*mec* type II but not the smaller SCC*mec* type IV is associated in MRSA strains with decreased toxin production, which may be more suitable for a hospital environment where antibiotic usage, ventilators and immunocompromised patients are commonplace (Collins *et al.*, 2010). Conversely, MRSA strains in the community can maintain high levels of toxin production by having a lower fitness burden associated with the smaller SCC*mec* type IV. SCC*mec* type I also reduces the fitness of the host strain in terms of growth rate and cell yield, which may be due to the ease with which PBP2a integrates into the cell wall synthesis complex (Lee *et al.*, 2007). Further studies are needed to test this hypothesis.

Arginine catabolic mobile element (ACME)

The ACME is a large MGE that may play an important role in the growth, transmission and pathogenesis of CA-MRSA. It was identified through genomic sequencing of FPR3757, a multidrug-resistant USA300 MRSA strain (Diep *et al.*, 2006). There is a high prevalence of ACME in *Staphylococcus epidermidis*, which suggests the origin for the element as well as evidence that ACME confers a selective advantage for colonization of human skin. Among the many ORFs in ACME, the two main gene clusters identified include the *arc* genes (*arcA*, *arcB*, *arcC* and *arcD)* and the *opp* genes (*opp-3A*, *opp-3B*, *opp-3C*, *opp3-D* and *opp3-E*). These genes are homologues of genes recognized to be virulence factors (Diep *et al.*, 2006).

The USA300 strain was shown to have superior fitness compared to an isogenic mutant that lacked ACME and SCC*mec* elements (Diep *et al.*, 2008). However, another study found no difference in virulence between USA300 strains and an isogenic ACME knockout in a rat model of skin infection and necrotizing pneumonia (Montgomery *et al.*, 2009). While there is a strong association between ACME and CA-MRSA isolates in the US (i.e. in USA300 strains) (Roberts *et al.*, 2011), it appears to be weaker in European countries, such as Italy (Sanchini *et al.*, 2011) and Spain (Marimón *et al.*, 2012), where USA300 is less common. ACME was first localized in the *S. aureus* genome of a USA300 strain downstream of SCC*mec* (Diep *et al.*, 2006). However, an HA-MRSA strain from Denmark (t024-ST8) clonally related to the USA300 strain was recently shown to contain an ACME located upstream of SCC*mec* between direct repeats (DRs) 2 and 3 (Bartels *et al.*, 2011). Most of the sequence between DR1 and DR3 was highly homologous to an *S. epidermidis* ACME composite island, further supporting *S. epidermidis* as the origin of this genetic

material. Another study identified ACME type II from *S. epidermidis* in the ST22-MRSA-IV clone, the predominant MRSA isolate in hospitals in Ireland (Shore *et al.*, 2011). The isolate had high-level resistance to mupirocin, which along with ACME enhances host tissue colonization by resisting nasal decolonization by mupirocin therapy. Another finding was that the novel ACME/SCC*mec*-CI element was located upstream of SCC*mec*, similar to the t024-ST8 strain from Denmark (Bartels *et al.*, 2011). This suggests that ACME may have been integrated into chromosomes before SCC*mec*. Further studies of ST8-MSSA isolates may determine whether this hypothesis is valid. Also, whether the presence of ACME provides a selective advantage to ST22-MRSA-IV and whether this ACME can spread to other MRSA strains remains to be elucidated.

Another potential benefit of ACME for its bacterial host is polyamine resistance. Polyamines, including Spm and Spd, are cations synthesized by living cells that exert bactericidal effects on *S. aureus* at physiological concentrations. Investigators found a gene encoding an Spm/Spd *N*-acetyltransferase within the USA300-specific ACME (Joshi *et al.*, 2011). Other strains of *S. aureus* exhibited sensitivity to polyamines, but acquisition of this ACME allowed the USA300 strain to circumvent polyamine hypersensitivity. Additional confirmatory studies are necessary to determine whether this gene impacts the virulence of the USA300 strain and whether it can be transmitted to other MRSA strains.

MRSA superantigens

Many species of *S. aureus* are capable of producing superantigens that cause serious toxinoses, including toxic shock syndrome and necrotizing pneumonia. These superantigens initiate a cytokine storm leading to a sepsis-like syndrome (Gordon & Lowy, 2008). Most superantigens are small secreted proteins of 20–28 kDa in size and share similar biochemical and structural properties (Thomas *et al.*, 2007). It is believed that all *S. aureus* superantigens are encoded by MGEs (Ono *et al.*, 2008). A recently identified core-genome-encoded superantigen, SEIX, was found to modulate the immune response in both human and animal models of disease pathogenesis (Wilson *et al.*, 2011). Using a rabbit model of necrotizing pneumonia, the authors found that SEIX produced by the USA300 strain of CA-MRSA contributed to lethality. In another study, MRSA isolates from patients at a university hospital were examined for SCC*mec* genes and superantigen genes by multiplex PCRs (Hu *et al.*, 2011). The superantigen genes *se* and *tst-1* were linked to SCC*mec* type I and type II, which may contribute to the biological fitness of MRSA.

In France, a strain of MRSA containing the *tst* gene that encodes toxic shock syndrome toxin 1 (TSST-1) has recently emerged (Durand *et al.*, 2006). It contains an SCC*mec* type I variant and has been named the ST5 Geraldine clone. In a large prospective study that involved strains of *S. aureus* from 104 laboratories in France, the ST5 Geraldine clone of MRSA was found to be more prevalent than the European ST80 clone (Robert *et al.*, 2011). Infections due to the ST5 Geraldine clone were acquired equally in the hospital and community, and they showed a wider range of clinical manifestations compared to the ST80 clone. However, there were relatively few cases of toxic shock syndrome, implying that TSST-1 is not the major virulence determinant of the ST5 Geraldine clone but rather an epidemiological marker for it. Ongoing surveillance is needed to monitor the spread of this clone, especially in light of its pattern of antibiotic resistance, which includes fusidic acid and possibly kanamycin and tobramycin (Durand *et al.*, 2006).

Biofilms in MRSA infections

The ability of MRSA to form biofilms is an important virulence mechanism that complicates infections, especially those involving foreign materials like catheters and prosthetic joints. One study found that between 2006 and 2007, 56 % of all device-related infections caused by *S. aureus* were MRSA infections in the US (Hidron *et al.*, 2008). Biofilms have been defined as surface-attached communities of cells encased in an extracellular polymeric matrix that are more resistant to antibiotics (Kiedrowski *et al.*, 2011). The enclosed bacteria are dormant and are recalcitrant to antibiotic therapy. They are also protected against the host's immune response. Once biofilm formation occurs, the easiest way to treat the infection is to remove the infected device. However, this can be challenging, especially if the patient is elderly or debilitated, there are limited alternative options such as venous access in individuals requiring chronic indwelling intravenous catheters, and the device is attached to a permanent fixture, such as a pacemaker or prosthetic implant. Biofilm formation by *S. aureus* occurs in multiple steps (see Fig. 1), starting with the adherence of the bacteria either directly to artificial surfaces or through host factors that act as bridging molecules such as fibrinogen or fibronectin (Schroeder *et al.*, 2009). Bacteria can adhere to components of the extracellular matrix of host tissues, leading to colonization. Adherence is promoted by protein adhesins of the microbial surface components recognizing adhesive matrix molecules (MSCRAMM) family (Foster & Höök, 1998). The next step involves proliferation of the bacteria and accumulation into a biofilm requiring intercellular adhesion (Schroeder *et al.*, 2009). This step is promoted by polysaccharide intercellular adhesin (PIA), which is synthesized by gene products encoded by the *icaADBC* operon. The biofilm-associated protein, which is encoded by the *bhp* homologue, is also involved in biofilm accumulation and was recently isolated in a strain of MRSA from a burn unit (Kateete *et al.*, 2011). MRSA transitions between planktonic and biofilm stages through quorum sensing (QS), defined as a multicellular response to coordinate expression of genes required for biofilm in a population density-dependent manner (Bordi & de Bentzmann, 2011). QS is encoded by the *agr* operon where *agrD* encodes the autoinducer. In a study of 168 strains of MRSA, 23 were from patients with device-related infections, 55 were from patients with non-device-related infections, and 90 were from

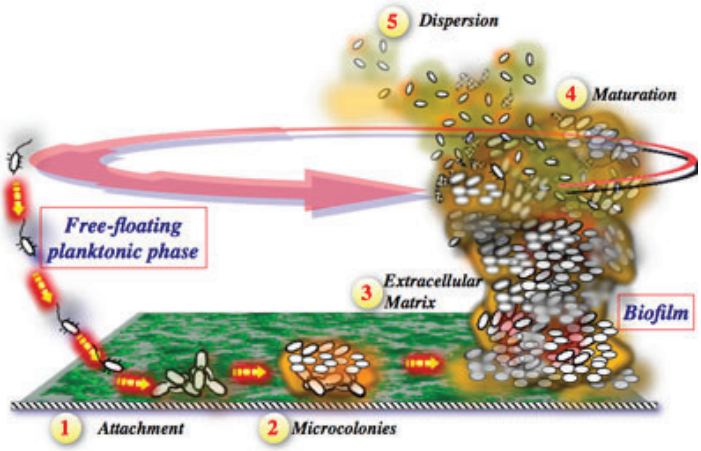

Fig. 1. Steps in MRSA biofilm formation. Reproduced with permission from J. M. Ghigo, Institut Pasteur, Paris, France.

asymptomatic nasal carriers (Kawamura *et al.*, 2011). The device group had a significantly higher prevalence of *agr-2* than the colonization group (78.3 % vs 34.4 %, $P=0.001$) and a higher biofilm index compared to *agr-1* and *agr-3*. Hence, type *agr-2* MRSA strains may be more likely to cause prosthetic device infections because of an enhanced ability to form biofilms.

Extracellular DNA (eDNA) has an important role in the matrix composition of a multitude of bacterial biofilms, including *S. aureus* (Izano *et al.*, 2008). The USA300 strain of CA-MRSA produces a biofilm whose matrix is composed of proteinaceous material and eDNA (Lauderdale *et al.*, 2010). The source of eDNA is believed to be chromosomal DNA that is released through cell lysis (Mann *et al.*, 2009). This study also demonstrated that staphylococcal thermonuclease degrades eDNA, resulting in biofilm dispersal. Recently, investigators used a mutant strain of CA-MRSA USA300 that lacks the ability to produce biofilms (CA-MRSA *sigB* mutant) to further characterize thermonuclease (Nuc) (Kiedrowski *et al.*, 2011). Levels of Nuc correlated inversely with biomass and Nuc activity levels were a strong predictor of biofilm formation across several different strains of *S. aureus*. The *sigB* mutant overproduced Nuc even in the presence of glucose supplementation, which is known to stimulate biofilm formation in *S. aureus* (Boles & Horswill, 2008). The overproduction of Nuc led to the degradation of eDNA and the inability to make biofilms. When Nuc was removed, the capacity to form biofilms was restored, although not completely. This may be due to the impact of extracellular protease activity, although further studies are needed to confirm this hypothesis.

MRSA (and other *S. aureus*) infections evoke a strong response from the immune system, with neutrophils providing the primary defence (Rigby & DeLeo, 2012). Although the ability of biofilms to protect *S. aureus* from the host immune response is well known, the exact mechanisms for this complex process are not clearly understood. Using a mouse model of catheter-associated biofilm infection, researchers demonstrated that *S. aureus* biofilms (using the USA300 LAC strain) are not recognized by Toll-like receptor (TLR) 2 and TLR9 receptors, which are part of traditional bacterial pattern recognition pathways (Thurlow *et al.*, 2011). TLRs are surface molecules on phagocytes and other immune cells involved in identifying microbial structures (like endotoxin) and generating signals that lead to the activation of innate immune responses. The primary response to *S. aureus* infection is thought to be a T helper 1 (Th1) type *in vivo* (Megyeri *et al.*, 2002). Recent studies evaluated the adaptive immune response to MRSA biofilms (using MRSA-M2, an ST30, *spa* type T019 and *agr* type III strain) in a mouse model and found that Th2 cell and T regulatory cell (Th2/Treg) responses protected against biofilm formation, while Th1/Th17 responses promote the development of chronic implant infection (Prabhakara *et al.*, 2011a, 2011b). Whether similar responses occur in humans requires further investigation. Results from these studies open avenues for developing immune adjuvant therapies (i.e. vaccines) that may help the immune system clear MRSA and other *S. aureus* infections. Future studies also need to be performed using mutants deficient in other surface colonization factors to determine their impact on staphylococcal biofilm formation.

A number of antibiotics and other compounds have been investigated for their anti-biofilm properties. Honey was shown to be bactericidal and able to penetrate MRSA biofilms (Merckoll *et al.*, 2009). Antibiotic-impregnated bone cement is commonly used to prevent post-operative infections after prosthetic joint placement. A quaternized chitosan derivative (26 %HACC) in polymethylmethacrylate (PMMA) bone cement inhibited biofilm formation in an MRSA isolate (strain ATCC 43300) better than gentamicin-loaded PMMA, significantly downregulated expression of *icaAD*, upregulated *icaR* which negatively mediates *icaAD* expression, and downregulated *mecA* (Tan *et al.*, 2012). In a study that evaluated a number of antiseptics, 10 % povidone-iodine was the only one that caused greater than 90 % reduction in both biofilm formation and dispersion (Aparecida Guimarães *et al.*, 2011). *N*-Acetylcysteine

was shown to decrease biofilm thickness in an MRSA isolate (a clinical strain not otherwise characterized) (Aslam & Darouiche, 2011). Among antibiotics, tigecycline reduced the level of transcription of *icaC*, which interfered with production of PIA leading to reduced intercellular adhesion in an MRSA strain (MRSA isolate 784, an EMRSA-16 strain isolated from a human wound infection in 2006 in Scotland), as well as upregulated genes for multiple adhesins including *fnbA*, *fnbB*, *cna* and *clfB* (Smith *et al.*, 2010). In a study that compared the anti-biofilm activity of vancomycin and moxifloxacin against MRSA, moxifloxacin demonstrated a 2.5 log reduction in the biofilm embedded bacterial counts while vancomycin failed to produce a 2 log reduction (Salem *et al.*, 2010). Indeed, further evidence of the poor activity of vancomycin against MRSA biofilm (using strain ATCC 43300) was demonstrated in experiments that combined vancomycin with rifampicin (Salem *et al.*, 2010). The combination showed *in vitro* antagonism against MRSA biofilm, which is concerning because this combination is often used in clinical practice to treat serious infections such as prosthetic valve MRSA endocarditis (Baddour *et al.*, 2005). This was further investigated in a study that compared the activity of daptomycin and vancomycin both alone and in combination with rifampicin and gentamicin against biofilm-producing MRSA strains (isolate B346846, with *agr* type I, *spa* type 17 and SCC*mec* type IV; and isolate B341002, with *agr* type II, *spa* type 2 and SCC*mec* type II) from patients with infective endocarditis (LaPlante & Woodmansee, 2009). Vancomycin did not achieve bactericidal activity at any of the time points tested. Daptomycin monotherapy demonstrated the best *in vitro* activity (as shown by declines in c.f.u. of bacteria) and was antagonized or delayed by rifampicin and gentamicin. Indeed, vancomycin treatment failures in MRSA infections are being increasingly reported (van Hal *et al.*, 2012). These may be due to rising mean inhibitory concentrations of vancomycin seen in some strains of MRSA, so-called 'MIC creep' (Liu *et al.*, 2011). In light of these new data, modification of existing clinical guidelines for the treatment of foreign body-associated infections may be warranted although additional *in vivo* studies are needed.

Antivirulence therapy

Agents directed against the virulence mechanisms of MRSA strains would have several advantages compared to antibiotics. First, there would be no selective pressure exerted on other non-pathogenic, commensal bacteria. Second, the associated toxicities of antibiotics (e.g. allergic reactions, nephrotoxicity and *Clostridium difficile* infection) may be avoided. Third, limiting antibiotics may decrease the development of drug-resistant bacteria. Combining antivirulence therapies with traditional antibiotics has the potential to change the paradigm of how MRSA infections are managed. Since bacterial survival is not impacted by the function of its virulence mechanisms, it is possible that resistance to antivirulence therapy would be slow to develop (Shoham, 2011).

One potential strategy is to inhibit the *agr* operon. *In vitro* experiments have shown that variants of autoinducing peptide (AIP) inhibit AgrC function (George *et al.*, 2008). An *in vivo* study demonstrated that administering AIP-2 concurrently with an *agr* type 1 strain reduced abscess formation (Wright *et al.*, 2005). However, *agr* inhibitors can promote biofilm formation, which could result in chronic *S. aureus* infections (Beenken *et al.*, 2010). Hence, further investigation on this approach is needed.

Another strategy for devices is the use of nanomaterials, defined as materials with at least one dimension less than 100 nm, to prevent the formation of biofilms (Taylor & Webster, 2011). Silver-lined urinary catheters and central venous catheters are used in clinical practice to lower the risk of health care-associated infections (Raad *et al.*, 2012). Decreasing the particle size of silver down to the nanometre range increases the surface area, which improves the antibacterial activity of the material (Taylor & Webster, 2011). Staphyloxanthin is a pigment of *S. aureus* that helps it resist reactive oxygen species such as those released by neutrophils. Early steps in staphyloxanthin production are similar to those in cholesterol production. A human squalene synthase inhibitor blocked staphyloxanthin biosynthesis *in vitro*, resulting in nonpigmented bacteria that were more susceptible to killing by human blood and clearance by the innate immune system in a mouse model (Liu *et al.*, 2008). Statins were shown to enhance *S. aureus* clearance by phagocytes through production of antibacterial DNA-based extracellular traps by human and murine neutrophils, macrophages and monocytes (Chow *et al.*, 2010).

Vaccines

The challenge of developing an effective anti-*S. aureus* vaccine has been an elusive goal for researchers over many years. For CA-MRSA infections, one specific target is PVL toxin, and antibody against it is under investigation as a potential vaccine. However, in a study on antibody levels against PVL in children with PVL-positive MRSA infections, neutralizing antibody against PVL was not protective against primary or recurrent CA-MRSA skin infections (Hermos *et al.*, 2010). Other investigators, using a murine model of dermatonecrosis, evaluated an agonist of human C5a called EP67 for its ability to induce host immunity against CA-MRSA (Sheen *et al.*, 2011). EP67 was effective in limiting the infection through the promotion of cytokine synthesis and neutrophil influx. This promising finding may warrant further investigation in humans.

Peptidoglycan (PG) comprises approximately 50 % of the cell wall of *S. aureus*. A PG-based vaccine against *S. aureus*, A170PG, was shown to be protective in a mouse model against several strains of MRSA including A174, A175, A176 and RIMD31092 (Capparelli *et al.*, 2011). The protection correlated with increased survival and reduced colonization and lasted at least 40 weeks. One caveat with this study is that the mouse strain used does not closely

mimic human infection because mice do not have pre-existing antibodies to *S. aureus*. In June 2011, Merck and Intercell announced the termination of phase II/III development of V170, a subunit vaccine containing the *S. aureus* antigen IsdB, which is a cell surface localized iron-regulated protein (Etz *et al.*, 2002). Safety concerns were cited due to an increase in overall mortality and multi-organ dysfunction in the vaccine recipients compared to those who received placebo. There is emerging consensus that future vaccines will need to contain multiple antigens (e.g. surface proteins, toxoids and capsular polysaccharides) and that the biological role of the cell-mediated immune response to MRSA infection will need to be better understood if ongoing efforts at vaccine development are to be successful (Patti, 2011; Daum & Spellberg, 2012).

Conclusions

MRSA infections remain a significant threat to human health into the second decade of the 21st century. Despite significant progress in understanding the pathogenesis of MRSA infection and the virulence mechanisms of MRSA strains, daunting challenges remain. The role of PVL in human MRSA infections remains controversial, but it appears that PVL is not likely to be a useful single target for vaccine development and is not likely the main factor determining the severity of CA-MRSA infections. Emerging technologies such as biomaterials that incorporate nanoparticles have the potential to impede the formation of MRSA biofilms, a key factor in prosthetic device-related infections. Host factors that lead to severe MRSA infections in certain individuals but more mild illness in others remain to be elucidated. Finally, further research is needed to unravel the complexities of virulence factor regulation and to determine the dynamics of how virulence factors are transmitted among different MSSA and MRSA strain types.

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