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Nat Rev Microbiol. Author manuscript; available in PMC 2017 November 30.

Published in final edited form as:

Nat Rev Microbiol. 2014 January ; 12(1): 49–62. doi:10.1038/nrmicro3161.

Adhesion, invasion and evasion: the many functions of the surface proteins of *Staphylococcus aureus*

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Abstract

Staphylococcus aureus is an important opportunistic pathogen and persistently colonizes about 20% of the human population. Its surface is 'decorated' with proteins that are covalently anchored to the cell wall peptidoglycan. Structural and functional analysis has identified four distinct classes of surface proteins, of which microbial surface component recognizing adhesive matrix molecules (MSCRAMMs) are the largest class. These surface proteins have numerous functions, including adhesion to and invasion of host cells and tissues, evasion of immune responses and biofilm formation. Thus, cell wall-anchored proteins are essential virulence factors for the survival of *S. aureus* in the commensal state and during invasive infections, and targeting them with vaccines could combat *S. aureus* infections.

Staphylococcus aureus is a commensal bacterium that can cause both superficial and invasive, potentially life-threatening, infections such as sepsis, endocarditis and pneumonia. Antibiotic treatment is often ineffective owing to the development of antibiotic-resistant strains, such as methicillin-resistant *S. aureus* (MRSA). MRSA is prevalent in hospitals, and hypervirulent MRSA strains have recently spread throughout the community[1–4]. *S. aureus* can express a broad range of virulence factors, including surface proteins that are covalently attached to peptidoglycan, which are known as cell wall-anchored (CWA) proteins. These surface proteins are crucial to the success of the organism as a commensal bacterium and as a pathogen (FIG. 1). The precise repertoire of CWA proteins on the surface varies among strains[5]: *S. aureus* can express up to 24 different CWA proteins, whereas coagulase-negative staphylococci such as *Staphylococcus epidermidis* and *Staphylococcus lugdunensis* express a smaller number[6,7]. Moreover, the expression of CWA proteins can be altered by growth conditions; for example, some proteins are expressed only under iron-limited conditions[8,9], whereas others are found predominantly on cells in the exponential[10] or stationary phases of growth[11]. Secretory signal sequences that are located at the amino termini direct the translated proteins to the secretory (Sec) apparatus in the membrane and are cleaved during

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Competing interests statement

The authors declare no competing interests.

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secretion. At their carboxyl termini, each of these proteins has a characteristic sorting signal, which facilitates their covalent anchorage to peptidoglycan (FIG. 2).

We propose to classify the CWA proteins into four groups on the basis of the presence of motifs that have been defined by structure–function analysis (FIG. 2; TABLE 1). The most prevalent group is the microbial surface component recognizing adhesive matrix molecule (MSCRAMM) family, which is defined by tandemly linked IgG-like folded domains. A theme that has emerged from the study of CWA proteins is that a single protein can carry out multiple functions. Perhaps this is not surprising, as proteins that are exposed on the surface of bacterial cells are in direct contact with the host and are subjected to selective pressure to carry out functions that are related to the colonization of host tissues and the evasion of host defences. The repertoire of CWA proteins that is available to *S. aureus* is limited, therefore many CWA proteins have evolved to have multiple roles. Moreover, these proteins show functional redundancy; for example, at least five CWA proteins bind the plasma glycoprotein fibrinogen and several promote adhesion to squamous epithelial cells. One consequence of this redundancy is that a null mutant that affects one CWA protein might only be partially defective in the studied function.

This Review examines how CWA proteins promote adhesion to the extracellular matrix (ECM) and to host cells, the invasion of host cells, the evasion of innate immune responses, perturb the adaptive immune response and can function as antigens in vaccines. We focus on those CWA proteins for which structural analysis has clarified the mechanism of ligand binding and protein function.

CWA protein architecture

All ORFs of CWA proteins contain a secretory Sec-dependent signal sequence at the amino terminus and a sorting signal — which comprises an LPXTG (Leu-Pro-X-Thr-Gly; where X means any amino acid) sortase cleavage motif, a hydrophobic domain and a stretch of positively charged residues — at the C terminus. The last two subdomains retain the protein in the membrane during secretion, which enables the sortase to bind and carry out its transpeptidase function (FIG. 2).

The MSCRAMM family

The acronym MSCRAMM was originally applied to surface proteins of *S. aureus* that mediate attachment to components of the host ECM, such as fibrinogen, fibronectin and collagen, at a time when it was thought that bacteria primarily attached to glycoconjugates present on cell surfaces[12]. In fact, many bacterial surface proteins are not MSCRAMMs, and some MSCRAMMs have additional functions other than promoting adhesion (TABLE 1). We now propose to use the term MSCRAMM to define a family of proteins on the basis of structural similarities and a common mechanism for ligand binding, which is mediated by two adjacent subdomains containing IgG-like folds[13]. This change is analogous to the revision of the term integrin, which was originally applied to mammalian cell surface receptors that recognized ECM components and promoted cell integration but has since been changed to include all proteins with the same structural organization even if they do not bind to the ECM. MSCRAMMs are also present in many Gram-positive bacteria, such as

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Staphylococcus pseudintermedius[14], coagulase-negative staphylococci[15,16], enterococci[17,18] and streptococci[19,20], some of which use similar binding mechanisms to the *S. aureus* proteins.

MSCRAMMs are characterized by at least two adjacent IgG-folded domains in the N-terminal A region. The archetypal MSCRAMMs are clumping factor A (ClfA) and ClfB from *S. aureus* and serine–aspartate repeat-containing protein G (SdrG) from S. *epidermidis.* All of these proteins bind fibrinogen using variants of the 'dock, lock and latch' (DLL) mechanism (see below). The A regions were shown by X-ray structural and biophysical analysis to be composed of three separately folded subdomains: N1, N2 and N3. N2 and N3 comprise the IgG-like folds. Other proteins in this family can be modelled with a high degree of certainty and are predicted to have A domains with a similar structure and to also bind ligands using the DLL mechanism (TABLE 1).

Linking the A regions to the wall-spanning region W is either region R (which is composed of Ser–Asp repeats (known as the SD region)) or a fibronectin-binding repeat domain, such as that found in fibronectin-binding protein A (FnBPA) or FnBPB, in which the fibronectin-binding repeat domain functions not only as a linker but also mediates avid ligand binding. These linker regions connect via a proline-rich domain that spans from the cell wall to the peptidoglycan-binding sorting signal. Thus, homologous A regions have become attached to flexible linkers that have different sequences and functions.

SdrC, SdrD, SdrE and bone sialo-binding protein (Bbp), which is another *S. aureus* MSCRAMM, have two or more repeated domains called B_{SDR} domains, which comprise 110–113 residues that are located between the A region and the flexible SD region[21]. The B_{SDR} repeats are folded separately and form a rigid rod that projects the A domain away from the cell surface and that is dependent on Ca^{2+} for structural integrity[22]. It is possible that B_{SDR} domains in the *S. aureus* proteins have ligand-binding activity that is similar to the related protein SdrF from *S. epidermidis*, in which B repeats have been reported to bind collagen IV (REF. 15).

Collagen adhesin (Cna), which is an *S. aureus* MSCRAMM protein that binds collagen, also has an A region at its N terminus that is divided into three subdomains (FIG. 2). However, Cna differs from the other MSCRAMMs as the ligand-binding domain is composed of the IgG-folded subdomains N1 and N2 rather than N2 and N3. The N1 and N2 subdomains of Cna are variants of the IgG fold and are closely related to the IgG-like subdomains N2 and N3 of ClfA[23]. This N-terminal region of Cna is linked to the cell wall-spanning W domain by a variable number of B_{CNA} repeated domains that differ in sequence from B_{SDR} domains[24]. Another difference is that Cna does not have a flexible stalk linking the repeated B domains to the wall-spanning region. Several other bacteria, including enterococci, streptococci and bacilli, express structural and functional homologues of Cna[17,25].

The NEAT motif family

Near iron transporter (NEAT) motif proteins are involved in haem capture from haemoglobin and help bacteria to survive in the host, where iron is restricted. Haem is transported via several CWA proteins, called iron-regulated surface (Isd) proteins, to a membrane

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transporter and then to the cytoplasm, where haemoxygenases release free iron[8,26]. The defining characteristic of Isd CWA proteins is the presence of one or more NEAT motifs, which bind either haemoglobin or haem. The structures of NEAT domains have been solved and the molecular mechanisms of ligand binding have been defined (reviewed in REFS 8,26,27). The ability of CWA Isd proteins to bind to other ligands is discussed below and summarized in TABLE 1.

Protein A: tandemly repeated three-helical bundles

Protein A is a multifunctional CWA protein that is ubiquitous in *S. aureus* and is often used in strain typing on the basis of variation in the DNA sequence-encoding region Xr. At the N terminus, protein A contains five homologous modules (known as EABCD; FIG. 2), each of which consists of single separately folded three-helical bundles[28,29] that can bind to several distinct ligands (TABLE 1). Located between this region and the cell surface is region Xr, which is composed of octapeptide repeats that are highly variable in number, followed by a constant region Xc.

Protein A is the only CWA protein of *S. aureus* that has the repeated three-helical bundles; however, the *S. aureus* binder of IgG protein (Sbi), which is non-covalently associated with lipoteichoic acid in the cell wall[30], contains four three-helical bundles, two of which have sequence similarity to protein A[31]. In addition, several small secreted proteins that are involved in immune evasion (such as staphylococcal complement inhibitor (SCIN) and extracellular fibrinogen binding protein (Efb)) contain single three-helical bundles[32].

The G5–E repeat family

S. aureus surface protein G (SasG) is closely related to the accumulation-associated protein (Aap), which is needed for biofilm formation in *S. epidermidis*. Notably, both proteins contain identical G5 domains in a tandem array that is separated by 50-residue sequences that are known as E regions[33,34]. G5 domains are characterized by five conserved glycine residues, and they adopt a β–triple helix–β-like fold that has no known ligand-binding function. In general, proteins that comprise highly similar domains in a tandem arrangement are prone to misfolding[35]. As the amino acid sequence of each G5 domain is identical, it is thought that alternating individually folded G5 and E regions is a mechanism to prevent protein misfolding. The G5–E domains of Aap and SasG become exposed on the surface of the bacteria either by proteolytic removal of the N-terminal A domain (in the case of Aap)[36] or by limited cleavage within the G5–E domains (in the case of SasG)[37].

MSCRAMM ligand-binding mechanisms

The mechanisms of ligand binding by MSCRAMMs, protein A and Isd proteins have been investigated by X-ray structural analysis of the proteins with and without bound ligands. In this section, we focus on ligand binding by MSCRAMMs. The binding mechanisms of protein A[38] and Isd proteins[8,26,27] have been recently reviewed.

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Dock, lock and latch mechanism

Analysis of the crystal structures of the minimum ligand-binding subdomains (N2–N3) of ClfA and ClfB from *S. aureus,* and SdrG from *S. epidermidis,* both in the apo form and in complex with ligand peptides[13,16,39], enabled the definition of a ligand-binding mechanism that is known as 'dock, lock and latch' (DLL)[16]. Ligands can dock to the open apo form and conformational changes create a closed form, in which the ligands are locked into place.

The DLL mechanism was originally described for SdrG of *S. epidermidis*. The N2 and N3 subdomains of this protein (and of ClfA and ClfB) are folded into a variant of the IgG fold (known as DEv-IgG), which is predominantly composed of two β-sheets that contain, in total, nine β-strands (for example, N2 contains A, B, E, D, D′, D″, C, F and G, whereas N3 contains A′, B′, E′, D′, D1′, D2′, C′, F′ and G′)[13] (FIG. 3a). The two subdomains are arranged in a specific orientation, which creates a trench between N2 and N3, in which ligands dock. The disordered ligand peptide aligns via β-strand complementation with the G ′ strand in the N3 subomain.

Ligand docking results in a redirection of the disordered extension of the N3 subdomain so that residues in this extension can interact with the ligand and lock it in place. Further propagation of the conformational change enables C-terminal residues of the N3 extension to be inserted into a trench in the N2 subdomain via β-strand complementation by forming an additional β-strand (known as the G″ β-strand) that aligns with the E β-strand in the N2 subdomain. This 'latch' further stabilizes the DEv-IgG–ligand complex. This event does not involve direct interaction with the ligand.

Ligand–peptide interactions with the G′ β-strand are mainly backbone–backbone interactions that involve hydrogen bonds. The nature of the side-chain interactions in the binding trench depends on the ligand sequence and the binding residues in the MSCRAMM; for example, binding of SdrG to fibrinogen involves a stretch of hydrophobic residues in the ligand peptide that is complemented by hydrophobic pockets in the 'locked' binding trench. The precise nature of these interactions determines binding specificity.

ClfA and ClfB bind to their peptide ligands (TABLE 1) using subtle variations of the DLL mechanism[13,39], in which, unlike the mechanism of SdrG, ligand binding involves parallel (rather than antiparallel) β-sheet complementation. As a result, the orientation of the ligand in the trench is inverted.

The SdrG and ClfB ligand-binding sequences are flanked by additional residues. As a consequence, only the open apo form of the proteins can accept the ligand. By contrast, both the closed and open forms of ClfA bind to its ligand. ClfA recognizes the extreme C terminus of the γ-chain of fibrinogen, which contains residues that can penetrate the hole that is formed by the lock region of the closed form.

Several MSCRAMMs can bind to two or more ligands; for example, in addition to binding to fibrinogen, ClfA also binds to complement factor I[40]. However it is not clear whether binding to factor I involves DLL. ClfB binds to the α-chain of fibrinogen and to loop regions of keratin K10 and loricrin by DLL[41–44]; it also binds dermokine[44]. The identification of

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several different ligands that bind to ClfB enabled the definition of the consensus ligand sequences GSSGXG (Gly-Ser-Ser-Gly-X-Gly) and GSSG (Gly-Ser-Ser-Gly) or STGXXG (Ser-Thr-Gly-X-X-Gly). FnBPA binds to fibrinogen and elastin by DLL, but structural models of the two ligands in complex are not available, therefore a consensus binding motif has not been defined[45]. Further detailed investigations of more MSCRAMM ligands will lead to a better understanding of how a single binding trench can accept several different ligands. Furthermore, it is probable that not all ligands bind to MSCRAMMs using DLL, and future studies should investigate additional binding mechanisms.

The collagen hug

Cna binds collagen using a variation of the DLL mechanism, which is known as the 'collagen hug'[23] (FIG. 3b) and uses the three aspects of DLL: ligand docking, locking and stabilization via latching. The N1- and N2-subdomain structures of Cna form IgG-like folds that have corresponding roles to N2 and N3 in DLL, with the N2 C-terminal extension forming the latch. The linker that connects the N1 and N2 domains of Cna is long and enables a hole to form in the interface between the two domains, which can accommodate a monomeric collagen triple-helix rod. The ligand docks into a shallow trench in the N2 domain, which is followed by a conformational change that forms the lock and enables the linker between N1 and N2 to wrap around the collagen molecule. After collagen is 'grasped' by the N1–N2 domain, the latching event takes place by β-strand complementation to stabilize the complex. Here, the lock residues are provided by the linker between the N1 and N2 subdomains and not by the C-terminal residues from the extension of the N3 subdomain.

CWA proteins as virulence factors

To experimentally show that a surface protein can act as a virulence factor and promote disease, the guidelines that were first articulated by Stanley Falkow as the 'Molecular Koch's Postulates' are helpful[46]. Isogenic mutants, along with complemented controls, are tested for virulence in models of infection; however, functional redundancy can make it difficult to detect a reduction in virulence in a mutant that is defective for a single factor. Most strains of *S. aureus* have two FnBPs and up to five proteins that can bind to fibrinogen. To overcome the problem of redundancy, a single CWA protein can be expressed alone in a surrogate host, such as *Lactococcus lactis*[41,47,48] or *Staphylococcus carnosus*[49]. Adding to the complexity of studying the contribution of CWA proteins to virulence, until recently it was not possible to genetically manipulate most clinical isolates, and studies were confined to a few well-characterized laboratory strains. Now, surface proteins of important clinical isolates can be studied, as a result of improvements in vectors for constructing mutations and a better understanding of ways to overcome restriction barriers to DNA uptake[50–52].

Besides these bacterial factors, differences among host species can also influence the results of infection studies. Therefore, studies of animal models of infection must be carefully interpreted, in particular if the surface protein in question has a considerably lower affinity for the animal compared with the human version of the ligand. For example, SdrG and IsdB do not bind to mouse fibrinogen[53] or haemoglobin[54], respectively, but genetically manipulated mice that express the human version of the protein can be used for *in vivo*

studies[54] (see TABLE 2 for an overview of results obtained in different models and diseases).

Animal studies of infection

The first CWA proteins that were experimentally shown to be virulence factors were protein A (in mouse sepsis and skin abscess)[55], FnBPA (in endocarditis) [56] and Cna (in mouse septic arthritis)[57]. In the first two cases, insertion mutations that inactivated gene expression and function were not complemented or reverted to wild type to show regained virulence. In the case of inactivation of Cna, complementation restored virulence in a study of ocular keratitis[58]. Despite these caveats, it is now widely accepted that each of these CWA proteins is important in pathogenesis.

A systematic study of the role of individual surface proteins in abscess formation in the mouse kidney enabled direct comparisons of factors that were expressed by a single bacterial strain (the laboratory strain Newman) in the same model and in the same mouse strain[59]. Bacteria that were defective for the CWA proteins SdrD, IsdB, ClfB, IsdA, IsdC, ClfA, Spa or SasG were intravenously inoculated to cause bacteraemia, and 5 days later the bacterial load and the number of abscesses in kidneys were measured. Compared with wild type, there was a >1 log reduction in the counts of viable mutant bacteria in kidney tissue; SdrD, IsdB, IsdA and Spa mutants also caused a reduced number of abscesses. This study indicates the importance of CWA proteins in experimental infections but is limited by the fact that only surface proteins that recognize the mouse versions of the ligands will be identified as virulence factors. Moreover, the study used the *S. aureus* strain Newman, which lacks Cna and has non-sense mutations in both *fnbA* and *fnbB* that result in the failure of the encoded proteins to be anchored to the cell wall[60]. In mouse models (of endocarditis[48] and mastitis[61]) that test the ability of FnBPs to promote bacterial uptake into non-phagocytic host cells, these proteins have been clearly demonstrated to be virulence factors even though the strain that was used (*S. aureus* strain 8325-4) only expresses them at low levels.

A role for FnBPs has also been shown in the pathogenesis of septic arthritis when *fnb* mutations were introduced into the mouse-pathogenic strain LS1 (REF. 62). A more comprehensive picture will be provided if bacterial strains (including current clinical strains) that express all surface proteins are compared and if studies are conducted using genetically manipulated mice that express the human version of the ligands. A recent study of strain ST239, which is the dominant *S. aureus* strain in the Far East, showed that the SasX protein confers virulence in mouse skin and lung infections[63]. Another study in patients with infected cardiac devices and bacteraemia identified three polymorphisms in FnBPs that increased their affinity for fibronectin, implying that there was *in vivo* selection for strains that have higher affinity for a ligand that coated the devices[64].

Nasal carriage and adhesion to squamous epithelium

Approximately 20% of the human population is permanently and persistently colonized by *S. aureus* in the moist squamous epithelium of the anterior nasal cavity[65]. The bacterium must compete with other commensal bacteria and avoid the innate immune defences of nasal secretions[66,67]. *S. aureus* actively replicates to avoid removal by the shedding of nasal cells

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and mucous flow. Microarray analysis of bacteria that were isolated from the nasal cavity indicates that bacteria grow exponentially in this environment and that they express high levels of mRNA encoding CWA proteins that are important in bacterial adhesion to squamous cells[68]. ClfB[41,69] and IsdA[70] promote nasal colonization in rodents and, in the case of ClfB, also in humans[71]. ClfB binds to the C-terminal domain of human and mouse keratin 10 (K10)[43], which is a major component of the interior of squamous cells and is exposed at the cell surface. ClfB recognizes a region of K10 that contains several Ω loops. These motifs are also present in loricrin, which is the dominant component of the cornified protein envelope of squames; consistent with this, ClfB binds loricrin *in vitro*[41]. Moreover, *S. aureus* has a decreased ability to colonize the nasal cavity of loricrin-knockout mice, which shows that, in mice, loricrin is an important ligand for ClfB. In addition, recombinant soluble loricrin blocks adhesion and colonization, which indicates that reducing nasal carriage in humans using inhibitors might be feasible[41].

IsdA also promotes the adhesion of *S. aureus* to squames. Indeed, colonization of the nasal cavity of cotton rats was reduced in a mutant that lacked IsdA and after immunization with recombinant IsdA[70]. Recombinant IsdA can bind to the cornified cell envelope proteins loricrin, involucrin and K10 *in vitro*[72] using its NEAT motif (FIG. 2) but the binding site (or sites) and mechanism of binding have not been determined.

Several other CWA proteins (such as SdrC, SdrD and SasG, as well as SasX, which is only expressed by ST239 strains)[63,73,74] promote adhesion to purified squames but the ligand or ligands that are involved are not known, and the ability of the proteins to promote colonization of the nares of rodents has not been reported.

Invasion of epithelial and endothelial cells

Until recently, *S. aureus* was not thought to be an intracellular pathogen. As well as being able to survive within neutrophils, which can function as a Trojan horse to promote dissemination[75], *S. aureus* can direct its uptake into cells that are not normally phagocytic, and host cells can be damaged from within by bacterial cytotoxins. In addition, intracellular bacteria are protected against attack from extracellular host defences, and intracellular *S. aureus* can persist in a semi-dormant state known as small colony variants, which renders them intrinsically resistant to antibiotic therapy[76]. FnBPs promote adhesion to the surface of mammalian cells and subsequent internalization[77–79].

The fibronectin-binding domains in the *S. aureus* CWA proteins FnBPA and FnBPB are located in an intrinsically disordered segment that is C-terminal to the fibrinogen-binding A region (FIG. 2). The binding motif is repeated up to 11 times, although the repeats have substantial sequence variations, which result in considerable variation in affinities for the fibronectin ligand[80].

Fibronectin is composed of three types of structural modules (known as type 1, type 2 and type 3 modules) and the N-terminal domain of fibronectin contains five type 1 modules, each of which is composed of a β-sandwich with two sheets that contain two and three strands, respectively[80]. The high-affinity fibronectin-binding motifs of FnBPs each bind to β-strands in the type 1 fibronectin modules using short antiparallel β-strand

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complementations; they act as a tandem β-zipper. Some hydrophobic and hydrophilic interactions between the FnBPs and fibronectin also contribute to binding. Individual short type 1 modules have a low affinity for FnBPs, but neighbouring tandemly arrayed fibronectin type 1 modules form multiple connections, resulting in a high affinity. The fibronectin-binding region of FnBPs is intrinsically disordered and consequently lacks discernible secondary structure. After immunization with the fibronectin-binding domain, the antibodies that have the highest affinity recognize epitopes in the β-zipper–fibronectin complex, which is only formed after the bacterium has attached to a ligand. These antibodies are immunodominant[81], but they do not block binding and are not protective.

Fibronectin also contains an arginine–glycine–aspartate sequence in type 3 module number 10, which is recognized by integrins, such as the abundantly expressed α5β1 integrin. Binding of fibronectin to FnBPs and its subsequent recognition by integrin α5β1 leads to internalization of the bacterium. At least one high-affinity fibronectin-binding repeat is required for internalization[82] in a process that involves the clustering of integrins on the host cell surface[83,84]. This interaction triggers phosphorylation at the C-terminal cytoplasmic domain of the integrin, which activates a signalling cascade that ultimately results in cytoskeletal rearrangements and uptake of bacteria into the cell by endocytosis[84]. Internalization has been shown in cultured cells *in vitro* and *in vivo* in a rat model of endocarditis — in which electron microscopy analysis of cardiac tissue revealed intracellular bacteria[48] — and in a mouse model of mastitis[61].

It has recently been found that IsdB directly binds to β3-containing integrins and that this interaction can promote bacterial adhesion to, and internalization by, mammalian cells[85]. Thus, it is possible that IsdB functions in concert with FnBPs to promote invasion via its direct interaction with integrins.

Evasion of immunity

Several CWA proteins interfere with innate and adaptive immune responses. Opsonization and phagocytosis requires IgG to bind to antigens on the bacterial cell surface. Clustered outwards-pointing IgG Fc regions are recognized by the soluble complement protein C1q in complex with C1s and C1r, which triggers activation of the classical complement pathway and C3 convertase. The convertase cleaves C3 and the resulting C3b protein covalently binds to the bacterial cell surface. Both IgG Fc domains and C3b are recognized by specific receptors on the surface of activated neutrophils, and opsonized bacteria are engulfed efficiently.

The binding of protein A to the Fc region of IgG results in the coating of the bacterial cell with IgG that is incorrectly oriented and therefore functionally impaired. Incorrectly oriented IgG cannot be recognized by neutrophil receptors and also prevents the activation of complement via the classical pathway. Consistent with this, protein A-deficient bacteria are more susceptible to neutrophil-mediated uptake and killing in the presence of serum opsonins[30,86] and have reduced virulence in mice[55,59,87].

ClfA and SdrE contribute to immune evasion by recruiting host regulators of complement and by promoting the destruction of C3b[40,88,89]. Cna binds to the collagenous domain of

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C1q and inhibits the classical complement pathway by interfering with the interaction of C1q with C1r[90]. Homologues in other bacteria have been shown to act as virulence factors in different animal models of infection, but it is unknown whether this effect is caused by their functions as adhesins or as immune-evasion factors[57,58].

ClfA is a potent virulence factor in mice after the intravenous injection of bacteria. It increases bacterial survival in the bloodstream, which promotes dissemination, and it increases abscess formation in internal organs, such as the kidney[59] and joints[91]. The increased survival of ClfA-expressing bacteria is dependent on fibrinogen binding[92]. Fibrinogen-coated bacteria are less likely to be cleared by neutrophils, although the molecular mechanisms that are involved are unclear.

Increasing inflammation

Protein A is an important virulence factor in the pathogenesis of experimental staphylococcal pneumonia in mice. It binds to tumour necrosis factor receptor 1 (TNFR1) on lung epithelial cells, which leads to the activation of intracellular signalling, the expression of chemoattractant cytokines (such as interleukin-8 (IL-8)) and to the recruitment of neutrophils[93]. This increases inflammation of the airway epithelium and thus contributes to tissue damage. Protein A, in combination with α-toxin[94,95], loosens tight junctions between airway epithelial cells and enables the dissemination of bacteria. However, it also binds to the epidermal growth factor receptor (EGFR)[96], which triggers activation of the disintegrin and metalloproteinase domain-containing protein 17 (ADAM17; also known as TNF-converting enzyme (TACE)), which cleaves and sheds TNFR1 into the airway lumen. This sequesters TNF and reduces the number of surface receptors, dampening the inflammatory response. Thus, protein A seems to trigger both pro-inflammatory and anti-inflammatory events, depending on the stage of the infection.

The hypervariable C-terminal region Xr of protein A also triggers inflammation. Extracellular protein A is endocytosed by an unknown receptor. This triggers interferon-β (IFNβ) expression, Janus kinase (JAK)–signal transducer and activator of transcription (STAT) signalling and IL-6 production. Mice that lack the IFNβ receptor are protected from lethal pneumonia caused by *S. aureus*[97].

Biofilm formation

Staphylococci are the most common cause of infections that are associated with indwelling medical devices, such as central venous catheters and prosthetic joints[98,99]. The ability to form multicellular communities known as biofilms is crucial to the success of bacteria in device-related infections and probably also has a role in many other types of infection[100,101]. Biofilm cells are resistant to phagocytosis and, as some cells are in a dormant state, it is difficult to target them with antibiotics. Until recently, the accumulation of bacteria during biofilm formation was attributed only to the polysaccharide intercellular adhesin (PIA), which is encoded by the *ica* operon[102], but it is now recognized that staphylococcal surface proteins can promote accumulation in an *ica*-independent manner. This is particularly relevant to MRSA as both hospital-associated and community-associated strains depend on proteins rather than polysaccharide for biofilm formation[103–105]. The CWA proteins that

participate in biofilm formation are biofilm-associated protein (Bap)[106], ClfB[107], FnBPs[103,104], SasC[108], SasG[37,74] and protein A[109]. Bap, SasG (which is a homologue of the *S. epidermidis* CWA protein Aap) and SasC are present only in subsets of isolates, whereas the remaining CWA proteins are widely distributed.

SasG- and Aap-dependent biofilm formation is the best understood biofilm formation process. In both cases, biofilm accumulation depends on zinc, which binds to Aap G5–E domains and thus facilitates the bridging of two Aap polypeptides[33]. Furthermore, the G5–E domains of Aap are predicted to form twisted rope-like structures, which — when connected to Aap proteins from other cells — could link the bacteria together (FIG. 4). A similar mechanism probably occurs during SasG-promoted biofilm accumulation[34,37]. When expressed at high levels on the cell surface, FnBPs[103], protein A[109] and SasC[108] can also promote biofilm formation; however, the mechanisms are not yet established[104]. For FnBP-mediated biofilm formation, the N2–N3 subdomains are required but the DLL mechanism is not involved[103,104]. It is possible that direct interactions between the A regions of FnBPs on adjacent cells could promote accumulation. Alternatively, FnBPs might bind to a ligand on the adjacent cell surface (FIG. 4). The identification of the binding site (or sites) on FnBPs (for example, by phage display)[110] will enable the design of inhibitors of the protein–protein interactions that occur in MRSA-biofilm formation, and thus might reduce the incidence of disease.

Clinical relevance of CWA proteins

As outlined above, *S. aureus* CWA proteins are important virulence factors and determine pathogenesis, thus their relevance to human diseases and clinical outcomes is an area of high interest. The variation of CWA proteins in clinical isolates and the targeting of these proteins with vaccines are two active areas of research.

Variation in CWA protein genes

The population of *S. aureus* comprises 11 dominant lineages, each of which has a unique combination of genes that encode CWA proteins[5]. Some are present in all lineages, whereas some are absent from a few or from the majority of lineages. Thus, many CWA proteins are not essential. In some cases this can be explained by functional redundancy. Sequence variation occurs in the functional domains of the CWA proteins and this variation is higher between lineages than within a lineage. The FnBP proteins show a high degree of variance: their A domains have greatly diverged, which has resulted in several isoforms[111,112]. However, the ligand-binding functions of each isoform seem to be retained. It is possible that variation in some surface proteins has resulted in altered ligand-binding affinity, which might affect the outcome of infections; for example, variations in the fibronectin-binding repeats of FnBPA are associated with varying ligand-binding affinities; *S. aureus* bacteraemia isolates that infect cardiac devices have a higher affinity for fibronectin than those that only cause bacteraemia[64]. The level and timing of CWA protein expression will also vary between strains or lineages, but this has not been studied yet. Detailed experimental investigation is needed to determine the consequences of variation between *S. aureus* lineages.

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CWA proteins as vaccines

Recombinant CWA proteins are potential antigens that could be used as vaccines to combat S. *aureus* infections. In laboratory animals, individual CWA proteins and combinations of CWA proteins have been shown to induce protective immunity, which is mediated by antibodies[91,113,114]. However, for unclear reasons, an IsdB vaccine that was protective in animals failed to protect patients from serious infections after cardiothoracic surgery in a Phase III trial[115,116]. One strategy to increase vaccine efficacy is to target several CWA proteins; for example, a combination of four CWA protein antigens resulted in superior protection in mice[114] than each antigen individually, which supports the use of a combination of antigens in vaccines for future clinical trials[116–118]. Furthermore, knowledge of the structure and mechanisms of ClfA and protein A ligand binding has informed the engineering of variants with substitutions that no longer bind to host proteins and that stimulate higher antibody titres and greater protective immunity[119,120]. Mice that were vaccinated with a protein A variant that lacked the ability to bind to IgG and IgM and that were subsequently challenged with *S. aureus* mounted an immune response to many antigens, which indicates that the immunosuppressive effect of protein A had been negated[120]. Stimulating humoral immunity alone is insufficient for protection in humans, so adjuvants that trigger a cell-mediated response involving T helper 1 (T_H1) and/or T helper 17 (T_H17) cells, as well as IL-17 and IFNγ-mediated recruitment of neutrophils will be important[116,118].

Discussion

CWA proteins provide crucial opportunities for bacteria to interact with the host and are indispensable for survival in the commensal state and during invasive infections. In this Review, we have categorized surface proteins primarily on the basis of common structural motifs and also on their functions, which have been established from *in vitro* studies and *in vivo* analysis in animal models (FIG. 1; TABLE 1; TABLE 2).

The function of many *S. aureus* surface proteins remains to be established and structural analysis will be key to providing insights into their roles. Although ligands for most CWA proteins have been described, we predict that many more will be discovered. This will be helped by improved phage-display technology combined with high-throughput DNA sequencing. This approach was validated using SdrG, for which ligands are known, and it was then successfully used to discover a ligand for SdrC, for which host targets were previously unknown[110]. The DLL mechanism of ligand binding is very adaptable. MSCRAMM proteins recognize a broad range of short, unfolded peptide ligands, and the ligand-binding trenches can accept ligands in either of two possible orientations. We envisage manipulating residues that are involved in ligand binding to increase affinity and alter specificity. This has already been achieved by changing Cna and adhesin of collagen (Ace) from *Enterococcus faecalis* residues in the ligand-binding loop between N1 and N2, which has resulted in an Ace variant that has a much higher affinity for type I collagen[121]. By contrast, molecules that have a lower affinity recognized a greater number of binding sites in the ligand. The discovery of molecules that have a higher affinity for the trenches

than natural ligands opens the possibility of developing novel therapeutics, for example, to abolish host cell adhesion, invasion and biofilm formation.

It is perhaps surprising that more is not known about how *S. aureus* CWA proteins interfere with the innate immune responses of the host, in particular how they regulate complement activation. This strategy is commonly used by other invasive bacteria, notably meningococci and streptococci[32]. Further work is needed to obtain a more detailed understanding of such interactions.

Most work on CWA proteins so far has been confined to a few laboratory strains. Studies must be extended to clinical strains, where there is considerable variation both in the repertoire of CWA proteins as well as sequence variation in binding domains. Furthermore, our understanding of the timing of expression and the abundance of particular proteins is likely to be revised; for example, the most abundant CWA protein on the communityassociated MRSA strain LAC is protein A[122], whereas hospital-associated MRSA strains express high levels of FnBPs in both the exponential and stationary phases of growth[104]. As described above, the affinity of FnBPs for fibronectin is important in determining whether a cardiac device will become infected[64].

In conclusion, surface proteins carry out a broad range of functions that are essential for the colonization of, and survival in, the host. Structural analysis has been crucial for defining the mechanistic basis of these phenomena and has provided a framework for the classification of this important group of bacterial proteins.

Acknowledgments

T.J.F. would like to acknowledge Science Foundation Ireland Programme Investigator grant 08/IN1/B1845. M.H. would like to acknowledge NIH grant AI 20624. The authors would like to thank D. Ravirajan for help with the on-line movie.

Glossary

Coagulase-negative staphylococci

Staphylococcus spp. (other than *Staphylococcus aureus*) that do not express coagulase and are less virulent

Extracellular matrix

The extracellular components of tissue that often provide structural support for cells

Fibronectin

A high-molecular-weight dimeric glycoprotein that is found in serum and in the extracellular matrix (ECM). It binds to integrins and to other components of the ECM

Oxidative burst

A respiratory burst that is produced by phagocytic cells. NADPH oxidase causes the rapid release of reactive oxygen species (superoxide and hydrogen peroxide)

Complement

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Proteins in serum that are activated by the presence of foreign antigens; a proteolytic cascade leads to the formation of the neutrophil opsosin C3b and the chemoattractant peptides C3a and C5a

Apo form

The form of a protein without bound ligand

Elastin

An elastic protein in connective tissue. It allows tissue to regain shape by stretching or contracting

Isogenic

A term used to describe strains that are characterized by identical genes

Squamous epithelium

The most superficial layer of stratified epithelium; it consists of flat, scale-like squamous epithelial cells (known as squames) that have a cornified envelope composed of proteins

Ω loops

Strings of glycine and serine residues in keratin 10 and loricrin, flanked by hydrophobic amino acids. Modelling suggests the formation of structures that are shaped like the Greek capital letter Ω

Fc regions

Fragment crystallizable regions at the tail of antibodies; they react with specific receptors on neutrophils and with the complement protein C1q to trigger the classical pathway of complement fixation

Classical pathway

One of three pathways for activating complement fixation. Requires clustered IgG molecules with their Fc regions pointing outwards to attract the hexameric complement protein C1q

Opsonin

A protein (antibody or complement protein) that enhances phagocytosis by neutrophils

α-toxin

A β-barrel pore-forming cytolysin that is secreted as a monomer and forms a heptamer in the membranes of susceptible cells; it is an important virulence factor

Tight junctions

Areas of close contact between the membranes of epithelial and endothelial cells; they are connected to the actin cytoskeleton

Phage-display technology

Bacteriophages that display libraries of peptides that are incorporated into capsid proteins. Individual particles that bind to ligands are enriched by ‘panning’. The peptide sequences are identified by DNA sequencing

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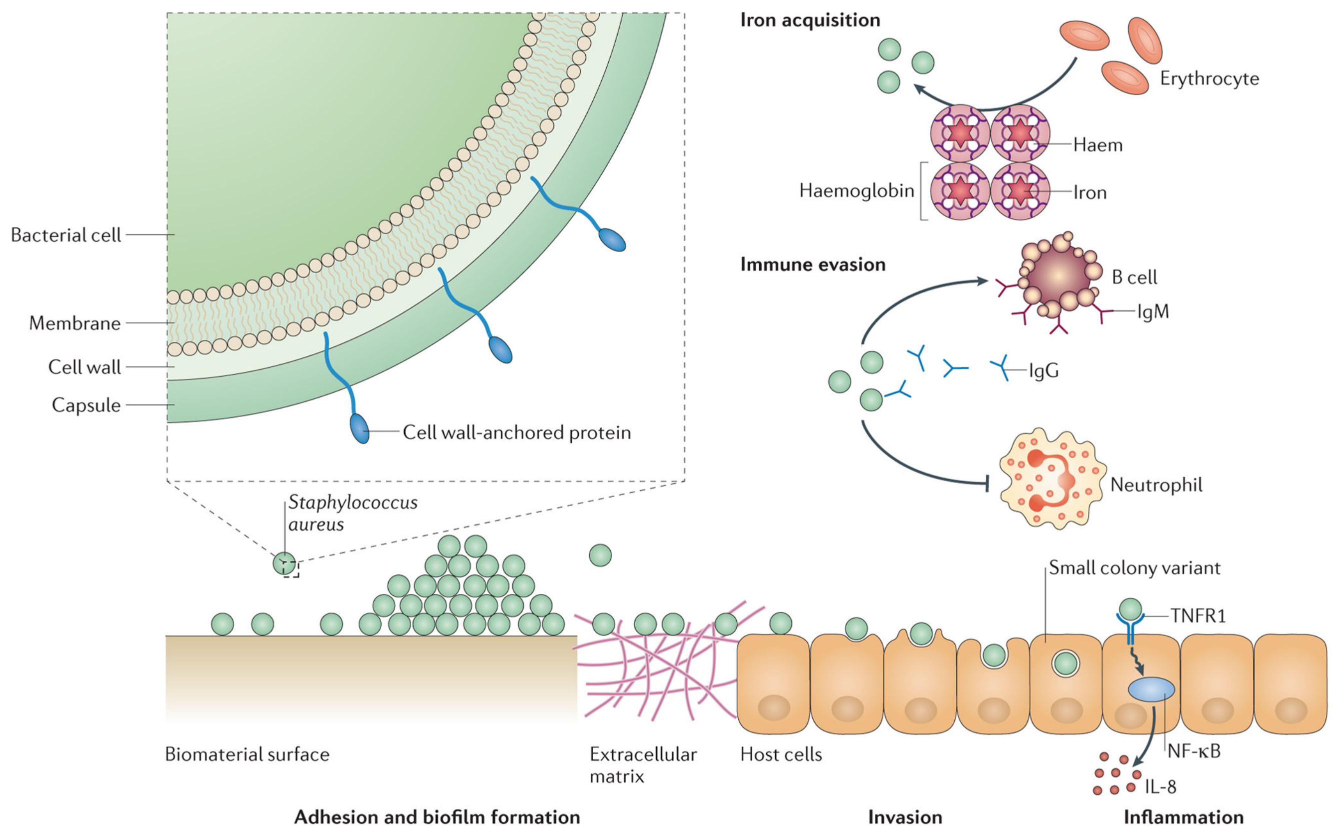

Figure 1. Functions of CWA proteins of *S. aureus*

The cell wall-anchored (CWA) protein iron-regulated surface determinant (Isd) binds haemoglobin and extracts and transports haem across the cell wall and membrane into the cytoplasm, where iron is released. Protein A acts as a superantigen for B lymphocytes and disrupts adaptive immune responses and immunological memory. Phagocytosis by neutrophils is inhibited by the binding of CWA proteins to IgG and other plasma proteins, by reducing the level of — or access by — neutrophil receptors to the complement opsonin C3b and, if engulfed, by inhibiting the oxidative burst. CWA proteins promote adhesion of *Staphylococcus aureus* to the extracellular matrix, to the surface of host cells and to biomaterial surfaces that are conditioned by the deposition of plasma proteins. Interactions between CWA proteins on adjacent cells contribute to the accumulation phase of biofilm formation. CWA proteins directly or indirectly interact with integrins and promote the invasion of non-phagocytic host cells. Intracellular bacteria can cause host cell apoptosis or necrosis, or they can enter a non-disruptive semi-dormant state known as small colony variants. By binding to and activating tumour necrosis factor receptor 1 (TNFR1) on host epithelial cells, protein A triggers the synthesis of cytokines (for example, interleukin-6 (IL-6)) and causes disruptive inflammation, which contributes to pathogenesis. NF-κB, nuclear factor-κB.

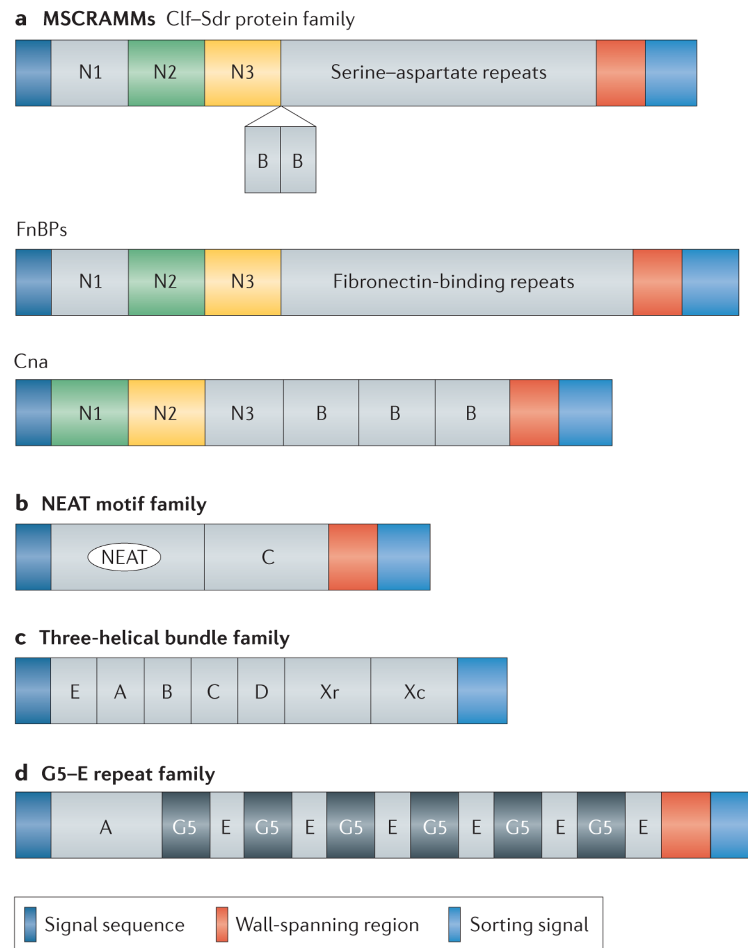

Figure 2. Classification of CWA proteins on the basis of structural motifs

The primary translation products of all cell wall-anchored (CWA) proteins contain a signal sequence at the amino terminus and a wall-spanning region and sorting signal at the carboxyl terminus. The CWA proteins that are depicted are those for which structural analysis has facilitated classification into four distinct groups. **a** | Microbial surface component recognizing adhesive matrix molecules (MSCRAMMs). The clumping factor (Clf)–serine–aspartate repeat (Sdr) group comprises proteins that are closely related to ClfA. ClfA and ClfB have a similar domain organization, whereas SdrC, SdrD and SdrE of *Staphylococcus aureus* and SdrF of *Staphylococcus epidermidis* contain additional B_{SDR} repeats that are located between the A domain and the serine–aspartate repeat R region. The N-terminal A region contains three separately folded domains, which are known as N1, N2 and N3. Structurally, N2 and N3 form IgG-like folds that bind ligands by the 'dock, lock and latch' (DLL) mechanism. Fibronectin-binding protein A (FnBPA) and FnBPB have A domains that are structurally and functionally similar to the A domain of the Clf–Sdr group. Located in place of the serine–aspartate repeat region are tandemly repeated fibronectin-binding domains (11 in FnBPA, 10 in FnBPB). The A region of the collagen adhesin (Cna) protein is organized differently to other MSCRAMMs, with N1 and N2 comprising IgG-like folds that bind to ligands using the 'collagen hug' mechanism. The A region is linked to the

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wall-spanning and anchorage domains by variable numbers of B_{CNA} repeats. **b** | Near iron transporter (NEAT) motif protein family. The iron-regulated surface determinant (Isd) proteins have one (for IsdA), two (for IsdB) or three (for IsdH) NEAT motifs that bind to haemoglobin or haem. The figure depicts IsdA, which has a C-terminal hydrophilic stretch that reduces cell surface hydrophobicity and contributes to resistance to bactericidal lipids and antimicrobial peptides. **c** | Three-helical bundle motif protein A. The five N-terminal tandemly linked triple-helical bundle domains (known as EABCD) that bind to IgG and other ligands are followed by the repeat-containing Xr region and the non-repetitive Xc region. **d** | G5–E repeat family. The alternating repeats of the G5 and E domains of *S. aureus* surface protein G (SasG) from *S. aureus* and accumulation-associated protein (Aap) from *S. epidermidis* link the N-terminal A region to the wall-spanning and anchorage domains. If the A domain is removed, the G5–E region can promote cell aggregation.

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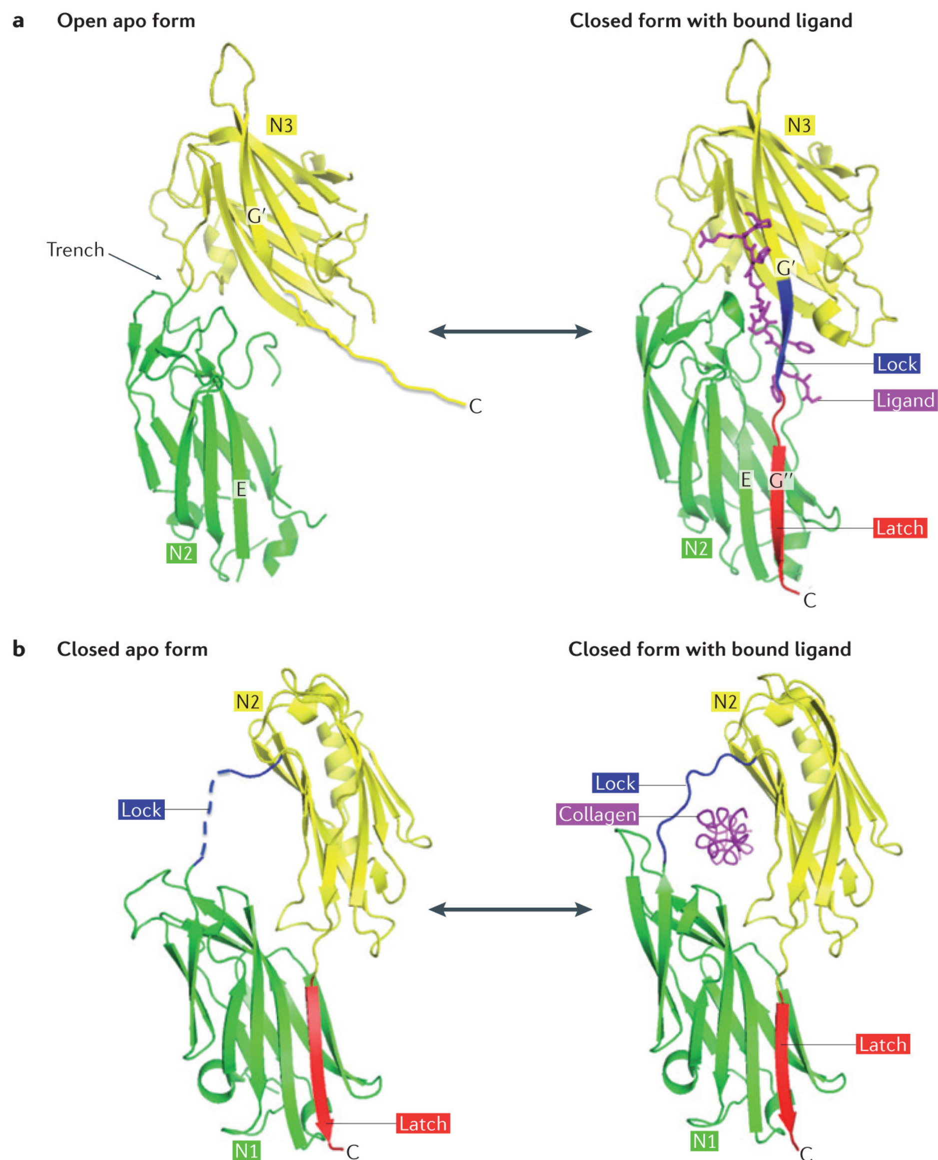

Figure 3. Mechanisms of ligand binding by MSCRAMM proteins

a | Dock, lock and latch (DLL) mechanism. The A region of the microbial surface component recognizing adhesive matrix molecule (MSCRAMM) protein in its open apo form has a wide trench between the N2 and N3 subdomains (apo serine–aspartate repeat-containing protein (SdrG); Protein Data Bank (PDB) reference: 1R19). The ligand peptide (purple) inserts into this trench and the MSCRAMM protein undergoes conformational changes to a closed form and locks the ligand in place (SdrG–ligand complex; PDB reference: 1R17). In the apo form, the disordered carboxy-terminal extension of the N3 subdomain is not part of the crystal structure. After ligand binding, this region forms the lock (blue) and the latch (red), thus locking the ligand in place. **b** | Collagen hug mechanism. In the apo form, collagen adhesin (Cna) is in an equilibrium between an open and closed form. The crystal structure shows the closed form with the empty ligand-binding trench covered by the lock (blue) (apo Cna; PDB reference: 2F68). The latch peptide (red) has

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undergone β-strand complementation with the latching sequence in N1. In the open form (not shown), the rope-like collagen triple helix docks into a trench that is located between the N1 and N2 subdomains. The conformational change back to the closed form captures (or 'hugs') the ligand (purple) using residues in the lock region (blue) (Cna–collagen complex; PDB reference: 2F6A).

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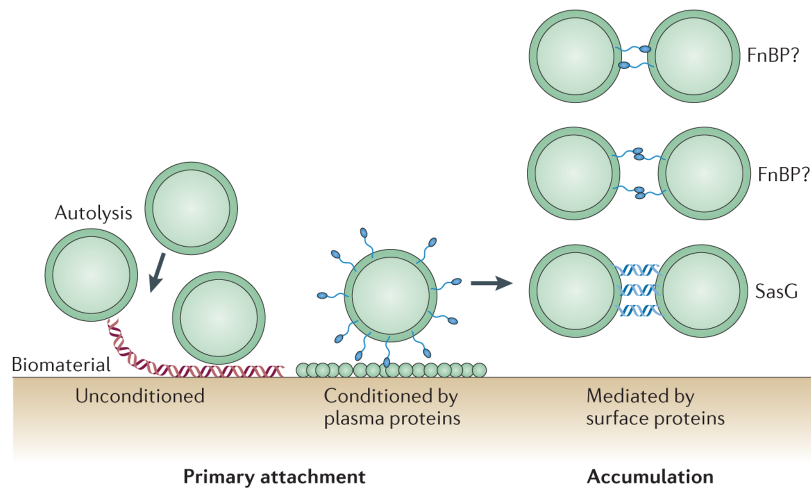

Figure 4. Roles of CWA proteins in biofilm formation

Cell wall-anchored (CWA) proteins promote attachment to surfaces that have been conditioned with host plasma proteins. Extracellular DNA that is released by autolysins can promote attachment to unconditioned surfaces. When the A domain of accumulation-associated protein (Aap) of *Staphylococcus epidermidis* or *Staphylococcus aureus* surface protein G (SasG) of *S. aureus* is removed, the G5–E repeats on adjacent cells can twist around each other in a Zn^{2+}-dependent manner to promote cell–cell aggregation and biofilm accumulation. Fibronectin-binding proteins (FnBPs) and other surface proteins promote accumulation either by homophilic protein–protein interactions or by binding to other ligands on neighbouring cells.

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Table 1

The main groups of cell wall-anchored proteins of *Staphylococcus aureus*

Protein group	Ligand and binding mechanism*	Function	Refs
MSCRAMMs			
Clumping factor A (ClfA)	Fibrinogen γ-chain carboxyl terminus; DLL	Adhesion to immobilized fibrinogen; immune evasion by binding soluble fibrinogen	13,123
	Complement factor I	Immune evasion; degradation of C3b	40,88
Clumping factor B (ClfB)	Fibrinogen α-chain repeat 5, keratin 10 and loricrin; DLL	Adhesion to desquamated epithelial cells; nasal colonization	39,41–44
Serine–aspartate repeat protein C (SdrC)	β-neurexin; DLL	Unknown	110
	Desquamated epithelial cells	Nasal colonization?	73
SdrD	Desquamated epithelial cells	Nasal colonization?	73
SdrE	Complement factor H	Immune evasion; degradation of C3b	89
Bone sialoprotein-binding protein (isoform of SdrE)	Fibrinogen α-chain; DLL	Adhesion to ECM	124
Fibronectin-binding proteins A (FnBPA) and B (FnBPB)	FnBPA A domain binds the C terminus of fibrinogen γ-chain and elastin; DLL. FnBPB A domain also binds fibronectin but not by DLL	Adhesion to ECM	45,125
	Fibronectin (FnBPA and FnBPB C-terminal repeats, β-zipper)	Adhesion to ECM; invasion	49,78–80
Collagen adhesin (Cna)	Collagen triple helix; collagen hug	Adhesion to collagen-rich tissue	23
	Complement protein C1q	Prevention of classical pathway of complement activation	90
NEAT motif family			
Iron-regulated surface protein A (IsdA)	Haem, fibrinogen, fibronectin, cytokeratin 10, loricrin (N-terminal NEAT-motif region)	Haem uptake and iron acquisition; adhesion to desquamated epithelial cells; resistance to lactoferrin	70,72,126,127
	Unknown ligand (C-terminal domain NEAT motif region)	Resistance to bactericidal lipids and antimicrobial peptides; survival in neutrophils	128
IsdB	Haemoglobin, haem (N-terminal NEAT motif region	Haem uptake and iron acquisition	27,129
	β3 integrins (NEAT motif regions)	Invasion of non-phagocytic cells	85
IsdH	Haem, haemoglobin (N-terminal and/or C-terminal NEAT motif region)	Haem uptake and iron acquisition	27,129
	Unknown ligand (N-terminal NEAT motif region)	Accelerated degradation of C3b	130
Three-helical bundle			
Protein A‡	IgG Fc, IgM Fab VH3 subclass, TNFR1 (three-helical bundle domain)	Inhibition of opsonophagocytosis ; B cell superantigen; inflammation	28,29,38,131,132,93,133
	von Willebrand factor	Endovascular infection; endocarditis	134
	Unknown ligand (region Xr)	Inflammation	97

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Protein group	Ligand and binding mechanism*	Function	Refs
G5–E repeat family			
S. aureus surface protein G (SasG) and plasmin-sensitive surface protein (Pls) (a SasG homologue in MRSA)	Unknown ligand (A domain)	Adhesion to desquamated epithelial cells	135,136
	Unknown ligand (G5–E repeats)	Biofilm formation	34,37
Structurally uncharacterized proteins			
Adenosine synthase A (AdsA)	Function not mediated by binding	Promotion of survival in neutrophils by inhibiting the oxidative burst	137,138
S. aureus surface protein X (SasX)	Unknown ligand	Biofilm formation; cell aggregation; and squamous cell adhesion	63
Serine-rich adhesin for platelets (SraP)	Salivary agglutinin gp340 and an unidentified ligand on platelets	Endocarditis; and endovascular infection	139,140
SasC	Unknown ligand	Promotes primary attachment and accumulation phases of biofilm formation	108
SasB, SasD, SasF, SasJ, SasK and SasL.	Unknown ligand	Putative LPXTG proteins identified from genome sequences. No known structure or function	141
Biofilm-associated protein (Bap)	gp96	Promotes biofilm formation; prevents invasion of mammary gland epithelial cells; and promotes aggregation on epithelial cell surfaces. Only found in bovine strains.	106,147

DLL, dock, lock and latch; ECM, extracellular matrix; Fab, antigen binding fragment; LPXTG, Leu-Pro-X-Thr-Gly (where X is any amino acid); MRSA, methicillin-resistant *Staphyloccocus aureus*; MSCRAMM, microbial surface component recognizing adhesive matrix molecule; NEAT, near iron transporter; *S. aureus*, *Staphylococcus aureus*; TNFR1, tumour necrosis factor receptor 1; VH3, variable heavy chain 3.

*Binding mechanism and/or region for those ligands for which structural or modelling and mutagenesis studies are available.

‡*S. aureus* binder of IgG protein (Sbi) has two related IgG-binding repeats but is anchored non-covalently to lipoteichoic acid (LTA) in the membrane and has no LPXTG motif[30]. *Staphylococcus pseudintermedius*[14] and *Staphylococcus hyicus*[142] have protein A orthologues.

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Table 2

Cell wall-anchored proteins as virulence factors

Role in colonization or infection	CWA proteins	Mechanism	Refs
Nasal or skin colonization	ClfB	Adhesion to loricrin on squames	41
	IsdA	Adhesion to squames	70
	SasX	Adhesion to squames	63
Endocarditis	ClfA	Adhesion to thrombus	143
	FnBPA	Adhesion to thrombus; invasion of adjacent endothelium	48
	ClfB	Adhesion to thrombus	144
	SraP	Adhesion to platelets; colonization of thrombus	139
Mastitis	FnBPs	Invasion of epithelial cells in mammary gland	61
Pneumonia	Protein A	Increased inflammation of lung epithelium	93
Foreign body infection	FnBPs	Promotion of MRSA biofilm; adhesion to intra-aortic patch	145,146
Ocular keratitis	Cna	Enhanced colonization and infection	58
Septic death; survival in blood	ClfA, protein A, IsdH, AdsA, SasX	Reduced opsonophagocytosis	55,59,63,87,91,130,138
Kidney abscess following survival in blood	ClfA, IsdA, IsdB, SdrD, ClfB, IsdC (Spa and SasG: modest effects)	Increased survival in the bloodstream prior to kidney infection	59
Septic arthritis	ClfA, Protein A	Enhanced survival in bloodstream prior to invasion of joint	83,87,91
	Cna	Enhanced survival in bloodstream; adhesion to cartilage within joint	57

AdsA, adenosine synthase A; Clf, clumping factor; Cna, collagen adhesin; FnBP, fibronectin-binding protein; Isd, iron-regulated surface; MRSA, methicillin-resistant *Staphylococcus aureus*; Sas, *S. aureus* surface protein; Sdr, serine–aspartate repeat-containing protein; Sra, serine-rich adhesin for platelets.