Protein A is released into the *Staphylococcus aureus* culture supernatant with an unprocessed sorting signal.

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Running Title: Release of Protein A by *S. aureus*.

Abstract

The immunoglobulin binding protein A (SpA) of *Staphylococcus aureus* is synthesized as a precursor with a C-terminal sorting signal. The sortase A enzyme mediates covalent attachment to peptidoglycan so that SpA is displayed on the surface of the bacterium. Protein A is also found in the extracellular medium but the processes involved in its release are not fully understood. Here we show that a portion of SpA is released into the supernatant with an intact sorting signal indicating that it has not been processed by sortase A. Release of SpA was reduced when the native sorting signal of SpA was replaced with the corresponding region of another sortase-anchored protein (SdrE). Similarly, a reporter protein fused to the sorting signal of SpA was released to a greater extent than the same polypeptide fused to the SdrE sorting signal. Released SpA protected bacteria from killing in human blood indicating that it contributes to immune evasion.

Introduction

Staphylococcus aureus is an important opportunistic pathogen causing serious invasive infections in the community and healthcare setting (1). Almost all clinical isolates of *S. aureus* express the major virulence factor staphylococcal protein A (SpA) (2). Protein A is located both on the surface of the bacterium and in the extracellular medium (3-6) and comprises four or five repeated immunoglobulin-binding domains (IgBDs) (7, 8). The IgBDs of SpA (Fig. 1) adopt a triple helical structure and can bind to the Fc region of IgG via helices I and II (9), and to the Fab region of human IgM of the subclass V_H3 via helices II and III (10). The binding of SpA to Fc and Fab domains contributes to *S. aureus* virulence in a mouse model of systemic infection (11). The interaction of SpA with IgM Fab triggers the proliferation and depletion of B cells (12) suppressing the development of adaptive immune responses. Thus infection with SpA-expressing bacteria does not provide protection against subsequent *S. aureus* infection (11). Protein A also inhibits phagocytic killing of *S. aureus* in human and mouse blood (11, 13). This process is likely to be dependent on the interaction of SpA with IgG Fc since *S. aureus* expressing a variant of SpA lacking the ability to recognise IgG Fc survives poorly in mouse blood, akin to a SpA-deficient mutant (11). The IgBDs of SpA also promote inflammation through their interaction with tumour necrosis factor receptor 1 (14). The Xr region of SpA (Fig. 1) comprises variable numbers of octapeptide repeats that contribute to inflammation by activating interferon-β signalling in airway epithelial and immune cells (15).

Protein A is synthesized as a precursor with an N-terminal signal sequence and C-terminal sorting signal (Fig. 1). The signal sequence is cleaved by signal peptidase during translocation of the precursor across the cytoplasmic membrane by the general secretory (Sec) pathway (16). The sorting signal comprises an LPETG motif, a hydrophobic membrane-spanning domain and, at the extreme C-terminus, a stretch of positively charged

residues (Fig. 1). The last two elements delay secretion across the membrane and facilitate recognition and cleavage by sortase A (17). Sortase A cleaves between threonine and glycine of the LPETG motif forming an acyl-enzyme intermediate capturing the C-terminal carboxyl group of the protein with its active site cysteine thiol (18). Acyl intermediates are relieved by the nucleophilic attack of the amino group of the pentaglycine crossbridge of lipid II (19). Following transglycosylation and transpeptidation, SpA becomes covalently anchored to peptidoglycan and is displayed on the surface of the bacterium (20).

A substantial amount of SpA is found in the extracellular medium (3-6). Released SpA can be detected in the skin lesions of mice infected with a USA300 strain of community-associated MRSA and in fluids recovered from patients with *S. aureus* infection (21). However, the processes involved in SpA release are not completely understood. Becker, *et al*. (4) described a mechanism whereby SpA is shed from the cell envelope of strain Newman into the culture medium following cleavage of the pentaglycine cross-bridge of peptidoglycan by the *S. aureus* glycyl-glycine endopeptidase LytM. The murein hydrolase LytN cleaves the amine bonds between N-acetylmuramic acid and the tetrapeptide side chains of peptidoglycan so that peptidoglycan fragments linked to SpA lack amino-sugars (4). This is hypothesised to allow released SpA to avoid activating nucleotide-binding and oligomerization domain containing protein 2 (NOD2) *in vivo* (4). The release of SpA is not completely inhibited in a *lytM*-deficient mutant of strain Newman indicating that LytM activity is not the only factor involved in the release of SpA (4). Here we identify a previously undescribed mode of SpA release by the USA300 strain LAC. We show that when this is interrupted, there is a significant reduction in the level of SpA release. We also investigate the biological significance of SpA release by studying the ability of extracellular SpA to promote *S. aureus* survival in human blood.

Materials and Methods

Bacterial strains and growth conditions. *S. aureus* was grown on Tryptic Soy Agar (Oxoid), or in BHI (Difco) broth at 37 °C. *Escherichia coli* was grown on Luria agar or broth (Difco). Cultures were supplemented with ampicillin (100 µg/ml, Melford Laboratories) or chloramphenicol (10 µg/ml), as required. Bacteria were diluted 1:200, washed in BHI and allowed to grow to the OD_{600} required. Strains harbouring the pRMC2 expression vector were grown to OD_{600} = 0.3 and induced with anhydrotetracycline (ATc) until OD_{600} = 1.2 was reached. Broth, where indicated, was supplemented with V8 (1 U/ml) and 3, 4-dichloroisocoumarin (DCI; 200 µM). Unless otherwise stated all reagents were obtained from Sigma.

Plasmid and strain construction. All strains and plasmids are listed in Table 2. Strain LAC* *spa* was constructed by transduction of *spa*::Kan^r by phage 85 into strain LAC*. LAC* *spa sbi* was constructed by transduction of *sbi*::Em^r into strain LAC* *spa*::Kan^r. Primer sequences are listed in Table S1. Cloning was carried out using the SLIC procedure as described by Li and Elledge (22). Primers for amplifying insert sequences contained 5' extensions with homology to the target vector. The complete *spa* gene from strain Newman (100% amino-acid sequence identity to the *spa* gene from LAC*) was amplified from genomic DNA by PCR using primers SpAF and SpAR (Table S1). Plasmid pRMC2 (23) was used as template for inverse PCR with primers pSLF and pSLR. Both amplimers were joined using SLIC to generate the plasmid pSpA, where *spa* is cloned between SacI and EcoRI sites of pRMC2.

DNA encoding the sorting signal of SdrE was amplified by PCR using primers RESF and RESR using genomic DNA from strain Newman as template. Plasmid pSpA was used as template for inverse PCR with primers dSSF and dSSR. The amplimers were joined using

SLIC to generate pSpAΩSdrESS

Similarly, to generate plasmids pD3D4-SpASS and pD3D4-SdrESS PCR was performed using primers dSSF and DWrR and plasmid pRMC2-*sbi*ΔD1D2 (13) as template. DNA encoding the SpA sorting signal was amplified using primers DSpF and SpAR and plasmid pSpA as template. DNA encoding the SdrE sorting signal was amplified using primers DSdF and RESR and plasmid pSpAΩSdrESS as template. PCR products were joined by SLIC to generate pD3D4–SpASS and pD3D4-SdrESS.

Plasmid pSpAΩSdrESS-DS was generated by amplifying 538 bp of DNA located downstream of the *spa* gene using primers SpADSF and SpADSR and genomic DNA from *S. aureus* LAC* as template and using primers pSLF and pSRER to amplify plasmid pSpAΩSdrESS. The PCR products were joined by SLIC so that the 538 bp fragment was incorporated into plasmid pSpAΩSdrESS directly downstream of the stop codon.

All plasmids were transformed into *E. coli* strain DC10B (24). Plasmids were isolated from DC10B, verified by DNA sequencing (Source Bioscience) using primers SEQF and SEQR and transformed (5 µg) into *S. aureus* made electrocompetent as previously described (25).

Deletion of the *lytM* gene was achieved by allelic exchange using pIMAY (24). Primers lytM-A and lytM-B were designed to amplify 509 bp of DNA located upstream and primers lytM-C and lytM-D amplified 512 bp of DNA located downstream of the *lytM* gene (Table S1). The upstream and downstream PCR products were denatured and allowed to reanneal via the complementary sequences in primers lytM-B and lytM-C and this was used as template in a second PCR using primers lytM-A and lytM-D. The amplimer was cloned into pIMAY (24) between KpnI and SacI restriction sites and the resulting plasmid (pIMAY::Δ*lytM*) was transformed into *E. coli* DC10B (24) and verified by DNA sequencing. The plasmid was transformed into *S. aureus* LAC* *spa sbi*, LAC* *sbi*, and Newman *sbi*

made electrocompetent and deletion of the *lytM* gene was achieved by allelic exchange as previously described (24). The resulting LytM-deficient mutants were confirmed by DNA sequencing of a PCR amplimer. The mutants were phenotypically identical to the parent strains in terms of growth rate and haemolysis on sheep blood agar (data not shown).

Strains LAC* *sbi* [SpAΩSdrESS], LAC* *sbi lytM* [SpAΩSdrESS] and Newman *sbi* [SpAΩSdrESS] were constructed by allelic exchange using pIMAY. Plasmid pIMAY (Monk *et al*., 2012) was used as template for PCR with primers (pIMAYF and pIMAYR). Primers pIREF and pIRER were used to amplify DNA encoding the SdrE sorting signal and 521 bp of DNA upstream and 538 bp of DNA downstream using plasmid pSpAΩSdrESS-DS as template. The PCR amplimers were joined by SLIC and transformed into *E.coli* DC10B (24). The resulting plasmid (pIMAY::SpAΩSdrESS) was transformed into *S. aureus* LAC* *sbi*, LAC* *sbi lytM* and Newman *sbi* made electrocompetent and replacement of DNA encoding the SpA sorting signal with DNA encoding the sorting signal of SdrE on the chromosome was achieved by allelic exchange as previously described (24). The resulting mutants were phenotypically identical to their respective parent strains in terms of growth rate and haemolysis on sheep blood agar (data not shown). The mutation was confirmed by DNA sequencing of a PCR amplimer.

Western Immunoblotting. To extract cell wall-associated proteins, cultures of *S. aureus* were harvested, washed in phosphate-buffered saline (PBS) and resuspended to an OD_{600} of 5 or 10 in lysis buffer (50 mM Tris/HCl, 20 mM $MgCl_2$, pH 7.5) supplemented with raffinose (30% w/v) and complete protease inhibitors (40 µl/ml, Roche). Cell wall proteins were solubilised by incubation with lysostaphin (100 µg /ml; AMBI, New York) for 8 min at 37°C. Protoplasts were removed by centrifugation at 16,000 × *g* for 5 min and the supernatant containing solubilised cell wall proteins was aspirated and boiled for 10 min in

final sample buffer. For supernatant fractions, bacteria were removed from cultures by centrifugation at 4,000 x *g* for 5 min and the supernatant was passed through a 0.2 μm filter. Following this, supernatant samples, where indicated, were concentrated using a 30,000 molecular weight cut off spin column (Millipore).

Proteins were separated on 7.5%, 10% or 12.5% (w/v) polyacrylamide gels, transferred onto polyvinylidene difluoride (PVDF) membranes (Roche) and blocked in 10% (w/v) skimmed milk proteins. Blots were probed with horseradish peroxidise (HRP)-conjugated rabbit anti-mouse IgG (1:2000 or 1:500, Dako), polyclonal rabbit anti-SdrE IgG (1:2000, 26), rabbit anti-V8 serum (a gift from Martin McGavin, 1:250) followed by HRP-conjugated protein A or rabbit anti-D3D4 IgG (1:500, 13) followed by goat anti-rabbit IgG-HRP. Biotin-labelled fibronectin was used in ligand affinity blots. Human fibronectin (0.5 mg/ml, Calbiochem) was incubated with biotin (2 mg/ml) for 20 min at room temperature. The reaction was stopped by addition of NH_4Cl (10 mM). Excess biotin was removed by dialysis against PBS overnight at 4°C. Blots were probed with biotin-labelled human fibronectin (15 μg/ml, Calbiochem) and HRP-conjugated streptavidin (0.5 μg/ml, Genscript). Reactive bands were visualised using the LumiGLO reagent and peroxide detection system (Cell Signalling Technology). Band quantification was performed using ImageQuant TL software (GE Healthcare).

Flow Cytometry. Cultures of *S. aureus* were washed once with PBS and once in a solution of bovine serum albumin (BSA, 0.1% w/v) and then adjusted to an OD_{600} of 0.3 in PBS. Bacteria were incubated with an equal volume of fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG (1:800 or 1:3200, Dako) for 30 min. Unbound antibody was removed by washing once in PBS. Bacteria were resuspended in formaldehyde (2% v/v) and bound IgG was detected using flow cytometry. Bacteria were gated on the basis of

forward and side scatter. The fluorescence intensity of 20,000 bacteria was analysed and the mean fluorescence was calculated.

Purification of SpA from *S. aureus* culture supernatants by affinity chromatography. *S. aureus* strain LAC* *sbi* was grown in BHI broth for 16 h to stationary phase. Bacteria were removed by centrifugation at 4,000 x *g* for 10 min and the supernatant was passed through a 0.2 µm filter. 150 ml of filtered supernatant was allowed to pass through a gravity-feed column packed with 4 ml bed volume IgG Sepharose (GE Healthcare). Briefly, the column was equilibrated with 16 ml of elution buffer (0.5 M HAc), followed by at least 20 ml of Tris-Saline Tween 20 (TST; 50 mM Tris buffer, pH 7.6, 150 mM NaCl and 0.05% Tween 20) until the column eluate was at neutral pH. The column was washed with 40 ml of TST, 10 ml of NH_4Ac (5 mM; pH 5.5), and SpA was eluted with 12 ml of elution buffer. The purified SpA was dialyzed against PBS at 4°C overnight. Protein purity was assessed by SDS-polyacrylamide gel electrophoresis (PAGE) and Western immunoblotting, and the protein concentration was determined with the BCA protein assay kit (Pierce). For N-terminal sequencing, samples were transferred to PVDF and sequencing was carried out by Abingdon Health Laboratory Services, UK.

Trypsin Digestion. Purified SpA (2 µg) was resuspended in a solution containing urea (6 M) and dithiothreitol (4 mM). The solution was heated at 60°C for 60 min. The sample was allowed to cool before iodoacetamide (15 mM) was added and incubated for 30 min at 37°C. A solution (120 µl) containing NH_4HCO_3 (50 mM; pH 7.8) and $CaCl_2$ (1 mM) was added to dilute the urea concentration to below 1 M. Sequencing Grade Modified Trypsin was added

(1 µg, Promega) and the samples were incubated at 37°C overnight. Prior to MS analysis the samples were cleaned using ZipTips (Millipore).

Liquid chromatography and tandem mass spectrometry (LC-MS/MS). The samples were run on a Thermo Scientific Q Exactive mass spectrometer connected to a Dionex Ultimate 3000 (RSLCnano) chromatography system. Peptides were resuspended in formic acid (0.1%). Each sample was loaded onto Biobasic Picotip Emitter (120 mm length, 75 µm ID) packed with Reprocil Pur C18 (1.9 µm) reverse phase media and was separated by an increasing acetonitrile gradient over 37 min at a flow rate of 250 nl/min. The mass spectrometer was operated in positive ion mode with a capillary temperature of 220°C, and with a potential of 2000 V applied to the frit. All data was acquired with the mass spectrometer operating in automatic data dependent switching mode. A high resolution (70,000) MS scan (300-2000 m/z) was performed using the Q Exactive to select the 12 most intense ions prior to MS/MS analysis using higher-energy collisional dissociation with stepped normalised collision energy.

Data Base Search. The raw data was *de novo* sequenced and searched against the Homo sapien subset of the Uniprot Swissprot database (to which the full-length amino acid sequence of SpA from strain LAC* was added) using the search engine PEAKS Studio 7 (Bioinformatics Solutions) for peptides cleaved with trypsin. Each peptide used for protein identification met specific Peaks parameters, i.e. only peptide scores that corresponded to a false discovery rate (FDR) of ≤1% were accepted from the Peaks PTM database search. The

Peaks De Novo results were filtered using an average local confidence (ALC) of ≥ 65% and peptide score of (-10lgP) of ≥ 15.

Enzyme-linked immunosorbant assay (ELISA). Microtitre plates (Nunc Maxisorp) were coated with chicken anti-protein A polyclonal IgY (1 µg /ml, Genscript) diluted in coating buffer (100 mM $NaHCO_3$, 34mM Na_2CO_3, pH 9.6) at 4°C overnight. Wells were washed five times with PBS and blocked with 100 µl of BSA (5% w/v, Fisher Scientific) at 37°C for 2 h. Supernatant samples were diluted (1:8 or 1:50) and 100 µl of each added into the appropriate well and incubated with shaking at room temperature for 1 h, and at 37°C with no shaking for 1 h. Wells were washed five times with PBS, 100 µl of mouse monoclonal biotin-conjugated anti-protein A IgG (1 µg /ml, Genscript) was added and incubated at 37°C for 1 h. Wells were washed five times with PBS, 100 µl of HRP-conjugated streptavidin (0.5 mg /ml, Genscript) was added and incubated at 37°C for 40 min. Wells were washed five times with PBS, 100 µl of 3,3',5,5'-tetramethylbenzidine liquid substrate solution was applied and incubated at room temperature with shaking for 10 min. The reaction was stopped by the addition of 50 µl of H_2SO_4 (2 M) and absorbance was read at 450 nm in an ELISA plate reader. Wells incubated with supernatants from LAC* *spa sbi*, LAC* *spa sbi* (pRMC2) or Newman *sbi* were included to account for background and absorbance readings for these wells was subtracted from the values obtained from the sample wells.

Whole blood survival assay. The ability of *S. aureus* to survive in whole human blood was studied as previously described (27). Bacteria were grown to OD_{600} = 1.2 in BHI broth, and washed twice in Roswell Park Memorial Institute media before being diluted to give 5 x 10^3 CFU/ml. Blood was obtained from healthy volunteers and treated with the anticoagulant

Hirudin (50 µg/ml, Refludan, Pharmion). 25 µl of bacteria was added to 475 µl of blood. Immediately 100 µl of each sample was added to 900 µl of ice cold endotoxin-free water, and 100 µl was plated out on TSA in triplicate to calculate input CFU. Tubes were incubated at 37°C with shaking (200 rpm) for 3 h. Following this, 100 µl of each sample was added to 900 µl of ice cold endotoxin-free water, and 100 µl of each was plated out on TSA in triplicate to calculate the number of recovered CFU. The percentage increase in CFU was determined by dividing the mean CFU after 3 h by the corresponding mean input CFU. Three independent experiments were performed using blood from three different donors. Ethical approval for the use of human blood was obtained from the TCD Faculty of Health Sciences ethics committee.

Statistical analysis. Statistical analysis was performed using Prism Graphpad 5 software. P values were calculated using Student's t-test.

Results

Release of protein A by *S. aureus* strain LAC*. Surface-displayed SpA becomes linked covalently to the *S. aureus* cell wall by sortase A (18, 28). Protein A is also released into culture supernatants (3-5). This study set out to investigate mechanisms of SpA release using an erythromycin-sensitive derivative of the USA300 strain LAC (LAC*) (29). Strain LAC expresses SpA at high levels (30, 31) and produces an additional Ig-binding protein (staphylococcal binder of immunoglobulin, Sbi) which associates with lipoteichoic acid in the cell envelope (32). Sbi is also found extracellularly (13).

In order to determine if LAC* releases SpA, proteins solubilised from the cell wall by lysostaphin treatment during protoplast formation (cell wall extract, CW) and culture supernatants (SN) were analysed by Western immunoblotting probing with HRP-conjugated rabbit IgG (Fig. 2A). To distinguish between Sbi and SpA, isogenic *spa* and *sbi* mutants and a double mutant (LAC* *spa sbi*) were used. A single band corresponding to SpA was detected in cell wall extracts from LAC* and LAC* *sbi* and was absent in extracts from LAC* *spa* and the LAC* *spa sbi* mutant (Fig. 2A). Consistent with previous findings, Sbi was not detected in cell wall extracts (Fig. 2A) since it is not solubilised by lysostaphin during protoplast formation (13).

Culture supernatants from LAC* and LAC* *sbi* contained a band corresponding to released SpA and this was absent from LAC* *spa* and LAC* *spa sbi* (Fig. 2A). As a control, the same cell wall extracts and supernatants were probed for SdrE, a cell wall associated protein (Fig. 2B) and V8, a secreted protease (Fig. 2C). The SdrE protein was detected only in the cell wall fraction (Fig. 2B) and V8 was detected only in supernatant samples indicating purity of the samples and equal loading of protein (Fig. 2B, 2C).

304 A band corresponding to extracellular Sbi was detected in supernatants from LAC*
305 and LAC* *spa* (Fig. 2A). Therefore to study the release of SpA, it was important to use the
306 LAC* *sbi* mutant to avoid interference from extracellular Sbi in culture supernatants. In
307 order to determine the proportion of total SpA that is released during growth of LAC* *sbi* in
308 brain heart infusion (BHI) broth, bands on a Western blot corresponding to SpA in cell wall
309 extracts and supernatants were quantified using densitometry (Fig. 2D). Released SpA was
310 expressed as a percentage of total SpA associated with the cell wall and supernatant.
311 Released SpA represented 6.5 % of total protein A from cultures grown to early exponential
312 phase (OD_{600} = 0.3) and 7.3% of total protein A from cultures grown to an OD_{600} of 1.2 (Fig.
313 2D).

314 Previously Becker, *et al*. (4) reported that the *S. aureus* endopeptidase LytM promotes
315 the release of SpA by cleaving within the pentaglycine cross-bridge of peptidoglycan. The
316 amount of SpA released into culture supernatants was reduced in a *lytM*-deficient mutant of
317 strain Newman (4). In order to determine if release of SpA by LAC* is promoted by LytM,
318 *S. aureus* culture supernatants from LAC* *sbi* and a LAC* *sbi lytM* mutant were examined by
319 Western immunoblotting. Less released SpA was detected in the supernatant of the LAC*
320 *sbi lytM* mutant (Fig. 3A, 3B). The amount of SpA on the bacterial surface and in culture
321 supernatants of a *lytM*-deficient mutant (LAC* *sbi lytM*) was quantified (Fig. 3C, 3D).
322 Bacteria were incubated with FITC-labelled rabbit IgG to detect surface located SpA by flow
323 cytometry. There was no significant difference in the amount of SpA displayed on the
324 surface of LAC* *sbi* and LAC* *sbi lytM* grown to the same optical density (OD_{600} = 0.3 or
325 OD_{600} = 1.2, Fig. 3C). However, there was a 34% reduction in the amount of SpA released
326 by LAC* *sbi lytM* compared to LAC* *sbi* when supernatants were harvested from bacteria
327 grown to an OD_{600} of 0.3 (Fig. 3D). LAC* *sbi lytM* grown to an OD_{600} of 1.2 released 11%
328 less SpA than LAC* *sbi* grown to the same optical density (Fig. 3D). These results show that

329 LytM contributes to the release of SpA by LAC* similarly to strain Newman (4, Fig. S1).
330 Given that released SpA constitutes 7% of total SpA (Fig. 2), a 34% reduction in release
331 would result in an undetectable change (~ 2%) in the amount of surface exposed SpA.

332 **Extracellular proteases are not required for the generation of released protein A.** In
333 agreement with studies performed by Becker, *et al.* (4) using strain Newman, we found that
334 release of SpA by the USA300 strain LAC* is partially dependent on the endopeptidase LytM
335 (Fig. 3D). However SpA release was not completely inhibited in a *lytM*–deficient mutant of
336 LAC* indicating that an alternative mechanism for the generation of released SpA exists. In
337 order to investigate if the generation of extracellular protein A in LAC* requires the activity
338 of proteases, the amount of SpA on the surface of LAC* and an isogenic mutant (LAC* PD)
339 lacking all extracellular proteases of *S. aureus* (V8, SplABCDEF, ScpA, SspB & aureolysin)
340 was compared. Surface-located SpA was detected with FITC-labelled IgG using flow
341 cytometry. The amount of SpA on the bacterial surface did not differ between LAC* and
342 LAC* PD at any of the growth phases tested (Fig. 4A). Cell wall extracts were prepared from
343 the same cultures and analysed by western immunoblotting. A band corresponding to SpA
344 was detected in the cell wall of all cultures (Fig. 4B). In order to study the generation of
345 released SpA by LAC* and LAC* PD, culture supernatants were examined by Western
346 immunoblotting. Released SpA was detected in the culture supernatants of bacteria at all
347 stages of growth (Fig. 4C). Less SpA was detected in the culture supernatant of a LytM-
348 deficient mutant compared to wild-type LAC* under the same conditions (Fig. 3A). No
349 major alterations in the level of released SpA was observed when supernatants from LAC*
350 were compared to LAC* PD indicating that extracellular proteases of *S. aureus* are not
351 responsible for the generation of released SpA (Fig. 4C).

352 Karlsson, et al. (33) implicated the *S. aureus* serine protease V8 in the cleavage of SpA
353 from the surface of the bacterium. However McGavin, *et al*. (34) could not replicate these

findings. Since LAC* PD is deficient in the *sspA* gene encoding V8 it seemed unlikely that V8 protease was responsible for the generation of released SpA by LAC*. In order to determine if the addition of purified V8 protease could promote the release of SpA, the LAC* PD strain was grown in broth with or without added V8. The concentration of V8 used for this experiment was previously shown to be sufficient to remove fibronectin binding proteins A and B (FnBPs) from the surface of *S. aureus* (34). As a control for V8 activity, the same samples were probed with biotin-labelled fibronectin to detect FnBPs. Very faint bands corresponding to FnBPs were detected in cell wall extracts from bacteria grown in broth containing V8 confirming that the protease was active under the conditions used (Fig. 4D). The effect of V8 was inhibited by the serine protease inhibitor 3,4-dichloroisocoumarin (DCI) confirming that the serine protease activity of V8 is responsible for removing FnBPs from the surface of *S. aureus*. In order to determine if V8 promotes the release of SpA, the same cell wall extracts were probed with HRP-conjugated rabbit IgG in a Western immunoblot. The integrity or abundance of cell wall-associated and released SpA (Fig. 4E) was not affected by incubation with V8 indicating that V8 does not promote the removal of SpA from the surface of *S. aureus*.

Identification of a protein A precursor with an unprocessed sorting signal in *S. aureus* culture supernatants. Extracellular proteases were not involved in the release of SpA by *S. aureus* LAC* (Fig. 4) while the glycyl-glycine endopeptidase LytM promoted some SpA release (Fig. 3). Since the release of SpA was not completely inhibited in a LytM-deficient mutant (Fig. 3D, (4) another mechanism of release must exist (4). In order to identify additional factors involved in SpA release, extracellular SpA was purified from the culture supernatant of strain LAC* *sbi* using affinity chromatography on IgG-sepharose. The purified protein was analysed using liquid chromatography tandem mass spectrometry (LC-MS/MS).

379 The sequence of eight of the peptides identified mapped to the extreme C-terminus of SpA
380 (Table 1). One peptide terminated with four successive glycines following the sequence
381 'LPET'. This was consistent with it originating from peptidoglycan-linked SpA and being
382 released by LytM cleavage (4). Interestingly, seven unique peptides with an intact LPETG
383 motif were identified (Table 1) indicating that the pre-protein had not been processed by
384 sortase A. This suggested that the protein did not derive from the cell wall and was not
385 released by LytM-mediated cleavage of the pentaglycine cross-bridge of peptidoglycan and
386 implied that SpA can be released by *S. aureus* prior to becoming covalently anchored to the
387 peptidoglycan. The five N-terminal residues of purified extracellular SpA were identified as
388 $_{37}$AQHDE$_{41}$ by N-terminal sequencing demonstrating that the signal peptide (residues 1-36)
389 had been removed by signal peptidase. In agreement with this, none of the peptides identified
390 by LC-MS/MS originated from the signal sequence (data not shown). Thus extracellular SpA
391 is processed by signal peptidase so that the N-terminal signal sequence is removed. A single
392 band corresponding to total released SpA was detected by SDS-PAGE gel or a Western blot
393 (Fig. S1). Unprocessed SpA with an intact sorting signal has a predicted molecular weight of
394 51,928.9 while SpA released from the cell wall by LytM is linked to fragments of
395 peptidoglycan of different lengths, the most abundant forms having predicted molecular
396 masses of between 52,555.4 and 54,151 (4). These different forms of extracellular SpA
397 cannot be distinguished since they co-migrate on an SDS-PAGE gel.
398

399 **Replacing the SpA sorting signal with the sorting signal of SdrE reduces release of SpA.**

400 The identification of SpA with an intact sorting signal in LAC* culture supernatants indicated
401 that SpA can be released from the bacterium without being sorted to the cell wall. This
402 strongly suggested a release mechanism independent of LytM activity. The sorting signal of
403 cell wall anchored surface proteins is essential for covalent linkage to cell wall peptidoglycan

(17, 35). Since some released SpA harbours an unprocessed sorting signal (Table 1) we set out to determine if altering the sorting signal of SpA might influence release of the protein. The sorting signal from SdrE, another cell wall-anchored protein of *S. aureus*, was exchanged with the SpA sorting signal to generate a chimera, SpA-SdrESS. The SdrE protein is located exclusively in the cell wall fraction of *S. aureus* and, in contrast to SpA, very little is detected in culture supernatants from LAC* (Fig. 2B).

In order to facilitate the manipulation of *spa*, the full-length *spa* gene was cloned into the anhydrotetracycline (ATc)-inducible expression vector pRMC2 to generate plasmid pSpA. A variant was constructed where DNA encoding the SpA sorting signal was replaced with DNA encoding the sorting signal from SdrE (pSpAΩSdrESS). Both plasmids were introduced into the LAC* *spa sbi* mutant. The level of SpA displayed on the surface of the bacteria was detected using FITC-conjugated rabbit IgG, and fluorescence was measured using flow cytometry. Fluorescence was not detected in the absence of ATc indicating that the promoter is tightly repressed when no inducer is present and SpA is not expressed (Fig. 5A). The amount of SpA displayed on the surface of *S. aureus* increased with increasing inducer concentration (Fig. 5A) and the level of SpA expressed by *S. aureus* carrying plasmid pSpA was identical to *S. aureus* carrying pSpAΩSdrESS at each ATc concentrations tested (Fig. 5A). This indicated that replacing the SpA sorting signal with the sorting signal from SdrE did not alter the levels of SpA displayed on the surface of *S. aureus*. To study released SpA, culture supernatants from LAC* (pSpA) and LAC* (pSpAΩSdrESS) were examined by Western immunoblotting probing with HRP-conjugated rabbit IgG (Fig. 5B). Densitometric analysis of relative band intensity indicated that LAC* carrying plasmid pSpAΩSdrESS released less protein A, approxiamately 65% as much as LAC* carrying plasmid pSpA (Fig. 5B). The relative amount of released SpA in culture supernatants was then quantified by ELISA (Fig. 5C). The amount of SpA released by bacteria carrying the plasmid

pSpAΩSdrESS was reduced to approximately 62% of the amount released by bacteria carrying pSpA (Fig. 5C). These data indicated that the release of SpA by *S. aureus* is reduced when the SpA sorting signal is replaced with the sorting signal of the wall-associated protein SdrE.

The C-terminal sorting signal of SpA allows release of cell wall-anchored proteins from the surface of *S. aureus*. The C-terminal sorting signal comprises an LPXTG motif followed by a hydrophobic domain and a positively charged tail (Fig. 1) and is essential for efficient anchoring of proteins to peptidoglycan (35). Since the release of SpA was reduced when the SpA sorting signal was replaced with the sorting signal of the wall-associated protein SdrE, we hypothesised that the sequence of the sorting signal might influence the release of SpA. Rather than replacing the native SdrE sorting signal with the sorting signal from SpA we instead generated chimeric proteins where the SpA or SdrE sorting signal was linked to a reporter protein. The advantage of this was that it allowed us to study the influence of the sorting signal alone on protein release. Chimeric proteins were generated where the D3D4 domains of Sbi were linked to the sorting signal of SpA or SdrE (Fig. 6A). Sbi is an envelope associated protein which does not become anchored to cell wall peptidoglycan (13). Plasmids pD3D4-SpASS and pD3D4-SdrESS each carried DNA encoding the Sbi signal sequence and D3D4 domains and the sorting signal from either SpA or SdrE, respectively. Cell wall extracts and supernatants were prepared from cultures of LAC* *spa sbi* carrying plasmids pD3D4-SpASS and pD3D4-SdrESS and analysed by Western immunoblotting using anti-D3D4 IgG. Both D3D4-SpASS and D3D4-SdrESS were detected in cell wall extracts (Fig. 6B) indicating that they had been sorted to the cell wall peptidoglycan. Less D3D4 protein was detected in culture supernatants from LAC* *spa sbi*

453 (pD3D4-SdrESS) (43% reduction as estimated by densitometry) than from LAC* *spa sbi*
454 (pD3D4-SpASS) (Fig. 6C). These data demonstrate that the release of a protein into *S.*
455 *aureus* culture supernatants depends on the sequence of its sorting signal.

456

457 **The release of SpA by LAC* is mediated by both LytM and the native SpA sorting**
458 **signal.** Taken together our data suggested that both the SpA sorting signal and the activity of
459 the glycyl-glycine endopeptidase LytM contribute to release of SpA by *S. aureus*. In order to
460 study the relative contribution of these factors, the sorting signal from SdrE was exchanged
461 with the SpA sorting signal on the chromosome of LAC* *sbi* and LAC* *sbi lytM* by allelic
462 exchange to yield strains LAC* *sbi* [SpAΩSdrESS] and LAC* *sbi lytM* [SpAΩSdrESS],
463 respectively. Protein A displayed on the surface of the bacteria was detected using FITC-
464 conjugated rabbit IgG. The level of SpA expressed by LAC* *sbi* [SpAΩSdrESS] and LAC*
465 *sbi lytM* [SpAΩSdrESS] was identical to the level expressed by LAC* *sbi* at each stage of
466 growth tested (OD_{600} = 0.3 and 1.2, Fig. 7A). The relative amount of released SpA in culture
467 supernatants was quantified by ELISA. The amount of SpA released by *S. aureus* LAC* *sbi*
468 [SpAΩSdrESS] grown to an OD_{600} of 0.3 was 21% less than the amount released by LAC*
469 *sbi* grown to an equal density (Fig. 7B). At an OD_{600} of 1.2, LAC* *sbi* [SpAΩSdrESS]
470 released 42% less SpA than LAC* *sbi* (Fig. 7B). When the sorting signal of SdrE replaced
471 the SpA sorting signal in a LytM-deficient mutant (LAC* *sbi lytM* [SpAΩSdrESS]) the
472 amount of SpA was reduced by 63% and 62% for bacteria grown to an OD_{600} of 0.3 and 1.2,
473 respectively compared to LAC* *sbi* grown to the same optical density (Fig. 7B). These data
474 demonstrate that the release of SpA by *S. aureus* is influenced by both the sorting signal and
475 LytM activity and that together these factors account for up to 63% of released SpA in strain
476 LAC*. Similar results were obtained using strain Newman *sbi* and Newman *sbi*
477 [SpAΩSdrESS] (Fig. S2)

Both cell wall-anchored and released SpA contribute to the survival of LAC* in whole human blood. Protein A contributes to the pathogenesis of invasive infection by protecting *S. aureus* from killing in blood (11, 13). Surface located SpA inhibits bacterial uptake by neutrophils (13, 36, 37) but the ability of released SpA to promote *S. aureus* survival in blood has not been examined.

Previously, Malachowa, *et al*. (38) demonstrated that transcription of the *spa* gene is highly upregulated when LAC is incubated in human blood. To investigate if SpA contributes to the ability of LAC* to resist killing in human blood, LAC* *sbi* and an LAC* *spa sbi* were incubated in blood and viable counts were used to determine bacterial survival. The LAC* *spa sbi* mutant demonstrated a reduced ability to survive in whole blood (143% of input inoculum recovered) compared to LAC* *sbi* (356% of input inoculum recovered, Fig. 8), showing that SpA protects LAC from phagocytic killing.

LAC* *sbi* expresses both cell wall-associated and released SpA (Fig 2). To investigate if the unprocessed form of released SpA increases the growth of *S. aureus* in blood, LAC* *sbi* [SpAΩSdrESS] was studied. This strain expresses similar levels of surface-associated SpA but less released SpA than LAC* *sbi* (Fig. 7) . LAC* *sbi* [pSpAΩSdrESS] grew significantly better in human blood compared to LAC* *spa sbi* (227% of input inoculum recovered) indicating that released SpA enhances the ability of *S. aureus* to survive and grow in blood. (Fig. 8). The effect of LytM-mediated SpA release on blood survival was not examined here since LytM is proposed to contribute to the release of all proteins linked to the pentaglycine crossbridge of peptidoglycan (4). Many of these proteins are important immune evasion factors and contribute to bacterial survival in blood (13, 39) and so the effects of LytM-released SpA could not be studied in isolation. In summary these data

indicate that released, as well as cell wall-associated SpA, protects *S. aureus* from killing in human blood.

Discussion

Protein A is a virulence factor in murine models of *S. aureus* kidney abscess formation, skin infection, pneumonia, sepsis and septic arthritis (15, 40-43). *S. aureus* produces both a cell wall associated and released form of SpA. Protein A becomes covalently linked to peptidoglycan by the action of sortase A. Here we demonstrate that SpA with an intact sorting signal is found in the culture supernatant indicating that a portion of the protein can be released without being processed by sortase A.

Becker, *et al*. (4) previously showed that the glycyl-glycine endopeptidase LytM cleaves within the pentaglycine crossbridge of peptidoglycan to release SpA. A *lytM* mutant of strain Newman released less SpA than wild-type bacteria (4). . In this study we demonstrate that LytM also contributes to the release of SpA by strain LAC*. Culture supernatants from a *lytM*-deficient mutant contained 34% less SpA than supernatants from wild-type LAC* grown to an OD_{600} of 0.3 and 11% less SpA at an OD_{600} of 1.2 (Fig. 3D). Thus it appears that LytM may have a greater influence on SpA release at early points in the growth phase.

We demonstrate that extracellular proteases of *S. aureus* do not mediate the release of SpA (Fig. 4). Despite conflicting evidence in the literature (34) it has been assumed that the *S. aureus* serine protease V8 cleaves SpA from the surface of *S. aureus* (44). However, this assumption was based on a single study with an uncharacterised strain (33). Our data demonstrate that V8 does not mediate SpA release. However, a previous study showed that there was a 2.1 fold increase in the amount of SpA in the cell wall of LAC* PD compared to wild-type LAC* (44) when the surface proteome of stationary phase cultures (15 hours of growth) was examined. Thus it is likely that V8 can affect the stability of SpA on the *S. aureus* surface to some degree.

545 The factors promoting SpA release reported in this study and by Becker, et al. (4) do
546 not account for all released SpA. Replacing the SpA sorting signal with that of SdrE in a
547 LytM-deficient mutant of LAC* reduced the amount of released SpA by up to 63% (Fig. 7B).
548 As proposed by Becker, *et al*. (4), it is possible that an unidentified autolysin or another
549 factor might be involved.A portion of SpA is released into the culture supernatant without
550 being processed by sortase. Replacing the native sorting signal from SpA with the sorting
551 signal of SdrE reduced the release of SpA. Why is a protein with the SpA sorting signal
552 released abundantly into the *S. aureus* culture supernatant while a protein with the SdrE
553 sorting signal is released to a lesser extent? Schneewind, *et al*. (35) demonstrated that
554 removing the charged tail of the sorting signal of SpA resulted in the release of the protein
555 into the culture medium instead of it becoming linked to the cell wall. Substitution of two
556 arginine residues with serine in the charged tail region resulted in a dramatic reduction in the
557 sorting of SpA to the cell wall (17). The spacing of the LPETG motif and positively charged
558 tail was also important since reducing the number of residues in the hydrophobic domain of
559 the sorting signal from 25 to 23 resulted in less protein being released into culture
560 supernatants (17). The SdrE sorting signal contains more positively charged residues at the
561 C-terminus (five rather than three) and has a slightly longer hydrophobic domain (one reside
562 longer) than the sorting signal from SpA (Fig. 5A). Thus it seems reasonable to hypothesise
563 that this is the reason why less protein is released when the native SpA sorting signal is
564 replaced with the sorting signal from SdrE.Released SpA can protect *S. aureus* from killing in
565 human blood (Fig. 8). Therefore the release of SpA is likely to contribute to the ability of *S.*
566 *aureus* LAC* to survive in the human bloodstream. The mechanism by which released SpA
567 protects bacteria from killing in blood warrants further investigation. Surface located SpA
568 has long been assumed to protect bacteria from opsonophagocytosis through its ability to
569 bind to the Fc region of IgG. However, recent work by Nordenfelt, *et al*. (45) suggested that

the majority of IgG is likely to be bound to SpA via the Fab region when bacteria are in the bloodstream. Falugi, *et al*. (11) showed that a variant of SpA lacking ability to recognise IgG Fc survived poorly in mouse blood. Therefore the ability of SpA to bind to IgG, or to another ligand which shares the same binding site on SpA, can protect bacteria from killing in blood. Further study will allow the mechanisms involved to be elucidated fully.

The release of SpA by *S. aureus* is likely to contribute to the ability of *S. aureus* to interfere with adaptive immunity through its binding to Fab region of human IgM on B cells. Becker, *et al*. (4) proposed that the removal of the amino sugars from cell wall-derived SpA is necessary so that released SpA will not be recognised by NOD2. If this is the case then the release of SpA with an unmodified C-terminus (intact sorting signal) represents a second strategy for the production of a soluble form of SpA that will not trigger host innate immune responses.

In summary we report that a portion of released SpA does not originate from the cell wall and is released following processing by signal peptidase but prior to cleavage by the sortase A enzyme Released SpA protects *S. aureus* from killing in human blood and the bacterium employs at least two independent strategies to ensure that SpA will be elaborated into the culture supernatant.

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765 **Figure Legends**

766 **FIG 1. Schematic representation of the domain organisation of protein A.**

767 Protein A consists of an N-terminal signal sequence (S) followed by up to five IgG-binding
768 domains (E – C), an antigenic variable region (Xr) and a cell wall-spanning region (Xc). The
769 Xc region harbours a LysM domain which can mediate non-covalent binding of proteins to
770 peptidoglycan. The sorting signal comprises an LPETG motif, a hydrophobic membrane
771 spanning region (M) and a positively-charged tail region (+).

772

773 **FIG 2. *Staphylococcus aureus* LAC* releases SpA.**

774 *S. aureus* LAC* was grown to an OD_{600} of 1.2. Cell wall extracts (CW) were diluted 1:5
775 prior to loading on the gel and supernatant samples (SN) were not diluted. Protein A was
776 detected using HRP-conjugated rabbit IgG (A), SdrE was detected using anti-SdrE IgG (B)
777 and V8 was detected using anti-V8 serum (C). *S. aureus* LAC* *sbi* was grown to an OD_{600} of
778 0.3 or 1.2 as indicated (D). Cell wall extracts were diluted 1:20 and supernatant samples
779 were not diluted. Size markers are indicated (kDa).

780

781 **FIG 3. LytM contributes to release of protein A by LAC***

782 A, B) Culture supernatants from LAC* *sbi* and LAC* *sbi lytM* grown to the OD_{600} indicated
783 were probed with HRP-labelled rabbit IgG in a Western immunoblot. Supernatants harvested
784 at OD_{600} = 0.3 (A) were concentrated 8-fold before loading on a gel and supernatants
785 harvested at OD_{600} = 1.2 (B) were concentrated 2-fold. Size markers are indicated (kDa).

786 C) Protein A on the surface of LAC* *sbi* and LAC* *sbi lytM* was detected using FITC-labelled
787 rabbit IgG and the fluorescence intensity was measured by flow cytometry. Values are

plotted as a percentage of the mean fluorescence intensity measured for LAC* *sbi* grown to an OD_{600} of 0.3. Bars represent the mean values and error bars indicate the SEM of three independent experiments.

D) Protein was captured from culture supernatants using chicken anti-SpA polyclonal IgY and detected using biotin-conjugated mouse monoclonal anti-SpA IgG followed by streptavidin-HRP in an ELISA. Values are expressed as a percentage of total released SpA measured for LAC* *sbi* grown to an OD_{600} = 0.3. Bars represent the mean percentage release from four independent experiments. Error bars represent the SEM. ** p = 0.006, *** p = 0.0003, n.s. = not significant, $p > 0.05$.

FIG 4. Extracellular proteases are not required for the release of protein A.

A) Protein A on the surface of LAC* and LAC* PD was detected using FITC-labelled rabbit IgG and the fluorescence intensity was measured by flow cytometry. Values are expressed as a percentage of the mean fluorescence intensity measured for LAC* harvested at OD_{600} = 0.3. Bars represent the mean values and error bars indicate the SEM of three independent experiments. n.s. = not significant, $p > 0.05$.

Cell wall extracts (CW, B) and culture supernatants (SN, C) from LAC* and LAC* PD grown to the OD_{600} indicated were probed with HRP-labelled rabbit IgG in a Western immunoblot. Supernatants harvested at OD_{600} = 0.3 were concentrated 8-fold, at OD_{600} = 0.6 concentrated 4-fold, and at OD_{600} = 1.2 concentrated 2-fold before loading on a gel.

LAC* PD was grown to an OD_{600} = 0.8 in broth alone or in broth supplemented with V8 (1U /ml) and DCI (200 µM) and cell wall extracts were probed with biotin-labelled fibronectin in a ligand affinity blot (D). Bound fibronectin was detected using streptavidin-HRP. The same

811 cell wall extracts and supernatants from the same cultures were probed with HRP-conjugated
812 rabbit IgG to detect protein A (E). Size markers are indicated (kDa).
813

814 **FIG 5. Release of protein A into *S. aureus* culture supernatants can be inhibited by**
815 **altering the sorting signal.**

816 A) Protein A on the surface of LAC* *spa sbi* (pSpA) (black bars) and LAC* *spa sbi*
817 (pSpAΩSdrESS) (white bars) was detected using FITC-labelled rabbit IgG and the
818 fluorescence intensity was measured using flow cytometry. Values are expressed as a
819 percentage of the mean fluorescence intensity measured for LAC* *spa sbi* (pSpA) grown in
820 broth supplemented with ATc (312.5 ng/ml). Bars represent the mean of three independent
821 experiments. Error bars represent the SEM. n.s. = not significant, $p > 0.05$.

822 B) LAC* *spa sbi* (pSpA) and LAC* *spa sbi* (pSpAΩSdrESS) were grown in broth
823 supplemented with ATc (312.5 ng/ml) and culture supernatants were probed with HRP-
824 conjugated rabbit IgG. Size markers are indicated (kDa).

825 C) Quantification of SpA in culture supernatants of LAC* *spa sbi* (pSpA) and LAC* *spa sbi*
826 (pSpAΩSdrESS) by ELISA. Bacteria were grown in broth supplemented with ATc (312.5
827 ng/ml) and SpA was captured from culture supernatants using chicken anti-SpA polyclonal
828 IgY. Bound SpA was detected using biotin-labelled mouse monoclonal anti-SpA IgG and
829 HRP-conjugated streptavidin in an ELISA. The absorbance at 450 nm was measured and
830 readings from wells incubated with culture supernatants from LAC* *spa sbi* (pRMC2) were
831 subtracted from the mean readings for LAC* *spa sbi* (pSpA) and LAC* *spa sbi*
832 (pSpAΩSdrESS) to account for background. Values for LAC* *spa sbi* (pSpA ΩSdrESS) are
833 expressed as a percentage of the values measured for LAC* *spa sbi* (pSpA). Bars represent
834 the mean of three independent experiments and error bars represent the SEM. ***$p < 0.0001$.

835

836 **FIG 6. Release of D3D4 reporter protein into *S. aureus* culture supernatants can be**
837 **inhibited by altering the sorting signal.**

838 A) Amino acid sequences of SpA and SdrE sorting signals. Amino acid coordinates are
839 indicated. Cell wall extracts (B) and culture supernatants (C) from LAC* *spa sbi* (pD3D4-
840 SpASS) and LAC* *spa sbi* (pD3D4-SdrESS) were probed with rabbit anti-D3D4 IgG.
841 Bound antibody was detected using HRP-conjugated protein A. Size markers are indicated
842 (kDa).

843

844 **FIG 7. Replacing the SpA sorting signal with the sorting signal from SdrE reduces**
845 **release of SpA by LAC*.**

846 A) Protein A on the surface of LAC* *sbi*, LAC* *sbi* [SpAΩSdrESS] and LAC* *sbi lytM*
847 [SpAΩSdrESS] was detected using FITC-labelled rabbit IgG and the fluorescence intensity
848 was measured using flow cytometry. Values are expressed as a percentage of the mean
849 fluorescence intensity measured for LAC* *sbi* grown to an OD_{600} of 0.3. Bars represent the
850 mean values and error bars indicate the SEM of three independent experiments. B) Protein
851 A was captured from culture supernatants using chicken anti-SpA polyclonal IgY and
852 detected using biotin-conjugated mouse monoclonal anti-SpA IgG followed by streptavidin-
853 HRP in an ELISA. The absorbance at 450 nm was measured and the mean reading from
854 wells incubated with culture supernatants from LAC* *spa sbi* were subtracted from the
855 readings for all other wells to account for background. Values are expressed as a percentage
856 of total released SpA measured for LAC* *sbi* grown to an OD_{600} = 0.3. Bars represent the

857 mean of four independent experiments. Error bars represent the SEM. ** $p = 0.007$, *** $p <$
858 0.0001, n.s. = not significant, $p > 0.05$.

859

860 **FIG 8. Released SpA protects *S. aureus* from killing in human blood.**

861 Washed bacteria were incubated in blood for 3 h at 37°C and the number of input and
862 recovered bacteria was calculated by viable counting. The percentage increase in CFU
863 (growth) of each strain was determined by dividing the mean CFU after 3 h by the mean
864 input CFU. Bars represent the mean percentage increase in CFU from three independent
865 experiments and error bars indicate the SEM. *** $p < 0.001$, *$p < 0.05$.

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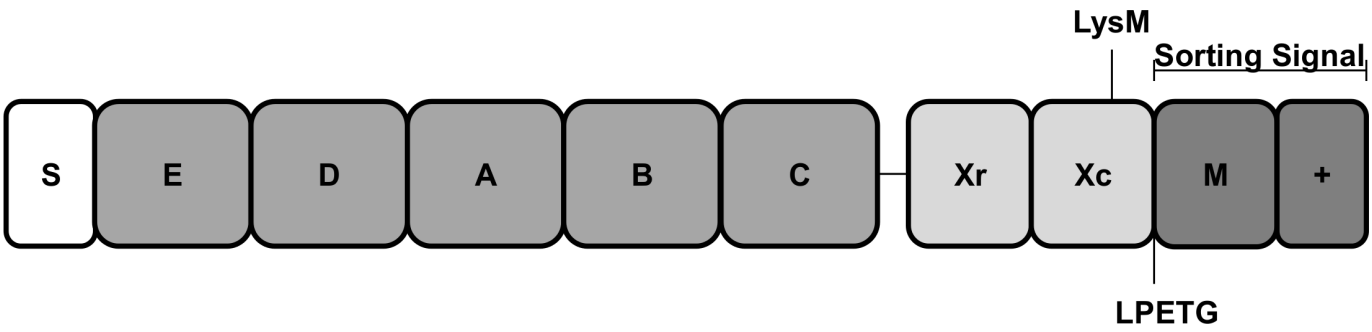
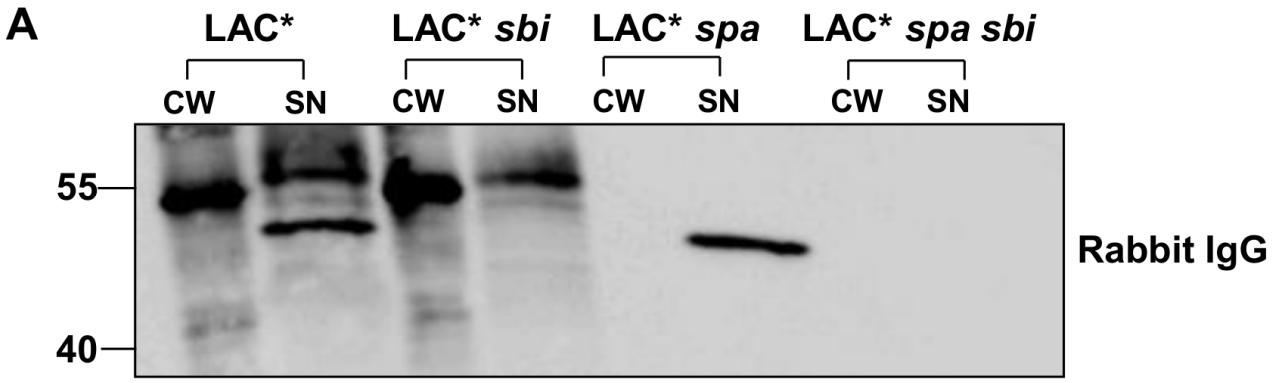
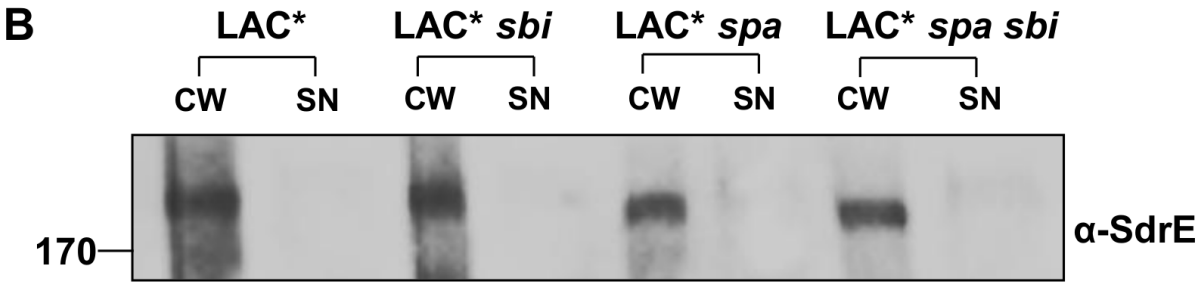
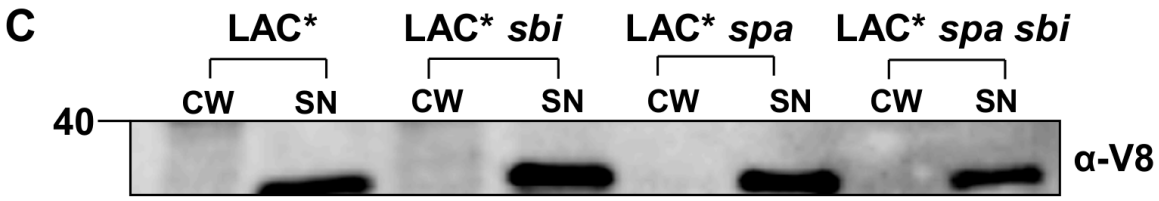
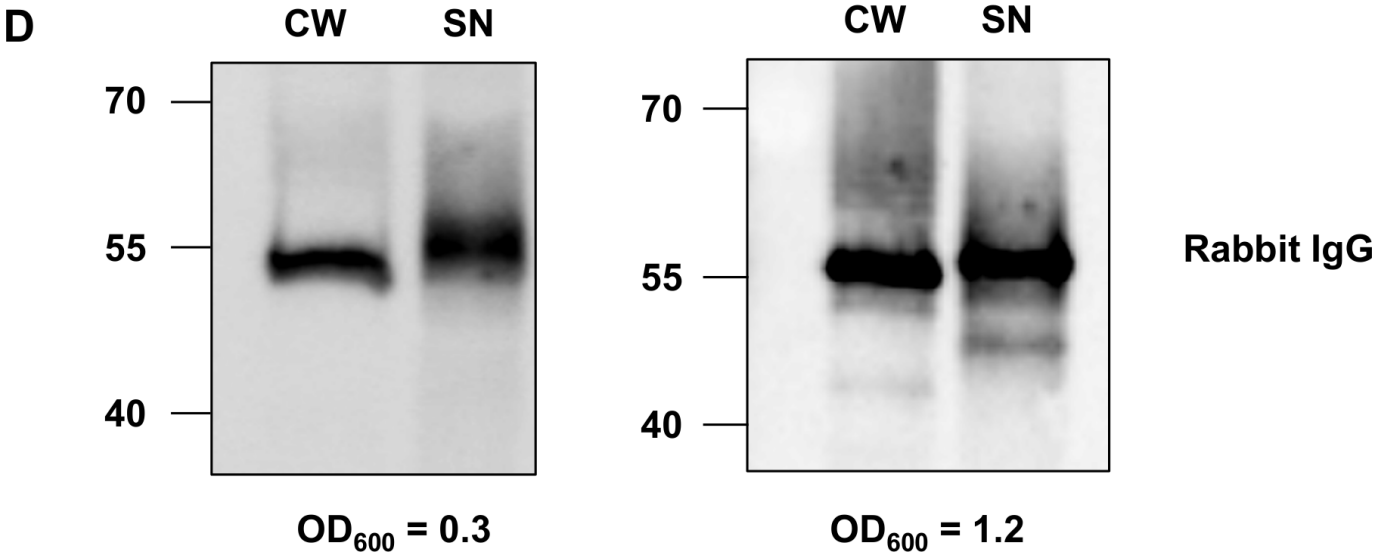
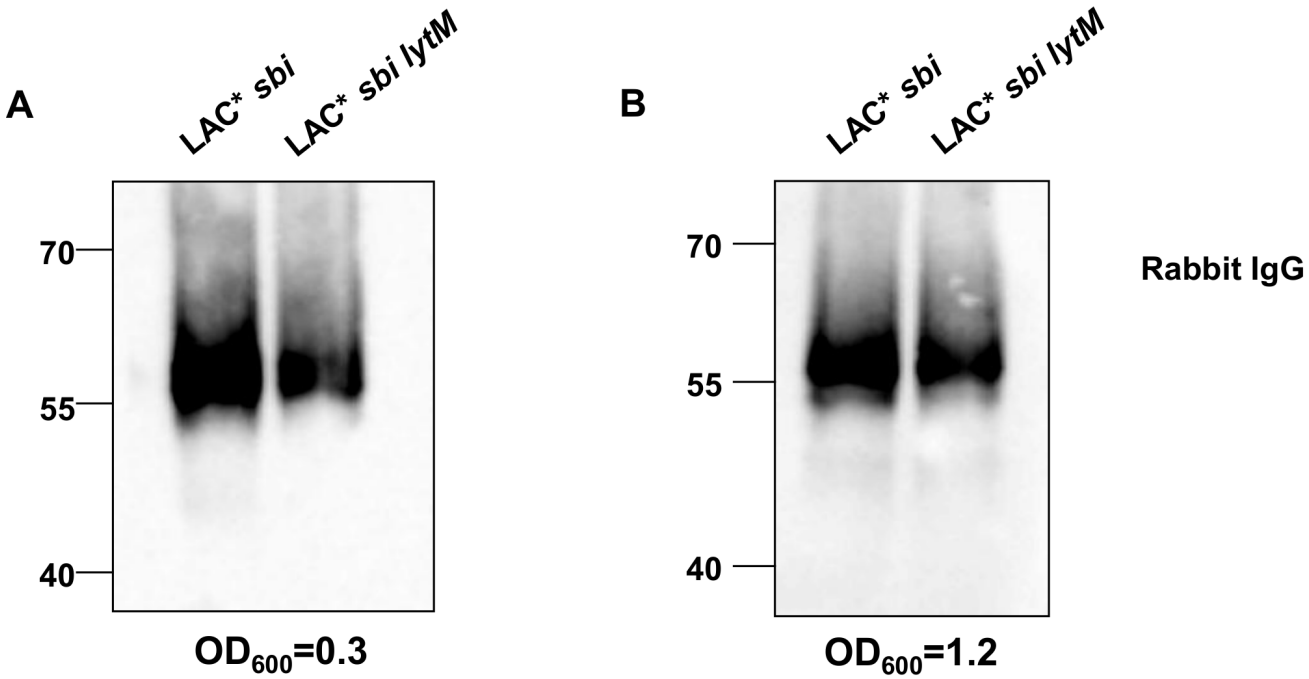


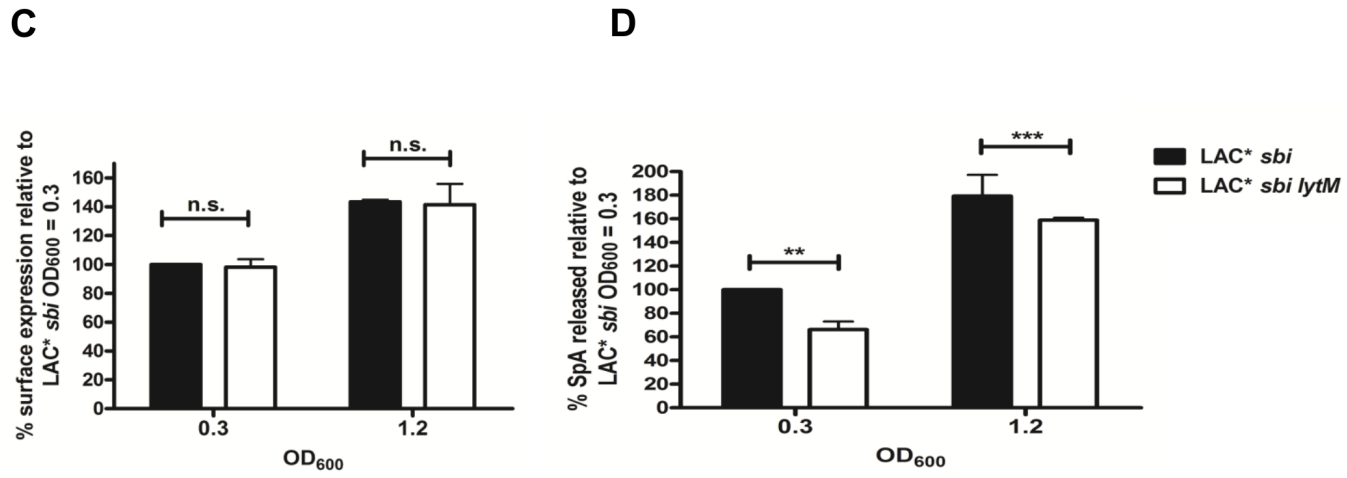


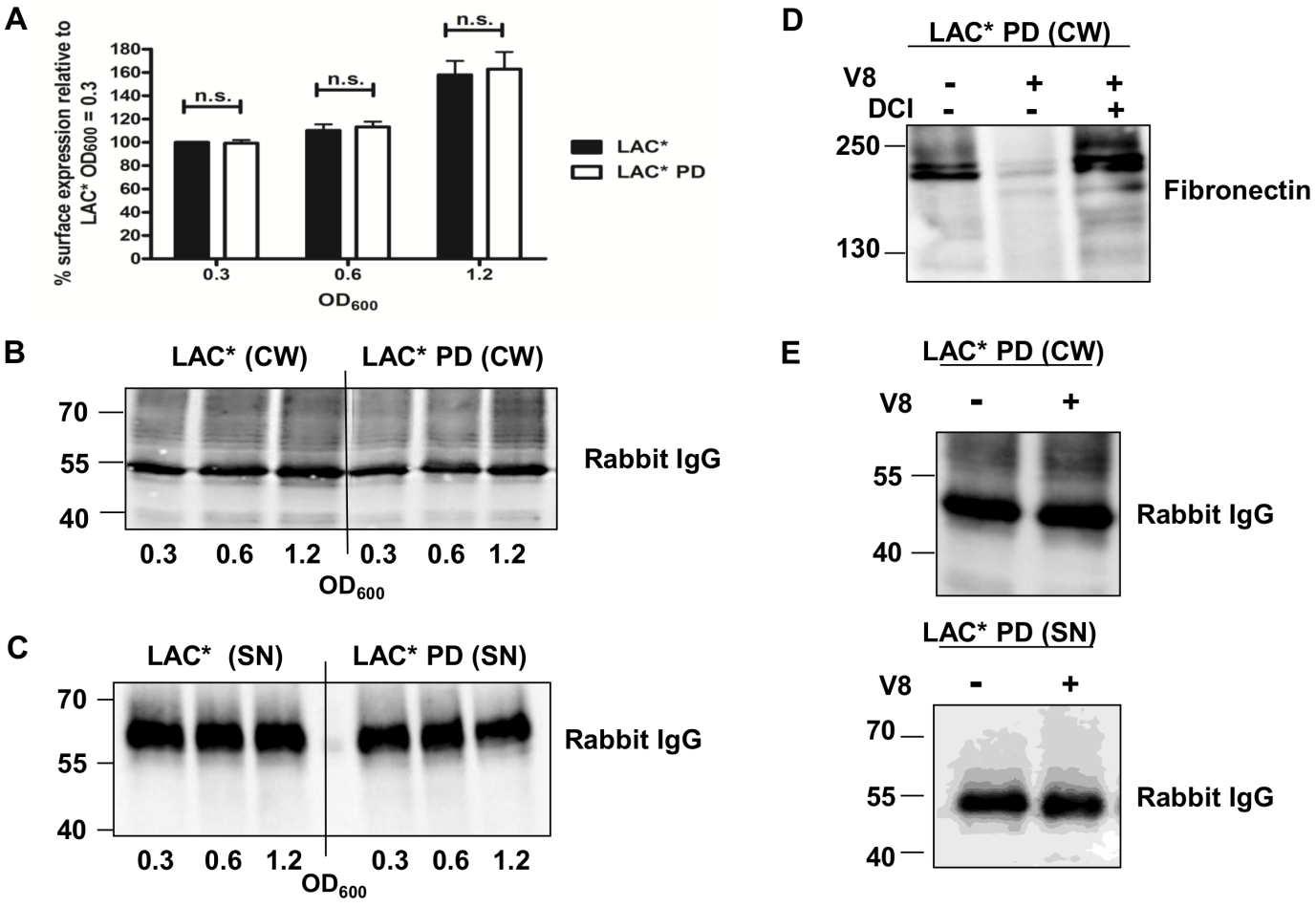


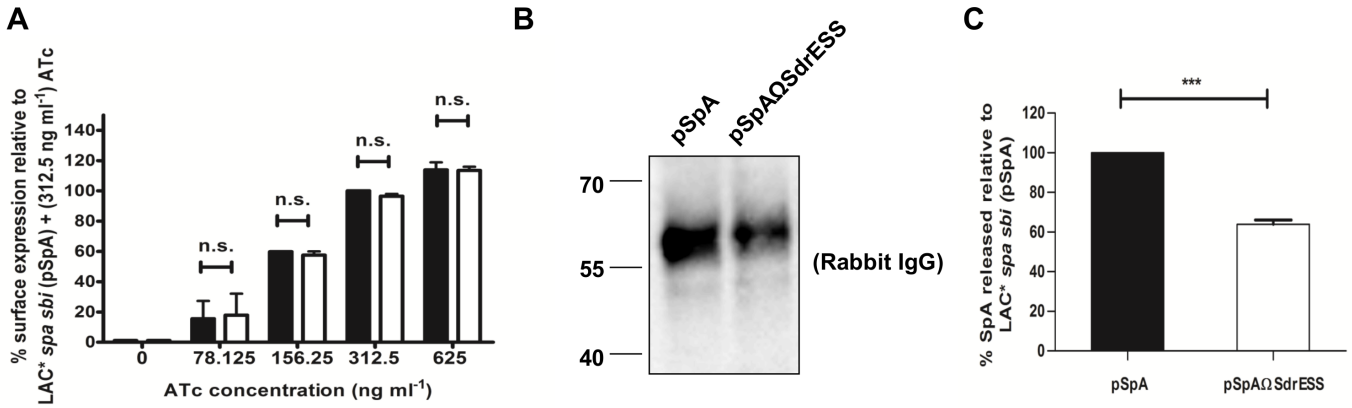








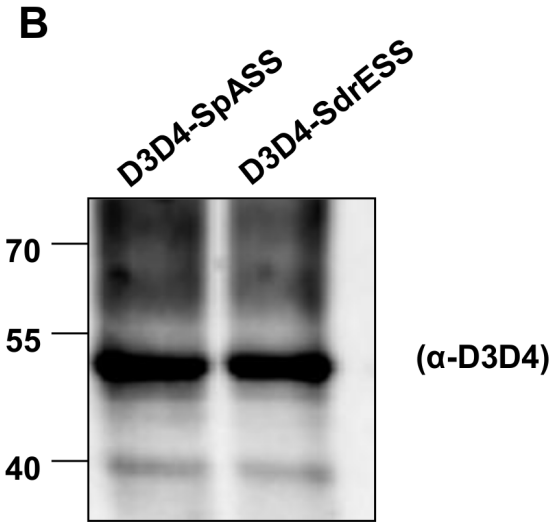


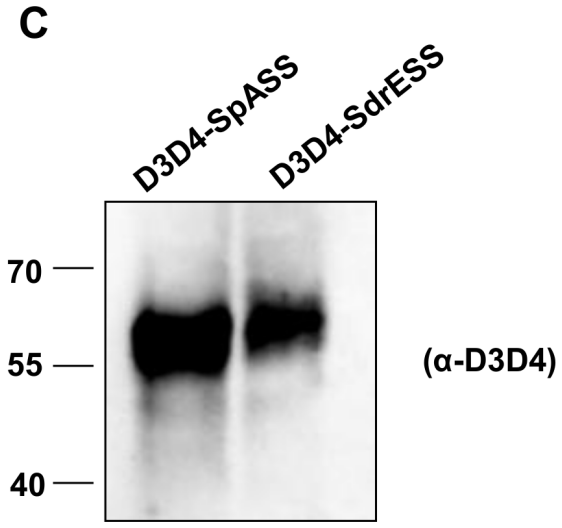


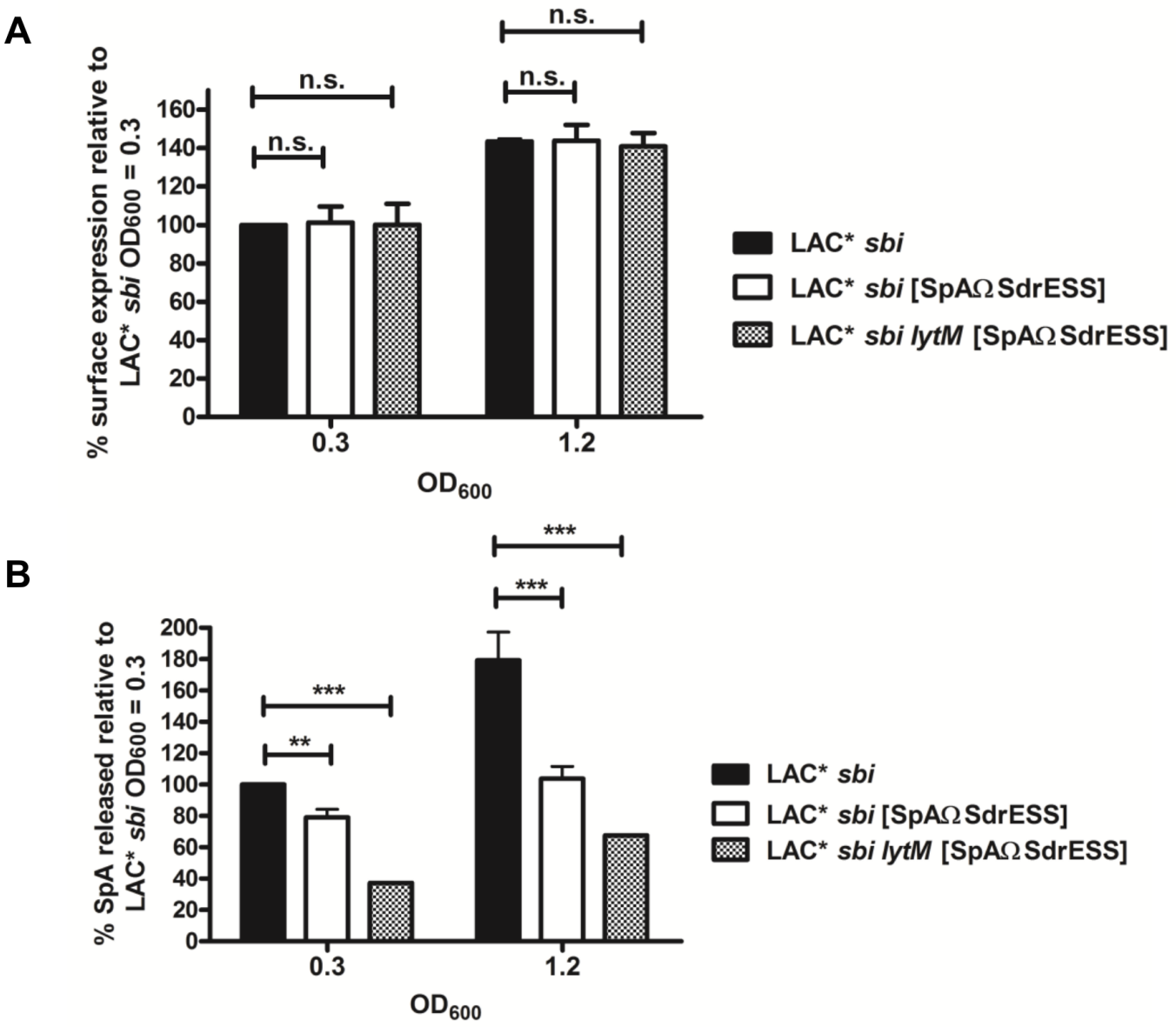
A

Sequences of sorting signals

SpA 474-508 LPETGEENP – FIGTTVFGGLSLALGAALLAGRRREL – –
SdrE 1117-1154 LPETGSENNGSNNATLFGGLFAALGSLLLFGRRKKQNK







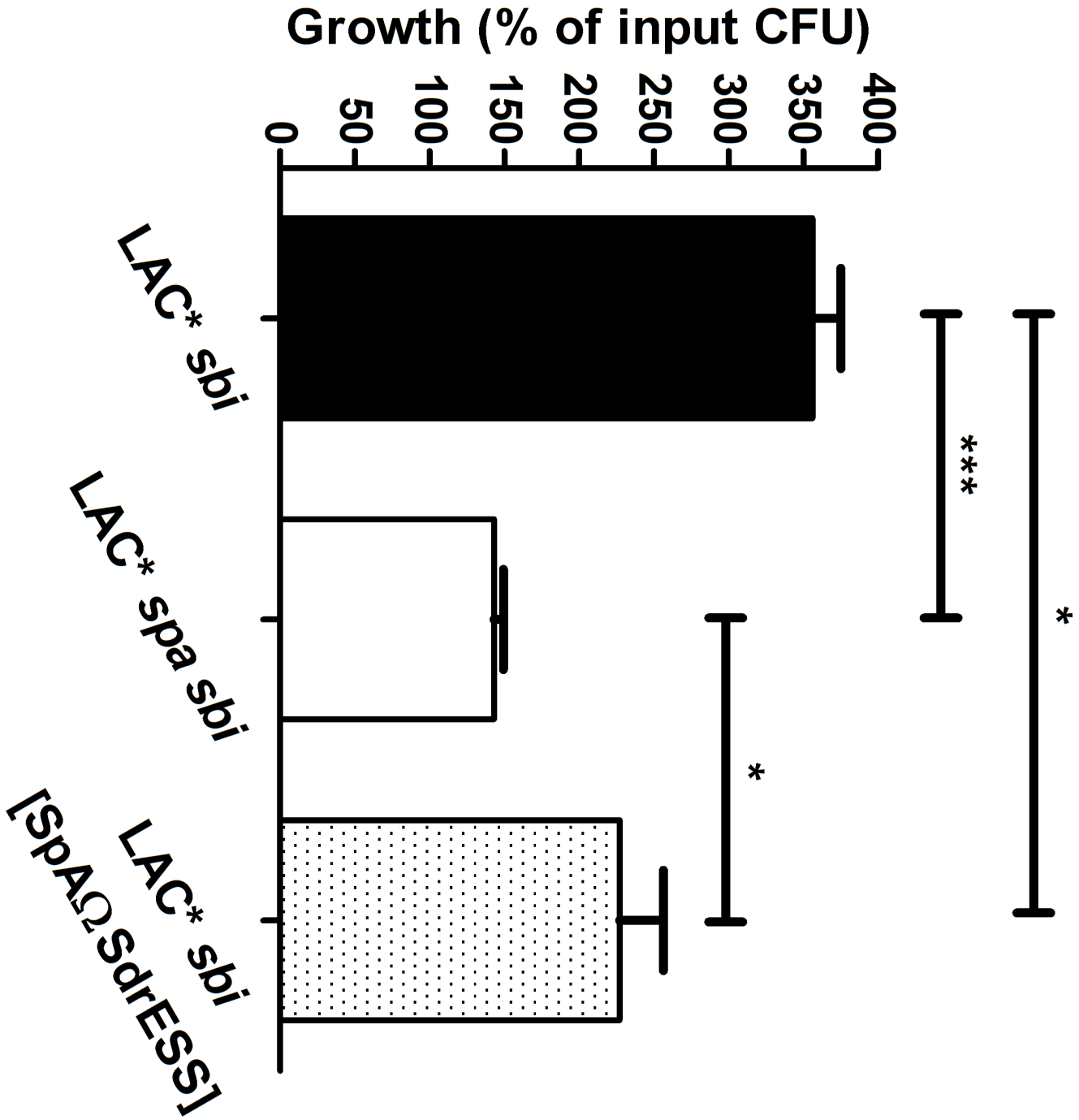

Growth (% of input CFU)
0
50
100
150
200
250
300
350
400
LAC* sbi
LAC* spa sbi
LAC* sbi [spAΩsdrESS]
***
*
*

TABLE 1. Peptides identified by liquid chromatography tandem mass spectrometry.

Peptide sequence	Number of peptides identified	Peaks peptide score -10lgP	Mass (Da)	m/z	Residue numbers
KAQALPETGEENPFI	1	44.48	1401.6411	701.8262	470-484
KAQALPETGEENPFIGTT	1	40.83	1704.7842	853.3968	470-487
KAQALPETGEENPFIGTTVFG	1	39.57	2019.9789	1010.9971	470-490
KAQALPETGEENPFIGTTVFGG	1	40.09	2077.0002	1039.5046	470-491
KAQALPETGEENPFIGTTVFGGL	1	36.39	2151.0483	1076.5337	470-492
KAQALPETGEENPFIGTTVFGGLSL	2	47.19	2351.1646	1176.5889	470-494
		47.76	2372.0847	791.7029	470-494
KAQALPETGGGG	1	47.56	956.4563	479.2358	470-478

TABLE 2. Bacterial strains and plasmids used in this study.

Strain	Description	Reference
S. aureus LAC*	Erythromycin-sensitive derivative of MRSA strain LAC. Clonal complex 8.	(29)
LAC* *spa*	Derivative of LAC* deficient in protein A. *spa*::Kan^r	This study
LAC* *sbi*	Derivative of LAC* deficient in Sbi. *sbi*:: Em^r.	(13)
LAC* *spa sbi*	Derivative of LAC* deficient in protein A and Sbi. *spa*::Kan^r, *sbi*::Em^r.	This study
LAC* PD	Protease-deficient derivative of LAC*. Δ*aur*, Δ*sspAB*, Δ*scp*, *spl*::Em^r.	(46)
LAC* *sbi lytM*	Derivative of LAC* *sbi* deficient in LytM. Δ*lytM*.	This study
LAC* *spa sbi lytM*	Derivative of LAC* *spa sbi* deficient in LytM. Δ*lytM*.	This study
LAC* *sbi* [SpAΩSdrESS]	Derivative of LAC* *sbi* where the SpA sorting signal has been replaced with the sorting signal of SdrE by allelic exchange.	This study
LAC* *sbi lytM* [SpAΩSdrESS]	Derivative of LAC* *sbi lytM* where the SpA sorting signal has been replaced with the sorting signal of SdrE by allelic exchange.	This study
Newman *sbi*	Derivative of S. aureus strain Newman; NCTC 8178, clonal complex 8. Deficient in Sbi. *sbi*::Em^r	(13)

Newman *sbi lytM*	Derivative of Newman *sbi* deficient in LytM. Δ*lytM*.	This study
Newman *sbi* [SpAΩSdrESS]	Derivative of Newman *sbi* where the SpA sorting signal has been replaced with sorting signal of SdrE by allelic exchange.	This study
E. coli DC10B	*dam*$^+$ Δ*dcm* Δ*hsdRMS endA1 recA1*	(24)
Plasmids		
pRMC2	Anhydrotetracycline-inducible expression vector. Amp^r, Cm^r.	(23)
pSpA	Plasmid pRMC2 containing the full-length *spa* gene.	This study
pSpAΔSS	Plasmid pSpA lacking DNA encoding the SpA sorting signal (residues 474-508).	This study
pSpAΩSdrESS	Plasmid pSpA where DNA encoding the SpA sorting signal (residues 474-508) has been replaced with DNA encoding the SdrE sorting signal (residues 1117-1154).	This study
pRMC2-*sbi*ΔD1D2	Plasmid pRMC2 containing DNA encoding the Sbi signal sequence (residues 1-40) and the D3D4 domains (residues 153-253).	(13)
pD3D4-SpA	Plasmid pRMC2 containing an in-frame fusion between DNA encoding Sbi D3D4 domains (residues 153-253) and DNA encoding the SpA sorting signal (residues 474-508).	This study
pD3D4-SdrESS	Plasmid pRMC2 containing an in-frame fusion	This study

	between DNA encoding Sbi D3D4 domains (residues 153-253) and DNA encoding the SdrE sorting signal (residues 1117-1154).	
pSpAΩSdrESS-DS	Plasmid pSpAΩSdrESS containing 538 bp of sequence downstream of the *spa* gene.	This study
pIMAY	Temperature-sensitive vector for allelic exchange, Cm^r.	(24)
pIMAY::Δ*lytM*	Plasmid for creating a *lytM* deletion mutant. Carries 509 bp of DNA from upstream and 512 bp of DNA from downstream of the *lytM* gene amplified from LAC.	This study
pIMAY::SpAΩSdrESS	Plasmid for replacing DNA encoding the sorting signal of SpA with DNA encoding the sorting signal of SdrE on the chromosome of LAC*.	This study