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The Sbi Protein Is a Multifunctional Immune Evasion Factor of *Staphylococcus aureus*▽

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The second immunoglobulin-binding protein (Sbi) of *Staphylococcus aureus* has two N-terminal domains that bind the Fc region of IgG in a fashion similar to that of protein A and two domains that can bind to the complement protein C3 and promote its futile consumption in the fluid phase. It has been proposed that Sbi helps bacteria to avoid innate immune defenses. By comparing a mutant defective in Sbi with mutants defective in protein A, clumping factor A, iron-regulated surface determinant H, and capsular polysaccharide, it was shown that Sbi is indeed an immune evasion factor that promotes bacterial survival in whole human blood and the avoidance of neutrophil-mediated opsonophagocytosis. Sbi is present in the culture supernatant and is also associated with the cell envelope. *S. aureus* strains that expressed truncates of Sbi lacking N-terminal domains D1 and D2 (D1D2) or D3 and D4 (D3D4) or a C-terminal truncate that was no longer retained in the cell envelope were analyzed. Both the secreted and envelope-associated forms of Sbi contributed to immune evasion. The IgG-binding domains contributed only when Sbi was attached to the cell, while only the secreted C3-binding domains were biologically active.

Staphylococcus aureus permanently colonizes the moist squamous epithelium of the anterior nares of approximately 20% of the population (45). Colonization is an established risk factor for the development of invasive disease in both the hospital and the community (27). Common community-acquired and nosocomial *S. aureus* infections include superficial skin lesions such as boils, abscesses, and impetigo, while invasive infections include septic arthritis, pneumonia, osteomyelitis, and endocarditis (13).

The ability of *S. aureus* to cause such a diverse range of infections is due in part to cell surface-associated proteins and extracellular toxins. Some cell surface-associated proteins bind to components of the extracellular matrix (ECM) of the host. These bacterial adhesins are called MSCRAMMs (*m*icrobial *s*urface *c*omponents *r*ecognizing *a*dhesive *m*atrix *m*olecules). Others promote evasion of host innate immune responses (12).

Protein A (Spa) was originally thought to be the only IgG-binding protein in *S. aureus*. Spa is a cell wall-anchored protein that consists of 4 or 5 homologous repeat domains of 56 to 61 residues that bind a variety of ligands, including IgG (Fig. 1) (30). A single Spa ligand-binding domain is composed of a three-helix bundle of antiparallel alpha-helices (7). The binding sites of the Fcγ region of IgG, von Willebrand factor, and tumor necrosis factor receptor-1 (TNFR-1) are located on helices 1 and 2, while the binding site of the V_H3 element of IgM is on helices 2 and 3 (34).

Phage display studies of *S. aureus* strain 8325-4 genomic DNA revealed a novel Ig-binding peptide that was later found to be part of Sbi (*s*econd *b*inding protein of *i*mmunoglobulin) (46, 47). At its N terminus are two IgG-binding domains (D1 and D2 [D1D2]) with sequence similarity to the IgG-binding domains of Spa (Fig. 1). Next are two independently folded domains (D3 and D4 [D3D4]) that bind to complement factor C3 (40). The C-terminal region of Sbi comprises a proline-rich repeat domain and a C-terminal domain rich in tyrosine and threonine that is likely to be involved in attaching the protein to the cell envelope (47).

Four surface components of *S. aureus* have been shown to promote evasion of neutrophil-mediated phagocytosis. Spa is known to inhibit opsonophagocytosis by binding IgG by the Fc region, which prevents classical complement fixation and recognition by the neutrophil Fc receptor (12). Clumping factor A (ClfA) is a major fibrinogen-binding surface protein. It is found predominantly on cells from the stationary phase of growth. ClfA binds to the γ-chain of fibrinogen via its N-terminal A-domain (29). *S. aureus clfA* mutants are significantly attenuated in murine models for sepsis and arthritis (22) and in a rat endocarditis model (36). Virulence is likely to be increased by bacterial cells becoming coated with fibrinogen, which inhibits deposition of, or access to, opsonins (18). In addition, ClfA can capture and activate serum complement regulator factor I and convert C3b to iC3b and C3d, resulting in the loss of opsonin (5, 17). This also prevents C3 convertase formation and terminal pathway activation (5).

The majority of clinical isolates of *S. aureus* produce serotype 5 or serotype 8 capsular polysaccharide (Cap) (33). Capsule expression reduces the uptake of bacteria by neutrophils in the presence of normal serum opsonins (28, 32). Although complement factors can accumulate on the cell wall surface beneath the polysaccharide layer, they are inaccessible to complement receptors on neutrophils.

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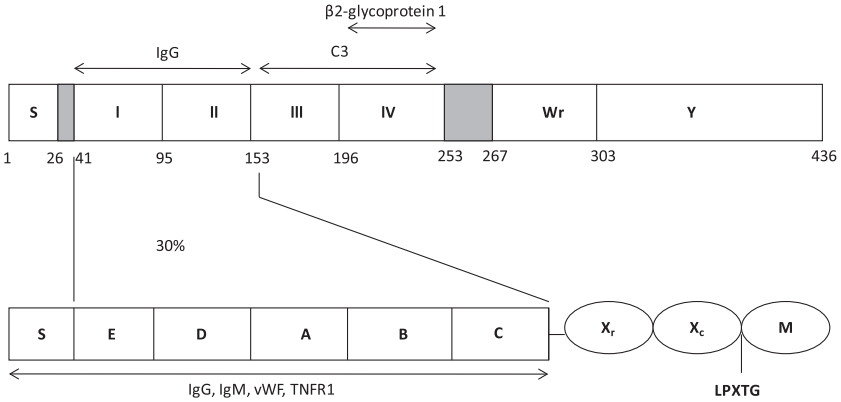

FIG. 1. Schematic diagrams of Spa and Sbi. The upper part of the figure shows Sbi and the lower shows Spa. S, signal sequence; D1 to D4 (I to IV), Sbi ligand-binding domains that have sequence similarity to IgG-binding domains of Spa (E, D, A, B, C); Wr and X_r, proline-rich C terminal domains; Y, C-terminal domain likely to be involved in membrane binding; LPXTG, wall anchoring motif; M, transmembrane domain.

The iron-regulated surface determinant protein IsdH is part of a complex of proteins that are expressed only under iron-restricted conditions. They extract heme from hemoglobin and transport it into the cytoplasm (43). *S. aureus isdH* mutants are engulfed more rapidly by human neutrophils in the presence of serum opsonins, survive poorly in fresh whole human blood, and are less virulent in a mouse model of sepsis (43). IsdH promotes accelerated degradation of the serum opsonin C3b by enhancing the conversion of C3b to C3d (43).

The cellular location of Sbi has not been addressed in previous publications. It has been assumed that it is anchored to the cell wall and is exposed on the cell surface, allowing it to bind IgG like protein A, but the C terminus lacks motifs associated with anchorage to the cell wall or membrane. In this paper, we analyze the cellular location of Sbi and demonstrate that it occurs both extracellularly and bound to the cell envelope. The cell-associated component fractionated with the cytoplasmic membrane but was also partly exposed on the cell surface. Also, we determine the extent to which Sbi promotes the evasion of phagocytosis and killing by neutrophils and compare this to the protection offered by Spa, ClfA, IsdH, and Cap.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Strains and plasmids are listed in Tables 1 and 2, respectively. *S. aureus* was routinely grown on tryptone soy agar (TSA) or broth (TSB) at 37°C with shaking. *Escherichia coli* strains were grown in L agar (Difco) or broth at 37°C with shaking. Antibiotics (Sigma) were added as required: chloramphenicol (Cm) at 10 μg/ml, erythromycin (Em) at 10 μg/ml, tetracycline (Tc) at 2 μg/ml, and kanamycin (Ka) at 50 μg/ml. Bacteria were also grown in RPMI 1640 (Sigma), an iron-deficient medium which was originally designed for the culture of human leukocytes (31). Starter cultures were diluted in 10 ml of RPMI to an optical density at 600 nm (OD_{600}) of 0.05 and grown at 37°C with shaking.

Isolation of an *sbi* mutation by allelic replacement. To inactivate the *sbi* gene, DNA fragments comprising 900 bp upstream and 740 bp downstream of *sbi* were amplified by PCR from genomic DNA and cloned together with an *ermC* cassette (from pTS*ermC*) between the HindIII-BamHI sites of plasmid pBluescript. The construct was then ligated to pTS*tet*K, a plasmid that is temperature sensitive for replication in *S. aureus*, and the resulting chimeric plasmid was designated pES2. pES2 was electroporated into *S. aureus* strain RN4220 and then transferred into *S. aureus* strain Newman to achieve integration of the *ermC* gene into the genome by homologous recombination by selecting on agar containing Em at 43°C. After several cycles of growth in broth at 28°C and at 43°C, colonies were selected on Em agar and tested for loss of Tc^r. The *sbi* mutation was validated by PCR and Western immunoblotting.

Complementation of the *sbi*::Em^r mutation. To complement the *sbi*::Em^r mutation, the *sbi* gene including the ribosomal binding site but lacking the promoter was amplified from the genomic DNA of strain Newman and cloned between the

TABLE 1. *S. aureus* strains used in this study

Strain	Relevant characteristic	Source or reference
RN4220	Restriction-deficient derivative of 8325-4	23
Newman	NCTC 8178	10
Newman *spa*	Newman *spa*::Ka^r	18
Newman *clfA*	Newman *clfA5* frameshift mutation (unmarked)	18
Newman *cap*	Newman *cap*::Tc^r	44
Newman *isdH*	Newman *isdH*::Em^r	43
Newman *spa clfA*	Newman *spa*::Ka^r *clfA5*	18
Newman *sbi*	Newman *sbi*::Em^r	This study
Newman *spa sbi*	Transduction of *sbi*::Em^r into Newman *spa*::Ka^r	This study
Newman *spa clfA cap*	Transduction of *cap*::Tc^r into Newman *spa*::Ka^r *clfA5*	This study
Newman *spa clfA cap isdH*	Allelic replacement of *isdH* in Newman *spa*::Ka^r *clfA5 cap*::Tc^r using pJH4*isdH2*	This study
Newman *spa clfA cap isdH sbi*	Transduction of *sbi*::Em^r into Newman *spa*::Ka^r *clfA5 cap*::Tc^r *isdH2*	This study
Newman *clfB*	Newman *clfB*::*lacZ* Em^r	11
MSSA 476	Clinical isolate	19
MRSA 252	Clinical isolate	19
COL	Clinical isolate	14
N315	Clinical isolate	24
USA300	Clinical isolate	8
P1	Clinical isolate	39
Cowan	Clinical isolate	15
LS-1	Clinical isolate	2
38	Clinical isolate st1	6
2	Clinical isolate st7	6
19	Clinical isolate st10	6
42	Clinical isolate st20	6
13	Clinical isolate st22	6
21	Clinical isolate st36	6
52	Clinical isolate st188	6

TABLE 2. Plasmids used in this study

Plasmid	Relevant characteristics	Source or reference
pBluescript II SK	*E. coli* cloning vector Amp^r	Stratagene
pTS*erm*C	*S. aureus* plasmid containing temperature-sensitive replicon; Em^r	21
pTS*tet*K	*S. aureus* plasmid containing temperature-sensitive replicon; Tc^r	
pES2	*S. aureus* shuttle plasmid; temperature-sensitive replicon; Ap^r in *E. coli*; Tc^r in *S. aureus*; sequences for creating *sbi*::Em^r mutation	This study
pRMC2	Derivative of the tetracycline-inducible expression vector pALC2073; anhydrotetracycline-inducible expression vector; Ap^r in *E. coli*; Cm^r in *S. aureus*	4
pRMC2-*sbi*	pRMC2 encoding full-length Sbi	This study
pRMC2-*sbi*ΔD1D2	pRMC2 encoding Sbi domains D3 and D4	This study
pRMC2-*sbi*ΔD3D4	pRMC2 encoding Sbi domains D1 and D2	This study
pRMC2-$sbi_{1–335}$	pRMC2 encoding Sbi_1 to Sbi_{335}	This study
pJH4*isdH*	Temperature-sensitive shuttle vector with *isdH2* mutations Y126A/H127E/F128A; EcoRV site codon 105	41

KpnI and BglII sites in pRMC2, an anhydrotetracycline-inducible vector of *S. aureus*, to create pRMC2*sbi*.

Construction of Sbi C-terminal truncates. pRMC2 plasmids that expressed N-terminal truncates of Sbi were generated by inverse PCR using either pRMC2*sbi* or pRMC2$sbi_{1–335}$ (which encodes Sbi_1 to Sbi_{335}) as the PCR template to create pRMC2*sbi*ΔD1D2, pRMC2*sbi*ΔD3D4, pRMC2sbi_{1-335}ΔD1D2, and pRMC2sbi_{1-335}ΔD3D4.

Cell fractionation. Solubilized cell wall proteins were obtained as follows. Stationary-phase cultures of *S. aureus* were harvested by centrifugation at 450 × *g* for 10 min at 4°C, washed in phosphate-buffered saline (PBS), and resuspended in a 1/20 volume of protoplast buffer (50 mM Tris-HCl, 20 mM $MgCl_2$, 30% [wt/vol] raffinose, pH 7.5) containing complete mini EDTA-free protease inhibitors (Roche). Cell wall proteins were solubilized by digestion with lysostaphin (200 μg/ml) at 37°C for 15 min. Protoplasts were harvested by centrifugation at 6,000 × *g* for 15 min and the supernatant was retained as the cell wall fraction. Protoplasts were sedimented by centrifugation at 6,000 × *g* and resuspended in protoplast buffer with protease inhibitors. Protoplast pellets were washed once and resuspended in ice-cold 50 mM Tris-HCl, pH 7.5, containing protease inhibitors and DNase (80 μg/ml). Protoplasts were lysed on ice by vortexing. The membrane fraction was obtained by centrifugation at 40,000 × *g* for 1 h at 4°C. The supernatant was retained as the cytoplasmic fraction. The pellet was washed once with ice-cold acetone and finally resuspended in 50 mM Tris-HCl, pH 7.5. The culture supernatant was filtered through a 0.45-μm filter, and proteins were concentrated by addition of a 1/20 volume of ice-cold 100 (wt/vol) trichloroacetic acid (TCA).

SDS-PAGE. Protein samples were diluted in final sample buffer (125 mM Tris-HCl, pH 6.8, 4% [wt/vol] SDS, 20% [vol/vol] glycerol, 10% [vol/vol] β-mercaptoethanol, and 0.002% [wt/vol] bromophenol blue) and boiled for 5 min. Samples were loaded onto acrylamide gels (3% stacking and 12% separating gel) and separated by electrophoresis (25) at 120 V, after which proteins were visualized by Coomassie blue staining or electroblotted onto polyvinylidene difluoride (PVDF) membranes (Roche) for Western immunoblotting.

Western immunoblotting. Proteins were electroblotted onto PVDF membranes (Roche) for 1 h at 100 V using a wet transfer cell (Bio-Rad). Membranes were incubated for 1 h at 4°C in TS buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl) containing 10% (wt/vol) skimmed milk (Marvel). Next, horseradish peroxidase (HRP)-conjugated antibody, HRP-IgG, or primary antibodies diluted in 10% (wt/vol) Marvel TS buffer were incubated with the membranes for 1 h at room temperature with shaking. Antibodies used were rabbit anti-ClfA, rabbit anti-IsdH, and HRP-conjugated rabbit anti-Sbi D3D4 WrY (a kind gift from M. Horsborough, University of Liverpool, United Kingdom). HRP-conjugated rabbit IgG (Dako) was used to detect Sbi and Spa. Unbound antibody was removed by three 10-min washes with TS buffer containing 0.01% Tween. Where necessary, secondary antibodies, HRP-conjugated goat anti-rabbit immunoglobulins (Dako) diluted in 10% (wt/vol) Marvel TS buffer, were then incubated with the membranes for 1 h at room temperature with shaking. Unbound secondary antibody was removed by washing three times with 0.01% Tween-TS buffer, and the bound HRP was developed with chemiluminesent substrate LumiGlo (New England BioLabs). Blots were exposed to X-Omat autoradiographic film (Kodak). The intensity of protein bands resulting from equal loadings of bacterial cell extracts or purified proteins was assessed visually.

Whole-cell immunoblots. Cells were washed twice in PBS and adjusted to an OD_{600} of 1. Doubling dilutions (5 μl) were dotted onto a nitrocellulose membrane (Protran). The membrane was blocked for 1 h with Marvel TS buffer. HRP-labeled rabbit IgG was diluted in Marvel TS buffer, incubated with the membrane for 1 h at room temperature with shaking, and washed 3 times with TS buffer to remove unbound antibody. The membrane was developed in the dark using the chemiluminescent substrate LumiGlo (New England BioLabs).

Whole-blood survival assays. The ability of *S. aureus* to survive in human blood was measured as previously described (43). Briefly, *S. aureus* strain Newman and its mutants grown in RPMI to stationary phase were diluted in PBS, and 100 μl (5×10^2 CFU) was added to 0.5 ml of fresh blood that was obtained from human healthy volunteers and that had been treated with 50 μg ml^{-1} the anticoagulant hirudin (Refludan, Pharmion srl, Rome, Italy). A portion of each culture was plated on agar to determine the input CFU. Tubes were incubated at 37°C with gentle rocking, and after 3 h serial dilutions were plated to determine the number of surviving CFU. To exclude the possibility that the viable counts were due to differences in the ability to grow in plasma, growth curves of each strain were calculated and were indistinguishable.

Isolation of human polymorphonuclear leukocytes. Fresh whole blood was obtained in sodium citrate-containing tubes from healthy volunteers and mixed with an equal volume of PBS. This mixture was centrifuged through step gradients of Histopaque (ρ = 1.077, Sigma) and Ficoll-paque (ρ = 1.119, Amersham), and polymorphonuclear leukocytes (PMNLs) were aspirated from the buffy coat between the Ficoll and Histopaque layers. Cells were washed in RPMI 1640 medium (containing 10 mM HEPES, 25 mM glutamine, and 0.05% [vol/vol] human serum albumin) (Gibco) and resuspended in water. After a 30-s osmotic shock to lyse contaminating red blood cells, 10× PBS was added and the cells were again washed in RPMI. PMNLs were counted in a hemocytometer (Bright Line, Neubauer) and adjusted to 5×10^6 cells ml^{-1} in RPMI. All reagents used in PMNL isolation were certified endotoxin free.

Phagocytosis. The phagocytosis assay was performed as previously described. *S. aureus* cells grown were grown for 18 h in RPMI 1640 medium and were washed twice in PBS and labeled with 30 μg ml^{-1} fluorescein isothiocyanate (FITC) in PBS for 1 h at 37°C with shaking. Cells were washed three times in PBS and enumerated in a Neubauer hemocytometer, adjusted to 1×10^9 CFU ml^{-1} in RPMI, and stored frozen at −20°C. Bacteria were thawed on ice and diluted to 5×10^7 CFU ml^{-1} in RPMI. Human serum was diluted in RPMI. Bacteria (50 μl) were opsonized in 10 μl of diluted serum for 10 min at 37°C, followed by the addition of 50 μl of prewarmed PMNLs and incubation at 37°C with vigorous shaking. The final bacterium:PMNL ratio was 10:1. Reactions were stopped after 5 to 15 min by the addition of 100 μl of ice-cold 2% (wt/vol) paraformaldehyde in PBS. The percentage of PMNLs bearing FITC-labeled bacteria (% phagocytosis) was determined by flow cytometric analysis of 5,000 cells by using a FACScan flow cytometer (Becton Dickinson). The percentage of internalized bacteria was determined by trypan blue quenching of extracellular fluorescence.

Determination of hemolytic activity. Qualitative evaluation of α-, β-, and δ-hemolysin production was carried out on sheep blood agar. Bacteria were streaked at a right angle to *S. aureus* RN4220, and the plate was incubated overnight. β-Hemolysin forms a turbid zone of hemolysis surrounding the vertical streak of RN4220. δ-Hemolysin and β-hemolysin are synergistic and produce a zone of clear hemolysis where they intersect.

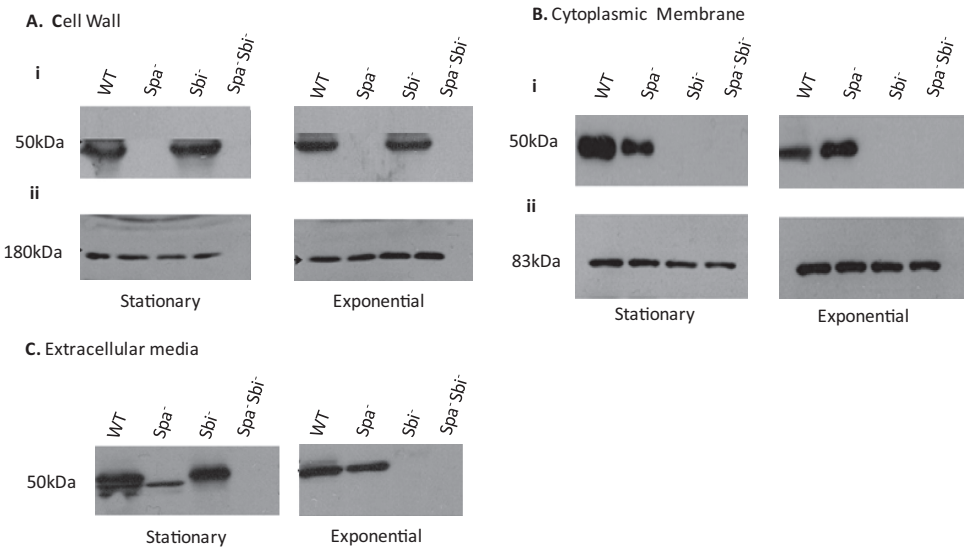

FIG. 2. Cellular localization of Sbi. Sbi and Spa have similar molecular masses and comigrate in SDS-PAGE minigels. Both proteins were recognized by HRP-IgG that bound to the Fc-binding domains. Cells from the stationary and exponential phases were tested. (A) Cell wall fractions. Gels were analyzed by Western blotting with HRP-labeled rabbit IgG or rabbit anti-SdrE antibody followed by HRP-labeled protein A. (B) Cytoplasmic membrane fraction. Gels were analyzed by Western blotting with HRP-labeled rabbit IgG or rabbit anti-EbpS antibody followed by HRP-labeled protein A. (C) Supernatant fraction probed with HRP-labeled rabbit IgG. These are representatives of 3 independent experiments.

RESULTS

Cellular location of Sbi. The Sbi protein does not contain domains that are found in proteins that are covalently or noncovalently associated with cell wall peptidoglycan or the cytoplasmic membrane. It is not clear from previous studies if Sbi is cell surface associated. This study set out to determine unambiguously which cellular compartment(s) carries Sbi and whether Sbi is expressed on the cell surface. To achieve this, a mutant defective in Sbi was constructed by allelic exchange to delete base pairs 20 to 1270 and replace them with an *ermC* cassette. The *sbi* mutation was combined with a mutation in the protein A (*spa*) gene. Cells were grown to the mid-exponential and stationary phases. After centrifugation to sediment cells, a sample of supernatant was concentrated 10-fold by TCA precipitation. The cells were adjusted to the same density in a high concentration of raffinose to protect protoplasts when the cell wall peptidoglycan was dissolved with lysostaphin. This solubilized any cell wall-associated proteins. The stabilized protoplasts were lysed, the membrane fragments were sedimented, and the supernatant was retained as the cytoplasmic fraction. Each fraction was analyzed by SDS-PAGE and immunoblotting. Detection of Spa and Sbi is complicated by the fact their molecular masses are very similar and that they both bind the Fc region of IgG. Identification of Spa and Sbi relied upon the comparison of mutants using HRP-conjugated rabbit IgG. Samples were also probed with antibodies specific for the cell wall-anchored protein SdrE (22) and the integral membrane protein EbpS (9), which serve as controls for both sample loading and the purity of fractions. These proteins are significantly different in size from Spa and Sbi and therefore can be well separated from them by SDS-PAGE.

The solubilized cell wall material probed with HRP-IgG had an ~50-kDa band present in the wild-type (WT) and *sbi* mutant samples which was missing in the *spa* mutant fraction. This is clearly protein A. The absence of any reactive protein in the *spa* mutant sample showed that Sbi is not a wall-anchored protein. Analysis of the membrane fraction revealed an ~50-kDa band in the wild-type and *spa* mutant samples that was absent from the *sbi* mutant (Fig. 2B). This is Sbi and strongly suggests that the protein is associated with the cytoplasmic membrane. The culture supernatant samples were also probed to reveal both Sbi and Spa in the samples from the stationary phase, but only Sbi was detected in the exponential-phase samples (Fig. 2C).

Expression of Sbi by clinical isolates. Our previous studies demonstrated that Sbi is associated with the cytoplasmic membrane of the laboratory strain Newman when cells were fractionated following protoplast formation. Here we examined 16 clinical isolates of *S. aureus* representing the genetic diversity of the species based on multilocus sequence typing (MLST) for expression of Sbi. The cytoplasmic membrane and culture supernatant were prepared and probed with HRP-conjugated rabbit anti-Sbi antibodies. The antibodies did not react with protein A in this fraction as indicated by the absence of any reactive protein band in the Spa^+ Newman Sbi^- mutant sample (Fig. 3). An ~50-kDa immunoreactive band was detected in the membrane fraction of all strains (except Cowan) and in the supernatant of all but four (TL210, LS-1, 52, and 21). There was considerable variation in the level of Sbi expressed, particularly in the culture supernatant.

Expression of antiphagocytic factors in iron-limited conditions. Next, we sought to determine if Sbi has immune evasion activity and to compare its role in immune evasion to that of known *S. aureus* antiphagocytic surface components protein A (Spa), clumping factor A (ClfA), iron-regulated surface determinant H (IsdH), and capsular polysaccharide (Cap). The abil-

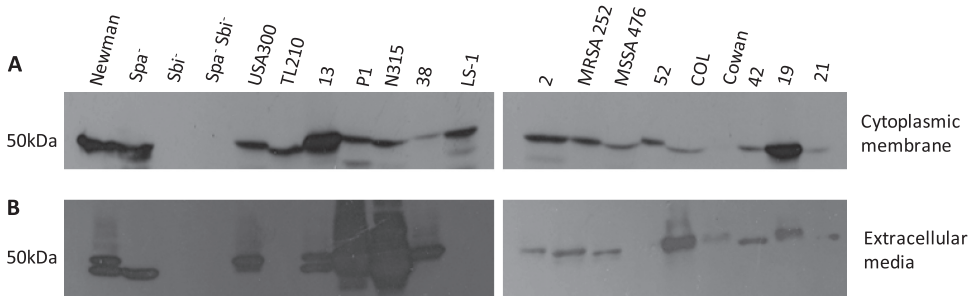

FIG. 3. Expression of Sbi by clinical isolates of *S. aureus.* Clinical isolates of *S. aureus* were analyzed by Western immunoblotting for the expression of Sbi in the cytoplasmic membrane (A) and the culture supernatant (B). Samples were separated by SDS-PAGE and analyzed by Western blotting with HRP-conjugated rabbit anti-Sbi antibodies.

ity of *S. aureus* grown in the iron-restricted medium RPMI and in human plasma to express the protein factors was tested by Western blotting of cell wall or cell membrane fractions. In each case, wild-type strain Newman was compared with the appropriate null mutant. The gels were probed with specific antibodies recognizing ClfA or IsdH, or with HRP-labeled IgG to detect Spa and Sbi. It is clear from data shown in Fig. 4 that each of the proteins was expressed when bacteria were grown in iron-limited medium.

Expression of *S. aureus* strain Newman antiphagocytic determinants in mutant strains. *S. aureus* is prone to acquiring mutations in the Agr global regulator (1, 42). In order to ensure that mutants affecting immune evasion factors remained Agr^+, each was tested for the expression of δ-toxin, a marker that is often used to reflect the integrity of Agr (1). Also, each mutant was tested in turn by Western immunoblotting to determine the levels of expression of the remaining antiphagocytic proteins. Each mutant expressed the same levels of the remaining antiphagocytic factors as the wild-type strain (Fig. 5A to D).

Expression of Sbi inhibits killing by neutrophils in whole human blood. In order to determine if expression of Sbi contributes to the survival of *S. aureus* in human whole blood, bacterial cells were incubated in blood obtained from several healthy donors. Hirudin was used as the anticoagulant in order to preserve complement activity. Protein A, ClfA, IsdH, and type 5 capsular polysaccharide are known to be important antiphagocytic factors, so the ability of the *sbi* mutant to survive was compared to those of *spa*, *clfA*, *isdH*, and *cap* mutants. This also allowed the relative contribution of each antiphagocytic factor in strain Newman to be compared under the same conditions, something that has never been done before. Furthermore, a mutant defective in *spa*, *clfA*, *cap*, and *isdH* was compared to a *spa clfA cap isdH sbi* mutant. None of the seven selected donors had detectable anti-Sbi, anti-Spa, anti-ClfA, or anti-IsdH antibodies as determined by enzyme-linked immunosorbent assay (ELISA) (data not shown). This is important because if a donor had antibodies against the antigen being tested, enhanced opsonization of the bacteria would occur, leading to greater phagocytosis. With wild-type *S. aureus* strain Newman, an average of 80% of the initial inoculum survived in blood for 3 h. The survival of the *sbi* and *spa* mutants was significantly impaired, with only 40% surviving (Fig. 6A). In contrast, the *isdH* and *clfA* mutants were severely compromised, with only 25% of the inoculum surviving. The survival of the *cap* mutant, although significantly impaired, was greater than that of the other mutants, with 60% of the initial inoculum surviving. The survival rates of the *spa clfA cap isdH* and *spa clfA cap isdH sbi* mutants were 20% and 10%, respectively (Fig. 6A). These experiments demonstrate that Sbi is important for the ability of *S. aureus* to survive in human blood. This is most likely because its ability to resist opsonophagocytosis by neutrophils was lost and the presence of Spa, ClfA and IsdH could not compensate.

It was important to show that the ability of strains to grow in plasma was not impaired. Bacteria were incubated in plasma

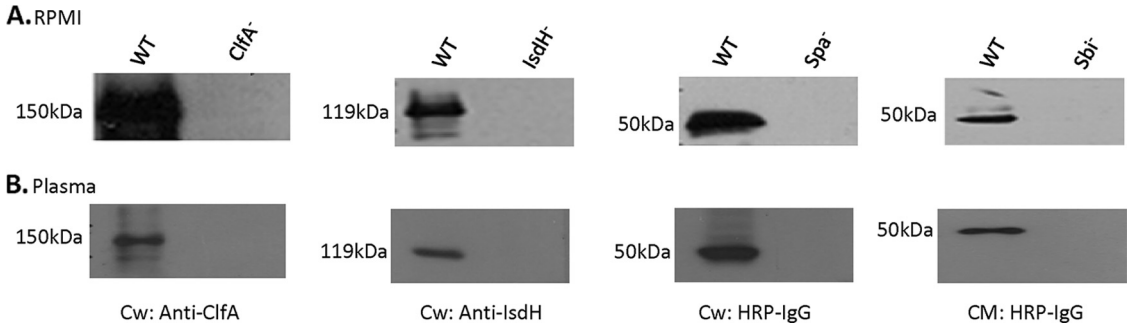

FIG. 4. Expression of antiphagocytic factors in iron-limited conditions. Bacteria were grown in RPMI (A) and human plasma (B). In each case, wild-type *S. aureus* strain Newman was compared with the appropriate null mutant. Solubilized cell wall (Cw)-associated proteins, or in the case of Sbi the cytoplasmic membrane (CM), were analyzed by SDS-PAGE and Western blotting by probing with rabbit antibodies recognizing ClfA or IsdH followed by HRP-labeled goat anti-rabbit immunoglobulin or with HRP-labeled rabbit IgG to detect Spa and Sbi.

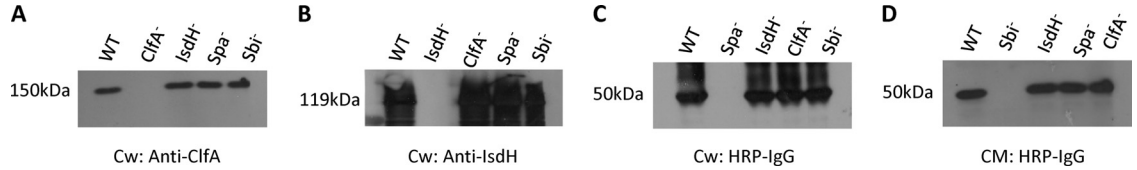

FIG. 5. Expression of *S. aureus* strain Newman antiphagocytic determinants in mutant strains. Each mutant was screened by SDS-PAGE and Western immunoblotting by probing with rabbit antibodies recognizing ClfA or IsdH followed by HRP-labeled goat anti-rabbit immunoglobulins (A and B) or with HRP-labeled rabbit IgG to detect Spa and Sbi (C and D).

for 3 h. All five single-null mutants and both of the multiple mutants grew to the same extent as the wild type, confirming previous results that growth in plasma resulted in cell numbers increasing to 130% of the initial inocula (43).

Phagocytosis by human neutrophils. In order to show that the expression of Sbi reduced the ability of human neutrophils to engulf *S. aureus* cells, strain Newman *sbi* was compared to the wild-type strain, to strain Newman defective in Spa, ClfA, IsdH, or Cap, and to a multiple mutant defective in all five factors in *in vitro* phagocytosis assays. IsdH and ClfA are established antiphagocyctic factors in this assay and thus represent positive controls in the experiment. Bacteria were grown in RPMI and incubated with purified human neutrophils from three healthy donors in the presence of 10% normal human serum (NHS) to provide opsonins. Wild-type *S. aureus* strain Newman resulted in 59% phagocytosis. The *spa*, *sbi*, *clfA*, *isdH*, and *cap* mutants were engulfed at rates significantly greater than those for the wild type, resulting in 75.3%, 72.1%, 82.4%, 78.5%, and 71.9% phagocytosis, respectively. In contrast, the multiple *spa clfA cap isdH sbi* mutant was severely compromised, resulting in 92.8% phagocytosis (Fig. 6B). This experiment is consistent with the results described for the whole-blood survival assays and confirms that the expression of Sbi is important for *S. aureus* to avoid opsonophagocytosis.

Cell-associated Sbi is surface exposed. In order to determine if Sbi is exposed on the cell surface, a Newman *spa* mutant and a strain expressing Sbi from the anhydrotetracycline-inducible expression vector pRMC2 were compared by whole-cell immunoblotting (Fig. 7A). Doubling dilutions of cell suspensions were probed with HRP-conjugated rabbit IgG that bound to the N-terminal domains D1D2 of Sbi (Fig. 7A). This showed that the IgG-binding domains of the cell-associated form of Sbi are exposed on the cell surface, despite fractionating with the cytoplasmic membrane. A truncated form of Sbi was expressed by pRMC2-*sbi* lacking domains D3D4, and this truncated Sbi also bound IgG but at a 4-fold-lower level, suggesting that the IgG-binding domains might be less accessible. The cytoplasmic membrane fractions of the wild type and truncates lacking D1D2 or D3D4 were probed with antibodies recognizing Sbi D3D4 WrY. This confirmed that the Sbi truncates were associated with the membrane and that they appeared to be expressed at the same level as the wild type.

Inhibition of killing in human blood by truncates of Sbi. To investigate if both the IgG-binding domains D1D2 and the C3-binding domains D3D4 of Sbi were required for protection against phagocytosis, bacteria expressing deletion mutants lacking D1D2 or D3D4 were incubated in human blood. Partial complementation of resistance to phagocytosis was conferred by both SbiΔD1D2 and SbiΔD3D4, with 51.25% and 52.5% survival, respectively, compared to 38.75% for strain Newman *sbi* (Fig. 7C). This demonstrates that both the IgG (D1D2) and C3 (D3D4) binding domains contribute to survival.

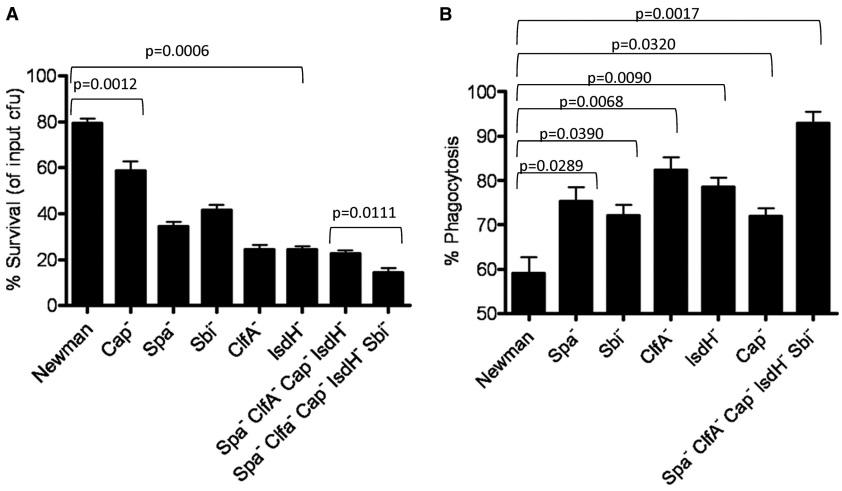

FIG. 6. Survival of *S. aureus* in blood and resistance to phagocytosis by human neutrophils. (A) Bacteria grown in RPMI were tested for their ability to survive 3 h of incubation in human blood. Surviving bacteria were measured by viable counting. Data are presented as the mean percentage of surviving CFU ± SD ($n = 7$). (B) Bacterial cells were labeled with FITC, adjusted to 5×10^7 CFU ml^{-1}, opsonized with 10% pooled human sera, and incubated with polymorphonuclear leukocytes from three individual donors. After the reaction was stopped, the percentage of PMNLs bearing fluorescent bacteria was measured by flow cytometry. Data are presented as the mean percentage of phagocytosis ± SD ($n = 3$).

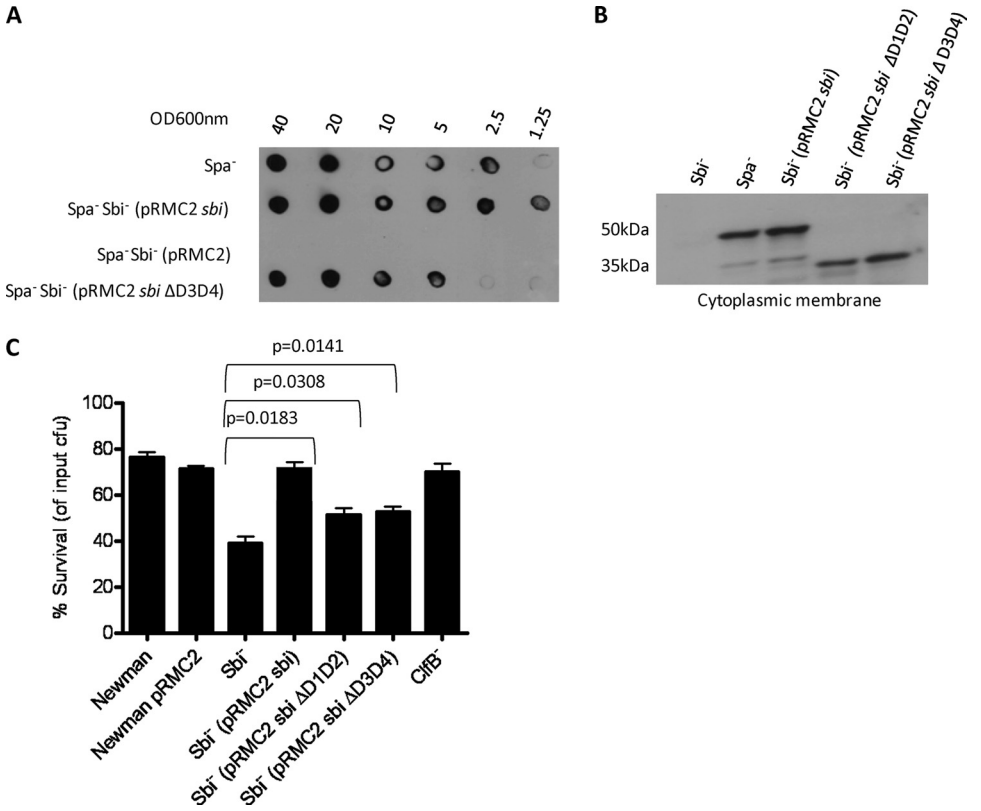

FIG. 7. Expression of Sbi truncates and survival in blood. (A) Serial dilutions of cells were applied to a nitrocellulose membrane and probed with HRP-conjugated rabbit IgG. (B) Cytoplasmic membrane fractions of Newman Spa$^-$, Newman Sbi$^-$ (pRMC2-*sbi*), Newman Spa$^-$ Sbi$^-$, Newman Sbi$^-$ (pRMC2-*sbi*ΔD1-D2), and Newman Sbi$^-$ (pRMC2*sbi*ΔD3-D4) were analyzed by SDS-PAGE and Western blotting by probing with HRP-labeled anti-Sbi D3D4 WrY antibodies. (C) Bacteria grown in RPMI were tested for their ability to survive 3 h of incubation in human blood. Surviving bacteria were measured by viable counting. Data are presented as the mean percentage of surviving CFU ± SD (n = 3).

Mutant plasmid pRMC2$sbi_{1–335}$, which expressed a variant of Sbi where the four binding domains were found only in the growth medium, was constructed (data not shown). It was interesting to determine if the exclusively extracellular form of Sbi was protective. Indeed, about 56.2% of the cells survived in blood, indicating that the extracellular form of the protein was partially protective (Fig. 8B). This implies that the wall-associated form is also active in immune evasion.

It has been suggested that Sbi domains D3D4, when secreted from the cell, can function in the futile consumption of C3. To study this further, pRMC2sbi_{1-335} was further manipulated to delete the regions encoding D1D2 or D3D4. These truncates were only found in the culture supernatant (Fig. 8A). Expression of the truncated protein comprising only secreted D1D2 failed to protect bacteria from phagocytosis, compared to the secreted D3D4 domains, which were partially protective (51.6% survival) (Fig. 8B). This suggests that only the D3D4 domains are biologically active when secreted.

DISCUSSION

Freshly isolated human blood treated with an anticoagulant that leaves the complement system intact is an excellent *in vitro* model for bacteremia. Small numbers of bacteria are incubated in whole blood that contains fully functioning neutrophils and normal levels of opsonins. Mouse models of *S. aureus* infection can be compromised because components of the murine innate immune defenses might not be recognized by staphylococcal immune evasion factors (16, 37).

It has been postulated from *in vitro* studies that Sbi is involved in innate immune evasion by *S. aureus*. Domains D1 and D2 at the N terminus of the elongated protein bind to the Fc region of human IgG in the same manner as protein A (47). If Sbi is exposed on the bacterial cell surface, it is likely that it will bind IgG by its Fc domain and inhibit opsonization and phagocytosis. Detailed biochemical and biophysical analysis of the interaction of domains D3 and D4 with complement protein C3 led to the hypothesis that extracellular Sbi could trigger futile consumption of the central component of complement activation (40). This could be an effective defense only if Sbi is extracellular and the D3D4 domains of any cell-bound Sbi are not exposed on the bacterial cell surface. Any surface-exposed Sbi D3D4 could trigger complement activation that would promote rather than prevent opsonization.

Until the study reported here, the precise cellular location(s) of Sbi was unknown and its role in immune evasion untested. Unexpectedly, cell-associated Sbi was found exclusively in the cytoplasmic membrane fraction when cells were treated with lysostaphin to create stable protoplasts. No Sbi was found among the solubilized proteins in the cell wall fraction. In contrast, protein A was detected only in the wall fraction, as

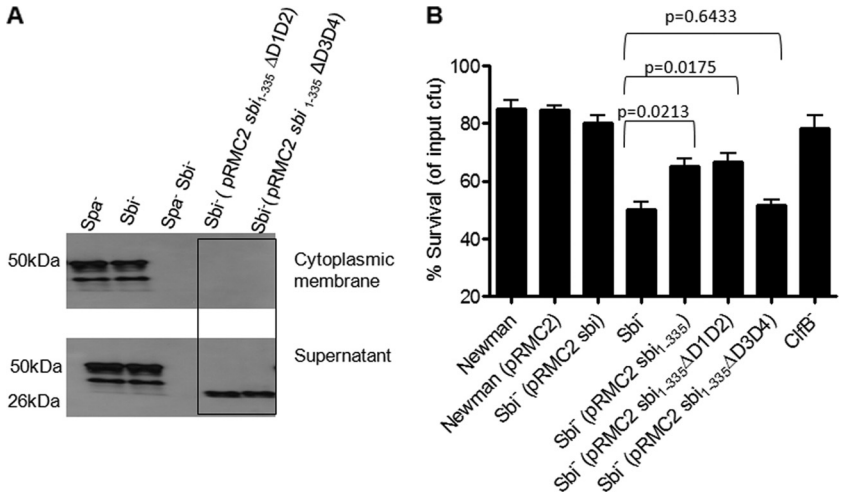

FIG. 8. Expression of secreted Sbi truncates and survival in blood. (A) Cytoplasmic membrane fractions of Newman Spa$^-$, Newman Sbi$^-$ (pRMC2-*sbi*), Newman Spa$^-$ Sbi$^-$, Newman Sbi$^-$ (pRMC2*sbi*$_{1–335}$ΔD1D2), and Newman Sbi$^-$ (pRMC2*sbi*$_{1–335}$ΔD3D4) were analyzed by SDS-PAGE and Western blotting by probing with HRP-labeled anti-Sbi antibodies. (B) Bacteria grown in RPMI were tested for their ability to survive 3 h of incubation in human blood. Surviving bacteria were measured by viable counting. Data are presented as the mean percentage of surviving CFU ± SD (n = 4).

expected for an LPXTG-anchored protein. Nevertheless, like Spa, the IgG-binding domains of Sbi were exposed on the surface of the cell. Thus, Sbi behaved similarly to internalin B of *Listeria monocytogenes* in cell fractionation studies (20). InlB is both extracellular and surface located, yet it fractionates with the cytoplasmic membrane by binding to lipoteichoic acid (LTA), a molecule which protrudes from the cell, allowing secreted extracellular InlB to bind to the surface (20).

This study confirms that Sbi contributes to the ability of *S. aureus* to evade opsonophagocytosis and to avoid being killed by neutrophils. This was shown primarily by measuring the survival of bacteria in fresh whole human blood and was supported by studies with purified human neutrophils. First, Sbi was compared with known surface-located immune evasion factors. Isogenic mutants defective in Sbi, ClfA, Spa, IsdH, and Cap each showed a reduced ability to survive in blood and were taken up more avidly by purified neutrophils *in vitro*. The protective effect of Sbi was also demonstrated by comparing a strain that was defective in Spa, ClfA, IsdH, and Cap with a mutant lacking all five factors.

Another question to be addressed was whether both the secreted and the cell surface-associated forms of Sbi contributed to immune evasion. *S. aureus* strains in which Sbi was expressed from an inducible promoter were constructed. The cloned *sbi* gene was mutated to express derivatives of Sbi that lacked residues in the C terminus required for retaining the protein in the cell envelope. Mutants of Sbi which lacked the IgG-binding domains D1D2 or the C3-binding domains D3D4 were also constructed. Survival of bacteria expressing truncated Sbi proteins led to the conclusion that both the envelope-associated and the secreted forms of the protein contributed to survival. However, an exclusively secreted form of Sbi that lacked D3D4 and contained only D1D2 did not protect, whereas a secreted protein carrying only D3D4 was protective. This implies that D1D2 domains provide protection only when anchored to the cell surface, whereas only the secreted D3D4 domains are biologically active.

We have relied on studies of bacterial survival in whole human blood to fulfill Koch's postulates at the molecular level for Sbi. Chabelskaya et al. (3) were unable to demonstrate that Sbi contributed to the survival of mice injected intravenously with *S. aureus*. It is important to note that it was difficult to demonstrate that Spa is a virulence factor in a similar mouse infection model. Statistically significant differences were seen only in the severity of arthritis on 1 day and only when groups of 30 mice were tested (35).

Differences in host specificity of other immune evasion factors have made it difficult to show loss of virulence in mouse infection models (38, 41). This illustrates the problems sometimes encountered when attempting to fulfill molecular Koch's postulates in murine models. This problem could be overcome by using an animal where staphylococcal immune evasion factors are more proficient. Alternatively, investigators have constructed transgenic mice that express the human version of a target protein or have murinized the pathogen by engineering a virulence factor that can recognize the murine ligand (26, 38, 41).

In summary, we have revealed that Sbi has an important role in determining the ability of *S. aureus* to avoid phagocytosis in human blood. We have shown that Sbi reduced the uptake of bacteria by human neutrophils in the presence of serum opsonins and promoted the survival of bacteria in human blood. We have established the relative contribution of each antiphagocytic factor of *S. aureus* to immune evasion under the same growth conditions and in the same genetic background. Moreover, we have shown that Sbi domains D1D2 and D3D4 as well as secreted Sbi are capable of supporting the antiphagocytic effects of the Sbi protein. An exclusively secreted form of Sbi that lacked D3D4 and contained only D1D2 did not protect, whereas a secreted protein carrying D3D4 did.

This implies that D1D2 domains provide protection only when anchored to the cell surface, whereas only the secreted D3D4 domains are biologically active. Our work, in combination with what is known about Sbi, suggests an important role for the protein in *S. aureus* immune evasion.

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